

Homologous down-regulation of the glucocorticoid receptor is influenced by the dimerization state of the receptor

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Declaration

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Abstract

Glucocorticoids (GCs) remain the mainstay therapeutic choice for the treatment of inflammation, and exert their potent anti-inflammatory effects via the glucocorticoid receptor (GR α). However, the chronic use of GCs, in addition to generating undesirable side-effects (e.g. hyperglycemia), results in homologous down-regulation of the GR α . This reduction in GR α protein levels has been coupled to a decrease in GC-responsiveness, in a number of psychological and pathological conditions, which may culminate in GC-acquired resistance, a major concern for chronic GC users. The current study investigated whether ligand-induced down-regulation of the GR α is influenced by the dimerization state of the receptor by transfecting human wild type GR α (hGRwt) or a dimerization deficient GR α mutant (hGRdim) into COS-1 cells. In addition, Compound A (CpdA), which abrogates GR dimerization, was used to mimic the effect of the hGRdim in HepG2 cells containing endogenous GR α . Furthermore, the ability of an endogenous mutant, mGRdim, to undergo ligand-induced receptor turnover was compared to that of the wild-type GR α , mGRwt, in MEF-mGRdim and MEF-mGRwt cells, respectively. Whole-cell-binding and Western blotting revealed that the hGRwt, but not the hGRdim, underwent homologous down-regulation following dexamethasone (Dex), a potent synthetic GC, and cortisol (F), an endogenous GC, treatment. In contrast, ligand-induced down-regulation of GR α was abolished by CpdA treatment or the use of hGRdim, suggesting a novel role for GR α dimerization in mediating receptor turnover. These findings from the COS-1 cells were supported by results from the HepG2 cells, and, in part, by results from the MEF cells. Moreover, the dimerization state of the GR α influenced the post-translational processing of the receptor, impacting its degradation via the proteasome. Specifically, 'loss' of GR α dimerization via CpdA treatment or the use of the dimerization deficient GR α mutant, restricted hyper-phosphorylation at Ser404, which has been coupled to increased GR α degradation, as well as restricted the interaction of GR α with the E3 ligase, FBXW7 α , thus hampering receptor turnover. Lastly, a model to mimic acquired GC resistance was established and tested. Results from these experiments demonstrated that prolonged GC treatment of mGRwt (i.e. 'gain' of GR α dimerization) leads to molecular GC resistance (i.e. GILZ) and clinical GC resistance (FKBP51), whilst maintaining the up-regulation of a metabolic gene (i.e. TAT). In contrast, 'loss' of GR α dimerization partially restricts acquired resistance, at a molecular and clinical level, whilst displaying an improved side-effect profile in terms of restricting the expression of a metabolic gene (i.e. TAT). These results expand our understanding of factors that contribute to GC-resistance and may be exploited clinically.

Opsomming

Glukokortikoïede (GK's) bly die staatmaker terapeutiese keuse vir die behandeling van inflammasie en oefen hul kragtige anti-inflammatoriese effekte uit via die glukokortikoïede reseptor ($GR\alpha$). Die chroniese gebruik van GK's, benewens die ontwikkeling van ongewenste newe-effekte (bv. hiperglisemie), lei ook tot 'n afregulering van die $GR\alpha$. Hierdie afname in $GR\alpha$ proteienvlakke word gekoppel aan 'n afname in GK-responsiwiteit, in 'n aantal sielkundige en patologiese toestande, wat kan lei tot GK-verworwe weerstand, 'n groot kommer vir chroniese GK-gebruikers. Die huidige studie ondersoek of ligand-geïnduseerde afregulering van die $GR\alpha$ beïnvloed word deur die dimerisasietoestand van die reseptor deur menslike wilde tipe $GR\alpha$ (hGRwt) of 'n dimerisasie-defektiewe $GR\alpha$ -mutant (hGRdim) in COS-1-selle te transfekteer. Daarbenewens is Compound A (CpdA), wat GR-dimerisasie ophef, gebruik om die effek van die hGRdim in HepG2-selle wat endogene $GR\alpha$ bevat, na te boots. Verder is die vermoë van 'n endogene mutant, mGRdim, om ligand-geïnduseerde reseptoromset te ondergaan, vergelyk met dié van die wild-tipe $GR\alpha$, mGRwt, onderskeidelik in MEF-mGRdim en MEF-mGRwt-selle. Heel-sel-binding Western klad het aangetoon dat die hGRwt, maar nie die hGRdim nie, homoloë afregulering ondersonaan na behandeling met deksametason (Dex), 'n kragtige sintetiese GK, en kortisol (F), 'n endogene GK. In teenstelling hiermee is ligand-geïnduseerde afregulering van $GR\alpha$ afgeskaf deur CpdA-behandeling of die gebruik van hGRdim, wat 'n splinternuwe rol vir $GR\alpha$ -dimerisasie in die bemiddeling van reseptoromset voorstel. Hierdie bevindings van die COS-1-selle is ondersteun deur die resultate van die HepG2-selle, en gedeeltelik deur die resultate van die MEF-selle. Verder het die dimeriseringstoestand van die $GR\alpha$ die post-translasie-modifisering van die reseptor beïnvloed, wat afbraak deur die proteasoom beïnvloed het. Spesifiek, 'verlies' aan $GR\alpha$ -dimerisasie via CpdA-behandeling of die gebruik van die dimerisasie-defektiewe $GR\alpha$ -mutant, het hiperfosforilering by Ser404, wat gekoppel is aan verhoogde $GR\alpha$ -afbraak, sowel as die interaksie van $GR\alpha$ met die E3-ligase, FBXW7 α , beperk wat dus die reseptoromset belemmer het. Laastens is 'n model om verworwe GK-weerstand na te boots daargestel en getoets. Resultate van hierdie eksperimente het getoon dat langdurige GK behandeling van mGRwt (dws 'wins van $GR\alpha$ dimerisasie) lei tot molekulêre GK weerstand (dws GILZ) en kliniese GK weerstand (dws FKBP51), terwyl die opregulering van 'n metaboliese geen (dws TAT) behoue bly. In teenstelling hiermee verminder 'verlies' van $GR\alpha$ -dimerisasie die verworwe weerstand, op molekulêre en kliniese vlak, terwyl 'n verbeterde newe-effekprofiel vertoon word in terme van die beperking van die uitdrukking van 'n metaboliese geen (dws TAT). Hierdie resultate brei ons begrip uit van faktore wat bydra tot GK-weerstand en kan klinies ontgin word.

I dedicate this thesis to my beloved family, Andrew, Diana, Kristin and David. Without your unlimited love and unwavering support, the completion of this thesis would not have been possible.

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Abbreviations

A	acetylation
ACTH	adrenocorticotrophic hormone
AD	atopic dermatitis
AF1	activation domain 1
AF2	activation domain 2
ALL	acute lymphoblastic leukaemia
ANOVA	one-way analysis of variance
AR	androgen receptor
BSA	bovine serum albumin
BZ	bortezomib
CBG	corticosteroid-binding globulin
CE	counting efficiency
ChIP	chromatin immunoprecipitation
CHIP	carboxy terminus of heat shock protein 70-interacting protein
CHX	cycloheximide
CIA	collagen-induced arthritis
CLP	cecal ligation and puncture
Co-IP	co-immunoprecipitation
COPD	chronic obstructive pulmonary disease
CpdA	Compound A
CRH	corticotrophin-releasing hormone
DBD	DNA-binding domain
DCC	dextran coated charcoal
DEPC	diethyl pyrocarbonate
Dex	dexamethasone
D-loop	dimerization loop
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DST	dexamethasone suppression test
DUB	de-ubiquitinating
DUSP1	dual specificity phosphatase 1
EAE	experimental autoimmune encephalomyelitis
EAN	experimental autoimmune neuritis
EDTA	ethylenediaminetetraacetic acid
ELS	early life stress
ER	estrogen receptor
EtOH	ethanol
F	cortisol
FBXW7α	F-box/WD repeat-containing protein 7
FCS	fetal calf serum
FKBP51/52	FK506 binding protein 5
FLS	fibroblast-like synoviocytes
F.O	fractional occupancy

FSS	forced swim stress
GAD	generalized-anxiety disorder
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCs	glucocorticoids
GFP	green fluorescent protein
GILZ	glucocorticoid–induced leucine zipper
GPCR	G-protein coupled receptor
GRα	glucocorticoid receptor alpha isoform
GRE	glucocorticoid response element
GSK3β	glycogen synthase kinase 3 β
Hdm2	human double minute 2
HPA	hypothalamic-pituitary-adrenal
Hsp	heat shock protein
HTC	hepatoma tissue culture
IBD	inflammatory bowel disease
IL-6	interleukin 6
ITP	immune thrombocytopenia
k	rate constant
Kd	ligand-binding affinity
LBD	ligand-binding domain
LPS	lipopolysaccharide
MD	major depression
Mdm2	murine double minute 2
MM	multiple myeloma
MMP	matrix metalloproteinase-1
MS	maternal separation
NCBI	National Center for Biotechnology Information
NCD	non-communicable disease
nGRE	negative glucocorticoid response element
NR3C1	nuclear receptor subfamily 3 group C member 1
NS	nephrotic syndrome
NSB	non-specific binding
NTD	N-terminal Domain
P	phosphorylation
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
Pen/Strep	penicillin and streptomycin
PLA	proximity ligation assay
PPS	preconception paternally stressed
PTMs	post-translational modifications
PTSD	post-traumatic stress disorder
RA	rheumatoid arthritis
RFI	relative fluorescence intensity
RSD	repeated social defeat
S	sumoylation

SDS	sodium dodecyl sulphate
SEDIGRAM	selective dimerizing GR α agonist/modulators
SEMOGRAM	selective monomerizing GR α agonist/modulators
SGRM	selective glucocorticoid receptor modifier
STUB1	STIP1 homology and U-Box containing protein 1
T	threonine
T 1/2	half-time
TA	triamcinolone acetonide
TAT	Tyrosine aminotransferase
TBS	Tris-buffered saline
TBS/T	Tris-buffered saline Tween
TE	Tris- ethylenediaminetetraacetic acid
TNFα	tumor necrosis factor alpha
TSG101	tumour susceptibility gene 101
UbcH7	Ubiquitin-Conjugating Enzyme 7
Ubq	ubiquitination
UPS	ubiquitin proteasome system
USA	United States of America
WHO	World Health Organization

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Chapter 1:

Introduction

1.1. Introduction

'Life is like riding a bicycle. To keep your balance, you must keep moving' Albert Einstein¹

In 2014, the World Health Organization (WHO) reported that 43% of deaths reported in South Africa were due to non-communicable diseases (NCDs)². Furthermore, an alarming 89% and 88% of deaths in the United Kingdom and United States, respectively, were as a result of NCDs³. NCDs, a collective term for a range of medical conditions, are classified as diseases that are non-infectious and non-transmissible from patient to patient, with the four main NCDs being cardiovascular disease, cancer, chronic respiratory disease and diabetes^{2,3}.

The progression of many of these NCDs is driven by chronic inflammation and they are often classified as inflammatory-linked or auto-immune. Chronic inflammation represents a profound prolonged increase in systemic inflammatory processes, which disrupts the system's homeostasis and which may ultimately result in an inability of the system to adapt and thus a 'point of no return'^{2,3}. The pathological manifestations associated with inflammatory diseases embody this 'point of no return'. Unlike chronic inflammation, acute inflammation, a brief inflammatory response, serves as a protective mechanism allowing for the organism to cope with temporary threats and for homeostasis of the biological system to be restored⁴. In terms of the magnitude of the inflammatory response, chronic inflammation is considered to be a low-level of inflammation, with subtle local and systemic signs⁵ (Fig. 1.1). In contrast, acute inflammation is characterised by a greater inflammatory response with prominent symptoms such as swelling, redness, pain and heat⁵ (Fig. 1.1).

The inflammatory response, which culminates in either chronic or acute inflammation, is intricately linked to the stress response. Thus, the inflammatory response activates the stress response and perturbing the stress response, via a stressor, may disrupt the balance of the inflammatory response⁴⁻⁶. The interrelatedness of these two responses or systems may be eloquently described by a 'bidirectional interaction'⁴⁻⁶ (Fig. 1.1). Broadly speaking, acute stress is a normal response to everyday life⁴. It often occurs following an unpredictable threat, such as an injury, and is associated with the fight-or-flight response. Activation of the stress response by an acute stressor results in the synthesis and secretion of physiological mediators, some of whose fundamental role is to suppress

inflammation, also termed immunosuppression⁴⁻⁶ (Fig. 1.1). Moreover, the acute stress response is short-lived and once the threat or challenge has been removed the stress-response is able to return to baseline or adapt to the stress⁴ (Fig. 1.1). Whilst acute stress responses are understood as adaptive reactions to overcome challenges and restore homeostasis, chronic stress, as a result of the repeated exposure to a physical or psychological stressor for a prolonged period of time, is often associated with enhanced inflammation and in most cases causes irreversible damage^{4,6-8} (Fig. 1.1). More specifically, chronic stress permanently alters endocrine-autonomic-immune signalling pathways, at both a central and peripheral level leading to inflammatory dis-inhibition and disease promotion^{4-7,9,10} (Fig. 1.1).

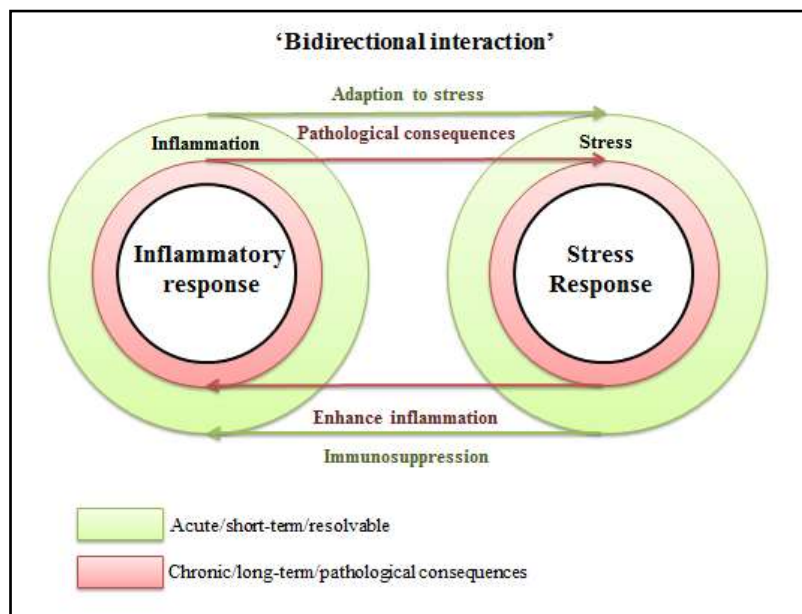


Figure 1.1: A 'bidirectional interaction' exists between the stress response and inflammation. The stress response is activated by stressors, which may be acute or chronic. Activation of the stress response stimulates signalling pathways to produce physiological mediators, which may act directly on the inflammatory response. In terms of acute stress, these physiological mediators function to reduce inflammation through immunosuppression, allowing for the biological system to adapt to the stressor and homeostasis to be restored. In contrast, repeated chronic stress, leads to continual activation of signalling pathways associated with the stress response. In turn, an excess in physiological mediators can have permissive or stimulatory effects on the inflammatory response, ultimately enhancing inflammation. It is this, prolonged chronic inflammation that has pathological consequences and in many cases drives the progression of a number of NCDs.

Central to the stress-response is the hypothalamic-pituitary-adrenal (HPA) axis, which functions to maintain homeostasis by modulating the inflammatory response and other systems within the body, including the metabolic, cardiovascular and reproductive systems^{11,12}. In order to mediate the regulation of multiple cellular processes within these systems, the finely-tuned HPA-axis coordinates the synthesis and secretion of glucocorticoids (GCs) into the periphery⁴. Broadly speaking, upon activation, in an ultradian/circadian manner or in response to internal or external threats, such as infection, pain, or stress¹³⁻¹⁶, the hypothalamus secretes corticotrophin-releasing hormone (CRH), which directly acts on the anterior pituitary gland, stimulating the release of

adrenocorticotrophic hormone (ACTH)^{15,17} (Fig. 1.2). ACTH then stimulates the release, into the blood stream, of endogenous GCs, such as cortisol (F), from the adrenal cortex, where they are synthesised^{15,18} (Fig. 1.2). Once in the blood these lipophilic molecules are transported to their target tissues and cells, bound to carrier proteins such as corticosteroid-binding globulin (CBG), where they then bind to their cognate receptor, the glucocorticoid receptor alpha isoform (GR α)¹⁹⁻²².

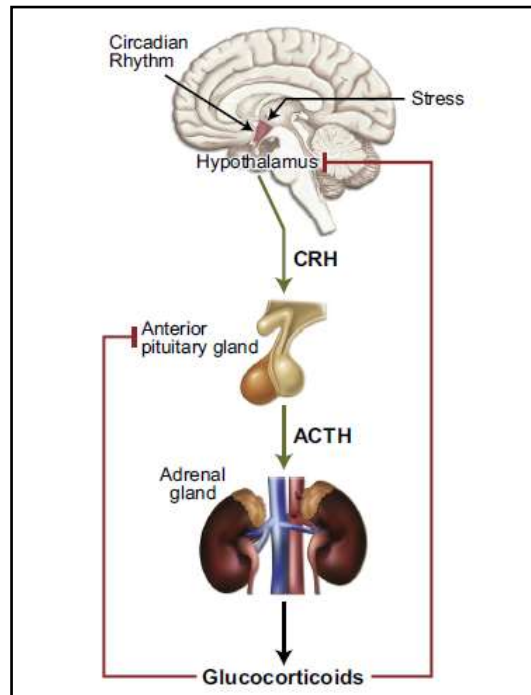


Figure 1.2: The HPA axis regulates the synthesis and secretion of GCs following activation by the circadian rhythm or stress. Figure from Oakley *et al.*¹⁵ where CRH refers to corticotrophin releasing hormone, and ACTH refers to adrenocorticotrophic hormone. Activation by these hormones of the anterior pituitary and adrenal gland, respectively, is represented by the green arrows. Conversely, the red lines indicate the inhibitory negative feedback loops of the GCs, via the GR α , onto the hypothalamus and anterior pituitary gland.

Stress-induced changes in HPA-axis signalling may result in the disruption of the homeostatic activity of the stress response, and an overall increase in the concentration of circulating GCs^{4,5}. This altered HPA axis signalling has a multitude of effects, disrupting homeostasis at both the central and peripheral level and directly impacting the inflammatory response, and subsequently the degree of inflammation within the system^{4-7,9,10} (Fig. 1.1). However, depending on the magnitude of the stressor and the length of time of exposure to a particular stressor, the system may or may not be able to adapt. To introduce the central and peripheral effects of altered HPA-axis signalling and the ability of the system to reassert homeostasis, a figure adapted from Romero *et al.*²³, which describes the Reactive Scope Model (Fig. 1.3), is used as framework.

Firstly, **central homeostasis** of the stress system via the HPA-axis is predominantly maintained via negative feedback of endogenous GCs, via the GR α , on the hypothalamus and anterior-pituitary

gland (Fig. 1.2). Under ultradian/circadian signalling these negative feedback loops function to ensure optimal HPA-axis signalling and to maintain the endogenous concentration of GCs within the range of predictive homeostasis²³ (Fig. 1.3). Acute stress such as from infection, pain or injury, stimulates HPA-axis activity leading to an increase in the concentration of circulating endogenous GCs^{4,7,23}. This increase in the GC concentration represents an adaptive reaction of the stress response employed to overcome challenges, in this case the acute stressor. The adjustment the system needs to make, in this case to the HPA-axis activity, in order to respond to unpredictable perturbations (e.g. acute stress) is referred to as reactive homeostasis²³ (Fig. 1.3). In the case of acute stress, it provides elasticity for the system to react and adapt to the stress-induced increase in HPA-axis activity and the subsequent increase GC concentration²⁴. As the system makes these necessary adjustments it undergoes ‘wear and tear’ through maintaining GC concentrations within this reactive homeostasis range²³. This ‘wear and tear’ may be described by the term allostatic load and refers to the cost incurred by the system to maintain stability through change (i.e. maintaining the GC concentration within the reactive homeostasis range)^{23,24} (Fig. 1.3).

The concept of allostatic load can be thought of in terms of one of Albert Einstein’s quotes: ‘***Life is like riding a bicycle. To keep your balance, you must keep moving***’, which suggests that maintaining homeostasis of a system (life), requires continual adaptation (peddling of opposing left and right legs) whilst expending energy (energy required to generate force on peddles). Furthermore, as with a tiring cyclist, this allostatic load will eventually drive a gradual decrease in the ability of the system to cope and ultimately reduce the threshold between reactive homeostasis and homeostatic overload, which may result in homeostatic failure²³ (Fig. 1.3). The latter is a consequence of sustained ‘wear and tear’ or a cumulative allostatic load and is often the result of repeated/prolonged stress activation, known as chronic stress.

Chronic stress encourages prolonged stress-induced changes in HPA-axis activity resulting in HPA axis hyper-activity²⁵⁻²⁷. Consequently, this leads to a prolonged surge in the concentrations of circulating endogenous GCs⁴. These elevated endogenous GC concentrations are driven above the reactive homeostasis range into a pathological range referred to as homeostatic overload²³ (Fig. 1.3). Unlike short-term acute stress, these prolonged stimulatory effects induced by chronic stress on the HPA axis, and significantly higher GC concentrations, in many cases, lead to pathological consequences. In addition to chronic stress, the therapeutic use of exogenous GCs, for the treatment of inflammation, may also promote an increase in the concentration of circulating GCs into the homeostatic overload range¹⁰ (Fig. 1.3). This GC excess may result in adverse side-effects, such as hyperglycaemia, through a GC-mediated increase in the expression of metabolic enzymes and the unnecessary excess mobilization of glucose. In the case of both chronic stress and prolonged

exogenous GC treatment, the dysregulation of the HPA axis is further compounded by deficient $GR\alpha$ -mediated negative feedback loops, which are unable to contest the homeostatic overload, in terms of GC concentration, leading to an insufficiency in HPA-axis suppression¹⁵ (Fig. 1.2 and 1.3). One of the proposed reasons for defective negative regulation of the HPA axis is the peripheral effects induced by prolonged GC excess. An example, and central theme to this study, is **ligand-induced $GR\alpha$ down-regulation** in peripheral tissues and cells²⁸.

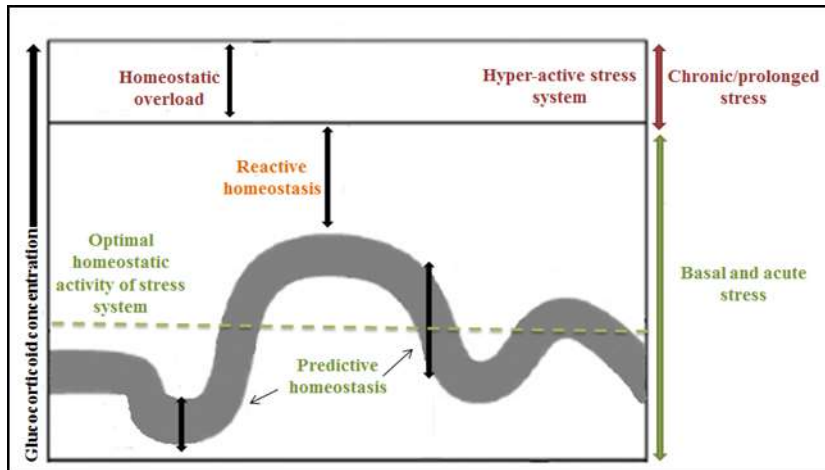


Figure 1.3: The homeostatic activity of the stress system may be described using an adapted version of the Reactive Scope Model by Romero *et al.*²³ Under basal conditions, optimal homeostatic activity of the stress-system occurs (dashed green line). Moreover, GCs are synthesised and secreted in a pulsatile manner following circadian/ultradian activation, resulting in slight changes in the GC concentration, which may be described as *predictive homeostasis* (thick grey line). Acute stress stimulates HPA-axis activity and drives an increase in the GC concentration to above the *predictive homeostasis* range (thick grey line). Subsequently, this perturbation encourages an adaption by the stress response, termed *reactive homeostasis*, which functions to counteract the stressor. In the case of chronic, prolonged stress, the stress system becomes hyperactive, driving a severe increase in GC concentration, which the system cannot counteract, and causing *homeostatic overload*. It is in this range where pathological consequences develop and disease progression ensues.

The GC/ $GR\alpha$ signalling pathway functions, in peripheral tissues and cells, to directly increase the expression of anti-inflammatory genes and decrease the expression of pro-inflammatory genes (Fig. 1.4). It is these two mechanisms, termed GC-mediated transactivation and transrepression, respectively, which collectively allow for the classification of GCs as powerful immunosuppressive physiological mediators, serving to counteract inflammation, and allowing for **peripheral homeostasis** to be maintained during optimal HPA axis signalling²⁹.

1. Specifically, GCs (e.g. endogenous GC, F) secreted by the adrenal gland (Fig. 1.2) are transported in the blood to tissue- or cell-specific sites, where they bind to their cognate receptor, the $GR\alpha$ ¹⁵.
2. This binding results in a consequent change in the receptor conformation and the dissociation of the bound inhibitory protein complex (including the heat shock proteins)¹⁵.
3. Subsequently, this conformational change facilitates receptor dimerization and then translocation of the GC- $GR\alpha$ complex from the cellular cytoplasm into the nucleus^{15,30}.

4. Once in the nucleus, the GC-bound GR α acts as a ligand-activated transcription factor to positively (i.e. transactivation) and negatively (i.e. transrepression) regulate the expression of a large cohort of GC-responsive genes¹⁵.
5. Following the modulation of GC-responsive gene expression, the GC-GR α complex is exported to the cytoplasm¹⁵.
6. Once exported it is targeted for degradation by the proteasome¹⁵.

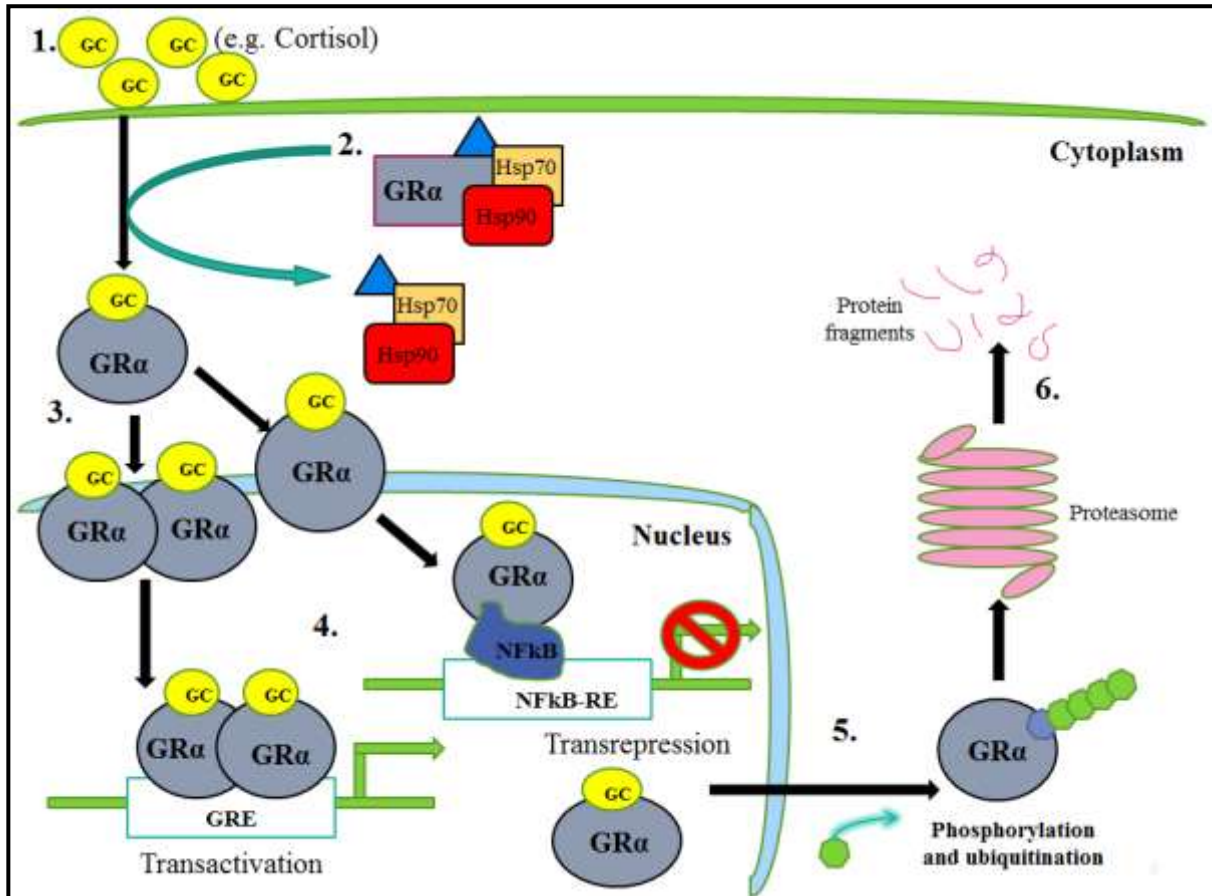


Figure 1.4: The GC/GR α signalling pathway. (1) GCs are transported in the blood to target tissues or cells where they diffuse across cell membranes into the cytoplasm and bind to the GR α . (2) Binding of GCs initiates a conformational change in the GR α and dissociation of an inhibitory protein complex. (3) The GC-GR α complex either dimerizes or remains as a monomer and translocates to the nucleus. (4) The GC-GR α complex binds to glucocorticoid response elements (GREs) or other transcription factor response elements (e.g. NF κ B-RE) to regulate the expression of GC-responsive genes. (5) Following transactivation or transrepression the GC-GR α complex is exported to the cytoplasm where it is targeted for degradation by post-translational modifications (e.g. phosphorylation and ubiquitination), via (6) the proteasome.

Under basal or acute stress conditions, degradation of the GR α , induced down-stream of ligand-binding, serves to protect the cell from continual GC/GR α signalling and maintains GC function within the normal reactive scope²³ (Fig. 1.3). In contrast, prolonged exposure to excess GCs, as a result of chronic stress or exogenous GC administration, not only alters the central homeostasis of the HPA-axis, but promotes enhanced GR α protein down-regulation in peripheral cells and tissues³¹⁻³⁷. In this case, receptor levels are significantly reduced, which results in a disintegration

of the negative feedback loop of GCs on the HPA axis and drives GC concentrations above the *reactive homeostasis* range and into *homeostatic overload*²³ (Fig. 1.3). Furthermore, due to the fact that GCs serve as endogenous anti-inflammatory molecules to combat inflammation, significant reductions in the GR α , through which they mediate their effects, have severe implications. In addition, pharmacologically, a significant reduction in GR α expression has been linked to a concomitant reduction in GC response and this decrease in GC sensitivity poses potential problems for the use of GCs as anti-inflammatory drugs³⁸⁻⁴³. Moreover, in some cases it leads to partial or complete acquired resistance to GC treatment and the subsequent deterioration of clinical control when treating a variety of auto-immune and inflammation-associated diseases (e.g. asthma and haematological cancers)^{15,44,45}. Studies have demonstrated that one of the ways in which the GC response may be maintained over time is through restoration of GR α expression, which may ultimately reverse acquired resistance to GC treatment⁴⁶⁻⁴⁸. One of the ways this has been achieved is through the use of Ginsenoside Rh1, which ameliorates the ligand-induced down-regulation of the GR α protein and reverses resistance, thereby ultimately restoring homeostasis at a cellular level⁴⁹.

CompoundA (CpdA) or 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride, is a synthetic analogue of a phenyl aziridine precursor that occurs in the shrub *Salsola tuberculatifomis* Botsch⁵⁰. Prolonged treatment with CpdA does not mediate wild type GR α down-regulation^{37,51,52}. This finding is in stark contrast to Dexamethasone (Dex), a potent GR α agonist used pharmacologically, shown by Visser *et al.*⁵² to result in a significant reduction in wild type GR α protein and mRNA expression over time, which supported the findings of several other studies in which Dex was used³¹⁻³⁷. Furthermore, CpdA has a non-steroidal structure and is known to have a dissociative behaviour when it comes to GC/GR α signalling, which allows for its classification as a selective glucocorticoid receptor modifier (SGRM)^{37,52}. Essentially, this dissociative behaviour refers to CpdA's ability to negatively down-regulate pro-inflammatory genes, mediating its well documented potent anti-inflammatory potential, without positively up-regulating genes often associated with adverse side-effects of GCs^{36,53-55}. More specifically CpdA efficiently mediates transrepression, but not transactivation, via the GR α . It is thought that the dissociative behaviour of CpdA may be as a result of its ability to prevent the formation of GR α dimers, for which Robertson *et al.*⁵⁶ along with others⁵³ provided a strong case by revealing that the dimerization abrogating capabilities of CpdA differed from the dimerization promoting capabilities of Dex. Taken together, these characteristics of CpdA sparked interest in a possible link between GR α dimerization and

GR α turnover, which in the current study is initially further investigated using a human dimerization deficient¹ mutant GR α , hGRdim⁵⁷.

The dimerization deficient mutant, hGRdim, was created by introducing a single amino acid exchange, of an alanine for a threonine at amino acid position 458, in the dimerization loop (D-loop) of the wild type human GR α ⁵⁷, thus producing a mutant with a single disrupted dimerization interface. The D-loop is located within the second zinc finger, found within the DNA-binding domain of the hGR α , which is involved in mediating receptor/DNA interactions⁵⁷. Based on previous findings, in which dimerization is inhibited through this D-loop amino acid exchange in another steroid receptor, the androgen receptor (AR)⁵⁸, it was widely accepted that the same mutation would prevent dimerization of the GR α . Classically, it is thought that GR α dimerization is a requirement for direct DNA-binding of the receptor to a glucocorticoid response element (GRE) and thus the reported reduced affinity of hGRdim for the GRE initially provided evidence⁵⁹ for its inability to successfully form GR α dimers. However, there is conflicting evidence surrounding the inability of hGRdim to dimerize^{56,60}. Despite this, hGRdim is still the most widely characterised and utilized dimerization deficient GR α mutant^{56,59} and, in the current study, will be used to substantiate the effects that a CpdA-induced ‘loss²’ of wild type GR α dimerization has on receptor turnover.

Thus, the current study **hypothesizes** that there is an **association between ligand-induced GR α dimerization and ligand-induced down-regulation of the GR α protein.**

To prove or disprove this hypothesis, the following questions were asked:

1. Is receptor dimerization a requirement for ligand-induced receptor turnover?
2. Mechanistically, how does the inability of the GR α to dimerize prevent ligand-induced down-regulation?
3. Following prolonged GC treatment do changes in GR α protein expression mediate downstream effects, through the modulation of GC-responsive gene expression?

In order to address the above questions the following **aims** were established, which are reflected in the results chapters 4, 5 and 6, respectively:

- **Chapter 4:** Investigate the role of ligand-induced receptor dimerization in mediating GR α protein turnover through the treatment of endogenous human GR α or transiently transfected wild-type (hGRwt) and dimerization-deficient mutant GR α (hGRdim) with dimerization

¹Deficient is defined by the Cambridge Dictionary as “not having enough of” and thus we will use this term as such throughout the thesis to describe an impaired, but not totally disrupted, dimerization ability.

² Loss is defined by the Cambridge Dictionary as “the fact you no longer have something or have less of something” and thus we have used the word to mean “less of” rather than “total loss” throughout the thesis.

promoting GCs, both endogenous (F) and synthetic (Dex), and a dimerization abrogating GC (CpdA).

- **Chapter 5:** Determine how the inability of GR α to dimerize impedes molecular mechanisms involved in ligand-induced GR α protein down-regulation, via proteasomal degradation, with specific reference to post-translational modifications such as phosphorylation and ubiquitination.
- **Chapter 6:** Explore how changes in the expression of both GR α mRNA and protein, due to a ‘loss’ or ‘gain’ of receptor dimerization, modulates the mRNA expression of a subset of GC-responsive genes, by establishing a model to mimic a continuum of acquired resistance to GC treatment.

In addition to the results chapters outlined above and the current chapter, **Chapter 1**, which highlighted the importance of homeostasis and provided background on the HPA-axis and more specifically, the GC/GR α signalling pathway, while furthermore describing results from previous studies, which provided a platform to launch this study, this thesis consists of 3 additional chapters, namely **Chapter 2, 3** and **7**. **Chapter 2**, a review article written for publication, aims to enlighten the reader about the broad spectrum of GC resistance, both congenital and acquired, while honing in on the idea of acquired GC resistance as a continuum and discussing factors which may affect this continuum of resistance. Important to note, is that some repetition of Chapter 1 is unavoidable as these concepts will be included in the manuscript for publication. **Chapter 3**, details the material and methods used in the current study, while **Chapter 7** discusses the findings of the current study. Here, an in depth analysis of all the results in the current study is presented by contextualizing the findings in terms of the current literature, stating the limitations of this study and providing future perspectives.

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Chapter 2:

“Acquired GC resistance”: reviewing the importance of glucocorticoid receptor expression.

2.1. Introduction

In 2014, the World Health Organization (WHO) reported that 43% of deaths reported in South Africa were due to non-communicable diseases (NCDs), with the four main NCDs being cardiovascular disease, cancer, chronic respiratory disease and diabetes^{1,2}. Furthermore, an alarming 89% and 88% of deaths in the United Kingdom and United States, respectively, were as a result of NCDs². The progression of many NCDs and psychological/pathological conditions are driven by chronic, persistent inflammation¹⁻³. Unlike acute inflammation, which serves as a protective mechanism allowing for homeostasis to be returned following a temporary threat, chronic inflammation represents a prolonged increase in the systemic inflammatory process, which continuously disrupts the system's homeostasis, ultimately resulting in an inability of the system to adapt^{3,4}. This sustained disruption in the homeostasis of the system, has a knock on effect, modulating a number of essential systemic signalling pathways, such as the hypothalamic-pituitary-adrenal (HPA) axis, which is central to the stress response⁴. Due to the interrelatedness of the stress and inflammatory response, chronic persistent inflammation, can be considered both a cause and a consequence of a prolonged disruption of the central HPA axis signalling pathway's homeostasis, which in turn has many peripheral effects, one of these being an increase in circulating glucocorticoids (GCs)^{3,5-8}.

Endogenous GCs are physiological mediators synthesised and regulated by the HPA-axis and secreted in an ultradian/circadian manner or in response to internal or external threats, such as infection, pain, or stress⁹⁻¹². GCs function within the body to regulate inflammation and maintain internal homeostasis of the biological system^{3,5-7,9,13,14}. To this day, exogenous GCs, designed to mimic the biological anti-inflammatory action of endogenous GCs, remain the mainstay therapeutic choice for the treatment of chronic inflammation as a result of disease and/or psychological or physical stress¹⁴⁻¹⁶. Currently one of the most widely prescribed

drugs in the world with an estimated 1.2% of the population of the United States of America (USA) using GCs^{17,18}. Whilst often efficient in curbing damaging inflammation it is believed that approximately 30% of all patients who require pharmacological GC treatment, experience a degree of damaging GC insensitivity¹⁹. Specifically, 4-10% of asthma patients, 30% of rheumatoid arthritis patients, almost all chronic obstructive pulmonary disease (COPD) and sepsis patients²⁰ and 10-30% of untreated acute lymphoblastic leukaemia (ALL) patients²¹ experience varying degrees of GC insensitivity.

Due to this stochastic response to GCs, within disease groups²²⁻²⁴ compounded by inter-individual and intra-individual variation in patient GC sensitivity as well as tissue-specific intra-individual GC-responsiveness among organs, tissues, cells and even within the same cell^{19,25}, research is now focused on developing diagnostic tools for determining GC sensitivity prior to treatment so that GCs may be used selectively in personalized therapeutic regimes^{19,26}. This will likely assist in limiting the generation of adverse side-effects and prevent the development of further GC insensitivity²⁷⁻³⁰.

This review revisits and highlights the importance of the glucocorticoid receptor alpha isoform (GR α), the receptor to which GCs bind and which mediate their biological effects, in GC sensitivity, specifically in terms of an acquired resistance to GC treatment. Primary focus is given to disease- or treatment associated reductions in receptor levels, which drive the development of GC insensitivity, as well as the molecular mechanisms involved in mediating receptor turnover at both the mRNA and protein level. Furthermore, methods to restore GR α protein expression and improve GC sensitivity are briefly detailed. To conclude, we propose the notion that the role of the conformation of the liganded receptor is somewhat undervalued and overlooked when considering ways in which GC resistance may be curbed, suggesting that this fundamental aspect of GC/GR α signalling requires further investigation and that the link between liganded-GR α conformation and receptor turnover should be considered as it may have implications in acquired GC resistance.

2.2. GC resistance

GCs, both endogenous and exogenous, mediate their biological effects via their ubiquitously expressed cognate receptor, GR α ^{17,31}. Briefly, the synthesis and secretion of GCs into the blood stream is tightly regulated by the HPA-axis¹⁷. Once in the bloodstream, delivery of these GCs to various tissues and cells as well as the activity and bioavailability of these small lipophilic molecules is further governed by transport proteins, cortisol-binding globulin

(CBG) and albumin¹⁷. Upon reaching the cell, GCs diffuse across the cell membrane and bind the intracellular GR α ¹⁷. Upon binding, the GR α undergoes a conformational change driving the dissociation of an inhibitory multi-protein complex consisting of, amongst others, Heat-shock protein 90 (Hsp90) and FK506 binding protein 51/52 (FKBP51/52), allowing for subsequent translocation of the GR α to the nucleus of the cell³². It is here where the GC-bound GR α mediates its biological effects via various mechanisms of transrepression or transactivation of a wide range of GC-responsive genes. Generally speaking, it is thought that the GC-mediated transrepression of pro-inflammatory genes is what provides the indispensable potent anti-inflammatory potential of GCs³². As specific details of the GC/GR α signalling pathway are not the focus of this paper, comprehensive reviews by Desmet *et al.*³², Ratman *et al.*³³, Vandevyver *et al.*³⁴ and Weikum *et al.*³⁵ are recommended.

Central to the ability of GCs to combat inflammation (i.e. patient sensitivity to GC treatment) is the requirement for a significant amount of functional GR α through which they may mediate their effects³⁶⁻³⁸, which we term the GR α ‘functional pool’. Importantly, there are a multitude of factors which can regulate the ‘functional pool’ of GR α , either at the level of the activity of the receptor and/or at the level of the amount (i.e. expression) of the GR α , thus ultimately contributing to GC resistance. In short, disruptions in GR α function are known to modulate the subcellular localization, ligand binding, and transactivation ability of the receptor²⁵, and are regulated by, amongst others, increases in additional GR isoforms (GR β and GR γ) due to alternative splicing events, inactivating GR α mutations, the inflammatory cytokine profile of the cellular microenvironment and mutations/polymorphisms in the ERK pathway, as eloquently reviewed by Nicolaidis *et al.*⁴, Oakley *et al.*^{11,14,39}, Merkulov *et al.*⁴⁰ and Patel *et al.*⁴¹. However, rather than altered GR α activity, the focal point of this review is ‘reviewing the importance of GR α expression’ with regards to GC resistance.

GC resistance is highly complex and multi-faceted and has been extensively identified and studied in health and disease⁴². Broadly speaking, GC resistance may be divided into two major groups: generalized or acquired GC resistance^{4,42}. Importantly generalized GC resistance is also referred to as systemic or primary resistance, whilst acquired GC resistance is also known as localized or secondary resistance. However, in this review we will only make use of the terms generalized and acquired GC resistance. Essentially, these two groups of GC resistance are distinctively different in terms of their occurrence within a biological system with the latter form affecting distinct tissues and/or cells and not present throughout the organism (or patient)⁴². However, with that said, central to both of these two types of GC

resistance is dysfunction of the GR α ‘functional pool’. In the next section we provide a short synopsis of generalized GC resistance whilst honing in on acquired GC resistance and the importance of GR α mRNA and protein expression in the development of this acquired GC insensitivity.

2.2.1. Generalized GC resistance

In terms of generalized GC resistance, two severe hereditary/familial conditions, termed Primary Generalised Glucocorticoid Sensitivity (PGGS) and Primary Generalized Glucocorticoid Resistance (PGGR), also at times referred to as Chrousos Syndrome and characterized by rare hereditary pathological point mutations of the GR gene²⁵ and others (e.g. ER22/23EK polymorphism)⁴², represent the extremities of GC responsiveness (i.e. hypersensitivity and hyposensitivity). These inactivating mutations lead to perturbations in the GR ‘functional pool’ by mainly altering receptor function (e.g. reduced transactivational capabilities, DNA-binding, ligand-binding affinity and abnormal nuclear translocation) but in some cases also GR α mRNA and/or protein expression^{4,25,42,43}. The effect of these mutations is considered generalized as it occurs throughout the biological system (i.e. in every cell)^{4,42}. In addition to these hereditary mutations, a number of acquired gene polymorphisms in the GR gene and additional genes (e.g. ER22/23EK polymorphism) are known to lead to GR α deregulation at the level of GR α activity, however these should not be confused with acquired GC resistance, addressed in the next section, as these acquired gene mutations still elicit a generalized effect, as in the case of the hereditary mutations⁴². With this generalized form of GC resistance falling beyond the scope of the current review, we advise reviews by Quax *et al.*⁴², Vandevyver *et al.*⁴⁴, Nicolaidis *et al.*^{4,25}, Charmandari *et al.*⁴⁵ and Beck *et al.*⁴³.

2.2.2. Acquired GC resistance

Unlike generalized GC resistance, localized, acquired GC resistance is often restricted to a specific tissue or cell type (i.e. immune cells), rendering these peripheral tissues/cells insensitive to circulating GCs (endogenous and/or exogenous) over time^{26,27,42}. Moreover, this form of GC resistance is significantly more common in the general population and has been linked to a number of psychological and pathological conditions/diseases (Table 1). An apt description for this form of GC resistance is a “consequence of a pathophysiological process”¹⁷, however, what this description excludes is the development of acquired GC resistance following prolonged GC treatment (Table 2). Acquired GC resistance has been identified and defined in a number of diseases/conditions and often develops after a period of

acceptable GC response (i.e. at the start of the therapeutic regime), posing a significant challenge for the clinical use of GCs^{27,42}.

2.2.3. Current diagnostic approaches for determining GC resistance

GC resistant patients often require higher GC doses for prolonged periods of time in order to efficiently combat chronic inflammation in a number of psychological and physical conditions, which likely leads to the generation of adverse side-effects (e.g. osteoporosis and hyperglycemia) and may compound GC insensitivity³⁷. With this in mind it is of utmost importance for practitioners to be able to evaluate the GC-responsiveness of individualized patients before devising a therapeutic regime; this will allow for GCs to be used selectively and permit personalized GC treatment, depending on patient sensitivity, resulting in the optimal therapeutic outcome of GC treatment¹⁹. Currently, a range of endocrine and biochemical methods are employed to determine GC insensitivity in patients or *in vitro*, respectively, however, at present no single, standardized method for determining patient sensitivity to GC treatment exists¹⁹.

In terms of systemic or generalized GC resistance, patients display clinical heterogeneity ranging from asymptomatic to mild and severe symptoms (e.g. hypertension, hirsutism, hyperandrogenism, fatigue and hypokalemia), eloquently reviewed by Nicolaidis *et al.*²⁵. Thus, initially, an in depth analysis into the family and personal history of the patient must be conducted in order to detect any history of clinical manifestations of GC resistance^{4,25}. Thereafter, concentrations of the endogenous GC, cortisol (F), should be monitored using protocols such as the cortisol awakening rise/response (CAR)⁴⁶ or the 24-hour urinary free cortisol (UFC)²⁵, as examples. Following which, a dexamethasone (Dex) suppression test (DST) or the more recent Dex/CRH suppression test is conducted to test the responsiveness of the central HPA axis, which is regulated by a GC-mediated negative feedback loop^{26,42}. Lastly, specific gene sequencing to detect possible mutations in the GR gene can be used to confirm the diagnosis of generalized GC resistance in a patient^{4,25}.

Localized or tissue-specific resistance is more difficult to diagnose than systemic or generalized resistance, which generally displays a 'clinical picture' of GC resistance^{4,25}. In general, patients are mostly asymptomatic and thus a range of in depth biochemical diagnostic approaches are required to determine the GC-responsiveness of specific tissue and/or cells^{19,26,47}.

Examples of these include:

1. Investigating GC-mediated inhibition of cell proliferation using various assays such as the BrdU incorporation lymphocyte steroid sensitivity assay (BLISS)¹⁹, a specific prognostic model measured by the Lille score⁴⁸, chemiluminescence and flow cytometry⁴⁹.
2. Measuring the responsiveness of GC mediated genes (e.g. interleukin 8 (IL-8), interleukin 17 (IL-17), matrix metalloproteinase-1 (MMP) or dual specificity protein phosphatase 1 (DUSP-1))⁵⁰⁻⁵²
3. The analysis of GR α function (e.g. ligand binding and nuclear translocation)
4. Lastly, and importantly for the current review, the mRNA and protein expression of the GR α receptor.

A wealth of data has been collected demonstrating differences in GR α mRNA and/or protein expression between steroid responders and non-responders in both normal versus disease states, as well as within disease states ‘with’ or ‘without’ GC therapy (Table 1 and 2). However, an increasing demand for more sensitive and specific GC response tests remains, to be able to quantify/identify individual patient GC insensitivity thus avoiding unnecessary high-doses of GCs and the afore mentioned consequences²⁷.

2.3. GR α expression in health and disease

In many, but certainly not all stress-related, psychological and pathological conditions, reductions in the expression (i.e. mRNA and/or protein) of GR α have been noted, relative to ‘normal’ or healthy patients^{40,42} (Table 2.1). These disease-associated reductions, of up to 80%, in the GR α ‘functional pool’ often produce GC-resistant forms within disease groups, which are exceptionally challenging to manage clinically^{40,42,53}. In addition to the disease-associated reductions in receptor mRNA and/or protein expression (i.e. generally mediated via endogenous GCs), treatment (i.e. with exogenous synthetic GCs) associated reductions in the GR α ‘functional pool’ are well documented (Table 2.2). Important to note is that it is often difficult to distinguish between ligand-induced GR α down-regulation as a pathological consequence of disease progression and exogenous treatment induced receptor turnover, as with some patients withholding GC treatment would not be ethical⁵⁴. Furthermore, many diseased GC resistant patients require higher GC doses for longer periods of time to elicit sufficient anti-inflammatory effects, which generally has been shown to compound the development of GC resistance, through further reducing the GR α ‘functional pool’, which is

neatly demonstrated by Andreae *et al.*⁵⁵. In this section, ligand-induced reductions in GR α expression at the mRNA or protein level, which are driven by stress or pathological or psychological conditions (Table 2.1) and/or by exogenous GC use as therapeutics (Table 2.2), are specifically reviewed.

2.3.1. Disease-associated reductions in GR α expression

2.3.1.1. Stress

There is a wealth of evidence implicating environmental effects such as physical, psychological or pre/post-natal stress, in the development of acquired GC resistance through modulation of GR α mRNA and/or protein expression, which has implications for the treatment of a number of stress-related disorders and potentially a number of chronic psychological or pathological conditions⁵⁶. Importantly, the modulation of GR α levels, induced by various stressors, is fundamentally dependent on the duration of the stressor, the environment in which the stress occurs, and lastly the individual's sensitivity to stress⁵⁷⁻⁶³. Generally speaking, stress leads to significant increases in circulating endogenous GCs, which subsequently modulates GR α expression in peripheral tissues or cells⁶⁴. Moreover, stress-induced, GC-mediated GR α down-regulation is tissue-specific⁵⁷⁻⁶². Whilst it is thought to be an adaptive mechanism employed by the cell to protect against the damaging effects of unrelenting stress, this reduction in GR α expression and GC response poses major challenges for the therapeutic use of GCs⁶⁴. In the next section we provide examples of these stress-induced effects on GR α mRNA and protein expression, honing in on cases where receptor expression is reduced (Table 2.1).

2.3.1.1.1. Pre/post-natal stress

Early life stress (ELS), which includes pre- and post-natal stress, may alter HPA axis signalling (reviewed by Van Bodegom *et al.*⁶⁵), which has prolonged consequences on GR α mRNA and/or protein expression, GC sensitivity and susceptibility to psychological conditions (i.e. depression) in adulthood, in rodents and humans⁶⁶⁻⁷⁵. A number of studies, which investigate post- and pre-natal stress (i.e. using maternal separation (MS) and preconception paternal stress (PPS) as ELS stressors) have demonstrated the downstream effects of ELS on GR α mRNA and/or protein expression⁷⁶⁻⁸¹.

Firstly, in terms of post-natal stress in humans, a convincing amount of evidence exists associating the effects of childhood trauma (i.e. physical abuse or adoption) with the

methylation status of the GR α promoter^{82–84}, however, only a handful of human studies, on receptor mRNA expression have been conducted^{71–73}. Specifically, in adult suicide completers, with a history of severe childhood abuse, GR α mRNA expression is drastically reduced in hippocampal regions of the brain, relative to controls^{71,72}. Moreover, this reduction in receptor mRNA expression was thought to be a consequence of childhood abuse mediated alterations in the HPA signalling pathway, and consequent increases in circulating GCs^{71,72}. Additionally a study by Perroud *et al.*⁷³, demonstrated pre-natal (i.e. rather than post-natal) stress in humans, such as maternal stress, led to transgenerational transmission of the maternal stress to offspring, in the form of biological alterations in HPA signalling, from a cohort of women who were exposed to the Tutsi genocide during pregnancy. Interestingly, rather than increases in GC concentrations, mothers and their children had reduced levels of the circulating endogenous GC, F, despite displaying significant reductions in GR α mRNA expression⁷³, suggesting that a direct link between an increase in GC concentration and reductions in receptor expression, should always be made with caution.

In terms of rodent post-natal stress models, a recent study by Woo *et al.*⁷⁶ demonstrated a decrease in GR α mRNA expression in rats as a direct consequence of MS as infants. This was supported by additional studies in which reductions in receptor mRNA expression in the frontal cortex and hippocampus of adult and adolescent rodents was noted, following ELS in the form of infant MS^{75,77}. Additionally, MS has been shown to decrease GR α mRNA expression in the dorsal medial prefrontal cortex and amygdala in rats^{70,78}, which is likely to have implications in lifelong maladaptive behaviour.

Similarly to post-natal stress, pre-natal stress in rodents, both maternal and paternal, is known to drive changes in GR α mRNA and/or protein expression in offspring^{79,80}. More specifically, in offspring of PPS rats (i.e. paternally stressed pups) a decrease in receptor mRNA expression in the hippocampi of these pups was documented relative to non-paternally stressed pups, with the pups displaying an increase in anxiety-like behaviour⁷⁹. Additionally, in a study by Mueller *et al.*⁸⁰, maternal stress was shown to increase the endogenous GC, corticosterone, in mice and reduce GR α protein expression in the dentate gyrus of offspring. This is supported by Bingham *et al.*⁷⁴ where exogenous prenatal corticosterone exposure, used to mimic the prenatal stress-induced increase in circulating endogenous GCs, reduced receptor protein expression in the medial prefrontal cortex, hypothalamus and hippocampus of adult offspring. Lastly, a recent study by Lan *et al.*⁸¹ demonstrated that pre-natal stress leads to reductions in GR α protein expression in the limbic region in fetuses.

2.3.1.1.2. Physical or psychological stress:

A large number of studies in rodent models, using various methods of inducing stress, have also demonstrated stress-induced reductions in both GR α mRNA and protein expression, in various tissues and/or cells, relative to non-stressed control groups (Table 2.1). Specifically, in terms of GR α mRNA, earlier studies by Paskitti *et al.*⁸⁵ and Karandrea *et al.*⁶³ demonstrated that restraint stress led to significant reductions in receptor mRNA expression in brain regions (i.e. dentate gyrus, hippocampus and hypothalamus) of rats. In support of these findings are more recent studies, which have confirmed stress-induced GR α mRNA down-regulation in rats^{76,86–90}. Interestingly, results from a study by Witzmann *et al.*⁸⁶ showed that prolonged restraint and psychological stress induced increases in the circulating endogenous GC (i.e. corticosterone) in adult rats resulted in a decrease in GR α mRNA expression in peripheral leucocytes, however. In another study where forced swim stress (FSS) was used as the stressor, reduced receptor mRNA expression in the dentate gyrus of rats, was noted, and thought to be mediated by the stress-induced increase in endogenous GCs⁶⁴. Additionally, Jung *et al.*⁸⁷ demonstrated that repeat social defeat (RSD) led to decreased GR α mRNA expression in splenic macrophages, which was later correlated to diminished GC sensitivity in these cells. More recently, Makhathini *et al.*⁸⁸ noted that significant increases in corticosterone levels resulted in a consequent decrease in GR α mRNA expression in the hippocampi of rats, following repetitive restraint stress (RRS). In terms of GR α protein expression, exposure to repeated water-immersion and restraint stress (WIRS) increased GCs and decreased receptor protein expression in oligodendrocytes in the corpus callosum of rats, relative to non-stressed controls⁸⁹. Moreover, restraint stress led to GR α protein reductions in the prefrontal cortex⁹⁰ and hippocampi⁷⁶ of rats and was thought to be GC-mediated. Additionally, in RSD a reduction in GR α protein led to diminished GC sensitivity⁹¹.

A fairly well accepted notion is that certain chronic physical, psychological and/or pre-/post-natal stressors can, in addition to encouraging the development of GC resistance, significantly increase susceptibility to severe psychological or pathological conditions^{92,93}. An example in support of this notion is a recent study by Han *et al.*⁹² where stress-induced hypercortisolemia mediated a decrease in the GR α protein expression of hippocampi and the hypothalamus of mice, which subsequently increased their susceptibility to psychological disorders (e.g. depression), relative to control and resilient mice. Another example is provided by Li *et al.*⁹³, where psychosocial stress, in the form of social disruption, reduced GR α mRNA and protein

expression in the lung tissues of asthmatic mice (i.e. in a murine model of allergic asthma), thereby decreasing GC sensitivity and driving asthma exacerbation, relative to the control asthmatic group not exposed to psychosocial stress.

In conclusion, this section provided examples of a range of stress-related studies with indisputable evidence for the importance of GR α expression in mediating GC sensitivity and briefly highlights GC-mediated down-regulation as one of the likely mechanisms in which severe, prolonged stress may drive the progression of a range of psychological, pathological and auto-immune or inflammatory-linked disorders, further explored in the next section^{56,94}.

2.3.1.2. Psychological conditions:

In many psychological disorders, including depression and schizophrenia, phenotypic overlap exists with patients displaying common symptoms such as flat affect, anhedonia and other negative symptoms⁹⁵. Similarly, a large cohort of patients, but not all^{96,97}, suffering from psychological disorders/conditions display consistent biological findings⁹⁸⁻¹⁰³, namely an increase in inflammation and hyperactivity of the HPA, which drives hypercortisolemia (i.e. an increase in circulating endogenous GC, F) with consequences for GR α expression, either at the mRNA and/or protein level, in peripheral tissues¹⁰⁴. Whilst it must be noted that vast heterogeneity in GR α expression exists in patients with psychological conditions¹⁰³⁻¹⁰⁵, the current review focuses on conditions/disorders which have been specifically linked to decreases in GR α mRNA and/or protein expression (Table 2.1). Specifically, a number of studies have demonstrated a reduction in GR α mRNA expression in patients suffering from major depression (MD) in various tissues of the brain including the hippocampus, prefrontal, temporal and entorhinal cortex, as well as in peripheral blood mononuclear cells (PBMCs)^{95,106,107}. Moreover, these reductions in receptor mRNA expression were also noted for patients with schizophrenia and bipolar disorder⁹⁵. In addition, a number of studies have provided evidence regarding patients suffering from post-traumatic stress disorder (PTSD) having reduced GR α mRNA expression, relative to healthy controls, in peripheral cells such as lymphocytes and PBMCs¹⁰⁸⁻¹¹¹. Interestingly, this reduction in receptor expression in peripheral PBMCs was found to be cumulative in PTSD patients with an increased trauma burden¹¹¹. Furthermore, in patients suffering from generalized-anxiety disorder (GAD), a negative correlation was made between circulating GC concentrations and GR α mRNA expression, which was subsequently shown to result in diminished GC sensitivity⁵³. Taken together, these studies highlight the importance of receptor expression in the pathophysiology

of various psychological disorders by providing examples of cases in which reduced GR α expression is noted, with implication in GC resistance. For more information on GC resistance in stress-induced psychopathologies, a review by Merkulov *et al.*⁴⁰, is recommended.

2.3.1.3. Pathological conditions

2.3.1.3.1. Auto-immune or inflammatory-linked conditions

It would appear that the existing literature detailing the disease-associated effects on GR α expression in a number of auto-immune and inflammatory conditions is somewhat contradictory, with differential effects on GR α mRNA and/or protein expression noted, relative to their normal or healthy counterparts. In addition, it seems considerably more difficult to tease apart whether modulations in receptor expression are a pathological consequence of the auto-immune disease (i.e. as in the case of many psychological disorders, often stress related) or as a result of prolonged GC treatment, which many of these patients require. Nevertheless, in this section we have highlighted cases in which reduced GR α mRNA and/or protein expression is noted in auto-immune or inflammatory-linked conditions, attempting to limit it to cases in which patients were not receiving treatment (Table 2.1).

Firstly, in atopic dermatitis (AD), a condition linked to inflammation of the skin, significant reductions in GR α mRNA expression in systemic cells (i.e. PBMCs) of AD sufferers, relative to healthy controls, have been reported by Inui *et al.*¹¹². Moreover, in the same study, reductions in receptor expression were correlated to an increase in resistance to both topical GC treatment and the systemic administration of a potent GC¹¹². To our knowledge, it would seem that this is the only study in which GR α mRNA expression and GC response have been comprehensively studied in AD¹¹². The importance of GR α mRNA expression in the auto-immune disease systemic lupus erythematosus (SLE), also commonly referred to as lupus, for which inflammation of the skin predominantly on the face is a common symptom, has also been documented and is reviewed by Bazso *et al.*¹¹³ with disease-associated reductions in GR α mRNA expression reported by more than one study^{114–117}. Specifically, a reduction in receptor mRNA expression has been reported in PBMCs^{114–116}. Additionally, a study by Guan *et al.*¹¹⁷ demonstrated that GR α mRNA expression was higher in sensitive patients before receiving GC treatment, suggesting that reductions in receptor mRNA expression, in this case, were likely to be disease-associated. In addition, in peripheral tissues and cells (i.e. leucocytes) of patients suffering from a resistant form of another auto-immune disease,

namely inflammatory bowel disease (IBD), the disease-associated effects on GR α mRNA expression were either absent (i.e. no change in receptor mRNA expression)¹¹⁸ or led to reductions in receptor mRNA expression¹¹⁹, relative to healthy controls. Thus this may suggest that GR α expression is not the primary determinant for GC sensitivity in IBD, reviewed by Creed *et al.*¹²⁰, however, a partial role for receptor mRNA and/or protein expression may exist. Lastly, in adult immune thrombocytopenia (ITP), a condition driven by an auto-immune response, reduced GR α mRNA and protein expression was correlated with GC resistance¹²¹.

In terms of asthma, the majority of patients demonstrate a good therapeutic response to GC therapy, however, there are two known forms of asthma which are steroid resistant (i.e. Type I and II), reviewed in Luhadia *et al.*¹²², which have been linked to defects in GR α function and expression^{123–125}. Type 1 has an increased GR β /GR α cellular ratio, due to low GR α protein expression compounded by an increase in the GR isoform, GR β , and has been linked to GC resistance¹²⁶, whilst Type II is linked to disease-associated reduction in only GR α protein expression, relative to normal controls (i.e. non-asthmatic patients)¹²⁷. With that said, it must be highlighted that an increase in GR α mRNA expression has also been detected in the PBMCs of asthmatic patients relative to healthy controls¹²⁸. These studies suggest that the amount of GR α present in peripheral tissues/cells, be it in part, plays some role in the responsiveness of asthma patients to therapeutic GCs (e.g. inhaled corticosteroids), which function to reduce asthmatic exacerbations and restrict symptoms associated with this inflammatory-based lung disease¹²². In another lung condition, COPD, studies have demonstrated reductions in GR α mRNA¹²⁹ and protein expression¹³⁰ in the lung tissue of COPD patients, relative to the lung tissue from healthy controls, which has been linked to GC resistance. Moreover, Hodge *et al.*¹³¹ showed that lymphocyte senescence in COPD patients is due to a decrease in GR α expression and that this loss is associated with an increase in disease severity.

In the two most common forms of arthritis, osteoarthritis and rheumatoid arthritis (RA), contradictory results have been documented for disease-associated effects on GR α expression. Interestingly, one study found that GR α mRNA expression decreases in RA patients receiving GC treatment but increases in untreated RA patients¹³² with another study showing a slight decrease in GR α protein expression in steroid-resistant RA¹³³. This is consistent with a decrease in GR α protein expression in the lymphocytes of RA patients, however, this decrease was not correlated to diminished GC sensitivity¹³⁴. In contrast, in

osteoarthritic (OA) human articular chondrocytes the reductions in both GR α protein and mRNA expression noted, relative to healthy controls, did result in reduced GC-responsiveness¹³⁵.

Lastly, in a rodent model, T-cells obtained from mice with experimental autoimmune encephalomyelitis (EAE), have reduced GR α mRNA expression, which was linked to diminished GC sensitivity in terms of GC-resistant apoptosis¹³⁶.

In summary, it is clear that reductions in GR α mRNA and/protein expression are noted in a number of auto-immune diseases or inflammatory-linked conditions, which, in many cases, results in GC resistant forms of these diseases (e.g. GC resistant ITP, asthma and COPD). This diminished GC sensitivity, in a number of these conditions, poses a significant therapeutic challenge for the use of GCs in the treatment of these conditions.

2.3.1.3.2. Cancer

GCs are a primary therapeutic choice in cancer for either their pro-apoptotic effects or their use as an adjuvant therapy, in combination with chemotherapeutic agents, to reduce symptoms such as inflammation, allergic reactions, pain and nausea, which may also be caused by the tumour itself¹³⁷. However, both the type of cancer cell as well as the GR α content of certain cancer cells is thought to play a significant role in mediating the response to GC treatment¹³⁷. It is fairly well documented that high GR α expression is associated with a good response to GC treatment in lung cancer¹³⁷⁻¹⁴². On the other hand, drastic reductions in GR α mRNA and/or protein expression, thought to be a pathological consequence of the tumorigenic process, in part, may lead to GC insensitivity. Specifically, a number of authors have detailed that a reduction in receptor expression negatively correlated to GC response¹³⁷⁻¹⁴²; here we provide a number of examples in which this correlation holds true (Table 2.1). In ALL, the leading cause of cancer related deaths in the children, reduced GR α protein expression has been associated with GC resistant forms of ALL¹⁴³⁻¹⁴⁷. In addition in multiple myeloma (MM), reductions in receptor mRNA, and in some cases protein expression^{148,149}, have been noted, leading to diminished GC sensitivity in terms of GC-mediated transactivation and apoptosis, rendering some MM cell lines GC resistant¹⁵⁰⁻¹⁵². Furthermore, a number of lung cancers (i.e. small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)) contain very little GR α content, both at the mRNA and protein level, and also display reduced GC sensitivity in terms of apoptosis¹³⁸⁻¹⁴². Similarly, Nasset *et al.*¹⁵³ also demonstrated significant reductions in GR α mRNA expression in breast cancer tissues

relative to normal breast tissue, which supported an earlier study by Lien *et al.*¹⁵⁴, where up to a 50% reduction in receptor mRNA expression in breast cancer tissues, was observed. Finally, in an early study by Vanderbilt *et al.*¹⁵⁵, differences in GR α mRNA and protein expression were shown to differentially modulate the GC response in a rat hepatoma cell line. In summary, these studies provide a strong case for the requirement of an adequate GR α ‘functional pool’ to mediate the indispensable pro-apoptotic and symptom-reducing effects of GCs in a number of malignancies.

2.3.1.3.3. Infection and other conditions

Additional cases of disease-associated reductions in GR α mRNA and/or protein expression have been documented in conditions such as sepsis, nephrotic syndrome (NS), keloid disease and stroke^{156–160}. Specifically, in septic patients reductions in receptor mRNA or protein expression, relative to healthy controls, was noted in neutrophils¹⁵⁷ and T-cells¹⁵⁶, respectively. Moreover, these reductions in receptor expression were negatively correlated to GC-responsive gene expression¹⁵⁷, an indication of an altered GC sensitivity. Although these studies on sepsis provide evidence for a link between disease-severity and the expression of GR α it must be noted that reports of this association are highly variable and thus for an in depth review on GR α expression and sepsis pathology a review by Dendoncker *et al.*¹⁷, is recommended. In terms of NS in children, the GR α protein expression was assessed before exogenous GC treatment in two patient groups, namely steroid-sensitive (SSNS) and those who had initial steroid resistance (SRNS)¹⁵⁸. Patients who were considered resistant before the start of GC therapy were reported to have reductions in GR α protein expression in PBMCs, which Hammad *et al.*¹⁵⁸ postulated may be one of the pathophysiological mechanisms of acquired GC resistance in these children. As with NS, patients with a skin condition known as keloid disease can be separated into two groups, namely non-responders (nRPs) or responders (RPs)¹⁵⁹. Before receiving GC therapy the nRPs were shown to have reduced GR α expression at both the mRNA and protein level in tissue isolated from keloid scars, which was associated with decreased GC sensitivity following treatment¹⁵⁹. Lastly, in an *in vitro* model for hypoxia (used to mimic stroke events), murine brain micro-vascular endothelial cells were exposed to O₂/glucose deprivation that resulted in significant reductions in GR α protein expression relative to normoxic (i.e. normal) cells, which was proposed to be the cause of a decrease in subsequent GC sensitivity¹⁶⁰.

To summarize, it is clear that disease-associated reductions in the GR α mRNA and/or protein expression are apparent in pathological conditions, including those that disrupt the function of vital organs, such as the kidneys and heart, as well as the skin. Moreover, these reductions in receptor expression are often associated with GC insensitivity.

2.3.2. Treatment-associated reductions in GR α expression mediated by exogenous GCs

Cases in which reductions in the GR α 'functional pool' as a pathological consequence of the progression of the disease/condition relative to healthy controls are often associated with increasing concentrations of endogenous GCs (i.e. F or corticosterone). However, the effects of treatment with exogenous GCs (e.g. Dex, triamcinolone acetonide (TA), hydrocortisone and various prednisolone-based steroids, budesonide and fluticasone propionate), used as mainstay therapeutics to combat inflammation in many of these diseases/conditions, on GR α expression have not yet been addressed and are reviewed in this section, with a focus on comparing receptor expression 'with GC treatment' to 'without GC treatment'. Important to note is that it is often difficult to discriminate between disease- and treatment induced reductions in receptor expression due to an inability to withhold treatment from patients with chronic inflammatory disorders⁵⁴. Nonetheless, using cellular models containing endogenous or transiently transfected GR α , a number of studies have demonstrated dose- and time-dependent effects of exogenous GCs (i.e. with GC treatment) on GR α mRNA and protein turnover, relative to untreated cells (i.e. without GC treatment), additionally, these studies have been supported by various *ex vivo* and *in vivo* experiments in which the effects of prolonged, chronic exogenous GC treatment on receptor expression were investigated. In Table 2.2, we provide examples of, specifically, reductions in GR α expression as a result of exogenous GC treatment.

Specifically, an early study by Rosewicz *et al.*¹⁶¹ demonstrated dose- and time dependent reductions (to approximately 50% relative to control in GR α mRNA expression by the synthetic GC, Dex, in human IM-9 lymphocytes and rat pancreatic acinar (AR42J) cells. This finding was supported by other studies, which confirmed the ability of various concentrations of Dex (10^{-10} to 10^{-6} M) to mediate receptor turnover at the mRNA level, and additionally, at the level of protein expression, in hepatoma tissue culture cells (HTC), HeLa and COS-1 cells at a specific time point (i.e. 24 hours¹⁶²) or over a treatment period of up to 72 hours¹⁶²⁻¹⁶⁹. Hoeck *et al.*¹⁶⁷ also detected a Dex-induced decrease of GR α expression at both the level of

mRNA and protein in NIH 3T3 cells with up to a 75% and 80% reduction in receptor mRNA and protein expression, respectively. Moreover, pulse-chase experiments, in the same study, revealed a decrease in GR α half-life from 8 hours to 3 hours following hormone treatment. Furthermore, another study using Chinese Hamster ovary-derived (CHO) cells stably transfected with GR α (MG/hGR) showed a rapid Dex-mediated (5 nM) time-dependent reduction GR α protein expression to 50% of control, within 5 hours of treatment¹⁶⁹. Moreover, even after 4 weeks of Dex treatment this decrease in receptor protein persisted and reached a maximal reduction in receptor protein levels of 70%¹⁶⁹. In terms of GR α mRNA expression, a 50% reduction, relative to control, was noted within 2 hours of treatment that was then followed by a rise in receptor mRNA expression and subsequent decline after 12 hours treatment with Dex¹⁶⁹. It was postulated that this ‘biphasic pattern’ observed at the GR α mRNA level may be due to the involvement of ligand-induced transcriptional, post-transcriptional and translation mechanisms in mediating receptor mRNA expression, which was not reflected at the protein level¹⁶⁹. Interestingly, a study by our own research group revealed a Dex-mediated decrease in GR α protein expression, but not mRNA, in mouse BWTG3 cells after 24 hours treatment, relative to an untreated control¹⁷⁰. This is supported by Kleinschnitz *et al.*¹⁶⁰, where no change in GR α expression was noted, however, a reduction in GR α protein to 39% was noted following Dex treatment, relative to the untreated control, in an *in vitro* model, namely the immortalized mouse brain capillary endothelial (cEND) cell line. In a recent study by Ramamoorthy *et al.*¹⁷¹ a 50 to 90% decrease in specifically nascent GR α mRNA was observed after Dex treatment in two human cell lines, namely the U2-OS and A459 cells. In line with this finding is an observed Dex-induced down-regulation of total GR α mRNA expression in human respiratory epithelial cells (BEAS-2B, A549, and primary nasal epithelial cells)¹⁷² and HeLa cells¹⁷³, where in the latter cell line a reduction to 57% of the control was observed, following prolonged treatment with Dex. Moreover, in a study using normal human liver (HL7702) cells, chronic Dex treatment down-regulated GR α expression at both the mRNA and protein level¹⁷⁴. These studies have detailed reductions in GR α expression at both the mRNA and protein level but did not address the relevance of these Dex-mediated reductions in receptor expression in terms of GC sensitivity. A study by Dekelbab *et al.*¹⁷⁵, however, suggests that GC sensitivity is compromised following chronic Dex treatment (i.e. over 72 hours), which results in GR α down-regulation in L6 muscle cells. Moreover, this study demonstrated rapid reductions in GR α protein expression following a mere 1 hour of treatment, however, no effect was noted at the level of GC-mediated transactivation at this early time point¹⁷⁵. Conversely, in a study

by Gossye *et al.*⁵² the significant reductions in GR α protein, following Dex treatment over 24 hours, were directly correlated to diminished GC sensitivity in fibroblast-like synoviocytes (FLS) cells. This was supported by a study by Li *et al.*⁵¹ that showed that prolonged Dex treatment (32 hours) resulted in reductions of GR α mRNA and protein in RAW 264.7 cells, which directly impacted the expression of GC-responsive genes. Sun *et al.*¹⁷⁶ also demonstrated significant reductions in the GR α protein expression of PBMCs, following 18 hours of Dex treatment, which resulted in a decrease in GC sensitivity, measured via GC-mediated apoptosis, highlighting the negative implications of long-term GC therapy in the development of GC resistance. Lastly, in a study in which prolonged Dex treatment of HeLa cells for 2 years led to a reduction in GR α mRNA and protein expression to below detectable levels, receptor overexpression by transfection of a GR α plasmid following the period of prolonged Dex treatment, was unable to restore GC sensitivity in this system¹⁷⁷.

A number of studies using *ex vivo* and *in vivo* models mirror results of Dex-mediated reductions in receptor expression obtained in cell lines, outlined above. In a variety of mouse tissues (liver, kidney, lung and heart) and rat liver tissue, prolonged treatment with Dex (up to 8 days) led to significant reductions in GR α mRNA and protein expression^{51,52,54,163,171,178–180}, which in some cases was associated with diminished GC sensitivity^{51,52}. In another *ex vivo* rodent study, using cultured mouse podocytes, both short- (i.e. 1 hour) and long-term (i.e. 5 days) treatment with various concentrations of Dex (1, 10 and 100 μ M), administered to mimic constant oral vs. pulse intravenous GC treatments, led to reductions in GR α protein, but not mRNA, expression, which altered podocyte GC-responsive gene expression¹⁸⁰. Additionally, in an *in vivo* study, chronic Dex administration for 3 days resulted in reductions in GR α protein expression in rat hippocampal neurons¹⁷⁹. More evidence of the ability of prolonged Dex treatment (i.e. 7, 14, 21 and 28 days) to reduce receptor mRNA and protein expression in the frontal cortex and hippocampus of male mice is seen in an article by Hu *et al.*¹⁷⁸. Moreover, in a human study in which Dex was administered in a therapeutic regime, of a total of four doses (4 mg per oral dose) every 6 hours, steady reductions in GR α protein expression in normal lymphocytes was observed, with the lowest levels in receptor protein expression detected 30 hours post-treatment⁵⁴.

In addition to the use of the synthetic GC, Dex, a number of studies, summarized in Table 2.2, have demonstrated the effects of the prolonged use of other exogenous GCs (such as, TA, hydrocortisone, various prednisolone based GCs, fluticasone propionate and budesonide) on GR α mRNA and/or protein expression. Specifically, in an *in vitro* study conducted in a

murine fibroblast-like cell line (i.e. L929 cells), treatment with TA (1 μ M over 96 hours) decreased GR α mRNA and protein expression¹⁸¹. Furthermore, an *in vivo* study demonstrated a reduction in GR α protein expression in the livers of rats treated with hydrocortisone (5 mg/100g body weight) intraperitoneally for 6 hours, relative to rats that did not receive hormone treatment¹⁸². In addition, treatment with prednisolone in human-derived HeLa cells, containing endogenous GR α , led to the suppression of receptor mRNA expression to 67% of the untreated control (100%)¹⁷³. Consistent with this result, is GC-mediated reductions in GR α mRNA expression noted *in vivo*, in liver tissues, following exogenous methylprednisolone treatment¹⁸³. Additionally, in an *in vivo* human asthmatic model, which investigated the effects of high doses of methyl-prednisolone (i.e. 120mg/ day for 10 days) on receptor expression in human blood monocytes, noteworthy reductions in GR α mRNA expression, relative to untreated asthmatic patients, was shown¹⁸⁴. Furthermore, a study addressing reductions in GR α expression in steroid-treated renal transplant patients, relative to non-treated patients, established that high doses of various methyl-prednisolone based therapeutics caused a 50% reduction in GR α protein expression in lymphocyte subpopulations, whilst prolonged low-dose therapy caused only an 11% reduction¹⁸⁵. Consistent with the disease-associated effects on GR α expression, reported in Table 2.1, Andreae *et al.*⁵⁵ detailed reductions in receptor protein expression in PBMCs of patients with auto-immune disease ‘without treatment’, relative to healthy controls, additionally reporting even lower GR α protein expression in patients receiving GC treatment. Specifically, both low-dose oral GC treatment (i.e. 0.01–0.3 mg/kg orally) and high-dose intravenous pulse therapy (10–15 mg/kg) resulted in significant reductions in receptor protein expression, however, a more drastic reduction was observed following the high dose regimen⁵⁵, suggesting a dose-dependent effect of GC therapy on GR α expression, also reported by Sanden *et al.*¹⁸⁶. Although another study did not find the same disease-associated effects of asthma on GR α protein expression, a similar reduction in receptor expression was observed in asthmatic children treated with a short course of prednisolone therapy, relative to asthmatic children who did not receive GC treatment¹⁸⁷. In support of this finding is a study where systemic GR α protein expression (i.e. in PBMCs) in patients with IBD not receiving prednisolone therapy, was not significantly different to normal controls, however, corticosteroid-treated IBD patients displayed lower receptor expression, relative to normal controls and patients without treatment¹⁸⁸. In a recent study, GR α mRNA expression was investigated in PBMCs as a biomarker of GC resistance in Vogt-Koyanagi-Harada (VKH) disease, an inflammatory-disease that affects the eye¹⁸⁹. A portion of these patients are GC

resistant, however, they do not display reduced GR α expression relative to the GC sensitive patients, before receiving treatment¹⁸⁹. With that said, following treatment with prednisolone a 0.7-fold decrease in GR α mRNA expression was noted in resistant patients, which was absent in the sensitive patients. This reduction in receptor expression was correlated to clinical predictive factors of GC resistance for this disease, including tinnitus, fundus pigmentation and chronic disease. Lastly, local treatment of healthy individuals with two additional GCs, fluticasone propionate or budesonide, resulted in a significant, dose-dependent reduction in GR α mRNA expression in nasal mucosal biopsy specimens¹⁹⁰.

Briefly, to summarize this section on GR α expression in health and disease, it is evident that many inflammatory-driven and auto-immune diseases drive reductions in receptor expression, as a pathological consequence of disease progression often associated with an increase in endogenous GCs. Moreover these disease-associated alterations in the GR α ‘functional pool’ may lead to the development of an acquired resistance to GC treatment. Additionally, in a number of cases, the use of exogenous GCs as therapeutics, to curb inflammation and partially retard the advancement of these psychological and pathological conditions, led to further reductions in GR α expression, which may ultimately hasten the development of acquired GC resistance, currently a major threat in the pharmaceutical industry. With the incidence of these conditions increasing and the burden of resistance to GC treatment mounting, it is of utmost importance to fully understand the molecular mechanisms involved in ligand-induced GR α down-regulation. To date, a number of GC-mediated molecular mechanisms which regulate the GR α ‘functional pool’ have been identified and are reviewed in the following section.

2.3.3. GC-mediated molecular mechanisms involved in reducing GR α expression

The wealth of data demonstrating disease- and/or treatment associated reductions in GR α expression underscores the physiological importance of fine-tuning GR α expression and encourages an urgency to comprehensively elucidate the multiple molecular mechanisms, employed by the cell, to tightly regulate the expression or ‘functional pool’ of this receptor^{44,64}.

The regulation of the GR α ‘functional pool’ may be described using a simple ‘push’ versus ‘pull’ mechanism where, when in a dynamic state of equilibrium and unperturbed, the synthesis of GR α is roughly equivalent to receptor turnover, and GR α expression occurs at a constant rate. Important to note is that the ‘push’, used by the current review, is governed by

two processes namely transcription and translation and whilst the ‘pull’ is defined by proteasomal-degradation (Fig. 2.1), specifically via the ubiquitin-proteasome pathway (UPS). Important to note is that the UPS generally regulates the degradation of intracellular proteins, whilst the lysosomal degradation pathway primarily degrades extracellular proteins and some cell surface proteins¹⁹¹. One can assume that perturbations in the balance or dynamic state of GR α regulation (i.e. ‘push’ is balanced by the ‘pull’) will most likely be due to alterations in GR α mRNA and/or protein expression. One of the ways in which the harmony of this dynamic state may be perturbed is via an increase in circulating endogenous⁶⁴ (i.e. disease-associated) or exogenous (prolonged, chronic use of therapeutics) GCs, which subsequently induces GC-mediated or ligand-induced (terms used interchangeably) GR α down-regulation.

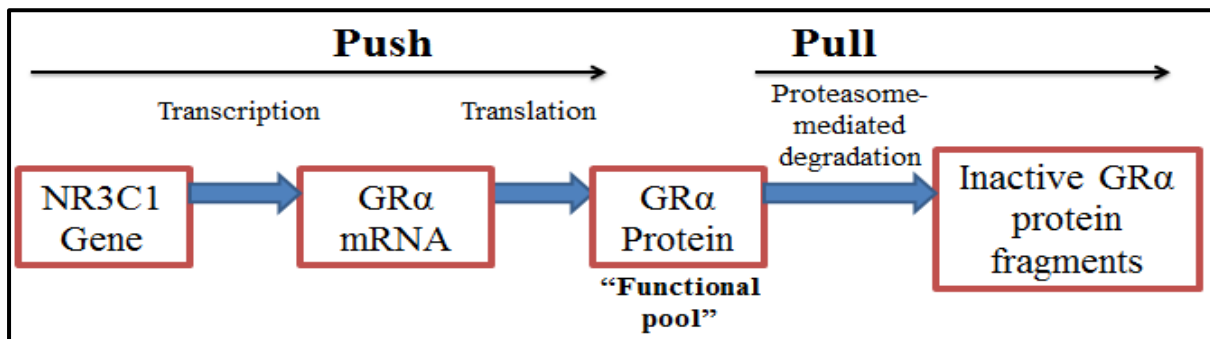


Figure 2.1: Specific regulation of the GR α protein ‘functional pool’, described by a simple ‘push’ vs. ‘pull’ mechanism.

Ligand-induced regulation of GR α expression is far from being straightforward but rather involves multiple layers of regulation at the epigenetic, transcriptional, post-transcriptional and post-translational levels^{14,42}. Moreover, at each ‘level’ of GR α regulation multiple molecular mechanisms are involved, which function in a highly specific manner to stabilize or, importantly for the current review, destabilize the receptor, thus contributing to the complexity of the finely-tuned GC/GR α signalling pathway and potentially advancing acquired GC resistance. In this section, we review the molecular mechanisms specifically known to reduce GR α mRNA and protein expression as a direct result of ligand-binding (summarized in Table 2.2). However, it must be noted, that various indirect or ligand-independent mechanisms^{4,14,42,44} (e.g. pro-inflammatory cytokines and the tissue-specific expression of additional GR isoforms as a result of alternative splicing) are also known to regulate GR α expression. For details on these ligand-independent mechanisms of GR α regulation, reviews by Vandevyver *et al.*⁴⁴ and Quax *et al.*⁴² are recommended.

2.3.3.1. GR α mRNA regulation

2.3.3.1.1. Epigenetic regulation

In terms of epigenetic regulation of GR α expression, DNA methylation¹⁹² of the GR α (NCR31) promoter³¹ has been identified as one of the major mechanisms involved in disease-associated acquired GC resistance^{53,64,71,72,77,79,80,140,153,193}, across species, and is often positively-correlated with an increase in circulating GC concentrations⁶⁴. In contrast to the positive-correlation with GC concentration, a ligand-induced increase in DNA methylation of the GR α promoter is generally negatively-correlated with GR α mRNA expression in both humans and mice, which is summarized in Table 2.3.

Generally speaking, extensive evidence exists implicating an increase in NCR31 DNA methylation in a consequent decrease in GR α mRNA expression⁷⁷ and possibly a corresponding reduction in GR α protein expression⁷⁹. A specific exonic sequence in the rat GR α gene was identified as a region that underwent substantial DNA methylation, following stressful events¹⁹². Specifically, increased DNA methylation at the exon 1₇ promoter, within the GR α gene, was shown to mediate a reduction in receptor mRNA expression in Wistar rats⁷⁷. Similarly, a study by Niknazar *et al.*⁷⁹ demonstrated a GC-mediated increase in the methylation status of the same exon (i.e. exon 1₇) in the GR α gene, which led to a decrease in the GR α mRNA expression, in adult male and female Wistar rats. Additionally, a GC-mediated increase in the methylation of exon 1₇ in the mouse GR α promoter region (–578 to –490 base pairs), resulted in a reduction in GR α protein expression⁸⁰. Lastly, following acute stress, an increase in DNA methylation at the exon 1₇ promoter was observed, as well as an up to 75% reduction in GR α mRNA expression in dentate gyrus neurons of male Wistar rats⁶⁴.

In support of these findings from the rat studies, are a number of human studies in which DNA methylation of the GR α gene, specifically at exon 1_F, exon 1_B, exon 1_H and exon 1_C in humans, has been shown to result in reduced GR α mRNA expression^{53,71,72,140,153}. Specifically, Mc Gowan *et al.*⁷² noted an increase in DNA methylation of the exon 1_F promoter in the GR α gene and consequent reductions in GR α mRNA expression, in the hippocampal regions of victims with a history of abuse. Similarly, a recent study by Wang *et al.*⁵³ detailed an increase in F in patients with GAD that resulted in DNA methylation of the exon 1_F promoter in the GR α gene, which led to reductions in receptor mRNA expression as well as diminished GC sensitivity. Additionally, Nessel *et al.*¹⁵³ investigated DNA

methylation of the GR α gene in breast cancer tissue, relative to normal tissue, and found an increase in the methylation status of the exon 1_B promoter, which correlated with a decrease in GR α mRNA expression in the cancerous tissue. Furthermore, Kay *et al.*¹⁴⁰ demonstrated an increase in DNA methylation at another site in the promoter, exon 1_C, where a 6% increase in GR α methylation, significantly reduced receptor protein expression by up to 50%. Lastly, changes in the methylation status of the exon 1_C and exon 1_H promoter of the GR α gene, have been shown to modulate GR α mRNA expression in the hippocampi of suicide completers⁷¹.

It must be noted that in some peripheral tissues and cells, a decrease in the differential methylation of the GR α promoter and a corresponding decrease in GR α mRNA expression has also been observed as reviewed by Tyrkstra *et al.*⁹⁷. However, although this finding highlights a level of complexity surrounding regulation of the GR α gene by DNA methylation, the effect is currently not well documented and requires further investigation. Nonetheless, although some ambiguity remains surrounding the emerging association between ligand-induced GR α gene methylation and receptor expression, what is clear is that in a broad number of psychological and pathological conditions this epigenetic mechanism is likely to contribute, to a certain degree, to the development of tissue-specific resistance to GC treatment as a result of GC-mediated reductions in the GR α mRNA expression.

2.3.3.1.2. Transcriptional regulation

It is well documented that in addition to GC-mediated transrepression of pro-inflammatory genes (i.e. IL-6), mainly via an indirect tethering mechanism¹⁹⁴, GR α can also inhibit GC-responsive gene expression by directly binding to a negative glucocorticoid response element (nGRE) in the promoter¹⁹⁵. Recently, in support of earlier studies by Burnstein *et al.*^{162,196,197}, which postulated that the GR α gene contained sequences sufficient for ligand-induced GR α mRNA down-regulation, a study by Ramamoorthy *et al.*¹⁷¹ successfully identified the presence of an nGRE in the GR α promoter. Subsequently, the ability of the receptor to regulate its own expression by binding to this nGRE present in the NCR31 promoter, following GC treatment, was demonstrated¹⁷¹. Furthermore, inhibition of transcription initiation of the GR α gene was shown to be the primary mechanism of this GC-mediated auto-regulatory loop, resulting in GC-mediated reductions in nascent GR α mRNA expression by up to 90% in some cells (e.g. A459 lung carcinoma cells)¹⁷¹. Specifically, following binding of the receptor to the intragenic response element, this process is mediated by a long-range interaction between the GC-bound GR α at an nGRE present in exon 6 and a NCOR1

repression complex, which is assembled at the transcription start site of the gene¹⁷¹. Additionally, the ability of the GR α to regulate its own transcription in a GC-dependent manner, was found to be neither species nor tissue-specific, and was consistent across human, rat and mouse cell lines, as well as in mouse tissues¹⁷¹. Whilst this paper¹⁷¹ convincingly demonstrates the GC-mediated auto-regulatory loop occurring via an nGRE in the GR α gene promoter, it appears to be the only paper to do so. In terms of acquired/disease-associated glucocorticoid resistance, this constitutive repression of the GR α gene, via the GC-mediated auto-regulatory feedback loop, is likely to be compounded by disease-associated increases in circulating endogenous GCs and chronic, prolonged therapeutic GC regimes¹⁷¹. Thus it would be of interest to ascertain the biological activity of other GCs, such as selective glucocorticoid receptor modifiers (SGRMs) via this GR α nGRE.

2.3.3.1.3. Post-transcriptional regulation

Unlike transcriptional regulation of the GR α gene that leads to modulations in nascent receptor mRNA expression, post-transcriptional regulation involves the destabilization of mature receptor mRNA, via the presence of adenylate uridylate (AU)-rich elements present in the 3'-untranslated region (UTR) of the GR α mRNA, which may ultimately affect receptor protein expression, presenting another level of regulation for fine-tuning GR α expression^{27,44,198,199}. One of the ways in which this can occur is through the regulatory role of micro-RNAs (miRNAs), a family of small non-coding RNAs, which bind to AU-rich regions in the UTR of target mRNAs. Unlike small interference RNAs (siRNAs), which function to degrade mRNA transcripts, miRNAs primarily prevent efficient translation of mRNA transcripts, however, can also induce degradation of these transcripts²⁰⁰⁻²⁰³.

The ability of miRNAs to regulate GR α mRNA expression has been shown to be mediated by circulating GCs, both as a result of disease-associated or stress-induced increases in endogenous GC concentrations, as well as by treatment with exogenous GCs, with implications in acquired resistance to GCs^{144,150,156,204-207} (Table 2.3). To date, a number of miRNAs have been identified and shown to target GR α mRNA transcripts across a number of species (i.e. rodents and humans)⁴⁴. A review by Vandevyver *et al.*⁴⁴ discussed the majority, but not all⁸⁷, of the miRNA target sites in the human GR α mRNA transcript and is recommended for an overview of these sites.

In terms of GR α mRNA expression, a study by Riester *et al.*²⁰⁵ experimentally confirmed that four miRNAs, namely miR-96, miR-101a, miR-142-3p and miR-433, are able to reduce the

receptor mRNA expression by up to 40% in mice. Recently, RSD has been shown to increase the expression of two additional miRNAs, namely miR-29b and miR-340-5p, which was inversely correlated to GR α mRNA expression in splenic macrophages, of mice⁸⁷. Additionally, a recent study demonstrated that FSS resulted in the increase of miR-124a, in a time-dependent manner, which was associated with a reduction in GR α mRNA expression, in male Wistar rats⁶⁴.

Important to reiterate is that miRNAs act on GR α mRNA, primarily blocking translation, and thus this is considered mRNA regulation, however, the effects may only be reflected at the level of the receptor protein as demonstrated by a study in which restraint stress in rats, led to an increase in the expression of the ubiquitously expressed miR-18, which subsequently lead to a decrease in GR α protein but not mRNA expression, in the paraventricular nucleus of these rats²⁰⁶. This finding is supported by that of Vreugdenhill *et al.*²⁰⁷ where miR-18 reduced GR α protein expression by almost 40% in rat neuroscreen cells (NS1). Furthermore, Shimizu *et al.*⁸⁹ revealed that a stress-induced increase in miR-124a led to a significant reduction in GR α protein expression in the corpus callosum of mice.

Furthermore, in humans, a GC-mediated increase in miR-124 and consequent decrease in GR α mRNA and protein expression has also been demonstrated in ALL cells²⁰⁴ and in T-cells of sepsis patients¹⁵⁶. An investigation by Tessel *et al.*¹⁵⁰ detailed the effects of the less commonly studied miR-130b on GR α protein expression. Specifically, overexpression of miR-130b led to significant decreases in GR α protein expression, however, unexpectedly, knockdown of this miR-130b did not alter GR α protein expression, in human MM cell lines¹⁵⁰. Moreover, although these experiments were conducted in the presence of Dex, it is not clear whether GC's directly mediate the expression of miR-130b¹⁵⁰. Lastly, an increase in miR-142-3p expression has been noted in GC-resistant ALL patients¹⁴⁴. Furthermore, this miRNA has been shown to target GR α mRNA resulting in a decrease in receptor protein expression¹⁴⁴. Unfortunately, in many of these studies, it is unclear whether the up to 80% increase in miRNA expression⁸⁷ is directly mediated via a disease-associated or stress-induced increase in circulating GC concentrations, however, one could postulate that a positive correlation exists between the two (i.e. an increase in GC concentrations and an up-regulation of miRNA expression).

2.3.3.2. GR α protein regulation

2.3.3.2.1. Post-translational regulation

In addition to GC-mediated GR α mRNA regulation, the receptor is also subjected to GC-mediated protein regulation in the form of post-translational modifications (PTMs) (i.e. phosphorylation, ubiquitination and sumoylation)^{11,16,44,208–213}. The nature and degree of these PTMs modulates both GR α function and expression, impacting GC-responsiveness in selective tissues and in some cases may contribute to an acquired GC resistance^{11,16,44,165,166,208–215}. For the sake of this review we will hone in on the PTMs which directly assist in GR α protein turnover, via the proteasome, in a GC-dependent manner, and thus for an overview of the effects of PTMs on GR α function we encourage additional comprehensive reviews^{11,16,44,208–213}.

With reference to GR α , the most widely studied and first PTM identified was phosphorylation⁴⁴. Since the initial discovery, a number of GR α phosphorylation sites have been identified (Fig. 2.2). Although basal phosphorylation may occur in a ligand-independent manner^{214,215}, hyper-phosphorylation at several of these sites is GC-mediated^{214,215} and is known to modulate GR α function (e.g. receptor ligand- and DNA-binding affinity, subcellular localisation, co-regulator interaction and gene regulation) as well as expression⁴⁴. Moreover, various kinases (e.g. p38, ERK, JNK, CDKs and GSK3 β) responsible for the specific phosphorylation of these sites, have been described⁴⁴.

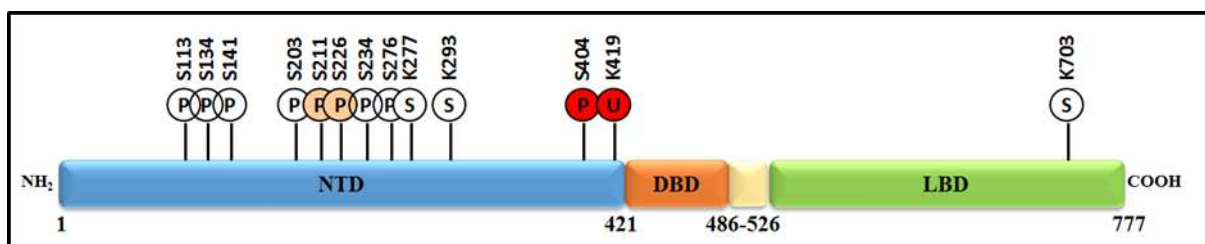


Figure 2.2: Post-translational modification sites of human GR α with focus pertaining to phosphorylation, ubiquitination and sumoylation, adapted from Vandevyver *et al.*⁴⁴. The human GR α protein consists of 777 amino acids (i.e. 1 to 777) and undergoes PTMS at numerous sites. Moreover, many of these PTM sites are contained within the N-terminal domain (NTD) (i.e. amino acids 1 to 421) of the receptor, with two present in close proximity of the DNA-binding domain (DBD) (i.e. amino acids 421 to 486). Specifically; phosphorylation (P) occurs at serine (e.g. S211, S226 and S404) residues, whilst ubiquitination (U) and sumoylation (S) occurs at lysine residues (i.e. K419 and K277, K293 and K703, respectively). Unlike the others, the K703 sumoylation site occurs within the ligand-binding domain (LBD) of the receptor (i.e. amino acids 526 to 777). Moreover, PTMs at these sites are known to modulate GR α function (white) or protein expression (red) and in some cases affect both receptor function and protein expression (pink).

Studies have demonstrated that GC-mediated GR α phosphorylation mediates receptor protein turnover, leading to reductions in GR α protein expression, and thus it is likely that the

phosphorylation status of GR α plays an important role in the effectiveness of GC treatments or overall GC sensitivity^{214,215}. A study by Webster *et al.*²¹⁶ demonstrated that multiple point mutations (i.e. at more than one site at a time such as Serine 212 to alanine, Serine 220 to alanine and Serine 234 to alanine) in the mouse GR α protein led to a loss of ligand-induced GR α protein turnover. Additionally, Avenant *et al.*²¹⁵, detailed the ability of GC-mediated hyper-phosphorylation at Ser211 and Ser226 to alter GR α half-life, in COS-1 cells transiently transfected with human GR α , using a panel of 12 ligands. Moreover, respectable correlations between ligand-selective phosphorylation and ligand-selective receptor turnover were made with potent synthetic GCs (i.e. Dex) inducing the greatest extent of receptor phosphorylation and thus considerably more GR α protein turnover²¹⁵. In another study by Galliher-Beckley *et al.*²¹⁴, a wealth of evidence was provided to support the notion that Dex-induced hyper-phosphorylation mediated by GSK3 β (i.e. the kinase responsible for phosphorylation at Ser404 for the human GR α and Ser412 for mouse GR α) at one of the more recently discovered phosphorylation sites, Ser404, enhances receptor turnover of the human GR α . Specifically, a mutant incapable of being phosphorylated at Ser404 and inhibition of phosphorylation at this site using a GSK3 β , the kinase responsible for phosphorylation at Ser404, kinase inhibitor (BIO) confirmed that restricting Dex-induced Ser404 hyper-phosphorylation of GR α resulted in an increase in receptor protein stability²¹⁴. Whilst considerable evidence exists for the effects of GR α phosphorylation on receptor function (i.e. nuclear translocation and transcriptional activity), reviewed by Anbalagan *et al.*²⁰⁸, Ramamoorthy *et al.*²¹⁷ and Vandevyver *et al.*⁴⁴, to our knowledge, it is only these few papers²¹⁴⁻²¹⁶, which directly demonstrate the ability of phosphorylation at Ser211, Ser226, Ser404 in human GR α ^{214,215} and at multiple sites (Ser212, Ser220 and Ser234) of the mouse GR α ²¹⁶, to impact receptor turnover.

GR α protein receptor turnover is proteasome-dependent and before successful delivery to the catalytic proteasome, the receptor requires tagging with a second PTM, namely ubiquitination, which occurs following phosphorylation^{165,166}. It was postulated that the inability of the GR α phospho-deficient mutants with mutations at multiple sites (i.e. Ser212, Ser220 and Ser234 of the mouse GR α ^{216,218}) to undergo degradation via the proteasome is due to inefficient phosphorylation-dependent ubiquitination, however, a lack of GR α ubiquitination was not demonstrated experimentally with these mutants. To date, only a single ubiquitination site for GR α has been identified and occurs within the PEST degradation motif at lysine 426 (K426) in mice and lysine 419 (K419) in humans. Mutations

at these specific residues restore GR α protein expression by restricting receptor protein turnover via the proteasome^{165,166}. A number of studies have demonstrated that that GC-mediated GR α protein turnover occurs via the proteasome, specifically, through the use of proteasome inhibitors (e.g. MG132, bortezomib, beta-lactone, and epoxomicin) GC-mediated down-regulation of the rodent and human GR α protein, was inhibited^{8,160,165,166,219–224}. However, unlike ubiquitination of some of the other nuclear receptors²²⁵, such as the estrogen (ER)²²⁶ and mineralocorticoid (MR)²²⁷, GR α ubiquitination is not as widely studied and only a handful of papers exist demonstrating GC-mediated GR α ubiquitination^{165,166,219,228}. Moreover, the notion that ubiquitination of GR α increases following GC treatment, seems to be controversial, with one paper demonstrating Dex-mediated increase in GR α ubiquitination²²⁸ and another research group noting a Dex-induced reduction, rather than an increase, in GR α ubiquitination in the presence of a MG132^{8,166}. It appears that the study of GC-mediated ubiquitination of GR α has, to some degree, been avoided, likely due to the difficulty of detection, however, it seems necessary for further research to be conducted in this specific area of GC/GR α signalling. The use of highly specific and sensitive assays, such as a Proximity-Ligation Assay (PLA), may provide more insight into GR α ubiquitination. Nevertheless, GR α ubiquitination and the ubiquitin-proteasome pathway/system have been shown to function cooperatively to control receptor degradation rates, ultimately contributing to the stringent regulation of overall GR α levels^{165,166}.

In addition to phosphorylation and ubiquitination a third PTM has been shown to regulate GR α function^{208,210,229–232} (i.e. inhibiting the transcriptional activity of the receptor) and, less frequently, promote receptor protein degradation in a GC-dependent manner²¹⁰. Similarly to ubiquitination, sumoylation is a dynamic, reversible process, which involves a multi-step, enzyme-catalysed reaction to mediate the covalent attachment of the SUMO protein to the protein of interest (i.e. GR α)²³³. Interestingly, unlike ubiquitin, there are a number of SUMO proteins, namely SUMO-1, SUMO-2/3. It is specifically the addition of SUMO-1, which is thought to drive GC-mediated protein turnover²¹⁰. Le Drean *et al.*²¹⁰ demonstrated that overexpression of SUMO-1 aids Dex-mediated receptor down-regulation, additionally implicating the proteasome in this degradation of the GR α protein, however, this paper does not detail at which site (i.e. lysine residue) this sumoylation occurs. To our knowledge, this is the only paper to describe the potential of sumoylation to regulate GR α protein expression.

Lastly, it is important to note there are a number of additional PTMS which the GR α protein is known to undergo, including acetylation, nitrosylation and oxidation, however, these are thought to modulate GR α function and, as of yet, no evidence exists for their ability to affect receptor protein stability, the focus of the current review. For this reason, reviews by Zhou *et al.*²¹¹, Duma *et al.*¹⁶, Nicolaides *et al.*²¹³, Vandevyver *et al.*⁴⁴, Anbalagan *et al.*²⁰⁸, Weikum *et al.*³⁵, Kadmiel *et al.*²¹² are recommended for more information on how these additional PTMS impact GR α activity.

2.3.3.3. Enzymes of the UPS that mediate GR α protein turnover

Importantly, proteasomal degradation of the substrate (i.e. GR α) requires rounds of ubiquitination, mediated by various enzymes of the UPS (Fig. 2.3), to form a poly-ubiquitin chain, which is then recognized by the proteasome resulting in substrate degradation. There are number of enzymes that form part of the UPS, which interact with the GR α protein (Fig. 2.3). These interactions are either ligand-independent or ligand-dependent, and function to regulate the expression or ‘functional pool’ of the receptor by mediating its turnover via the ubiquitin-dependent proteasomal degradation pathway^{165,166}. As regulators of GR α expression these co-regulator/GR α interactions have implications in GC sensitivity and thus we briefly review some examples. Importantly, GR α interacts with a wide range of additional co-factors proteins, however, many of these other interactions are associated with mediating GR α function rather than expression and will not be discussed^{14,234–241}.

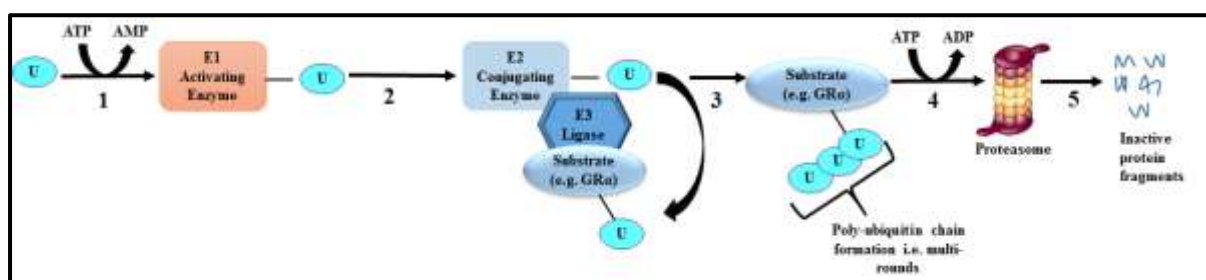


Figure 2.3: The ubiquitination of a substrate requires multiple rounds of a multi-step enzyme process before being targeted to the proteasome. 1. Ubiquitin (U) is activated by an activating enzyme (E1) in an energy (ATP) dependent manner. 2. The activated U molecule is then transferred to E2, a conjugating enzyme. 3. E3 binds the substrate and the E2 and the transfer of the activated U molecule from E2 to the substrate occurs. 4. This is repeated, until a poly-ubiquitinated chain is formed and the ubiquitinated substrate is then actively (i.e. ATP-dependent) delivered to the proteasome. 5. The catalytically active proteasome recognizes and degrades the substrate to produce inactive protein fragments.

The binding of two enzymes associated with the UPS, namely the inactive E2 conjugating enzyme, tumour susceptibility gene 101 (TSG101)²⁴² or the E3 ligase, carboxy-terminus of heat shock protein 70-interacting protein²⁴³ (CHIP), to the GR α protein does not require prior

ligand binding. Moreover, whilst binding of CHIP to GR α is unaffected by GC treatment²¹⁹, the formation of the TSG101/GR α complex only occurs in the absence of ligand-binding (i.e. to the unliganded GR α)²⁴⁴. Specifically, TSG101 binds to the N-terminal region of the hypo-phosphorylated unliganded receptor, both of which (i.e. unliganded GR α and TSG101) are located in the cytoplasm, and prevents protein turnover of the unliganded GR α , by acting as a dominant negative regulator of ubiquitination due to its catalytically inactive characteristic^{244,245}. Knockdown experiments in which TSG101 was targeted, demonstrated an increase in the protein instability of the hypo-phosphorylated form of GR α , thus suggesting a role for TSG101 in protecting the unliganded GR α from receptor turnover²⁴⁴. Interestingly a mutant receptor, incapable of even undergoing basal phosphorylation (i.e. as is the case with unliganded GR α), namely GR(S203A/S211A), showed enhanced interaction with TSG101²⁴⁴, which demonstrates that the association of GR α with TSG101 is dependent on the GR α phosphorylation status. Unlike TSG101, CHIP appears to not be dependent on the phosphorylation status of GR α , but rather associates with the receptor regardless of the presence of ligands, and thus the phosphorylation status, however, its presence in the cell is vital for GC-mediated GR α protein turnover²¹⁹. Interestingly, overexpression of CHIP in HT22 cells, where steady-state receptor levels were unaffected by prolonged hormone treatment, is able to restore GC-mediated down-regulation of the GR α protein via the ubiquitin-dependent proteasome degradation pathway, confirming a role for this E3 ligase in mediating receptor turnover and altering GR α expression²¹⁹.

Another interesting UPS enzyme, in terms of GC-mediated receptor turnover is F-box/WD repeat-containing protein 7 (FBXW7 α), an E3 ubiquitin ligase which requires the presence of a CDC4 phosphodegron motif²⁴⁶ in the substrate. Furthermore, this catalytically active E3 ligase requires preceding substrate phosphorylation, at this motif (i.e. the CDC4 phosphodegron motif of the GR α), in order to mediate phosphorylation-dependent ubiquitination and subsequent proteasomal degradation via the proteasome²⁴⁷. Specifically, FBXW7 α binding to GR α is primarily dependent on GSK3 β -mediated phosphorylation at Ser404²¹⁴, which then targets the GR α for ubiquitination and proteasomal degradation²⁴⁸. To demonstrate this Malyukova *et al.*²⁴⁸ demonstrated an increase in GR α protein expression following the inactivation of FBXW7 α , via mutations, as well as noting that a GR α phosphorylation mutant (S404A) was incapable of GC-mediated ubiquitination, which partially restricted its degradation via the proteasome. From this evidence, it is clear that

FBXW7 activity and expression has implications for GC sensitivity by regulating GC-mediated reductions in the GR α 'functional pool'.

Ubiquitin-Conjugating Enzyme (UbcH7), an E2 ligase, is a known co-regulator of steroid hormone receptors²⁴⁹ and, in terms of GR α , is known to directly interact with the C-terminus of the receptor with this interaction enhanced in the presence of the synthetic GC, Dex²⁵⁰. Moreover, UbcH7 has been shown to modulate GR α activity and, more importantly expression, by targeting the receptor protein for GC-mediated proteasome degradation²⁵⁰. Immunofluorescence studies have elucidated that UbcH7 is predominantly co-localized with liganded-GR α in the cell's nucleus allowing for the interaction between these two proteins to take place, however, some UbcH7 expression in the cytoplasm does exist²⁵⁰. In a study by Garside *et al.*²⁵⁰, over-expression of a dominant negative form of UbcH7 preserved GR α expression, through increasing the stability of the receptor and restricting GC-mediated protein turnover, via the proteasome, which confirmed UbcH7 as a key regulator of GR α protein expression and supported a role for UbcH7 in mediating GC sensitivity²⁵⁰.

Lastly, another recognized UPS enzyme involved in the regulation of GR α protein expression is the E3 ligase, murine double minute 2 (i.e. Mdm2 or Hdm2, the human homologue²⁵¹). Unlike the other the other enzymes, Mdm2 relies on the presence of p53 to form a trimeric complex with GR α to mediate receptor protein turnover, both in the presence and absence of hormone, via the ubiquitin-dependent proteasomal pathway²³⁶. More specifically, the interaction of GR α and p53²⁵¹ requires the ligase activity of Mdm2²²² or Hdm2²²⁸ for the successful ubiquitination and turnover of the GR α protein. A study by Sengupta *et al.*²²⁸ demonstrated that treatment of human umbilical endothelial cells with Dex enhanced GC-mediated ubiquitination of GR α in the presence of all three proteins (i.e. GR α , p53 and Hdm2). Furthermore, disruption of the interaction of p53 with Hdm2 prevented Dex-induced ubiquitination of GR α and p53²²⁸. Of interest is another study by Kinyamu *et al.*²²² where treatment with estrogen (i.e. the classical ligand for the ER) mediated GR α protein turnover, moreover, this study found that p53 and Mdm2 were interdependent and the presence of both these proteins was required for estrogen-mediated ER protein down-regulation, via the proteasomal degradation pathway²²².

2.4. Restoring GR α expression and revisiting the relevance of receptor conformation:

Combining all the evidence presented in this review, it is clear that significant reductions in GR α expression, both at the mRNA and protein level, whether disease-associated (Table 2.1), treatment-associated (Table 2.2) or both (i.e. disease-associated reductions compounded by exogenous GC use) are implicated in acquired GC resistance. Specifically, stress-induced (i.e. physical and psychological) reductions in the GR α ‘functional pool’, in many cases, results in altered behavioural effects (i.e. cognitive dysfunction and anxiety-like behaviour), which may, over time, drive the development of a number of psychological conditions (i.e. major depression, schizophrenia and bipolar disorder) and promote acquired GC resistance. Furthermore, reductions in the GR α ‘functional pool’ associated with the progression of pathological conditions (i.e. auto-immune or inflammatory-linked conditions, cancer and sepsis) encourage a wide range of GC-resistant forms of these conditions (i.e. GC resistant asthma, ITP and COPD) (Table 2.1). With the increasing incidence of these psychological and pathological conditions and the advancing threat of acquired GC resistance, a dire need for the development of novel GC therapeutics to combat chronic inflammation without eliciting GC resistance, exists.

In order to develop novel GC therapeutics to restrict the development of acquired GC resistance, it is of utmost importance to fully elucidate the complex nature of GC/GR α signalling, with focus pertaining to the molecular mechanisms involved in promoting GR α turnover. In recent years, a number of molecular mechanisms (i.e. DNA methylation, mRNA regulation and post-translational modifications) involved in reducing GR α expression have been uncovered. Moreover, strategies to restore receptor expression and maintain the GR α ‘functional pool’, through exploiting these molecular mechanisms, through combinatorial treatments with compounds (i.e. bortezomib (BZ)) have been explored and in some cases utilized in a clinical setting^{51,89,160,223,252,253}.

Ligand-induced GR α down-regulation can be prevented through the use of proteasome inhibitors, such as MG132, used in tissue culture cells, and BZ, used clinically^{252,253}. Moreover, the use of BZ, a Food and Drug Administration (FDA) approved therapeutic²⁵⁴, has been shown to restore GC sensitivity through preventing receptor turnover^{160,223}. Specifically, in a model of hypoxic blood brain barrier (BBB) damage, O₂/glucose deprivation drives significant reductions (i.e. approximately 80% reduction) in GR α protein

expression, however, treatment with BZ restores receptor expression to approximately 90% in the absence of Dex and 50%, in the presence of Dex, relative to the control (100%). Importantly, this increase in GR α protein expression was directly correlated to an increase in GC sensitivity, in this model¹⁶⁰, and mice that were treated with BZ in combination with Dex, several hours after a stroke was induced, showed a reduction in the development of brain edema, which Dex alone could not restrict, likely due to the reductions in the GR α protein expression induced by O₂/glucose deprivation and compounded by Dex treatment¹⁶⁰. Additionally, a study by Lesovaya *et al.*²²³ demonstrated the ability of BZ to increase the anticancer activities of GCs, by resulting in the accumulation GR α protein expression through proteasomal inhibition. Whilst these studies provide support for proteasome inhibitors in restoring GC sensitivity by blocking receptor turnover, one must keep in mind that, the proteasome and UPS as a whole is fundamental for the finely tuned regulation of ubiquitous proteins²⁵⁵ and thus, one of the major limitations of using proteasome inhibitors, in the clinical setting, is the specific targeting of these inhibitors to prevent the degradation of particular proteins (i.e. GR α).

In another study by Shimizu *et al.*⁸⁹, stress induced significant down-regulation (i.e. over 30%) of GR α protein expression, relative to non-stressed controls (100%), was noted. Interestingly, treatment of stressed mice with a compound called Yokukansan (YKS), a Japanese herbal medicine for the treatment of psychiatric and psychological symptoms^{256,257}, was able to normalize GR α protein expression, counteracting the stress-induced receptor turnover by approximately 20%⁸⁹. Moreover, this study was able to elucidate the mechanism of action of how YKS prevented GR α protein turnover⁸⁹. Specifically, YKS led to significant reductions, almost 50%, in the expression of miR-124, a miRNA known to target GR α mRNA preventing its translation to GR α protein. These results suggests that the ability of YKS to restore receptor expression occurs at the post-transcriptional level, specifically through targeting of miR-124⁸⁹. To our knowledge, this is one of the few studies to successfully determine the molecular underpinnings of a compound's ability, in combination with GCs, to prevent reductions in the GR α 'functional pool'.

Another compound, namely Ginsenoside Rh1⁵¹ (one of the major active compounds of Ginseng, a highly valued herb, specifically in Asia²⁵⁸) has been shown to restore GR α expression, however, the exact molecular mechanisms involved in the mechanism of action of this compound in restricting receptor turnover, is not entirely understood. Specifically, Li *et al.*⁵¹ demonstrated that co-treatment of RAW 264.7 cells with Dex and Ginsenoside Rh1,

prevented reductions in GR α mRNA expression, relative to Dex treatment alone, thus potentiating Dex's anti-inflammatory potential, specifically in prolonged treatments. In addition Ginsenoside Rh1 was able to restrict Dex-induced GR α protein turnover (i.e. to 34% of control) and partially restore GR α protein expression to 66% of the control⁵¹. Whilst in this study, the ability of Ginsenoside Rh1 was found to require mRNA transcription and new protein synthesis, suggesting its ability to transcriptionally and post-transcriptionally regulate GR α expression, the exact molecular mechanism of how this compound mediated regulation at these levels, remains to be elucidated.

Generally, studies have focused on restoring the GR α 'functional pool' through preventing or restricting ligand-induced (i.e. endogenous or exogenous GCs) receptor turnover, at the mRNA and protein level. However, could one approach this from a different angle? Specifically is the development of a ligand, which binds to the GR α and selectively modulates the receptor conformation to allow for a maintained anti-inflammatory potential but reduced receptor turnover, viable? An idea that supports this notion, is that of 'biased ligands' by Lutrell *et al.*²⁵⁹ in G-protein coupled receptor signalling (GPCR). It is well documented that ligand binding to a receptor initiates a conformational change in the receptor that dictates the down-stream effects, however, Lutrell *et al.*²⁵⁹ makes the argument that through the use of 'biased' ligands one may be able to selectively modulate the 'conformational ensemble', thus driving the 'conformational equilibrium' towards a particular state. In terms of GC/GR α signalling, the idea of selectively encouraging a specific GR α conformation to maintain the beneficial anti-inflammatory effects of GCs (via transrepression) whilst reducing the risk of generating side-effects (via transactivation), has previously been demonstrated through the development of dissociated GCs or SGRMs. Moreover, a recent review by De Bosscher *et al.*¹⁹⁴ proposed a concept called the SEDIGRAM concept, further explored in Chapter 7, which essentially refines the idea of SGRMs by proposing that ligands able to drive monomeric GR α conformation may be more beneficial in prolonged treatment regimens for the treatment of chronic inflammation, due to their ability to transrepress pro-inflammatory genes without up-regulating metabolic genes associated with GC-mediated GR α transactivation, which have been linked to side effects. However, what was not addressed in this review, are the ligand-selective effects of GR α conformation on receptor turnover, with implications in acquired GC resistance. It is this notion, with focus pertaining to the dimerization state of GR α (i.e. dimer versus monomer), which forms the central theme of the current study.

2.5. Conclusions

To conclude, acquired GC resistance, due to reductions in the GR α ‘functional pool’, is an ever increasing therapeutic challenge for the pharmaceutical industry and occurs ubiquitously throughout a number of psychological and pathological conditions. In recent years a number of the molecular mechanisms which underpin these GC-mediated reductions in the GR α ‘functional pool’ have been elucidated, with attempts to counteract GC-mediated receptor turnover being made through combinatorial treatment of GCs with other compounds which disrupt transcriptional, post-transcriptional and post-translation GR α regulation. Whilst in some cases these strategies have proved fruitful, they are not without limitations (i.e. preventing all protein degradation). Thus, we believe the idea of ‘biased ligands’ and the overlooked and possibly undervalued importance of GR α conformation, with particular reference to the receptor’s dimerization state, requires investigation, specifically in terms of the ability of the ligand-induced dimerization state of the receptor to influence its turnover.

Table 2.1: Disease-associated reductions in GR α expression

Type of condition (general)	Broad category of disease condition	Species	Specific stress/condition/disease	Tissue/cells	GR α mRNA expression	GR α protein expression	Implications for GC sensitivity	References
Stress	Pre/post-natal stress	Humans	Pre-natal stress Childhood adversity/abuse leading to adult suicide	^a PBMCs Hippocampus	Reduced	^b N.C	^c N.D	71–73
		Rodents	Early Life Stress (ELS) (i.e. maternal separation (MS) and pre-conception paternal stress (PPS))	Hippocampus, amygdala, limbic regions of brain dentate gyrus	Reduced	Reduced	Cognitive dysfunction, altered behavioural affects, increase in anxiety-like behaviour, anhedonia	74–81
	Physical or psychological stress	Rodents	Restraint stress, psychological stress, forced swim stress (FSS), repeated social defeat (RSD), repetitive restraint stress (RSS), water-immersion and restraint stress (WIRS)	Hippocampus, amygdala, hypothalamus, cerebellum, splenic macrophages, splenocytes, peripheral leucocytes, oligodendrocytes of corpus callosum, prefrontal cortex, lung tissues	Reduced	Reduced	More susceptible to psychological disorders, asthma exacerbations, diminished GC sensitivity	63,64,76,85–93
Psychological condition	Psychological conditions	Humans	Major depression (MD), Schizophrenia, Bipolar disorder Post-traumatic stress disorder (PTSD) General anxiety disorder (GAD)	Hippocampus, prefrontal-, temporal- and entorhinal cortex, PBMCs, lymphocytes	Reduced	N.D	Diminished GC sensitivity Treatment-resistant depression	53,95,106–111
Pathological conditions	Auto-immune or inflammatory linked conditions	Human	Atopic Dermatitis (AD)	PBMCs	Reduced	N.D	GC resistant to topical treatment and systemic administration of potent corticosteroid	112
			Systemic lupus erythematosus (SLE)	PBMCs	Reduced	N.D	Diminished GC sensitivity	115–117
			Inflammatory bowel disease (IBD)	PBMCs	Reduced	N.C	Impaired GC response	119
			Adult immune thrombocytopenia (ITP)	PBMCs	Reduced	Reduced	GC resistant ITP	121
			Asthma	PBMCs, cells from skin biopsies of patients	N.D	Reduced	GC resistant asthma	126,127
			Chronic obstructive pulmonary disease (COPD)	PBMCs, lymphocytes, lung tissue	Reduced	Reduced	GC resistant COPD	129–131,260
			Arthritis	Chondrocytes and lymphocytes	Reduced	Reduced	Steroid resistant arthritis	133–135
	Rodents	Experimental encephalomyelitis (EAE)	T cells	Reduced	Reduced	GC-resistant apoptosis	136	
	Cancer	Human	Acute Lymphoblastic Leukaemia (ALL) Multiple myeloma (MM) Small-cell Lung Cancer (SCLC), Non-small cell lung cancer (NSCLC), Breast cancer	B-lineage leukaemia, T-ALL resistant, lymphoblasts, T-leukemic, Multiple myeloma, human carcinoma, lung adenocarcinoma cells, breast tissue	Reduced	Reduced	GC resistant ALL GC resistant MM and diminished GC sensitivity (transactivation and GC-mediated apoptosis) GC resistant SCLC	21,138–154
			Rodents	Liver cancer	HTC cells	Reduced	Reduced	Reduced sensitivity to Dex
	Infection and other conditions	Human	Sepsis	Neutrophils and T-cells	Reduced	Reduced	Diminished GC sensitivity	156,157
			Idiopathic nephrotic syndrome (NS)	PBMCs	NC	Reduced	Steroid resistant Nephrotic syndrome (SRNS)	158
			Keloid disease	Keloid tissue	Reduced	Reduced	Diminished GC sensitivity	159
Rodents		Stroke	mouse brain capillary endothelial cells (cEND)	N.C	Reduced	Diminished GC sensitivity	160	

^a Peripheral blood mononuclear cells (PBMCs), ^b No change in GR α expression (mRNA or protein) (N.C), ^c Not detected (N.D)

Table 2.2: Treatment-associated reductions in GR α expression mediated by exogenous GCs

Exogenous GC	<i>in vitro/ex vivo/in vivo</i>	Treatment conditions		Cells/tissues	GR α mRNA expression	GR α protein expression	Implications in GC sensitivity	References
		Concentration	Time					
Dex	^a <i>in vitro</i>	Various Dex doses (10 ⁻¹⁰ to 10 ⁻⁶ M)	Generally up to 72 hours with one study continuing treatment for up to 4 weeks and one for up to 2 years	Human IM-9 lymphocytes and rat pancreatic acinar (AR42J) cells Hepatoma tissue culture (HTC), HeLa, COS-1, cells NIH 3T3 cells, Chinese Hamster ovary-derived (CHO) cells, BWTG3 cells Mouse brain capillary endothelial (cEND) cells, U2-OS and A459, Human respiratory epithelial cells (BEAS-2B) Normal human liver (HL7702) cells L6 muscle cells, Fibroblast-like synoviocytes (FLS), RAW264.7 cells Peripheral blood mononuclear cells (PBMCs)	Reduced	Reduced	Not all papers determined implications in GC sensitivity but the ones that did, demonstrated diminished GC sensitivity	51,52,160-177
	^b <i>ex vivo</i> or ^c <i>in vivo</i>	5 μ M, 20 μ g or 1mg/kg - 5mg/kg body weight	Up to 48 hours, 3 to 28 days	Variety of mice and rat tissues (liver, kidney, lung and heart), Culture mouse podocytes Rat hippocampal neurons Mice frontal cortex and hippocampus tissue Human lymphocytes	Reduced	Reduced	Not all papers determined implications in GC sensitivity but the ones that did, demonstrated diminished GC sensitivity	51,52,54,163,171,172,178-180
Triamcinolone acetonide (TA)	<i>in vitro</i>	1 μ M	Up to 96 hours	L929 cells (a fibroblast-like cell line)	Reduced	Reduced	^d N.D	181
Hydrocortisone	<i>in vivo</i>	Intraperitoneally 5 mg/100g body weight	6 hours	Liver tissue	N.D	Reduced	Altered GC sensitivity	182
Various prednisolone-based steroids	<i>in vitro</i>	10 ⁻⁵ M	0 to 24 hours	HeLa	Reduced	N.D	N.D	173
	<i>in vivo</i>	120mg/kg ^e Low-dose and 1 x mega dose; 0.01-0.3 mg/kg orally or 10-15 mg/kg ^f i.v. pulse therapy; 1mg/kg body weight	10 days ^e Daily (oral) or 3 doses; 4-6 weeks (i.v)	Liver tissue Human blood monocytes Lymphocyte subpopulations PBMCs	Reduced	Reduced	Diminished GC sensitivity GC resistance based on clinical predictive factors for GC resistance (i.e.. fundus depigmentation and chronic disease in ^g VKH)	55,183-189
Fluticasone propionate (FP) and budesonide (BUD)	<i>in vivo</i>	Up to 400 μ g/day of FP or 800 μ g/day of BUD was administered intranasally, given as a morning and evening dose	Up to 2 weeks	Nasal mucosa biopsy	Reduced	N.D	N.D	190

^a *in vitro*: GC treatment of transiently, stably transfected or endogenous GR α in tissue culture cells. ^b *ex vivo*: GC treatment of endogenous GR α in cells/tissues derived directly from animals in a tissue culture assay. ^c *in vivo*: Subjects (rodents or patients) treated with GCs with cells/tissues retrieved and assayed (i.e. GC treatment does not occur in tissue culture). ^d Not detected (N.D). ^e See Berki *et al.*¹⁸⁵ for details. ^f Intravenous therapy (i.v). ^g Vogt-Koyanagi-Harada (VKH) disease¹⁸⁹.

Table 2.3: GC-mediated molecular mechanisms involved in reducing GR α expression

Level of regulation	Molecular mechanism	Species	GR α mRNA expression	GR α protein expression	Reference
Epigenetic	DNA methylation of GRα gene <ul style="list-style-type: none"> Rodents: exon 1₇ Humans: exon 1_F, exon 1_C, exon 1_B, exon 1_H 	Rodent	Reduced	Reduced	64,77,79,80
		Human	Reduced	Reduced	71,72,140,153
Transcriptional	GRα gene regulation via ^anGRE <ul style="list-style-type: none"> present in exon 6 	Human	Reduced	^b N.D	164,171,197
Post-transcriptional	miRNA <ul style="list-style-type: none"> Rodents: miR-96, miR-101a, miR-142-3p, miR-433, miR-29b, miR-340-5p, miR-18 and miR-124a Humans: miR-124, miR-130b and miR-142-3p 	Rodent	Reduced	Reduced	64,87,89,205–207
		Human	Reduced	Reduced	144,150,156,204
Post-translational	Phosphorylation <ul style="list-style-type: none"> Rodents: <ul style="list-style-type: none"> ^c multiple mouse mutations (Ser212, Ser220 and Ser234) hyper-phosphorylation at Ser412 Humans: hyper-phosphorylation at Ser211, Ser226 and Ser404 	Mouse	N.A	Decreased	214,216
		Human	N.A	Decreased	214,215
	Ubiquitination <ul style="list-style-type: none"> Rodents: K426 Humans: K419 Proteasome degradation (i.e. use of proteasome inhibitors) <ul style="list-style-type: none"> Rodents: MG132 or bortezomib (BZ) Humans: MG132 or BZ 	Human	N.A	Decreased	166,220–224
		Mouse	N.A	Decreased	8,160,165,166,219
	Sumoylation <ul style="list-style-type: none"> Specific site unknown 	Human	N.A	Decreased	210

^aNegative glucocorticoid response element (nGRE), ^bNot detected (N.D), ^cNot applicable (N.A) as effects exerted on GR α protein

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Chapter 3:

Materials and methods

3.1. General

3.1.1. Test Compounds

The test compounds, also referred to as ligands, used in this study, included a potent synthetic GR α agonist, Dex (11 β ,16 α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione), and endogenous GC, F (11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione-17-hydroxycorticosterone), which were purchased from Sigma Aldrich (South Africa). Additionally, and a non-steroidal dissociative GC, CpdA (2-(4-acetoxyphenyl)-2-chloro-*N*-ethylethylammonium chloride) was synthesized as previously described¹ or purchased from Enzo Life Sciences (South Africa). All stock solutions were prepared in ethanol to a final concentration of 1 M and stored at -20°C.

3.1.2. Plasmids

Throughout this study the focus was on the hGRwt and hGRdim GR α plasmids, however, a number of mutation-specific and species-specific GR α plasmids were also investigated. In addition, an ubiquitin construct containing a HA-tag was utilized for the investigation of GR α ubiquitination. Lastly, the current study also made use of two GR α plasmids, which each contained a green-fluorescent protein tag (GFP), namely GFP-hGRwt and GFP-hGRdim.

Details of all plasmids, their sources and references are to be found in Table 3.1.

3.1.3. Cell culture and transfections

Cell lines that were used in this study, included an African Green monkey kidney epithelial cell line (COS-1 cells), a human hepatoma derived cell line (HepG2 cells) and two mouse embryonic fibroblast cell lines (MEF-mGRwt and MEF-mGRdim)². The COS-1 cells were purchased from American Type Culture Collection (USA) whilst the HepG2 and MEF cells were kind gifts from Barbara Burkhart (NIEHS, Research Triangle Park, USA) and Jan Tuckerman (University of Ulm, Germany), respectively.

The COS-1 cells were used to investigate the behaviour of the transiently transfected hGRwt and hGRdim plasmids because COS-1 cells are known to have little or no endogenous GR α . The HepG2 cell line was used to study the behaviour of endogenous human GR α and thus did not require transient transfections. The MEF cell lines, isolated from GR^{wt} and GR^{dim} mice as previously described², allowed for comparison of the dimerization deficient mouse GR α (MEF-mGRdim) and wild type GR α (MEF-mGRwt) in an endogenous setting.

3.1.3.1. Maintenance of cell lines

All cell lines were maintained in high glucose (4.5 g/ml) Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, South Africa) supplemented with 10% fetal calf serum (FCS) (Merck, South Africa), 1.5 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate and 100 IU/ml penicillin and 100 μ g/ml streptomycin (1% Pen/Strep) (Sigma-Aldrich, South Africa). For the HepG2 cells, additional L-glutamine was added to a final concentration of 2 mM. The four cell lines were maintained at a temperature of 37^oC, 90% humidity and 5% CO₂ in T75 tissue culture flasks (Lasec, South Africa). All cell lines were tested for mycoplasma and found to be negative.

3.1.3.2. Transfections and cell culture during experiments

Cells were seeded into tissue culture plates in supplemented DMEM, and all experiments were conducted on cells with a passage number between 1 and 35.

Approximately 24 hours later, once COS-1 cells had reached 60-70% confluency, specific plasmids (Table 3.1) were transiently transfected using either FuGENE 6 or XtremeGENE HP Fugene transfection reagents (1 μ g DNA: 2 μ l FuGENE) as described by manufacturer (Roche, Germany) (Table 3.2). For transfections, double the amount of the hGRdim plasmid (ng/ μ l) was transfected in relation to that of the hGRwt, because of hGRdim's lower expression efficiency. Due to the fact that endogenous GR α levels were analysed in HepG2 and MEF cells, they did not require GR α plasmid transfection.

During experiments COS-1 cells were induced with compounds (see details in figure legends) in unsupplemented DMEM, whilst HepG2 and MEF cells were induced in DMEM supplemented with 1% Pen/Strep and 10% charcoal-stripped FCS, due to the fact that these cells did not grow well in unsupplemented medium unlike the COS-1 cells, which was either purchased from Merck (South Africa) or stripped using a dextran coated charcoal (DCC)-based stripping buffer.

3.1.3.3. Stripping of FCS using DCC-based stripping buffer

The DCC-based stripping buffer contained activated charcoal and dextran from *Leuconostoc* species. (100:1 w/w ratio) purchased from Sigma Aldrich (South Africa), which was dissolved in a solution containing 0.25 M sucrose, 1.5 mM MgCl₂ and 10 mM HEPES. The solution was placed overnight on a stirrer at 4°C overnight. The next day the stripping buffer was pelleted by centrifugation at 500 x g for 10 minutes at room temperature, in 50 ml tubes. The supernatant was then discarded and 50 ml of FCS was added to the pelleted charcoal. The tubes were then placed on a shaker overnight at 4°C to ensure sufficient mixing. The next day the 50 ml tubes were centrifuged at 2000 x g for 30 minutes, to pellet the charcoal. Following centrifugation, the supernatant (i.e. the steroid-stripped FCS) was collected and subsequently filter sterilized using a VacuCap® 90 PF filter unit (PALL Scientific, South Africa), aliquoted and stored at -20°C until used.

3.2. Determining GR α protein concentration and analysing ligand-induced GR α protein down-regulation

Throughout this study two different techniques, namely Western blotting (using various antibodies Table 3.3) and competitive whole cell GR α -binding were employed to determine the cellular GR α protein expression levels in the three cell lines (COS-1, HepG2 and MEF cells), both in the absence and presence of test compounds and/or inhibitors cycloheximide (CHX) and MG132 (Table 3.4). In the case of the Western blotting, GR α protein expression was normalised to a loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in order to ensure equal protein loading between treatment conditions. Additionally, for a more quantitative analysis on GR α protein expression whole cell GR α -binding was used to calculate the amount of receptor in terms of fmol/mg protein.

3.2.1. Western blotting

After appropriate seeding, transfections and treatments (see figure legends and Table 3.2), cells were washed once with ice-cold phosphate buffered saline (PBS) and harvested in sodium dodecyl sulphate (SDS) bromo-phenol blue reducing buffer (100 mM Tris-HCl pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) β -mercaptoethanol and 0.1% (w/v) bromophenol blue). Once lysates were prepared they were boiled for 10 minutes at 95°C, separated on 7.5% or 10% Mini-Protean TGX precast gels (BioRad, South Africa) using the BioRad MiniProtean gel system and power pack (BioRad, South Africa). The PageRuler

Prestained Protein Ladder (#26616, Thermo Scientific, USA) was also loaded to determine if the protein of interest was the correct size. Following protein separation in the gels, the proteins were then transferred to a Hybond-ECL nitrocellulose membrane (AEC Amersham, South Africa). Once transferred, membranes were blocked for 1 hour in 10 % milk powder or 5% bovine serum albumin (BSA) purchased from Sigma (South Africa). Membranes were then washed once with 1 x Tris-buffered saline Tween (TBS-T) and probed with primary antibodies (Table 3.3) overnight at 4⁰C or at room temperature for 1 hour. Blots were then washed 1 x 15 minutes with TBS-T, 1 x 5 minutes with TBS-T and 1 x 5 minutes with Tris-buffered saline (TBS) and then incubated with secondary antibodies (Table 3.3), for 1 hour. Subsequently the blots were immersed in either Pierce or BioRad ECL Western blotting detection agents (Thermo Scientific (USA) and BioRad (South Africa), respectively, for five minutes, and exposed to FUJI Medical X-Ray Film (Africa X-ray Industrial and Medical (AXIM, South Africa) or alternatively, bands were visualized using the chemiluminescence setting on the MyECL Imager (Thermo Scientific, USA). For protein normalisation, GAPDH as a loading control was probed (see figure legends and Table 3.3) and UNSCANIT or the MyECL Image analysis Version 2.0 software used to calculate the intensity of the bands. Results were expressed as the intensity of the band for the protein of interest normalized to the band for the loading control protein. In each experiment, compound treated samples were expressed, as a percentage, relative to the solvent (EtOH), unless otherwise stated (see figure legends), which was set at 100%.

3.2.2. Whole cell GR α -binding

In addition to Western blotting, the ligand-induced effects on GR α protein expression were also investigated using whole cell GR α -binding. For this a whole cell GR α binding experiment was conducted at each time point to determine GR α protein expression. For whole cell binding the optimal incubation time for binding equilibrium to be reached, needs to be determined. This has previously been determined for the COS-1 cells³ but not for the MEF cells. Thus, in the current study a whole cell binding experiment was conducted to determine the time to equilibrium in the MEF cells. Lastly, in both the COS-1 cells and the MEF cells (MEF-mGRwt and MEF-mGRdim) a homologous competitive binding experiment, with Dex, were conducted. This allowed for the ligand binding (K_d) affinity of Dex for the GR α , in these cells, to be determined as well as the cellular GR α concentration (fmol/mg protein).

3.2.2.1. Determining time to equilibrium in MEF cells

MEF-mGRwt cells were seeded (Table 3.2) and 24 hours later were cells steroid starved in charcoal-stripped FCS with 1.5 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate and 1% Pen/Strep (Sigma-Aldrich, South Africa), for an additional day. Following steroid starvation cells were incubated with 20 nM [³H]-Dex (with a specific activity of 77 Ci/mmol, purchased from AEC Amersham) (total binding) or 20 nM [³H]-Dex and an excess of unlabelled Dex (10 μM) (non-specific binding) for 0, 2 or 4 hours at 37⁰C and 5% CO₂. After incubation, cells were placed on ice and washed 3 times with ice-cold 0.2% PBS-BSA, for 15 minutes per wash, to remove unbound ligand, and then washed quickly (another three times) with ice-cold PBS to remove albumin. Cells were then lysed with 100μl passive lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-ethylenediaminetetraacetic acid (EDTA) and 1.44 nM at room temperature and allowed to go through a freeze-thaw cycle. Thawed lysates (100 μl/well) were added to 1ml of scintillation fluid/well (Thermo Scientific, USA) and the counts per minute (cpm) were determined using a scintillation counter (Beckman LS 6500 Beta-Scintillation counter). Specific binding (cpm) was calculated by subtracting the non-specific binding from total binding (total binding - non-specific binding = specific binding) and plotted, against time. One-phase exponential association curves were fitted to the data to determine an incubation time required to reach equilibrium in the MEF-mGRwt cells. This was then applied to the MEF-mGRdim cells.

3.2.2.2. Determining GRα protein expression (cpm/mg protein) following a time course of ligand-induction, in COS-1 cells

Cells, COS-1, were seeded, transfected (hGRwt and hGRdim) and treated with ligands for various lengths of time (see figure legends and Table 3.2). Following, which cells were washed 3 times, for 15 minutes each, with pre-warmed PBS supplemented with BSA, to a final concentrations of 0.2% (BSA-PBS) (to remove bound and free ligand). Subsequently, cells were quickly washed 3 times with pre-warmed PBS to remove the BSA. To determine the GRα expression following different lengths of ligand treatments, a whole cell GRα binding experiment was done for each time point. These whole cell binding experiments were performed as previously described⁴ with the following changes; unsupplemented DMEM containing 20 nM [³H]-Dex (with a specific activity of 68 Ci/mmol) in the absence (total binding) or presence (non-specific binding) of 10 μM unlabelled Dex, was added to wells (500 μl/well) and incubated at 37⁰C for 4 hours. After incubation cells were placed on ice and

washed three times with ice-cold 0.2% BSA-PBS for 15 minutes, to remove unbound ligand, followed by three washes with ice-cold PBS to remove BSA. Cells were then lysed, underwent a freeze-thaw cycle and cpms determined using a scintillation counter. Total protein concentration was determined using the Bradford method⁵, which was used to normalize results (cpm/mg protein) and specific binding was then calculated. Specific binding of lysates from solvent (EtOH) treated cells was set at 100% (dotted line) for each time point and % specific binding of lysates from compound treated cells were then determined relative to solvent (EtOH), at each time point, and plotted. All experiments were tested for ligand depletion and counting efficiency (CE), which was less than 10% and approximately 43%, respectively.

3.2.2.3. Homologous competitive binding to determine ligand affinity (Kd) and Bmax

For this experiment, cells were seeded, transfected (save the MEF cells) and steroid starved in charcoal-stripped FCS, 24 hours prior to the homologous competitive whole cell binding experiment (see figure legends and Table 3.2). This homologous binding experiment can only be conducted when the labelled ligand and the competing unlabelled ligand are chemically identical and allows for the determination of receptor number and affinity. To do this, cells were incubated in unsupplemented DMEM (500 μ l/well) with either 20 nM [³H]-Dex or 40 nM [³H]-Dex (specific activity of 77 Ci/mmol) containing solvent (EtOH) or increasing concentrations of unlabelled Dex (10^{-11} to 10^{-5} μ M) at 37°C for 4 hours. Following incubation, cells were washed, lysed, underwent a freeze-thaw cycle and cpms determined using a scintillation counter. Total protein concentration was determined using the Bradford method⁵, which was used to normalize total binding results (cpm/mg protein), which were then plotted against increasing concentrations of unlabelled Dex (logM). The data was then fit with a global-fitting one-site homologous non-linear regression curve⁶, which then provided one shared value for the binding affinity (logKd), the maximum binding of ligand to receptors (Bmax) and the measure of non-specific binding (NSB) from all sets of data. These values were then used to calculate the total cellular GR α concentration in terms of fmol/mg protein, in the next section.

3.2.2.4. Calculating the total cellular GR α concentration (fmol/mg) protein

Using the Bmax values (cpm/mg protein) derived from the homologous competitive binding experiments in combination with the reported CE (%) and the specific activity of the labelled [³H]-Dex (77 Ci/mmol), as well as the amount of disintegrations per minute (dpms), which is

2.22×10^{12} in one Curie (Ci), one can calculate the GR α concentration of cells (fmol/mg protein), using the equation below:

$$fmol/mg\ protein = \frac{B_{max}}{(2.22 \times 10^{12})(CE)(SA)} \div 10^{12} \quad (1)$$

A step-by-step breakdown of this equation with the units for each value is now described.

Firstly, the units for Equation 1 are as follows:

$$fmol/mg\ protein = \frac{(cpm/mg\ protein)}{(dpms/Ci)(\%)(Ci/mmol)} \div 10^{12} \quad (1)$$

Next, the maximal binding (B_{max}) with units of cpms/mg protein was converted to dpms/mg protein using Equation 2 and the experimentally determined counting efficiency (CE).

$dpms/mg\ protein = \frac{(cpms/mg\ protein)}{CE}$	(2)
--	-----

Or rather

$cpms/mg\ protein = dpms/mg\ protein (CE)$	(2)
--	-----

Therefore, substituting Equation 2 into Equation 3 we now have the following, where the units for (CE) cancel each other out:

$fmol/mg\ protein = \frac{(dpms/mg\ protein)}{(dpms/Ci)(Ci/mmol)} \div 10^{12}$	(3)
---	-----

These units then cancel out to provide Equation 4:

$fmol/mg\ protein = mmol/mg\ protein \div 10^{12}$	(4)
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To get fmol/mg protein from mmol/mg protein we divide by a factor 10^{12} , which then provides the GR α concentration of the cell in **fmol/mg protein**.

3.3. Investigating the interactions of GR α and components of the ubiquitin-proteasome system (UPS), with reference to the localisation of these interactions.

To further dissect ligand-induced down-regulation of the GR α protein at the proteasome level a number of additional techniques were used. Firstly, to evaluate phosphorylation of the GR α protein, Western blotting (as described in 3.2.1) was conducted with an antibody specific for the GR α phosphorylated at serine 404 (Table 3.3). Additionally, the role of inhibiting phosphorylation at this site, using BIO (a GSK3 β inhibitor) (Table 3.4) was also investigated with Western blotting. Subsequently, to investigate GR α ubiquitination and the interaction of GR α with certain UPS enzymes a small-interference RNA (siRNA) knockdown experiment and a number of co-immunoprecipitation's (Co-IP) were conducted. Lastly, to further investigate the nature of these interactions, with selective components of the UPS, as well as the specific subcellular localisation of these interactions, immunofluorescence and a proximity-ligation assay was utilized.

3.3.1. siRNA transfection

The next day, after cells were seeded (Table 3.2), cells were transfected using HiPerfect Transfection reagent (Qiagen, Germany) with (8 μ l: 40 nM siRNA) non-silencing control (NSC) (40 nM) or a siTSG101 cocktail (40 nM), consisting of a mix of four siTSG101 RNAs (Table 3.5) (Qiagen, Germany) in Opti-MEM[®] Reduced Serum Medium with GlutaMAX[™] Supplement (Thermo Scientific, USA). The transfection mix was added directly to the cells, after a single wash with pre-warmed PBS, and incubated for 4 hours at 37^oC. After incubation, 500 μ l of complete medium (DMEM with 5% FCS and 1% Pen/Strep) was added to the cells and incubated overnight at 37^oC. The next day, cells were transfected with either the hGRwt or hGRdim plasmids using the FuGENE or XtremeGENE HP transfection reagent (Table 3.2) and incubated for an additional 24 hours. Following treatment with compounds (see figure legends), cells were lysed in 100 μ l of SDS bromophenol blue reducing buffer and subjected to Western blotting using GAPDH as a loading control (Table 3.3).

3.3.2. Co-IP

For Co-IP, once cells had been seeded, transfected (Table 3.1 and 3.2) and treated with compounds (see figure legends), they were washed three times with 10 ml of ice-cold PBS

and then lysed in 500 μ l of RIPA Buffer (#R2078, Sigma Aldrich, South Africa) supplemented with a Complete Mini Protease Inhibitor Tablet (Roche, Germany). The dishes were placed at -20°C overnight. Following a freeze and thaw cycle, cells were harvested and centrifuged at $16000 \times g$ for 5 minutes. The supernatant was collected and the pellet discarded. For inputs, 9 μ l of the supernatant per condition was set aside and 3 μ l of 5 x SDS-BB buffer added. Protein A/G PLUS-Agarose beads (#sc-2003, Santa Cruz Biotechnology, Germany) were pre-blocked using the following protocol; beads were centrifuged at $1500 \times g$ for 5 minutes at 4°C and washed with 1 ml IP dilution buffer (0.01% SDS, 20 mM Tris pH 8, 1.1% Triton-X-100, 167 mM NaCl, 1.2 mM EDTA, 1 x protease inhibitor tablet and made up to 10 ml with H_2O) and centrifuged again at $1500 \times g$ for 5 minutes at 4°C . Following centrifugation, 1 ml IP dilution buffer was added to 500 μ l of beads along with 100 μ g of Salmon Sperm DNA (11 mg/ml stock) (Thermos Scientific, USA) and rotated on a rotating wheel for 1 hour at 4°C . Subsequently, beads were centrifuged at $1500 \times g$ for 5 minutes at 4°C and approximately 500 μ l of beads were re-suspended in 500 μ l IP buffer to produce a 50% slurry, which was kept at 4°C . Following pre-blocking of the beads, the sample supernatant was pre-cleaned with 15 μ l of 50% slurry and rotated for 1 hour to minimize non-specific binding to the beads. Once pre-cleaned, samples were centrifuged at $5500 \times g$ for 1 minute and the supernatant collected to which the specific antibody (Table 3.3) was added. The supernatant, with antibody (Table 3.3), was placed at 4°C to rotate overnight. After incubation with antibody, 20 μ l of pre-blocked 50% bead slurry was added to the supernatant, incubated at 4°C on a rotating wheel for 45 minutes and then precipitated at $5500 \times g$ for 1 minute. The supernatant was then aspirated and the beads washed 6 times with wash buffer 1 (WB1) (0.1% SDS, 1% Triton x100, 2 mM EDTA, 20 mM Tris pH8 and 500 mM NaCl). After the last wash, the supernatant was aspirated and 25 μ l of 2 x SDS bromo-phenol blue reducing buffer was added to the beads, which was then boiled at 95°C for ten minutes, to elute the immunoprecipitated proteins. Inputs and immunoprecipitation samples were centrifuged at 14 000 rpm for 5 minutes and the supernatants were subjected to Western blotting using antibodies (Table 3.3).

3.3.3. Investigating the direct interaction GR α and UPS components with reference to subcellular localisation

As detailed in Table 3.2, cells were seeded, transfected, re-plated where necessary (see figure legends) and treated. Subsequently, cells were washed three times with ice-cold PBS, fixed

and permeabilized. To do this, cells were incubated (at 37 °C) with a 1:1 ratio of 4% paraformaldehyde and unsupplemented DMEM, for ten minutes. Following incubation, cells were washed 3 times with warm PBS for 5 minutes at a time and then incubated (at 37 °C) with 4% paraformaldehyde (300 µl/well in an 8 well chamber) for another 10 minutes. Once this incubation was completed, cells were washed again with warm PBS (3 times) for 5 minutes at a time, which completed the fixing. Once fixed, cells were permeabilized with 0.02% Triton-X for 6 minutes (300 µl/well in an 8 well chamber) at room temperature. Once permeabilized, cells underwent final washes (3 times with warm PBS for 5 minutes at a time) and then stored in PBS (300 µl/well in an 8 well chamber) at 4 °C, ready for immunofluorescence or proximity-ligation assay (PLA).

3.3.3.1. Subcellular localisation of GR α and UPS components using

Immunofluorescence

After permeabilization, cells were blocked with 5% PBS-BSA (300 µl/well) for 1 hour at room temperature. Cells were then washed 3 times with 1.5% PBS-BSA (300 µl/well) for 5 minutes per wash and incubated with primary antibody, diluted in 5% PBS-BSA (100 µl/well), for 1 hour at room temperature. Subsequently, cells were washed 3 times with 1.5% PBS-BSA (300 µl/well) for 5 minutes at a time and incubated with Alexa Fluor secondary antibodies (Table 3), diluted in 5% PBS-BSA (100 µl/well), for 1 hour at room temperature. Following incubation with primary and Alexa secondary antibodies (Table 3.3), cells were washed 3 times with PBS. For nuclei visualisation a stock solution of 10mg/ml Hoechst 33258 stain (Sigma Aldrich, South Africa) was diluted (1:1000) in distilled water and 300 µl/well added to the cells, which were then incubated for 5 minutes at room temperature. Finally, cells were washed 3 times with ice-cold PBS (300 µl/well) and stored in PBS at -20 °C until imaged using the LSM780 confocal microscope (Zeiss, Germany).

3.3.3.2. Image acquisition and analysis

The LSM780 confocal microscope with ELRYA PS1 super-resolution platform (Zeiss, Germany) was used for image acquisition. The microscope is equipped with a GaAsP detector, for signal collection. The settings for the signal detection for each experiment are detailed below:

Immunofluorescence

- Alexa488 (from 490 to 552 nm)

- Alexa594 (from 611 to 733 nm)
- DAPI (from 410 to 473 nm)

PLA (only) with untagged GR α plasmids:

- Duolink amplification signal (from 611 to 733 nm)
- DAPI (from 414 to 522 nm)

PLA and GFP-tagged GR α plasmids:

- Duolink amplification signal (from 611 to 733 nm)
- GFP (from 499 to 561 nm)
- DAPI (from 414 to 522 nm)

The 405nm, 488 nm and 561 nm lasers, with their appropriate beam splitters (MB405 and MBS488/561) were used for exciting of the three respective fluorophores. Additionally, a LCI “Plan-Apochromat” 63x/1.4 Oil DIC objective was used. Moreover, the laser power and detection gains were optimised to prevent ‘bleedthrough’ and the image resolution was set at 1024 x1024. Z-stacks were acquired and presented as maximum intensity projections.

Lastly, the specific methods of quantification for the subcellular localisation, co-localisation and interaction, using PLA, of GR α and the selective UPS components are detailed in Chapter 5.

3.3.3.3. Co-localisation of GR α and UPS components

Confocal images acquired following immunofluorescence were used to determine the co-localization of GR α with proteins of the UPS. Co-localization, which refers to co-compartmentalization or interaction of two molecules (or proteins), can be defined by the ‘existence of spatial overlap between two molecules’⁷⁻⁹. Each of these molecules are detected using different Alexa fluorophores with minimal overlap in their emission spectra, which recognize primary antibodies bound to the proteins of interest (Table 3.3), producing a two-channel image (i.e. red (561 nm) and green (488 nm)). Using the powerful ZENN 2012 software analysis, which utilizes specialized algorithms the degree of co-localisation between two molecules can be detected and visualized. This software displays the co-localisation of two proteins, using white pixels containing both red and green intensities, in a 2D-scattergram, an example of which is also detailed in Costes *et al.* (2004)⁹. In order to clarify random colour overlap between the two fluorophores and confirm co-localization, thresholds based on single stains were implemented. Using the visual interpretation of an experienced

researcher, Lize Engelbrecht (Central Analytical Facility (CAF), Stellenbosch University), the optimal threshold intensities for each channel (or colour), where pixels do not show any spatial correlation, were determined and applied. Thresholds for the channels, red or green, and therefore the individual proteins, were determined individually and used as a ‘cut off’ between specific staining and non-specific staining. The region where both individual channels were above their respective thresholds, was defined as the co-localisation region⁹.

For the quantification of the co-localisation signal, the weighted co-localization coefficients were determined, using the defined thresholds and the ZENN 2012 software analysis to rank the pixel intensities in each channel, and plotted.

3.3.3.4. Interaction of GR α and UPS components using a PLA

To confirm the interaction of GR α with components of the UPS, a highly sensitive technique was used, namely the Duolink proximity ligation assay (Sigma Aldrich, South Africa) (Fig. 3.1)¹⁰. A brief description of how this assay works is outlined in Chapter 5, with the details of the assay provided in the current section.

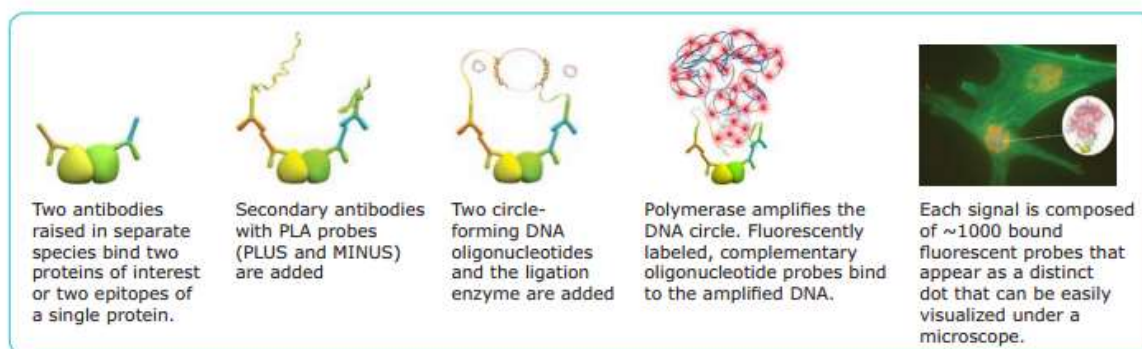


Figure 3.1: A simple description of the Duolink PLA taken from Sigma Aldrich¹⁰

For this assay, the same protocol detailed in Section 3.3.3 and 3.3.3.1 was followed to just after the primary antibody incubation. Unlike with the immunofluorescence, after primary antibody incubation cells were washed 3 times with 1.5% PBS-BSA (300 μ l/well) for 5 minutes at a time (at room temperature). Subsequently, Duolink PLA (PLUS and MINUS) probes (Sigma Aldrich, South Africa) were diluted at a 1:5 ratio in 5% PBS-BSA just before use, vortexed and added to the cells, which were then incubated in a pre-heated humidity chamber, for 1 hour at 37⁰C. Following incubation with probes, cells were washed twice with 1 x Wash Buffer A (Sigma Aldrich, South Africa), for 5 minutes at a time. After washing, the Duolink ligation stock (Sigma Aldrich, South Africa) was diluted in high quality distilled water, at a ratio of 1:5 and then the ligase (1 U/ μ l) (Sigma Aldrich, South Africa) was added

to the reaction mix at a ratio of 1:40 just before addition to the cells. The cells were incubated, in a pre-heated humidity chamber, with the ligation mix for 30 minutes at 37°C and subsequently washed with 1 x Wash Buffer A twice, for 2 minutes each. Once ligation was completed, the Amplification stock (Sigma Aldrich, South Africa) was diluted at a ratio of 1:5 in high quality distilled water and the polymerase (Sigma Aldrich, South Africa) added to the solution at 1:80. The mix was then immediately applied to the cells and incubated for 100 minutes at 37°C in a pre-heated humidity chamber. Following amplification the cells were washed with 1 x Wash Buffer B (Sigma Aldrich, South Africa) twice for 10 minutes and then in 0.01% x Wash Buffer B for a further minute. For nuclei visualisation a stock solution of 10 mg/ml Hoechst 33258 stain (Sigma Aldrich, South Africa) was diluted (1:1000) in distilled water and 300µl/well added to the cells and incubated for 5 minutes at room temperature. Finally, cells were washed 3 times with ice-cold PBS (300 µl/well) and stored in PBS at 4°C for imaging using the LSM780 confocal microscope. The PLA signal is visualised, as a fluorescent red 'spot', which can then be quantified.

3.4. Mimicking acquired GC resistance in a cellular model, the MEF cells

Throughout this study, changes in the expression of the GR α protein are noted across all cell lines (COS-1, HepG2 and the MEF cell lines). Additionally, studies have suggested that these changes in GR α expression (both at the protein and mRNA level) may have down-stream effects on GR α mediated gene expression^{11,12}, which has a number of biological implications, such as the development of acquired GC resistance. With this in mind, the current study sought to mimic acquired GC resistance using a cellular model (MEF cells) as detailed in Section 6.2.2 to investigate down-stream GR mediated gene expression.

3.4.1. Establishing a working model

The experimental procedure that was followed is neatly illustrated in a diagram (Figure 6.3), which provides details on compound treatments (short-term versus long-term) employed in order to mimic acquired GC resistance.

3.4.2. RNA isolation

Following specific treatments, cells were washed 3 times with ice-cold PBS and harvested for RNA. RNA isolation, cells were lysed with 400 µl Tri Reagent (Sigma, South Africa) and stored at -80°C to undergo freeze/thaw cycle. Once samples had thawed and been transferred to microcentrifuge tubes, to which 80 µl of chloroform (Sigma Aldrich, South Africa) was

added. Samples were then vortexed vigorously for 20 seconds and kept at room temperature for 5 minutes. Lysates were centrifuged at 4°C for 20 minutes at 14 000 rpm and 160 µl of the clear supernatant was added to chilled microcentrifuge tubes. Next, 200 µl of isopropanol (Merck, South Africa) was added to each clear sample, vortexed, incubated at room temperature for 10 minutes and then stored at -20°C. The next day samples were centrifuged at 4°C for 20 minutes at 14 000rpm. The supernatant was then discarded and the pellet washed 3 x with 75% ethanol-Diethyl pyrocarbonate (DEPC) water and then allowed to air-dry for ten minutes before dissolving it in 10 µl of DEPC treated water. RNA concentrations and quality (260/280 ratio) was measured using the NanoDrop 1000 (Thermo Scientific, USA). The integrity of the RNA isolated was checked by running 0.5 µg of RNA on a 1% agarose formaldehyde gel (for 50 ml: 0.5 g agarose in 36 ml DEPC-treated water, 5 ml 10 x 3-(N-morpholino) propanesulfonic acid (MOPS) buffer and 9 ml formaldehyde) in 1x MOPS buffer, 10 x dilution of 10 x MOPS buffer (0.4 M MOPS, pH 7, 0.1 M sodium acetate and 0.01 M EDTA, pH 8).

3.4.3. cDNA synthesis

cDNA was synthesised from 1µg of RNA using the ImProm-II reverse transcription system (Promega, USA) according to the manufacture's protocol.

3.4.4. Real-time PCR (RT-PCR)

Specific primers (Table 3.6) were designed using National Center for Biotechnology Information (NCBI) and then purchased from Integrated DNA Technologies (USA). Primers were dissolved in 1 x Tris-EDTA (TE) buffer to produce a primer stock solution (100 µM) from which the working solution (10 µM) of each primer was made. RT-PCR was conducted to measure the expression of various genes using the cDNA prepared. A final reaction volume of 10 µl consisting of 1 µg cDNA, 0.3 µl FWD primer and 0.3 µl REV primer (final primer concentration of 0.333 nM), 3.4 µl of DEPC water and 5 µl of SYBR green ABI PRISM (Kappa Biosystems, South Africa).

The annealing temperatures for the primer sets were optimized and the primer efficiencies were determined (Table 3.6) by preparing a standard curve from a 5 x dilution series of prepared cDNA. Primer efficiencies obtained were between 1.87-2.18 (Table 3.6) and were calculated using Equation (1)¹³. The expression of the genes of interest was normalized to 18S (reference gene) amplification and calculated according to Equation (2)¹³ and expressed

as a fold induction or reduction relative to solvent (without tumor necrosis factor alpha (TNF α)), unless otherwise stated (see figure legends).

$Primer\ efficiency = 10^{\left(\frac{-1}{slope}\right)}$	(5)
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$Relative\ gene\ expression = \frac{(expression\ target\ gene)^{\Delta CP\ of\ gene\ of\ interest\ (solvent-sample)}}{(expression\ of\ refence\ gene)^{\Delta CP\ of\ reference\ gene\ (solvent-sample)}}$	(6)
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3.5. Statistical analysis

Statistical analysis throughout this study was conducted using the GraphPad Prism software, Version 5. Specifics of the statistical analyses used, including post-tests, are detailed in the figure legends. The extent of the statistical significance is indicated using numerical symbols (e.g. *, # and \$), with the approximate value of these symbols also indicated in the figure legends. Non-significant results are indicated by 'ns'.

Table 3.1: Plasmids used throughout this study

	Plasmid	Description	Kind gift from	Reference
Human	pRS-hGR α (hGRwt)	Human wild type GR α	Prof. R. Evans, Salk Institute for Biological Studies, California, USA	14
	pHisGRA458T (hGRdim)	GR α dimerization deficient mutant with single mutation of alanine to a threonine (at amino acid 458) in D-loop	Prof. K. De Bosscher, University of Ghent, Belgium.	2
	hGR(N454D/A458T)	GR α mutant with two mutations, within the D-loop at amino acid 458 and flanking the D-loop at 454	Prof. A. Cato, Institute of Toxicology and Genetics (ITG), Germany.	15
	hGR(D4X)	GR α mutant containing the A458T mutation and 3 other mutations, nearby, thought to be important for GR α dimerization		15
	hGR α (R477H)	A naturally occurring GR α mutant known to cause generalized GC resistance	Prof. R. Kofler, Innsbruck Medical University, Austria.	16
	pEGFP-C2-GR (GFP-hGRwt)	Green Fluorescent-tagged (GFP) human wild type GR α	Prof. S Okret, Karolinska Institute, Sweden	17
	pEGFP-C2-GR477T (GFP-hGRdim)	Green Fluorescent-tagged (GFP) GR α dimerization deficient mutant with single mutation of alanine to a threonine (at amino acid 458) in D-loop	Prof. S Okret, Karolinska Institute, Sweden	17
Mouse	pcDNA-mGRwt	Mouse wild type GR α		
	pcDNA-mGR(A458T) (mGRdim)	GR α dimerization deficient mutant with single mutation of alanine to a threonine (at amino acid 458); homologous to hGRdim	Prof. S. Bodine, University of California, Davis, USA.	18
Other	HA-tagged Ubiquitin	Ubiquitin plasmid for overexpression ubiquitin	Prof. D Bohmann, AAB Institute of Biomedical Sciences, New York, USA.	19

Table 3.2: Details of transfection conditions

Type of dish	Cell type	Cell number (cells/well)	Transient transfection	Transfection agent	Amount of pDNA (ng/well)	Plasmid transfected	Experiment	Chapter	Figure	Other
24 well	COS-1	5 x 10 ⁴	Yes	XTremeGENE HP	200	hGRwt	WCB	4	4.3	-
	COS-1	5 x 10 ⁴	Yes	Fugene 6	200	hGRwt	WCB	4	4.4 and 4.5A	-
	COS-1	5 x 10 ⁴	Yes	XTremeGENE HP	200	hGRwt, hGR(R477H), hGRdim(N454D/A458T), hGRdim (D4X), mGRwt and mGRdim	WB	5	5.18A and B	-
	COS-1	5 x 10 ⁴	Yes	XTremeGENE HP	400	hGRdim	WB	5	5.18A	-
	MEF-mGRwt and MEF- mGRdim	5 x 10 ⁴	No	-	-	-	WCB	6	6.1 and 6.2	-
	COS-1	5 x 10 ⁴	Yes	Fugene 6	400	hGRdim	WCB	4	4.5C	-
12 well	COS-1	5 x 10 ⁴	Yes	Fugene 6	400	hGRwt	WB	4	4.5B, 4.7A and B, 4.8A and B	-
	COS-1	5 x 10 ⁴	Yes	Fugene 6	800	hGRdim	WB	4	4.5D, 4.7A and C, 4.8A and C	-
	COS-1	5 x 10 ⁴	No	-	-	-	WB	4	4.9A	-
	HepG2	5 x 10 ⁴	No	-	-	-	WB	4	4.5D, 4.7D, 4.8D and 4.9B	-
	COS-1	1 x 10 ⁵	Yes	Fugene 6	400	hGRwt	WB	5	5.4A	-
	COS-1	1 x 10 ⁵	Yes	Fugene 6	800	hGRdim	WB	5	5.4B	-
	HepG2	1 x 10 ⁵	No	-	-	-	WB	5	5.4C and D	-
	COS-1	1 x 10 ⁵	Yes	XTremeGENE HP	400	hGRwt	WB	5	5.5B	-
MEF-mGRwt and MEF- mGRdim	1 x 10 ⁵	No	-	-	-	WB, cDNA synthesis and RT-PCR	6	6.4, 6.5, 6.6, 6.7, 6.8, 6.9 and 6.10	-	
10 cm	COS-1	1 x 10 ⁶	Yes	XTremeGENE HP	3000	hGRwt	Co-IP and WB	5	5.5C, 5.5D, 5.5E and 5.5F	-
	COS-1	1 x 10 ⁶	Yes	XTremeGENE HP	300	HA-tagged ubiquitin	Co-IP and WB	5	5.5F	-
	COS-1	1 x 10 ⁶	Yes	XTremeGENE HP	3000	hGRwt	Immunofluorescence and PLA	5	5.6A and B, 5.8A and B, 5.9A and B, 5.10A and B, 5.12A and B, 5.13A and B	COS-1 cells were replated into 8 well chambers (3 x 10 ⁴ cells/well), 24 hours after transfection and before compound treatment.
	COS-1	1 x 10 ⁶	Yes	XTremeGENE HP	3000	GFP-hGRwt and GFP-hGRdim	PLA	5	5.14, 5.15, 5.16 and 5.17	COS-1 cells were replated into 8 well chambers (3 x 10 ⁴ cells/well), 24 hours after transfection and before compound treatment.
8 well chamber	COS-1	3 x 10 ⁴	Yes	XTremeGENE HP	200	hGRdim	Immunofluorescence and PLA	5	5.7A and B, 5.8A and C, 5.9A and C, 5.11A and B, 5.12A and C, 5.13A and C	COS-1 cells were directly transiently transfected into 8-well chamber and not re-plated.

* Whole cell Grabinning (WCB), Western blotting (WB), co-immunoprecipitation (Co-IP) and Proximity-Ligase Assay (PLA)

Table 3.3: Antibodies used throughout this study

Target protein	Size (kDa)	Target species	Primary antibody (1 ^o)	1 ^o antibody dilution	Secondary antibody (2 ^o)	2 ^o antibody dilution	Application
GRα	97	All human	sc-8992 ^a	1:1000	Goat anti-rabbit IgG-HRP, sc-2030 ^a	1:10 000	Western blotting
	94	All mouse	sc-8992 ^a	1:1000	Goat anti-rabbit IgG-HRP, sc-2030 ^a	1:10 000	Western blotting
	97	Human	Ab2768 ^b	1:500	Goat Anti-Mouse IgG H&L (Alexa Fluor® 594) (ab150116) ^b	1:500	Immunofluorescence
	97	Human	Ab2768 ^b	1:500	Duolink kit ^a	-	PLA
GAPDH	37	Human and mouse	sc-47724 ^a	1:500	Goat anti-mouse IgG-HRP, sc-2005 ^a	1:5000	Western blotting
pSer404-GRα	97	Human	Gift from J.Cidlowski ^c	1:500	Goat anti-rabbit IgG-HRP, sc-2030 ^a	1:10 000	Western blotting
p53	53	Monkey	#2524 ^d	1:1000	Goat anti-mouse IgG-HRP, sc-2005 ^a	1:5000	Western blotting
TSG101	55	Monkey	ab83 ^b	1:500	Goat anti-mouse IgG-HRP, sc-2005 ^a	1:5000	Co-immunoprecipitation and Western blotting
CHIP/STUB1	35	Monkey	ab168768 ^b	1:500	Goat anti-mouse IgG-HRP, sc-2005 ^a	1:5000	Co-immunoprecipitation and Western blotting
Anti-HA tag (ChIP grade)	-	-	ab9110 ^b	1:1000	Goat anti-rabbit IgG-HRP, sc-2030 ^a	1:10 000	Co-immunoprecipitation and Western blotting
Ubiquitin	8	Monkey	ab7780 ^b	1:200	Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150077) ^b	1:500	Immunofluorescence
	8	Monkey	ab7780 ^b	1:200	Duolink kit ^a	1:500	PLA
FBXW7α	79	Monkey	ab109617 ^b	1:500	Goat anti-rabbit IgG-HRP, sc-2030 ^a	1:5000	Co-immunoprecipitation and Western blotting
	79	Monkey	ab109617 ^b	1:500	Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150077) ^b	1:500	Immunofluorescence
	79	Monkey	ab109617 ^b	1:500	Duolink kit ^a	-	PLA

^a Santa Cruz, USA. ^b Abcam, USA. ^c National Institute of Environmental Health Sciences, USA. ^d Cell Signalling, USA.

Table 3.4: Other reagents used throughout this study

Reagent	Application	Catalogue number	Final concentration
MG132	Proteasome inhibitor	M7449 ^a	1 µM
CHX	Translation inhibitor	C4859 ^a	1 µM
BIO	GSK3β inhibitor	361550 ^b	5 µM
TNFα	Pro-inflammatory cytokine	T7539	0.02 µg/ml

^a Sigma Aldrich, South Africa. ^b Merck, Germany.

Table 3.5: Target sequences of siRNA ‘cocktail’ for TSG101 knockdown

Product name	Catalogue number	Target sequence
TSG101_6 ^a	SI02664529	ACCCGTTTAGATCAAGAAGTA
TSG101_7 ^a	SI02664522	CAGCTGAGGGCACTAATGCAA
TSG101_8 ^a	SI02655184	CAGTTTATCATTC AAGTGTA A
TSG101_12 ^a	SI04437398	ATGGTTACCCGTTTAGATCAA

^a Qiagen, Germany.

Table 3.6: Primers used throughout this study

Species	Gene	Protein	5'-3' Forward primer sequence	5'-3' Reverse primer sequence	Optimized annealing Temperature (°C)	Product size	Primer efficiency
Mouse	Nr3c1	GRα	AAA GAG CTA GGA AAA GCC ATT	TCA GCT AAC ATG TCT GGG AAT TCA	55	182	2.18
Mouse	Tsc22d3	GILZ	AAT GCG GCC ACG GAT G	GGA CTT CAC GTT TCA GTG GAC A	56	166	1.87
Mouse	Tat	TAT	TCG GCT CTG CTG GAG GCA CT	TCT ACC GCA GGG CGT GAG GT	55	289	1.91
Mouse	Fkbp5	FKP51	GCTGGCAAACAACACGAGAG	GAGGAGGGCCGAGTTCATT	58	107	1.91
Mouse	Il6	IL-6	ATGCTGGTGACAACCACGGCC	AGCCTCCGACTTGTGAAGTGGA	55	189	1.94
Mouse	Rn18s	18S	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG	55	151	1.9

*All primers were designed using NCBI and then purchased from Integrated DNA Technologies (USA)

3.6. References

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Chapter 4:

Receptor dimerization is a requirement for ligand-induced down-regulation of GR α

4.1 Introduction

Synthetic GCs continue to be the preferred therapeutics for the treatment of diseases associated with chronic inflammation, however, these prolonged GC treatment regimens are continuously confronted by two major limitations, namely, the generation of undesirable side-effects and the development of an acquired resistance to GC treatment¹⁻⁵.

Considering that 1 – 2% of the global population is currently receiving long-term, high dose GC treatment, and that a 34% increase in the incidence of GC treatment was noted between 1989 and 2008, these limitations (i.e. undesirable side-effects and acquired GC resistance) have major implications for the pharmaceutical industry⁶⁻⁸. These implications include: increased morbidity rates, an increase in financial costs and concomitant economic impact (treating adverse side-effects and providing higher GC doses to overcome resistance) and overall, a poorer quality of life for the patients^{6,8-10}.

Over recent years, research has focused on investigating and developing SGRMs, which in essence aim to maintain a potent anti-inflammatory potential whilst having an improved side effect profile, and a number have been used in clinical trials^{11,12}. Whilst SGRMs are proving somewhat successful in curbing the generation of undesirable side-effects¹³⁻¹⁶, there is still the lingering issue of developing acquired resistance to GC treatment following prolonged GC use.

Perturbations in the ‘functional pool’ of GR α protein may result in resistance, both inherited and acquired. Many studies have demonstrated a direct correlation between the ability of a patient to respond to GC treatment and the amount of functional GR α protein¹⁷⁻¹⁹. The amount of functional GR α protein (‘functional pool’) may be altered at a functional level (i.e. GR α gene mutations) or at an expression level (i.e. the level of the ‘pool’ or how much is available in the ‘pool’)²⁰⁻²⁴. Fundamental to the current study, is the latter, changes in the level of the ‘pool’, which broadly refers to an increase or decrease in GR α protein expression. A reduction in GR α protein expression may lead to acquired GC resistance, a major limitation encountered in treating chronic inflammation, and thus it is necessary to understand the factors affecting GR α protein down-regulation.

Regulation of protein expression may be described by a simple ‘push’ vs. ‘pull’ mechanism, which refers to the dynamic state of protein synthesis and degradation, respectively, working together to regulate cellular protein levels, such as those of the GR α protein (Fig. 4.1). The ‘push’ is governed by two processes, namely, transcription and translation, whilst the ‘pull’ is defined by protein degradation pathways, such as lysosomal and ubiquitin-proteasome mediated degradation (Fig. 4.1).

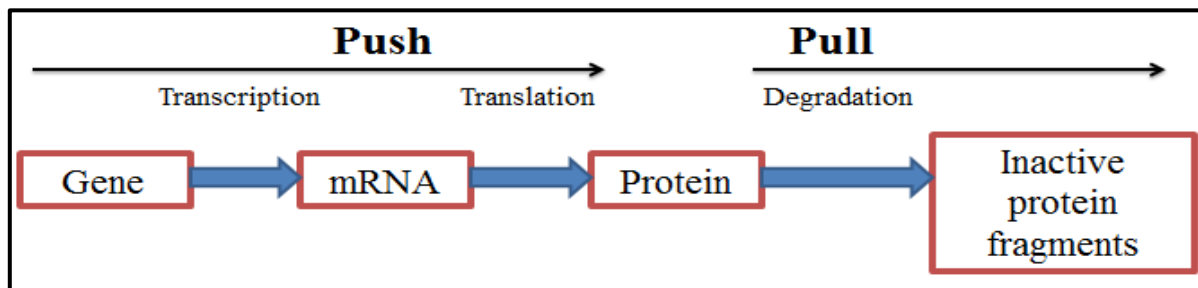


Figure 4.1: General regulation of protein ‘pool’ described by a simple ‘push’ vs. ‘pull’ mechanism.

Honing in on GR α , the focus of the current study, one can assume that disturbing this dynamic state of regulation (i.e. the ‘push’ vs. ‘pull’ mechanism), will most likely result in altered GR α mRNA and/or GR α protein expression (Fig. 4.2). One of the ways in which the dynamic state of regulation may be perturbed is through binding of certain cognate GR α ligands to GR α and several studies have indeed shown that treatment with GCs, particularly Dex, results in altered, predominantly reduced, GR α expression at both the mRNA and protein level^{25–33}.

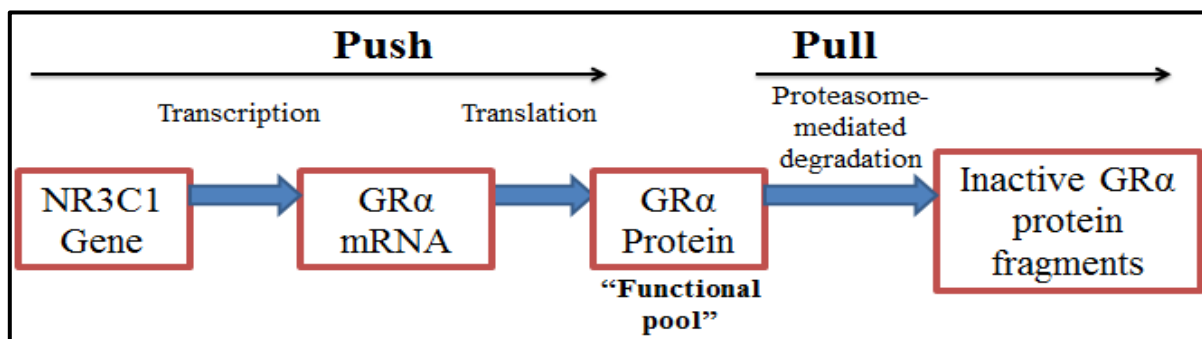


Figure 4.2: Specific regulation of the GR α protein ‘functional pool’, described by a simple ‘push’ vs. ‘pull’ mechanism.

Firstly, at the mRNA level, Dex treatment has been shown to reduce both nascent and mature GR α mRNA expression, by altering GR α gene transcription and affecting mRNA stability, respectively^{30,34–38}. Interestingly, the effect of Dex treatment on GR α mRNA expression is somewhat conflicting and a number of studies have reported GR α mRNA up-regulation, following Dex treatment^{39,40}. Secondly, at the protein level, it is well accepted that Dex treatment results in down-regulation of the GR α protein^{22,28–30,32,41,42}. Dex treatment results in a robust reduction in GR α protein expression, directly reducing the ‘functional pool’ of GR α available to perform its biological function. Although it is well accepted that the rate of GR α protein down-regulation is

increased following Dex treatment, basal GR α protein down-regulation of the unliganded receptor, which occurs at a significantly slower rate, should not be disregarded⁴¹.

Consistently, and in stark contrast to Dex treatment, a number of studies have shown that CpdA, a SGRM, does not result in GR α down-regulation at either the mRNA or protein level^{30,32}. Furthermore, following treatment with CpdA, the GR α protein is reported to have a half-life similar to that of the unliganded GR α protein^{30,41}. Interestingly, CpdA does not result in GR α dimerization, in contrast to Dex treatment^{33,43}, which hints at a possible role for ligand-induced GR α dimerization in ligand-induced GR α protein down-regulation.

These thought provoking effects of CpdA treatment on GR α dimerization and GR α down-regulation provided a concrete platform for the current study, which investigated the effect of ‘gain’ or ‘loss’ of GR α dimerization on ligand-induced GR α protein down-regulation. It was hypothesised that ‘gain’ of GR α dimerization following Dex treatment of the human wild type GR α (hGRwt) would lead to GR α protein down-regulation. In contrast, ‘loss’ of dimerization, either as a result of CpdA treatment of hGRwt or through the use of a dimerization deficient mutant (hGRdim), would not result in ligand-induced GR α protein down-regulation.

Broadly speaking, the current study started off by analysing how the ‘functional pool’ of GR α is altered by GC treatment by focussing on the type of ligand, concentration of ligand, treatment time and the relevance of GR α dimerization in ligand-induced receptor down-regulation. CpdA treatment was used as a molecular tool to abrogate dimerization of the wild-type GR α . In addition, the role of GR α dimerization in ligand-induced GR α protein down-regulation was confirmed using the dimerization deficient mutant, hGRdim⁴⁴.

Furthermore, process specific inhibitors were used to establish which process, the ‘push’ (transcription and translation) or the ‘pull’ (proteasomal degradation), is primarily responsible for regulating ligand-induced receptor turn over. Lastly, to rule out a possible direct and general effect of CpdA treatment on cellular protein turnover, the degradation of a short-lived tumour suppressor protein, p53, was investigated and compared in the absence and presence of CpdA⁴⁵.

A deeper understanding of the possible association between dimerization and down-regulation of GR α may provide more insight into GC resistance. Whilst ligand-induced GR α protein down-regulation is thought to be an essential cellular function^{17,30,33}, it may have pathological consequences in patients receiving high dose GC treatment for prolonged periods of time.

4.2 Results

4.2.1 GR α protein down-regulation is ligand and dose-dependent

It is fairly well documented that the synthetic GR α ligand, Dex, induces GR α protein down-regulation in a number of cellular systems^{22,27–32,42}, however, only a handful of studies have investigated the effect of prolonged treatment of F, the endogenous ligand for GR α ^{32,46}. Furthermore, most previous studies have evaluated effects at a single ligand concentration, which may drastically limit the interpretation of results.

To explore differences between two GR α dimerization promoting ligands, Dex and F, and to substantiate previously reported effects on ligand-induced GR α protein down-regulation, COS-1 cells transiently transfected with hGRwt were treated with Dex or F. Furthermore, to obtain additional information, like potency and efficacy, a dose-response (10^{-11} M – 10^{-5} M) experiment, at a single treatment time of 24 hours, was conducted. Potency (IC₅₀) refers to the concentration (nM) of ligand required to reduce hGRwt protein expression by 50%, while the efficacy describes the percentage of maximal reduction of hGRwt expression.

Both Dex (Fig. 4.3A; red) and F (Fig. 4.3A: green) treatment led to ligand-induced GR α protein down-regulation. Specifically, the efficacies for Dex and F were 41.3% and 45.5% (Fig. 4.3B), respectively, which translated into a 58.7% reduction in hGRwt expression after Dex treatment that was not significantly different from the 54.5% reduction induced by F treatment (Fig. 4.3D). A clear shift to the right was observed for the F graph, which was reflected in a significantly ($p < 0.01$) 50-fold lower potency for F (20nM) when compared Dex (0.4nM) (Fig. 4.3C and D).

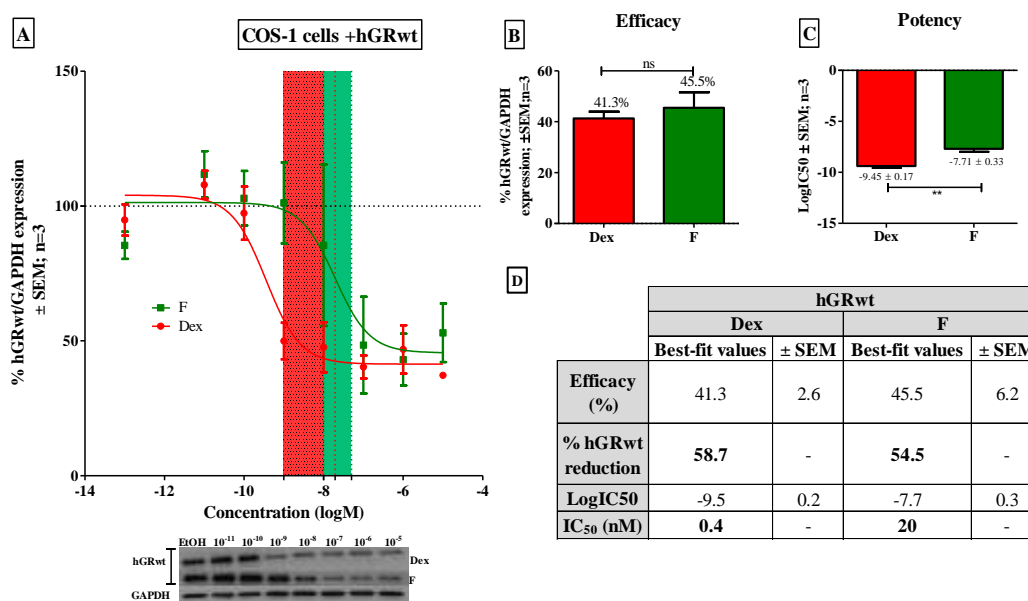


Figure 4.3: hGRwt protein down-regulation is ligand and dose-dependent. COS-1 cells were seeded into a 24 well plate (5×10^4 cells/well) and transfected the next day with hGRwt using a transfection agent. Following 24 hours incubation, cells were treated with either solvent (EtOH) or varying concentrations (10^{-11} M to 10^{-5} M) of Dex and F for 24 hours. Thereafter, hGRwt protein expression was assessed by Western blotting where GAPDH was probed to ensure equal protein loading. The Western blot shown (A, inset) is representative of three independent experiments. For quantification (A), the intensity of the hGRwt and GAPDH bands was determined using UNSCANIT, the hGRwt expression was then normalised to GAPDH expression and expressed as a percentage (average \pm SEM) of hGRwt expression in the presence of solvent (EtOH), which was set at 100% (dotted line). Physiological concentrations of Dex (red; 1-20 nM) and F (green; 10-50 nM) are indicated by shaded areas. Efficacy (B and D) and potency (C and D) of GCs for hGRwt down-regulation were determined. For statistical analysis, an unpaired t-test was used to evaluate the effects of ligands on the efficacy (ns, $p > 0.05$) and the potency (**, $p < 0.01$).

A dose response curve also allowed for the evaluation of effects at a range of concentrations, such as the physiological concentrations of Dex (Fig 4.3A: red shaded area) or F (Fig 4.3A: green shaded area). In a physiological setting, it is clear that hGRwt protein down-regulation will occur. Specifically, free plasma F levels range from 10nM to 50nM during the circadian cycle in non-stressed individuals^{31,47,48}, which would correspond to a 15 – 39% reduction, while the free plasma Dex levels range from 1– 20 nM following a low dose administration⁴⁹, which correlates with a 43 – 60 % reduction in hGRwt expression.

In order to eliminate the effect of ligand concentration in subsequent experiments, fractional occupancy (F.O) of hGRwt by Dex and F was calculated using Kd values (8nM and 91nM) from He *et al.*⁵⁰ (Table 4.1). F.O refers to the fraction of ligand-bound receptors relative to the total receptor ‘pool’, bound and unbound, and may be calculated using the following equation:

$$\text{Fractional occupancy} = \frac{[\text{Ligand}]}{[\text{Ligand}] + [\text{Kd}]}$$

Table 4.1: Calculating the fractional occupancy of Dex and F for hGRwt

Concentration (logM)	Ligand concentration (nM)	% F.O ^b of Dex for hGRwt	% F.O ^b of F for hGRwt	Fold difference ^c
-9	1	11	1	10
	8^a	50	8	6
-8	10	56	10	6
	91^a	92	50	2
-7	100	93	52	2
-6	1000	99	92	1
-5^d	10000	>99	99	1

^a Kd values for Dex (8 nM) and F (91 nM) provided by He *et al.*⁵⁰. ^b Fractional occupancy was calculated using highlighted Kd values⁵⁰ and equation above table and is expressed as a percentage. ^c The fold difference was calculated by dividing the percentage fractional occupancy for Dex by the percentage fractional occupancy for F. ^d The ligand concentration used in subsequent experiments to ensure > 99% fractional occupancy for both GRα ligands.

To ensure comparable F.O of the hGRwt, saturating concentrations of Dex and F (10 μ M), which corresponded to a F.O of $\geq 99\%$ for both ligands, was used in subsequent experiments.

In summary, hGRwt protein expression was markedly down-regulated by the dimerization promoting GCs, Dex and F, which resulted in a decrease in the level of the ‘functional pool’ of GR α . Results represented in Fig. 4.3 highlight that GR α protein down-regulation is ligand- and dose-dependent, occurring over a wide range of GC concentrations, including the physiological ranges of the GCs tested. Additionally, in both cases (cells treated with Dex and F) GR α down-regulation is observed when the F.O is around 10%.

4.2.2 Rate of GR α protein degradation is altered in a ligand-selective manner

The extent of GR α protein down-regulation is ligand and dose-dependent (Fig. 4.3), however, previous studies have suggested that the unliganded GR α protein also undergoes degradation, and that GC treatment, especially with dimerization promoting GCs, such as Dex and F, accelerates the rate of GR α protein turnover^{26,28,34,51}. In contrast, treatment with CpdA, which abrogates dimerization^{33,43}, induces minimal receptor turnover, with a degradation rate comparable to unliganded GR α ³².

To analyse and compare unliganded and liganded GR α protein down-regulation over time (2 – 72 hours), COS-1 cells transiently transfected with hGRwt, were treated with solvent (EtOH), Dex, F or CpdA (10 μ M). To determine, differences in hGRwt protein down-regulation induced by ligands over time, half-life ($t_{1/2}$) and rate constant (K) values were calculated. The rate constant, K (cpm/hour), a measure of the reduction in hGRwt protein per hour, was used to calculate the half-life (hours), which refers to the time required for a 50% reduction in hGRwt protein expression following treatment and was calculated using the following equation:

$$t_{1/2} = \frac{\ln(2)}{K}$$

From the results, it is clear that the unliganded hGRwt protein was degraded in a time-dependent manner (Fig. 4.4A) and has a half-life of 70 hours (Fig. 4.4B). Furthermore, the dimerization promoting GCs, Dex and F, significantly ($p < 0.01$) increased receptor turnover (Fig. 4.4A), resulting in a decreased half-life of hGRwt of 21 and 22 hours, respectively (Fig. 4.4B). For both GCs, maximal hGRwt protein down-regulation was reached following 48 hours of treatment. Unlike Dex and F treatment, hGRwt protein down-regulation seemed to be virtually absent following treatment with the dimerization abrogating SGRM, CpdA (Fig 4.4A).

The K values indicated a significant ($p < 0.001$) increase in the rate of hGRwt protein degradation, from 0.01 cpm/hour for the unliganded receptor, to 0.03 cpm/hour following Dex and F treatment

(Fig. 4.4C). On the other hand, a decrease in the rate of hGRwt protein down-regulation was observed following CpdA treatment (0.004 cpm/hour) (Fig. 4.4A).

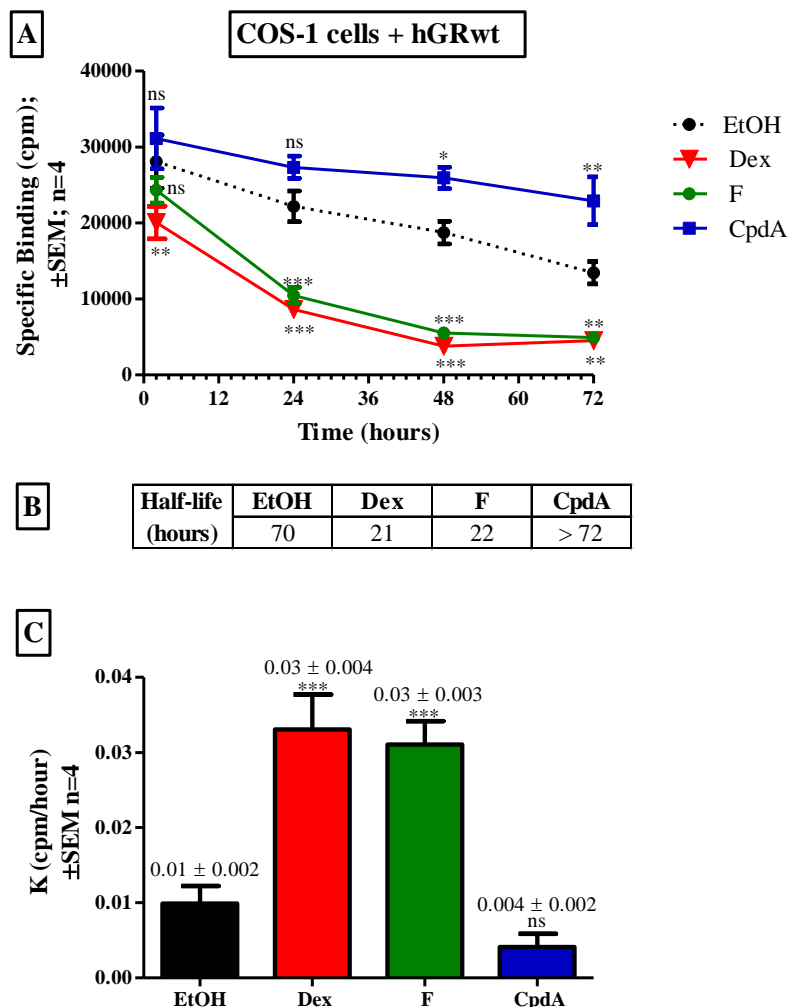


Figure 4.4: Rate of hGRwt protein degradation is altered in a ligand-selective manner. COS-1 cells were seeded into a 24 well plate (5×10^4 cells/well) and transfected the next day with hGRwt using a transfection agent. Following 24 hours incubation, cells were treated with solvent (EtOH) or the GCs, Dex or F, or CpdA (10^{-5} M) for 2 to 72 hours. Thereafter, whole cell GR α -binding (A) was conducted using 20nM [3 H]-Dex. Once lysed, hGRwt expression was detected via a scintillation counter and specific binding values (cpm) were plotted against time. Whole cell GR α -binding results shown are representative of four independent experiments (average \pm SEM), conducted in triplicate. Statistical analysis was conducted using a two-way analysis of variance (ANOVA) followed by a Bonferroni post-test comparing each time point to solvent (EtOH) (ns, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). Half-lives (B) and rate constants (C) were calculated using non-linear regression one-phase dissociation decay analysis. For statistical analysis of rate constants, one-way ANOVA followed by a Bonferroni's post-test was conducted comparing K (cpm/hour) values to solvent (EtOH) (ns, $p > 0.05$, ***, $p < 0.001$).

To summarize, the unliganded hGRwt protein underwent basal down-regulation. Moreover, the rate of receptor turnover increased and the half-life of the hGRwt protein decreased, relative to the unliganded hGRwt, following treatment with the dimerization promoting GCs, Dex and F, but not the dimerization abrogating SGRM, CpdA.

4.2.3 GR α dimerization is required for down-regulation of the GR α protein

So far, the current study has established that the extent (Fig. 4.3) and rate (Fig. 4.4) of hGRwt protein down-regulation is ligand-selective. Of note was the ability of CpdA treatment to abrogate basal hGRwt degradation (Fig. 4.4), in agreement with its previously published ability to preserve receptor expression relative to the unliganded GR α ³⁰⁻³². This characteristic of CpdA treatment, combined with its capacity to prevent or even abrogate^{33,43} GR α dimerization, sparked interest in a possible role for GR α dimerization in ligand-induced receptor down-regulation.

To determine the effects of ‘gain’ or ‘loss’ of GR α dimerization on GR α protein down-regulation, both hGRwt (capable of forming dimers following treatment with GR agonists) and hGRdim, a dimerization deficient mutant⁴⁴, were evaluated in COS-1 cells, in the presence of dimerization promoting (Dex or F) or abrogating (CpdA)^{33,43} GR α ligands. In addition, the effect of ‘loss’ (CpdA) or ‘gain’ (Dex and F) of GR α dimerization on hGRwt protein down-regulation was confirmed in HepG2 cells, containing endogenous human GR α (hGR α). In each figure, whole cell GR α -binding (Fig.4.5A and C) and Western blotting (Fig.4.5E), the values for unliganded GR α at each time point was set at 100% and represented by a dotted line (i.e. liganded GR α expression was normalised to unliganded GR α at each time point). This explains why the unliganded receptor degradation observed in Fig.4.4A is not apparent in these figures.

‘Gain’ of hGRwt dimerization via dimerization promoting GCs, Dex and F (10 μ M), induced a significant ($p < 0.001$) reduction in hGRwt protein expression over time (Fig. 4.5A and B). To validate the findings obtained with transiently transfected hGRwt, the effect of Dex and F treatment on endogenous hGR α protein expression was evaluated in HepG2 cells, which also showed significant ($p < 0.05$) reduction in receptor expression (Fig. 4.5E). Specifically at 24 hours, Dex and F treatment reduced the ‘pool’ of transiently transfected hGRwt to a mere 38 and 46% of the unliganded GR α (100%), respectively (Fig. 4.5A and B), while similarly, but slightly less robustly, endogenous hGR α protein expression in HepG2 cells was reduced to 52% and 69%, respectively (Fig. 4.5E). Furthermore, transiently transfected hGRwt protein expression was further reduced to 18% and 23% with Dex and F, respectively, reaching a maximum reduction in receptor expression after 48 hours (Fig. 4.5A and B).

In stark contrast, ‘loss’ of GR α dimerization through the use of hGRdim resulted in no significant reduction of hGRdim protein expression, following Dex and F (10 μ M) treatment (Fig. 4.5C and D). In support of the hGRdim result, CpdA treatment, known to abrogate GR α dimerization^{33,43}, of the hGRwt did not result in a reduction in hGRwt protein expression (Fig.4.5A and B). In addition, due to the lack of an endogenous dimerization deficient mutant in the HepG2 cells, CpdA treatment was used as a molecular tool to mimic the effect of ‘loss’ of dimerization^{33,43} (Fig. 4.5E). As with

the transiently transfected hGRwt, CpdA treatment did not lead to down-regulation of the endogenous hGR α protein (Fig. 4.5E).

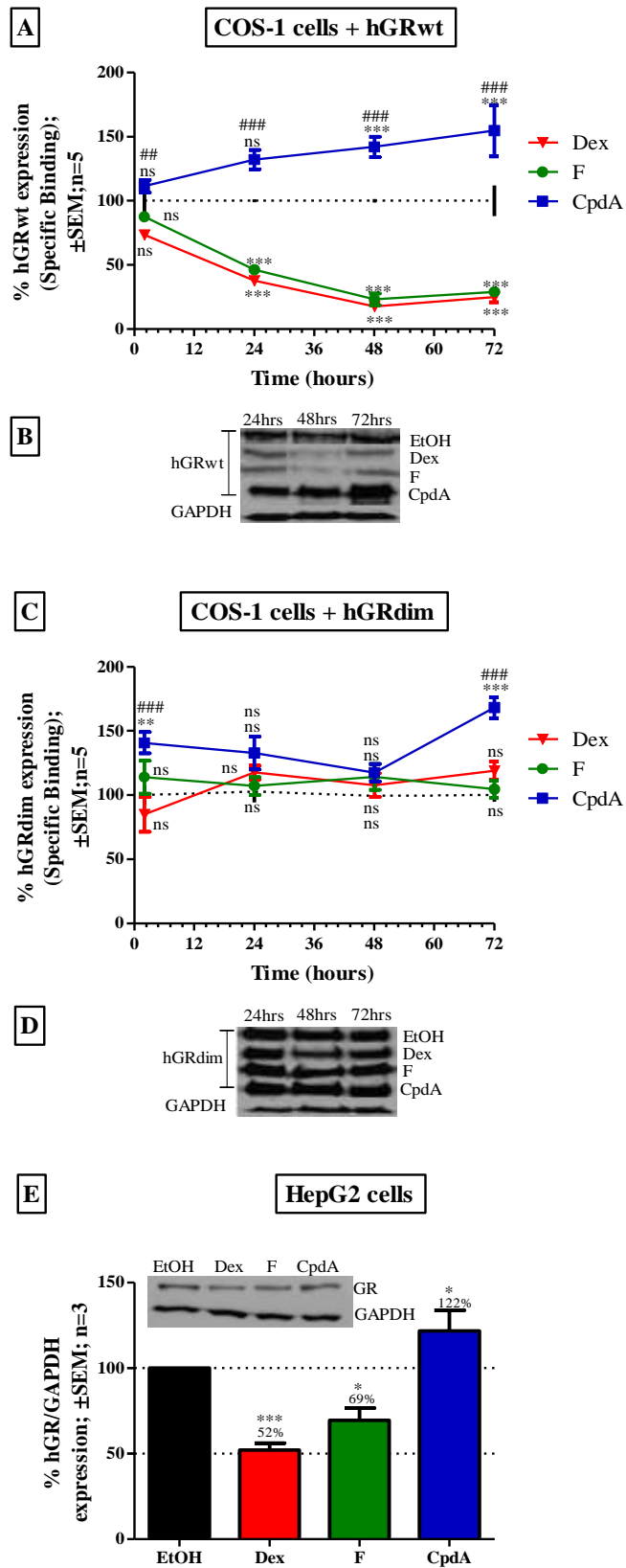


Figure 4.5: GR α dimerization is required for ligand-induced GR α protein down-regulation. COS-1 cells were seeded into a 24 well plate (5×10^4 cells/well) and transfected the next day with hGRwt (A and B) and hGRdim (C and D), using a transfection agent. Following 24 hours incubation, cells were treated with Dex, F or CpdA (10^{-5} M) for 2-72 hours. Thereafter hGRwt (A) and hGRdim (C) expression was monitored via whole cell GR α -binding, using 20nM [3 H]-Dex. Whole cell GR α -binding results shown are representative of five independent experiments (average \pm SEM), conducted in triplicate. In the graphs, the dotted line represents GR α (hGRwt or hGRdim) expression in presence of solvent (EtOH) and is set at 100%. Statistical analysis for (A) and (C) was conducted using a two-way ANOVA followed by a Bonferroni post-test comparing experimental values to solvent (EtOH) (ns, $p > 0.05$, **, $p < 0.01$, ***, $p < 0.001$) or to Dex (##, $p < 0.01$, ###, $p < 0.001$). GR α protein expression, hGRwt (B) and hGRdim (D) were confirmed by Western blotting. The 24 hour treatment time point was repeated, with all ligands, in the HepG2 cells (containing endogenous hGR α) (E, inset), which were seeded into a 12 well plate (5×10^4 cell/well). GR α expression was assessed using Western blotting where GAPDH was probed to ensure equal protein loading. Western blots shown are representative of three independent experiments. For quantification (E), the intensity of the hGR α and GAPDH bands was determined using UNSCANIT and hGR α levels were then normalised to GAPDH expression and expressed as a percentage (average \pm SEM) of hGR α expression in presence of the solvent (EtOH), which was set at 100% (dotted line). Statistical analysis for (E) was conducted using a one-way ANOVA with a Dunnett post-test comparing experimental values to solvent (EtOH) (*, $p < 0.05$, ***, $p < 0.001$).

To our knowledge, this is the first time that the ability of the hGRdim to undergo ligand-induced down-regulation has been investigated and collectively, the results obtained in the current study strongly suggest that the dimerization state of the GR α protein is important for its efficient ligand-induced turnover. Thus ‘loss’ of GR α dimerization through monomer favouring GR α (i.e. hGRdim and CpdA treated hGRwt), in contrast to ‘gain’ of GR α dimerization (i.e. Dex or F treated hGRwt), may be evading molecular mechanisms involved in regulating ligand-induced receptor turnover. Despite the fact that it was clear that the conformation of the receptor (dimer versus monomer) affected the sensitivity to the ligand-induced reduction of the ‘functional pool’ of GR α protein, it was not yet clear from these results at which level: ‘push’ or replenishment (protein synthesis) versus ‘pull’ or depletion (protein degradation), respectively, ‘loss’ of GR α dimerization exerted its effects.

4.2.4 ‘Push’ versus ‘pull’ mechanism

Cellular protein expression is maintained through a dynamic state of synthesis and degradation, which may be described by a simple ‘push’ versus ‘pull’ mechanism (Fig.4.1). Specifically for the GR α , the ‘push’ involves transcription of the GR α gene (NR3C1)^{52,53} to produce GR α mRNA and the subsequent translation of this GR α mRNA to form the GR α protein (Fig.4.2). The GR α protein may then be subjected to degradation (‘pull’), which a number of studies have suggested occurs via the proteasome^{22,28,29,54} (Fig.4.2).

Having established a role for GR α dimerization in ligand-induced GR α protein down-regulation, it seemed necessary to delve deeper, in an attempt to elucidate at what level, transcription, translation or degradation, ‘loss’ of GR α dimerization was preventing down-regulation and maintaining the ‘functional pool’ of GR α . To do this, two inhibitors, CHX and MG132 (1 μ M), were used to inhibit translation and proteasomal degradation, respectively (Fig.4.6). Following treatment with the inhibitors, cells were treated with Dex, F or CpdA (10 μ M) and the effect of translational inhibition

by CHX (Fig.4.7) or proteasomal inhibition by MG132 (Fig.4.8) on ligand-induced GR α degradation determined.

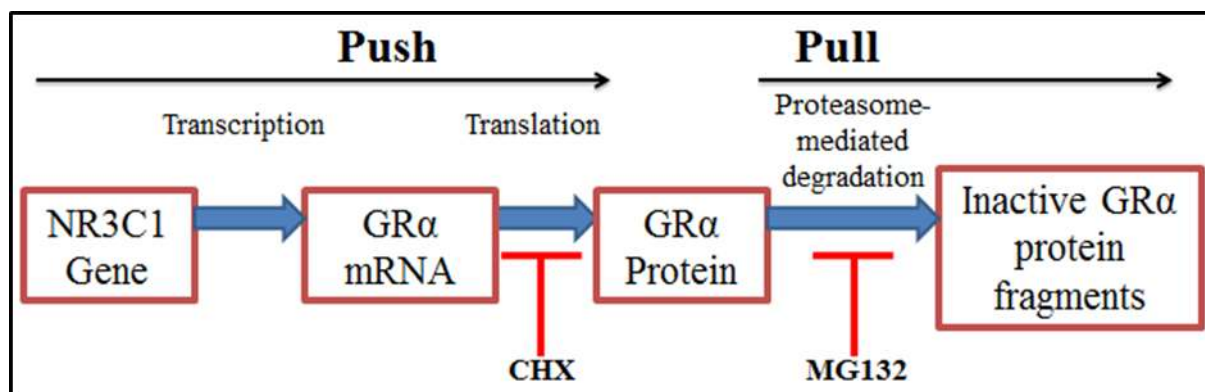


Figure 4.6: Specific regulation of GR α described by a simple ‘push’ vs. ‘pull’ mechanism, including the inhibitors used, CHX⁵⁵ and MG132, to inhibit translation and proteasome degradation, respectively.

4.2.4.1. Ligand-induced down-regulation of the GR α protein is unaffected by inhibiting new protein synthesis

Previous studies have noted ligand-induced transcriptional and translational regulation of the GR α ^{30,34,36–38,56,57}, indicating that the ligand-bound GR α is able to down-regulate its own production by altering the expression of its gene, NR3C1. In addition, ligand-bound GR α may alter the stability of its mRNA³⁷. Both of these scenarios result in a decrease in GR α mRNA, which may be reflected at the GR α protein level as a reduction in the GR α protein ‘pool’. When investigating the translational regulation of the GR α mRNA, one may look at the effects on the GR α mRNA or on the GR α protein ‘pool’ (Fig. 4.6). In the current study, it was decided to firstly investigate the effects of inhibiting translation, with CHX⁵⁵, on the extent of ligand-induced GR α protein down-regulation.

To investigate the effects of inhibiting translation on ligand-induced GR α protein down-regulation, COS-1 cells, transfected with either hGRwt (Fig.4.7A and B) or hGRdim (Fig.4.7A and C), and HepG2 cells, containing endogenous hGR α (Fig.4.7D), were treated with solvent (EtOH), Dex, F or CpdA (10 μ M) in the absence or presence of CHX.

If there was significant regulation of the GR α ‘pool’ at the level of the ‘push’ (transcription or translation) one would expect less ligand-induced GR α protein down-regulation when translation was inhibited. On the other hand, if regulation at this level did not contribute significantly to the overall ligand-induced reduction in the ‘pool’ of receptor, the extent of ligand-induced GR α protein down-regulation would remain the same in the presence of CHX.

As Figure 4.4 suggests, unliganded hGRwt underwent basal receptor turnover in the absence of CHX, thereby reducing the hGRwt protein ‘pool’. This effect was absent in Fig.4.7A and B because

the normalised hGRwt protein expression for the solvent (EtOH) in the absence of CHX (-CHX) was set at 100% and represented by a dotted line. To determine whether the GR α protein undergoes further down-regulation in the presence of CHX, unliganded hGRwt and hGRdim protein expression was assessed in the absence and presence of CHX. As Fig. 4.7A demonstrates, inhibiting translation with CHX, led to a further non-significant 15.8% reduction in hGRwt, but not hGRdim, protein expression.

Having addressed the effect of translational inhibition on unliganded hGRwt and hGRdim protein down-regulation, the effect of CHX on ligand-induced GR α protein down-regulation was investigated. Important to note, however, GR α protein expression was normalised relative to unliganded GR α 'pool' in absence or presence of CHX, which was set at 100%.

Firstly, the ability of dimerization promoting GCs Dex and F, to induce hGRwt or endogenous hGR α protein down-regulation was confirmed in the absence of CHX (Fig. 4.7B and D). Secondly, inhibiting translation with CHX had no significant effect on the level of Dex or F mediated hGRwt or endogenous hGR α protein down-regulation (Fig.4.7B and D). Specifically, Dex treatment reduced transiently transfected hGRwt protein expression to 62% and 64%, in the absence and presence of CHX, respectively (Fig. 4.7B). Likewise, following Dex treatment, endogenous hGR α protein expression was reduced to 51% and 60%, in the absence and presence of CHX, respectively (Fig. 4.7D). Almost identical results in the absence or presence of CHX were also observed for F via hGRwt and endogenous hGR α (Fig. 4.7B and D).

In the case of a 'loss' of receptor dimerization, using transiently transfected hGRdim in the COS-1 cells (Fig. 4.7C), or the molecular tool, CpdA treatment, for hGRwt and endogenous hGR α (Fig. 4.7B and D), inhibiting translation, with CHX, had no significant ($p > 0.05$) effect on receptor protein expression.

In conclusion, inhibiting translation had no significant effect on the extent of ligand-induced GR α protein down-regulation for hGRwt, hGRdim or the endogenous hGR α . Thus, these results provided reliable evidence that regulation of GR α protein expression via transcriptional and post-transcriptional processes was negligible in the test systems, COS-1 and HepG2 cells, used in this study. At this point it was postulated that the majority of ligand-induced GR α protein down-regulation was mediated via the 'pull' or post-translational processes (e.g. proteasomal degradation).

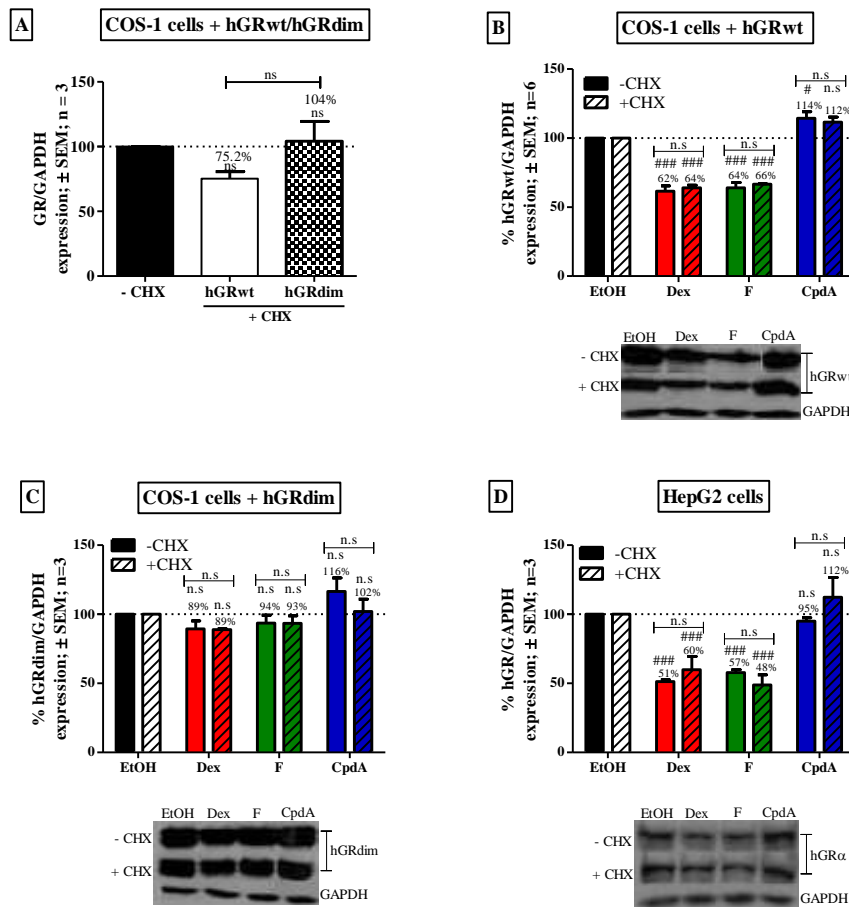


Figure 4.7: Ligand-induced GR α protein down-regulation is unaffected by new protein synthesis. COS-1 cells were seeded in a 12 well plate (5×10^4 cells/well) and transfected the next day with either hGRwt (A and B) or hGRdim (A and C) using a transfection agent. Following 24 hours incubation, cells were treated with solvent (EtOH) or $1\mu\text{M}$ CHX for 1 hour and, in the absence (- CHX) or presence of CHX (+ CHX), with solvent (EtOH) or the compounds, Dex, F and CpdA (10^{-5}M), for a further 16 hours. GR α protein expression was assessed by Western blotting, where GAPDH was probed to ensure equal protein loading. The Western blots shown (B, C and D inset) are representative of three independent experiments. For quantification (A, B, C and D), the intensity of the GR α and GAPDH bands was determined using UNSCANIT and the GR α expression was then normalised to GAPDH expression expressed as a percentage (average \pm SEM). Firstly, in the presence of solvent (EtOH), the effect of CHX (+ CHX) on unliganded GR α protein expression was investigated (A) and compared to GR α of expression in the absence of CHX (- CHX). Thereafter, the effect of CHX (+ CHX) on the extent of hGRwt (B), hGRdim (C) and endogenous hGR α (D) ligand-induced down-regulation was investigated. The dotted line, in all graphs, represents GR α expression in the absence (- CHX) and/or presence of CHX (+ CHX) and in the presence of solvent (EtOH) and is set at 100%. To analyse the effects of the ligands on GR α expression in the absence (- CHX) and presence (+ CHX) of CHX, statistical analysis was conducted using a one-way analysis of variance (ANOVA) with a Bonferroni post-test comparing GR α expression post Dex, F and CpdA-treatment to the solvent (EtOH) ($\#$, $p < 0.05$, $\###$, $p < 0.001$) (B, C and D). To evaluate the significance of adding CHX (+ CHX) on the extent of ligand-induced GR α protein down-regulation, a two-way ANOVA was used followed by a Bonferroni post-test (ns, $p > 0.05$) (B, C and D).

4.2.4.2. Ligand-induced down-regulation of the GR α protein occurs predominantly via the proteasome

Ligand-induced GR α protein down-regulation was unaffected by inhibiting protein synthesis ('push'). Worded differently, the effect of the ligand bound GR α on down-regulation of the GR α gene (NR3C1) and GR α mRNA stability appeared to be negligible in the test systems, COS-1 and HepG2 cells (Fig.4.7B, C and D), used. This result suggested that ligand-induced regulation of the 'functional pool' of GR α most likely occurs predominantly at the level of the 'push' or proteasomal degradation.

Indeed, there is strong evidence demonstrating that unliganded and ligand-induced GR α protein down-regulation occurs via a proteasome-dependent protein degradation pathway^{22,28,29,54,58,59}. Thus, a possible role for the proteasome in mediating ligand-induced receptor turnover, in the test systems used in the current study, was investigated.

To explore the role of the proteasome, the effects of inhibiting proteasomal degradation, with the proteasome inhibitor MG132, on ligand-induced GR α protein down-regulation was investigated. COS-1 cells, transfected with either hGRwt (Fig.4.8A and B) or hGRdim (Fig.4.8A and C), and HepG2 cells, containing endogenous hGR α (Fig.4.8D), were treated with solvent (EtOH), Dex, F or CpdA (10 μ M) in the absence or presence of MG132.

If GR α protein down-regulation occurs predominantly via the proteasome, one would expect that inhibiting proteasomal degradation with MG132 would prevent receptor turnover.

Figure 4.4 suggests that unliganded hGRwt underwent basal receptor turnover in the absence of MG132, thereby reducing the protein 'pool' of hGRwt. To determine whether basal GR α protein down-regulation was mediated via the proteasome, unliganded hGRwt and hGRdim protein expression was assessed in the absence and presence of MG132 (Fig. 4.8A). Clearly, inhibiting proteasomal degradation, with MG132, resulted in a significant ($p < 0.01$) 5.6-fold increase in basal hGRwt expression and a non-significant 2.3-fold increase in basal hGRdim protein expression (Fig. 4.8A), compared to in the absence of MG132.

Having addressed the effect of proteasomal inhibition on unliganded hGRwt and hGRdim protein down-regulation, the effect on ligand-induced GR α protein down-regulation in the presence of MG132 was investigated. Important to note, GR α protein expression was normalised relative to unliganded GR α 'pool' in absence or presence of MG132, which was set to 100%.

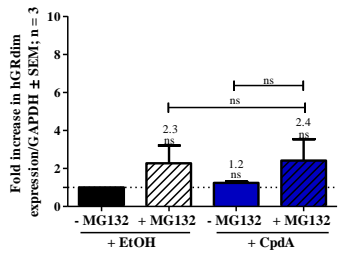
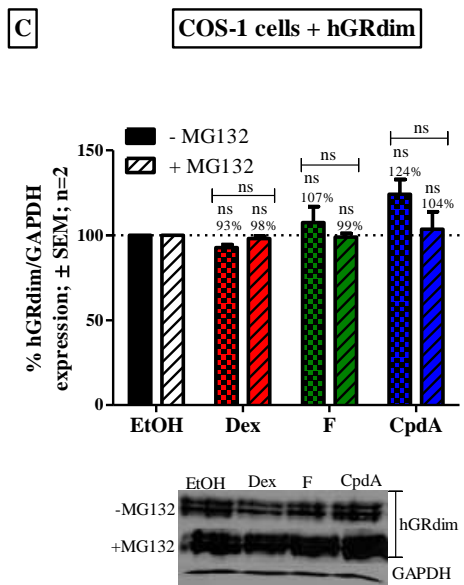
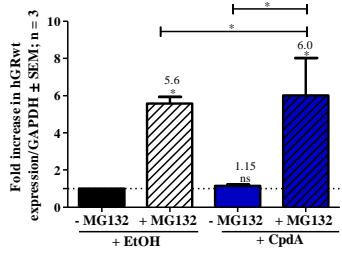
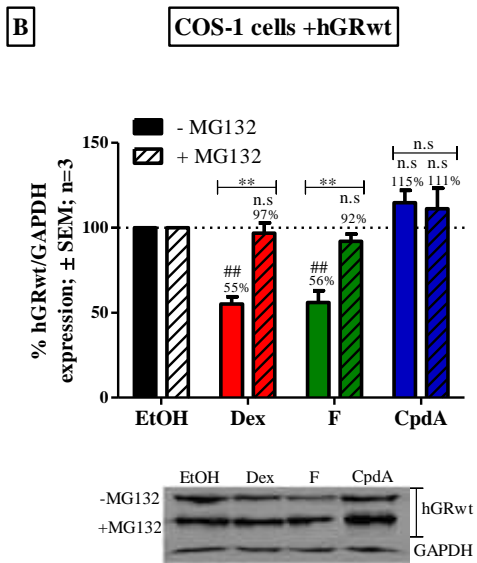
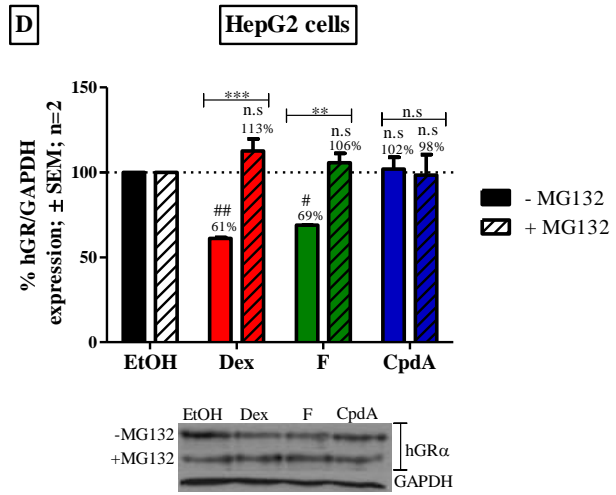
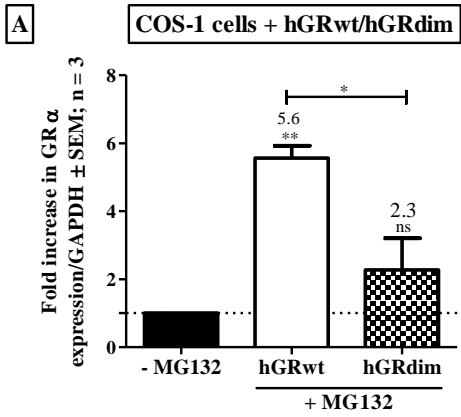


Figure 4.8: Ligand-induced GR α protein down-regulation occurs predominantly via the proteasome. COS-1 cells were seeded in a 12 well plate (5×10^4 cell/well) and transfected the next day with either hGRwt (A and B) or hGRdim (A and C) using a transfection agent. Following 24 hours incubation, cells were treated with solvent (DMSO) or $1\mu\text{M}$ proteasome inhibitor (MG132) for 1 hour and then, in the absence (- MG132) or presence of MG132 (+ MG132), treated with solvent (EtOH) or the compounds Dex, F and CpdA (10^{-5}M) for 16 hours. GR α protein expression was assessed by Western blotting, where GAPDH was probed to ensure equal protein loading. The Western blots shown (B, C and D inset) are representative of three independent experiments. For quantification (A, B, C and D), the intensity of the GR α and GAPDH bands was determined using UNSCANIT and then the GR α expression was normalised to GAPDH expression and expressed as a percentage (average \pm SEM). Firstly, the effect of MG132 (+ MG132) on unliganded GR α protein expression was investigated (A) and compared to GR α of expression in the absence of MG132 (- MG132). Thereafter, the effect of MG132 (+ MG132) on the extent of hGRwt (B), hGRdim (C) and endogenous hGR α (D) down-regulation was investigated. The dotted line, on all graphs, represents the fold increase in GR α expression in the presence of solvent (EtOH) and/or absence of MG132 (- MG132) and is set at 1-fold or 100%. To demonstrate that the effects of ligands on GR α expression in the absence (- MG132) and presence (+ MG132) of MG132, statistical analysis was conducted using a one-way analysis of variance (ANOVA) with a Bonferroni post-test comparing to control (- MG132, EtOH) (n.s, $p > 0.05$; #, $p < 0.05$; ##, $p < 0.001$). To analyse the significance of adding MG132 on the extent of ligand-induced GR α protein down-regulation, a two-way ANOVA was used followed by a Bonferroni post-test (ns, $p > 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

Firstly, the ability of dimerization promoting GCs, Dex and F, to induce hGRwt and endogenous hGR α protein down-regulation was confirmed in absence of MG132 (Fig. 4.8B and D). Secondly, inhibiting proteasomal degradation (i.e. in the presence of MG132) completely abolished Dex and F mediated hGRwt and endogenous hGR α protein down-regulation (Fig. 4.8B and D). Specifically, Dex treatment significantly ($p < 0.01$) reduced transiently transfected hGRwt protein expression to 55% of the solvent (EtOH) treated hGRwt expression in the absence of MG132 but was unable to reduce hGRwt expression (97%) in the presence of MG132 (Fig. 4.8B). Likewise, following Dex treatment, endogenous hGR α expression was significantly ($p < 0.01$) reduced to 61% of the solvent (EtOH) in the absence of MG132 but in the presence of MG132, hGR α protein expression was similar (113%) to that of the solvent (EtOH) (Fig. 4.8D). An almost identical result was also observed for F via hGRwt and the endogenous hGR α (Fig. 4.8B and D).

‘Loss’ of GR α dimerization using transiently transfected hGRdim in the COS-1 cells (Fig. 4.8C), or the molecular tool, CpdA treatment, for the transiently transfected hGRwt and endogenous hGR α (Fig. 4.8B and D), prevented ligand-induced receptor down-regulation in the absence MG132. Furthermore, blocking proteasomal degradation by MG132 did not significantly affect hGRdim expression (Fig. 4.8C) and following Dex treatment, hGRdim expression was maintained at 93% and 98%, in the absence and presence of the inhibitor, MG132, respectively (Fig. 4.8C). An almost identical result was observed following F treatment (Fig. 4.8C). In addition, transiently transfected hGRwt and endogenous hGR α expression was not significantly altered following CpdA treatment, whether in the absence or presence of MG132 (Fig. 4.8B and D).

Lastly, it is important to note the absolute and significant ($p < 0.05$) difference between transiently transfected hGRwt expression following CpdA treatment in the absence (1.15-fold) and presence (6-fold) of MG132 (Fig. 4.8B inset), which was masked in Figure 4.8B. Furthermore, a slight but significant ($p < 0.05$) increase (5.6-fold versus 6-fold) was observed when comparing unliganded

and CpdA-treated hGRwt in the presence of MG132 (Fig. 4.8B inset). In contrast, when investigating the absolute difference between hGRdim protein expression following CpdA treatment in the absence (1.2-fold) and presence of MG132 (2.4-fold) (Fig. 4.8C inset) a considerably lower fold increase in receptor expression was noted. In addition no significant increase (2.3-fold versus 2.4-fold), relative to unliganded hGRdim (- MG132), was observed when comparing unliganded and CpdA-treated hGRdim in the presence of MG132 (Fig. 4.8C inset).

In summary, the unliganded hGRwt, but not hGRdim, underwent significant basal protein down-regulation, which was mediated by the proteasome. Furthermore, the ligand-induced (Dex and F) down-regulation of transiently transfected hGRwt and endogenous hGR α protein was abolished by the proteasome inhibitor, MG132. Additionally, inhibiting proteasomal degradation did not alter CpdA-treated hGRwt (Fig. 4.8B) or endogenous hGR α (Fig. 4.8D) protein expression nor did it affect Dex and F treated hGRdim (Fig. 4.8C) protein expression.

Taken together, the last two figures (Fig.4.7 and 4.8) provide substantial evidence that the proteasomal degradation pathway plays a predominant role in orchestrating the turnover of the GR α protein. At this point, it was assumed that it was at this level of proteasome degradation that hGRdim and CpdA-treated hGRwt were evading degradation due to their monomeric-favouring GR α conformations.

4.2.5 CpdA treatment does not affect proteasome function

To rule out the possibility of a direct effect of CpdA treatment on proteasomal function, the degradation of the p53 protein was investigated in the absence and presence of CpdA. The p53 protein is known to have a short half-life⁵⁶, which has been reported to be between 3 and 22 hours in different cells⁵⁷, and is degraded via the proteasome. If CpdA treatment was directly disrupting the proteasome function, one could assume that the p53 protein expression would not be reduced in the presence of CHX.

COS-1 cells (Fig. 4.9A) and HepG2 cells (Fig. 4.9B), were treated with solvent (EtOH) or the translation inhibitor, CHX, to prevent new protein synthesis of p53. Subsequently, cells were treated with solvent (EtOH) or CpdA (10 μ M) and p53 expression analysed using Western blotting. Inhibiting p53 protein synthesis, by blocking translation with CHX, resulted in a significant ($p < 0.05$) reduction in p53 protein to 49% of expression in the absence of inhibitor, suggesting successful p53 protein turnover (Fig.4.9A). Similar results were seen in HepG2 cells (Fig. 4.9B).

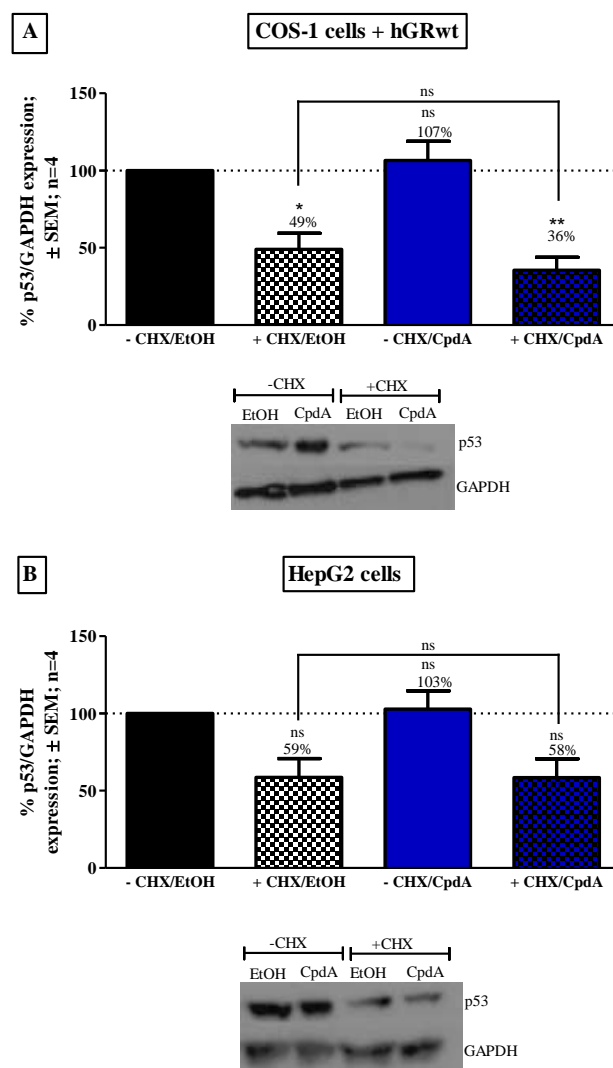


Figure 4.9: CpdA treatment does not affect proteasome function. COS-1 (A) and HepG2 cells (B) were seeded in a 12 well plate (5×10^4 cells/well). Following 24 hours incubation, cells were treated with EtOH (solvent) or $1\mu\text{M}$ CHX for 1 hour and, in the absence (- CHX) or presence of CHX (+ CHX), with EtOH (solvent) or CpdA (10^{-5}M) for 16 hours. The p53 protein expression was assessed using Western blotting, where GAPDH was probed to ensure equal protein loading. The Western blots shown below the graphs (A and B inset) are representative of three independent experiments. For quantification, the intensity of the p53 and GAPDH bands was determined using UNSCANIT and subsequently the p53 expression was normalised to GAPDH expression and expressed as a percentage (average \pm SEM) of p53 expression in the presence of solvent (EtOH), which is set at 100% (dotted line). To demonstrate the effects of CHX and CpdA on p53 expression, statistical analysis comparing all treated p53 protein expression to -CHX/EtOH (control) was conducted using a one-way analysis of variance (ANOVA) with a Bonferroni Multiple comparisons post-test (ns, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$).

In the absence of CHX but in the presence of CpdA, p53 protein expression (107%) was comparable to solvent (EtOH) treated expression (100%), in the absence of CHX. Moreover, and most importantly, in the presence of CHX and CpdA, p53 protein expression was reduced to 36%, a reduction not significantly different from that of the solvent (EtOH) in the presence of the inhibitor. Similar results were seen in HepG2 cells (Fig. 4.9B).

Together, these results suggest that p53 was as efficiently degraded following CpdA treatment as in its absence, and confirmed that CpdA does not directly inhibit proteasome function but rather prevents GR α degradation by inducing a preferential monomeric conformation of the GR α .

4.3 Discussion

Overcoming inflammation, specifically in a number of autoimmune diseases, is gradually becoming more and more difficult. Currently, a number of challenges exist in managing chronic inflammation, but central to the current study is the development of GC acquired resistance.

GC sensitivity is directly proportional to the “functional pool” of GR α available within a cell, tissue or individual^{5,17–19,55}. This GR α ‘functional pool’ may be affected by various factors, the focus of this study, however, is ligand-induced down-regulation of the GR α protein^{26,27,29,38,41–43,58}.

Although, the ability of certain ligands to alter the GR α ‘functional pool’ via autologous down-regulation is well documented^{26,27,29,38,41–43,58}, the factors (such as receptor conformation induced by cognate GR α ligands) that influence the extent of this ligand-induced receptor down-regulation are not entirely understood.

In essence, the current chapter established a novel and fundamental role for ligand-induced receptor conformation (i.e. dimer vs. monomer) in determining the extent of receptor turnover. Using two simple concepts, namely ‘gain’ or ‘loss’ of GR α dimerization, this study cements a link between receptor dimerization and GR α protein down-regulation.

The premise of a ‘loss’ of receptor dimerization preventing GR α protein turnover originated from two interesting, previously published, characteristics of the SGRM, CpdA. Specifically, that CpdA treatment does not lead to receptor turnover^{30,32} or dimerization^{33,43}. In fact, CpdA had been shown to abrogate ligand-independent dimers⁴³ resulting in a severe ‘loss’ of GR α dimers. These findings provided a platform from which this study was launched, which confirmed a role for GR α dimerization in receptor turnover. Throughout, the effects of a ‘loss’ of GR α dimerization, either ligand-induced (CpdA) or through use of a mutant (hGRdim), were compared to the effects of a ‘gain’ of receptor dimerization on GR α protein turnover.

Results comparing the ability of dimerization promoting (Dex and F) and dimerization abrogating (CpdA) ligands to mediate ligand-induced GR α protein down-regulation of the wild type receptor, hGRwt, yielded some interesting details with regards to the efficacy and potency of these ligands to induce receptor turnover but also provided results on the rate of degradation and consequent half-life of the GR α protein, following prolonged GC treatment.

In agreement with previous published results^{30,41,60}, ligand-induced ‘loss’ of receptor dimerization through dimerization abrogating CpdA treatment, did not lead to the same extent of GR α protein down-regulation as dimerization promoting, Dex and F treatment did in COS-1 cells transiently transfected with hGRwt (Fig. 4.4A, Fig. 4.5A and B) or in HepG2 cells (Fig. 4.5E) containing endogenous GR α . In fact the extent of down-regulation of the unliganded receptor was significantly greater than that of the CpdA treated hGRwt after 48 and 72 hours, respectively (Fig. 4.4A). This was reflected in the difference of the half-life values obtained for the unliganded and CpdA treated hGRwt where the current study reported a half-life for hGRwt of over 72 hours following CpdA treatment, which was slightly higher than that of the unliganded hGRwt (70 hours) (Fig. 4.4B). In contrast, Avenant *et al.*⁴¹ notes a slightly lower GR α half-life (42 hours) following CpdA treatment, relative to the unliganded receptor (44 hours).

The half-life (70 hours) of the unliganded hGRwt determined in the current study (Fig. 4.4B) was higher than the unliganded GR α half-life reported by Avenant *et al.*⁴¹. It was also higher than the reported half-lives of unliganded GR α (between 16 - 22 hours) in studies using other cell lines, namely rat HTC cells³⁴, U2OS osteosarcoma cells and A459 cells⁶¹, and other GR α species (i.e. mouse/rat constructs or endogenous human GR α)^{27,28}. This discrepancy may be attributed to the use of translation inhibitors such as doxycycline or CHX^{27,28,34,61}, which were not used in this experiment in the current study.

In stark contrast to CpdA, ligand-induced ‘gain’ of dimerization by dimerization promoting GCs, Dex and F, accelerated GR α protein down-regulation and led to an increase in the rate of receptor turnover and a reduction in the receptor half-life (Fig. 4.4B and C). Specifically, Dex and F treatment significantly increased the rate of hGRwt protein down-regulation by 3-fold, relative to the unliganded receptor turnover. In terms of half-lives, the absolute values of GR α half-lives (Fig. 4.4B), following treatment with Dex (21 hours) and F (22 hours), in the current study, were considerably higher than previously reported values, which range from between 3 and 12 hours, post ligand treatment^{26,27,34,42,61}. However, the 3.3- (Dex) and 3.5-fold (F) reduction, relative to the unliganded receptor, in the hGRwt protein half-lives obtained, corresponded well to the 4.4- (Dex) and 3.5-fold (F) reduction reported by Avenant *et al.*⁴¹. Furthermore, the 3.3-fold decrease noted in the hGRwt half-life after Dex treatment is comparable to additional studies, which suggested fold decreases of 2 to 3-fold.

At saturating concentrations of the dimerization promoting GCs, Dex and F, no notable differences in their ability to induce maximal ligand-induced GR α protein down-regulation were observed (Fig. 4.3B). However, a 50-fold difference in the potencies of Dex (0.4 nM) and F (20 nM) (Fig. 4.3C) to facilitate ligand-induced GR α protein down-regulation was revealed using a dose-response curve

(Fig. 4.3A). One could argue that this may be due to differences in the ligand binding affinities of these two GCs. Indeed Dex has been reported to have a considerably higher affinity than F for GR α ⁵⁰. Specifically in a study by He *et al.*⁵⁰ Dex had an 11-fold higher affinity for the GR α than F, with K_d values for these GCs, determined as 8 and 91 nM, respectively (Table 4.2). This difference in the reported ligand binding affinities of Dex and F was not unforeseen as synthetic ligands have been designed to mimic and augment the biological action of endogenous GCs. However, this difference in affinity cannot be directly correlated to the difference in the potencies of these GCs to induce down-regulation because of a non-linear increase in the receptor F.O with increasing ligand concentrations (Table 4.1). Therefore to address this, the F.Os of hGRwt by Dex and F were calculated at a single ligand concentration (1 nM) and the difference observed in the F.Os was directly compared to the difference in the percentage of down-regulation observed at the same concentration of ligand (Table 4.2). Thus, treatment with ligands (1 nM) resulted in 11 and 1% of hGRwt occupied by Dex and F, respectively, which suggested an 11-fold higher occupancy of hGRwt by Dex at this single ligand concentration (Table 4.2). One would expect a consequent 11-fold increase in the ability of Dex to induce hGRwt receptor turnover, however this was not the case. Treatment with Dex and F (1 nM) resulted in an 86 and 2% reduction in hGRwt protein expression, respectively (Table 4.2). Thus, a considerably larger 43-fold difference in the ability of Dex (1 nM) to induce receptor turnover, after treatment for 24 hours, was noted (Table 4.2).

Table 4.2: Correlation of the extent of hGRwt protein down-regulation with fractional occupancy of Dex and F

	Dex	F	Fold difference ^e
K_d (ligand binding nM)^a	8	91	11
IC₅₀ (down-regulation nM)^b	0.4	20	50
% F.O at 1nM ligand concentration^c	11	1	11
hGRwt down-regulation at 1 nM (%)^d	83	2	43

^a K_d values for Dex (8 nM) and F (91 nM) provided by He *et al.*⁵⁰. ^b The potency (IC₅₀) values for hGRwt protein down-regulation mediated by either Dex or F from Figure 4.3. ^c Fractional occupancy at 1nM ligand concentration using K_d values⁵⁰. ^d Percentage hGRwt protein down-regulation, relative to maximal down-regulation 100%, following ligand treatment (1 nM), for 24 hours. ^e The fold difference was calculated by dividing the values for Dex (first column) by the values for F (second column).

One could postulate that at lower, non-saturating ligand (1 nM) concentrations Dex treatment resulted in a slightly different receptor conformation, when compared to that of F treatment, which resulted in a higher degree ('gain') of GR α dimerization that promoted in GR α protein down-regulation thus explaining the augmented extent of down-regulation. This postulation is supported by findings that suggests that the GR α antagonist, RU486, is less capable of inducing GR α dimers⁶² and also not as efficient as Dex at mediating receptor turnover⁴¹. In contrast, at saturating ligand concentrations (10 μ M), one could assume that maximal Dex and F-induced receptor dimerization and consequent receptor turnover occurred, which is supported by the similar reported efficacies for

these GCs, in the current study. These findings seem to support a role for the extent of GR α dimerization in mediating receptor turnover, however, the current study did not directly compare the degree of GR α dimerization induced by Dex or F and correlate this to the extent of receptor turnover, but rather through the investigation of ligand-induced receptor turnover of a dimerization deficient mutant, hGRdim, elegantly confirmed a role for GR α dimerization in receptor turnover.

‘Loss’ of dimerization, through the use of hGRdim (Fig. 4.5C and D), prevented the dimerization promoting ligands (Dex and F) from inducing receptor turnover, unlike with hGRwt (Fig. 4.5A and B) and the endogenous wild type hGR α (Fig. 4.5E). This novel finding reinforces the link between GR α dimerization and ligand-induced GR α protein down-regulation.

With acquired resistance to GC treatment gaining traction and in many cases posing major clinical challenges in treating chronic inflammation, the elucidation, by the current study, of this role of receptor dimerization in receptor turnover provides novel molecular insights into areas (i.e. receptor conformation) which may be targeted in an attempt to reverse or counteract resistance. One could postulate, in the case of a ligand-induced ‘loss’ of GR α dimerization, that GC sensitivity may be restored through maintaining the receptor ‘functional pool’, as the ability of patients to respond to GC treatment has been associated with higher GR α protein expression^{1,17,19}, however, extensive studies investigating responsiveness, in terms of GC responsive gene expression, would need to be conducted with dimerization abrogating GR α ligands.

Throughout this study a high degree of reproducibility, as illustrated in Table 4.3, was maintained. In independent experiments, the percentage of hGRwt protein expression following ligand treatment for 24 hours, at saturating concentrations, ranged from 39 to 41% for Dex, 46 to 48% for F and 123 to 124% for CpdA (Table 4.3). Specifically, the notable average reduction in hGRwt protein expression to 40% and 47%, following Dex and F treatment, respectively, was not unlike the 55% (Dex) and 60% (F) reduction in receptor expression observed by Avenant *et al.*⁴¹, using almost identical experimental conditions. These highly reproducible findings in the COS-1 cells were mirrored in the endogenous system, the HepG2 cells, where hGR α protein expression was reduced to 52% (Dex) and 69% (F) post-treatment with ligands (10 μ M) (Table 4.3). In agreement with these findings, although marginally different, a study using L β T2 cells, containing endogenous GR α showed a similar ligand-induced decrease in endogenous GR α protein expression to 60% (Dex) and 50% (F)⁴¹. Similar Dex and F mediated down-regulation of GR α has also been confirmed in a number of other test systems, under different treatment conditions^{28,29}. This ability to produce such reproducible results with the wild type receptor, suggested a robust system in which the role for GR α dimerization in receptor turnover was established.

When investigating at what level, namely the ‘push’ or the ‘pull’, abrogation of receptor dimerization prevented GR α down-regulation, results in the current study, strongly implicated the proteasome in orchestrating reductions in the GR α ‘functional pool’ through ligand-induced (specifically dimerization promoting Dex and F) receptor degradation of transiently transfected hGRwt (Fig. 4.8B) and endogenous hGR α (Fig. 4.8D). With the use of a proteasome degradation inhibitor, MG132, Dex and F-induced receptor turnover (driven by a ‘gain’ of GR α dimerization) of hGRwt and endogenous hGR α was completely abolished, and receptor expression was maintained (Fig. 4.8B and D). These findings are in accordance with previous studies, which have suggested the GR α is degraded via a proteasome-dependent pathway^{22,28,29,54}. Specifically, in COS-1 cells, transfected with wild type mouse or human GR α constructs^{28,29}, as well as in GrH2 cells²², a rat hepatoma cell line, and CHIP6B cells⁶³, derived from mouse hippocampal cells. In the case of a ‘loss’ of receptor dimerization via CpdA treatment of hGRwt (Fig. 4.8B) and endogenous hGR α (Fig. 4.8D) or use of hGRdim (Fig. 4.8C), receptor expression remained unaffected in the presence of the proteasome inhibitor. In addition, the possibility that CpdA had a direct inhibitory effect on the function of the proteasome was ruled out by investigating, for the first time, the effect of CpdA treatment on p53 turnover.

Table 4.3: The percentage of GR α protein expression remaining after ligand treatment in different independent experiments

		% GR α expression remaining following ligand treatment								
		COS-1		HepG2	COS-1		HepG2	COS-1		HepG2
		hGRwt	hGRdim	hGR α	hGRwt	hGRdim	hGR α	hGRwt	hGRdim	hGR α
Fig.	Time (hrs)	Dex (10 μ M)			F (10 μ M)			CpdA (10 μ M)		
4.3	24	41	-	-	46	-	-	-	-	-
4.4	24	39	-	-	47	-	-	123	-	-
4.5	24	39	118	52	48	107	69	124	133	122
4.7	16	62	89	51	64	94	57	114	116	95
4.8	16	55	93	61	56	107	69	115	124	102

Interestingly, down-regulation of unliganded hGRwt was also impeded in the presence of MG132, which suggested that basal receptor turnover is also mediated in a proteasome-dependent manner as is demonstrated by a 5.6-fold increase observed in the unliganded hGRwt protein expression in the presence of MG132 (Fig. 4.8A). These results are substantiated by a study from Yemelyanov *et al.*⁶⁴ in which another proteasome inhibitor, bortezomib (BZ), was used with GR α transfected LNCaP cells, PC3 cells, D4541, PCa cells, CMET-T lymphoblastic leukaemia cells and NCEB mantle cell lymphoma cells^{59,64}. Although a slight increase (2.3-fold) in unliganded hGRdim protein expression (Fig. 4.8A) was noted in the presence of MG132, this increase was significantly ($p <$

0.05) less than that observed for unliganded hGRwt, which suggests that although basal turnover of hGRdim via the proteasome occurs, it is not as drastic as unliganded hGRwt protein turnover and is likely due to hGRdim's impaired ability to form GR α dimers^{33,43}.

The fact that unliganded and ligand-induced GR α down-regulation relies on a dimeric form of the GR α and occurs predominantly via the proteasome, was supported by results obtained in the current study following translational inhibition, by CHX, which indicated that protein synthesis (i.e. 'push') did not affect the extent of receptor turnover. Moreover, any ligand-induced GR α protein down-regulation occurring at the level of the 'push' was insufficient to significantly alter the extent of the receptor 'functional pool', in the current test systems, COS-1 and HepG2 cells. In terms of basal receptor turnover, a decrease in hGRwt protein expression but not hGRdim expression was noted in the presence of CHX (Fig. 4.7A). This was to be expected, as the 'pool' of hGRwt was no longer in a dynamic state of synthesis ('push') and degradation ('pull') but rather being subjected only to degradation and in addition highlighted the inability of the 'pull' or degradation process to act on the 'pool' of, predominantly monomeric, hGRdim protein.

Taken together, the results in the current chapter propose that efficient degradation of the GR α protein relies on a dimeric conformation of the GR α (Fig. 4.10). Furthermore, results implicated the proteasome in regulating GR α protein expression, which suggests that the majority of ligand-induced GR α down-regulation occurs at the level of the 'pull' with minimal regulation occurring at the 'push' level. Understanding at what level the 'loss' of GR α dimerization prevents receptor turnover, provides insight for future therapeutic drug development to combat chronic inflammation. Whilst dimerization promoting GCs may be efficient for short-term treatment, they gradually become ineffective during long-term treatment regimes, with a loss in responsiveness through a reduction in the receptor 'functional pool'. Thus, one way to prevent the development of acquired GC resistance may be to design ligands, which fully or partially disrupt GR α dimerization and thus ultimately maintain receptor expression. These novel ligands could potentially be used alone or in combination with current potent anti-inflammatories, such as Dex, to reach and sustain desired therapeutic outcomes. In addition, understanding the role of receptor dimerization in ligand induced down-regulation could provide additional information when assessing congenital resistance to GC treatment, in patients with naturally occurring GR α mutations.

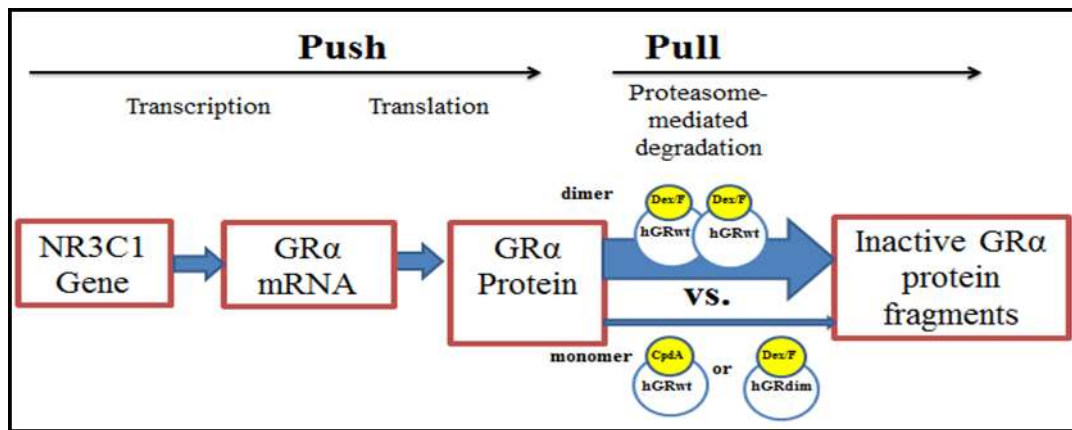


Figure 4.10: Ligand-induced ‘gain’ of GR α dimerization is a pre-requisite for efficient receptor turnover, which occurs predominantly via the proteasome.

4.4 Conclusion

Collectively, the initial results of the current study led to the discovery of a novel requirement for ‘gain’ of GR α dimerization for effective ligand-induced GR α protein down-regulation. In addition, ligand-induced receptor turnover was found to occur predominantly via the proteasomal degradation pathway, while translational regulation by GR α ligands in the test systems used in the current study is negligible. Lastly, evidence was provided that rules out the possibility of a direct effect of CpdA on proteasome function.

Taken together, results from the current chapter established an important factor for ensuring successful ligand-induced receptor turnover, namely GR α dimerization. These findings contribute to the understanding of one of the ways in which acquired GC resistance develops. This is particularly important as acquired GC resistance is gaining traction and hampering the battle against chronic inflammation. The level of GR α dimerization could potentially be exploited pharmacologically to overcome acquired GC resistance, however, a deeper understanding of the molecular mechanisms involved in the link between GR α dimerization and ligand-induced GR α protein down-regulation, is required. The next chapter delves deeper into these molecular mechanisms associated with proteasomal degradation.

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Chapter 5:

‘Loss’ of receptor dimerization modulates post-translational processing of the GR α and its turnover.

5.1. Introduction

The degradation of many intracellular proteins is mediated by the proteasome degradation pathway or UPS¹. This system consists of a large number of components, which function collectively to ensure the highly specific nature of the UPS¹. In general, proteins are tagged through covalent PTMs), which provide the signal for recognition by the catalytic proteasome that subsequently mediates the degradation of the protein substrates¹ (Fig. 5.1).

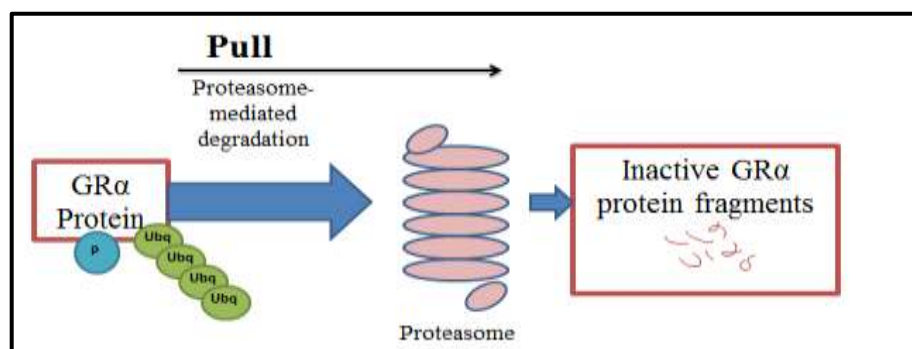


Figure 5.1: The GR α protein is post-translationally modified by PTMs such as phosphorylation (P) and ubiquitin (Ubq), leading to subsequent recognition and degradation by the proteasome, into inactive protein fragments.

Like many other cellular proteins, both unliganded and liganded GR α undergoes a number of PTMs including acetylation, sumoylation, and particularly important for this study, phosphorylation (P) and ubiquitination (Ubq)²⁻⁴. These PTMs are known to modulate GR α activity and function at a number of levels, and essentially contribute to the extensive heterogeneity observed in GR α signalling⁵.

Specifically, phosphorylation, the first identified⁶ and most common⁷ PTM, involves the covalent attachment of a phospho group to specific residues (i.e. serine and threonine) on a protein (i.e. GR α) by enzymes referred to as kinases⁸. The GR α undergoes intense phospho-regulation, predominantly at several identified serine residues but it can also occur at other residues, such as threonine⁹ (Fig. 5.2). One of the more recently discovered sites of GR α phosphorylation is of a serine residue at

amino acid position 404 (Ser404) in humans, which is mediated by a kinase known as glycogen synthase kinase 3 β (GSK3 β)⁸. It is thought that basal GR α phosphorylation at Ser404 occurs, but that treatment with certain ligands (e.g. Dex) induces hyper-phosphorylation of the receptor at this site⁸. Additionally, this ligand-induced hyper-phosphorylation at Ser404 has been shown to decrease overall GR α stability through priming the receptor for recognition by other UPS components, followed by additional tagging and subsequent proteasome degradation⁸.

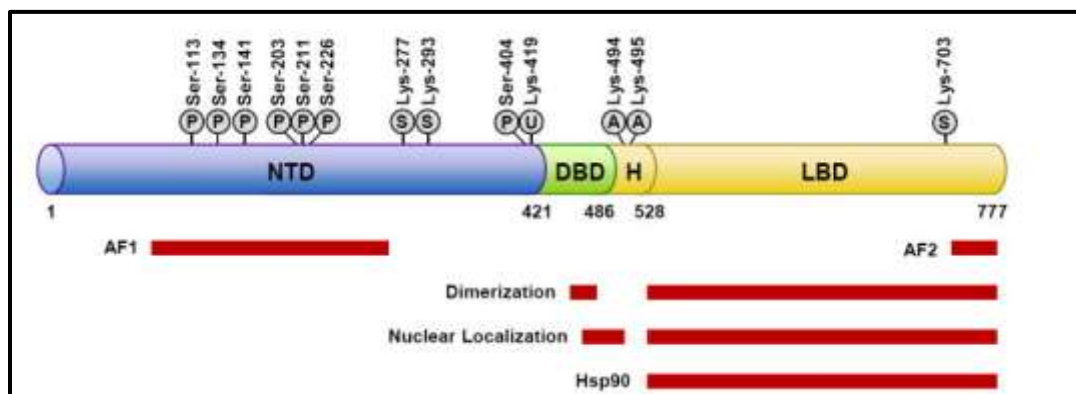


Figure 5.2: The domain structure of the human GR α and sites of post-translational modifications. A figure from Oakley et al.¹⁰ shows the N-terminal Domain (NTD, blue), the DNA-binding domain (DBD, green), the hinge region (H, yellow) and the ligand-binding domain (LBD, yellow). Represented (in red) are specific regions for activation domains (AF1 and AF2), the dimerization domain, the nuclear localization domain and the domain that regulates the interaction of GR α with Hsp90. Additionally, sites of PTMs are indicated for phosphorylation (P), sumoylation (S), ubiquitination (U) and acetylation (A).

Often, PTMs occur in a sequential manner. It is thought that the PTM, ubiquitination, is dependent on preceding protein phosphorylation¹. Unlike phosphorylation, ubiquitination of a protein requires a number of enzymes, with a hierarchy of specificity for the protein substrate, and occurs in a multi-step manner¹¹. Briefly, ubiquitin, a small (8kDa) regulatory protein, is activated in an energy dependent two-step process by constitutively active E1 activating enzymes¹¹ (Fig. 5.3). Following activation of the ubiquitin molecule, a member of the second group of enzymes, termed E2 conjugating enzymes, then bind the activated ubiquitin molecule and E1 activating enzyme complex (or intermediate) and mediate the transfer of the active ubiquitin to the E2 conjugating enzyme, forming a second intermediate¹¹ (Fig. 5.3). Subsequently, the ubiquitination of a protein substrate is completed by E3 ligases, which are highly substrate specific. This step involves the binding of a specific E3 ligase to the protein substrate, which then mediates the transfer of the activated ubiquitin molecule from the E2 conjugating enzyme to a specific residue, often a lysine, on the substrate¹¹. The binding of the highly specific E3 ligase to the target protein is thought to be mediated in both a ligand-independent and ligand-dependent manner¹². This process is then repeated and once multiple rounds of ubiquitin conjugation have occurred and a poly-ubiquitin chain is generated, the substrate is recognised by the large catalytically active proteasome, which mediates the subsequent degradation of the substrate into inactive protein fragments (Fig. 5.3).

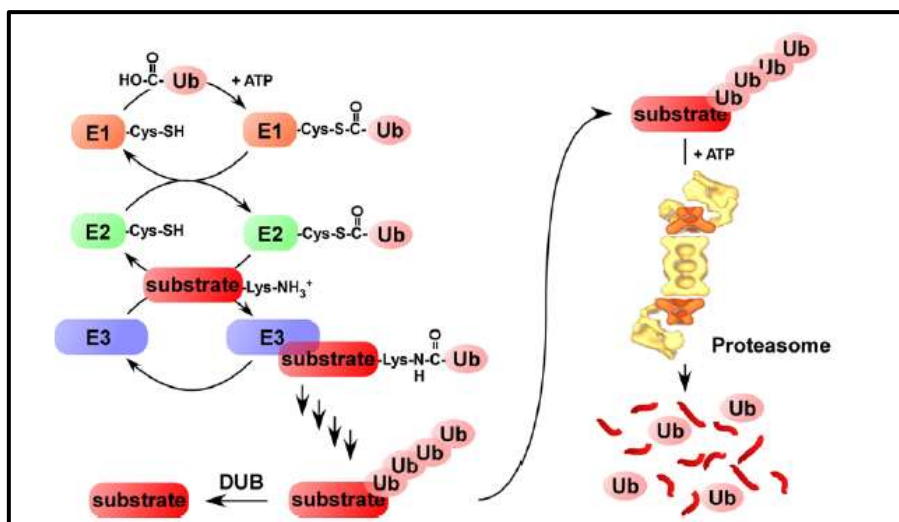


Figure 5.3: The ubiquitination of a substrate is a multi-step process (left) and results in proteasome degradation (right). A figure from Lee *et al.*¹² shows the steps and enzymes involved during substrate ubiquitination. Ubiquitin (Ubq) is activated by an activating enzyme (E1), the activated ubiquitin molecule is then transferred to E2, a conjugating enzyme. E3 binds the substrate and the transfer of the activated ubiquitin molecule from E2 to the substrate occurs. This is repeated, until a poly-ubiquitinated chain is formed and then recognised and degraded by the catalytically active proteasome to produce inactive protein fragments. In some cases, proteins are de-ubiquitinated by de-ubiquitinating enzymes (DUB).

In regards to GR α , a number of these enzymes related to the ubiquitination process, have been identified^{13–18}. In the current study, three of these enzymes known to interact with unliganded and liganded GR α were investigated; namely the E2 conjugating enzyme tumour susceptibility gene 101 (TSG101)¹⁸, and the E3 ligases, namely the carboxy terminus of heat shock protein 70-interacting protein (CHIP) (also referred to as STIP1 homology and U-Box containing protein 1 (STUB1))¹⁹ and, F-box/WD repeat-containing protein 7 (FBXW7 α)²⁰.

TSG101:

TSG101 belongs to a group of catalytically inactive enzymes, termed the ubiquitin E2 variants (UEV), which resemble classical E2 conjugating enzymes, with high sequence specificity, but are incapable of catalysing ubiquitin transfer^{15,21}. Interestingly, although catalytically inactive, TSG101 is still able to bind ubiquitin but this is thought to occur at a different site to classical E2 conjugating enzymes²¹. As mentioned, phosphorylation in general modulates GR α function at a transcriptional level but more importantly at the level of GR α protein stability¹⁵. TSG101 is known to interact with the non- or hypo-phosphorylated GR α , ultimately stabilizing the receptor and preventing its degradation¹⁵.

CHIP:

CHIP is known to be a proteasome-targeting factor or, more specifically, an E3 ligase and is considered an important regulatory enzyme functioning to assist the degradation of mis-folded or damaged cellular proteins, via the proteasome^{19,22,23}. Interestingly, in addition to its ability to

mediate the addition of ubiquitin to various client proteins, CHIP is also known to be able to ubiquitinate itself²². As an E3 ligase, this enzyme has a high specificity for its substrates, which are heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90) client proteins, one of which is the GR α ^{13,22,23}. Furthermore, a confirmed role for CHIP in regulating GR α protein turnover via the proteasome, has been confirmed, both in the absence and presence of GCs¹³.

FBXW7 α :

FBXW7 α , an F-box protein is, like CHIP, an E3 ligase, which mediates the degradation of substrates in an ubiquitin-dependent manner¹⁴. Moreover, this E3 ligase is thought to specifically require prior GSK3 β -dependent phosphorylation of the substrate, at a site referred to as a CDC4 phosphodegron consensus motif¹⁴. Phosphorylation, at this specific motif, allows for FBXW7 α to bind its substrate with a high specificity¹⁴.

Recently, GR α was found to be a novel client of FBXW7 α ¹⁴. As previously mentioned, GR α undergoes GSK3 β - dependent phosphorylation at Ser404 and this influences receptor stability⁸. Interestingly, the amino acid sequence that borders this serine residue is known to bear a resemblance to the highly conserved phosphodegron consensus motif and it is here that the E3 ligase, FBXW7 α , is known to bind¹⁴. Subsequently, a ligand-dependent increase in the binding of FBXW7 α to GR α mediates ubiquitination and proteasomal degradation¹⁴. Whilst it is clear that in the presence of a potent dimerization promoting GC, Dex, this interaction between GR α and FBXW7 is well established¹⁴, it remains to be understood whether dimerization abrogating CpdA, can induce an interaction between FBW7 α and GR α .

Ubiquitin:

Following interaction with the E3 ligases, ubiquitin, a small 76 amino acid protein is covalently attached to its substrates. This process of ubiquitin-tagging is repeated until a poly-ubiquitin chain is formed on the substrate, which is then recognized by the proteasome, which then mediates the degradation of the substrate into inactive protein fragments^{1,11,12,24–28}.

Ubiquitination of the GR α occurs at a single site, a conserved lysine residue at amino acid position 419 in humans, which falls within the PEST (a peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) degradation) motif²⁹(Figure 5.2). Interestingly, this site K419 occurs slightly upstream from the phosphorylation site S404, known to regulate binding of FBXW7 α ¹⁴. Wallace *et al.*^{29,30}, elegantly demonstrated that mutation of the lysine found within this PEST element resulted in the abrogation of ligand-induced GR α protein down-regulation via the proteasome. These findings provide evidence for ubiquitination as a requirement to prime the protein for subsequent recognition and degradation by the proteasome, the ultimate site of

proteolysis in the UPS. Whilst extensive evidence is available for ubiquitination of other steroid receptors (e.g. the AR)³¹⁻³⁴, it would seem the literature available on GR α ubiquitination is restricted to a handful of papers^{29,30,35}.

It is well understood that the UPS serves as a vital ‘switch-off’ mechanism to prevent continual signalling by prolonged hormone treatment, and to fine-tune the magnitude and duration of its response, through degradation of its cognate receptor, such as GR α ¹². From Chapter 4, it was established, that the dimerization state of GR α is an important factor for ligand-induced receptor turnover via the proteasome but a description of how a ‘loss’ of dimerization prevented receptor turnover at a molecular level was absent. It is this, which the current chapter will focus on.

Thus, generally speaking, the current chapter investigates how ‘gain’ or ‘loss’ of GR α dimerization modulates the post-translational processing of the receptor, more specifically, by investigating the PTMs, phosphorylation and ubiquitination, as well as the interaction of GR α with other components of the UPS (e.g. E2 and E3 enzymes). Also within this chapter, the ligand-induced turnover of a number of other GR α mutants is investigated, as well as the ability of the dimerization abrogating ligand, CpdA, to rescue Dex-mediated GR α protein down-regulation.

Elucidating the exact molecular mechanisms, in terms of the post-translational processing of the receptor, of how a ‘loss’ of GR α dimerization prevents ligand-induced receptor turnover, may highlight steps in the UPS pathway, which could be exploited pharmacologically for the development of novel dimerization abrogating GR α ligands that do not induce receptor turnover and maintain receptor levels, even following prolonged GC treatment. This has major implications in terms of acquired GC resistance and the battle against counteracting chronic inflammation and curbing the progression of auto-immune diseases.

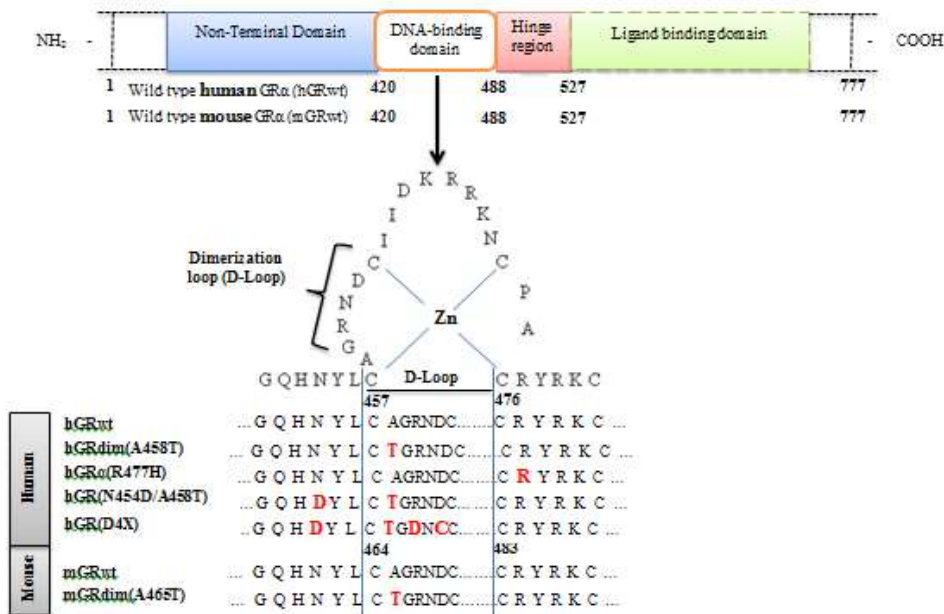
5.2. Results

5.2.1. Ligand-induced GR α protein down-regulation is prevented in a mutation specific manner

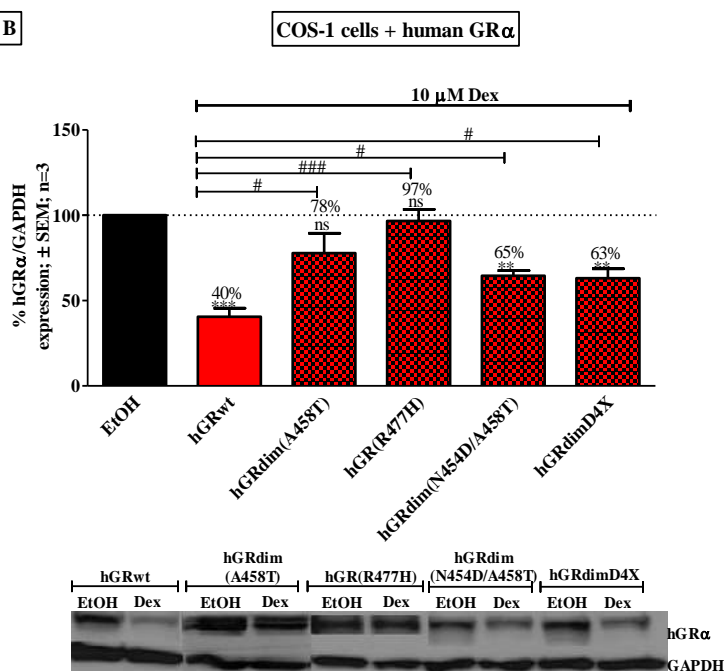
In Chapter 4, a novel requirement for GR α dimerization in ligand-induced GR α protein down-regulation was established. More specifically, this link between receptor dimerization and GR α protein turnover was investigated by comparing the human wild type GR α to one dimerization deficient mutant namely hGRdim, with a single point mutation at amino acid position 458 (Fig. 5.4A). At this point, it seemed necessary to determine whether this requirement for GR α dimerization in receptor turnover is dependent on mutations at a specific site and/or whether it is species specific.

To do this, a number of other GR α mutant plasmids thought to be impaired in their dimerization capabilities, including a natural occurring mutant and a mouse wild type and dimerization deficient mutant GR α plasmid (Table 3.1 and Fig. 5.4A), were used³⁶⁻³⁹. Western blotting was conducted following treatment of transiently transfected GR α plasmids, in COS-1 cells, with Dex (10 μ M) for 24 hours, to detect changes in GR α expression.

A



B



C

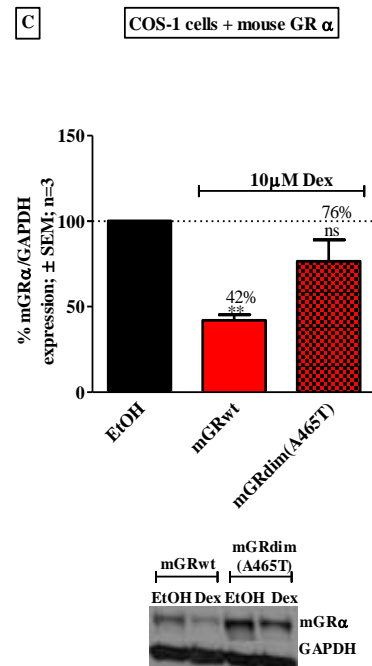


Figure 5.4: Ligand-induced GR α protein down-regulation is prevented in a mutation specific manner. COS-1 cells were seeded into a 24 well plate (5×10^4 cells/well) and transfected the next day with one of the GR α plasmids in (A) namely; hGRwt, hGRdim(A485T), hGR(R477H), hGR(N545D/A458T), hGR(D4X), mGRwt or mGRdim (A465T) using a transfection agent. Following 24 hours incubation, cells were treated with solvent (EtOH) or Dex (10^{-5} M) for 24 hours and GR α protein expression determined by Western blotting where GAPDH was probed to ensure equal protein loading. Western blots shown below, the quantification graphs for human (B) and mouse (C) are representative of three independent experiments. For quantification, the intensity of the GR α and GAPDH bands were determined using UNSCANIT and GR α levels were then normalised to GAPDH expression. For each plasmid, GR α expression following Dex treatment was expressed as a percentage (average \pm SEM) of GR α expression in presence of the solvent (EtOH), which was set at 100% (dotted line). For simplicity, only a single bar representing GR α expression in the presence of the solvent (EtOH) is depicted in (B and C). Statistical analysis for was conducted using a one-way ANOVA with a Dunnett post-test comparing experimental values to solvent (EtOH) (ns, $p > 0.05$, *, $p < 0.05$, ***, $p < 0.001$) or a t-test to compare the mutants to hGRwt in the presence of Dex (B) (#, $p < 0.05$ and ###, $p < 0.001$).

Consistent with previous results from Chapter 4 (Table 3), hGRwt protein expression was significantly ($p < 0.001$) reduced to 40%, relative to solvent (EtOH), following treatment with Dex, whilst hGRdim expression was not significantly ($p > 0.05$) affected (Fig. 5.4B). Interestingly, the natural human mutant GR α , hGR(R477H), did not undergo ligand-induced GR α protein down-regulation similarly to the classical dimerization deficient mutant, hGRdim (Fig. 5.4B). In contrast to these two mutants, two additional GR α mutants, hGRdim(N545D/A458T) and hGRdimD4X, underwent significant ($p < 0.01$) Dex-induced GR α protein down-regulation (Fig. 5.4B). Whilst hGR(N545D/A458T) and hGR(D4X) expression was reduced to 65 and 63%, respectively, relative to the solvent (EtOH), following treatment with Dex for 24 hours, the extent of receptor turnover with these two mutants was significantly ($p < 0.05$) less than that observed with hGRwt (Fig. 5.4B). One could postulate that this is due to the mutation at A458T present in both hGRdim(N545D/A458T) and hGRdimD4X.

Additionally, results obtained with the transiently transfected mouse wild type, mGRwt, and classical mouse dimerization deficient mutant, mGRdim(A465T) (Fig. 5.4C), were similar to that obtained with the hGRwt and hGRdim plasmids, in COS-1 cells (Fig. 5.4B). Following 24 hours Dex treatment, mGRwt levels were significantly ($p < 0.01$) reduced to 42%, whilst mGRdim(A458T) levels were not significantly down-regulated (Fig. 5.4C).

In summary, Dex-induced human GR α protein down-regulation is prevented in a dimerization deficient, mutation specific manner. Furthermore, it seems as though, with the human GR α , the mutation of an alanine residue to a threonine at position 458, which has been shown to result in a ‘loss’ of receptor dimerization⁴⁰, contributes to a loss of receptor degradation in the case of hGRdim(N545D/A458T) and hGRdimD4X, but that the additional mutations in these GR α mutants (Fig.5.4A) may be unable to restrict Dex-mediated receptor degradation. Interestingly, the mutation of the natural mutant, hGR(R477H), occurs outside the D-loop and does not contain the mutation of an alanine residue to a threonine at position 458. Although it has been shown that this mutant has a reduced ability to bind DNA, which is generally mediated via a GR α dimer, the ability of this

mutant to dimerize has not yet been demonstrated and requires further investigation. Lastly, Figure 5.4C confirms that the novel requirement for GR α dimerization in ligand-induced receptor turnover, established in the previous chapter, is not restricted to a single species.

5.2.2. GR α and FBXW7 α

Having provided further evidence for a link between GR α dimerization and ligand-induced GR α protein degradation, the next step was to further investigate the molecular mechanisms involved in mediating receptor turnover and how these molecular mechanisms are modulated by different GR α conformations (i.e. dimer versus monomer).

Recently, GR α has been identified as a novel substrate for FBXW7 α , which forms part of a larger complex, termed the SCF (Skp1/Cul1/F-box) E3 ubiquitin ligase complex, whose primary function is to carry out substrate ubiquitination and delivery of this ubiquitin-tagged substrate to the proteasome for subsequent degradation¹⁴. With the degradation of GR α being demonstrated as proteasome dependent, it seemed necessary to determine how the dimerization state of GR α influences the interaction of the receptor with FBXW7 α .

In addition, many recent studies have highlighted the importance of not only investigating protein expression and protein-protein interactions but also determining the subcellular localisation of these interactions, to provide further insight into molecular mechanisms occurring within the cell^{41,42}. Thus, the current study, before determining the effect of GR α conformation (i.e. dimer versus monomer) on the nature of the interaction of the receptor with the E3 ligase, FBXW7 α , investigated how the subcellular localisation and co-localisation of this interaction, was affected by a 'loss' or 'gain' of GR α dimerization.

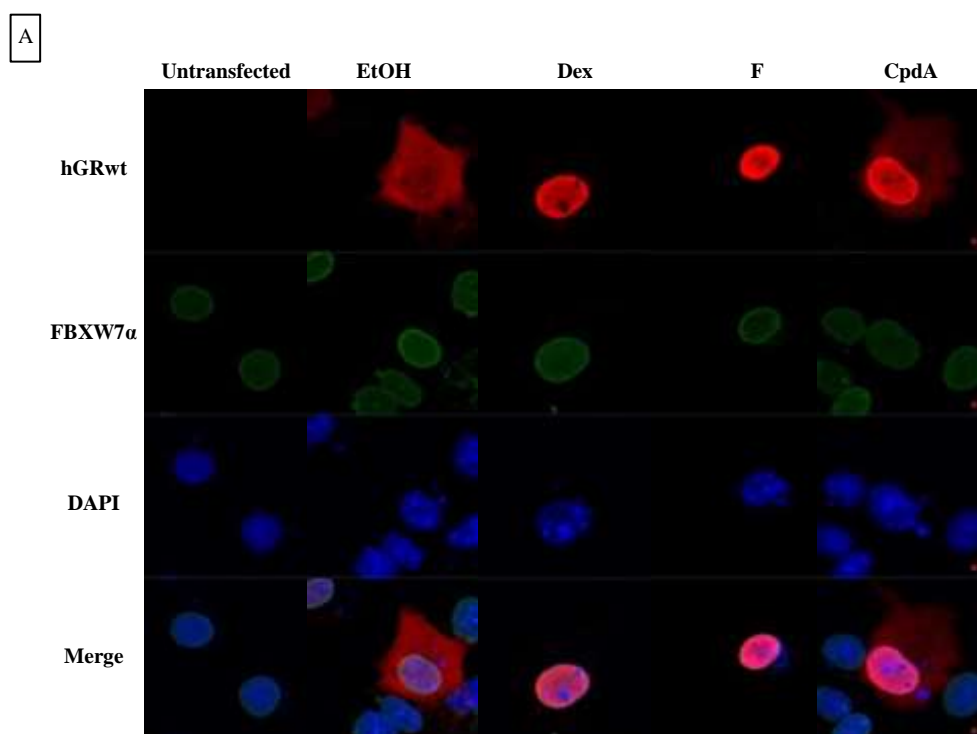
COS-1 cells were seeded in a 10cm dish (1×10^6 cells/well) and the next day transfected with GR α plasmids (i.e. hGRwt, hGRdim, GFP-hGRwt or GFP-hGRdim) (Table 3.1 and 3.2). Cells were then treated with solvent (EtOH), Dex, F or CpdA ($10 \mu\text{M}$) for 3 hours and then lysed, following which a co-IP was conducted, or after 24 hours, cells were then re-plated into an 8-well chamber (3×10^4 cells/well) in preparation for immunofluorescence or PLA. It must be noted, that for hGRdim, COS-1 were plated into an 8-well chamber (4×10^4 cells/well) and hGRdim directly transfected into the chambers (i.e. without the re-plating step) (Table 3.2). Once the cells had settled in the 8-well chambers (48 hours post-transfection) they were treated with solvent (EtOH), Dex, F or CpdA ($10 \mu\text{M}$) for 3 hours. Following treatment, COS-1 cells were fixed, permeabilized and immunofluorescence, or PLA was performed using antibodies specific to GR α and FBXW7 α (Table

3.3), to determine the subcellular localisation and co-localisation of GR α and FBXW7 α , as well as the interaction of these two proteins.

5.2.2.1. Ligand-dependent subcellular localisation of hGRwt modulates its co-localisation with endogenous FBXW7 α .

From the results, notable differences in the subcellular localisation of hGRwt, are noted (Fig. 5.5) following treatment with compounds, for 3 hours. Firstly, in terms of GR α , no GR α expression was detected in untransfected COS-1 cells (Fig. 5.5A). In contrast, in COS-1 cells that had been transiently transfected with hGRwt, obvious red fluorescence was observed, suggesting successful transfection of hGRwt plasmid (Fig. 5.5A).

From the quantification (Fig. 5.5B), it is clear that the unliganded hGRwt receptor (i.e. solvent (EtOH)) is evenly distributed throughout the cytoplasm and the nucleus ($p > 0.05$). In contrast, treatment with the dimerization promoting synthetic GC, Dex, and endogenous GC, F, resulted in total nuclear translocation of hGRwt, with little to no receptor expression detected in the cytoplasm ($p < 0.001$) (Fig. 5.5B). A significant (b, $p < 0.001$) decrease in cytoplasmic hGRwt and a significant increase (c, $p < 0.001$) in nuclear hGRwt expression were noted following Dex and F treatment, relative to the unliganded hGRwt (Fig. 5.5B).



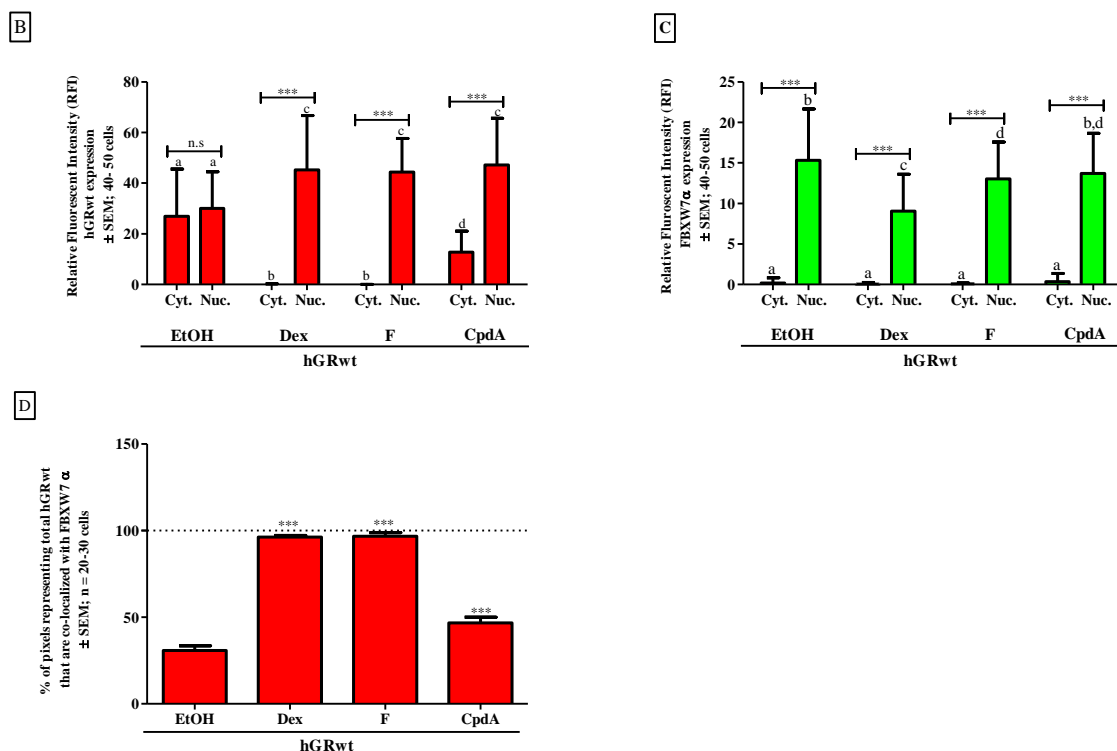


Figure 5.5: Ligand-dependent subcellular localisation of hGRwt modulates its co-localisation with endogenous FBXW7 α . COS-1 cells were seeded into a 10 cm dish (1×10^6 cells) and transiently transfected the next day with hGRwt using a transfection reagent. Following 24 hours incubation, cells were re-plated into 8 well chambers (3×10^4 cells/well) (Table 3.2) and the next day treated with solvent (EtOH), Dex, F or CpdA (10^{-5} M) for 3 hours. Thereafter, cells were fixed, permeabilized, and immunofluorescence conducted, with antibodies specific for GR α and FBXW7 α . Cells were then imaged using a confocal microscope. A representative image (A) illustrates the individual subcellular localisation of hGRwt (red, first row) and FBXW7 α (green, second row), as well as the position of the cell's nucleus (blue DAPI stain, third row) with the merge representing an overlay of all three channels (red, green and blue). For the quantification of the subcellular localisation of hGRwt and FBXW7 α the relative fluorescence intensity (RFI) of the red (hGRwt) (B) or green (FBXW7 α) (C) pixels was calculated for individual cells and plotted. In addition, the co-localisation of these two proteins, in terms of hGRwt (D), was determined using the weighted co-localisation coefficients, where the horizontal dotted line represents 100% co-localisation of hGRwt with FBXW7 α . Statistical analysis was conducted using a two-way ANOVA followed by a Bonferoni's post-test comparing experimental values to solvent's (EtOH) cytoplasm for hGRwt (B) or FBXW7 α (C) (for a,b,c,d and e letters that are the same represent no significant difference between values whilst letters, which are different are significantly different from each other $p < 0.05$) or comparing the cytoplasmic and nuclear expression of hGRwt (B) or FBXW7 α (C) within a treatment group (ns, $p > 0.05$ and ***, $p < 0.001$). For co-localisation (D), a one-way ANOVA with a Tukey's post-test was used, comparing experimental values to the solvent (EtOH) (***, $p < 0.001$).

Similarly, treatment with the dimerization abrogating GC, CpdA resulted in significant ($p < 0.001$) translocation of hGRwt from the cytoplasm to the nucleus (Fig. 5.5B). However, unlike with Dex and F treatment, a substantial amount of hGRwt still resided in the cytoplasm following treatment with CpdA (Fig. 5.5B). With that said, the amount of hGRwt present in the cytoplasm following CpdA treatment was significantly less (d, $p < 0.001$) than that observed following treatment with solvent (EtOH).

From the quantification (Fig. 5.5B), it is clear that the unliganded hGRwt receptor (i.e. solvent (EtOH)) is evenly distributed throughout the cytoplasm and the nucleus ($p > 0.05$). In contrast, treatment with the dimerization promoting synthetic GC, Dex, and endogenous GC, F, resulted in

total nuclear translocation of hGRwt, with little to no receptor expression detected in the cytoplasm ($p < 0.001$) (Fig. 5.5B). A significant (b, $p < 0.001$) decrease in cytoplasmic hGRwt and a significant increase (c, $p < 0.001$) in nuclear hGRwt expression were noted following Dex and F treatment, relative to the unliganded hGRwt (Fig. 5.5B). Similarly, treatment with the dimerization abrogating GC, CpdA resulted in significant ($p < 0.001$) translocation of hGRwt from the cytoplasm to the nucleus (Fig. 5.5B). However, unlike with Dex and F treatment, a substantial amount of hGRwt still resided in the cytoplasm following treatment with CpdA (Fig. 5.5B). With that said, the amount of hGRwt present in the cytoplasm following CpdA treatment was significantly less (d, $p < 0.001$) than that observed following treatment with solvent (EtOH)

When investigating the subcellular localisation of FBXW7 α , little to no FBXW7 α expression was detected in the cytoplasm, with significant ($p < 0.001$) nuclear localisation of FBXW7 α occurring across the different treatment conditions (Fig. 5.5C). Interestingly, a notable decrease (c, $p < 0.001$) in the nuclear FBXW7 α expression is observed following treatment with Dex. Furthermore, a slight yet, significant (d, $p < 0.05$), decrease in the nuclear expression of this protein is also detected after treatment with F relative to the solvent (EtOH) (Fig. 5.5C). This F-induced decrease in nuclear FBXW7 α expression was not as severe as that of the Dex-induced decrease in nuclear FBXW7 α expression, and was comparable to that after treatment with CpdA (Fig. 5.5C).

Lastly, Figure 5.5D demonstrates the co-localisation of hGRwt and FBXW7 α , importantly with reference to hGRwt. In other words, the quantification graph (Fig. 5.5D) depicts the percentage (%) of the total hGRwt protein expression, which co-localizes with FBXW7 α . From this graph, it is clear that, in the case of unliganded hGRwt, only 30% of the total hGRwt protein expression co-localizes with FBXW7 α (Fig. 5.5D). This suggests that a large portion of the hGRwt expression (approximately 70%) does not occupy the same space as FBXW7 α , likely due to the cytoplasmic nature of unliganded hGRwt and the predominantly nuclear localisation of FBXW7 α . However, promoting hGRwt dimerization and its nuclear translocation, through Dex and F treatment, results in a significant ($p < 0.001$) increase in the co-localisation of hGRwt and FBXW7 α , with almost all (just less than 100%) of hGRwt occupying the same space as FBXW7 α (Fig. 5.5D). Similarly, treatment with dimerization abrogating CpdA, resulted in a significant ($p < 0.001$) increase (47%) in the amount of hGRwt expression co-localised with FBXW7 α , relative to the unliganded receptor (30%). However, the percentage co-localisation of these two proteins, following CpdA treatment, was half of that noted following Dex and F treatment but significantly greater ($p < 0.001$) than that of the solvent (EtOH) and directly reflects CpdA's diminished ability to induce nuclear translocation of hGRwt to where the predominantly nuclear FBXW7 α resides.

5.2.2.2. Ligand-dependent subcellular localisation of hGRdim modulates its co-localisation with endogenous FBXW7 α .

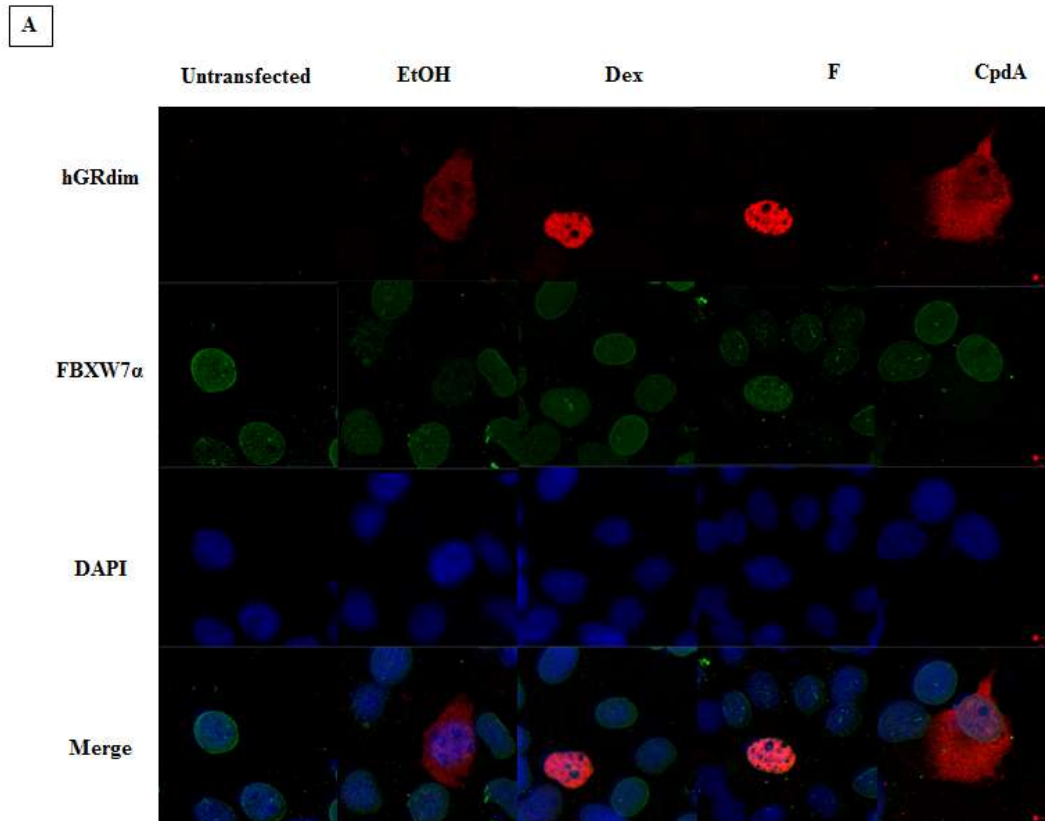
Having demonstrated that a ‘loss’ of GR α dimerization, through CpdA treatment, did not drastically inhibit nuclear translocation of the receptor, the ability of the dimerization deficient mutant, hGRdim, to translocate to the nucleus following GC treatment, was investigated, in order to confirm that the mutation did not significantly alter the nuclear translocation of GR α . The subcellular localisation and co-localisation of hGRdim and FBXW7 α was determined and quantified as in Section 5.2.2.1, of the current chapter, but instead of hGRwt, hGRdim was used for this section.

Similarly to the hGRwt experiment (Fig. 5.5) no GR α expression was detected in untransfected COS-1 cells, but in cells that had been transiently transfected with hGRdim obvious red fluorescence was observed, suggesting that successful transfection of the hGRdim plasmid was achieved (Fig. 5.6A). In addition, as with hGRwt (Fig. 5.5B), the unliganded hGRdim is evenly distributed throughout the cytoplasm and the nucleus ($p > 0.05$) (Fig. 5.6B). Moreover, like hGRwt (Fig. 5.5B), treatment with Dex and F, resulted in total nuclear translocation of hGRdim with little receptor expression detected in the cytoplasm ($p < 0.001$) (Fig. 5.6B). Likewise, relative to the unliganded hGRdim, Dex and F treatment induced a significant (b, $p < 0.001$) decrease in cytoplasmic hGRdim and a corresponding increase (c, $p < 0.001$) in the nuclear localization of hGRdim (Fig. 5.6B). Interestingly, and in contrast to hGRwt (Fig. 5.5B), treatment with dimerization abrogating GC, CpdA, did not induce significant ($p > 0.05$) translocation of hGRdim from the cytoplasm to the nucleus (Fig. 5.6B) and hGRdim remained evenly distributed throughout the cytoplasm and nucleus, similarly to the unliganded receptor (a, $p > 0.05$) (Fig. 5.6B).

In terms of FBXW7 α , FBXW7 α expression was found to be predominantly nuclear ($p < 0.001$) as in Figure 5.5C (Fig. 5.6C). However, the significant decrease in the nuclear FBXW7 expression, relative to the unliganded hGRwt, observed following Dex and F treatment of hGRwt (Figure 5.5C) was absent in COS-1 cells transfected with hGRdim (Fig. 5.6C). Lastly, CpdA treatment also did not significantly ($p > 0.05$) alter the nuclear localization of FBXW7 α in cells where hGRdim was present (Fig. 5.6C).

With regards to co-localisation of hGRdim and FBXW7 α , a similar co-localisation pattern to that of hGRwt and FBXW7 α (Fig. 5.5D), was observed for hGRdim and FBXW7 α (Fig. 5.6D), however, with some notable differences. Specifically, Figure 5.6D suggests that in the absence of ligand, approximately 44% of the total hGRdim protein expression co-localizes with FBXW7 α , which is slightly more than the 30% reported in the previous result for hGRwt (Fig. 5.5D). As with hGRwt (Fig. 5.5) Dex and F treatment resulted in a significant ($p < 0.001$) increase in the co-localisation of

the mutant GR α and the predominantly nuclear FBXW7 α , relative to the unliganded hGRdim (Fig. 5.6D). Specifically, about almost all (just less than 100%) of hGRdim is found co-localised with FBXW7 α , in the nucleus (Fig. 5.6D). In contrast to the results obtained for hGRwt (Fig. 5.5D), treatment with CpdA, did not significantly affect the co-localisation of FBXW7 α with hGRdim, relative to unliganded hGRdim (Fig. 5.6D) and reflects the inability of CpdA treatment to induce nuclear translocation of hGRdim, also observed in Figure 5.6B.



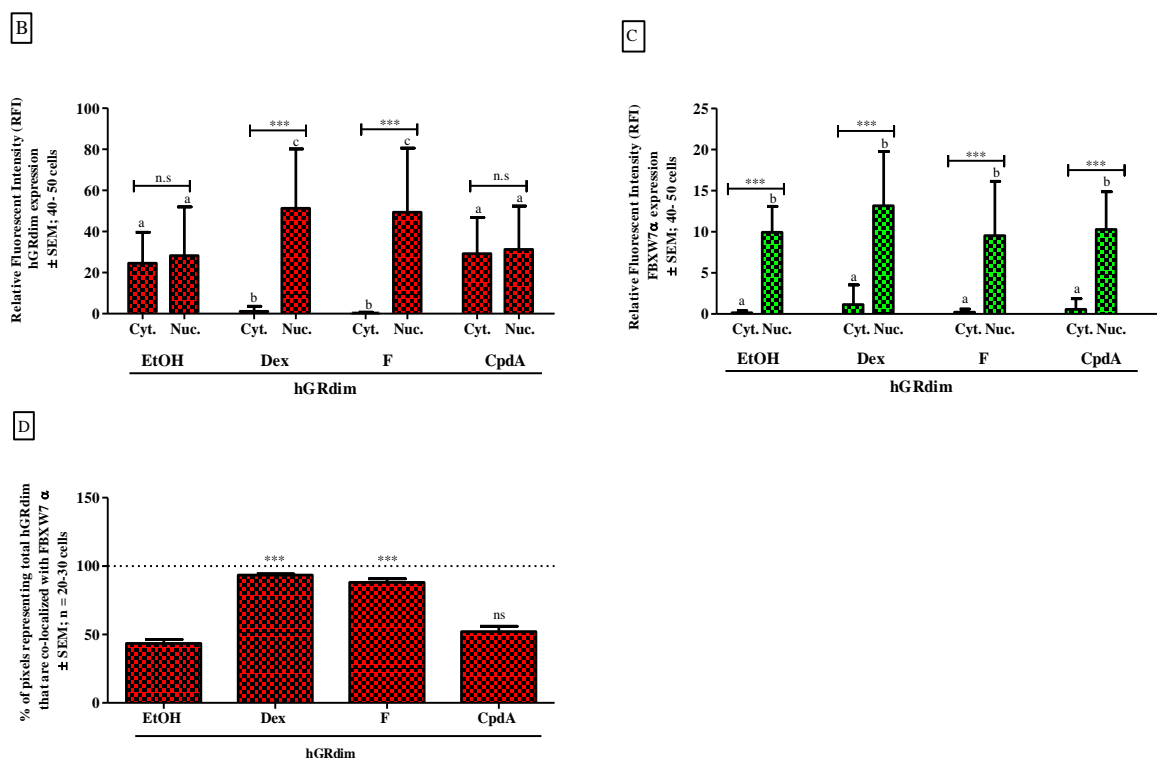


Figure 5.6: Ligand-dependent subcellular localisation of hGRdim modulates its co-localisation with endogenous FBXW7α. COS-1 cells were seeded into a 10 cm dish (1×10^6 cells) and transiently transfected the next day with hGRdim using a transfection reagent. Following 24 hours incubation, cells were re-plated into 8 well chambers (3×10^4 cells/well) (Table 3.2) and the next day treated with solvent (EtOH), Dex or CpdA (10^{-5} M) for 3 hours. Thereafter, cells were fixed, permeabilized, and immunofluorescence conducted, with antibodies specific for GRα and FBXW7α. Cells were then imaged using a confocal microscope. A representative image (A) illustrates the individual subcellular localisation of hGRdim (red, first row) and FBXW7α (green, second row), as well as the position of the cell's nucleus (blue DAPI stain, third row) with the merge representing an overlay of all three channels (red, green and blue). For the quantification of the subcellular localisation of hGRdim and FBXW7α the relative fluorescence intensity (RFI) of the red (hGRdim) (B) or green (FBXW7α) (C) pixels was calculated for individual cells and plotted. In addition, the co-localisation of these two proteins, in terms of hGRdim (D), was determined using the weighted co-localisation coefficients, where the horizontal dotted line represents 100% co-localisation of hGRdim with and FBXW7α. Statistical analysis was conducted using a two-way ANOVA followed by a Bonferoni's post-test comparing experimental values to solvent's (EtOH) cytoplasm for hGRdim (B) or FBXW7α (C) (for a,b,c,d and e letters that are the same represent no significant difference between values whilst letters, which are different are significantly different from each other $p < 0.05$) or comparing the cytoplasmic and nuclear expression of hGRdim (B) or FBXW7α (C) within a treatment group (ns, $p > 0.05$ and ***, $p < 0.001$). For co-localisation (D), a one-way ANOVA with a Tukey's post-test was used, comparing experimental values to the solvent (EtOH) (***, $p < 0.001$).

5.2.2.3. 'Loss' of GRα dimerization modulates its interaction with FBXW7α.

Having demonstrated ligand-induced changes in the subcellular localisation and co-localisation of GRα (hGRwt and hGRdim) with the E3 ligase, FBXW7α, the current study determined whether a 'loss' of receptor dimerization influenced the ability of FBXWα to interact with GRα using a co-immunoprecipitation assay and PLA.

Results from the co-immunoprecipitation assay demonstrated that a 'gain' of hGRwt dimerization via the potent, synthetic dimerization promoting GC, Dex, leads to a significant ($p < 0.05$) increase in the association of hGRwt and E3 ligase, FBXW7α (Fig. 5.7A). Furthermore, a slight increase in

this interaction is noted following treatment with F, however, this increase was not significant ($p > 0.05$) (Fig. 5.7A). In stark contrast to the dimerization promoting GCs, ‘loss’ of GR α dimerization, following treatment with dimerization abrogating GC, CpdA, did not induce interaction of the receptor with FBXW7 α , relative to the basal interaction (i.e. solvent (EtOH)) (Fig. 5.7A). Moreover, in one of the two experiments conducted, no interaction between FBXW7 α and hGRwt was noted following CpdA treatment, as seen in the representative Western blot (Fig. 5.7A, inset and Fig. S2), which suggests CpdA treatment, may abrogate the basal interaction between these two proteins.

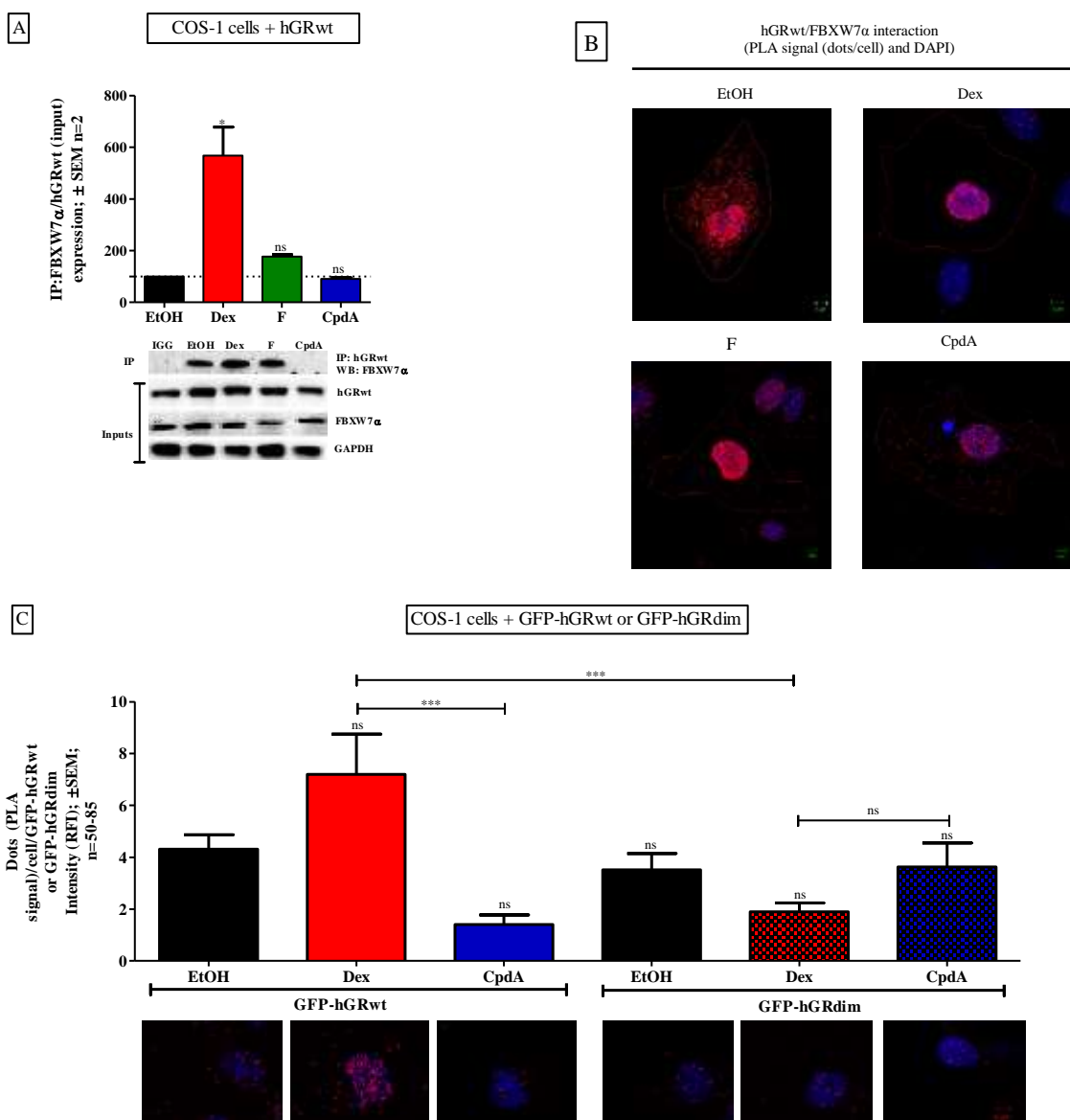


Figure 5.7: ‘Loss’ of GR α dimerization modulates its interaction with FBXW7 α . COS-1 cells were seeded, transfected and in some cases re-plated in 8 well chambers. Following 24 hours incubation, cells were treated with solvent (EtOH), Dex, F or CpdA (10^{-5} M) for 3 hours. For the co-IP (A) experiment, cells were lysed after compound treatment and FBXW7 α was immuno-precipitated with hGRwt. Western blotting was conducted to determine protein expression and GAPDH was probed to ensure equal protein loading. A representative blot of three independent experiments is shown. For quantification, the intensity of, hGRwt, FBXW7 α and GAPDH were determined using the MyECL Image Software Analysis. Moreover, the hGRwt/FBXW7 α interaction was normalised to hGRwt input expression and dotted line on the graph representative of hGRwt/FBXW7 α interaction in the presence of solvent (EtOH), and is set at 100%. For the PLA (B and C), following treatment cells were fixed, permeabilized and PLA conducted using specific antibodies for GR α and FBXW7 α , after which cells were imaged. A representative image of individual cells from the hGRwt and FBXW7 α (B) and GFP-tagged GR α and FBXW7 α (C, below graph) experiment, is shown. In these representative images the PLA signal is observed as distinct red ‘spots’ and the cell’s nucleus is depicted by the blue DAPI stain (B and C, inset below graph). For quantification of the GFP-tagged GR α and FBXW7 α interaction (C), the PLA signal (dots/cell) was quantified using the IMAGEJ Software and normalized to the GR α concentration (i.e. GFP-signal (RFI)), which was determined using the ZENN 2012 Software Analysis, and plotted. Statistical analysis was conducted using a one-way ANOVA followed by a Bonferoni’s post-test (A) comparing experimental values to solvent (EtOH) and the same statistical analysis was used with a Tukey’s post-test (C) comparing experiment values to GFP-hGRwt solvent (EtOH, (ns, $p > 0.05$ and *, $p < 0.05$). Additionally, t-tests were used in (C) to compare effects of Dex and CpdA via GFP-hGRwt and the effects of Dex via GFP-hGRdim, relative to GFP-hGRwt and lastly, the effects of Dex via GFP-hGRdim, relative to the unliganded GFP-hGRdim (ns, $p > 0.05$ and ***, $p < 0.001$).

At this point it was postulated that the GR α monomer (CpdA-bound hGRwt) and FBXW7 α interaction may be weaker or more transient than the interaction of FBXW7 with a GR α dimer (Dex-bound or F-bound hGRwt) due to the fact that ‘loss’ of dimerization did not severely alter the co-localisation of GR α with FBXW7 α (Fig. 5.5D and 5.6D), relative to a ‘gain’ of GR α dimerization, and the lack of interaction between these two proteins demonstrated in Figure. 5.7A, following CpdA treatment. Thus, to further explore the interesting result in Figure 5.7A, a PLA was conducted.

Unexpectedly, results from the initial PLA experiment, using hGRwt (Fig. 5.7B), contradicted the results from the co-immunoprecipitation assay (Fig. 5.7A). Specifically, the greatest interaction of hGRwt and FBXW7 α was noted following treatment with solvent (EtOH) and, in contrast to Figure. 5.7A, treatment with Dex, F or CpdA appeared to reduce the interaction of hGRwt and FBXW7 α (Fig. 5.7B). This is evident from the fewer fluorescent red spots observed following treatment with compounds (Fig. 5.7B). Worth noting is that this experiment was repeated using the dimerization deficient mutant and little to no PLA signal was detected (data not shown).

Essentially, the major limitation of the PLA experiment with hGRwt in Figure 5.7B was the inability to account for cells that had been successfully transiently transfected with the hGRwt plasmid. Thus, to overcome this challenge, it was decided to repeat the PLA experiment making use of green fluorescently tagged (GFP) GR α plasmids, namely GFP-hGRwt and GFP-hGRdim. This allowed for simultaneous visualisation of GR α (green) expression and the PLA signal (GR α /FBXW7 interaction, red) (Fig. 5.7C). For quantification, the PLA signal for each cell was normalised to the GR α content in the same cell and it is this normalized PLA signal (Dots/cell/RFI)

which was then plotted (Fig. 5.7C), thus accounting for possible changes in GR α concentration, due to potential differences in the transfection efficiency.

From the results, and in support of the co-immunoprecipitation result (Fig. 5.7A) and co-localisation results (Fig. 5.5D), promoting dimerization of GFP-hGRwt through treatment with Dex, resulted in an increase in the association of the wild-type receptor with FBXW7 α , although this was not significant ($p > 0.05$) (Fig. 5.7C). Moreover, 'loss' of dimerization of GFP-hGRwt, following CpdA treatment, restricted the interaction of the receptor with the E3 ligase (Fig. 5.7C), as in Figure 5.7A. Interestingly, no significant ($p > 0.05$) ligand-induced changes in the interaction of the dimerization deficient GR α mutant, GFP-hGRdim with FBXW7 α , were observed relative to the unliganded GFP-hGRwt (i.e. solvent (EtOH)) (Fig. 5.7C). In addition, the ability of dimerization promoting Dex to induce an interaction between GR α and FBXW7 α was significantly ($p < 0.001$) restricted with the use of GFP-hGRdim, relative to GFP-hGRwt (Fig. 5.7C) and is not a result of a lack of Dex-induced co-localisation between GFP-hGRdim and FBXW7 α as shown in Fig. 5.6D.

5.2.2.4. GR α concentration modulates the interaction of GFP-hGRwt with FBXW7 α

During imaging of cells, it was apparent that transient transfection of the untagged GR α plasmids did not produce a homogenous population of COS-1 cells with regards to GR α concentration (Fig. S3), but rather a population of cells that ranged from low to medium/high GR α concentration. As the cellular concentration of GR α is known to modulate GC action, such as nuclear mobility and GR α -mediated transcription^{43,44}, the current study set out to determine whether the cellular receptor concentration altered the ability of GFP-hGRwt to interact with FBXW7 α .

To do this, the cell population (cell number 1-79) was plotted from lowest to highest GFP-hGRwt concentration (RFI) with their correlating PLA signal (dots/cell), which represents the GFP-hGRwt and FBXW7 α interaction (Fig. S5). This graph demonstrated that increasing cellular GFP-hGRwt concentration results in a concurrent decrease in PLA signal, suggesting a role for GR α concentration in modulating the interaction of the receptor with the E3 ligase, FBXW7 α . To further investigate this notion, cells transiently transfected with GFP-hGRwt, were grouped into two categories, based on the total cellular GFP intensity (RFI), low GR α (RFI < 25) and medium/high GR α (RFI > 25) concentration, which was calibrated using FITC beads and the confocal microscope by a member of our research group lab (unpublished) (Fig. S6).

From the results, it is evident that at **low** cellular concentrations of GFP-hGRwt, dimerization promoting Dex significantly ($p < 0.001$) induces GFP-hGRwt/FBXW7 α interaction, whilst dimerization abrogating CpdA does not ($p > 0.05$) (Fig. 5.8A). Moreover, the amount of GFP-

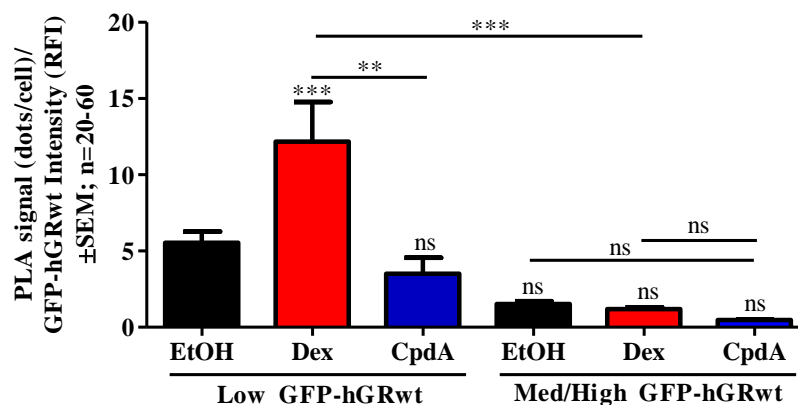
hGRwt/FBXW7 α interaction induced by Dex is significantly ($p < 0.01$) different to that of CpdA (Fig. 5.8A).

At **medium/high** cellular GFP-hGRwt concentrations, no increase ($p > 0.05$) in the interaction of the receptor with FBXW7 is noted across the treatment conditions (Fig. 5.8D). Additionally, no significant difference ($p > 0.05$) between the ability of Dex and CpdA to induce this interaction is noted, in cells with medium/high GFP-hGRwt concentrations (Fig. 5.8D). Interestingly, notable differences were observed when comparing the ability of dimerization promoting Dex to induce GFP-hGRwt/FBXW7 interaction in cells containing low GFP-hGRwt, to those which contain medium/high levels of GFP-hGRwt (Fig. 5.8B and D). Specifically, Dex does not seem capable ($p < 0.001$) of inducing GFP-hGRwt/FBXW7 interaction in cells with medium/high GFP-hGRwt expression (Fig. 5.8D).

Taken together, results from this section on the subcellular localisation, co-localisation and interaction of GR α with the E3 ligase, FBXW7 α suggest the following:

Firstly, the unliganded GR α occurs both in the cytoplasm and the nucleus and that ‘loss’ of receptor dimerization, through the use of the mutant (hGRdim) does not affect the subcellular localisation of the unliganded receptor. Secondly, ‘gain’ of hGRwt dimerization through treatment with dimerization promoting GCs, Dex and F, resulted in complete nuclear translocation of hGRwt and almost (slightly less than hGRwt) complete nuclear translocation of hGRdim, where FBXW7 α resides, confirming that the mutation does not drastically affect the subcellular localisation of this monomeric GR α or its co-localisation with FBXW7 α . Thirdly, in this study, treatment with the dimerization abrogating compound, CpdA, induced nuclear translocation of hGRwt but failed to do the same for the dimerization deficient mutant. Thus, a significant portion of the hGRwt monomers (as a result of CpdA treatment), but not hGRdim monomers following CpdA treatment, were found in the nucleus co-localised with FBXW7 α .

A



B

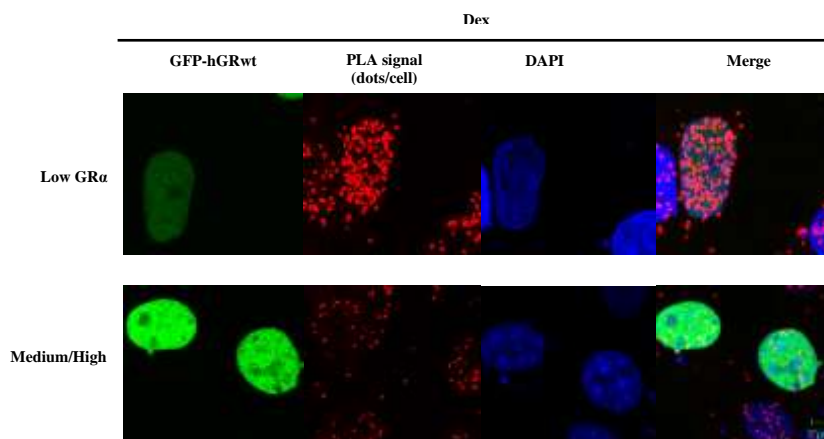


Figure 5.8: GR α concentration modulates the interaction of the receptor with FBXW7 α . COS-1 cells were seeded into a 10 cm dish (1×10^6 cells) and transiently transfected the next day with GFP-hGRwt (A and B) using a transfection reagent. Following 24 hours incubation, cells were re-plated into 8 well chambers (3×10^4 cells/well) and the next day treated with solvent (EtOH), Dex or CpdA (10^{-5} M) for 3 hours. Following treatment cells were fixed, permeabilized and PLA conducted using specific antibodies for GR α and FBXW7 α . For the characterisation of cells based on GR α content, COS-1 cells transiently transfected with GFP-hGRwt were grouped, based on the total cellular GFP intensity (RFI) with their corresponding PLA signal (dots/cell), into two categories; low GR α (RFI < 25) and medium/high GR α (RFI > 25) content, which was calibrated using FITC beads and the confocal microscope (Fig. S6). For the quantification of the GFP-tagged GR α and FBXW7 α interaction (A), the PLA signal (dots/cell) was quantified using the IMAGEJ Software and normalized to the GR α concentration (i.e. GFP-signal (RFI)), which was determined using the ZENN 2012 Software Analysis, and plotted. Statistical analysis was conducted using a one-way ANOVA followed by a Tukey's post-test comparing all experimental values (ns, $p > 0.05$, **, $p < 0.01$ and ***, $p < 0.001$). A representative image (B) depicting the effect of GFP-hGRwt concentration on the interaction of GFP-hGRwt and FBXW7 α , following treatment with Dex, where the PLA signal is observed as distinct red 'spots', GFP-hGRwt concentration by the green GFP signal and the cell's nucleus is depicted by the blue DAPI stain. Lastly, the merge is an overlay of all three channels (red, green and blue).

In terms of the interaction of GR α and FBXW7 α , 'loss' of GR α dimerization, either following CpdA treatment or by the use of the dimerization deficient GR α , restricts an increase in the interaction of the receptor with the E3 ligase, relative to the basal interaction. Together, results from two different experiments, namely co-IP and PLA, solidify a role for GR α dimerization in mediating interaction with the E3 ligase, FBXW7 α . Moreover, apart from GR α conformation (i.e. monomer versus dimer), the cellular concentration of the receptor itself seems to modulate its interaction with FBXW7 α .

To our knowledge, this is the first time the ability of receptor conformation, and cellular concentration, to modulate the GR α interaction with the E3 ligase, FBXW7 α , has been investigated. These findings highlight the importance of receptor conformation in mediating interactions with certain UPS components and furthermore, identify a novel cellular effect regulated by changes in GR α concentration. This finding may have implications for the rate of ligand-induced GR α degradation.

5.2.3. Loss of GR α dimerization restricts hyper-phosphorylation at Serine 404.

The highly specific interaction of FBXW7 α with its substrates requires prior substrate phosphorylation of a CDC4 phosphodegron motif, at serine or threonine residues, generally mediated by a class of enzymes, termed GSK^{45,46}. Specifically, in terms of GR α , Malyukova *et al.*¹⁴ demonstrated that the interaction of FBXW7 with GR α is dependent on GSK-mediated hyper-phosphorylation at Ser404. Interestingly, hyper-phosphorylation of GR α at this site has also been directly linked to a decrease in protein stability and increased degradation of GR α via the proteasome⁸. With this in mind, it seemed necessary to investigate whether the novel requirement for GR α dimerization in mediating the interaction of GR α with FBXW7 α , established in the current study, would be effectuated at the level of hyper-phosphorylation at Ser404.

To investigate whether a ‘loss’ of GR α dimerization restricted phosphorylation of GR α at Ser404, in turn preventing receptor turnover and maintaining GR α stability, COS-1 cells were transiently transfected with hGRwt or hGRdim and treated with solvent (EtOH), Dex, F or CpdA (10 μ M). Phospho-Ser404 GR α levels were then determined by Western blotting using a phospho-Ser404 specific anti-body (Table 3.3). This was repeated in HepG2 cells containing endogenous GR α . In addition, using a GSK3 β inhibitor (BIO), we inhibited the Dex-induced hyper-phosphorylation at Ser404, in the HepG2 cells, and determined whether this inhibition would restrict receptor turnover and partially restore endogenous GR α protein levels.

‘Gain’ of hGRwt dimerization via dimerization promoting GR α ligands, Dex and F (10 μ M) resulted in a significant ($p < 0.001$) hyper-phosphorylation, at Serine404, of transiently transfected hGRwt to 153 and 140%, respectively (Fig. 5.9A). A similar, significant ($p < 0.001$) increase in pSer404-hGR α (145%) expression was observed with endogenous hGR α , in the HepG2 cells, following Dex treatment (Fig. 5.9C). Although not significant, hyper-phosphorylation (125%), at Ser404, of endogenous hGR α was also noted, after treatment with F (Fig. 5.9C).

In stark contrast, ‘loss’ of GR α dimerization through the use of the mutant, hGRdim, did not result in any hyper-phosphorylation of hGRdim at Ser404, following Dex and F treatment (Fig. 5.9B). In fact, pSer404-hGRdim seemed to be completely absent and even basal phosphorylation of transiently transfected hGRdim at this site was undetectable (Fig. 5.9B), unlike with the hGRwt (Fig. 5.9A). In support of the hGRdim result, CpdA treatment, known to abrogate GR α dimerization^{47,48}, reduced hyper-phosphorylation of transiently transfected hGRwt (Fig. 5.9A) and endogenous hGR α at Ser404 (Fig. 5.9C). More specifically, pSer404-hGRwt expression following CpdA treatment was not significantly different from basal pSer404-hGRwt expression, in the presence of solvent (EtOH), but significantly different from the hyper-phosphorylation of hGRwt at this site, following Dex ($p < 0.001$) and F treatment ($p < 0.001$) (Fig. 5.9A). This result was

consistent for endogenous hGR α in the HepG2 cells (Fig. 5.9C). In addition, inhibition of the Dex mediated hyper-phosphorylation at Ser404 (on the endogenous hGR α), using the GSK3 β inhibitor, BIO, resulted in significant ($p < 0.05$) restoration of GR α protein levels (Fig. 5.9D). A slight, yet insignificant, increase is also noted in receptor levels following treatment with F and BIO (Fig. 5.9D). Lastly, no significant change was noted in hGR α protein levels following treatment with dimerization abrogating, CpdA, and the kinase inhibitor, BIO (Fig. 5.9D).

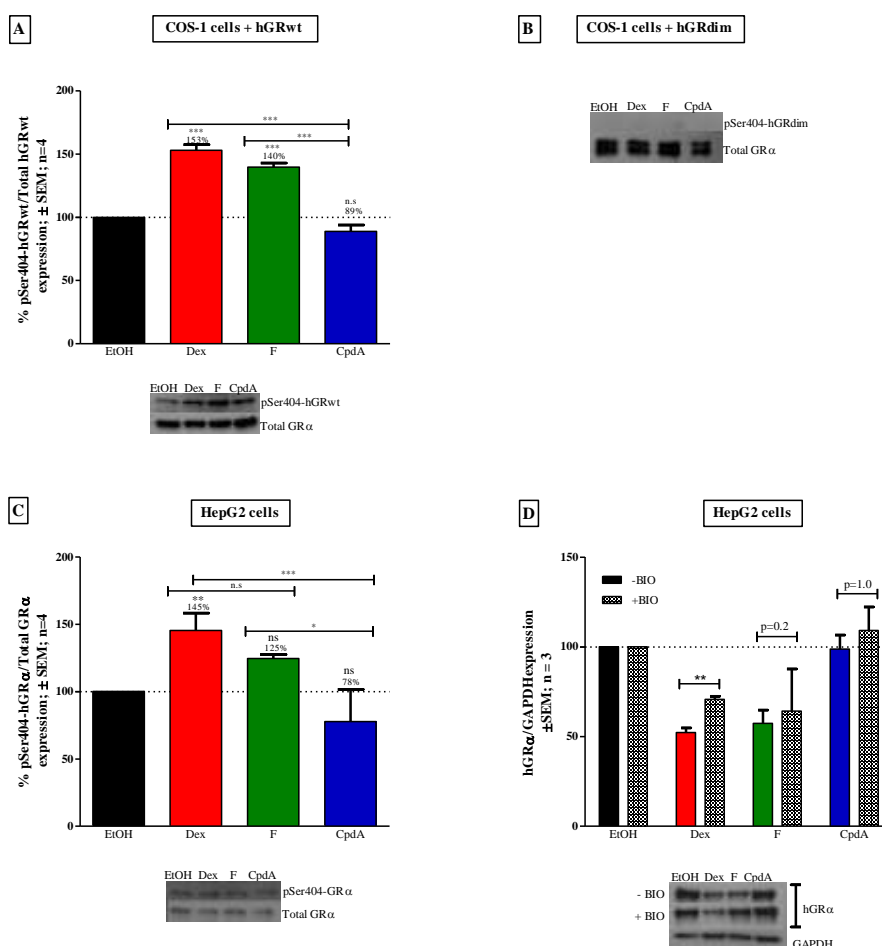


Figure 5.9: Monomeric GR α , due to CpdA binding or use of hGRdim mutant, does not undergo hyper phosphorylation at pSer404. COS-1 cells were seeded in a 12 well plate (1×10^5 cell/well) and the next day transfected with either hGRwt (400ng/well) (A) or hGRdim (800ng/well) (B) using FuGENE 6 transfection agent (see materials and methods). Following 24 hours incubation, cells were treated with compounds Dex, F and CpdA (10^{-5} M) for 2 hours and subsequently lysed in 80 μ l/well RIPA buffer (see materials and methods). This experiment was repeated in HepG2 cells seeded in a 12 well plate (1×10^5 cell/well), containing endogenous GR α (C). Additionally, HepG2 cells were treated with 5 μ M BIO for 1 hour and then with compounds (D). All pSer404-GR levels were detected using Western blotting, blots were quantified (graph) using UNSCANIT and a representative figure from a single experiment (positioned below graph, except in the case of hGRdim) is shown. Blots were then stripped, using a stripping buffer (see materials and methods), and re-probed for total GR α protein content for normalization. In the case of (D), total hGR α was detected and normalized to loading control GAPDH. For statistical analysis, of hGRwt (A) and endogenous hGR α (C), a one-way analysis of variance (ANOVA) followed by a Bonferoni's Multiple comparison post-test (ns, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$) was conducted. Statistical analysis of hGRdim could not be conducted due to the absence of any visible pSer404-GR α levels. A t-test was conducted comparing hGR α protein levels in the absence or presence of BIO, following Dex, F or CpdA treatment in the last figure. The dotted line represents values in presence of control (EtOH) and is set at 100% for each experiment.

To summarize, restricting phosphorylation at Ser404 of dimeric GR α , through the use of a kinase specific inhibitor, partially stabilizes the receptor levels thereby confirming the role of phosphorylation at this site in mediating receptor turnover. In addition, these findings suggest that a lack of phosphorylation at Ser404, due to a ‘loss’ of receptor dimerization, through CpdA treatment or use of hGRdim, likely restricts the interaction of hypo-phosphorylated GR α with the E3 ligase, FBXW7 α , as this interaction is dependent on phosphorylation at Ser404¹⁴. This, to our knowledge, is the first time the link between GR α conformation (i.e. monomer versus dimer) and hyper/hypo-phosphorylation at Ser404 and interaction with FBXW7 α , has been investigated. This finding, in combination with the findings in Section 5.2.2, demonstrate how a ‘loss’ of receptor dimerization modulates the post-translational processing of the GR α and explains, in part, one of the ways in which ligand-induced down-regulation of predominantly monomeric GR α is prevented. Furthermore, confirming a role for receptor dimerization in ligand-induced GR α down-regulation, initially described in Chapter 4.

5.2.4. ‘Loss’ of hGRwt dimerization stabilizes the interaction of the receptor with TSG101, increasing receptor stability.

In addition to FBXW7 α , there are other enzymes involved in the UPS, which are known to affect GR α stability, namely CHIP and TSG101. Like FBXW7 α , CHIP is an E3 ligase known to interact with GR α , targeting it for proteasome degradation, however, unlike FBXW α , CHIP has been shown to interact with both the unliganded and liganded receptor^{13,22,23}. In contrast, TSG101 resembles an E2 conjugating enzyme, however it is catalytically inactive and unable to catalyse the transfer of ubiquitin to an E3 ligase for the tagging of the substrate^{15,21}. Moreover this enzyme, TSG101, is thought to stabilize the non- or hypo-phosphorylated form of GR α ¹⁵. Thus, having demonstrated that a GR α monomer is less capable than a GR α dimer of interacting with FBXW7 α (Fig. 5.7), it was decided to investigate whether a ‘loss’ of receptor dimerization, following treatment of hGRwt with CpdA, influenced the interaction of GR α with either CHIP or TSG101, relative to a ‘gain’ of hGRwt dimerization, following Dex and F treatment.

Firstly, to investigate the interaction of hGRwt with CHIP or TSG101 following treatment with dimerization promoting, Dex and F, or dimerization abrogating, CpdA, COS-1 cells were transiently transfected with hGRwt and treated with solvent (EtOH), Dex, F or CpdA (10 μ M). Subsequently, a co-IP followed by Western blotting was conducted.

In terms of the co-immunoprecipitation results, ‘gain’ of hGRwt dimerization (via Dex treatment) slightly, but not significantly ($p > 0.05$), altered the association of the receptor with the E3 ligase, CHIP, however F and CpdA treatment did not ($p > 0.05$) (Fig. 5.10A). One could postulate that this

E3 ligase is exchanged for FBXW7a, following Dex treatment. Similarly, an increase in hGRwt dimerization, following Dex treatment, resulted in a slight decrease ($p > 0.05$) in the association of the receptor with the catalytically inactive, TSG101 (Fig. 5.10B). Moreover, ‘loss’ of hGRwt dimerization, as a result of CpdA treatment, appeared to stabilize the interaction of the receptor with TSG101 relative to unliganded hGRwt in one of the two experiments (Fig. 5.10B, see blot) but no conclusive deductions can be made and this interaction requires further investigation (Fig. 5.10B). Although the quantification of the effect of CpdA on the hGRwt/TSG101 interaction was not significantly ($p > 0.05$) different to that of the unliganded receptor, the Western blot (Fig. 5.10B, inset) seemed to show a potential increase in the interaction of these two proteins.

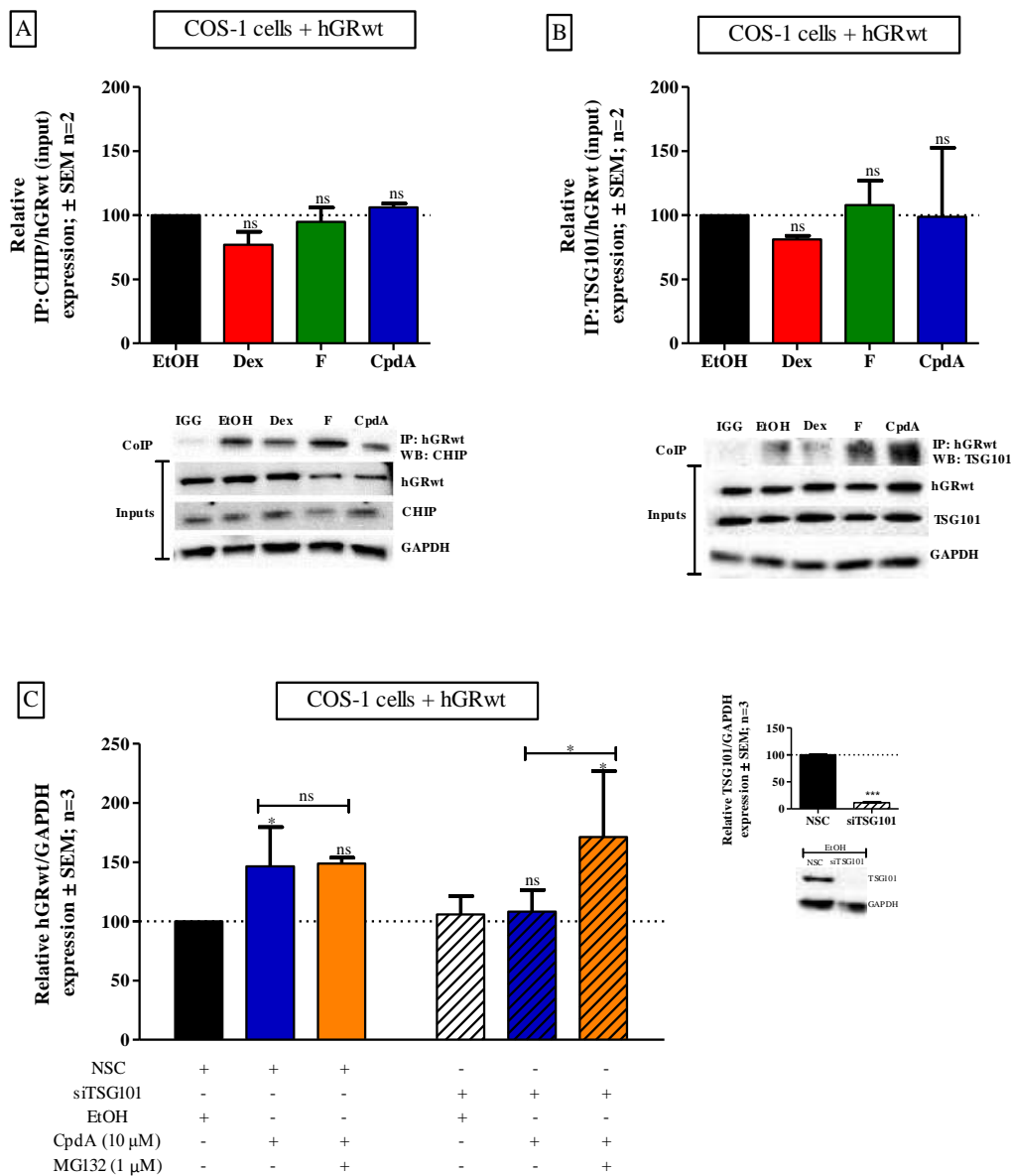


Figure 5.10: ‘Loss’ of hGRwt dimerization stabilizes the interaction of the receptor with TSG101, increasing receptor stability. COS-1 cells were seeded and the next day transfected with hGRwt (Table 3.2). For the co-IP (A and B) experiment, following 24 hours incubation, cells were treated with solvent (EtOH), Dex, F or CpdA (10^{-5} M) for 3 hours and then lysed. CHIP (A) or TSG101 (B) was immuno-precipitated with hGRwt using specific antibodies (Table 3.3). In the knockdown experiment (C), cells were transfected with either a non-silencing control (NSC) or siRNA targeting TSG101 (siTSG101) a day after hGRwt transfection, and then treated with solvent (EtOH) or CpdA (10 μ M), for 24 hours. Additionally, to block proteasome degradation, pre-treatment for 1 hour with MG132 (1 μ M) was conducted, followed by CpdA (10 μ M) treatment for 24 hours. Following co-IP and knockdown, Western blotting was conducted to determine protein expression and GAPDH was probed to ensure equal protein loading. A representative blot of two or three independent experiments is shown. For quantification (A and B), the interaction of hGRwt and CHIP or TSG101 was calculated by determining the intensity, using the MyECL Image Software Analysis, of CHIP or TSG101 (after IP), which was normalized to total hGRwt (i.e. intensity of hGRwt input). The dotted line on the graph represents the interaction of hGRwt with CHIP or TSG101 in the presence of solvent (EtOH), and is set at 100%. Statistical analysis was conducted using a one-way ANOVA or t-test (A and B) and a two-way ANOVA (C) with a Bonferoni’s post-test comparing all experimental values (ns, $p > 0.05$ and *, $p < 0.05$) or an unpaired t-test (C, inset) (***, $p < 0.001$).

‘Loss’ of GR α dimerization, following CpdA treatment, prevented hyper-phosphorylation of GR α at Ser404 (Fig. 5.9), in part stabilizing hGRwt. Furthermore, TSG101 is known to associate with non- or hypo-phosphorylated GR α , protecting it from degradation via the proteasome¹⁵. Taken together, one would expect that TSG101 is important in CpdA’s ability to maintain GR α protein levels. Thus, to further investigate a possible role of TSG101 in CpdA-induced stability of the GR α , a TSG101 knockdown experiment was conducted (Fig. 5.10C).

COS-1 cells were transiently transfected with hGRwt and a non-silencing control (NSC) or siRNA targeting TSG101 (Fig. 5.10C). Cells were then treated with solvent (EtOH) or CpdA (10 μ M) for 24 hours, or MG132 (1 μ M) for 1 hour followed by CpdA (10 μ M) for 24 hours (Fig. 5.10C). Results from the knockdown experiment, using siRNA targeting TSG101, demonstrate that endogenous TSG101 was significantly ($p < 0.001$) eliminated from the COS-1 cells (Fig. 5.10C, inset). Importantly, in the presence of endogenous TSG101 (i.e. NSC samples) and consistent with previous results in the current study, 24 hours of CpdA treatment leads to an increase ($p < 0.05$) in GR α protein levels, relative to solvent (EtOH) (Fig. 5.10C). Furthermore, pre-treatment with MG132, had no effect on CpdA’s ability to maintain receptor levels, when TSG101 was present in the cells (Fig. 5.10C). In contrast, in COS-1 cells where TSG101 had been depleted, CpdA did not result in a significant increase in GR α protein levels, relative to the solvent (EtOH), suggesting a level of receptor turnover in the presence of CpdA and absence of TSG101 (siTSG101) (Fig. 5.10C). This was confirmed by the addition of MG132, the proteasome inhibitor, which significantly ($p < 0.05$) prevented GR α protein turnover even in the absence of the stabilizing TSG101 protein (siTSG101) (Fig. 5.10C).

In summary, consistent with literature, results suggest the association of CHIP with GR α occurs in the presence and absence of GCs¹³. Furthermore, although not significant, ‘gain’ of GR α dimerization, through treatment with Dex, may reduce the association of the receptor with the stabilizing TSG101 protein, whilst ‘loss’ of dimerization, via CpdA treatment, may stabilize this

interaction, however, this requires further investigation. Results do suggest, however, that TSG101 is in some way required for CpdA's ability to prevent receptor turnover, which is evident from the TSG101 knockdown experiment.

5.2.5. GR α ubiquitination

Results from the current study, and others^{29,30,49,50}, have demonstrated that the GR α is degraded primarily via the proteasome. Furthermore, in order for the proteasome to successfully mediate the degradation of protein substrates, in this case GR α , the substrate must be tagged by a poly-ubiquitin chain to allow for substrate recognition by the proteasome^{1,11,12,24-28}. This formation of the poly-ubiquitin chain via the covalent attachment of numerous ubiquitin molecules to the substrate is mediated by a hierarchy of enzymes, one of them being FBXW7 α ^{11,14}.

The current study has shown that a 'loss' of receptor dimerization influences the interaction of the receptor with components of the UPS (i.e. FBXW7 α), in turn affecting GR α stability.

To determine whether a 'loss' of GR α dimerization modulated the ubiquitination status of the receptor, the current study investigated whether a 'loss' of GR α dimerization modulated the subcellular and co-localisation of GR α and ubiquitin, using immunofluorescence. Additionally, the effect of GR α conformation (i.e. monomer versus dimer) on the ubiquitination status of the receptor was investigated using co-immunoprecipitation and PLA.

5.2.5.1. Ligand-dependent subcellular localisation of hGRwt modulates its co-localisation with endogenous ubiquitin. 5.2.5.1

The subcellular and co-localisation of hGRwt and ubiquitin was determined as in Section 5.2.2.1 (for hGRwt and FBXW7 α).

From the results, it is clear successful transient transfection of the hGRwt plasmid was achieved (Fig. 5.11A). This is evident when comparing the immunofluorescence for hGRwt (red) between the untransfected COS-1 cells and the cells transiently transfected with hGRwt (Fig. 5.11A). From the quantification, results from cells containing the hGRwt plasmid showed that the unliganded hGRwt is more nuclear than cytoplasmic, following 6 hours treatment with solvent (EtOH) (importantly, 3 hours longer than in FBXW7 α experiment in Figure 5.5) ($p < 0.001$) (Fig. 5.11B). As expected, treatment with dimerization promoting synthetic GC, Dex, and endogenous GC, F, resulted in total nuclear translocation of hGRwt, with little to no receptor detected in the cytoplasm ($p < 0.001$) (Fig. 5.11B). A significant (c, $p < 0.001$) decrease in cytoplasmic hGRwt but, unlike Figure 5.5B which noted an increase, no change (b, $p > 0.05$) in the nuclear hGRwt localization is observed following Dex and F treatment, relative to the unliganded hGRwt (Fig. 5.11B). A possible

explanation for this is the difference in the periods of compound treatment between the two experiments (i.e. 3 versus 6 hours). In addition, treatment with dimerization abrogating GC, CpdA, resulted in significant ($p < 0.001$) translocation of hGRwt from the cytoplasm to the nucleus, however, a portion (approximately 30%) of hGRwt remained in the cytoplasm (Fig. 5.11B). Although, following CpdA treatment, the hGRwt cytoplasmic localization was similar to that of the unliganded hGRwt, the nuclear localization of hGRwt was in fact greater (d, $p < 0.05$) than that of the unliganded receptor (Fig. 5.11B). In terms of ubiquitin, ubiquitin expression was found to be predominantly nuclear ($p < 0.001$) (Fig. 5.11C) across all treatments.

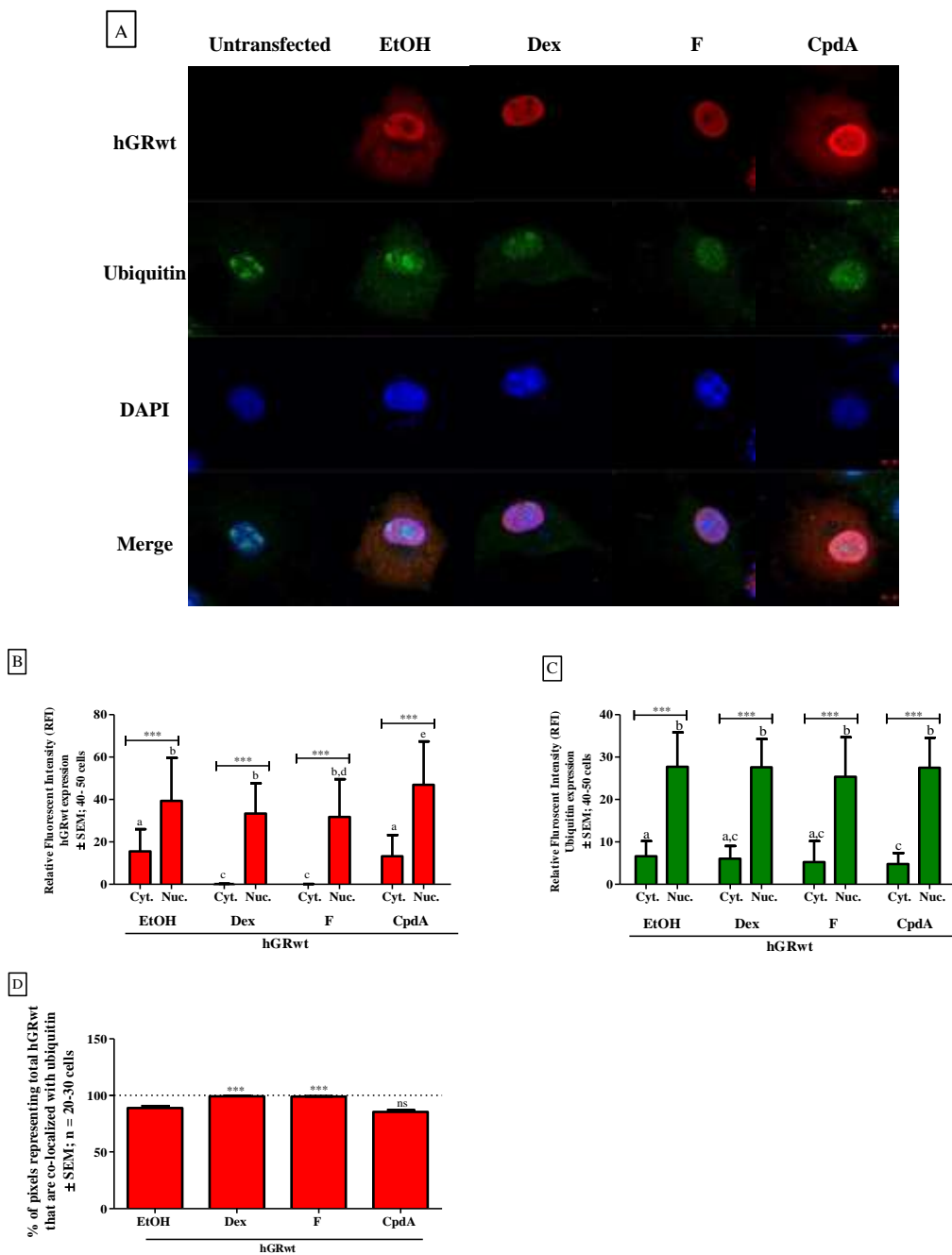


Figure 5.11: Ligand-dependent subcellular localisation of hGRwt modulates its co-localisation with endogenous ubiquitin. COS-1 cells were seeded into a 10 cm dish (1×10^6 cells) and transiently transfected the next day with hGRwt using a transfection reagent. Following 24 hours incubation, cells were re-plated into 8 well chambers (3×10^4 cells/well) (Table 3.2) and the next day treated with solvent (EtOH), Dex or CpdA (10^{-5} M) for 6 hours. Thereafter, cells were fixed, permeabilized, and immunofluorescence conducted, with antibodies specific for GR α and ubiquitin. Cells were then imaged using a confocal microscope. A representative image (A) illustrates the individual subcellular localisation of hGRwt (red, first row) and ubiquitin (green, second row), as well as the position of the cell's nucleus (blue DAPI stain, third row) with the merge representing an overlay of all three channels (red, green and blue). For the quantification of the subcellular localisation of hGRwt and ubiquitin the relative fluorescence intensity (RFI) of the red (hGRwt) (B) or green (ubiquitin) (C) pixels was calculated for individual cells and plotted. In addition, the co-localisation of these two proteins, in terms of hGRwt (D), was determined using the weighted co-localisation coefficients, where the horizontal dotted line represents 100% co-localisation of hGRwt with ubiquitin. Statistical analysis was conducted using a two-way ANOVA followed by a Bonferroni's post-test comparing experimental values to solvent's (EtOH) cytoplasm for hGRwt (B) or ubiquitin (C) (for a,b,c,d and e letters that are the same represent no significant difference between values whilst letters, which are different are significantly different from each other $p < 0.05$) or comparing the cytoplasmic and nuclear expression of hGRwt (B) or ubiquitin (C) within a treatment group (ns, $p > 0.05$ and ***, $p < 0.001$). For co-localisation (D), a one-way ANOVA with a Tukey's post-test was used, comparing experimental values to the solvent (EtOH) (***, $p < 0.001$).

Lastly, Figure 5.11D demonstrates the co-localisation (i.e. where these proteins occupy the same space) of these two proteins, hGRwt and ubiquitin, importantly with reference to hGRwt. In other words, the quantification graph (Fig. 5.11D) depicts the percentage (%) of the total hGRwt protein, which co-localizes with ubiquitin. From this graph, it is clear that, a large proportion (approximately 89%) of the unliganded hGRwt protein co-localizes with ubiquitin (Fig. 5.11D). This is due to the ubiquitous, but more nuclear, expression of ubiquitin throughout the cell (i.e. cytoplasm and nucleus). With that said, promoting hGRwt dimerization and its nuclear translocation, through Dex and F treatment, results in a significant ($p < 0.001$) increase in the co-localisation of hGRwt and ubiquitin, with almost all (just less than 100%) of hGRwt now occupying the same space as ubiquitin (Fig. 5.11D). In contrast, treatment with dimerization abrogating CpdA (85%) did not significantly ($p > 0.05$) modulate the co-localisation of hGRwt and ubiquitin, relative to the unliganded receptor (89%). This is likely due to CpdA's diminished ability to induce nuclear translocation of hGRwt to where the majority of the cellular ubiquitin expression occurs (i.e. the nucleus).

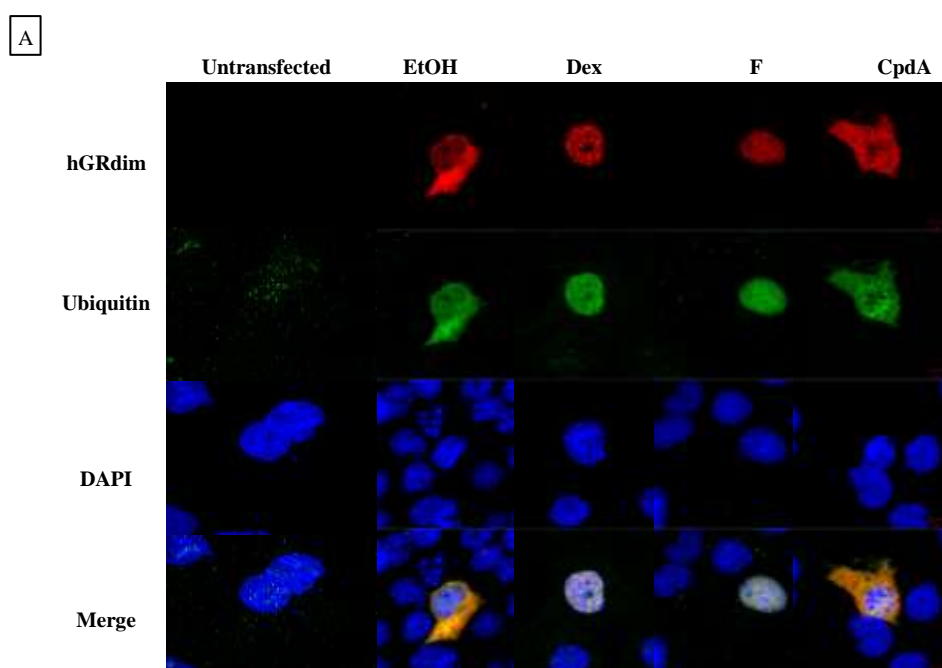
5.2.5.2. Ligand-dependent subcellular localisation of hGRdim does not modulate its co-localisation with endogenous ubiquitin.

The subcellular and co-localisation of hGRdim and ubiquitin was determined as in Section 5.2.2.2 (for hGRdim and FBXW7 α).

As with hGRwt in Figure 5.11, successful transient transfection of the hGRdim plasmid was achieved (Fig. 5.12A). Moreover, in cells containing the hGRdim plasmid and treated with solvent (EtOH) for 6 hours, an even subcellular distribution of the unliganded hGRdim, across the cytoplasm and nucleus, was noted (Fig. 5.12A and B). As expected, treatment with Dex and F resulted in total nuclear translocation of hGRdim, with negligible receptor levels detected in the

cytoplasm ($p < 0.001$) (Fig. 5.12A and B). In addition, and unlike hGRwt (Fig. 5.11A and B), treatment with the dimerization abrogating ligand, CpdA did not induce ($p > 0.05$) translocation of hGRdim from the cytoplasm to the nucleus (Fig. 5.12A and B) consistent with results in Figure 5.6.

Interestingly, and in stark contrast to results obtained with hGRwt (Fig. 5.11C), ubiquitin expression in COS-1 cells transfected with hGRdim and treated with solvent (EtOH) or compounds (Dex, F or CpdA) appeared to vary considerably (Fig. 5.12C). From the image it appears that there is less endogenous ubiquitin expression in these COS-1 cells (Fig. 5.12A) in comparison to the endogenous ubiquitin expression in Figure 5.11A, however, this is not the case. Rather microscope settings were altered in this figure (Fig. 5.12A) to take into account, what appears to be, an increase in ubiquitin expression in cells transfected with hGRdim. Moreover, the quantification of the subcellular localisation of ubiquitin in these cells generally mimicked the subcellular localisation of hGRdim in Figure 5.12B. Specifically, in cells containing unliganded hGRdim, ubiquitin expression was prominent in both the cytoplasm and the nucleus, with significantly ($p < 0.01$) more so in the nucleus (Fig. 5.12C). Additionally, treatment with Dex and F, appeared to induce an unexpected increase in nuclear and decrease in cytoplasmic expression of ubiquitin, relative to cells containing unliganded hGRdim (Fig. 5.12C), suggesting that these GCs are modulating the subcellular localisation and potentially the expression of ubiquitin in what seems to be an hGRdim dependent manner. Conversely, CpdA treatment did not ($p > 0.05$) modulate the subcellular localisation or expression of ubiquitin, relative to cells treated with solvent (EtOH) (Fig. 5.12C).



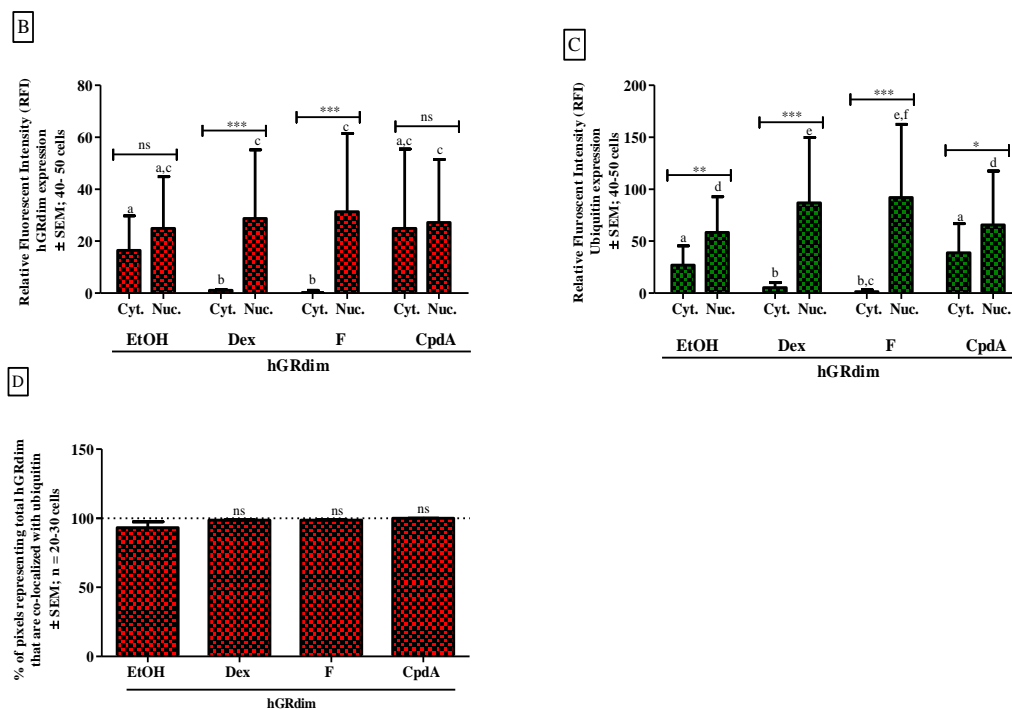


Figure 5.12: Ligand-dependent subcellular localisation of hGRdim does not modulate its co-localisation with endogenous ubiquitin. COS-1 cells were seeded into a 10 cm dish (1×10^6 cells) and transiently transfected the next day with hGRdim using a transfection reagent. Following 24 hours incubation, cells were re-plated into 8 well chambers (3×10^4 cells/well) (Table 3.2) and the next day treated with solvent (EtOH), Dex or CpdA (10^{-5} M) for 3 hours. Thereafter, cells were fixed, permeabilized, and immunofluorescence conducted, with antibodies specific for GR α and ubiquitin. Cells were then imaged using a confocal microscope. A representative image (A) illustrates the individual subcellular localisation of hGRdim (red, first row) and ubiquitin (green, second row), as well as the position of the cell's nucleus (blue DAPI stain, third row) with the merge representing an overlay of all three channels (red, green and blue). For the quantification of the subcellular localisation of hGRdim and ubiquitin the relative fluorescence intensity (RFI) of the red (hGRdim) (B) or green (ubiquitin) (C) pixels was calculated for individual cells and plotted. In addition, the co-localisation of these two proteins, in terms of hGRdim (D), was determined using the weighted co-localisation co-efficients, where the horizontal dotted line represents 100% co-localisation of hGRdim with and ubiquitin. Statistical analysis was conducted using a two-way ANOVA followed by a Bonferoni's post-test comparing experimental values to solvent's (EtOH) cytoplasm for hGRdim (B) or ubiquitin (C) (for a,b,c,d and e letters that are the same represent no significant difference between values whilst letters, which are different are significantly different from each other $p < 0.05$) or comparing the cytoplasmic and nuclear expression of hGRdim (B) or ubiquitin (C) within a treatment group (ns, $p > 0.05$ and ***, $p < 0.001$). For co-localisation (D), a one-way ANOVA with a Tukey's post-test was used, comparing experimental values to the solvent (EtOH) (***, $p < 0.001$).

Lastly, Figure 5.12D demonstrates the co-localisation of hGRdim and ubiquitin, with reference to hGRdim. From the graph, it is clear that no ligand-induced changes in the co-localisation of hGRdim and ubiquitin occur, with almost all (just less than 100%) of the receptor co-localised with ubiquitin, across treatments (Fig. 5.12D). This is in contrast to the result obtained for hGRwt and ubiquitin in Figure 5.11.

5.2.5.3. Decreased GR α ubiquitination is observed following treatment with dimerization promoting GCs.

Previously, a 'loss' of receptor dimerization influenced the ability of FBXW7 α to interact with GR α . Moreover, the current study demonstrates that this is due to a difference in the

phosphorylation status between a GR α dimer a monomer. As ubiquitin, is considered the ‘recognition signal’ for the degradation of the receptor, via the proteasome, it was decided to determine whether a ligand-induced ‘loss’ of receptor dimerization alters the ubiquitination status of GR α .

To do this, an initial co-immunoprecipitation assay was conducted followed by PLA. For the co-IP, due to a lack of a high quality ubiquitin antibody at the time of this experiment, COS-1 cells were transiently co-transfected with HA-tagged ubiquitin and hGRwt, and treated with solvent (EtOH) and Dex (10 μ M) and the pull-down conducted with a highly specific HA antibody, to determine ubiquitinated GR α (Fig. 5.13A). Unlike the co-IP, the PLA was done using a highly specific ubiquitin anti-body, which became commercially available to investigate the interaction of endogenous ubiquitin with hGRwt (Fig. 5.13B).

In terms of the co-IP results, an unexpected decrease in hGRwt ubiquitination was observed, following treatment with dimerization promoting Dex, relative to unliganded hGRwt (solvent (EtOH)) (Fig. 5.13A). Moreover, results from the PLA, supported the co-IP result (Fig. 5.13B). Specifically, that promoting hGRwt dimerization through Dex and F treatment appeared to reduce the amount of ‘fluorescent red spots’, suggesting a decrease in GR α ubiquitination (Fig. 5.13B). One could postulate that this decrease in hGRwt ubiquitination is due to dimeric GR α already being degraded, by the proteasome, following 6 hours of Dex and F treatment and is supported by the reduction in hGRwt expression, noted in the hGRwt input of the co-IP in Figure 5.13A.

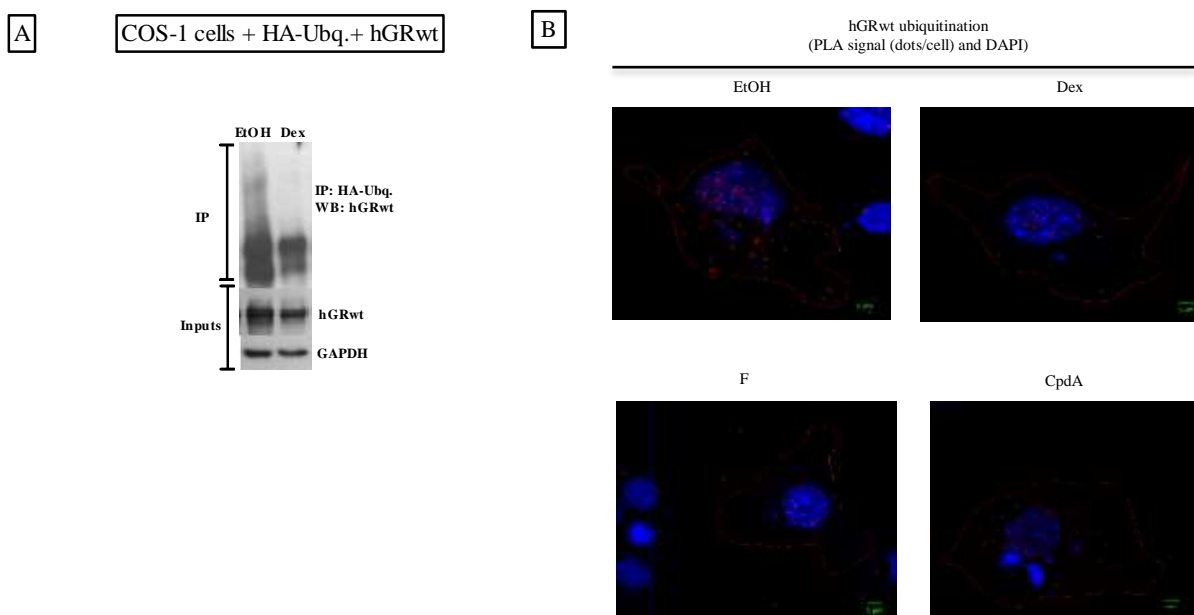


Figure 5.13: Decreased GR α ubiquitination is observed following treatment with dimerization promoting GCs. COS-1 cells were seeded, transfected and in some cases re-plated in 8 well chambers. Following 24 hours incubation, cells were treated with solvent or compounds for 6 hours. For the co-IP (A) experiment, after treatment with solvent (EtOH) or Dex (10^{-5} M) cells were lysed and ubiquitinated hGRwt was immuno-precipitated with using an HA-antibody. Western blotting was conducted to determine protein levels and GAPDH was probed to ensure equal protein loading. For the PLA (B), following treatment with solvent (EtOH), Dex, F or CpdA (10^{-5} M), cells were fixed, permeabilized and PLA conducted using specific antibodies for GR α and ubiquitin. A representative image of individual cells demonstrating hGRwt ubiquitination, is shown, where the PLA signal is observed as distinct red ‘spots’ and the cell’s nucleus is depicted by the blue DAPI stain.

Interestingly, and in contrast to Dex and F treatment, ‘loss’ of hGRwt dimerization, though CpdA treatment, seemed to induce ubiquitination of the receptor, however, the fluorescent spots appeared lighter, relative to the unliganded hGRwt (Fig. 5.13B). This suggests that monomeric GR α is in fact ubiquitinated, regardless of its lack of hyper-phosphorylation at Ser404 and interaction with the E3 ligase, FBXW7 α .

Taken together, these results suggest that both unliganded and liganded hGRwt are able to undergo ubiquitination. With that said, less hGRwt ubiquitination is observed following treatment with Dex and F, which is likely due to significant degradation occurring at this time point. Of interest, is whether blocking degradation through the use of the proteasome inhibitor, MG132, would alter the observed receptor ubiquitination, following Dex and F treatment for 6 hours? The use of MG132 should be considered in further investigations of GR α but is beyond the scope of the current study. Lastly, the ubiquitination of hGRdim should be investigated to gain further insight on the link between receptor dimerization and ubiquitination. Unfortunately, due to time and financial constraints, the PLA experiment could not be repeated using the GFP-tagged GR α plasmids as in Figure 5.7C.

5.2.6. Co-treatment with CpdA lessens the extent of ligand-induced GR α protein down-regulation, thereby partially restoring GR α levels.

It is clear that the conformation of GR α is vital for ligand-induced receptor turnover at the protein level. More specifically, that dimeric GR α , formed following treatment with dimerization promoting, Dex and F, is more susceptible to down-regulation and undergoes significant ligand-induced protein down-regulation. In contrast, monomeric GR α generated following CpdA treatment (or the use of hGRdim) evades degradation by modulating post-translational modifications of the GR α and altering interactions of GR α with components of the proteasome system, thus maintaining receptor levels.

Up to now, the ligand-selective effects of Dex (dimerization promoting) and CpdA (dimerization abrogating) on hGRwt protein down-regulation have been investigated by treating COS-1 cells, transiently transfected with hGRwt, with either Dex (10 μ M) or CpdA (10 μ M) alone. To

investigate whether CpdA treatment, in combination with Dex, could minimize the extent of the Dex-induced down-regulation of the transiently transfected hGRwt protein, Western blotting was conducted following two co-treatments, namely Dex (1 nM) and CpdA (10 μ M) or Dex (10 μ M) and CpdA (10 μ M) for 24 hours.

Consistent with previous results (Chapter 4, Table 3), Figure 5.14 suggests that hGRwt protein expression is significantly ($p < 0.001$) reduced to 34%, relative to the solvent (EtOH), following 24 hours treatment with a high Dex concentration (10 μ M). As one would expect, based on previous findings in the current study, treatment with a lower concentration of Dex (1 nM) led to a slightly less, yet significant ($p < 0.01$) reduction in hGRwt protein expression to 52%, relative to solvent (EtOH) (Fig. 5.14), suggesting a dose-dependent effect. Moreover, CpdA (10 μ M) treatment for 24 hours lead to a slight ($p > 0.05$) increase in receptor expression relative to the solvent (EtOH), a result that is comparable to previous results in the current study (Chapter 4, Table 3).

Interestingly, treatment of transiently transfected hGRwt with CpdA (10 μ M) in combination with Dex (1 nM) significantly ($p < 0.05$) diminished receptor turnover, relative to Dex-induced hGRwt down-regulation, by maintaining hGRwt protein levels at 90% of solvent (EtOH) (Fig. 5.14). A similar but more significant ($p < 0.01$) effect was observed following treatment with CpdA (10 μ M) in combination with the higher concentration of Dex (10 μ M) (Fig. 5.14).

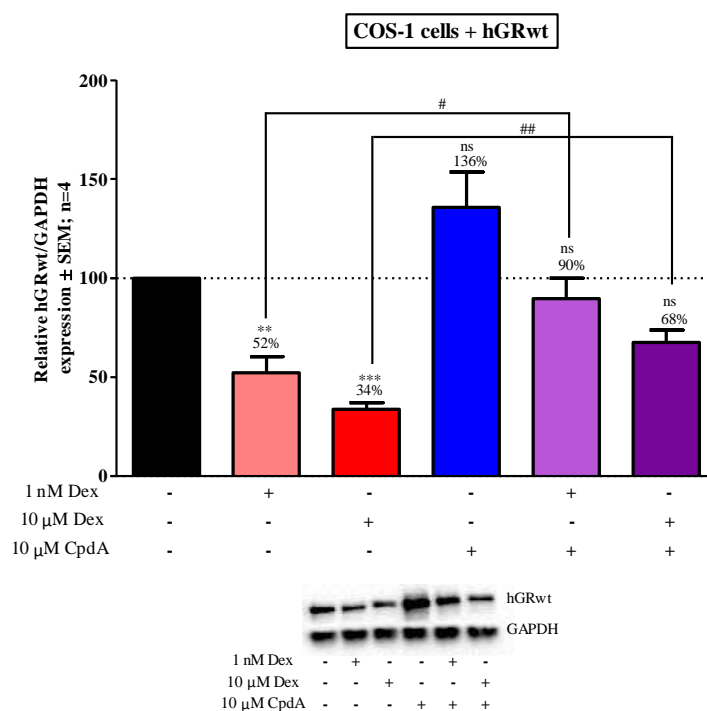


Figure 5.14: Treatment of Dex with CpdA partially restores hGRwt protein levels. COS-1 cells were seeded into a 24 well plate (5×10^4 cells/well) and transfected the next day with hGRwt using a transfection agent. Following 24 hours incubation, cells were treated individually with solvent (EtOH), Dex (1 μ M), Dex (10 μ M) or CpdA (10 μ M) or with CpdA (10 μ M) in combination with either Dex (1 μ M) or Dex (10 μ M) for 24 hours. hGRwt protein expression was confirmed by Western blotting where GAPDH was probed to ensure equal protein loading. Western blot shown (inset) is representative of four independent experiments. For quantification, the intensity of the hGRwt and GAPDH bands were determined using My ECL Image Analysis software and hGRwt levels were then normalised to GAPDH expression. For each treatment condition, hGRwt expression as expressed as a percentage (average \pm SEM) of hGRwt expression in presence of the solvent (EtOH), which was set at 100% (dotted line). To determine the effect of each treatment condition on hGRwt protein expression relative to the solvent (EtOH), statistical analysis for was conducted using a one-way ANOVA with a Bonferroni post-test (ns, $p > 0.05$, *, $p < 0.05$, **, $p < 0.001$ and ***, $p < 0.001$). To assess the ability of CpdA to preserve hGRwt expression in the presence of Dex a t-test was conducted comparing hGRwt expression following Dex treatment (either 1 μ M or 10 μ M) alone to Dex treatment in combination with CpdA (10 μ M) ($^{\#}$, $p < 0.05$, $^{\#\#}$, $p < 0.01$).

Taken together, results in Figure 5.14 suggest that CpdA, when combined with Dex, has the ability to restrict Dex-induced hGRwt protein down-regulation to a certain extent. One could postulate that this is due to CpdA's ability to abrogate Dex-induced hGRwt dimers. More specifically, that CpdA in combination with Dex, results in a lower degree of hGRwt dimerization than Dex alone does, which partially prevents hyper-phosphorylation at Ser404 and interaction with FBWX7 α , two processes, results in the current chapter have highlighted as fundamental for receptor turnover, however, this requires investigation.

5.3. Discussion

The GC/GR α signalling pathway is subjected to layers of regulation, which function to co-ordinate the localization, activity and ultimately expression of various proteins. Central to this pathway is the GR α , whose concentration is an important determinant in GC sensitivity^{5,51–54}.

It is well documented that ligand-induced down-regulation of this receptor occurs^{29,30,47,55–60}. Moreover, in Chapter 4, a novel requirement for GR α dimerization in mediating ligand-induced receptor turnover, specifically via the proteasome degradation pathway, was described. This finding provided further insight into the GC/GR α signalling pathway and encouraged further investigation into the molecular mechanisms (i.e. post-translational processing of the receptor) involved in preventing ligand-induced GR α down-regulation of predominantly monomeric GR α , which was explored in the current chapter.

Essentially, the current chapter begins by determining whether the requirement for GR α dimerization in receptor turnover is dependent on a mutation at a specific site and whether it is species specific. Having provided further evidence for this link between receptor dimerization and protein turnover, this study demonstrates the ability of GR α conformation (i.e. monomer versus dimer) to modulate the post-translational processing of the receptor, as well as the interaction of the GR α with enzymes associated with the UPS, which ultimately, in part, affected the extent of receptor turnover mediated via the proteasome.

A factor common to all GR α mutants, with the exception of the natural mutant, is the single amino acid exchange of an alanine to a threonine within the D-loop of the DNA-binding loop (Fig. 5.4A). All GR α mutants, regardless of species and the presence of additional single point mutations, displayed a reduced receptor protein turnover, following treatment with Dex. Specifically, the mutation of an alanine residue to a threonine at position 458 (human) and 465 (mouse) is fundamentally important in restricting ligand-induced GR α degradation (Fig. 5.4). Moreover, additional mutations, such as an asparagine to an aspartate at position 454, in some of these GR α mutants (i.e. hGR (N545D/A458T) and hGR(D4X)) partially restores Dex-mediated receptor turnover. Additionally, unlike some of the other GR α mutants, the natural mutant hGR α (R477H), whose mutation occurs outside of the D-loop, did not undergo significant Dex-induced GR α protein down-regulation. Generally speaking, these mutants display a reduced ability to bind DNA and thus a reduced ability to induce transactivation of GC-responsive genes^{36,37,40,61,62}. These characteristics suggest these mutants are likely to be monomeric, however, apart from the classical dimerization deficient mutants (hGRdim and mGRdim), little evidence for the ability of these mutants to form dimers exists. Recently a study by Presman *et al.*⁶³ produced a mouse GR α mutant, which contains the classical dimerization deficient mutant (A465T) as well as an additional mutation, an isoleucine to an alanine substitution at position 634 (I634A), in the ligand-binding domain of the receptor, which is severely compromised in its ability to form receptor dimers and mediate the transactivation of GC responsive genes. With the current study having demonstrated a novel link between GR α dimerization and receptor turnover, it remains to be elucidated, and is of significant interest, whether this mutant, termed GRmon⁶³, is capable of undergoing ligand-induced protein turnover.

Recently, GR α was identified as a novel substrate for a highly specific E3 ligase, FBXW7 α . This protein is an f-box protein, which functions as part of an SCF (Skp1/Cul1/f-box) type of E3 ubiquitin ligase complex, which as a whole, is responsible for targeting various proteins for proteasomal degradation¹⁴. Furthermore, the expression of FBXW7 α has been shown to be capable of regulating the stability of the receptor, in a ligand-dependent manner¹⁴. Specifically, in cells where an FBXW7 α deficiency exists, a decrease in the extent of receptor turnover and a corresponding increase in GR α stability, in the presence of Dex, are observed¹⁴. Moreover, silencing FBXW7 α in HEK293 and HeLa cells, in the presence of Dex, led to an increase in GR α protein levels, similarly to the noted increase in receptor levels following proteasome inhibition with MG132, confirming FBXW7 α 's role in mediating GR α protein turnover¹⁴. Furthermore, in CHX-chase experiments where FBXW7 α had been silenced, notable increases in the half-lives of the GR α protein in Peer (from 3.8 hours to 5.2 hours) and K652 (7.5 hours to 9.8 hours) leukemic cell lines, were noted, even following treatment with Dex¹⁴. Lastly, in HCT116-FBXW7 knockout

cells, reconstituting the FBXW7 α expression in these cells led to an almost 3-fold ligand-induced decrease in the half-life of the GR α protein¹⁴.

Importantly, in order for FBXW7 α to mediate successful ligand-induced GR α turnover, a physical interaction between the E3 ligase and the receptor is required¹⁴. In terms of receptor conformation, ‘gain’ of GR α dimerization, via treatment with dimerization promoting Dex (10 μ M for 1 hour) induces the interaction of transiently transfected GR α and FBXW7 α in HEK293 cells, promoting its degradation by the proteasome¹⁴. In the current study, it was found that the greatest Dex-induced interaction between hGRwt and FBXW7 α occurred after 3 hours treatment with Dex (Fig. 5.7A). Having established the optimal treatment conditions, using dimerization promoting Dex treatment, for the formation of the GR α /FBXW7 α complex, the effect of a ‘loss’ of GR α dimerization on the ability of a GR α monomer to interact with the catalytically active E3 ligase, FBXW7 α , which to our knowledge had not yet been elucidated, was investigated.

Broadly speaking, results in which CpdA treatment was used to abrogate receptor dimerization, suggested that a ‘loss’ of GR α dimerization restricts interaction of the receptor with FBXW7 α (Table 1). More specifically, unlike dimerization promoting Dex and F, CpdA did not induce an increase in the interaction of GR α and FBXW7 α , relative to the unliganded receptor, in either the co-immunoprecipitation or the PLA experiment, where COS-1 cells were transiently transfected with hGRwt or GFP-hGRwt respectively (Table 1 and Fig. 5.7). Additionally, ‘loss’ of GR α dimerization, as a result of CpdA treatment, appeared to partially reduce interaction beyond the basal interaction of the unliganded receptor, which is likely explained by CpdA’s ability to abrogate existing unliganded GR α dimers⁴⁸.

In support of the CpdA results, ‘loss’ of GR α dimerization through the use of mutant (GFP-hGRdim) restricted Dex’s ability to induce interaction of the receptor and FBXW7 α (Table 1 and Fig. 5.7), in contrast to GFP-hGRwt. A similar result was obtained for the untagged hGRdim in the PLA experiment (data not shown); however, this may simply be due to differences in the expression between untagged hGRwt and hGRdim (Fig. S1). In terms of the co-IP results for untagged hGRdim (data not shown), no detectable FBXW7 α expression was noted following pull-down using a GR α anti-body and Western blotting. This may be due to the lack of hGRdim and FBXW7 α interaction, however, one cannot exclude the lower expression of the hGRdim in the COS-1 cells as a possible reason for this lack of interaction.

The interaction of FBXW7 α with its substrates has been shown to require prior phosphorylation at serine or threonine residues of the substrate^{45,46}. Of particular interest, for the current study, is the way in which FBXW7 α interacts with GR α , namely via a CDC4 phosphodegrom consensus sequence⁸. Interestingly, the amino acid sequence which flanks a recently discovered GR α

phosphorylation site, Ser404, resembles this highly conserved CDC4 phosphodegron motif and a study by Malyukova *et al.*¹⁴ demonstrated that the interaction of FBXW7 α with GR α , is dependent on hyper-phosphorylation at this novel site¹⁴. Generally, phosphorylation of the GR α , which is considered a phospho-protein with phosphorylation occurring at a number of different sites (Fig. 5.2), is known to affect the transcriptional response, subcellular localization and protein-protein interactions of the receptor with co-regulators⁹. Additionally, Galliher-Beckley *et al.*⁸ demonstrated that ligand-induced hyper-phosphorylation at Ser404 modulates GR α stability by encouraging an increase in receptor degradation. With this in mind, it seemed necessary to investigate whether the novel requirement for GR α dimerization in mediating the ligand-induced interaction of GR α and FBXW7 α , established in the current study, would be effectuated at the level of Ser404 phosphorylation, which then in combination modulates the protein stability of the receptor.

Indeed, a ‘loss’ of GR α dimerization, through treatment with dimerization abrogating CpdA, significantly restricted phosphorylation of the receptor (Table 1 and Fig. 5.9). By setting Dex-induced hyper-phosphorylation at Ser404 as 100%, the current study reports a substantial reduction in Ser404 phosphorylation to 58% in hGRwt transfected COS-1 cells and 54% in the endogenous system (i.e. HepG2 cells), following CpdA treatment (Fig. 5.9A and C). Interestingly, this was not the first time CpdA’s ability to restrict GR α phosphorylation had been demonstrated. In a study by Avenant *et al.*⁶⁴, treatment of transiently transfected COS-1 cells, with dimerization abrogating CpdA restricted phosphorylation of GR α relative to Dex (100%) at two additional sites, namely Ser226 (14%) and Ser211 (24%). This was confirmed in LBT2 cells containing endogenous GR α , where CpdA treatment restricted phosphorylation at Ser226 and Ser211 (< 25%), relative to Dex⁶⁴. Interestingly, both these sites have also been shown to be linked to receptor turnover, albeit to a lesser extent.⁶⁴

Additionally, results using the dimerization deficient mutant, hGRdim, to replicate the ‘loss’ of GR α dimerization observed with CpdA treatment, demonstrated phosphorylation at Ser404 was in fact undetectable across all treatment conditions (Table 1 and Fig. 5.9B). A study by Galliher-Beckley *et al.*⁸ demonstrated that a mutant (S404A) incapable of being phosphorylated at Ser404, much like hGRdim, had an increased half-life, relative to the wild type GR α , following Dex treatment. Furthermore, to explain the increased half-life of the Ser404 mutant, Galliher-Beckley *et al.*⁸ speculated that the lack of phosphorylation at Ser404, causes GR α to adopt different conformations, relative to the wild-type GR α . Using a trypsin digest experiment, this group demonstrated that the mutant (incapable of Dex-induced hyper-phosphorylation at Ser404) did indeed adopt a different conformation to that of the wild-type GR α , following Dex treatment⁸. From the results, in the current study, it would seem probable that the conformation of the Ser404 mutant

is predominantly monomeric, however, this requires further investigation. Furthermore, although we believe that the conformation of GRdim is likely to restrict the Dex-induced phosphorylation at Ser404, we cannot exclude that the mutation itself may affect the interaction of the mutant with the GSK enzyme responsible for phosphorylation, at this site.

In contrast to the lack of phosphorylation of the monomeric GR α , ‘gain’ of GR α dimerization (induced by the binding of dimerization promoting GC’s, Dex and F) often results in maximal hyper-phosphorylation of GR α at a number of phosphorylation sites⁶⁴, but not all (i.e. Ser203)⁴⁹. Avenant *et al.*⁶⁴ showed a Dex and F-mediated maximal GR α phosphorylation at Ser226 (100 and 122%, respectively) and Ser211 (100 and 91%, respectively). This finding is in line with the 91% F-induced GR α phosphorylation at Ser404, relative to Dex (100%), in the current study (Fig. 5.9A). Furthermore, a study which identified the novel site, Ser404⁸, reported a Dex-induced increase, between 1.5 to just over 2-fold, in GR α phosphorylation at Ser404, relative to the basal phosphorylation of the unliganded GR α (EtOH), in a number of cell lines either with transfected GR α (i.e. U2OS cells) or endogenous GR α (i.e. HeLa, MG-63, A549 and HepG2 cells). This result is similar to the Dex-induced (1.5-fold) increase observed in the current study in both the COS-1 (transiently transfected with hGRwt) and HepG2 cells (Fig. 5.9A and C). Lastly, using the GSK3 β inhibitor (BIO), Galliher-Beckley *et al.*⁸ demonstrated a 94% inhibition of the Dex-induced phosphorylation at Ser404, however, no investigation into the direct effect of this BIO inhibition on GR α stability, was made. Results from the current study, demonstrated that inhibition of GR α phosphorylation at Ser404 using BIO⁸ could partially block Dex-induced receptor down-regulation, confirming the role of ligand-induced hyper-phosphorylation at Ser404 in driving receptor turnover (Fig. 5.9D).

Table 5.1: Summary of results on GR α phosphorylation at Ser404 and receptor interaction with FBXW7 α

		Unliganded	Ligand-induced 'gain' of GR α dimerization		Ligand-induced 'loss' of GR α dimerization			
PTM or interaction	GR α	EtOH	Dex	F	CpdA	Cell line	Technique	Figure
pSer404	hGR α	+	++	++	+/-	HepG2	WB	5.9C
	hGRwt	+	++	++	+/-	COS-1	WB	5.9A
FBXW7 α	hGRwt	+	+++	++	+/-	COS-1	Co-IP	5.7A and B
	GFP-hGRwt	+	++	N.D	-	COS-1	PLA	5.7C
Mutant-induced 'loss' of GRα dimerization								
pSer404	hGRdim	U.D	U.D	N.D	U.D	COS-1	WB	5.9B
FBXW7 α	GFP-hGRdim	+	-	N.D	+	COS-1	PLA	5.7C

*In the table U.D refers to undetected and N.D to not detected (i.e. experiment was not done), '+' suggests and increase and '-' a decrease (+++ > ++ > + > +/- > -), Western blotting (WB), co-immunoprecipitation (Co-IP) and proximity-ligation assay (PLA).

Taken together, these results, in combination with previous findings^{8,64} confirm that a ‘loss’ of GR α dimerization restricts hyper-phosphorylation of GR α at Ser404, which consequently modulates the

interaction of the receptor with the catalytically active, FBXW7 α . This is likely to be one of the molecular mechanisms in which monomeric GR α evades proteasomal degradation.

An essential requirement for the degradation of proteins via the proteasome is substrate ubiquitination, which occurs in a multi-step manner, via a number of enzymes¹¹. Tagging of a protein substrate with ubiquitin is completed by E3 ligases (e.g. FBXW7 α), which bind the protein substrate and mediate the transfer of the activated ubiquitin molecule from another UPS enzyme (i.e. an E2 conjugating enzyme) to the substrate¹¹. Specifically, in terms of GR α , interaction of the receptor with FBXW7 α is an essential step linking preceding GR α phosphorylation to subsequent GR α ubiquitination, at a single ubiquitin site^{29,30}, and consequent proteasomal degradation⁸. For this reason, the current study investigated whether differences, dependent on GR conformation (i.e. dimer versus monomer), in the ubiquitination status of the GR α exist, following ligand treatment for 6 hours. This length of treatment was based on results from the current studies' FBXW7 α experiment, optimization (data not shown) and previous studies, which have investigated GR α ubiquitination^{13,30,65}.

Unfortunately, results from our ubiquitination experiments proved to be somewhat inconclusive. Whilst a Dex-induced increase in the ubiquitination status of wild type GR α was expected, a significant decrease was noted (Fig. 5.13). It must be noted, that in the current study, MG132 was not employed to block proteasome degradation before 6 hours compound treatment in the ubiquitin experiment and is something which should be considered in future investigations. A likely reason for the decrease in GR α ubiquitination, following Dex treatment, is significant ligand-induced GR α degradation after 6 hours (approximately 40% reduction in receptor levels according to Figure 4.5A). Unlike other steroid receptors^{16,66}, very little evidence exists for a significant-ligand induced increase in GR α ubiquitination, with a number of studies rather showing a decrease in GR α ubiquitination, even in the presence of proteasome inhibition using MG132^{30,65}. A study by Wallace *et al.*³⁰ demonstrated an obvious reduction in the ubiquitination status of GR α following treatment with dimerization promoting Dex (100 nM for 16 hours) even in the presence of the proteasome inhibitor, MG132, using similar conditions to that of the current study, namely COS-1 cells transiently transfected with untagged hGRwt and ubiquitin (Fig. 5.13A). In a study by Wang *et al.*⁶⁵, GR α ubiquitination occurred in both the absence and presence of Dex (1 μ M) for 18 hours and MG132 (5 μ M), in HT22 cells with endogenous GR α and transiently transfected ubiquitin. Interestingly, and in contrast to Wallace *et al.*³⁰, a lack of receptor turnover is observed in HT22 cells⁶⁵, even though ubiquitination of both the unliganded and liganded (i.e. Dex treated) GR α occurs. A reason provided for this lack of receptor turnover in the HT22 cells, is that GR α has the capacity to be ubiquitinated, however, its degradation is hampered at the level of delivery of the

ubiquitinated receptor to the proteasome, mediated by E3 ligases, or at the level of proteasome activity⁶⁵. In terms of the current study, this could explain why GR α ubiquitination is observed following treatment with dimerization abrogating GC, CpdA (Fig. 5.13B) and suggests monomeric GR α is capable of being ubiquitinated, however, it may not efficiently engage with the proteasome thus evading degradation. Moreover, taking into account that monomeric GR α restricts the interaction of the receptor with E3 ligase, FBXW7 α , this demonstration of GR α ubiquitination following treatment with CpdA supports the notion of monomeric GR α stabilizing an interaction with other UPS enzymes. In support of this idea is the ligand-dependent switching of ubiquitin dependent proteasome degradation for the ER, which has been successfully demonstrated⁶⁷. Specifically, CHIP binds the ER and mediates its ubiquitination and turnover in the absence of ligand, however, binding of estrogen to the ER results in dissociation of CHIP, thus it is postulated that ligand-induced ER degradation occurs via another ubiquitin-proteasome mediated pathway and likely another E3 ligase⁶⁷. In support of this finding is the fact that unliganded ER degradation is inhibited in cells where CHIP is absent, however, ligand-induced ER turnover in these cells still occurs⁶⁷. One could postulate that predominantly monomeric GR α ^{47,48} associates with a complex involving CHIP and the catalytically inactive E2-conjugating enzyme, TSG101¹⁵, whereas dimeric GR α , following treatment with Dex or F, preferentially associates with FBXW7 α , rather than CHIP or TSG101.

GR α 's association with the catalytically inactive E2 conjugating enzyme, TSG101, is known to be dependent on the phosphorylation status of the receptor¹⁵. More specifically, TSG101 has been shown to associate with the hypo-phosphorylated (at Ser203 and Ser211) form of GR α where, in the absence of ligand it binds to GR α , protecting it from degradation¹⁵. Interestingly, although results from the current study suggest that CpdA treatment results in predominantly monomeric, hypo-phosphorylated GR α , it did not induce a significant increase in the association of GR α and TSG101 (Fig. 5.10B). With that said, knockdown of the stabilizing TSG101 protein resulted in a 1.5-fold reduction in CpdA-treated GR α expression (Fig. 5.10C), a slightly lower fold destabilization (2.9-fold) than that reported for the non-phosphorylatable mutant (GR α S203A/S211A) used in the Ismail *et al.*¹⁵ study, following TSG101 knockdown, suggesting, that unlike the GR α mutant S203A/S211A, CpdA treatment of wild type GR α might allow for a degree of basal phosphorylation of the GR α at Ser404 and other sites (i.e. Ser203 and 211) also supported by Figure 5.9A and C. Furthermore, the ability of proteasome inhibition (MG132) to impede GR α destabilization by approximately 1.5-fold, following CpdA treatment in the absence of TSG101 (Fig. 5.10C), is similar to the 2-fold increase in the stability of the non-phosphorylatable mutant in the presence of MG132, but absence of TSG101¹⁵. These results with TSG101 provide food for

thought with regards to how the conformation of hypo-phosphorylated GR α may dictate the associations of the receptor with other UPS enzymes (i.e. TSG101).

Recent evidence has demonstrated a link between the nuclear shuttling of proteins and protein turnover^{41,68}. Thus in addition to determining the nature of the interaction of GR α with FBXW7 α (i.e. using PLA), it was necessary to consider the subcellular and co-localization of GR α with proteins of the UPS, as well as, elucidate how a 'loss' of receptor dimerization may affect the co-localization of GR α and UPS proteins, in subcellular compartments. This provided further insight into the regulatory molecular mechanisms involved in ligand-induced receptor down-regulation.

In terms of the receptor, the unliganded GR α is largely cytoplasmic⁶⁹, however, in support of the findings in Figure 5.5 and 5.6, some studies report that the unliganded GR α is evenly distributed throughout the cytoplasm and the nucleus, with the overall subcellular localization reflecting both the rate of active nuclear import as well as passive export of receptor export⁴³. Moreover, the subcellular localisation of the unliganded GR α is influenced by the receptor concentration within the cell^{43,44}. In both cases, it is thought that the unliganded GR α is sequestered, to a certain extent, in the cytoplasm bound to a multi-protein complex, whose primary function is to mask the receptor's nuclear localization signal (NLS) and maintain the receptor in a favourable conformation for ligand-binding⁷⁰⁻⁷². Interestingly, although considered a transcriptional regulator¹⁵, TSG101, is predominantly cytoplasmic⁷³. Moreover, it is postulated that the interaction of TSG101 and GR α , via the N-terminus of the receptor, provides a mechanism in which the unliganded GR α is protected from degradation¹⁵. In contrast to TSG101's cytoplasmic nature, the E3 ligase, FBXW7 α , is specifically located within the peri-nuclear space of a cell, also referred to as the nucleoplasm¹⁴. This is evident from the subcellular localization results, of the current study, which depict a miniscule amount of FBXW7 α expression in the cytoplasm with almost all FBXW7 α expression found in the nucleus (Fig. 5.5 and 5.6). Additionally, in Figure 5.5A and 5.6A, a prominent 'ring' of FBXW7 α expression around the cell's nucleus, is observed, and although it appears as if FBXW7 α is found within the nucleus, one must keep in mind that these images represent an overview of a range of images taken throughout the cell. Thus what appears to be nuclear expression of this E3 ligase is likely the expression of FBXW7 α found in the peri-nuclear space at the top and bottom of the cell. Furthermore, due to the nuclear localization of FBXW7 α and the predominantly cytoplasmic nature of unliganded GR α , it makes sense that little co-localization of these two proteins occurs, in the absence of GC treatment (Fig. 5.5D and 5.6D). In Figure 5.7B fluorescent red 'spots', indicating association between GR α and FBXW7 α , were detected in the cytoplasm, which is unexpected due to the nuclear nature of FBXW7 α . As the interaction of FBXW α with GR α is dependent on dimerization and phosphorylation at Ser404⁸, it is likely a small population of

ligand-independent GR α dimers in the cytoplasm⁴³ and that the signal detected outside the nucleus reflects basal interaction of the unliganded receptor with FBXW7 α . Moreover, the PLA assay is highly sensitive and involves the amplification of a signal (i.e. red 'spot') unlike immunofluorescence, which is potentially the reason why no FBXW7 α was detected in the cytoplasm using immunofluorescence.

Unlike the unliganded GR α , promoting receptor dimerization, with Dex and F treatment, shifts the subcellular localization of GR α , to predominantly nuclear (Fig. 5.5A and B). Thus, 'gain' of receptor dimerization following Dex and F treatment, reflects a point in time (i.e. 3 hours post-treatment) where the import rate is significantly higher than the export rate. A study by Robertson *et al.*⁴³, demonstrates that 50% of GR α is localized within the nucleus after just 4 to 5 minutes, following treatment with Dex and F (1 μ M), although nuclear import is thought to be ligand and concentration dependent⁴³. Moreover, a maximal nuclear localization of the wild-type GR α of approximately 95% was reached, 1 hour post-treatment with Dex⁴³. This finding supports those of the current study where almost all (98.6%) of the GR α protein expression is found in the nucleus, following treatment with Dex and F (Fig. 5.5 A and B). The nuclear localization of GR α is initiated via a conformational change in the receptor, induced by ligand-binding, and the consequent dissociation of the inhibitory multi-protein complex⁷⁰⁻⁷². It may be that TSG101 is found within this multi-protein complex, however, the exact positioning of TSG101, requires further investigation. Nevertheless the ligand-bound GR α moves away from the cytoplasmic TSG101, toward the nucleus, and as a result of this movement of GR α , an increase in the co-localization of the ligand-induced dimeric GR α with FBXW7 α , occurs (Fig. 5.5D), suggesting that this interaction is likely to occur in the nucleus/nucleoplasm. As mentioned the interaction of FBXW7 α with GR α is dependent on hyper-phosphorylation of the receptor at Ser404, and although basal Ser404 phosphorylation of GR α appears to be cytoplasmic, hyper-phosphorylation of this site is thought to occur within the nucleus⁸. The fact that the enzyme, which is responsible for mediating phosphorylation of GR α at this site, namely GSK-3 β , is a predominantly nuclear kinase, provides further support for this suggestion⁸. Taken together, a ligand-induced 'gain' of GR α dimerization drives the receptor into the nucleus of the cell where it undergoes phosphorylation at Ser404, which subsequently encourages the binding of FBXW7 α ultimately leading to its degradation.

GR α 's ability to dimerize (i.e. 'gain' of receptor dimerization), although not an absolute requirement for nuclear translocation, has been shown to influence the nucleo-cytoplasmic shuttling of the receptor, with a 'loss' of GR α dimerization influencing the extent of nuclear import of the receptor⁴³. Interestingly, a study by Robertson *et al.*⁴³ suggests the effect a 'loss' of GR α dimerization has on the half-life (i.e. time taken for 50% nuclear distribution) and maximal nuclear

import of the receptor, is similar when preventing dimerization through CpdA treatment of the wild-type receptor or Dex treatment of the dimerization deficient mutant. Results from the current study, slightly challenge these findings with different maximal nuclear localization values reported for monomeric CpdA-treated hGRwt and monomeric Dex-treated hGRdim, approximately 73% and 98% respectively (Fig. 5.5 and 5.6). A possible reason for this discrepancy is the different treatment periods of 1 hour⁴³ versus 3 hours. Thus, CpdA treatment may be, in part, hampering GR α protein turnover by sequestering a portion of the receptor population in the cytoplasm, away from GSK-3 β -mediated hyper-phosphorylation at Ser404 and preventing interaction with FBXW7 α . This notion is supported by the finding in the current study, which demonstrates knockdown of the largely non-nuclear TSG101 (known to associate with the hypo-phosphorylated GR α), which results in a 26% reduction in CpdA-hGRwt protein expression (i.e. by proteasomal degradation). This is comparable to the small portion of GR α residing in the cytoplasm, following CpdA treatment (Fig. 5.5). However, with that said, a much larger proportion of monomeric CpdA-treated GR α and almost all monomeric Dex-treated hGRdim are found in the nucleus, which eludes to the fact that the slight differential subcellular localisation of GR α monomers and the co-localisation of these monomers with UPS components cannot be the sole reason for monomeric GR α 's increased stability. In other words, the lack of interaction between monomeric GR α and FBXW7 α is not only due to these two proteins existing in different subcellular compartments within the cell. Although the ability of the GR α phosphorylation mutant (at Ser404)⁸ to dimerize has not been investigated, the subcellular localization of this mutant has been described, with total nuclear import observed, following 1 hour of Dex. This finding further confirms that differential subcellular localization of GR α is not the primary method of evading receptor turnover, though, its contribution should not be disregarded, and that it is rather the modulated post-translational processing of the GR α which protects the receptor from ligand-induced down-regulation.

Finally, the current study demonstrates that the dimerization abrogating GC, CpdA, is able to partially restore GR α protein levels, in the presence of the dimerization promoting GC, Dex (Fig. 5.14). One could postulate that CpdA treatment is in some way modulating the dimerization status of GR α by, in part, preventing the formation of total GR α dimers, induced by Dex. This in turn may be restricting the post-translational processing of the receptor, ultimately stabilizing it to a degree. To fully understand the effect of Dex and CpdA on GR α , it would be necessary to investigate and compare the dimerization state of the receptor relative to GR α expression, following Dex and CpdA treatment, alone or in combination. A possible method to do this is the Number and Brightness Assay, eloquently described by Presman *et al.*⁶³.

5.4. Conclusion

In conclusion, results from this chapter provide further evidence for a novel requirement for GR α dimerization in mediating ligand-induced down-regulation of the receptor, which was established in Chapter 4. Additionally, the effects of a 'loss' of GR α dimerization on the post-translational processing of the receptor, as well as the interaction of the receptor with UPS components, was highlighted. Essentially, two molecular mechanisms, namely phosphorylation of GR α at Ser404 and interaction of the receptor with E3 ligase, FBXW7 α , are restricted when a population of GR α monomers rather than dimers exists. Moreover, results from the CHIP and TSG101 experiments provide food for thought with regards to their association with differential GR α conformations; however, these interactions require further investigation. Lastly, to our knowledge, this is the first time a link between GR α conformation and the post-translational processing of the receptor has been established.

5.5. References

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5.6. Supplementary Figures



Figure S1: ‘Loss’ of GR α dimerization modulates its interaction with FBXW7 α (Repeat 2). COS-1 cells were seeded, transfected. Following 24 hours incubation, cells were treated with solvent (EtOH), Dex, F or CpdA (10^{-5} M) for 3 hours. For the co-IP experiment, cells were lysed after compound treatment and FBXW7 α was immune-precipitated with hGRwt. Western blotting was conducted to determine protein expression and GAPDH was probed to ensure equal protein loading. A representative blot of three independent experiments is shown.



Figure S2: COS-1 cells express different concentrations of hGRwt following transient transfection. COS-1 cells were seeded into a 10 cm dish (1×10^6 cells) and were transiently transfected the next day with hGRwt using a transfection reagent. Following 24 hours incubation, cells were re-plated into 8 well chambers (3×10^4 cells/well) and the next day treated with solvent (EtOH) for 3 hours. Thereafter, cells were fixed, permeabilized and immunofluorescence conducted using a GR α antibody. Following immunofluorescence, cells were imaged using a confocal microscope.

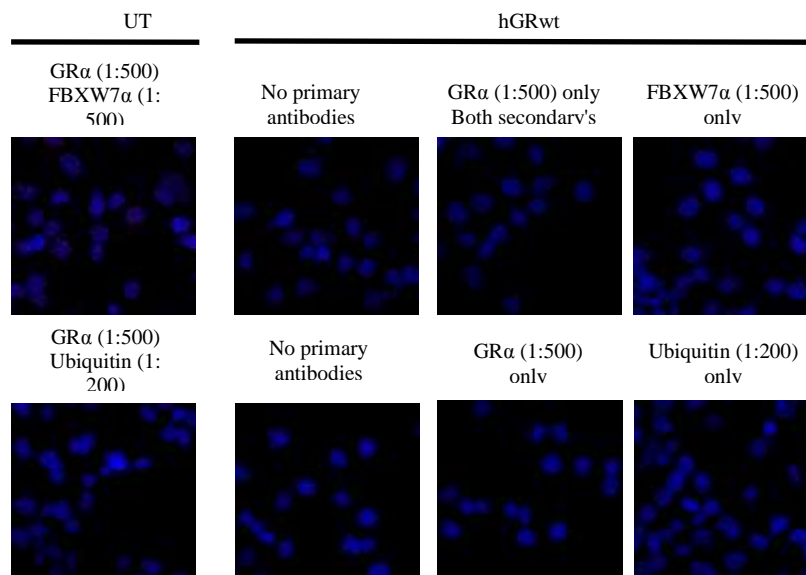


Figure S3: Control figure for PLA depicting no cross-reactivity of primary antibodies. COS-1 cells were seeded, left untransfected or transfected with hGRwt and re-plated in 8 well chambers. Following 24 hours incubation, cells were treated with solvent (EtOH) for 3 hours. For the PLA, following treatment cells were fixed, permeabilized and PLA conducted using specific primary antibodies for GR α together with FBXW7 α or ubiquitin (first column), with no primary antibodies (second column), with only the GR α primary antibody (third column) or with FBXW7 α or ubiquitin primary antibodies only (fourth column), after which cells were imaged. A representative image of individual cells is shown. In this representative image if the PLA signal was positive it would be observed as distinct red 'spots' and the cell's nucleus is depicted by the blue DAPI stain. From the images one can see our negative controls were successful.

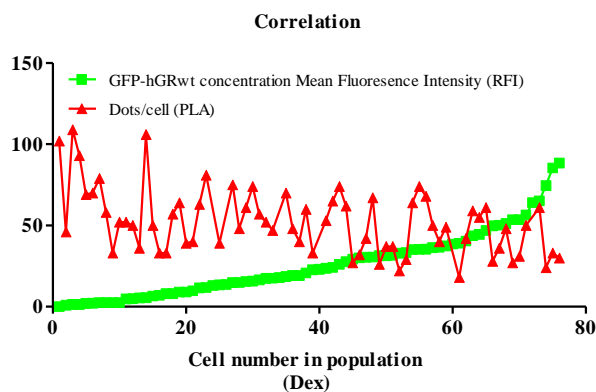


Figure S4: Increasing GR α concentrations result in a concurrent decrease in its interaction with FBXW7 α . COS-1 cells were seeded into a 10 cm dish (1×10^6 cells), and were transiently transfected the next day with GFP-hGRwt using a transfection reagent. Following 24 hours incubation, cells were re-plated into 8 well chambers (3×10^4 cells/well) and the next day treated with solvent (EtOH) for 3 hours. Thereafter, cells were fixed, permeabilized, and immunofluorescence (green) and PLA conducted (red) after which cells were imaged using a confocal microscope). For the quantification of the interaction of GR α (GFP-hGRwt) and FBXW7 the dots/cell (red) was quantified using IMAGEJ software, whilst the GR α (GFP-hGRwt) concentration was calculated in terms of relative fluorescence intensity (RFI), using the ZENN software for individual cells. The cell population (1 to 79 individual cells) was plotted from low to high GR α concentration (i.e. increasing GFP (RFI) signal) (indicated by green on graph) with each cells corresponding GR α /FBXW7 α interaction (dots/cell).

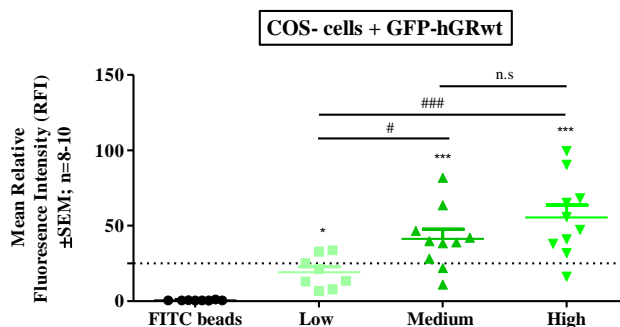


Figure S5: Quantifying GFP-hGRwt concentration ranges using FITC beads and FACS (unpublished work by member of our research group). COS-1 cells were seeded into a 10 cm dish (1×10^6 cells) and the next day, were transiently with 4000ng of mGRwt using the DEAE-dextran method. After 72 hours, cells were re-suspended in 20% FCS medium and sorted into low, medium and high GR α concentrations, relative to calibrated FITC beads by means of FACS. Cells were then replated in 8 well chambers in individual populations (i.e. low GR α /well, medium GR α /well and high GR α /well) and allowed to recover for 3 hours before imaging using a confocal microscope. Approximate GR α concentration per population group was then determined (RFI) and plotted. Statistical analysis was conducted using a one-way ANOVA followed by a Newman-Keuls Multiple Comparison post-test comparing all experimental values (RFI) (calibrated FITC beads, low, medium and high GFP-hGRwt) to each other (ns, $p > 0.05$, *, $p < 0.01$ and ***, $p < 0.001$) and (ns, $p > 0.05$, #, $p < 0.05$ and ###, $p < 0.001$).

Chapter 6:

Preserving GR α expression through ‘loss’ of receptor dimerization: relevance in a working model for acquired GC resistance.

6.1. Introduction

The functionality and amount (i.e. ‘pool’) of GR α available within a cell, is vital for GCs to efficiently elicit their biological actions¹⁻³. As GCs are instrumental in regulating homeostasis, metabolism and inflammation, perturbations in the GC/GR α signalling pathway have been shown to have severe implications, which often results in metabolic and immunological complications⁴⁻⁶.

One of the ways in which the GC/GR α signalling pathway is disrupted is by prolonged GC treatment that may lead to adverse side effects⁴⁻⁷ and/or acquired GC resistance^{2,8-11}, which may be as a result of severe reductions in the ‘functional pool’ of GR α . The development of acquired resistance to GC treatment, poses a major threat for the pharmaceutical industry and research is focusing on elucidating ways in which improved sensitivity to GCs can be achieved in prolonged GC treatment regimes⁴⁻⁶.

Ligand-induced down-regulation of GR α has been shown to occur at the mRNA and protein level¹²⁻²². Specifically, reductions in the mRNA expression of GR α is thought to occur as a result of ligand-induced GR α mRNA destabilization^{13,23,24} or repression of the GR gene itself^{15,25,26} (via a nGRE situated in the GR α promoter^{16,27}). Furthermore, ligand-induced GR α protein down-regulation is thought to occur at the proteasomal level^{18,19,22,28}. As GCs elicit their effects via the GR α , which functions as a ligand-dependent transcription factor to ‘positively’ or ‘negatively’ regulate GC-responsive gene expression, one could speculate that changes in GR α mRNA and/or protein expression would directly modulate the mRNA expression of GC-responsive genes.

Positive’ up-regulation (transactivation) of GC-responsive genes, following GC treatment, has largely been thought to be mediated via a GR α dimer (via direct DNA-binding to

GREs)²⁹. On the other hand ‘negative’ down-regulation (transrepression) of such genes is generally thought to be regulated by a GR α monomer (via an indirect tethering mechanism to transcription factors such as Nf κ B)²⁹ (Fig. 6.1). Although this duality does exist, the distinction between these modes of GC-mediated gene regulation is thought to be considerably more complex than initially described²⁹ (e.g. repression can occur via direct DNA-binding to a nGRE²⁸). Additional models are eloquently described in a recent review by Vandevyver *et al.*³¹.

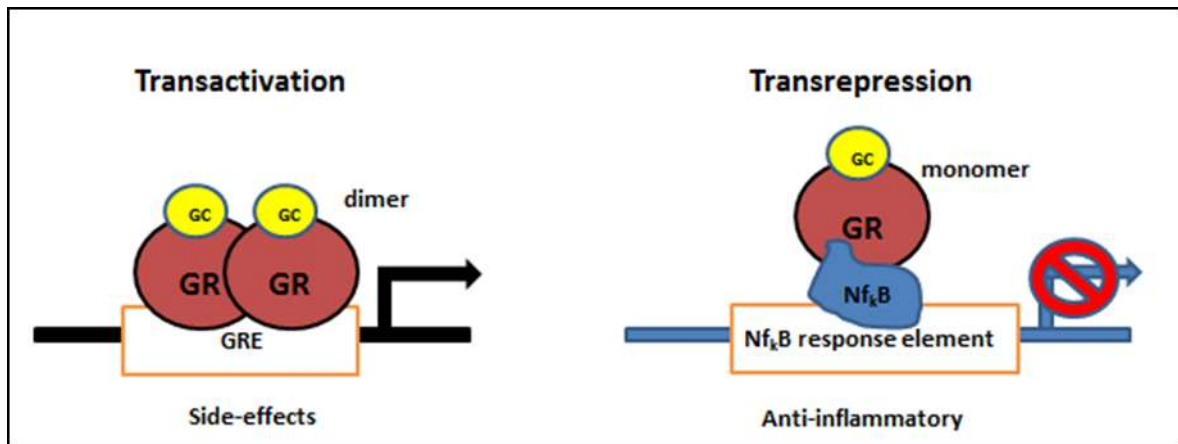


Figure 6.1: GR α signalling can lead to either transactivation or transrepression of GC-responsive genes. Classically, transactivation is mediated via direct binding of a GR α dimer to a glucocorticoid response element (GRE), leading to an increase in gene expression. In contrast transrepression is mediated via the tethering of a GR α monomer to a transcription factor (e.g. Nf κ B), which is bound to its respective response element, in turn inhibiting gene expression.

The current study selected genes from these two broad categories, namely transactivation (GILZ, TAT and FKBP51) and transrepression (IL-6), based on their functional relevance to metabolism, inflammation and acquired GC resistance, to investigate the biological implications of altered GR α protein expression, with general information on these genes given below. In addition, effects at the GR α gene level were determined by determining GR α mRNA expression and changes in the receptor expression at the mRNA level were compared to changes in GR α protein expression.

GR α -mediated transactivation:

GILZ

Glucocorticoid-induced leucine zipper (GILZ) is ubiquitously expressed in a wide range of tissues and cell types, including a number of cells, which form part of the immune system^{32,33}. GILZ is a well-known GC-inducible gene and is thought to be highly responsive, with its mRNA expression significantly up-regulated following GC treatment³³⁻³⁵. Up-regulation of this gene is thought to occur via classical GR α -mediated gene transactivation,

which involves the binding of a GR α dimer to multiple GREs present in the proximal region of the GILZ promoter^{36–40}. Although up-regulation of GC-responsive genes is commonly associated with an increase in metabolic activity and the subsequent development of adverse side-effects (e.g. hyperglycaemia), a GC-induced increase in GILZ mRNA expression has recently been shown to partially contribute to the anti-inflammatory potential of GCs³³.

TAT

Tyrosine aminotransferase (TAT) is expressed predominantly in the liver and plays a prominent role in gluconeogenesis⁴¹. The expression of this metabolic enzyme is induced via GCs^{42–44}, which leads to activation of the GR α and subsequent binding of GR α dimers to multiple GREs in the proximal region of the TAT gene promoter^{42,45–47}. GC-induced TAT up-regulation is required for regulating glucose metabolism under normal conditions⁴⁸. However, prolonged GC treatment and severe up-regulation of TAT has been associated with adverse side-effects and may result in metabolic disorders such as hyperglycaemia⁴⁹.

FKBP51

FK506 binding protein 5 (FKBP51)⁵⁰ is an immunophilin, which is widely expressed in a number of cell types. Higher expression of this immunophilin has been detected in metabolically active tissues, such as muscle and adipose, with lower levels observed in pancreatic, stomach and spleen tissues^{51,52}. The ligand-activated GR α is thought to directly increase FKBP51 expression, through binding to two putative GRE-containing regions in the FKBP51 gene^{53,54}. An increase in FKBP51 expression has been associated with cellular desensitization to GC treatment (or acquired GC resistance), via a negative-feedback loop. Specifically, the FKBP51 protein acts as a molecular chaperone for GR α and is thought to sequester GR α in the cytoplasm, hampering its translocation into the nucleus^{55–58}. This mechanism may serve as a protective mechanism to protect the cell from overstimulation by GCs, through restricting GC/GR signalling, however, elevated FKBP51 expression has also been linked to acquired GC resistance in a number of disease states such as asthma^{59–62}.

GR α -dependent transrepression:

IL-6

Interleukin 6 (IL-6) is a pro-inflammatory cytokine whose expression is elevated during infection by pathogens^{63–65} or through stimulation by the pro-inflammatory cytokine, tumour necrosis factor alpha (TNF α)^{66–69}. The IL-6 promoter contains a number of regulatory regions, including binding sites for NF κ B and AP-1⁶⁹. Binding of transcription factors (NF κ B

and AP-1) to the IL-6 promoter, which can be triggered by TNF α through a signalling cascade, activates the IL-6 promoter and increases IL-6 expression⁶⁹. GR α -mediated trans-repression of IL-6, via tethering of the GR α to pro-inflammatory transcription factors, specifically NF κ B and AP-1⁷⁰ bound to their respective response elements, inhibits the expression of pro-inflammatory genes, such as IL-6 (Fig. 6.1). It is this inhibition that confers the potent immunosuppressive effects of GC treatment^{8,31,71}.

In Chapter 4 of the current study, a novel role for receptor dimerization in ligand-induced GR α protein down-regulation was established, in COS-1 cells transiently transfected with hGRwt and hGRdim and HepG2 cells containing endogenous hGR α (Fig. 4.5). Specifically, a 'loss' of dimerization was achieved through treatment of hGRwt and endogenous hGR α with the dimerization abrogating ligand, CpdA, or through the use of the dimerization deficient mutant, hGRdim, which prevented receptor turnover. Subsequently Chapter 5, elucidated the molecular mechanisms involved in preventing receptor turnover of predominantly monomeric GR α and, in addition, found that this novel requirement for GR α dimerization in receptor protein turnover was not species specific but was in fact mirrored using a transiently transfected dimerization deficient mutant of mouse origin, namely, mGRdim(A465T) (Fig. 5.4). Although the HepG2 cells allowed for the investigation of the effect of a 'loss' of dimerization with endogenous hGR α on receptor protein turnover, using CpdA as a molecular tool to mimic the transiently transfected hGRdim mutant, this cellular system did not provide a means to determine the effects of a 'loss' of dimerization using an endogenous dimerization deficient mutant.

For this reason, additional cell lines, namely MEF-mGRwt and MEF-mGRdim, which are immortalised mouse embryonic fibroblasts⁷², were introduced. These cell lines were originally isolated from wild type GR α (GR^{+/+}) and GR α dimerization deficient (GR^{dim/dim}) mice, respectively⁷². Specifically, the GR^{dim/dim} mice were generated by introducing a single point mutation in the dimerization loop (D-loop), situated in the DNA-binding domain of the GR α gene, at amino acid position 465⁷². It must be noted for simplicity the MEF-mGRwt and MEF-mGRdim cell lines are referred to as mGRwt and mGRdim, respectively, throughout this chapter and should not be confused with the transiently transfected mGRwt and mGRdim, in Chapter 5.

Importantly, these immortalised cell lines allowed for the investigation and comparison of the effects of a ‘gain’ or ‘loss’ of GR α dimerization (via an endogenous mutant), respectively, on receptor turnover in an endogenous system. Moreover, the MEF-mGRwt and MEF-mGRdim cells provided a system in which the effects exerted by changes in the ‘functional pool’ of GR α , (observed in Chapter 4) at the level of GC-responsive gene expression could be determined.

Using these cell lines, it was hypothesised that a ‘loss’ of GR α dimerization through the use of mGRdim will prevent ligand-induced GR α protein turnover, following treatment with dimerization promoting Dex. Furthermore, by preventing receptor turnover through a ‘loss’ of dimerization (i.e. via treatment of mGRwt with CpdA or the use of mGRdim), the ‘functional pool’ of GR α will preserve its ability to efficiently mediate GC-responsive gene expression, even following prolonged GC treatment. In contrast, a ‘gain’ of GR α dimerization via treatment of mGRwt with Dex will encourage receptor turnover, leading to drastic reductions in the GR α ‘functional pool’, ultimately impairing its ability to mediate GC-responsive gene-expression, following prolonged GC treatment. The latter lack of a GC response or increase in GC insensitivity has major implications for combatting inflammation in patients with chronic inflammation.

In general, the current chapter begins by characterizing the MEF cells in terms of the initial GR α content or ‘functional pool’ and binding affinity for Dex of mGRwt and mGRdim. Subsequently, the novel requirement for GR α dimerization in mediating receptor turnover (established in Chapter 4) was investigated by comparing the effects of dimerization promoting Dex on endogenous mGRwt and mGRdim protein expression, using Western blotting and whole cell GR α -binding. Having demonstrated that a ‘loss’ of GR α dimerization restricts dose-dependent Dex-induced mGRdim protein turnover, further investigation into the ligand-dependent effects of Dex and dimerization abrogating, CpdA, alone or in combination, on GR α protein and mRNA expression, was conducted. Using an adapted experimental protocol from Li *et al.*⁷³ the time-dependent effects of these GCs (i.e. short-versus long-term pre-treatment) on the mGRwt and mGRdim protein and mRNA expression, was determined. Once the ligand-selective and time-dependent effects of the GCs had been established for mGRwt and mGRdim, the responsiveness, in terms of GC-responsive gene expression, of the working model or GC-responsive system established to mimic GC acquired resistance, was tested. It must be noted that TNF α , was used throughout the experimental protocol designed for establishing a working model to mimic acquired GC

resistance (Fig. 6.4). The reason for this being that all the genes (i.e. those mediated via transactivation and transrepression) could be analysed from the same sample. With that said, the effects of TNF α on basal GR α protein and mRNA expression, as well as the effect of TNF α on basal gene expression of GC-responsive genes, is addressed.

Lastly, due to the fact that prolonged GC treatment (as opposed to short-term GC treatment) has been associated with the development of GC acquired resistance, the main focus of the current chapter is to mimic acquired resistance to GC treatment. Thus, using the established working system (Fig. 6.4), we investigated how the ligand-induced changes in the GR α ‘functional pool’, or lack thereof, re-directed or re-shaped the GC-responsive gene expression, at the mRNA level, following short-term versus long-term GC pre-treatment.

Integral to this chapter, is the notion that short-term GC pre-treatment reflects normal GC signalling, whilst long-term GC pre-treatment represents the development of acquired GC resistance (Fig. 6.4), a relevant and significant pharmaceutical challenge.

To conclude, demonstrating a role for GR α dimerization in ligand-induced receptor turnover, using an endogenous mutant, will substantiate results from previous chapters in the current study. Furthermore, elucidating how changes in the GR α ‘functional pool’ modulate the expression of GC-responsive genes, provides further insight into the potential effects of prolonged GC treatment and the development of GC insensitivity. Lastly, establishing that the maintenance of the GR α ‘functional pool’ through abrogation or ‘loss’ of GR α dimerization, preserves the anti-inflammatory potential of GC/GR signalling, provides novel possibilities in which the GC/GR α signalling pathway may be exploited pharmacologically.

6.2. Results

6.2.1. Characterizing MEF-mGRwt and MEF-mGRdim cells with regards to GR α expression and Dex-induced receptor turnover

Having introduced two new cellular systems, namely the MEF-mGRwt and MEF-mGRdim cells, in the current chapter, it is necessary to characterize these cells based on their GR α content and their ligand-binding affinity for Dex. This allows for the ruling out of differences in the initial GR α ‘functional pool’ and differences in the Dex-binding affinities as reasons for observed differences between the two cells lines.

In addition, once characterized, the novel requirement for GR α dimerization in mediating Dex-induced receptor protein turnover, established in Chapter 4 (i.e. COS-1 cells transiently

transfected with hGRwt and hGRdim or HepG2 cells containing endogenous hGR α) and Chapter 5 (i.e. COS-1 cells transiently transfected with mGRwt and mGRdim), was investigated. Importantly, unlike in the transiently transfected COS-1 cells, this system (i.e. the MEF-mGRwt and MEF-mGRdim cells) allows for the effective regulation at the transcriptional (or ‘push’) level to be investigated. Moreover, the effects of a ‘loss’ of GR α dimerization on the ligand-induced down-regulation of an endogenous mutant, mGRdim, can be determined using the MEF-mGRdim cells.

6.2.1.1. Determining the level of GR α expression in the MEF-mGRwt and MEF-mGRdim cells

Using whole cell GR α -binding the current chapter started with determining the optimal incubation time for binding equilibrium to be reached using an association binding time-course, which was conducted in the MEF-mGRwt cells. Optimal conditions, with regards to incubation time, were applied to the MEF-mGRdim cells and whole cell competitive GR α -binding experiments were then conducted in both cell lines, after which global fitting curves were fitted to the homologous competitive binding data. From these curves, the MEF-mGRwt and MEF-mGRdim cells were characterised according ligand-binding affinities (Kd) for Dex for mGRwt and mGRdim, respectively. Next, in combination with obtained Bmax values, from the curves, the GR α concentration (fmol/mg protein), was calculated.

From the association binding time course, which was conducted at 0, 2, 4, 6 and 8 hours, using 20 nM [³H]-Dex, the incubation time required to reach binding equilibrium was determined to be 2 hours for the MEF-mGRwt cells (Fig.6.2A). Therefore, 2 hours incubation was selected for further whole cell competitive GR α -binding experiments in MEF-mGRwt and MEF-mGRdim cells.

Data obtained from the homologous competitive GR α -binding experiments provided Kd and Bmax values, which were required to determine the initial GR α ‘function pool’, for mGRwt and mGRdim in the MEF-mGRwt and MEF-mGRdim cells, respectively (Fig. 6.2B and C). No significant difference between the Kd values of Dex for mGRwt (5.6 nM) and mGRdim (8.9 nM) was observed (Fig. 6.2C). Similarly, the Bmax (cpm/mg protein) value, representative of maximal GR α binding, of 2959 cpm/mg protein for the mGRwt was not significantly different from the 2815 cpm/mg protein noted for the mGRdim (Fig.6.2C). Using these Kd and Bmax values the concentration of GR α (fmol/mg protein) in the MEF-mGRwt and MEF-mGRdim cell lines was calculated (Fig.6.2C). No difference in the initial GR α content or

‘functional pool’ of GR α , was found, between the two cell lines (Fig. 6.2C). Specifically, the calculated concentrations of GR α in the MEF-mGRwt and MEF-mGRdim cells, were 40.7 and 38.7 fmol/mg protein, respectively, which corresponds to the defined low GR α expression (67.0 fmol/mg protein) designated by a previous member in our lab⁷⁴.

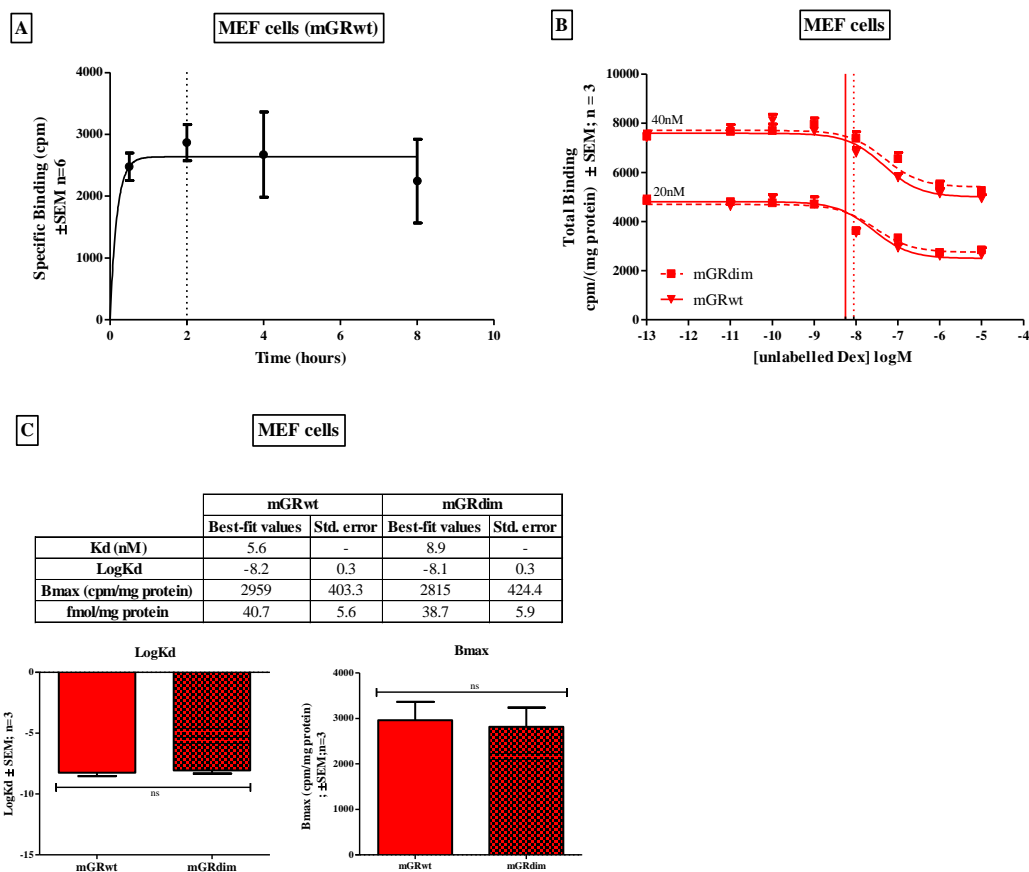


Figure 6.2: Determining GR α concentration in MEF-mGRwt and MEF-mGRdim cells. MEF cells (i.e. MEF-mGRwt or MEF-mGRdim cells) were seeded into a 24 well plate (5×10^4 cells/well) and 24 hours later, cells were steroid starved. To determine the time to binding equilibrium (A), whole cell GR α -binding was conducted in the MEF-mGRwt cells, using 20 nM [3 H]-Dex. Cells were then lysed and cpms were detected via a scintillation counter with specific binding values (cpms) calculated and the plotted against time (in hours). Whole cell GR α -binding results shown are representative of six independent experiments (average \pm SEM), conducted in triplicate. The dotted line, on the graph (at 2 hours), represents the incubation time chosen for subsequent experiments. Having established the time required for binding equilibrium to be reached, competitive whole cell GR α -binding (B) using two concentrations of [3 H]-Dex, namely 20 and 40 nM, and increasing concentrations of unlabelled Dex (10^{-13} to 10^{-5} nM), was conducted. Once lysed, total binding (cpm) values were detected via a scintillation counter, normalised to total protein concentration (cpm/mg protein) and plotted. Whole cell GR α -binding results shown are representative of three independent experiments (average \pm SEM), conducted in triplicate. The solid and dotted lines, on the graph at -8.2 and -8.1, represent the logKd values for mGRwt and mGRdim, respectively, which were determined and plotted (C) along with the Bmax values. For statistical analysis, an unpaired t-test was used to evaluate differences in ligand binding affinities (logKd) of Dex for mGRwt and mGRdim and the Bmax values (ns, $p > 0.05$). In addition, using these values, the concentration of GR α in the MEF-mGRwt and MEF-mGRdim cell lines, was calculated (fmol/mg protein).

To ensure comparable fractional occupancy of Dex for mGRwt and mGRdim, the fractional occupancies were calculated at all ligand concentrations (Table 6.1). Comparable fractional

occupancy of 99% for Dex via mGRwt and mGRdim was noted following treatment with 1 μ M Dex and thus this Dex concentration was used in subsequent experiments (Table 6.1).

Table 6.1: Calculating the fractional occupancy of Dex for mGRwt and mGRdim.

Ligand concentration		% F.O ^c of Dex for mGRwt	% F.O ^d of Dex for mGRdim	Fold difference ^e
(logM)	(nM)			
-9	1	15	10	2
	5.6 ^a	50	39	1
	8.9 ^b	62	50	1
-8	10	64	53	1
-7	100	95	92	1
-6 ^f	1000	99	99	1
-5	10000	>99	>99	1

^a Kd value of Dex (5.6 nM) for mGRwt as established in Figure 6.1. ^b Kd value of Dex (8.9 nM) for mGRdim as established in Figure 6.1. ^c Fractional occupancy of Dex for mGRwt was calculated using Kd value^a and equation in Chapter 4 and expressed as a percentage. ^d Fractional occupancy of Dex for mGRdim was calculated using Kd value^b and equation in Chapter 4 and expressed as a percentage. ^e The fold difference was calculated by dividing the percentage fractional occupancy of Dex for mGRwt by the percentage fractional occupancy of Dex for mGRdim. ^f The ligand concentration used in subsequent experiments to ensure > 99% fractional occupancy for both GR α ligands.

6.2.1.2. Endogenous mouse GR α protein turnover is dose-dependent and influenced by receptor dimerization

To confirm and substantiate the novel requirement for receptor dimerization in GR α turnover, both MEF cell lines were treated with Dex in a dose-dependent manner (10^{-13} M – 10^{-5} M) for 24hrs, after which GR α expression was measured, using Western blotting. From these dose-response curves, the potencies (IC₅₀) and efficacies of Dex-induced GR α protein down-regulation of mGRwt and mGRdim was determined. In support of the results obtained using Western blotting, competitive whole-cell GR α binding following 1 μ M (10^{-6} M) Dex treatment for 24 hours was conducted.

In agreement with results obtained in the COS-1 cells transiently transfected with hGRwt (Fig. 4.3 and 4.4), the mGRwt protein, in the MEF-GRwt cells, undergoes protein down-regulation following 24 hours treatment with the dimerization promoting GC, Dex (Fig. 6.3). Moreover, this Dex-induced down-regulation of the mGRwt protein is dose-dependent (Fig. 6.3A).

In contrast to mGRwt, which is capable of forming receptor dimers, ‘loss’ of dimerization via the use of mGRdim protein in the MEF-GRdim cells, results in a partial loss of ligand-induced GR α protein down-regulation (Fig. 6.3). Although, the mGRdim protein does undergo protein down-regulation, the extent of this down-regulation was significantly ($p <$

0.01) less than that of the mGRwt. This is reflected by the difference in efficacy values, which are 27.4% for mGRwt and 50.9% for mGRdim, following 24 hours of Dex treatment (10 μ M) (Fig. 6.3B). In addition, no significant difference ($p > 0.05$) between the potencies of Dex to induce receptor turnover of mGRwt and mGRdim, namely 1.1 nM and 1.5 nM respectively, was observed (Fig. 6.3C and D). Using Western blotting, the extent of Dex-induced down-regulation of the endogenous mouse GR α (Fig. 6.3B) was found to be greater than that of the transiently transfected mGRwt and mGRdim, where receptor protein levels were reduced to 42% and 76%, respectively (Fig. 5.4C). A possible explanation for this observed difference between the COS-1 cells (i.e. transiently transfected with mGRdim) and the MEF-mGRdim cells is the lack of effective regulation at the transcriptional (or ‘push’) level (Fig. 4.2). Unlike the transiently transfected mGRdim, which is controlled via a constitutive promoter, the endogenous mGRdim is regulated by the endogenous GR α promoter.

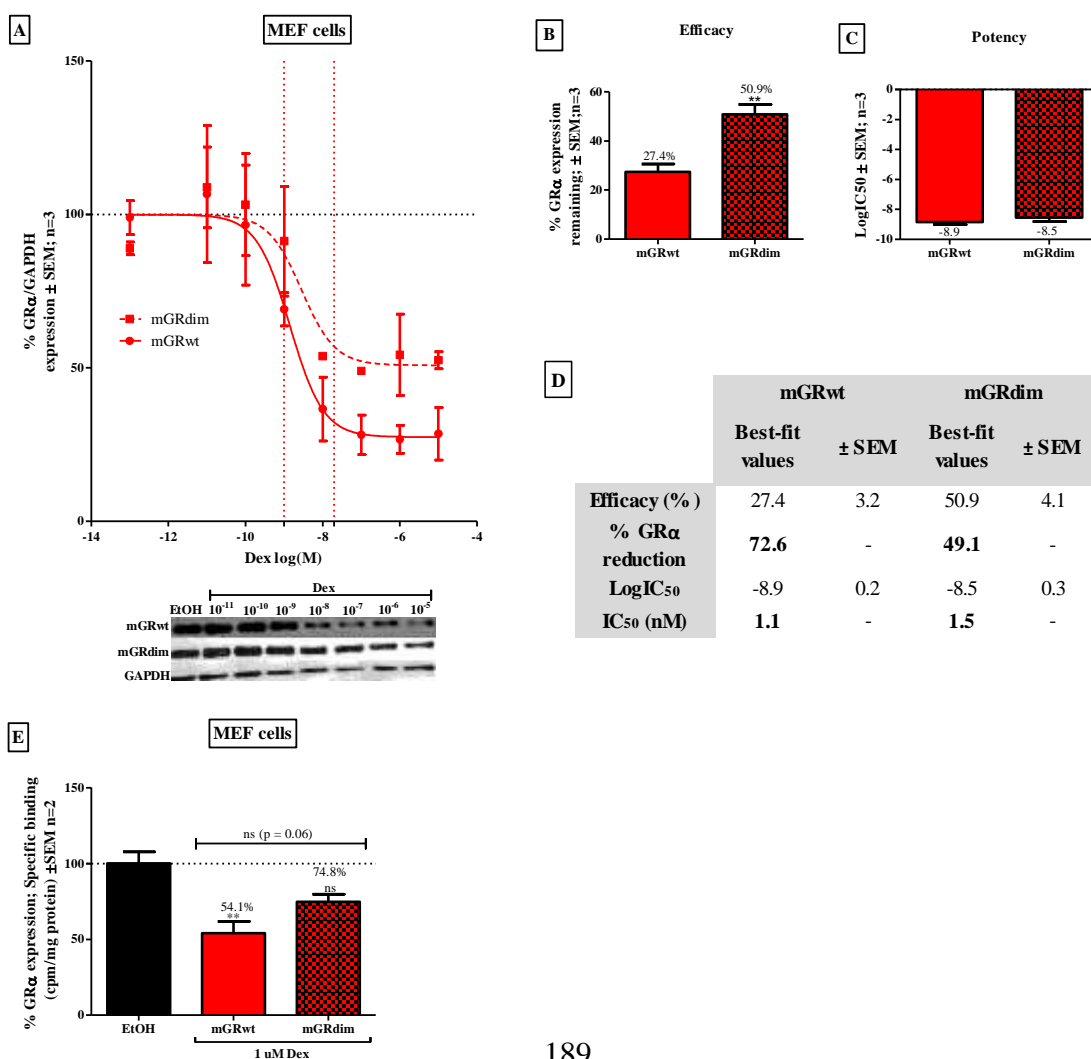


Figure 6.3: Endogenous mouse GR α protein turnover is dose-dependent and influenced by receptor dimerization. MEF-mGRwt and MEF-mGRdim cells were seeded into a 24 well plate (5×10^4 cells/well) and 24 hours later, cells were steroid starved. Following 24 hours incubation, cells were treated with either solvent (EtOH) or varying concentrations (10^{-11} M to 10^{-5} M) of Dex for 24 hours. Thereafter, mGRwt and mGRdim protein levels were assessed by Western blotting where GAPDH was probed to ensure equal protein loading. The Western blot shown (A, inset) is representative of three independent experiments. For quantification (A), the intensity of the mGRwt, mGRdim and GAPDH bands were determined using the My ECL Image Analysis software, and the mGRwt and mGRdim expression normalised to GAPDH expression and expressed as a percentage (average \pm SEM) of mGRwt or mGRdim expression in the presence of solvent (EtOH), which was set at 100% (dotted line). The physiological concentration range of Dex (1 – 20 nM) is indicated by vertical lines (red). Efficacies (B and D) and potencies (C and D) of Dex for mGRwt and mGRdim protein down-regulation were determined. For statistical analysis, an unpaired t-test was used to evaluate the differences in the efficacy (**, $p < 0.01$) and the potency ($\log IC_{50}$) (ns, $p > 0.05$) of Dex via mGRwt and mGRdim. Competitive whole cell GR α -binding (E) was conducted using 20 nM [3 H]-Dex and unlabelled Dex (10 μ M) (as in the COS-1 cells). Cells were lysed and cpm were detected via a scintillation counter and used to calculate specific binding values, which were normalised to total protein concentration (cpm/mg protein). In the graph the dotted line represents GR α expression (mGRwt and mGRdim) in the presence of the solvent (EtOH) and is set at 100%, with the mGRwt and mGRdim expression, following treatment with Dex (1 μ M) for 24 hours, calculated as a percentage relative to the solvent (EtOH). Whole cell GR α -binding results shown are representative of two independent experiments (average \pm SEM), conducted in triplicate. For statistical analysis, an unpaired t-test was used to evaluate differences in receptor expression, following Dex treatment (1 μ M) (**, $p < 0.01$)

Lastly, the results from the whole cell GR α -binding (i.e. in the MEF cells) suggest mGRwt protein levels were more significantly ($p < 0.01$) reduced to 54.1%, than the mGRdim protein levels which were reduced to 74.8% of the EtOH control following 24 hours treatment with Dex (1 μ M) (Fig. 6.3E). The extent of down-regulation observed here is less than that observed in the Western blotting (Fig. 6.3B), however, one must keep in mind these are different techniques and whole cell GR α -binding using transiently transfected mGRwt and mGRdim, was not conducted in Chapter 5 and thus a comparison cannot be made.

Collectively, these initial results obtained with the endogenous mouse GR α in the MEF-mGRwt and MEF-mGRdim cells, substantiates a requirement for GR α dimerization in Dex-induced receptor protein turnover, initially established in Chapter 4. Whilst a ‘loss’ of GR α dimerization in COS-1 cells transiently transfected with hGRdim completely abolished receptor turnover, at the protein level, in Chapter 4 (Fig. 4.5), a slight yet non-significant Dex-induced reduction in receptor expression was noted in Chapter 5, in COS-1 cells transiently transfected with mGRdim. In comparison, ‘loss’ of GR α dimerization, through the use of the endogenous mutant mGRdim in the MEF-mGRdim cells, only partially restricted Dex-induced GR α protein turnover, in the endogenous system used in the current chapter, and is likely due to additional regulation at the transcriptional level.

GR α concentration has been shown to modulate receptor function⁷⁴, thus, importantly, due to the fact that no significant differences in the GR α concentration between the MEF-mGRwt and MEF-mGRdim cells, or in the binding affinities for Dex for mGRwt and mGRdim, were

observed, it is likely that any differences in ligand-induced GR α down-regulation are, most likely, to be due to the dimerization state of the receptor.

Furthermore, to our knowledge this is the first time the MEF-mGRwt and MEF-mGRdim cells have been characterised with regards to GR α concentration, affinity of Dex for endogenous mGRwt or mGRdim and subsequent ability of these GR α 's to undergo Dex-induced protein down-regulation.

6.2.2. Establishment and validation of a model to mimic acquired GC resistance, using an adapted experimental protocol⁷³.

Previous studies have suggested that the ability of a patient to respond to GC treatment is often proportional to the magnitude of the GR α 'functional pool' within cells and tissues^{1,3,75}. The current study, in combination with others^{18,19,28}, has demonstrated that GR α protein down-regulation occurs predominantly via the proteasome following treatment with dimerization promoting GCs (i.e. Dex), resulting in a significant reduction in the size of the GR α 'functional pool'. In addition, the extent of ligand-induced GR α protein down-regulation is not only dependent on the type and concentration of ligand, but is also dependent on the length of time of the GC treatment (Fig. 4.3 and 4.4). Of utmost importance is that, amongst other factors^{17,76-79}, this ligand-induced reduction in the GR α expression, following prolonged treatment with certain GCs, may result in an acquired resistance to GC treatment and poses a major threat in the treatment of inflammatory disorders.

Having characterized the MEF-mGRwt and MEF-mGRdim cells with regards to initial receptor expression and Dex-induced receptor turnover, the current chapter aims to further elucidate the biological implications of altered GR α expression. Specifically, how ligand-induced changes in GR α expression, as a result of either 'gain' or 'loss' of receptor dimerization, modulate down-stream GC-responsive gene expression. A brief reminder that the subset of genes chosen for this study were chosen for the following reasons: TAT for its potential link to adverse side-effects, GILZ, which is known to have an anti-inflammatory potential, FKBP51 for its association with GC resistance and IL-6 for its pro-inflammatory potential, which is counteracted by GC signalling.

To do this an experimental protocol used in a study by Li *et al.*⁷³ was adapted (Fig. 6.4) and employed, in the current study. Firstly, MEF-mGRwt or MEF-mGRdim cells were plated in 12 well plates (1×10^5 cells/well) (Fig. 6.4, Day 1), the next day (Fig. 6.4, Day 2) cells were

steroid starved using charcoal-stripped FCS and 24 hours later (Fig. 6.4, day 3) pre-treated with GR α ligands alone, Dex (1 μ M) or CpdA (10 μ M) and in combination, Dex (1 μ M) and CpdA (10 μ M) (Fig. 6.4). Pre-treatment with GR α ligands followed either a short-term protocol (i.e. 2 hours) on the fourth day (Fig. 6.4, Day 4) or a long-term protocol (i.e. 24 hours) beginning the day before (Fig. 6.4, Day 3), to alter the ‘functional pool’ of GR α . At this point, a necessary reminder is that the short-term pre-treatment protocol was designed to reflect acute GC/GR α signalling, whilst, the long-term pre-treatment protocol was designed to reflect prolonged, chronic GC treatment, which has been associated with the development of acquired resistance to GC treatment.

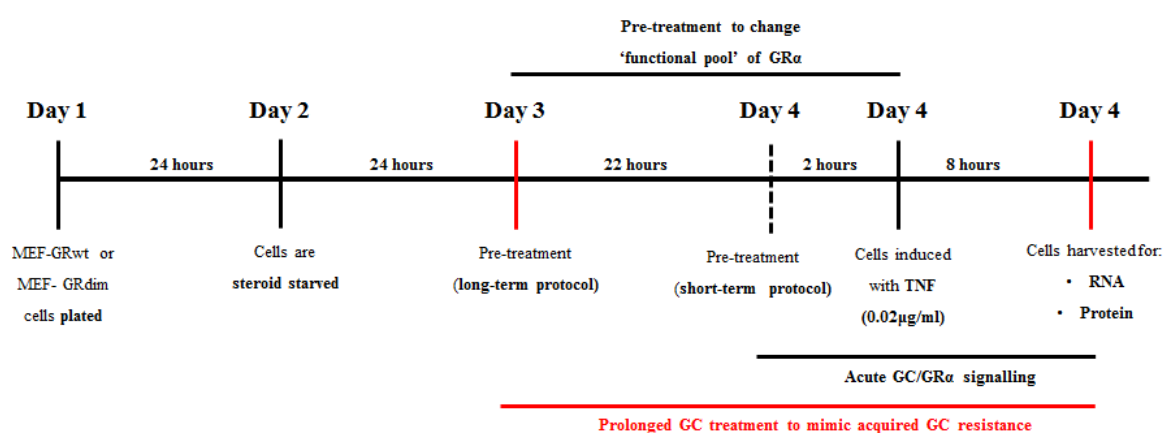


Figure 6.4: The experimental protocol adapted from Li *et al.*⁷³ used, in the current study to establish a GC-responsive system in order to mimic acquired GC resistance.

Following pre-treatment with GR α ligands, cells were induced with the pro-inflammatory cytokine, TNF α (0.02 μ g/ml), for an additional 8 hours (Fig. 6.4). Important to note, is that TNF α was added in the presence of the GR α ligands and was used to induce the expression of the pro-inflammatory gene (i.e. IL-6). Whilst the presence of TNF α was not required for the other genes investigated (GR α , GILZ, TAT and FKBP51), it was included to allow for the analysis of all genes from a single sample. However, any potential effects of TNF α on these other genes were addressed. Additionally, a second important detail is that the GR α ligands were not removed following pre-treatment and thus essentially resided on the cells for 10 and 32 hours for the short-term and long-term protocols, respectively (Fig. 6.4). Lastly, following treatment with GR α ligands and TNF α , the cells were harvested (Fig. 6.4, Day 4) and GR α protein levels and GR α , GILZ, TAT, FKBP51 and IL-6 mRNA expression determined using Western blotting and real-time PCR, respectively (Fig. 6.4).

6.2.2.1. Modulation of endogenous mGRwt and mGRdim expression in model of acquired GC resistance

In order to successfully assess how changes in GR α expression modulate GC-responsive gene expression, it was of utmost importance to initially establish differences between the expression of the GR α 'functional pool' following short-term (i.e. representing acute GC/GR α signalling) and long-term GC pre-treatment (i.e. chronic GC treatment, which may cause resistance). This allowed for changes at the level of GC-responsive gene expression to be correlated to changes in the concentration of the receptor.

To begin, the current study addressed the effect of TNF α on mGRwt and mGRdim protein and mRNA expression (Fig. 6.5). Once the effects of TNF α on GR α expression, had been accounted for, the ligand-selective effects of the dimerization promoting GC, Dex, and the dimerization abrogating GC, CpdA, alone or in combination, on mGRwt and mGRdim protein and mRNA expression, were determined (Fig. 6.6). Following which, the time-dependent effects of GC treatment (i.e. short-term versus long-term pre-treatment) on mGRwt and mGRdim, at both the protein and mRNA level, were detailed (Fig. 6.6). Lastly, the GC-mediated effects on mGRwt protein and mRNA expression were directly compared to those exerted on mGRdim to substantiate evidence for GR α dimerization as a requirement for ligand-induced protein turnover (Fig. 6.7).

6.2.2.1.1. The effect of pro-inflammatory cytokine, TNF α , on GR α protein and mRNA expression.

From the results, it is clear that treatment with the pro-inflammatory cytokine, TNF α , significantly increased GR α protein expression to 116% ($p < 0.001$) and 147% ($p < 0.001$), in mGRwt and mGRdim cells, respectively, relative to GR α protein expression in the absence of TNF α (100%) (Fig. 6.5A). Furthermore, a significantly ($p < 0.01$) higher increase in GR α protein expression, following TNF α treatment, was observed in the mGRdim cells when compared to GRwt (Fig. 6.5A). In contrast, TNF α treatment had no significant ($p > 0.05$) effect on GR α mRNA levels (Fig. 6.5B) in either the MEF-mGRwt or the MEF-mGRdim cell line. Interestingly, the fact that the effect of TNF α treatment on GR α protein expression (Fig. 6.5A) was not reflected at the GR α mRNA level (Fig. 6.5B) suggests that TNF α does not influence the expression of the GR α gene but may be, in part, involved in the stabilization of the GR α protein.

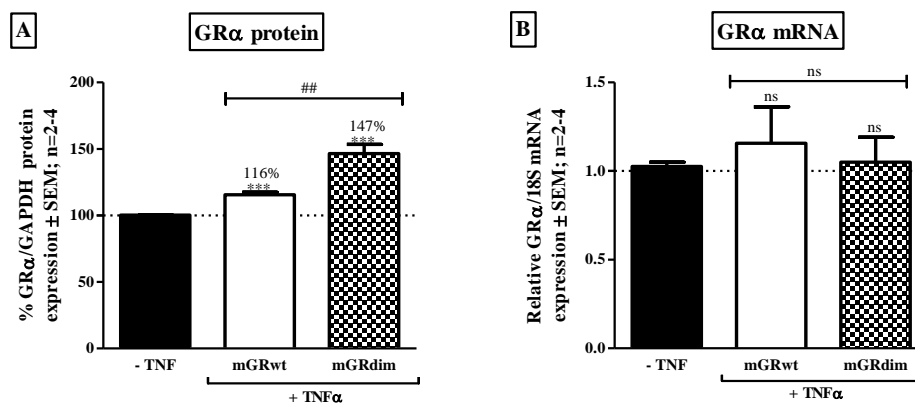


Figure 6.5: The effect of TNF α on GR α protein and mRNA expression in MEF cells. MEF-mGRwt and MEF-mGRdim cells were seeded into a 12 well plate (1×10^5 cells/well). Following 24 hours incubation, cells were steroid starved. Following steroid starvation, 48 hours later, cells were treated with solvent (EtOH) or TNF α (0.02 μ g/ml) for 8 hours. Thereafter, mGRwt and mGRdim protein expression was assessed by Western blotting where GAPDH was probed to ensure equal protein loading. For quantification of GR α protein (A), the intensities of the mGRwt, mGRdim and GAPDH bands were determined using MyECL Image Analysis software and subsequently the mGRwt and mGRdim expression was normalised to GAPDH expression and expressed as a percentage (average \pm SEM) of mGRwt or mGRdim expression in the absence of TNF α , which was set at 100% (dotted line). For quantification of GR α mRNA expression (B), RT-PCR was conducted and the expression of the GR α gene, normalized to that of the reference gene, 18S, calculated using Equation 2. GR α mRNA expression in the presence of TNF α is represented as a fold increase (average \pm SEM) relative to in the absence of TNF α , which is represented by the dotted line and set at 1. For statistical analysis, an unpaired t-test was used to evaluate the effect of TNF α on mGRwt or mGRdim protein (***, $p < 0.001$) and mRNA expression (ns, $p > 0.05$), as well as to determine differences between mGRwt and mGRdim protein (##, $p < 0.01$) and mRNA expression (ns, $p > 0.05$), in the presence of TNF α .

With the effects of TNF α on GR α protein and mRNA expression being accounted for in the current figure (Fig. 6.5), the time-dependent and ligand-selective induced changes in GR α expression were determined relative to receptor expression in the presence of TNF α in all subsequent experiments. Important to note is that for the rest of this chapter the solvent refers to EtOH plus TNF α .

6.2.2.1.2. Modulation of the GR α ‘functional pool’ is ligand-selective and time-dependent, at both the protein and mRNA level, in MEF-mGRwt and MEF-mGRdim cells

In terms of ‘gain’ of dimerization through treatment of endogenous mGRwt with the dimerization promoting Dex, a significant ($p < 0.001$) reduction in mGRwt protein following short-term pre-treatment resulted in the retention of only 58% of the initial ‘functional pool’ of GR α protein (100%) (Fig. 6.6A). As expected, long-term Dex pre-treatment promoted a further significant ($p < 0.001$) reduction in mGRwt protein expression ending up with a mere 29% mGRwt protein remaining, which is significantly ($p < 0.05$) lower than that of the short-term protocol (Fig. 6.6A). This finding supports the idea that chronic, prolonged GC

treatment further reduces the GR α protein ‘functional pool’, also demonstrated in Chapter 4 with transiently transfected hGRwt. In addition, these results obtained at the level of mGRwt protein expression were reflected at the level of mRNA expression, where short-term Dex pre-treatment reduced, although not significantly, mGRwt mRNA expression to 0.7, relative to the solvent (Fig. 6.6B). Similarly ($p > 0.05$) to the short-term pre-treatment, long-term pre-treatment with Dex reduced mGRwt mRNA expression to just under 0.7, specifically 0.66, which, unlike the short-term Dex pre-treatment, was found to be significantly different to that of the solvent (Fig. 6.6B). Interestingly, unlike the transiently transfected COS-1 cell system in Chapter 4, this endogenous system (i.e. the MEF-mGRwt cells) highlights Dex-induced regulation of the GR α ‘functional pool’ at the mRNA level (i.e. ‘push’) (Fig. 4.2).

In contrast to the dimerization promoting GC, Dex, a ‘loss’ of mGRwt dimerization as a result of treatment with the dimerization abrogating GC, CpdA, significantly ($p > 0.05$) restricted GR α protein turnover, following both short-term and long-term pre-treatment (Fig. 6.5A). Specifically, in support of results obtained in the COS-1 cell system transiently transfected with hGRwt (in Chapter 4), endogenous mGRwt protein levels were maintained at 94% and 89% of initial levels, post short and long-term CpdA pre-treatment, respectively (Fig. 6.6A). Interestingly, and in contrast to what was observed at the protein level, mGRwt mRNA expression was significantly reduced, relative to the solvent, to 0.6 ($p < 0.01$) and 0.4 ($p < 0.001$), following short and long-term pre-treatment, respectively, with CpdA (Fig. 6.6B). This result sparks interest in the ability of a GR α monomer, induced by CpdA treatment, to regulate its own gene expression, through ligand-induced down-regulation, which to our knowledge has not yet been investigated.

Interestingly, and in contrast to results obtained with transiently transfected hGRwt in the COS-1 cells (Fig. 5.14), the presence of CpdA (in the combination pre-treatment) was unable to protect mGRwt protein expression from Dex-induced receptor turnover, relative to Dex pre-treatment alone (Fig. 6.6A). Although results in Figure 6.6A do suggest a protective trend, it is not significant ($p > 0.05$). Specifically, both short ($p < 0.01$) and long-term ($p < 0.001$) pre-treatment with the Dex and CpdA combination, resulted in significant reductions in mGRwt protein levels, to 61% and 48%, respectively which is a smaller reduction than seen with Dex alone (58% and 29%, respectively) relative to solvent (Fig. 6.6A). From the mGRwt mRNA expression results (Fig. 6.6B), a significant ($p < 0.001$) reduction, to 0.3 and 0.4, relative to solvent, was noted following short and long-term pre-treatment, respectively, with the Dex and CpdA combination pre-treatment. Furthermore, results suggest that pre-

treatment with the combination of Dex and CpdA, unexpectedly, further reduced ($p < 0.05$) mGRwt mRNA expression relative to Dex alone, following short and long-term pre-treatment (Fig. 6.6B). Taken together, it appears that at the level of GR α protein expression, the dimerization abrogating GC, CpdA, opposes the effects of the dimerization promoting GC, Dex.

In contrast, at the level of ligand-induced GR α mRNA regulation, Dex and CpdA work cooperatively to reduce receptor mRNA expression.

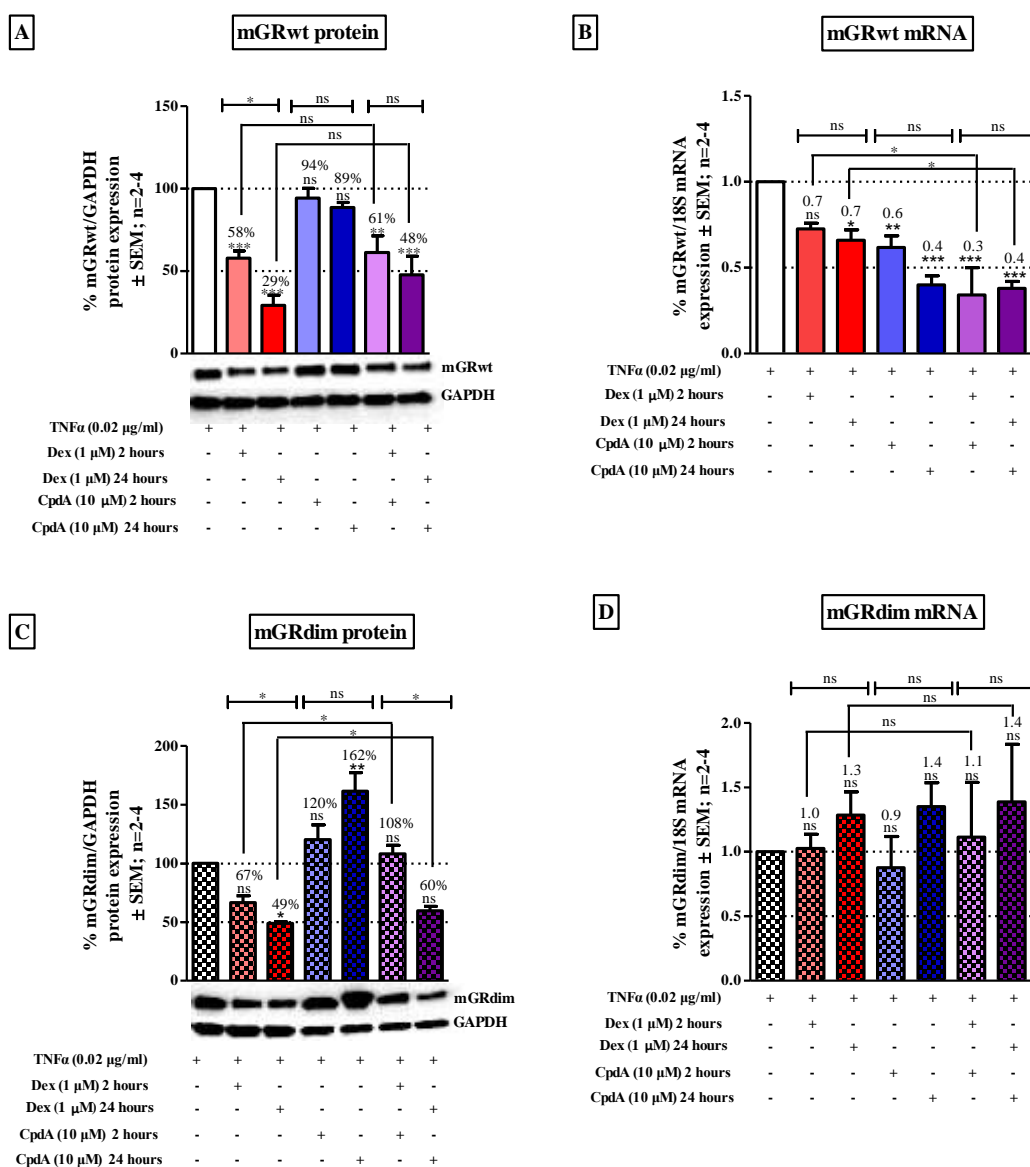


Figure 6.6: Modulation of the GR α ‘functional pool’ is ligand-selective and time-dependent, at both the protein and mRNA level. MEF-mGRwt and MEF-mGRdim cells were seeded into a 12 well plate (1×10^5 cells/well). Following 24 hours incubation, cells were steroid starved. After steroid starvation, 24 hours later, cells were pre-treated with solvent (EtOH), Dex (1 μ M), CpdA (10 μ M) or Dex (1 μ M) and CpdA (10 μ M) combined for either short (2 hours, lighter bars) or longer (24 hours, darker bars) periods of time. Following pre-treatment with GR α ligands, cells were treated with TNF α (0.02 μ g/ml) for an additional 8 hours. Thereafter, mGRwt and mGRdim protein expression was assessed by Western blotting where GAPDH was probed to ensure equal protein loading. Western blot shown for mGRwt (A, inset) and mGRdim (C, inset) are representative of two to four independent experiments. For quantification of mGRwt (A) and mGRdim (C) protein expression, the intensities of the mGRwt, mGRdim and GAPDH bands were determined using MyECL Image Analysis software and subsequently the mGRwt and mGRdim expression normalised to GAPDH expression and expressed as a percentage (average \pm SEM) of GR α expression in the presence of solvent (EtOH + TNF α), which was set at 100% (dotted line). For quantification of mGRwt (B) and mGRdim (D) mRNA expression, RT-PCR was conducted and the expression of the GR α gene (mGRwt or mGRdim), normalized to that of the reference gene, 18S, and calculated using Equation 2. GR α mRNA expression, following short- or long-term pre-treatment with test compounds in the presence of TNF α is represented as a fold increase or decrease, relative to the solvent (EtOH + TNF α), which is represented by the dotted line and set at 1. For statistical analysis, a one-way ANNOVA followed by a Tukey post-test was used to evaluate the time-dependent and ligand-selective effects of the GR α ligands on mGRwt and mGRdim protein (ns, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$) and mRNA expression (ns, $p > 0.05$, *, $p < 0.05$).

‘Loss’ of dimerization through the use of the endogenous mutant, mGRdim, partially restricted the ability of dimerization promoting Dex to induce receptor turnover, following short-term pre-treatment (Fig. 6.6C). Specifically, mGRdim protein expression was reduced to 67% of initial (100%) mGRdim ‘functional pool’, following short-term Dex pre-treatment, however, this reduction was not significant ($p > 0.05$). Furthermore, long-term Dex treatment led to a significant ($p < 0.05$) reduction, to 49%, in mGRdim protein expression, relative to solvent (Fig. 6.6C). In stark contrast, this modest Dex-induced reduction in the mGRdim protein expression was not reflected at the mRNA level (Fig. 6.6D), suggesting that treatment with dimerization promoting ligand, Dex, is unable to induce any significant ($p < 0.05$) reduction in mGRdim mRNA expression, following either short or long-term pre-treatment (Fig. 6.6D).

Briefly, taking into account results obtained with mGRwt following Dex and CpdA treatment, it appears that at the level of GR α protein expression ligand-induced monomeric GR α (i.e. ‘loss’ of GR α dimerization through CpdA treatment of mGRwt) is the most efficient at evading proteasomal degradation. Furthermore, monomeric GR α as a result of the mutation (i.e. ‘loss’ of GR α dimerization through mGRdim following treatment with Dex) can partially restrict Dex-mediated receptor protein turnover, however, not as efficiently as the CpdA-induced GR α monomer. Lastly, dimeric GR α (i.e. ‘gain’ of GR α dimerization through mGRwt following treatment with Dex) is the most readily degraded via the proteasome. Interestingly, at the level of ligand-induced GR α mRNA regulation, a differential pattern is observed with the greatest reduction in receptor mRNA observed

following treatment of mGRwt with CpdA, followed by treatment of mGRwt with Dex and lastly, treatment of mGRdim with Dex, the latter which did not modulate GR α expression at all.

Whilst mGRwt protein levels were maintained following short-term CpdA pre-treatment (Fig. 6.6A), a slight increase to 120%, relative to solvent, was noted for the mGRdim when treated with CpdA (Fig. 6.6B). A further significant ($p < 0.01$) increase in mGRdim protein levels, to 162%, relative to solvent, was observed following long-term pre-treatment with CpdA, however, this up-regulation is not significantly different from the increase after short-term CpdA pre-treatment (Fig. 6.6B). Consistent with results obtained following treatment with Dex, neither short nor long-term pre-treatment of mGRdim with CpdA, resulted in significant changes of mGRdim at the mRNA level (Fig. 6.6D).

In the case of the combination pre-treatment, CpdA was able to significantly ($p < 0.05$) safeguard the mGRdim protein from Dex-induced receptor protein turnover (Fig. 6.6C). The mGRdim protein expression was significantly ($p < 0.05$) increased, relative to that of the Dex alone pre-treatment, to 108% and 60%, respectively, following short and long-term co-treatment with Dex and CpdA (Fig. 6.6C). Similarly to the results obtained with individual Dex or CpdA treatments, the combination pre-treatment of Dex and CpdA had no significant effect on mGRdim mRNA expression (Fig. 6.6D).

The results in Figure 6.6 highlight the ligand-selective (i.e. Dex and CpdA alone or in combination) and time-dependent effects (i.e. short versus long-term pre-treatment) of receptor turnover in the individual MEF-mGRwt and MEF-mGRdim cell lines. Whilst these findings hint at a requirement for GR α dimerization in ligand-induced receptor turnover, in these cells, they do not confirm this role by directly comparing whether the effects observed via mGRwt are significantly different to those observed via mGRdim, which is addressed in the next section.

6.2.2.1.3. Validating the model of acquired resistance to GC treatment, in terms of ligand-induced alterations in GR α ‘functional pool’, and investigating a role for dimerization by directly comparing mGRwt and mGRdim.

In order to validate the model of acquired GC resistance, significant differences in mGRwt protein expression following short-term and long-term Dex pre-treatment, needed to be obtained. Following short-term Dex pre-treatment (i.e. acute GC/GR α signalling) mGRwt

protein expression was significantly ($p < 0.001$) reduced to 58%, relative to solvent, however, importantly, a significantly greater ($p < 0.05$) reduction in mGRwt protein expression (i.e. to 29%) was noted, following long-term Dex pre-treatment (i.e. prolonged GC treatment) (Fig. 6.6A). Thus, these findings validated the model established to mimic acquired GC resistance.

Moreover, no significant difference between the mGRwt and mGRdim receptor expression was observed following short-term Dex pre-treatment, indicative of acute GC/GR α signalling. Conversely, 'loss' of GR α dimerization, through the use of the mutant, mGRdim, partially but significantly ($p < 0.01$) restricted ligand-induced GR α protein down-regulation, relative to 'gain' of GR α dimerization via mGRwt, following long-term pre-treatment with dimerization promoting Dex (Fig. 6.7A). Specifically, although mGRdim expression was reduced (49% of the 'functional pool' remaining post-treatment) approximately 20% more GR α expression was noted post long-term Dex pre-treatment, relative to the mGRwt (29% of the 'functional pool' remaining post-treatment) (Fig. 6.7A). These results confirm a requirement, be it partial, for GR α dimerization in receptor turnover at the protein level, notably, following prolonged GC treatment.

In support of a role for GR α dimerization in mediating ligand-induced GR α protein turnover, demonstrated using mGRdim, were results obtained with mGRwt following treatment with dimerization abrogating GC, CpdA^{80,81} (Fig. 6.7A). No significant ligand-induced reduction in mGRwt or mGRdim protein levels, relative to solvent, was noted, following treatment with CpdA (Fig. 6.7A). Furthermore no significant ($p > 0.05$) difference between mGRwt and mGRdim protein expression following short-term CpdA pre-treatment was observed (Fig. 6.7A), while in contrast, long-term CpdA pre-treatment of the mutant, mGRdim, resulted in a significant ($p < 0.01$) increase in receptor protein expression (162% of solvent) relative to mGRwt (89% of solvent (Fig. 6.7A). A possible explanation for this is that binding of CpdA to mGRdim stabilizes a different GR α monomer species than the Dex-bound mGRdim monomer, which may be able to form weak and transient dimers⁸², however this requires further investigation.

In terms of ligand-induced regulation at the mRNA level, Dex pre-treatment (i.e. short and long-term) was capable of significantly ($p < 0.05$) reducing mGRwt mRNA expression relative to solvent, while 'loss' of GR α dimerization, through use of the mGRdim mutant, completely abolished Dex's ability to induce GR α down-regulation of mRNA expression (Fig. 6.7B). Unlike with mGRwt, after short- and long-term Dex pre-treatment mGRdim expression was maintained (1.0-fold) or slightly increased (1.3-fold), relative to the solvent

(Fig. 6.7B). One could postulate, that this lack of Dex-induced GR α down-regulation of receptor mRNA expression may be due to the reduced ability of mGRdim to form dimers⁷².

However, results from monomeric GR α as a result of CpdA treatment argue against the requirement of a GR α dimer for ligand-induced reductions in receptor mRNA expression (Fig. 6.7B). Specifically, at the level of mRNA expression, no significant ($p > 0.05$) difference was noted between mGRwt and mGRdim, following short-term pre-treatment with the dimerization abrogating SGRM, CpdA (Fig. 6.7B). In contrast, long-term pre-treatment with CpdA, led to a reduction in mGRwt mRNA expression (0.4-fold), which was significantly different ($p < 0.001$) to the slight increase noted in mGRdim mRNA expression (1.4), following the same pre-treatment conditions (Fig. 6.7B). These findings suggest ligand-induced GR α down-regulation of the GR α gene and/or mRNA destabilization occurs via a CpdA-bound mGRwt monomer but not via a Dex or CpdA-bound mGRdim monomer.

In summary, Figure 6.7B identifies a novel, mandatory requirement for GR α dimerization in ligand-induced receptor turnover at the transcriptional and post-translational level by directly comparing the effects of Dex via mGRwt (i.e. 'gain' of GR α dimerization) to the effects of Dex via the mutant, mGRdim (i.e. 'loss' of GR α dimerization) at both the protein and mRNA level. Additionally, whilst it has previously been postulated that CpdA bound to mGRwt is a similar monomeric species to Dex bound to mGRdim⁸¹, results from the current study highlight significant novel differences between the CpdA-bound mGRwt monomer and the Dex-bound mGRdim, specifically at the level of ligand-induced GR α mRNA regulation. A possible reason for these differences may be due to varying degrees of a 'loss' of GR α dimerization between monomeric CpdA bound mGRwt and monomeric Dex-bound mGRdim, which is further explored in the discussion of this chapter. Specifically, CpdA has a potent ability to abrogate existing GR α dimers⁸¹, whilst speculation surrounding the ability of the dimerization deficient mutant to form GR α dimers, does exist⁸².

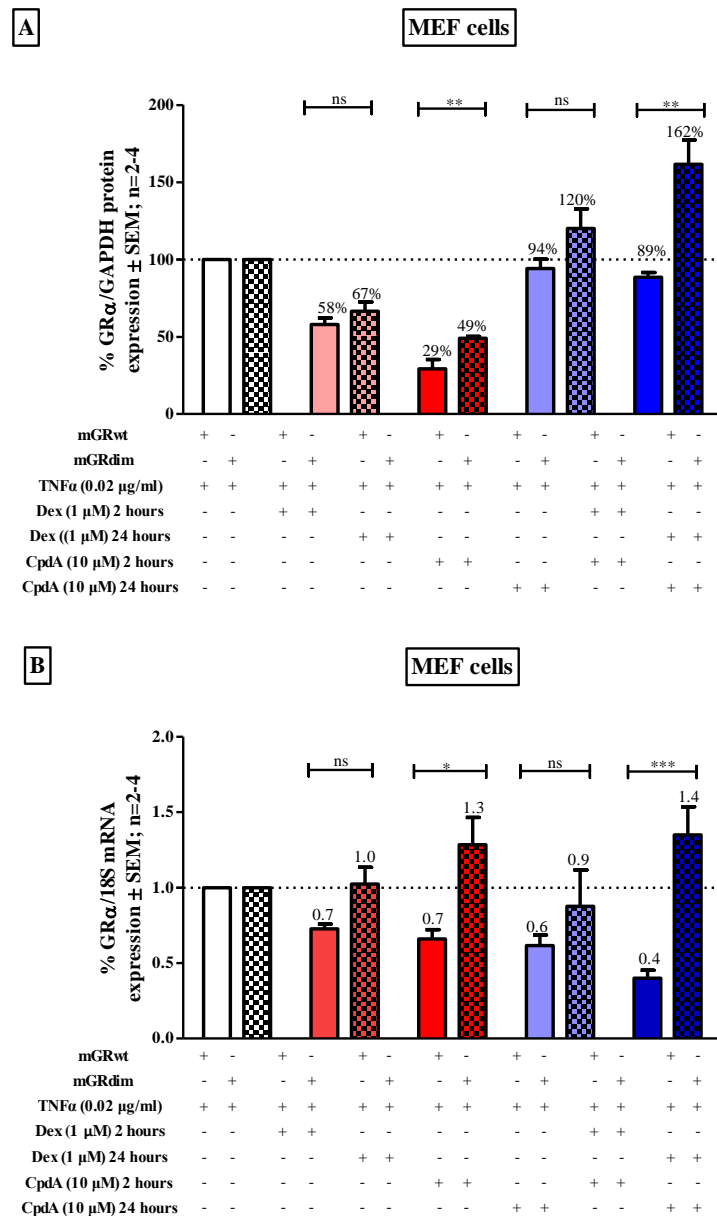


Figure 6.7: ‘Loss’ of GRα dimerization influences receptor turnover at the protein and mRNA level. Data was used from Figure 6.6 and for statistical analysis, a one-way ANOVA followed by a Tukey post-test was used to evaluate and compare the time-dependent and ligand-selective effects of the GRα ligands between mGRwt and mGRdim at the protein (ns, $p > 0.05$, **, $p < 0.01$), and mRNA level (ns, $p > 0.05$, *, $p < 0.05$, ***, $p < 0.001$).

6.2.2.2. Determining the responsiveness of the system under acute GC/GRα signalling conditions, represented by short-term GC pre-treatment

Having demonstrated that GRα expression is modulated in a ligand-selective, time- and dimerization dependent manner at both the protein and mRNA expression level, it was necessary to first determine the extent of acute GC/GRα signalling (i.e. following short-term

pre-treatment), which occurs by using the experimental protocol (Fig. 6.4), in the MEF cells, and is quantified by measuring the ligand-induced changes in the mRNA expression of the GC-responsive genes (i.e. GILZ, TAT, FKBP51 and IL-6). Essentially, in this section, the responsiveness of the system (or established working model) is determined with focus on the ligand-selective effects of GR α ligands, alone or in combination, in the context of 'gain' or 'loss' of GR α dimerization.

Firstly, the effect of the pro-inflammatory cytokine, TNF α , on basal GC-responsive gene expression is addressed. Secondly, the previously published effects of Dex on GC-responsive gene expression^{35,42,53,69} were confirmed via mGRwt in the MEF-mGRwt cells, to ensure the responsiveness of the system and to confirm acute GC/GR α signalling. Subsequently, these effects were compared to the effects of Dex on GC-responsive gene expression via mGRdim, in the MEF-mGRdim cells, to establish whether genes were regulated via GR α dimers or monomers. Next, the effects on GC-responsive gene expression, mediated via monomeric CpdA-bound mGRwt, were compared to that of monomeric Dex-bound mGRdim. Lastly, the ligand-selective effects, on GC-responsive gene expression, of Dex and CpdA in combination, via mGRwt and mGRdim were compared to the effects elicited by these GCs individually.

6.2.2.2.1. The effect of the pro-inflammatory cytokine, TNF α , on basal GC-responsive gene expression

From the results, it is clear that TNF α treatment, on its own, significantly reduced GILZ mRNA expression to 0.4 ($p < 0.001$) and 0.6-fold ($p < 0.05$), relative to solvent (EtOH) in the MEF-mGRwt and MEF-mGRdim cells, respectively (Fig. 6.8A). Moreover, no significant ($p > 0.05$) change in TAT (Fig. 6.8B) or FKBP51 (Fig. 6.8C) mRNA expression is observed in either of the cell lines, following treatment with TNF α . Lastly, as expected, TNF α treatment leads to a significant ($p < 0.001$) 4-fold increase in IL-6 mRNA expression in the MEF-mGRwt cells and a slightly lower fold increase (2.4-fold) in IL-6 mRNA expression in the MEF-mGRdim cells (Fig. 6.8D). For all the genes, the effect of TNF α on GC-responsive gene expression in the MEF-mGRwt cells was not significantly ($p > 0.05$) different to the effect observed in the MEF-mGRdim cells (Fig. 6.8A, B, C and D).

It was necessary to address the effects of TNF α on GC-responsive gene expression, in order to account for possible TNF α -induced changes in mRNA expression in subsequent experiments. Thus, important to note, is that potential ligand-induced GR α effects on GC-

responsive gene expression, observed in the context of ‘gain’ or ‘loss’ of GR α dimerization, are normalised to changes observed in the presence of solvent plus TNF α , in latter sections.

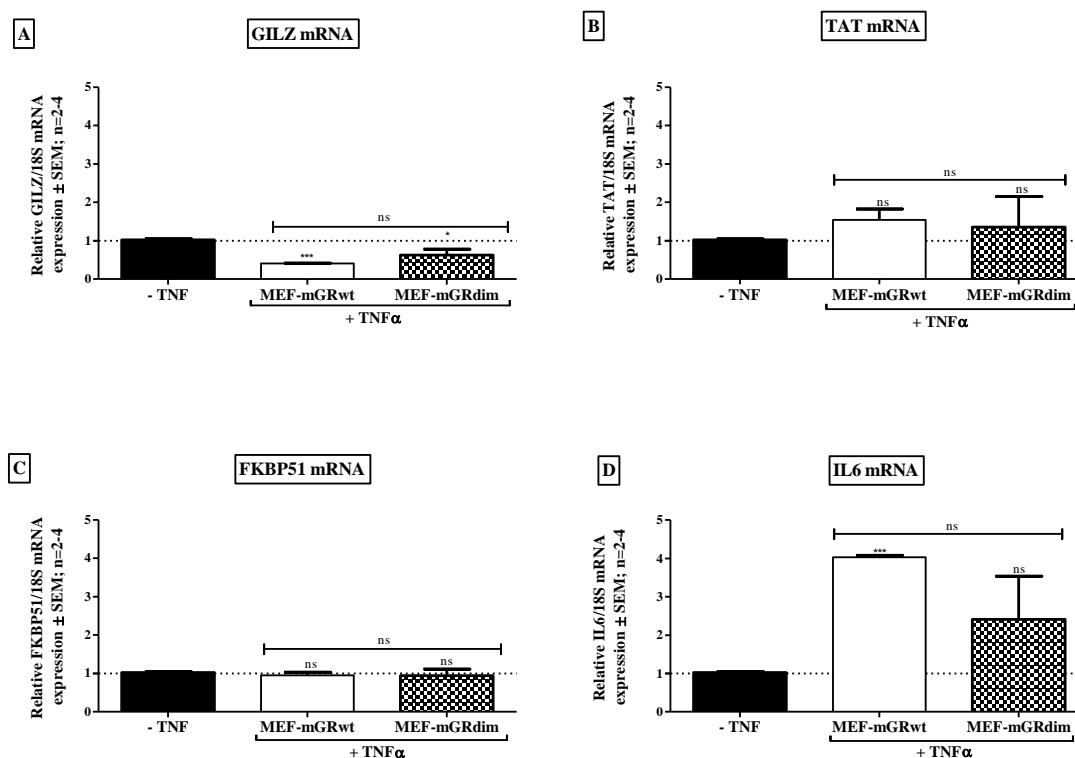


Figure 6.8: The effect of the pro-inflammatory cytokine, TNF α , on basal GC-responsive gene expression. MEF-mGRwt and MEF-mGRdim cells were seeded into a 12 well plate (1×10^5 cells/well). Following 24 hours incubation, cells were steroid starved. After steroid starvation, 48 hours later, cells were treated with EtOH or TNF α (0.02 μ g/ml) for 8 hours. Thereafter, the mRNA expression of basal GC-responsive genes was determined in the MEF-mGRwt and MEF-mGRdim cell lines by performing RT-PCR. The GILZ (A), TAT (B), FKBP51 (C) and IL-6 (D) basal mRNA expression was calculated using Equation 2 and normalized to that of the reference gene, 18S. TNF α induced changes in basal GC-responsive gene expression was expressed as a fold increase or decrease (average \pm SEM) relative to the gene expression of the genes of interest in the absence of TNF α , which was set at 1 (dotted line). For statistical analysis, an unpaired t-test was used to evaluate and compare the TNF α -induced effects on basal GC-responsive gene expression in the MEF-mGRwt or MEF-mGRdim cell lines, relative to gene expression in the absence of TNF α lines (ns, $p > 0.05$, *, $p < 0.05$, ***, $p < 0.001$), as well as to compare the TNF α -induced effects on basal GC-responsive gene expression between the MEF-mGRwt and MEF-mGRdim cells ($p < 0.05$).

6.2.2.2. GC-responsive gene expression is ligand-selective under acute GC/GR α signalling conditions (i.e. short-term pre-treatment)

Essentially, results from this section demonstrate that the working model established, using the experimental protocol in Figure 6.4, is GC-responsive, following short-term Dex pre-treatment.

Firstly, Dex induces transactivation of GC-responsive genes, via mGRwt, leading to a significant increase in GILZ ($p < 0.001$), TAT ($p < 0.01$) and FKBP51 ($p < 0.001$) mRNA

expression to 6.6- (Fig. 6.9A), 13.3- (Fig. 6.9B) and 4.0-fold (Fig. 6.9A), respectively. Moreover, IL-6 mRNA expression is down-regulated, to 0.7-fold relative to solvent, following short-term pre-treatment with Dex (Fig. 6.9D). These findings confirm that the working model reacts as expected and that a GR α dimer, induced following treatment of mGRwt with Dex, is capable of both inducing (i.e. via transactivation) and repressing (i.e. via transrepression) a number of GC-responsive genes.

In contrast to a 'gain' of receptor dimerization, using the wild type receptor, mGRwt, 'loss' of GR α dimerization, through the use of the mutant, mGRdim, restricts GC-mediated transactivation of GC-responsive genes, whilst maintaining its ability to induce transrepression of the pro-inflammatory cytokine, IL-6. Specifically, unlike in the MEF-mGRwt cells, Dex short-term pre-treatment was unable to significantly ($p > 0.05$) up-regulate GILZ (1.6-fold, Fig. 6.9A) and TAT (2.3-fold, Fig. 6.9B) mRNA expression, in the MEF-mGRdim cells. Additionally, a significant difference between the Dex-induced GILZ ($p < 0.01$) mRNA levels are noted between the two cell lines (Fig. 6.9A). Although not significant ($p > 0.05$) a similar trend is observed for TAT (Fig. 6.9B) when directly comparing MEF-mGRwt and MEF-mGRdim cells, following short-term Dex pre-treatment. Interestingly, FKBP51 is significantly ($p < 0.01$) increased after Dex treatment in the MEF-mGRdim cells, however, this increase is not as prominent as, and significantly ($p < 0.01$) lower, than that observed in the MEF-mGRwt cells (Fig. 6.9C). In terms of the Dex-mediated transrepression of IL-6, via mGRdim, a 'loss' GR α dimerization did not significantly ($p > 0.05$) affect the ability of Dex-mediated transrepression of the IL-6 gene and resulted in an equal reduction in IL-6 mRNA expression in both cell lines (Fig. 6.9E). This finding is not unexpected as, in general, GC-mediated transrepression of GC responsive genes is thought to be mediated via a GR α monomer^{80,81}.

Once the requirement for GR α dimerization in regulating the expression of the GC-responsive genes, (GILZ, TAT, and FKBP51 but not IL-6) had been determined, the effect of a 'loss' of mGRwt dimerization as a result of CpdA treatment was compared to the effect of a 'loss' of dimerization via the mutant, mGRdim, following Dex treatment. CpdA treatment, failed to induce significant up-regulation of the GC-responsive genes GILZ (1.0-fold), TAT (1.5-fold) and FKBP51 (1.0-fold) via mGRwt (Fig. 6.9A, B and C). Although CpdA pre-treatment was unable to induce GR α -mediated transactivation, most likely due to its ability to abrogate mGRwt dimerization^{80,81}, CpdA was capable of significantly ($p < 0.05$) reducing IL-6 mRNA expression to 0.6-fold (Fig. 6.9D). Essentially, these CpdA-mediated effects via

mGRwt are comparable and not significantly different ($p > 0.05$) to the Dex-mediated effects via mGRdim for all genes (GILZ, TAT and IL-6), except FKBP51 (Fig. 6.9A, B, C and D). Important to note, is that CpdA short-term pre-treatment of mGRdim also did not lead to significant ($p > 0.05$) transactivation of GILZ (Fig. 6.9A), TAT (Fig. 6.9B) or FKBP51 (Fig.6.9C), however, did result in significant ($p < 0.05$) transrepression of IL-6 (0.6-fold), similar to the effects observed via mGRwt (Fig. 6.9D).

Now that the effects of the individual GR α ligands, namely Dex or CpdA, on GC-responsive gene expression, via mGRwt or mGRdim, have been addressed, these effects were investigated following short-term pre-treatment of these ligands in combination, in both MEF-mGRwt and MEF-mGRdim cells. Combining the dimerization promoting GC, Dex, and the dimerization abrogating GC, CpdA, did not result in a significant ($p > 0.05$) increase in GILZ (1.2-fold) or FKBP51 (1.2-fold) mRNA expression (Fig. 6.6A and C), similar to the effects observed for CpdA alone, but unlike the effects observed for Dex short-term pre-treatment, in the MEF-mGRwt cells. In contrast, significant ($p < 0.05$) up-regulation of TAT (7.5-fold), via mGRwt, was noted following short-term pre-treatment with the combination of GR α ligands (Fig. 6.9B). However, although this increase in TAT mRNA expression (7.5-fold) is significantly ($p < 0.05$) higher than that induced by CpdA alone (1.5-fold) it appears to be not significantly ($p > 0.05$) different to the increase following Dex short-term pre-treatment (13.3-fold). Unlike for transactivation, the combination treatment resulted in transrepression of IL-6 that was higher, but not significantly different, to that of CpdA or Dex alone via mGRwt (Fig. 6.6E). As with the individual short-term pre-treatments of mGRdim with GR α ligands, the combination pre-treatment did not lead to significant ($p > 0.05$) transactivation of GILZ (Fig. 6.9A), TAT (Fig. 6.9B) or FKBP51 (Fig.6.9C), however, did result in significant ($p < 0.01$) transrepression of IL-6 (0.4-fold) in MEF-mGRdim cells (Fig. 6.9D).

Taken together, results from this section, confirmed that the working system established using the experimental protocol (Fig. 6.4) is GC-responsive, with GC-mediated transactivation and transrepression occurring as one would expect under acute GC/GR α signalling conditions^{71,83,84}.

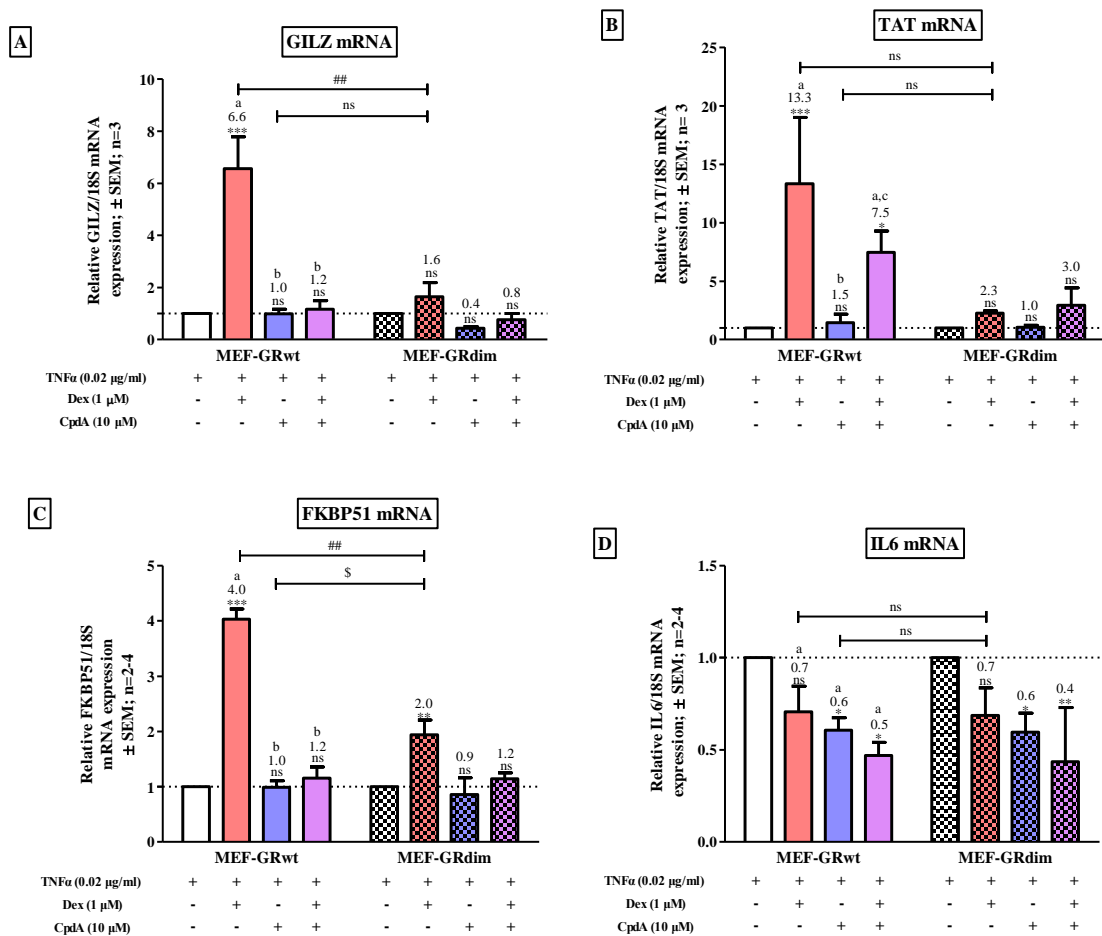


Figure 6.9: GC-responsive gene expression is ligand-selective under acute GC/GR α signalling conditions (i.e. short-term pre-treatment). MEF-mGRwt and MEF-mGRdim cells were seeded into a 12 well plate (1×10^5 cells/well). Following 24 hours incubation, cells were steroid starved. After steroid starvation, 24 hours later, cells were pre-treated with solvent (EtOH), Dex (1 μ M), CpdA (10 μ M) or Dex (1 μ M) and CpdA (10 μ M) combined, for 2 hours. Following pre-treatment with GR α ligands, cells were treated with TNF α (0.02 μ g/ml) for an additional 8 hours. Thereafter, the mRNA expression of GC-responsive genes was determined, in the MEF-mGRwt and MEF-mGRdim cell lines, by performing RT-PCR. The GILZ (A), TAT (B), FKBP51 (C) and IL-6 (D) mRNA expression was calculated using Equation 2 and normalized to that of the reference gene, 18S. Ligand-selective changes in GC-responsive gene expression was expressed as a fold increase or decrease (average \pm SEM) relative to the gene expression of the genes in the presence of the solvent (EtOH + TNF α), which was set at 1 (dotted line), for each cell line. For statistical analysis, a one-way ANOVA with a Tukey's post-test was used to evaluate the effects of GR α ligands (following short-term pre-treatment) on GC-responsive gene expression in the MEF-mGRwt or the MEF-mGRdim cells, relative to solvent (EtOH + TNF α) (ns, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). Furthermore, the letters a, b, c are used to indicate significant ($p < 0.05$) differences between bars within the MEF-mGRwt or MEF-mGRdim cells. An unpaired t-test was used to determine the differences between Dex pre-treatment via mGRwt or mGRdim (ns, $p > 0.05$, ##, $p < 0.01$) on GC-responsive gene expression. Lastly, differences between GC-responsive gene expression induced by CpdA pre-treatment of mGRwt and Dex pre-treatment of mGRdim were also determined using an unpaired t-test (ns, $p > 0.05$, \$, $p < 0.05$).

6.2.2.3. Evaluating acquired GC resistance at a molecular level (i.e. GC-responsive gene expression) following prolonged GC treatment.

It is well documented that a number of chronic auto-immune diseases require long-term and high-dose GC treatment to combat the severe inflammation associated with these illnesses

and that, following prolonged GC treatment, it is likely that acquired resistance to GC treatment may develop as a result of, in part, drastic reductions in GR α expression^{2,8-11}.

Currently, a need exists for the development of new therapeutic strategies to alleviate GC resistance either by reducing the treatment time or by developing novel GR α ligands that potentiate the anti-inflammatory effects of existing therapeutic GCs, like Dex⁸⁵⁻⁸⁷. One of the ways in which the development of GC insensitivity may be circumvented, is by preserving GR α expression⁷³.

Up to now the current study has established, tested, and validated a GC-responsive system, using an adapted experimental protocol (Fig. 6.4) from Li *et al.*⁷³, in order to investigate the down-stream effects (i.e. the modulation of GC-responsive gene expression) of ligand-induced changes in the GR α expression, however, the down-stream effects of drastic changes in receptor expression, which occur following prolonged GC treatment (i.e. long-term pre-treatment), have not yet been addressed. Thus, in essence, the aim in this section was to evaluate the expression of GC-responsive genes under conditions that mimic acquired GC resistance (i.e. long-term GC treatment) and to determine whether inducing predominantly monomeric GR α could aid in restoring GC sensitivity, by maintaining receptor expression.

To begin this section, the ligand-selective effects on a cohort of GC-responsive genes chosen due to their relevance in resistance, inflammation or the generation of adverse side-effects, associated with GC signalling, were investigated (i.e. GILZ, TAT, FKBP51 or IL-6). Specifically, the ligand-selective effects, via GR α , on these genes following long-term GC pre-treatment were compared to the effects on these genes after short-term GC pre-treatment, by measuring the mRNA expression of these genes, in the MEF-mGRwt (Fig. 6.10) or MEF-mGRdim cells (Fig. 6.11). Once the effects of prolonged GC treatment on GC-responsive gene expression had been determined in each individual cell line, the effect of a 'loss' of GR α dimerization via the mutant in the MEF-mGRdim cells on GC-responsive gene expression, was directly compared to that of the wild type in the MEF-mGRwt cells (Fig. 6.12), to assess whether prevention of GC acquire resistance can be achieved via predominantly monomeric GR α .

6.2.2.3.1. Prolonged GC treatment results in GC acquired resistance, at the molecular level, for one, but not all the GC-responsive genes.

Previously, the current study demonstrated that long-term pre-treatment with dimerization promoting Dex in the MEF-mGRwt cells, resulted in a significantly ($p < 0.05$) greater reduction (up to 20% more) in receptor expression, relative to short-term treatment (Fig. 6.6A). Interestingly, results in Figure 6.10A, suggest a concomitant decrease in the mRNA expression of GILZ following long-term pre-treatment relative to the mRNA expression of GILZ post short-term Dex pre-treatment. Specifically, long-term Dex treatment significantly ($p < 0.05$) reduced GILZ mRNA expression to 4.6-fold, relative to GILZ mRNA expression post short-term Dex pre-treatment (6.6-fold) (Fig. 6.10A). Surprisingly, and in contrast to GILZ, no significant ($p > 0.05$) reduction in TAT and FKBP51 mRNA expression was observed following long-term Dex pre-treatment (Fig. 6.10B and C). Furthermore, one would expect an increase in IL-6 mRNA expression, as a result of a decrease in mGRwt protein, following long-term Dex pre-treatment, however this is not the case (Fig. 6.10D).

In stark contrast to long-term Dex pre-treatment, long-term treatment with dimerization abrogating CpdA did not alter the expression of the GR α 'functional pool', relative to solvent (Fig. 6.6A). Specifically, mGRwt expression was maintained at 94 and 89% relative to solvent following short- and long-term pre-treatment, respectively (Fig. 6.6A). Thus, approximately 60% more GR α is available to induce down-stream effects (i.e. modulate GC-responsive gene expression), following long-term CpdA pre-treatment (89%), relative to long-term Dex pre-treatment (29%).

As one would expect, due to the monomeric nature of CpdA-bound mGRwt and its inability to transactivate GC-responsive genes^{12,56,88}, no significant ($p > 0.05$) changes in GILZ (Fig. 6.10A), TAT (Fig. 6.10B) or FKBP51 (Fig. 6.10C) mRNA expression were noted following long-term CpdA pre-treatment, as also observed for short-term CpdA pre-treatment. Additionally, no significant change in the mRNA expression of IL-6 (Fig. 6.10D) was observed, between two different periods of pre-treatments (i.e. short-term versus long-term pre-treatment). One could speculate that this is due to similar mGRwt protein levels detected between the short- and long-term pre-treatments with CpdA (Fig. 6.6A). On the other hand, one would expect, relative to prolonged Dex-mediated transrepression of IL-6 via mGRwt, more CpdA-mediated transrepression of IL-6 due to the 60% more mGRwt available (Fig. 6.6A) to transrepress this gene, following prolonged CpdA treatment, however this is not the

case (Fig. 6.10D).

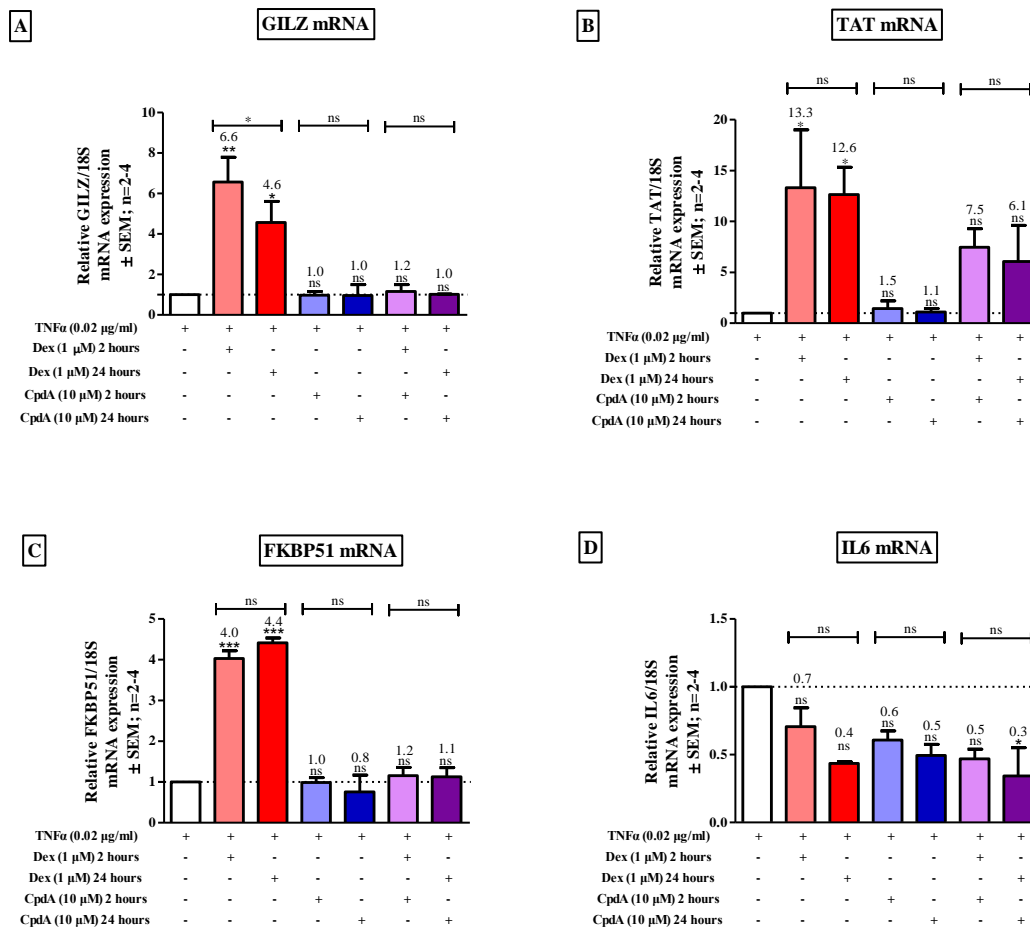


Figure 6.10: Prolonged GC treatment modulates the gene expression profile of GC-responsive genes. MEF-mGRwt cells were seeded into a 12 well plate (1×10^5 cells/well). Following 24 hours incubation, cells were steroid starved. After steroid starvation, 24 hours later, cells were pre-treated with solvent (EtOH), Dex (1 μ M), CpdA (10 μ M) or Dex (1 μ M) and CpdA (10 μ M) in combination, for 2 hours (short-term protocol; light bars) or 24 hours (long-term protocol, dark bars). Following pre-treatment with GR α ligands, cells were treated with TNF α (0.02 μ g/ml) for an additional 8 hours. Thereafter effects of short- or long-term pre-treatment on the mRNA expression of GC-responsive genes, was determined in the MEF-mGRwt cell line by performing RT-PCR. The GILZ (A), TAT (B), FKBP51 (C) and IL-6 (D) mRNA expression was calculated using Equation 2 and normalized to that of the reference gene, 18S. Ligand-selective and time-dependent changes in GC-responsive gene expression were expressed as a fold increase or decrease (average \pm SEM) relative to the gene expression of the genes in the presence of the solvent (EtOH + TNF α), which was set at 1 (dotted line). For statistical analysis, a one-way ANOVA with a Tukey's post-test was used to evaluate the effects of GR α ligands on GC-responsive gene expression in the MEF-mGRwt cells, relative to TNF α alone, at both pre-treatment times. In addition, an unpaired t-test was used to determine the direct effect of long-term (24 hours) pre-treatment on ligand-induced GC-responsive gene expression, relative to the effect observed post short-term (2 hours) pre-treatment (ns, $p > 0.05$ and *, $p < 0.05$).

Lastly, in terms of the combination pre-treatment, both short- and long-term pre-treatment with Dex and CpdA in combination significantly reduced mGRwt protein expression (Fig. 6.6A). Moreover, no difference in the extent of mGRwt protein turnover between the two periods of combination pre-treatment existed, although reductions in mGRwt expression in the combination pre-treatment were slightly less than with Dex alone (Fig. 6.6A). This lack of difference between the GR α 'functional pool's', following short- and long-term pre-treatment of GCs in combination, was reflected at the level of GC-responsive gene expression, where no significant change in the mRNA expression of GILZ (Fig. 6.10A), TAT (Fig. 6.10B), FKBP51 (Fig. 6.10C) or IL-6 (Fig. 6.10D) was observed.

6.2.2.3.2. Prolonged GC treatment does not modulate the gene expression profile of GC-responsive genes via mGRdim

Previously, the current study demonstrated that long-term pre-treatment with dimerization promoting Dex in the MEF-mGRdim cells, led to a significant reduction to 49% in mGRdim expression, relative to short-term Dex pre-treatment (Fig. 6.5C), however, this reduction was not as drastic as that observed with mGRwt (i.e. 29%, relative to solvent) following prolonged Dex pre-treatment (Fig. 6.5A). In terms of GC-responsive gene expression, a very slight, but insignificant reduction reflects the change in mGRdim expression at the level of GILZ mRNA expression from 2.1- to 1.9-fold, when comparing short-term and long-term Dex pre-treatment, respectively, in the MEF-mGRdim cells (Fig. 6.10A). Moreover, unlike GILZ, no significant change in the expression of the other genes was observed following long-term pre-treatment with Dex, relative to short-term Dex pre-treatment (Fig. 6.10B, C and D).

Interestingly, long-term treatment of mGRdim with dimerization abrogating CpdA resulted in a significant ($p < 0.01$) increase in the protein expression of mGRdim to 162%, relative to solvent, however this increase was not significantly ($p < 0.05$) greater than that mediated by short-term CpdA pre-treatment (i.e. 120%) (Fig. 6.6C). Moreover, prolonged treatment of mGRdim with CpdA (i.e. long-term pre-treatment) mediated a slight but not significant ($p < 0.05$) increase in the mRNA expression of GILZ, TAT and FKBP51 (Fig. 6.11A, B and C), relative to short-term pre-treatment. Furthermore, an interesting observation is made with the effect of a CpdA-mediated increase in mGRdim expression on IL-6 expression (Fig. 6.11D). Specifically, following long-term pre-treatment with CpdA, mGRdim's ability to transrepress IL-6 is, in part, diminished relative to the short-term CpdA pre-treatment. One could

postulate that this observed effect is due to a lack of CpdA-mediated mGRdim nuclear translocation, which was demonstrated by the current study in Chapter 5 (Fig. 5.6A), however, it does not explain the slight CpdA-mediated increase in .in TAT and FKBP51, via mGRdim (Fig. 6.11B and C) nor the effects of short-term CpdA pre-treatment on IL-6 (Fig. 6.11D).

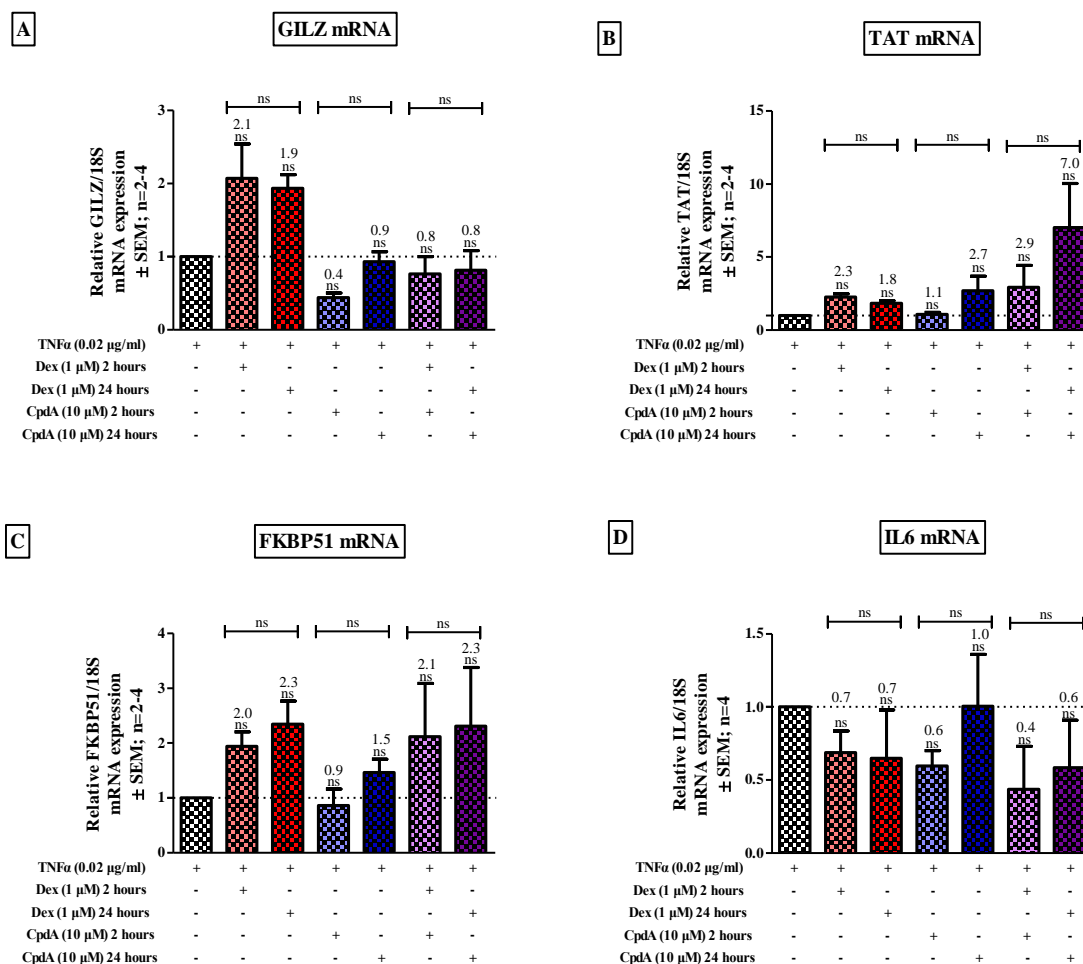


Figure 6.11: Prolonged GC treatment does not modulate the gene expression profile of GC-responsive genes via mGRdim. MEF-mGRdim cells were seeded into a 12 well plate (1×10^5 cells/well). Following 24 hours incubation, cells were steroid starved. After steroid starvation, 24 hours later, cells were pre-treated with solvent (EtOH), Dex (1 µM), CpdA (10 µM) or Dex (1 µM) and CpdA (10 µM) in combination, for 2 hours (short-term protocol; light bars) or 24 hours (long-term protocol, dark bars). Following pre-treatment with GRα ligands, cells were treated with TNFα (0.02 µg/ml) for an additional 8 hours. Thereafter effects of short or long-term pre-treatment on the mRNA expression of basal GC-responsive genes, was determined in the MEF-mGRdim cell line, by performing RT-PCR. The GILZ (A), TAT (B), FKBP51 (C) and IL-6 (D) mRNA expression was calculated using Equation 2 and normalized to that of the reference gene, 18S. Ligand-selective and time-dependent changes in GC-responsive gene expression were expressed as a fold increase or decrease (average ± SEM) relative to the gene expression of the genes in the presence of the solvent (EtOH + TNFα), which was set at 1 (dotted line). For statistical analysis, a one-way ANOVA with a Tukey's post-test was used to evaluate the effects of GRα ligands on basal GC-responsive gene expression in the MEF-mGRdim cells, relative to TNFα alone, at both pre-treatment times. In addition, an unpaired t-test was used to determine the direct effect of long-term (24 hours) pre-treatment on ligand-induced GC-responsive gene expression (ns, $p > 0.05$, *, $p < 0.05$, ***, $p < 0.001$), relative to the effect observed post short-term (2 hours) pre-treatment (ns, $p > 0.05$).

In the case of the combination pre-treatment, a significant ($p < 0.05$) difference in mGRdim protein expression between the short-term (108%) and long-term pre-treatment (60%) was observed (Fig. 6.6C). However, this difference in mGRdim protein expression is not reflected by a significant difference in GC-responsive gene expression for any of the genes (Fig. 6.11), although prolonged pre-treatment of Dex and CpdA in combination appears to potentially affect the mRNA expression of TAT (Fig. 6.11B).

6.2.2.3.3. ‘Loss’ of GR α dimerization results in reduced GC-mediated transactivation, but not transrepression.

To directly compare the effects of a ‘loss’ of dimerization, and thus preserved GR α expression, results were combined from both cell lines (Fig. 6.12). In terms of GC-responsive gene expression no significant ($p > 0.05$) differences, due to the use of mGRdim, across all genes (GILZ, TAT, FKBP51 or IL-6) were noted, following short- and long-term pre-treatment with CpdA alone or in combination with Dex (Fig. 6.12).

In contrast, significant differences ($p < 0.01$ and $p < 0.001$) in the mRNA expression of GILZ (Fig. 6.12A), TAT (Fig. 6.12B) and FKBP51 (Fig. 6.12C), but not of IL-6 (Fig. 6.12D), following both short-term and long-term pre-treatment with Dex, were noted, when mGRdim was utilized. Although, in terms of IL-6 mRNA expression, ‘loss’ of dimerization, through the use of mGRdim, did not significantly modulate Dex’s ability to transrepress IL-6 (Fig. 6.12D), ‘loss’ of dimerization through CpdA treatment of mGRwt appeared to be more efficient (0.5- fold) at transrepressing IL-6, relative to mGRdim (1.0- fold), following long-term CpdA pre-treatment (Fig. 6.12D). This is likely due to the difference in the subcellular localisation of CpdA-bound mGRwt and CpdA-bound mGRdim, which was observed in Chapter 5 (Fig. 5.6A).

To briefly summarize this current section, the use of mGRdim significantly modulated the Dex-mediated GC-responsive gene expression of genes mediated via GR α transactivation, however, did not alter GR α mediated transrepression of the pro-inflammatory cytokine, IL-6, relative to mGRwt. Additionally, ‘loss’ of GR α dimerization, following short-term or prolonged CpdA treatment or the use of the mutant, mGRdim, rarely re-shaped the GC-responsive gene expression profile, relative to mGRwt. These findings suggest, ‘loss’ of GR α dimerization, through the use of mGRdim, may restrict the development of adverse side-effects (i.e. from an up-regulation of metabolic genes such as TAT) but retain the beneficial anti-inflammatory potential of GCs, via GR α -mediated transrepression.

Taken altogether, results from this entire section, where a GC-responsive system was established, tested and validated to mimic an acquired resistance to GC treatment, provided some interesting findings.

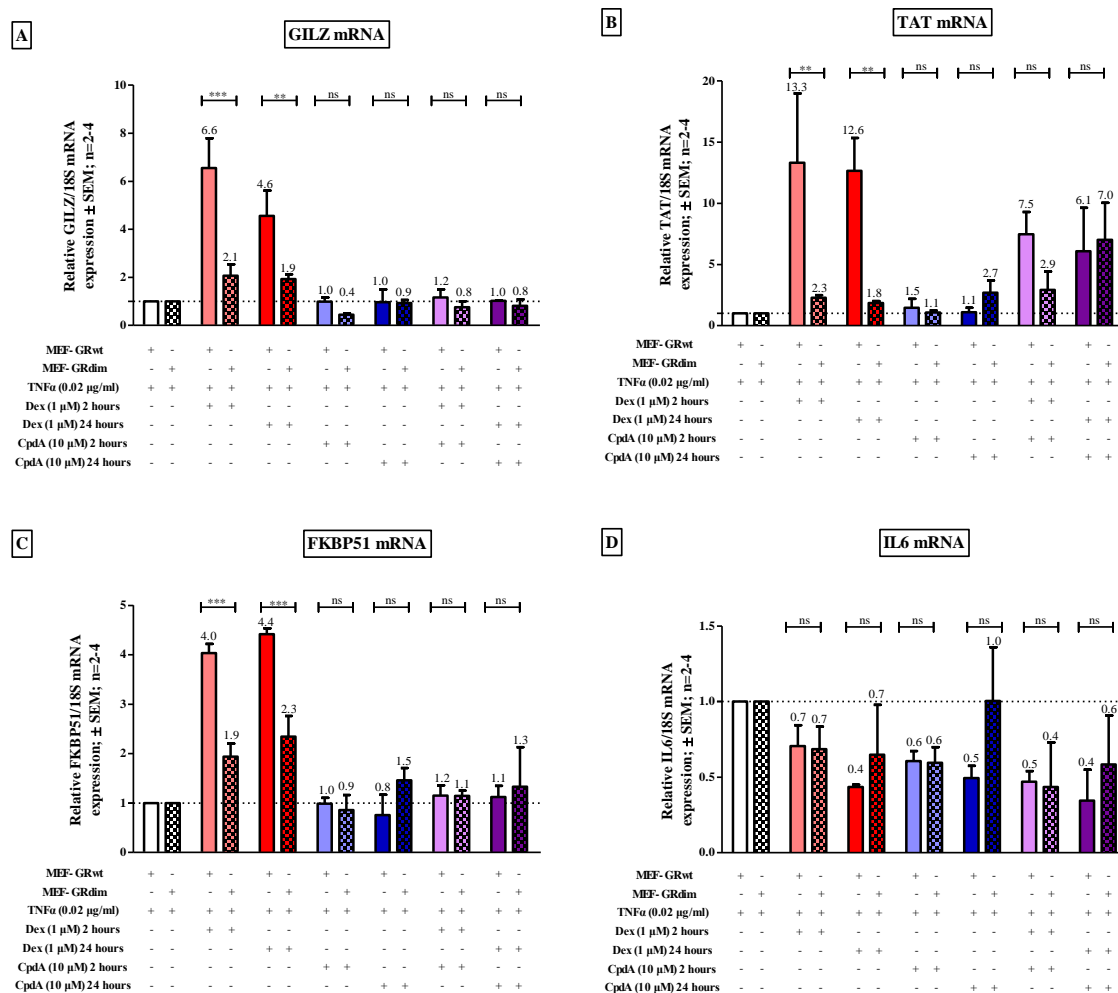


Figure 6.12: ‘Loss’ of GR α dimerization results in reduced GC-mediated, transactivation but not transrepression, in MEF-mGRdim cells. Data was used from Figure 6.10 and 6.11 and for statistical analysis, a two-way ANOVA with a Bonferroni’s post-test was used to evaluate the effects of a ‘loss’ of GR α dimerization on basal GC-responsive gene expression by comparing effects via mGRwt and mGRdim (ns, $p > 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

Briefly, having provided further evidence for a role for receptor dimerization in mediating ligand-induced GR α protein turnover, using an endogenous dimerization deficient mutant, mGRdim, and CpdA treatment of mGRwt, a novel mandatory requirement for GR α dimerization in ligand-induced receptor turnover at the level of mRNA expression was noted for mGRdim, but not for the dimerization abrogating GC, CpdA, in the current section. Specifically, significant differences in the ability of the CpdA-bound mGRwt monomer and the Dex-bound mGRdim monomer to regulate expression of the GR α gene, NR3C1^{15,16,25,26},

and/or affect GR α mRNA stability, resulting in alterations in total GR α mRNA expression, were observed.

The current study, went on to demonstrate that drastic reductions in receptor expression (as a result of prolonged GC treatment) driven by a ‘gain’ of mGRwt dimerization through treatment with dimerization promoting Dex, significantly modulated the expression of GILZ, but not TAT or FKBP51. Furthermore, contrary to what was expected, this reduction in wild type GR α , following long-term pre-treatment with Dex, did not reduce the ability to trans-repress the pro-inflammatory IL-6 gene. In terms of acquired resistance to GC treatment, these results highlight gene-specific effects and, in the case of the current study, suggest that acquired GC resistance at the molecular level, is likely to develop due to a decrease in the expression of the anti-inflammatory gene GILZ, rather than an increase in the expression of the pro-inflammatory gene, IL-6, following long-term Dex pre-treatment. The fact that no decrease in the responsiveness of TAT and FKBP51 expression was observed, even after prolonged GC treatment has considerably reduced the level of the GR α ‘functional pool’ available to regulate gene expression, suggest that these gene do not appear to have implications for acquired GC resistance, importantly, at the molecular level. However, maintained expression of TAT and FKBP51 even after prolonged GC treatment has implications for the generation of adverse side-effects and clinical resistance associated with GC signalling, which is further explored in the discussion of this chapter.

In contrast to reduced GR α protein expression, as a consequence of a ‘gain’ of receptor dimerization, preserving GR α expression, even after long-term GC pre-treatment, due to ‘loss’ of dimerization through CpdA treatment or dimerization deficient mutant GR α , does not result in an increase in the transactivation of GC-responsive genes GILZ, TAT or FKBP51. This is consistent with previous results, which suggests GC-mediated GR α transactivation occurs predominantly via a GR α dimer²⁹ and thus increasing predominantly monomeric GR α expression would not affect transactivation of such GC-responsive genes. This finding is beneficial in terms of genes such as TAT and FKBP51, which are linked to side-effects and resistance, respectively^{2,53,58}. However, the restricted increase in the expression of the anti-inflammatory gene, GILZ, due to the presence of predominantly monomeric GR α is disadvantageous for counteracting inflammation. Surprisingly, maintaining GR α expression did not result in greater trans-repression of the pro-inflammatory gene, IL-6.

Lastly, gene-specific effects were observed when investigating the effects between pre-treatment with the individual GCs and the GCs in combination. This may be linked to the degree of receptor dimerization when both the dimerization promoting Dex and the dimerization abrogating GC, CpdA, were added together. Taken together, the ability of CpdA to partially maintain GR α expression (Fig. 6.6), combined with its ability to mediate successful transrepression whilst somewhat restricting Dex-mediated transactivation of GC-responsive genes (Fig. 6.9), all in the presence of Dex, provide evidence for the potential ability of CpdA to counteract the adverse effects of Dex, following prolonged GC treatment, which may be exploited pharmacologically.

6.3. Discussion

GCs continue to remain the preferred drug choice for the treatment of inflammation even though prolonged GC treatment results in adverse side effects and, more importantly for the current study, acquired resistance to GC treatment^{2,8-11}.

Over the past years, research has focused on identifying GR α ligands, which maintain vital immunosuppressive effects but demonstrate an improved side-effect profile²⁹, however, acquired resistance to GC treatment has often been overlooked when developing novel therapeutics. With the incidence of patient insensitivity increasing⁴⁻⁶, and many individuals requiring higher GC doses in order to combat inflammation associated with various disease-states, it seems the recent shift in research focus towards understanding and exploiting the molecular mechanisms involved in the development of resistance, is essential.

Ligand-induced GR α down-regulation, following prolonged GC treatment, is one of the many factors that contribute to the development of GC insensitivity or an acquired resistance^{12,18-20,25,57,89,90}. Previous chapters, in the current study, identified receptor conformation, specifically GR α dimerization, as a novel requirement for mediating this ligand-induced GR α protein down-regulation (Chapter 4) and elucidated how receptor conformation affects the molecular mechanisms associated with proteasomal degradation (Chapter 5). Whilst these findings hinted that targeting receptor dimerization and maintaining GR α expression may serve as a potential mechanistic approach to reverse or prevent acquired GC resistance, they did not directly address the biological implications of altered GR α expression, in terms of acquired GC resistance. Specifically, how maintaining the GR α 'functional pool' through a 'loss' of receptor dimerization modulates the expression of GC-responsive genes, generally associated with acquired GC resistance. In an attempt to evaluate acquired resistance to GC

treatment, at the molecular level, the current chapter established and tested a GC-responsive system, or working model, using an adapted experimental protocol from Li *et al.*⁷³.

Firstly, and of utmost importance, the ability of our working model to mimic acquired resistance was validated by demonstrating further reductions in mGRwt expression, following long-term Dex pre-treatment (i.e. prolonged GC treatment to mimic acquired resistance), relative to short-term Dex pre-treatment (i.e. acute GC/GR α signalling).

Having established and validated the model, the current study went on to demonstrate the development of an acquired resistance to GC treatment, at the molecular level (i.e. GILZ), following long-term pre-treatment of mGRwt, relative to short-term pre-treatment, with dimerization promoting Dex. Moreover, 'loss' of GR α dimerization, through treatment of mGRwt or the use of mGRdim, did not display the same effects on GILZ mRNA expression (i.e. in terms of molecular resistance), due to the fact that GC-mediated transactivation of GILZ requires direct binding of a GR α dimer to the promoter of this gene.

Additionally, an acquired resistance to GC treatment, at the molecular level, was not demonstrated for the other genes, namely TAT, FKBP51 and IL-6, via mGRwt. However, these results provided evidence for the development of GC acquired resistance, following prolonged GC treatment, in terms of clinical GC resistance (FKBP51 and IL-6), as well as the generation of adverse side-effects (i.e. TAT). Furthermore, 'loss' of GR α dimerization, through treatment of mGRwt or the use of mGRdim, restricted up-regulation of TAT and FKBP51, whilst maintaining the ability to transrepress IL-6 (with the exception of long-term CpdA pre-treatment via mGRdim). Suggesting, 'loss' of GR α dimerization may limit the development of acquired GC resistance at a clinical level, as well as the development of adverse side-effects. Taken together, these findings highlight the importance of considering the effects of ligands on GR α conformation when developing novel therapeutics, especially for prolonged therapeutic regimens.

Specifically, 'gain' of mGRwt dimerization mediated by long-term Dex pre-treatment encouraged a significantly greater reduction in the mGRwt 'functional pool', to a mere 29%, relative to the short-term Dex pre-treatment (58%), which subsequently resulted in a loss of responsiveness of the GRE-drive gene, GILZ (Fig. 6.6A and 6.10A). Specifically, a significantly blunted GILZ response (4.6-fold) in GILZ expression, following long-term Dex pre-treatment, was noted, relative to the 6.6-fold increase in GILZ mRNA expression induced by short-term Dex pre-treatment (Fig. 6.10A). This approximate 1.4-fold reduction in GILZ

mRNA expression, in the current study, is supported by the work of Gossye *et al.*⁹⁰ in which an approximate 1.3-fold reduction (a 22-fold to 17-fold increase, relative to solvent) in the mRNA expression of another GRE-driven gene, glutamine synthetase (GS), was noted, following prolonged exposure to Dex (32 hours). In addition, the responsiveness of the GILZ gene (i.e. in the short-term Dex pre-treatment protocol) in the working model, used in the current study, is comparable to the responsiveness of this gene in other studies. Following, short-term pre-treatment, which was aimed to represent acute GC/GR α signalling, a 6.6-fold increase in the expression of GILZ, was obtained (Fig. 6.10A). In line with this finding, a study by Ronacher *et al.*²⁰ demonstrated a 6-fold increase, relative to solvent, in endogenous GILZ expression in stably transfected U20S osteosarcoma cells after two hours treatment with Dex. Moreover, Wang *et al.*⁹¹ reported a slightly lower, up to 5-fold, increase in GILZ mRNA expression, relative to solvent, following up to 6 hours treatment with a lower concentration of Dex (100 nM), in RAW264.7 cells.

Interestingly, and unlike GILZ, the expression of another GC-responsive gene, investigated in this study, namely FKBP51, was not inhibited by the significantly reduced mGRwt 'functional pool', following prolonged pre-treatment with Dex. Rather a slight, yet insignificant, increase in FKBP51 expression from 4.0-fold to 4.4-fold, following short- and long-term pre-treatment, respectively, was observed (Fig. 6.10C). This finding correlates well with previous studies, which demonstrated that a reduction in GR α expression was insufficient to affect the ability of GR α to induce 'positive' up-regulation (GR α -mediated transactivation) of certain GC-responsive genes^{73,90}. Specifically, when GR α expression was low, following 24 hour (long-term) pre-treatment with Dex (1 μ M), the mRNA expression of the GRE-driven dual specificity phosphatase 1 (DUSP1) was not significantly different from the mRNA expression noted when GR α expression was significantly higher after just 2 hours pre-treatment with Dex⁷³. Moreover, in a study which investigated the relative expression of FKBP51 as a function of time in a lymphoblast cell line, no decrease in FKBP51 mRNA expression, was noted after 24 hours GC treatment⁹². Lastly, in a study by Gossye *et al.*⁹⁰, the expression of the GC-inducible pro-collagen C-endopeptidase enhancer 2 (POLCE2) was found to be significantly higher in conjunction with a reduction in receptor expression.

In terms of an acquired resistance to GC treatment, results from the GILZ and FKBP51 mRNA expression experiments, support the fact that prolonged GC treatment encourages the development of GC insensitivity. Importantly, GC sensitivity may be described at the molecular level (i.e. diminished GILZ mRNA expression, via GR α , following prolonged GC

treatment) or at a clinical, more indirect level (i.e. rather than diminished GC-responsive gene expression, mRNA levels are maintained, after prolonged treatments (e.g. FKBP51) but this maintained mRNA expression has implications in acquired GC resistance). Specifically, due to the fact that GILZ is thought to contribute to the anti-inflammatory potential of GCs³³, a reduction in GILZ expression as a direct result of a reduction in GR α (i.e. acquired GC resistance at the molecular level), may promote inflammation. On the other hand, an increase in the expression of FKBP51, which was noted following long-term Dex pre-treatment in the current study, has been shown to encourage cellular desensitization to GC treatment^{59–62}, via a negative feedback loop in which the resultant protein of the FKBP51 mRNA expression sequesters GR α in the cytoplasm^{55–58}. Although the effects of a reduction in the mGRwt expression on GC-responsive gene expression appear to be gene-specific, these findings provide evidence that prolonged GC treatment (i.e. specifically with Dex) is a significant contributor to the development of clinical acquired GC resistance.

In contrast, acquired resistance to GC treatment was not achieved at the molecular level for IL-6, in the established model of the current study (Fig. 6.10D). Unexpectedly, the reduced ‘functional pool’ of mGRwt (29%) which was driven by long-term Dex pre-treatment, was still able to efficiently transrepress the expression of the pro-inflammatory cytokine, IL-6, from 1.0-fold in the presence of the solvent to 0.4-fold (Fig. 6.10D). These findings contradict results from Gossye *et al.*⁹⁰ where reduced GR α expression impeded the GC-mediated immunosuppressive effects on the pro-inflammatory mediators, IL-6, interleukin 8 (IL-8) and macrophage chemoattractant protein-1 (MCP-1) in FLS cells isolated from patients with active RA. Additionally, Li *et al.*⁷³ elegantly demonstrated that reduced GR α protein expression impeded the GC-mediated immunosuppressive effects for a number of other genes regulated by GR α , namely interleukin 17 (IL-17), MMP and TNF α . A potential reason for observed differences between the current study and the literature, is the greater differences (i.e. approximately more than 50%) in GR α expression observed between short- and long-term protocols in the literature^{73,90} whereas, although a significant difference in the mGRwt ‘functional pool’ between short- and long-term Dex pre-treatment, 58% and 29% respectively, were noted in the current study, it may be that this difference in receptor expression was not enough to modulate the GC-responsiveness of IL-6.

Lastly, in terms of the generation of adverse side-effects (e.g. hyperglycaemia) associated with prolonged GC treatment, results from the effect of a reduced mGRwt ‘functional pool’ on the expression of the metabolic gene, TAT, demonstrated that the remaining mGRwt

expression (29%), post long-term pre-treatment with Dex, was sufficient to drive the 'positive' up-regulation of the metabolic gene, TAT. Moreover, no significant difference between the GC-mediated increase in TAT mRNA expression following short- and long-term pre-treatment, which was 13.3-fold and 12.6-fold, respectively, was detected (Fig. 6.10B). This finding may provide a possible explanation for why some patients have been shown to develop resistance, whilst still displaying adverse side-effects⁹³.

Results highlighted gene specific effects in term of acquired GC resistance at the molecular level, with a small subset of GC-responsive genes investigated, and collectively, validated the growing concerns associated with prolonged GC treatment, namely the generation of undesirable side-effects (e.g. hyperglycaemia) and, more importantly for this study, the development of an overall clinical acquired resistance to GC treatment as a result of drastic reductions in the GR α 'functional pool'.

Whilst a 'gain' of mGRwt dimerization driven by long-term pre-treatment with dimerization promoting Dex resulted in drastic reductions in the mGRwt 'functional pool', a 'loss' of mGRwt dimerization following long-term pre-treatment with dimerization abrogating Cp dA maintained mGRwt protein expression at 89%, relative to the solvent (Fig. 6.6A). Neither short- nor long-term Cp dA pre-treatment resulted in the transactivation of the GRE-driven genes GILZ, TAT and FKBP51, relative to solvent (Fig. 6.10A, B and C). Due to the fact that Cp dA treatment does not result in GC-mediated transactivation of genes, via GR α , it is difficult to elucidate the role of maintained GR α expression, relative to the reduced GR α 'functional pool' following Dex treatment (Fig. 6.10A, B and C). However, in terms of GC-mediated transrepression, via GR α , both short- and long-term Cp dA pre-treatment reduced IL-6 expression to 0.6- and 0.5-fold, respectively, relative to the solvent (Fig. 6.10D). The lack of difference in the Cp dA-mediated transrepression of IL-6 between the two periods of treatment (i.e. short- and long-term pre-treatment), is likely due to the similar level of the mGRwt 'functional pool', noted in Figure 6.6A. Specifically, following short- and long-term Cp dA pre-treatment, the mGRwt 'functional pool' was reported to be 94% and 89% respectively, relative to the solvent, which is set at 100% (Fig. 6.6A). With that said, we postulated that, relative to Dex-treatment, greater Cp dA-mediated transrepression of IL-6 would occur due to the maintenance of the GR α 'functional pool' (Fig. 6.6A), following Cp dA treatment; however, this was not the case.

Taken together, results suggest that treatment with dimerization abrogating Cp dA, short- or long-term, in terms of the generation of adverse side-effects (e.g. hyperglycaemia), may be

more beneficial than treatment with dimerization promoting Dex. Additionally, with regards to acquired GC resistance, CpdA's inability to transactivate the anti-inflammatory gene, GILZ, could be unfavourable. However, CpdA's ability to prevent an increase in FKBP51 expression, which is associated with the development of patient insensitivity to GCs, in combination with its ability to maintain transrepression of the pro-inflammatory cytokine, IL-6, corroborates CpdA's powerful immunosuppressive effects even after prolonged treatment. Having addressed the effects of a CpdA-induced 'loss' of GR α dimerization, the effects of a 'loss' of dimerization, on GC-responsive gene expression, through the use of the endogenous mGRdim mutant, are now discussed.

Previously, Robertson *et al.*⁸¹, suggested that the effects of CpdA on certain aspects of the GC/GR α signalling pathway was elicited through a 'loss' of receptor dimerization, as CpdA treatment of wild type GR α was comparable to those mediated by Dex treatment of the dimerization deficient mutant GR α , in a transiently transfected system. This idea was confirmed by results from the current study in Chapter 4, with regards to ligand-induced GR α protein down-regulation, where results obtained with monomeric CpdA-hGRwt were similar to those obtained with monomeric Dex-bound hGRdim. With that said, Figure 4.5 does show a CpdA-induced up-regulation of hGRwt, which was not noted for hGRdim, following treatment with Dex. Importantly, results from Chapter 5, further sparked interest in differences between CpdA-bound hGRwt and Dex-bound hGRdim, in terms of subcellular localisation and Ser404 phosphorylation, however, further investigation of these effects at the proteasomal level was beyond the scope of this project.

Interestingly, the current chapter highlighted and confirmed significant differences between CpdA-bound mGRwt and Dex-bound mGRdim at the level of GC-mediated GC-responsive gene expression, using the endogenous mutant, mGRdim, in the MEF-mGRdim cells. Generally speaking, results confirmed that although these GR α species are thought to be similar in conformation (i.e. monomeric) they are not identical, and differ at the level of GC-responsive gene expression and GR α regulation.

Firstly, in contrast to transiently transfected COS-1 cells (Chapter 4), where hGRwt and hGRdim protein expression was maintained, following CpdA and Dex treatment, respectively, results, from the current chapter demonstrated that, following treatment with Dex, endogenous mGRdim underwent partial receptor protein turnover. Specifically, 'loss' of GR α dimerization through the use of mGRdim was unable to fully restrict mGRdim protein turnover, resulting in 49% remaining of the mGRdim 'functional pool' present following

long-term treatment with Dex (Fig. 6.6C). Importantly, although this Dex-induced down-regulation was significant it was not as potent as the long-term Dex-induced mGRwt protein turnover (Fig. 6.7A).

In contrast, and dissimilar to Dex via mGRdim (Fig. 6.6C), at no point did a CpdA-induced 'loss' of endogenous wild type GR α dimerization (i.e. mGRwt) drive significant receptor turnover at the protein level (Fig. 6.6A). Furthermore, prolonged CpdA treatment resulted in a significant increase in mGRdim protein expression (162%), relative to the solvent (Fig. 6.6C). This finding suggests an additive 'loss' of receptor dimerization effect on evading proteasomal degradation when CpdA treatment is combined with the use of mGRdim, and highlights potential differences between the monomeric conformation of CpdA-bound mGRwt, Dex-bound mGRdim and CpdA-bound mGRdim, which will be discussed at a later point.

In support of the observed differences between monomeric CpdA-mGRwt and Dex-bound mGRdim at the level GR α protein regulation, are differences which were observed at the level of ligand-induced alterations in GR α mRNA expression, which may be due to ligand-induced alteration in the regulation of the GR α gene and/or mRNA destabilization. Whilst ligand-induced down-regulation of the GR α gene is fairly well characterized for classical GCs, such as Dex^{16,94}, the effects of CpdA on GR α gene regulation is not as well characterized. In the current study significant down-regulation of 60% and 40% of mGRwt mRNA expression was noted, post short- and long-term CpdA pre-treatment, respectively (Fig. 6.6B). In support of this finding, Drebet *et al.*⁹⁵, using a myofibroblast cell line (CT5.3hTERT) demonstrated an approximate 50% reduction in GR α mRNA expression, following 24 hours (i.e. between the short- and long-term pre-treatment used in the current study) treatment with CpdA. Interestingly, in the same study⁹⁵, this CpdA-induced reduction in GR α mRNA expression was abolished following 48 hours of CpdA treatment. Moreover, in a study by a previous member in our lab¹², no CpdA-induced down-regulation of endogenous GR α mRNA in BWTG3 cells, was observed, following 24 hours treatment. Furthermore, the current study demonstrates that, unlike CpdA via mGRwt, neither Dex nor CpdA treatment was able to reduce mGRdim mRNA expression (Fig. 6.6D). To our knowledge, this is the first time ligand-induced GR α mRNA regulation has been investigated in the MEF-mGRdim cells.

Recently the specific mechanism by which Dex mediates down-regulation of GR α mRNA expression has been established and involves the binding of a ligand-activated GR α

monomer, to a nGRE in the promoter of the GR α gene (NR3C1)¹⁶. One could postulate, from results in the current study, that monomeric mGRwt induced by CpdA treatment is able to coordinate ligand-induced regulation of GR α mRNA expression by potentially the direct DNA-binding of CpdA-bound GR α to the nGRE, present in the GR α gene, in the MEF-mGRwt but not the MEF-mGRdim cells. In contrast, it would seem that the Dex-bound mGRdim monomer is unable to directly interact with this DNA regulatory region (i.e. the nGRE) within the GR α gene promoter.

Lastly, gene expression results, in the current study, supports previous results^{12,56,81,88} that demonstrate CpdA's ability to restrict the transactivation of simple GREs, via GR α , which requires a GR α dimer, as a result of CpdA's potent ability to abrogate receptor dimerization^{80-82,96} (Fig. 6.6A, B and C). In most cases (i.e. GILZ and TAT), a 'loss' of dimerization through the use of mGRdim mirrored CpdA's effect via mGRwt, by restricting Dex's well-established ability to drive GR α mediated GRE-gene expression (Fig. 6.9A, and B). However, in the case of FKBP51, a significant difference was noted between the ability of CpdA bound mGRwt and Dex bound mGRdim to regulate the expression of this gene (Fig. 6.9C). Specifically, a slight, yet significant, 2.0-fold increase in FKBP51 expression, following short-term Dex pre-treatment of mGRdim, was noted (Fig. 6.9C). These findings, once again, encourage the notion of differences between the monomeric conformation of CpdA bound to mGRwt and Dex bound to mGRdim.

Taken together, a possible explanation for the observed differences between CpdA-bound mGRwt and Dex-bound mGRdim, namely at the level of GR α protein and mRNA regulation as well as at the level of GC-responsive gene expression, is that CpdA bound to mGRwt produces a slightly different monomeric conformation to that of Dex-bound to the mutant mGRdim. Through modelling, CpdA has been shown to bind to the conventional site in the ligand-binding pocket of GR α , however, speculation exists that it is capable of binding at a second site in the GR-LBD^{29,97}, which is supported by the unconventional competitive binding curves generated from ligand-binding assays with CpdA^{20,96}. This possible binding of CpdA to a second site, in contrast to the classical binding of Dex to a single, well characterised ligand binding site in the LBD, most likely induces a different GR α conformation, which is supported by its inability to induce receptor dimerization^{80,81} and may encourage the binding of additional coregulators²⁰.

Whilst it is well accepted that CpdA abrogates GR α dimerization and serves as a useful tool to study the effects of a 'loss' of dimerization, the idea that dimerization deficient mutants

cannot undergo homo-dimerization have recently been challenged^{82,98,99}. Pioneering work with the dimerization deficient mutant, mGRdim, indicated that this GR α variant had a significantly impaired ability to mediate transactivation of GC-responsive genes^{72,100}, however its lack of ability to form GR α dimers was not thoroughly investigated. Moreover, it must be noted that although studies have continued to demonstrate the inability of mGRdim to mediate GRE-driven gene transcription, studies using recent technology (i.e. the Number and Brightness Assay) to study receptor dimerization in live cells, has brought to light the ability of the classical dimerization deficient mutant, mGRdim, to form receptor dimers^{82,98}. Although mGRdim has been shown to form GR α dimers, the extent of dimer formation is significantly less pronounced than for the wild-type GR α ^{82,98,101}.

6.4. Conclusion

In conclusion, the current study postulates that the conformation of the GR α is fundamental in influencing the ability of the receptor to mediate GC-induced transactivation and transrepression, which is supported by another study by a member from our research group⁸¹, as well as its novel ability to influence receptor turnover both at the mRNA and protein level. For this reason, encouraging a 'loss' of dimerization via treatment with dimerization abrogating GCs, such as CpdA, and elucidating the dimerization capabilities of mutations associated with GC resistance¹⁰² should be primarily considered when developing novel therapeutics for the treatment of chronic inflammatory diseases.

6.5. References

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Chapter 7:

Discussion

7.1. Introduction and overview of results

To briefly summarize, chronic stress or prolonged exogenous GC treatment disrupts the central homeostatic nature of GC signalling (Chapter 1) and often results in various peripheral effects, one of which is the tissue-specific reductions in the GR α ‘functional pool’. This reduction in the GR α ‘functional pool’ may ultimately underlie or drive the development of an acquired GC resistance and result in the progression of many psychological and pathological conditions (Chapter 2). Using various methodologies (Chapter 3), the current study elucidated a novel requirement for GR α dimerization in mediating ligand-induced reductions in the GR α ‘functional pool’ (Chapter 4). Moreover, the previously postulated notion¹ that different GR α conformations may be differentially modified post-translationally was explored, and, in general, evidence was provided that the GR α monomer evades receptor turnover by differential modulation of the post-translational processing of the receptor (Chapter 5). Lastly, the biological implications of altered GR α expression were investigated in terms of molecular and clinical GC resistance using a validated endogenous model to mimic acquired resistance to GC treatment (Chapter 6). To encapsulate the findings of this study in their entirety, we have constructed a proposed model, which also incorporates information from previous published studies (Fig. 7.1).

7.1.1. Proposed model

1. Unliganded hGRwt (Fig. 5.5) and hGRdim (Fig. 5.6) are primarily cytoplasmic bound to an inhibitory chaperone complex consisting of, amongst other factors (e.g. Hsp90)², the inactive E2 conjugating enzyme of the UPS, TSG101. TSG101 associates with the hypo-phosphorylated, unliganded receptor in the cytoplasm, protecting it from proteasomal degradation³.
2. Upon ligand binding, dissociation of the inhibitor chaperone complex occurs^{2,4}. It appears, in the case of ‘gain’ of hGRwt dimerization, following treatment with Dex and possibly F, dissociation of CHIP may occur, while ‘loss’ of dimerization through CpdA treatment of hGRwt may preserve the hGRwt/CHIP complex but further investigation is required (Fig. 5.10A). Nonetheless, from the current study, we believe that TSG101 remains bound to the hypo-phosphorylated CpdA-bound hGRwt with evidence to support this idea in the TSG101 knockdown experiment (Fig. 5.10C). Moreover, it may or may not translocate with the CpdA-bound hGRwt into the nucleus, however this requires further investigation. Furthermore, the

ability of predominantly monomeric hGRdim to associate with TSG101 following ligand binding, remains to be elucidated.

3. In the case of the dimerization promoting GCs, Dex and F, ligand-dependent dimerization of hGRwt occurs (i.e. 'gain' of GR α dimerization)⁵. In contrast, binding of the dimerization abrogating GC, CpdA, restricts dimerization^{5,6} and preferentially produces GR α monomers (i.e. 'loss' of GR α dimerization). Similarly, treatment of the dimerization deficient mutant, hGRdim, with Dex, F or CpdA, reduces GR α dimer formation, and thus hGRdim remains mostly monomeric.
4. Following ligand-binding the GC/GR α complex translocates from the cytoplasm to the nucleus (Fig. 5.5, 5.6, 5.11 and 5.12). Important to note is that the unliganded GR α , although predominantly cytoplasmic, can translocate to the nucleus, which may be due to ligand-independent dimerization⁷. Translocation of the hGRwt dimer, is rapid, occurring within minutes⁷, with almost no cytoplasmic hGRwt detected following treatment with Dex or F (Fig. 5.5). In the same manner, be it to a slightly reduced extent⁷, Dex and F encourage almost total nuclear localisation of hGRdim (Fig. 5.6). Interestingly, CpdA treatment is not as efficacious as Dex or F at inducing nuclear translocation of hGRwt (Fig. 5.5) and is incapable of facilitating significant movement of the hGRdim monomer from the cytoplasm to the nucleus (Fig. 5.6).
5. Once in the nucleus, the predominantly nuclear kinase GSK3 β ^{8,9} induces hyper-phosphorylation of the dimerized Dex- and F-bound hGRwt at Ser404 (Fig. 5.9), a site linked to GR α stability¹⁰. In contrast, 'loss' of GR α dimerization, through CpdA treatment of hGRwt or the use of the mutant, hGRdim, restricts hyper-phosphorylation at this site (Fig. 5.9). In fact, no Ser404 phosphorylation of hGRdim was observed across all treatments (Fig. 5.9B).
6. In the case of 'gain' of hGRwt dimerization, phosphorylation at Ser404 (Fig. 5.9), facilitates binding of the E3 ligase, FBXW7 α (Fig. 5.7), a predominantly nuclear E3 ligase (Fig. 5.5 and 5.6), which then mediates hGRwt ubiquitination¹¹. However, before the binding of FBXW7 α to its substrate (i.e. hGRwt), ubiquitin is activated and transferred to the E3 ligase by additional enzymes, namely ubiquitin activating E1's and conjugating E2's (see small dotted box Fig.7.1 and refer to Fig. 5.3 in Chapter 5 for details). Unlike with 'gain' of hGRwt dimerization, 'loss' of GR α dimerization (via CpdA treatment of hGRwt or the use of hGRdim) restricts, be it partially, the binding of the E3 ligase, FBXW7 α , (Fig. 5.7) thought to be a direct result of the absence of hyper-phosphorylation at Ser404 (Fig. 5.9), which is required for binding of FBXW7 α ¹¹. Moreover, we believe that this lack of FBXW7 α binding directly impacts ubiquitination of the GR α monomer, however, concede that we were unable to convincingly establish this and thus it requires further investigation (Fig. 5.13). Interestingly, we expected an increase in GR α ubiquitination via 'gain' of GR α dimerization (i.e. Dex treatment of hGRwt),

however, we observed a decrease in hGRwt ubiquitin following Dex and F (Fig. 5.13) postulate this is due to hGRwt that is already being degraded, following 6 hours treatment (Fig. 4.4). Furthermore, whether GR α ubiquitination occurs before or after DNA binding remains to be elucidated (see next step) as a wealth of evidence is available for the transcriptional role of the UPS system and DNA-proteolysis, reviewed by Maneix *et al.*¹²

7. GCs then mediate their biological effects through modulating the expression of a wide range of GC-responsive genes, either through the direct DNA-binding of a GR α dimer to GREs (e.g. Dex-bound hGRwt to GILZ) (Fig. 6.9A) or the tethering of a monomer to another transcription factor (i.e. NF κ B) bound to its response element (NF κ B-RE) (e.g. IL-6) (Fig. 6.9D). Importantly, we have only detailed the classic methods of GR α -mediated GC-responsive gene expression, in this model, but others (i.e. tethering via an nGRE) (Fig. 6.7B) do exist¹³.
8. Following the activation or repression of GC-responsive genes, the GR α dissociates from the DNA or transcription factor and is recycled for another round of transcription.
9. Alternatively, GR α is exported to the cytoplasm, where classically it is thought to be degraded (not demonstrated in our proposed model), with evidence for this notion available¹⁴. However, evidence for the presence of proteasomes^{15–20}, as well as FBXW7 α (Fig. 5.5 and 5.6) in the nucleus has also been provided and thus, we believe that (in our proposed model), following gene transcription, liganded hGRwt dimers but not GR α monomers, are targeted for the proteasome, within the nucleus. Moreover, that ligand-induced down-regulation of specifically a hGRwt dimer, but not a monomer, into inactive protein fragments, likely occurs in the nucleus or potentially even at the DNA, termed DNA-proteolysis¹², providing a means of rapid recycling of receptors at the promoters of GC-responsive genes to tightly regulate the duration and intensity of the GC response (see Section 7.3).
10. In terms of unliganded hGRwt degradation (Fig. 4.4) a basal level of phosphorylation at Ser404 is observed, which is absent with the unliganded hGRdim (Fig. 5.9). Moreover, due to the fact that we observed unliganded hGRwt turnover (Fig. 4.4), we postulate that a ‘switch’ between TSG101 and CHIP, potentially reliant on ligand-independent dimerization⁷, may occur, and that it is the binding of the E3 ligase, CHIP, which targets the unliganded receptor for degradation. We concede, however, that additional experiments for TSG101 and CHIP are required. Moreover, investigation into whether the unliganded receptor is degraded in the nucleus or the cytoplasm remains to be elucidated.

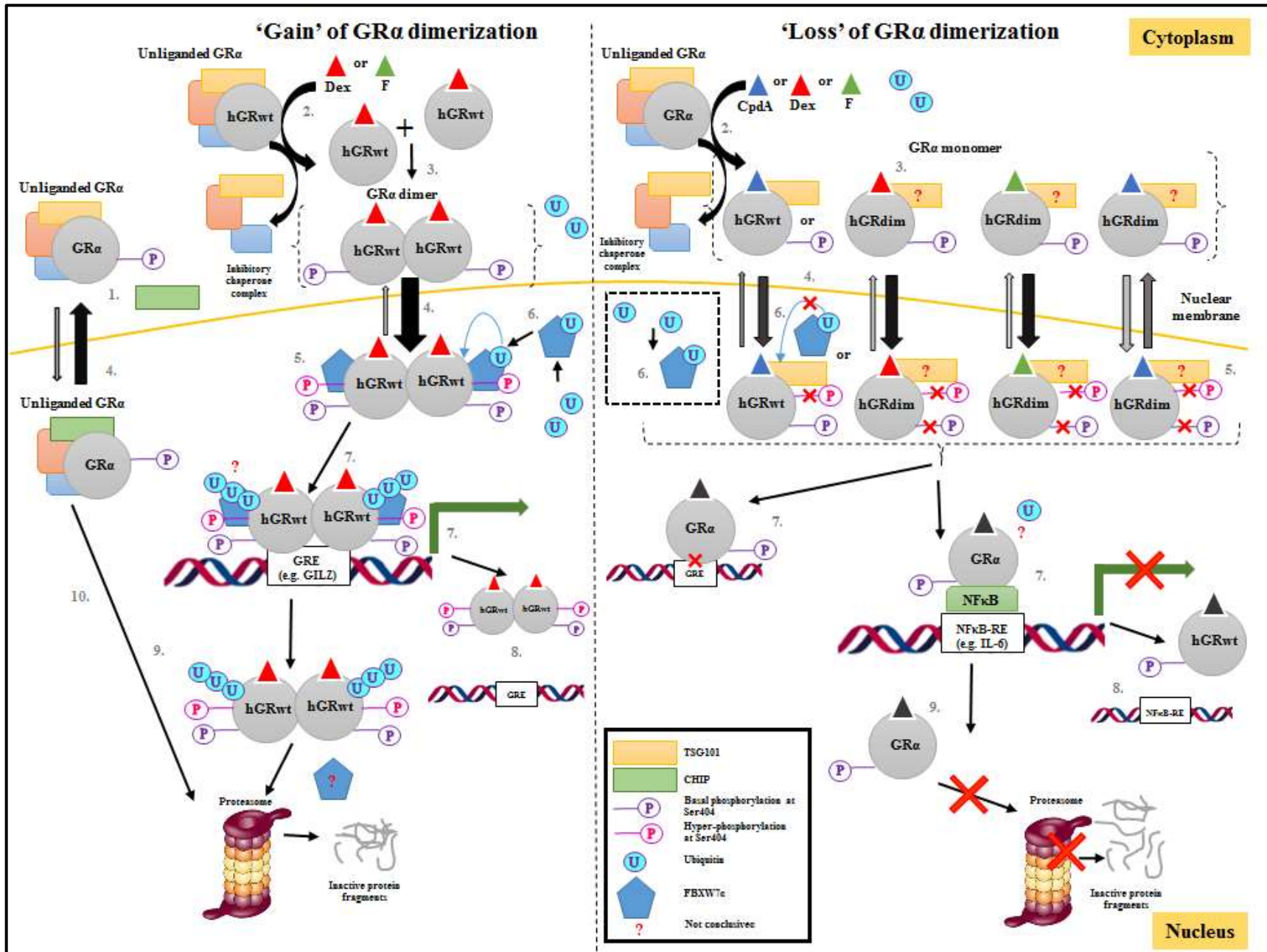


Figure 7.1: Proposed model comparing the effects of ‘gain’ and ‘loss’ of GR α dimerization in GC/GR α signalling (see text for details and solid box on model, for definitions). **1.** Unliganded, hypo-phosphorylated GR α is primarily cytoplasmic bound to an inhibitory chaperone complex, which includes the inactive E2 ligase, TSG101. **2.** Ligand binding causes dissociation of the inhibitor chaperone complex. **3.** Upon ligand binding the hGRwt dimerizes (i.e. ‘gain’ of GR α dimerization); however this is not the case following CpdA binding or the use of hGRdim (i.e. ‘loss’ of GR α dimerization). **4.** The GC/GR α complex translocates to the nucleus. Important to note is that the unliganded GR α , although predominantly cytoplasmic, can translocate to the nucleus and that the thickness of the arrows refers to extent of movement. **5.** Once in the nucleus, the dimerized hGRwt undergoes hyper-phosphorylation at Ser404. In contrast, ‘loss’ of GR α dimerization restricts hyper-phosphorylation at this site. **6.** In the case of ‘gain’ of hGRwt dimerization, phosphorylation at Ser404 facilitates binding of the E3 ligase, FBXW7 α , which binds ubiquitin (small dotted box) and mediates hGRwt ubiquitination. Unlike ‘gain’ of GR α dimerization, ‘loss’ of GR α dimerization restricts, be it partially, the binding of the E3 ligase, FBXW7 α . **7.** GCs then mediate their biological effects through modulating the expression of a wide range of GC-responsive genes, either through the direct DNA-binding of an hGRwt dimer to GREs or the tethering of a monomer to a transcription factor (i.e. NF κ B) bound to its response element (NF κ B-RE). **8.** Following the activation or repression of GC-responsive genes, the GR α dissociates from the DNA or transcription factor and is recycled for another round of transcription, or **9.** targeted for degradation by the proteasome, in the case of a ‘gain’ of hGRwt dimerization, but not in the case of a ‘loss’ of GR α dimerization (i.e. CpdA treatment of hGRwt or the use of hGRdim). **10.** Degradation of the unliganded receptor.

Having described the proposed model, emanating, in part, from results obtained in the current study (Fig. 7.1), in essence, this chapter aims to further consolidate results obtained in the current study with the existing literature by presenting a ‘bird’s eye view’ of the significance of this research in terms of novel therapeutics for combatting acquired GC resistance by highlighting, the somewhat, overlooked and undervalued importance of GR α conformation, specifically in terms of the dimerization state of the receptor. Additionally, the short-comings of the current study are also noted and this thesis is concluded with some enticing future perspectives, which are briefly discussed.

7.2. The importance of GR α conformation with focus pertaining to the dimerization state of the receptor

GC use for the treatment of inflammatory linked- or driven conditions is imperative, but is not without failings of significance, namely the generation of adverse side-effects and in some cases the development of acquired GC resistance. Generally, it is in long-term GC treatment regimens where the harmful effects exerted by GCs begin to exceed their beneficial effects as therapeutics. For this reason, many researchers over the years have directed their research efforts into trying to ‘tip the scales’ towards favouring the potent immunosuppressant capacity of GCs.

A number of strategies have been employed to improve the therapeutic use of GCs with focus pertaining to ligands displaying an increased efficacy and/or a defined functional selectivity^{13,21,22}.

Recently, a review by Luttrell *et al.*²³ has reiterated the complexity of G-protein coupled receptor (GPCR) signalling, brought to light by the ‘emerging paradigms of pluridimensional efficacy and functional selectivity’, and developed the idea of ‘biased ligands’. The concepts outlined in his review can be fruitfully transposed to GC/GR α signalling, providing a fresh perspective from which

to approach the development of novel therapeutic GCs, whilst substantiating the current study's reasoning for the importance of considering receptor dimerization. Thus, throughout the following discussion, these concepts from Luttrell *et al.*²³ provided a platform on which the current study builds, through the consolidation of its results.

7.2.1. 'Biased ligands'

Luttrell *et al.*²³ makes a strong case that the biological responses which arise from the interaction of a ligand with its cognate receptor are all encoded for at that single point of contact (i.e. within the receptor's LBD) with a change in receptor conformation being the initial consequence of ligand-binding. Analogous to the 'binding' of a baton to a conductor's hand, which sets the stage for a symphony of music driven by the 'conformation' the baton and conductor adopt together. Recently, in GPCR signalling, the simplified idea that ligands act merely as 'on' and 'off' switches to modulate receptor conformations to and from 'active' and 'inactive' states, has been revised with more complex concepts, such as the idea of a 'conformational ensemble', being proposed. Luttrell *et al.*²³ suggests that the binding of a ligand to a receptor differentially affects downstream signalling through modulating the 'conformational ensemble', then driving the 'conformational equilibrium' towards a particular state.

Traditionally ligands have been classified as agonists (partial or full) or antagonists, however, recent evidence has challenged this central dogma with concepts of 'functional selectivity' or 'biased ligands' suggesting ligand-receptor interactions are not as simple as once considered^{13,21-23}. Endogenous ligands are generally considered as full agonists based on the fact that they have coevolved with endogenous receptors to stabilize active receptor conformations, most favourable for eliciting the desired physiological biological response²³. On the other hand, 'biased ligands' as eloquently stated by Luttrell *et al.*²³: 'are novel pharmacologic entities that possess the unique ability to qualitatively change receptor (i.e. GPCR) signalling', in effect creating 'new receptors'. In other words 'biased ligands', which select specific receptor conformations with efficacy distinct profiles from that of endogenous ligands, modify biological responses (via various signalling pathways such as the GPCR or GC/GR α signalling pathway), in peripheral tissues or cells, towards a more beneficial outcome, which ultimately, is the underlying fundamental reason for the development of pharmaceutical agents.

In terms of GC/GR α signalling one could argue that this notion of 'biased ligands' has already been explored in terms of SGRMs), designed and developed as potentially improved GC-based therapeutics, on the basis of being capable of functionally separating GC-mediated transactivation

from transrepression, generally linked to adverse side-effects and the anti-inflammatory potential of GCs, respectively^{13,21}.

Recently, De Bosscher *et al.*¹ has developed a concept called ‘The SEDIGRAM concept’, which is derived from the selective GR α modulation by SGRMs. Rather than focusing specifically on the ability of synthetic ligands to transrepress versus transactivate GC-responsive genes, De Bosscher *et al.*¹ suggests that due to the vast and complex nature of GC/GR α signalling, one should investigate and develop novel GC therapeutics based on their ability to resolve acute versus chronic inflammation, proposing that these different processes of inflammation demand different therapeutic measures. Moreover, based on *in vivo* models^{6,13,24–29}, which utilized the dimerization abrogating GC, CpdA, or the dimerization deficient mutant, GRdim, mice, this review goes on to postulate that dimeric GR α (i.e. via selective dimerizing GR α agonists/modulators or SEDIGRAMs) is essential for the resolution of acute inflammation, whilst encouraging predominantly monomeric GR α (i.e. by selective monomerizing GR α agonists/modulators or SEMOGRAMs) may be more beneficial for the treatment of chronic inflammation¹.

In the next section the *in vivo* pharmacological evidence for biased ligand behaviour, provided by the use of CpdA or the GRdim mice model, that gave rise to the SEDIGRAM concept¹, is briefly summarized, whilst consolidating results from the current study. Additionally, we explore the effects of receptor concentration on GR α dimerization and provide an opinion on how this may influence the extent or rate of receptor turnover.

7.2.1.1. Pharmacological evidence for biased ligand behaviour with the dimerization abrogating GC, CpdA

The use of the SGRM or dimerization abrogating GC, CpdA, has proved relatively fruitful in combatting inflammation in a number of *in vivo* inflammatory mouse and rat models^{6,13,24–29}, discussed here. Specifically, in the zymosan-induced inflammatory paw mouse model, CpdA was found to be as effective as Dex at reducing paw swelling with both compounds reducing paw swelling by approximately 50%, relative to PBS-treated mice²⁵. Additionally, CpdA’s ability to attenuate collagen-induced arthritis (CIA) in DBA/1 mice²⁶, has been demonstrated. In support of these findings, Dewint *et al.*⁶ also showed CpdA’s ability to restrict the development of arthritis, relative to control mice, using the CIA model. Specifically, although less efficacious than Dex treatment where 100% of the joints presented normal histopathological features, in contrast CpdA treatment resulted in 79% of the joints demonstrating normal histopathological features, relative to the PBS-treated control mice where a mere 33% had normal histopathological features⁶. More recently, Rauner *et al.*²⁷, using the same CIA model demonstrated CpdA’s ability to suppress

inflammation specifically in terms of reduced disease activity, paw swelling and temperature, and although not as potent as Dex (100 µg/mouse) at reducing these factors (i.e. 72%, 22% and 10% respectively), CpdA (300 µg/mouse) treatment significantly reduced these factors by 43%, 12% and 7%, respectively. In a rat model of experimental autoimmune neuritis (EAN), CpdA attenuated the build-up of macrophages and lymphocytes, demyelination, as well as the increase in the mRNA expression of pro-inflammatory molecules in sciatic nerves²⁸. Additionally, CpdA treatment was able to reduce muscle inflammation in the forelimbs and hind limbs of *mdx* mice (i.e. a model to study muscle dystrophy) by approximately 40%, relative to control, which was comparable to the prednisolone-induced reduction in inflammation²⁹. Lastly, in C57Bl/6 mice induced with EAE^{24,30}, treatment of these mice with 5 mg/kg and 1.5 mg/kg CpdA severely hampered disease progression, however higher doses of CpdA (> 15mg/kg) proved to be lethal, providing evidence for CpdA's narrow therapeutic window²⁴.

Furthermore, CpdA's ability to restrict inflammation in these *in vivo* models has been shown to occur without generating adverse side-effects (e.g. hyperglycaemia and hyper-insulemia)^{25,26,28,30}. More specifically, unlike Dex, which led to a significant increase in glucose concentrations to 150 mg/dl, CpdA did not trigger a significant increase in blood glucose concentrations (i.e. approximately 100 mg/dl), relative to control mice (i.e. 120 mg/dl), which is indicative of a reduced risk for the development of hyperglycaemia²⁵ and was unable to induce hyper-insulinemia, relative to Dex-treated mice²⁶. Moreover, the inability of CpdA to induce hyper-insulinemia has also been noted in a model of EAE³⁰. Whilst Dex treatment induced an increase in insulin to 5 ng/ml, relative to the PBS-treated control mice (i.e. 1 ng/ml), CpdA treatment showed no effect on insulin levels³⁰. Unlike prednisolone, CpdA has also been shown to partially prevent the destruction of bone and cartilage in the joints of mice⁶ and demonstrated an ameliorative effect on markers of muscle dystrophy²⁹. Lastly, in terms of immune-inflammatory diabetes, CpdA was found to exert a protective effect by maintaining blood glucose levels around 10 mM, relative to untreated multiple low doses of streptozotocin (MLDS)-induced mice where a steady increase in blood glucose from 10 mM to 25 mM, was noted³¹.

These *in vivo* findings are supported by results from the current study in Chapter 6, which demonstrate CpdA's ability to combat inflammation without generating undesirable side-effects, at a molecular level in an *ex vivo* model, namely the MEF cells. Specifically, similarly to Dex, CpdA was able to reduce the expression of the pro-inflammatory cytokine, IL-6, via GR α , without inducing the expression of the GRE-driven metabolic gene, TAT. It must be noted, however, that this is not the first time this dissociated characteristic of CpdA has been demonstrated in an *ex vivo* model, with a number of studies, including one by a member of our research group³², having

previously shown similar behaviour in both *ex vivo* and *in vitro* models, many of which are eloquently reviewed by Sundahl *et al.*¹³

7.2.1.2. Pharmacological evidence for biased ligand behaviour through the use of the dimerization deficient mutant, GRdim

The ability of SGRMs to differentiate between the transactivation and transrepression of GC-responsive genes, is thought to be associated with their ability to induce different conformations of the receptor (specifically a GR α dimer versus a monomer) to which they bind^{5,6}. Support for this concept of ‘biased ligands’ or SGRMs in GC/GR α signalling originated from early transfection studies³³ using the dimerization deficient mutant, GRdim. Specifically, Heck *et al.*³³ demonstrated that introducing a single-point mutation in the D-loop of the GR α (i.e. to disrupt the dimerization capabilities of the receptor) was sufficient to dissociate GC-mediated GR α transrepression from transactivation. Based on these findings, in a study by Reichardt *et al.*³⁴, dimerization deficient mutant homozygotic GRdim mice were created and found to be viable, however, they were defective in the capacity of GRdim to bind DNA and mediate the transactivation of GRE-driven genes yet maintained their immunosuppressive abilities via transrepression of pro-inflammatory genes.

Essentially, these GRdim mice from Reichardt *et al.*³⁴ provided the first *in vivo* evidence for the ability of GR α to repress inflammation, both local and systemic, following GC treatment, without the requirement for the receptor to directly bind to DNA, which has subsequently been followed up by a number of other *in vivo* studies^{35–37}. Treatment of these GRdim mice³⁴ with lipopolysaccharides (LPS), to mimic bacterial infection and stimulate a systemic response, initially drove increases in TNF α (i.e. 1 hour after LPS treatment), which was subsequently counteracted by a significant increase in the endogenous GC, corticosterone, 3 hours after treatment. In this study GRdim mice³⁴ were as efficient as wild-type mice at counteracting the LPS-induced effects on TNF α , once again confirming that DNA-binding is not a requirement for regulating inflammation via GR α mediated transrepression. Additionally, in a model of acute inflammation in which inflammation (i.e. oedema) was induced by means of the topical administration of a phorbol ester, namely phorbol 12-myristate 13-acetate (PMA), GRdim mice were as efficient as wild-type GR α mice, following Dex treatment, at inhibiting the influx of inflammatory cells and, additionally, completely restricting an increase in IL-6 serum concentrations (0 pg/ml), relative to PMA-induced mice without Dex treatment (100 pg/ml)³⁵. In support of these findings, are results from the current study in Chapter 6, which demonstrate that Dex-mediated transrepression of IL-6 occurs via mGRwt and mGRdim in the MEF-mGRwt and MEF-mGRdim cells, respectively.

However, in a study by Kleiman *et al.*³⁶, which made use of two different models of sepsis, specifically LPS bolus-induced septic shock and cecal ligation and puncture (CLP), unlike GRwt mice, GRdim mice were found to be highly susceptible to sepsis, an-inflammatory-linked condition³⁶. Unlike TNF α , down-regulation of IL-6 and IL-1 β was severely diminished in GRdim mice even though similar increases in endogenous corticosterone, relative to wild-type mice, were noted, following stimulation with LPS or CLP³⁶. Moreover, GRdim mice demonstrated a reduction in survival rate with a mere 30% of these mice surviving, compared to the approximately 80% of wild-type mice surviving, 80 hours post stimulation, suggesting that GR α dimerization is a requirement for survival in models of sepsis and that the inability to reduce the prolonged expression of IL-6 and, more so, IL-1 β , likely contributes to the inability of GRdim mice to survive under these septic conditions³⁶.

Lastly, in terms of the metabolic side-effects of GC signalling, a study by Frijters *et al.*³⁷ investigated and compared the GC-responsive gene expression profiles in the liver tissue of wild-type and GRdim mice, following prednisolone treatment. Overall, results showed that, whilst prednisolone-mediated transactivation of a small subset of genes was completely abolished, the transactivation of other GC-responsive genes such as TAT and FKBP51, was significantly reduced (i.e. on average approximately 33% in GRdim mice relative to wild-type mice), however, importantly, not completely abolished in GRdim mice³⁷. In support of this finding is a literature search reported and summarized by Frijters *et al.*³⁷, which noted that the GC-induced expression of TAT was increased by 4.1-fold in wild-type mice and 1.6-fold in GRdim mice. This corroborates well with the 4.0-fold and 2.0-fold Dex-mediated increase in TAT expression, in MEF-mGRwt and MEF-mGRdim cells, reported by the current study.

The effects of the dimerization abrogating GC, CpdA, and the use of the dimerization deficient mutant, GRdim, in these *in vivo* models confirm that tipping the scale towards a GR α monomer, as opposed to a GR α dimer, appears to be, in general, beneficial for the treatment of inflammation as well as providing an improved side-effect profile, supporting the notion that selectively modulating receptor conformation through 'biased ligands', may be useful. However, there are limitations when it comes to the use of CpdA as a dimerization abrogating GC, as well as limitations with the dimerization deficient GR α mutant model. Specifically, although CpdA demonstrates a potent anti-inflammatory potential in these *in vivo* models, its stability issues³⁸ and its high propensity for alkylation as well as its narrow therapeutic window, make it an unlikely candidate for therapeutic use in a clinical setting¹³. Furthermore, although mice harbouring the dimerization mutation (i.e. GRdim) are as viable as GRwt, under most inflammatory conditions, however, they are highly sensitive to septic conditions³⁴. Additionally, unlike CpdA via GRwt, Dex via GRdim is still able to

mediate a level of GC transactivation⁷, which is supported by the FKBP51 results of the current study). It may be that the ability of GRdim to mediate the transactivation of certain genes is due to the ability of GRdim to form alternative, potentially unstable, receptor dimers via the LBD dimerization domain³⁹.

7.2.1.3. The SEDIGRAM concept¹

7.2.1.3.1. Combatting inflammation versus the generation of adverse side-effects

Collectively, the pharmacological evidence provided by the use of the dimerization abrogating GC, CpdA, and the dimerization deficient mutant, GRdim, gave rise to a novel concept, namely the SEDIGRAM concept proposed recently in a review by De Bosscher *et al.*¹. In support of Luttrell *et al.*²³ and the idea of ‘biased ligands’, central to the SEDIGRAM concept, is the idea of selectively modulating alterations in the conformations of the receptor or changing the ‘conformational equilibrium’, through the binding of specific GR α ligands¹. The development of these novel ligands, able to modulate receptor conformation, expand the ‘pharmacological toolbox’ not only at a molecular level, specifically, by GR α ligands that are capable of dissociating between transactivation (i.e. generally mediated via a GR α dimer and associated with adverse side-effects) and transrepression (i.e. generally mediated via a GR α monomer giving rise to the potent anti-inflammatory potential of GCs) of GC-responsive genes, but also, as De Bosscher *et al.*¹ suggests at a clinical level in terms of the treatment of acute versus chronic inflammation.

Essentially, this idea that different inflammatory processes (i.e. acute versus chronic) may call for different measures has been brought to light by the notion that dissociating GC-mediated transactivation from transrepression may not be sufficient in acute GC treatment regimes, due to the fact that a number of the anti-inflammatory actions of GCs are mediated via GC-mediated GR α transactivation⁴⁰. Thus in terms of short-term acute inflammation, it may be better ‘to paint with two brushes’ both via GC-mediated transactivation of anti-inflammatory genes and transrepression of pro-inflammatory genes, at the risk of certain short-term side-effects (i.e. increases in blood glucose). However, in long-term treatment of persistent chronic inflammation the beneficial anti-inflammatory potential of classical GCs is outweighed by the adverse side-effects (i.e. the development of hyperglycaemia), driven by GRE-mediated transactivation, thus posing a major threat to the well-being of the patient.

De Bosscher *et al.*¹ suggests that for short-term acute inflammation it may be more beneficial to encourage receptor dimerization through the use of SEDGRAs (i.e. GCs which show an increased dimerizing potential) or SEDGRMs (i.e. molecules that may act alone or in combination with GCs to increase the dimerization potential of GR α). Essentially, encouraging GR α dimer formation,

importantly in short-term treatment regimes, will allow for transrepression of pro-inflammatory cytokines (i.e. IL-6 and TNF α) but also GRE-driven transactivation of anti-inflammatory genes (i.e. GILZ), even if it should concomitantly increase the risk for adverse side-effects (i.e. an increase in glucose concentration). This suggestion aligns itself well with results from the current study in terms of the dimerization state of the receptor and GC-responsive gene expression. Specifically, in the model established in Chapter 6, we successfully demonstrated that short-term treatment with the dimerization promoting GC, Dex, induces the expression (6.6-fold) of the anti-inflammatory mediator GILZ, relative to the solvent, whilst repressing the expression of the pro-inflammatory cytokine, IL-6, 0.7-fold, and in addition induces the expression of the metabolic gene, TAT, 13.3-fold. Similarly to Dex, restricting GR α dimerization through treatment with the dimerization abrogating GC, CpdA, or the use of the dimerization deficient mutant, mGRdim, restricts the expression of IL-6, 0.6-fold and 0.7-fold, respectively, however, monomeric GR α is incapable of mediating an increase in the expression of the anti-inflammatory gene, GILZ. Thus the potent ability to combat acute inflammation, in short-term treatment protocols, from both angles (i.e. ‘to paint with two brushes’ via GC-mediated transactivation and transrepression) has somewhat been lost with monomeric GR α .

In contrast, the use of SEMOGRAMs (i.e. GR α agonists or molecules, which act alone or in combination with GCs to favour monomeric receptor formation over its dimerized counterpart) may prove to be the way forward for the treatment of long-term chronic inflammation, as suggested by De Bosscher *et al.*¹. Thus in prolonged GC treatments the anti-inflammatory potential of GCs may be slightly compromised, through the use of a GR α monomer, to prevent the generation of adverse side-effects¹. In support of this, results from the current study demonstrate that long-term treatment of CpdA was as capable as Dex at reducing the expression of IL-6 (i.e. 0.5-fold and 0.4-fold, respectively); however, even long-term treatment with CpdA could not stimulate GILZ expression. More importantly, and in stark contrast to Dex (12.6-fold), long-term treatment of CpdA (1.1-fold), did not result in an increase in the expression of TAT. Similarly, long-term Dex-treatment of the dimerization deficient GR α mutant inhibited IL-6 expression (0.7-fold) without significantly inducing transactivation of TAT (1.8-fold).

To briefly summarize, the SEDIGRAM concept provides new insight into the fact that the GRE-driven anti-inflammatory effects of GCs via a GR α dimer may be more beneficial for the treatment of short-lived acute inflammation, even though certain side-effects such as an increase in blood glucose, may occur temporarily. However, for the treatment of persistent chronic inflammation, which requires pro-longed exogenous GC treatment, the less potent anti-inflammatory potential of GCs, via predominantly monomeric GR α transrepression, may be more beneficial, mainly because

the increase in metabolic gene expression and thus side-effects are restricted. Whilst De Bosscher *et al.*¹ addresses selectively modulating the dimerization state of GR α in terms of the indispensable anti-inflammatory effects of GC signalling and the possible generation of adverse side-effects, she does not discuss the potential benefits of encouraging monomeric GR α in prolonged GC treatment regimes in terms of acquired GC resistance, with focus pertaining to maintaining GR α expression.

7.2.1.3.2. Acquired resistance to GC treatment

In this section we make use of results from the current study, in combination with a study by the De Bosscher research group²⁶, to strengthen the idea that in prolonged GC treatment regimens it may also be more beneficial to encourage predominantly monomeric GR α through ‘biased ligands’, referred to as SEMOGRAMs by the De Bosscher review¹, to combat acquired GC resistance. Specifically, evidence endorsing the advantageous use of monomeric GR α , as opposed to dimeric GR α , in terms of acquired resistance to GC treatment is provided and discussed, essentially building on the SEDIGRAM concept.

It is well documented that chronic exogenous GC treatment or chronic stress over a prolonged period of time results in the development of an often, tissue-specific acquired resistance to GC treatment. Many studies^{41–46}, including our own, have demonstrated that one of the ways in which this steady increase in GC insensitivity occurs is through ligand-induced down-regulation of GR α expression, both at the mRNA and protein level, which throughout the current study has been referred to as the GR α ‘functional pool’.

Chronic treatment with Dex *in vitro*, *ex vivo* and even *in vivo*, has been shown to reduce the GR α ‘functional pool’ from anywhere between 10 and 90 %, relative to an untreated control (see Chapter 2, Table 2.2). Moreover, in the current study, using three cellular models, namely the transiently transfected COS-1 cells and the HepG2 and MEF-mGRwt cells, which express endogenous GR α , 24 hours of treatment with the dimerization promoting GC, Dex, led to a 50 to 70% reduction in GR α protein expression (Chapter 4 and 6). Additionally, in a model established to mimic acquired GC resistance (Chapter 6), we successfully demonstrated that the more significantly reduced GR α ‘functional pool’, relative to the receptor expression following short-term Dex treatment, drove resistance at a molecular level. Specifically, diminished GC sensitivity was noted when comparing the expression of the anti-inflammatory gene GILZ, following long-term Dex treatment (i.e. 4.6-fold) with GILZ expression following short-term treatment (i.e. 6.6-fold).

In stark contrast, ‘loss’ of GR α dimerization, via CpdA treatment or the use of the dimerization deficient mutant (both hGRdim in the transiently transfected COS-1 cells and endogenous mGRdim in the MEF-mGRdim cells) restricted receptor turnover. More specifically, prolonged CpdA

treatment (24 to 32 hours) resulted in an increase in expression of wild-type GR α to approximately 130% and 122% in the COS-1 and HepG2 cells, respectively, whilst maintaining mGRwt expression in the MEF-mGRwt cells at 89%, relative to the solvent. In terms of the mutant, and unlike with wild-type GR α capable of Dex-induced dimerization, prolonged Dex-treatment was unable to induce significant down-regulation of hGRdim in the COS-1 cells, over a 72 hour period. Moreover, ligand-induced down-regulation of the endogenous mGRdim in the MEF-mGRdim cells was partially restricted (49%) relative to the solvent and more importantly, significantly restricted relative to mGRwt (29%), following prolonged treatment with Dex. Whilst the ability of CpDA treatment to maintain GR α expression has been demonstrated before^{26,32,46}, to our knowledge, this is the first time the ability of the dimerization deficient mutant to restrict receptor turnover has been noted. These results, as a whole, provide substantial evidence for the ability of ligand-induced GR α down-regulation to be influenced by the dimerization state of the receptor

Taken together, this novel requirement for GR α dimerization in mediating receptor turnover has significant implications for the development of an acquired resistance to GC treatment, especially in prolonged treatment regimes. Moreover, with the SEDIGRAM concept in mind, we support the notion that selectively modulating GR α conformation through ‘biased ligands’ (or. SEMOGRAMs) to preferentially induce GR α monomers is likely to be beneficial for combatting persistent chronic inflammation, not only by reducing the generation of adverse side-effects, but also, by maintaining GR α expression and GC sensitivity in the long run.

7.2.1.4. Receptor concentration influences the dimerization state of GR α

Up to now, the influence of receptor dimerization on ligand-induced GR α turnover has been addressed, specifically in terms of acquired resistance to GC treatment, however, we have not yet discussed how the dimerization state of the receptor may be influenced by factors, other than ‘biased ligands’. A study by a member of our research group has elucidated the effects of receptor concentration on GR α dimerization^{7,47}, which is briefly outlined in this section. This notion that GR α concentration can modulate receptor dimerization has interesting implications for the extent and rate of receptor turnover, both in the absence and presence of ligands.

It is well established that inter-individual differences in GR α concentration exist and that various disease states have been associated with increased or decreased receptor concentrations, within peripheral cells and tissues (for details and specific references we refer you to Table 2.1 in Chapter 2 of the current study). Additionally, intra-individual tissue-specific differences in GR α concentration are noted between the cells and tissues of healthy individuals. To illustrate the range of GR α concentration in cells, PBMCs have been reported to have a receptor concentration on the

lower end of the scale, of 4.1 fmol/mg protein⁴⁸, while in contrast skin cells have been reported to have a much higher GR α concentration of 893 fmol/mg protein⁴⁹.

Recently, a study by a member of our research group, noted that changes in GR α concentration may have profound effects on a number of aspects of GC/GR α signalling (i.e. ligand binding affinity, nuclear localisation and transcriptional response) but of utmost importance and relevance for the current study, is the ability of increasing receptor concentrations to modulate the dimerization state of GR α , demonstrated through an increase in positive co-operative binding⁷. Using an established physiologically relevant model, with three distinct GR α concentrations (i.e. low, medium and high), Robertson *et al.*⁷ investigated whether receptor dimerization was a pre-requisite for co-operative ligand binding, building on the established idea that positive co-operative binding due to increasing GR α concentrations is associated with an enhanced capacity to form receptor dimers⁵⁰⁻⁵². Put differently, positive co-operative ligand binding represents ligand-independent GR α dimerization.

Indeed, Robertson *et al.*⁷ demonstrated that positive co-operative ligand binding occurs at medium GR α concentrations (144 – 152 fmol/mg protein), but not low receptor concentrations (i.e. 41 – 67 fmol/mg protein) for the wild-type, but not the dimerization deficient GR α mutant. Specifically, increasing wild-type GR α concentrations from low to medium resulted in an increase in the Hill coefficient from 1.08 to 1.57, which was absent with increasing hGRdim concentrations⁷. Additionally, an increase in the ligand-binding affinity (K_d) from low (49.1 nM) to medium (23.9 nM) was observed with increasing wild-type GR α concentrations, however, remained unchanged for the dimerization deficient GR α , thus suggesting that ligand-binding was non-co-operative with increasing GR α concentrations of the mutant⁷. Thus, it was concluded that an increase in co-operative ligand binding to a GR α dimer occurs with increasing receptor concentrations and in order for this to occur a concentration-dependent increase in ligand-independent dimerization must occur for the wild-type receptor. These findings provide interesting food for thought on the effect of GR α concentration on the extent and rate of ligand-induced receptor turnover.

In Chapter 6, the GR α concentration was reported to be 40 fmol/mg protein and 38.7 fmol/mg protein in the MEF-mGRwt and MEF-mGRdim cells, respectively, which corroborates well with the reported low range of GR α concentration (i.e. 41 – 67 fmol/mg protein), reported by Robertson *et al.*⁷. Moreover, no difference between the ligand binding affinity of Dex for mGRwt and mGRdim, was reported at this receptor concentration in the current study, which is line with the similar ligand-binding affinities for the wild-type and mutant at the low concentration of receptor, noted by Robertson *et al.*⁷. With that said, the current study went on to observe differences in the extent or efficacy of Dex-induced receptor turnover between mGRwt and mGRdim, confirming a requirement for GR α dimerization in mediating receptor turnover. In terms of the effects of

increasing GR α concentration on positive co-operative ligand binding (i.e. reflected by an increase in the ligand-binding affinity for the wild-type receptor) and ligand-independent dimerization, it would be of interest to determine whether an increase in GR α concentration would modulate the extent and rate of receptor turnover, both in the absence and presence of ligand. One could postulate that, in the absence of ligand, increasing GR α concentration would drive ligand-independent receptor dimerization, ultimately encouraging receptor down-regulation.

Taken together, the results from Robertson *et al.*⁷ provided a link between GR α concentration and ligand-independent dimerization of the wild-type GR α , but not the dimerization deficient mutant, at medium receptor concentrations, which the current study builds on by demonstrating a requirement for dimerization in mediating receptor turnover. With vast ranges of physiological concentrations of GR α reported *in vivo* in different tissues and cells as well as in different disease states, these findings have implications for characterising the extent and rate of receptor turnover, by endogenous and exogenous GCs, in these various conditions. Moreover, the ability to characterize the extent and rate of ligand-induced turnover, based on GR α concentration and the dimerization state of the receptor, in a disease- or tissue-specific manner, may prove fruitful for the development of novel GC therapeutics to combat acquired GC resistance.

7.3. Where does receptor turnover take place?

In this section, we delve a little deeper into the spatial effects of the subcellular localisation of GR α and how this may modulate its turnover.

Back in the late 1970s and early 1980s the proteasome degradation pathway was thought to primarily function as a means to clear cells of mis-folded or damaged proteins through their conversion into inactive protein peptides⁵³. However, more and more evidence is unveiling the highly complex nature of the UPS system. Specifically, that the UPS has been shown to intricately be involved in the transcriptional regulation of a number of genes¹².

Classically, in terms of GR α signalling, it has been thought that following GC-mediated transcriptional regulation, the receptor dissociates from the DNA and is either recycled in the nucleus for another round of GC-mediated transcription or is exported to the cytoplasm, where the proteasome resides, and degraded, with evidence to support these ideas available¹⁴. However, recent studies have demonstrated a fundamental role for the UPS in finely tuning the expression of this ligand-activated transcription factor, within the nucleus and in some cases at the DNA (i.e. termed DNA proteolysis), thus regulating the expression of down-stream GC-responsive genes¹². As Maneix *et al.*¹² describes, the rapid elimination (i.e. through proteasomal degradation) of 'spent

transcription factors (i.e. GR α) resets the promoter and allows new copies of transcription factors to bind'.

In addition to demonstrating that GR α concentration modulates the binding affinity of a ligand for the GR α and the dimerization state of the receptor Robertson *et al.*⁴⁷ detailed the effects of receptor concentration on the subcellular localisation of GR α and its nuclear mobility, which in light of nuclear proteolysis, may also have relevance for the extent and rate of receptor turnover. Unlike nuclear import of GR α , export of the receptor following ligand-treatment is considerably slower⁴⁷. Specifically, following Dex-withdrawal, Robertson *et al.*⁴⁷ demonstrated, using immunofluorescence, that it takes approximately 13.3 hours for the wild type GR α to passively be exported back to the cytoplasm. Specifically, Robertson *et al.*⁴⁷ shows 30% of receptor in the cytoplasm after 6 hours, however, one must remember that in this experiment ligand-withdrawal was conducted and cells washed prior to the nuclear export determination. In contrast, the current study reported no wild-type GR α expression in the cytoplasm, following 6 hours of treatment, with saturating concentrations of Dex; however a 33 % reduction in total (i.e. nuclear and cytoplasmic) GR α protein expression was noted in Chapter 4. Thus, we believe, in the current study, that 'gain' of GR α dimerization following 6 hours treatment with a high concentration of Dex (10 μ M), and importantly without withdrawal, encourages receptor turnover, specifically in the nucleus (and potentially at the DNA).

The postulation that ligand-induced down-regulation of dimeric GR α occurs in the nucleus, at saturating Dex concentrations, is supported by findings, which suggest that the proteasome is predominantly nuclear but is also found in the cytoplasm¹⁵⁻²⁰. Moreover, the E3 ligase, FBXW7 α , which is known to regulate GC-mediated GR α turnover is primarily positioned at the nuclear membrane⁵⁴, which the current study confirms (Fig. 5.5 and 5.6). On the other hand, it is possible that basal degradation of the unliganded GR α , reported in the current study (Fig. 4.4), and by others⁴⁶, occurs in the cytoplasm and is regulated by the predominantly cytoplasmic E3 ligase, CHIP^{55,56}. Moreover, that 'loss' of dimerization, through the use of CpdA or the dimerization deficient GR α mutant, prevents nuclear degradation of GR α , specifically through restricting hyper-phosphorylation at Ser404 and the interaction of the receptor with FBXW7 α , in the nucleus. Interestingly, in the current study, CpdA was unable to mediate translocation of hGRdim (Fig. 5.6). This lack of nuclear translocation in combination with the fact that this species is predominantly monomeric likely preserves GR α levels more than the unliganded GR α .

This notion that nuclear receptors may be degraded in different subcellular compartments is not a novel one with a study demonstrating that the degradation of ER α , occurs both in the cytoplasm and the nucleus, depending on the ligand to which it is bound⁵⁷. However, to our knowledge, the spatial

identification of exactly where ligand-induced GR α down-regulation and where the physical interaction of the GR α with the proteasome occurs, has not been thoroughly investigated.

Taken together, we believe that going forward careful consideration needs to be given not only to the temporal effects, but also the spatial effects, of ligand-induced down-regulation of the GR α and suggest that the dimerization state of the receptor could potentially play a role in where receptor turnover takes place within the cell.

7.4. Short-comings of study and avenues to explore

Although the current study has provided a wealth of evidence for the importance of GR α conformation, specifically dimerization, in mediating the extent of ligand-induced receptor turnover, upon reflection there are a few short-comings which require mentioning and are outlined in this section. Moreover, potential strategies to overcome these drawbacks, in future studies, are provided and discussed.

Essentially, the most topical aspect is the ability of the dimerization deficient GR α mutant (GR α^{dim}) to form GR α homodimers. The notion that this dimerization deficient mutant has a reduced capacity to form GR α dimers originates from a study by Reichardt *et al.*³⁴ where a point mutation was introduced in the D-loop of the GR α gene in mice to create viable GR α^{dim} mutant mice, from which subsequent cell lines (i.e. MEF-mGR α^{dim} cells) have been derived. These mice harbouring the D-loop mutation, were found to lack the capacity to mediate GRE-driven transactivation of GC-responsive genes³⁴, which at the time was thought to occur via the direct DNA binding of dimeric GR α , whilst maintaining the transrepressive function of pro-inflammatory genes, via predominantly monomeric GR α . This classical dissociation of transactivation and transrepression and its association with the dimerization state of the receptor has since been challenged⁴⁰. Nonetheless, it was this primary characteristic of the mutant GR α in combination with the position of the point mutation (i.e. in the D-loop), which fortified the idea of the mutant's reduced ability to dimerize, however, to our knowledge; this was never directly investigated experimentally, in these early studies^{33,34}. For this reason controversy surrounding the dimerization capabilities of GR α^{dim} exists, with some papers suggesting it has the potential to fully dimerize following treatment with Dex^{39,58}. With that said, a number of studies have also demonstrated that whilst GR α^{dim} may not be completely deficient in its ability to dimerize, its affinity to form GR α dimers is, in part, reduced⁵⁹ and is postulated to be dependent on receptor concentration⁷. Specifically, Robertson *et al.*⁵ demonstrated that wild type GR α bound to dimerization abrogating GC, CpdA, displayed similar characteristics to Dex bound to GR α^{dim} in terms of their ability to mediate receptor nuclear translocation and the expression of GC-responsive genes, suggesting that these similarities were due

to a reduced ability to undergo ligand-induced dimerization. Moreover, that with increasing GR α concentrations, positive co-operative binding (where receptor dimerization is a pre-requisite) was observed for GRwt but not for GRdim⁷. In addition, the current study observes significant differences in the ability of GRwt and GRdim to undergo ligand-induced GR α down-regulation. Lastly, a recent study by Yamamoto *et al.*⁵⁹ investigated and compared the affinity (Kd) of receptor homodimerization between wild type GR α and the dimerization deficient mutant in living cells, using Fluorescence Cross-Correlation Spectroscopy (FCCS). Results from this study⁵⁹, confirmed that the tendency of the mutant toward a monomeric conformation in the nucleus following treatment with Dex, was higher than that of its wild type counterpart, and concluded that the mutation in the D-loop of GR α was sufficient to impair the formation of GR α dimers, in living cells, however, these results could not disregard the ability of a part of the mutant to form dimers *in vitro*, as suggested by Presman *et al.*^{39,58}, and thus proposed a model⁵⁹ of differential pathways of GR α dimerization, taking into account receptor subcellular localisation, termed the dynamic monomer pathway, and the static dimer pathway.

In addition to the D-loop, a second dimerization site present in the ligand-binding domain (LBD) of GR α has been identified and mutation of this site (I634A), in addition to the conventional D-loop mutation (A458T), has been conducted to produce a receptor species, termed ‘GRmon’, which has been proven to be incapable of forming receptor dimers³⁹. One could postulate that it may be via this second-site that the dimerization deficient GR α mutant is able to form dimers, following GC treatment. Thus, in terms of the current study, it would be of interest to determine the ability of a GRmon to undergo ligand-induced receptor turnover. On the basis of results from the current study we would postulate a continuum of GR α dimerization states (GRwt > GRdim > GRmon) and would expect this to be reflected in a continuum of efficiency of ligand-induced receptor turnover (GRwt > GRdim > GRmon).

The previously proposed idea that the effects elicited by CpdA via the wild type GR α may represent those elicited by Dex via the dimerization deficient mutant, GRdim,⁵ appears to be more complex than originally assumed, with the current study highlighting differences between these species in terms of GR α mRNA regulation and GC-responsive gene expression (i.e. FKBP51). Specifically, CpdA via mGRwt was capable of down-regulating GR α mRNA expression, whereas Dex via mGRdim could not and, unlike CpdA via mGRwt, Dex via mGRdim could partially induce the expression of FKBP51. Keeping in mind that the GR α LBD has also been associated with receptor dimerization, and that CpdA is capable of competing with Dex in terms of ligand binding to GR α ^{5,7}, could it be that binding of CpdA to the LBD of the GR α abrogates receptor dimerization⁵ at this additional site, unlike the GRdim mutant in which the D-loop mutation occurs in the DBD and only

is thought to partially impair the ability of GR α to dimerize? Is it possible that CpdA-bound to GRwt is a different monomeric species to Dex bound to GRdim? In order to investigate this it would be necessary to repeat experiments from the current study with GRmon from Presman *et al.*³⁹.

An additional challenge, in the current study, was the difference in the expression levels of hGRwt and hGRdim, specifically in the transiently transfected COS-1 system. As GR α concentration has been shown to modulate certain aspects (i.e. nuclear translocation of the receptor) of GC/GR signalling pathway⁴⁷, it is necessary to address these differences. Essentially, one cannot disregard the potential effect of a higher cellular hGRwt concentration, relative to hGRdim, on the overall dimerization state of the receptor and its subsequent ligand-induced receptor turnover, which the current study did not account for in Chapter 4. With that said, introducing the use of the GFP-tagged GR α in Chapter 5 allowed for the influence of receptor concentration on the post-translational processing of the receptor, in terms of the interaction with FBXW7 α , to be addressed in the PLA. Going forward, the use of the MEF cells (i.e. used in Chapter 6) may be a more appropriate system to investigate the post-translational processing of GR α and its interaction with various co-regulators, involved in the UPS, due to the equimolar expression of the wild type and mutant GR α , in the MEF-mGRwt and MEF-mGRdim cells, respectively.

Lastly, it was difficult to draw concrete conclusions from the ubiquitin experiments in the current study, with no notable differences in GR α ubiquitination observed between the unliganded and the ligand-bound receptor. It may be that the unliganded and liganded GR α are ubiquitinated to similar degrees in preparation for proteasomal degradation, however, associate with different E3 ligases (i.e. CHIP or FBXW7 α). In support of this, is the fact, illustrated in the current study, that the unliganded GR α does undergo receptor turnover but at a slower rate than the liganded GR α (i.e. Dex bound GR α)⁴⁶ and that, to date, there has only been a single ubiquitin site documented for GR α ^{60,61}. However, to fully elucidate differences in the ubiquitination status of GR α , in the absence and presence of ligands, one should consider re-doing the PLA experiments conducted in the present study, using proteasome inhibitors (i.e. MG132) and possibly de-ubiquitinating (DUBs) enzyme inhibitors, to prevent receptor turnover and to preserve the poly-ubiquitination chains, respectively. Additionally, one should not exclude how the dimerization state of the receptor influences other PTMs, such as sumoylation, which has also been implicated in GR α turnover⁶². To end this section, we believe that the involvement of CHIP and TSG101 in receptor turnover and their associations with either a GR α dimer or monomer also require further investigation, with results from the current study providing the 'first green shoots' for how receptor conformation regulates interactions of the receptor with enzymes associated with the UPS.

7.5. Future perspectives

As we delve deeper into the depths of GC/GR α signalling so the complexity of this signalling pathway is uncovered. Becoming more and more apparent is the importance of GR α conformation as a fundamental aspect of GC/GR α signalling, induced by selective or ‘biased ligands’. As Luttrell *et al.*²³ suggests, it the binding of the ligand to the receptor (i.e. GR α) that dictates ‘the rest of the symphony’. Recent studies, in combination with the current study, have started revising and re-considering the importance of GR α conformation and the possibility of selectively dictating the ‘conformational equilibrium’ through ‘biased ligands’, to combat chronic inflammation in various psychological and pathological conditions, whilst preventing the generation of adverse side-effects and the development of acquired resistance to GC treatment^{1,21,23,59}.

In terms of GC resistance, we provide support for the idea that preferentially selecting for GR α monomers and maintaining receptor expression may be the way forward for combatting chronic inflammation in prolonged GC treatments regimes. We propose the idea of a ‘continuum of resistance’ (Fig. 7.2) where encouraging GR α dimerization, not only may have negative implications in terms of the generation of adverse side-effects^{63,64}, but also drives reductions in the GR α ‘functional pool’, which potentially encourages a decrease in GC sensitivity. Furthermore, in addition to the use of compounds^{45,65–67} which may stall receptor turnover (i.e. proteasome inhibitors or compounds such as Ginsenoside⁴⁵), we believe that disrupting dimerization through ‘biased ligands’, in a tissue-specific manner, may be a fruitful approach for developing tailored treatments to counteract the development of acquired GC resistance in a number of disease states. Moreover, an in depth characterization of the dimerization capabilities⁶⁸ of GR α mutants⁶⁹ may provide more insight into generalized GC resistance and assist in the treatment of these rare, pathological conditions.

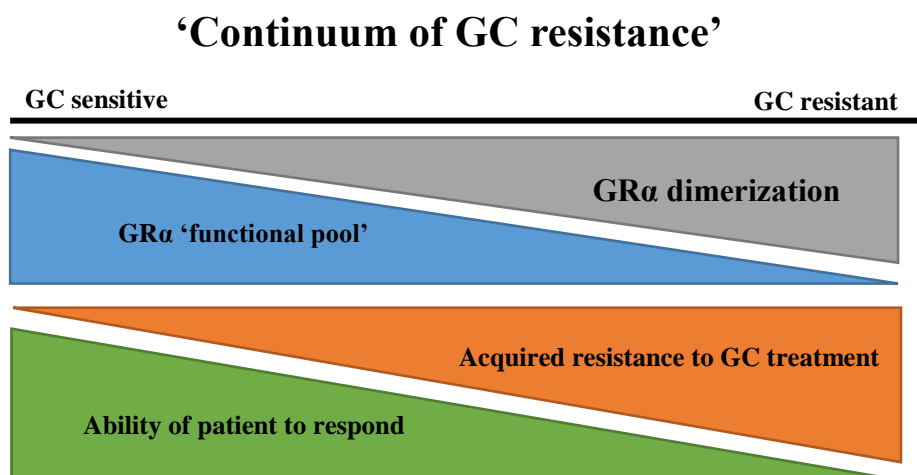


Figure 7.2: A ‘continuum of GC resistance’. As GR α dimerization increases, so increased ligand-induced receptor turnover of the GR α ‘functional pool’, both at the mRNA and protein level, occurs. These significant reductions in receptor turnover, in many cases, drive the development of an acquired resistance to GC treatment and so the ability of a patient to respond to GC treatment diminishes, posing a major threat to the pharmaceutical industry, in combatting chronic inflammation.

7.6. Final conclusion

Bringing it altogether, the current study has identified a novel and fundamental role for GR α dimerization in mediating ligand-induced down-regulation of the receptor, through treatment with the dimerization abrogating GC, CpdA, and supported by the use the dimerization deficient GR α mutant, using a number of cellular models (i.e. the COS-1, HepG2, MEF-mGRwt and MEF-mGRdim cells). Additionally, we uncovered the molecular mechanisms involved in mediating how a ‘loss’ of GR α dimerization prevents receptor turnover, by demonstrating that the post-translational processing of GR α (i.e. specifically phosphorylation at Ser404 and the interaction of the receptor with the E3 ligase, FBXW7 α) is influenced by the dimerization state of the receptor. Lastly, we established and tested a model to mimic acquired GC resistance, in which ligand-induced GR α turnover is thought to play a significant role, contributing to the idea proposed by De Bosscher *et al.*¹, that selecting for GR α monomers in prolonged GC regimens may be the way forward, however unlike De Bosscher *et al.*¹, we make this argument in terms of acquired GC resistance.

7.7. References

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