EFFECT OF MODULATORS OF INFLAMMATION ON HEPATIC ACUTE PHASE PROTEINS AND METABOLIC ENZYMES

Jacobus Albertus Koch Visser

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at Stellenbosch University.

Supervisor:
Prof. Ann Louw
Department of Biochemistry

Co-Supervisor:
Dr. Carine Smith
Department of Physiological Sciences

March 2010

Declaration:

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2010

SUMMARY

Crosstalk exists between the stress- and immune-system and this crosstalk has pharmacological importance in the use of glucocorticoids (GCs) as anti-inflammatory drugs for diseases such as asthma and arthritis. The focus of studies on this crosstalk has mainly been on the effects of GCs on immune function. The effect of the immune system on GC action, especially in the periphery, is not as well studied. The liver plays an important role in inflammation and stress in producing the acute phase proteins (APPs) required for the resolution of inflammation as well as in producing systemic glucose, through gluconeogenesis, required to fuel the stress responses. Understanding effects of stress and inflammation and their interplay in the liver is thus not only useful to expand our understanding of these systems but could also have clinical applications in understanding the side-effects associated with pharmacological use of GCs. CpdA has been identified as a selective glucocorticoid receptor (GR) modulator (SEGRM) in that it is able to repress genes but is not capable of activating genes via the GR. This attribute suggests that CpdA has the potential to be developed as an anti-inflammatory drug that displays fewer side effects. The current study investigated and compared effects of dexamethasone, a potent GR agonist, and CpdA, in the presence and absence of interleukin 6 (IL6), on the glucocorticoid receptor, three metabolic enzyme genes, involved in gluconeogenesis, and three APP genes. The metabolic enzyme genes investigated were tyrosine amintotransferase (TAT), phosphoenolpyruvate carboxykinase (PEPCK), and gamma glutmayltransferase (GGT), while the APP genes were serum amyloid A (SAA), C-reactive protein (CRP), and corticosteroid-binding globulin (CBG). The study investigated effects at the protein level, using Western blotting and ELISA assays, the protein activity level, using enzyme activity assays and whole cell binding, and at the mRNA level, using quantitive polymerase chain reactions (qPCR), in a mouse hepatoma cell line (BWTG3). The study showed that dexamethasone (Dex) and IL6 generally have divergent effects on the GR and metabolic enzymes

in that Dex down-regulated GR and up-regulated metabolic enzymes, while IL6 up-regulated GR and down-regulated metabolic enzymes, and that their functions are convergent for the acute phase proteins in that both up-regulatd positive APPs and down-regulated negative APPs. In contrast to Dex, CpdA up-regulated the GR and down-regulated the metabolic enzymes, while, similarly to Dex, it up-regulated positive APPs and down-regulated negative APPs. Our results for Dex and IL6 are supported by previous work in the literature. Our study is, however, unique, in combining the investigation of three metabolic enzymes with three APPs in addition to investigating GR levels in a single system under the same experimental conditions. Furthermore our results with CpdA have several novel aspects, such as down-regulation of metabolic genes, which contribute to the growing body of knowledge concerning this unusual GR ligand and its possible pharmacological applications.

OPSOMMING

Kruiskommunikasie bestaan tussen die stres– en die immuunsisteem en hierdie kruiskommunikasie is van farmakologiese belang vir die gebruik van glukokortikoïede (GKe) as anti-inflammatoriese medikasie vir siektes soos asma en artritis. Tot dusver was die fokus van studies oor hierdie kruiskommunikasie hoofsaaklik op die effek van GKe op immuunfunksie. Die effek van die immuunsisteem op GK werking, veral in die periferie, is nie so goed bestudeer nie. Die lewer speel ’n belangrike rol in inflammasie en stres deurdat dit die akute fase proteïene (AFPs) produseer wat benodig word vir die resolusie van inflammasie en omdat dit ook sistemiese glukose produseer, d.m.v. glukoneogenese, wat benodig word om die stres reaksie te dryf. ’n Beter insig in die effek van stres en inflammasie sowel as hul interaksie in die lewer is dus handig, nie net om ons begrip van hierdie sisteme te verbeter nie, maar ook omdat dit kliniese toepassing kan hê deurdat dit ons begrip van die newe-effekte wat gepaard gaan met die farmakologiese gebruik van GKe verbeter. Verbinding A (CpdA) is geïdentifiseer as ’n selektiewe glukokortikoïed reseptor (GR) moderator (SERGM) omdat dit die vermoë het om gene te onderdruk maar nie te aktiveer d.m.v. die GR. Hierdie eienskap dui op die potensiaal van CpdA om ontwikkel te word as ’n anti-inflammatoriese middel met minder newe-effekte. Die huidige studie het die effekte van dexamethasone, ’n sterk GR agonis, en CpdA, beide in die teenwoordigheid en afwesigheid van interleukin 6 (IL6), op die GR, drie metaboliese ensiem gene wat betrokke is by glukoneogenese, sowel as drie APP gene, ondersoek en vergelyk. Die metaboliese ensiem gene wat ondersoek is, is tirosien aminotransferase (TAT), fosfoenolpirovaat karboksikinase (PEPCK), en gamma glutamieltransferase (GGT), terwyl die APP gene serum amiloïede A (SAA), C-reaktiewe proteïen (CRP), en kortikosteroïed bindings globien (CBG) was. Die studie het die effekte in ’n muis hepatoma sellyn (BWTG3) op die proteïen vlak, deur van Western blotting en ELISA essays gebruik te maak, die proteïen aktiwiteits vlak, deur van ensiem aktiwiteits essays en vol-sel binding gebruik te maak, sowel as op die mRNA vlak, deur van kwantitatiewe polimerase ketting reaksie (qPCR) gebruik te maak, ondersoek. Die studie

toon dat dexamethasone (Dex) en IL6 in die algemeen divergente effekte het op die GR en metaboliese ensieme deurdat Dex GR af-reguleer en die metaboliese ensieme op-reguleer, terwyl IL6 die GR op-reguleer en die metaboliese ensieme af-reguleer, en dat hulle funksies konvergerend is vir die APPs deurdat beide positiewe APPs opreguleer en negatiewe APPs afreguleer. In teenstelling met Dex het CpdA die GR op-gereguleer en die metaboliese ensieme af-gereguleer terwyl dit, soos Dex, die positiewe APPs op-gereguleer en die negatiewe APPs af-gereguleer het. Ons resultate vir Dex en IL6 word ondersteun deur vorige werk in die literatuur. Ons studie is wel uniek omdat dit die ondersoek van drie metaboliese ensieme kombineer met die ondersoek van drie APPs, sowel as GR vlakke in ’n enkele sisteem onder dieselfde eksperimentele kondisies. Verder het ons resultate met CpdA verskeie nuwe aspekte, soos die af-regulering van metaboliese gene, opgelewer wat bydra tot die groeiende poel van kennis oor hierdie ongewone GR ligand en die moontlike farmakologiese gebruik daarvan.

ACKNOWLEDGEMENTS

Prof. Ann Louw, my supervisor, thank you for believing in me and for allowing me the opportunity to pursue this study. Your patience, guidance and understanding in difficult times were invaluable and greatly appreciated.

Dr. Carine Smith, thank you for your valuable input and helping me to understand my findings in a "bigger" physiological context.

To the members of the Louw and Africander research groups, thank you for all your support en encouraging words. The laughs, countless cups of coffee and little distractions have more value than what could be given credit to.

Carmen Langeveldt, thank you for your impeccable tissue culture work, encouragement and understanding rushed emergency orders had to be placed.

The South African National Research Foundation (NRF), thank you for financial support.

Employees of the Central Analytical Facility (CAF), Stellenbosch University, thank you for all the technical support during the qPCR studies as well as emotional support on days when the DNA would not amplify.

Thank you to all my family and friends for your emotional support and for putting up with my moods and ramblings.

ALPHABETICAL LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AF-1	Activation factor 1
AF-2	Activation factor 2
α-KG	α-ketoglutarate
AMP	Adenosine monophosphate
AP-1	Activator protein-1
APP	Acute phase protein
AR	Androgen receptor
AVP	Arginine vasopressin
cAMP	Cyclic AMP
CNS	Central nervous system
CpdA	Compound A, a selective selective GR modulator
CBG	Corticosteroid binding globulin
C/EBP	CCAAT/enhancer binding protein
CRE	Cyclic AMP-response element
CREB	cAMP response element binding protein
CRF	Corticotrophin releasing factor
CRH	Corticotrophin releasing hormone
CRP	C-Reactive Protein
DBD	DNA-binding domain
DEPC	Diethyl pyrocarbonate
Dex	Dexamethasone
DMEM	Dulbecco’s modified eagles’s medium

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
E	Epinephrine
ELISA	Enzyme linked immunosorbent assay
ER	Extrogen receptor
ERK	Extracellular-regulated kinase
FCS	Fetal calve serum
Gab1	Grb2-associated binder-1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GDP	Guanosine diphosphate
GGT	Gamma glutamyltransferase
GHA	Glutamohydromaxic acid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GSH	Glutathione
GTM	General transcription machinery
hGR	Human glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HSP	Heat shock protein
IL6	Interleukin 6
IL-6R	Interleukin 6 receptor
JAK	Janus kinase
LBD	Ligand binding domain
LC	Locus ceruleus

MAPK	Mitogen activated protein kinase
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide hydrate
NE	Norepinephrine
NF-κB	Nuclear factor-κB
nGRE	Negative glucocorticoid response element
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
pHBA	p-hydroxybenzoic acid
pHPP	p-hydroxyphenylpyruvate
PI3K	Phophoinositide 3-kinase
PLP	Pyridoxal-5'-phosphate
POMC	Pro-opiomelanocortin
PR	Progesterone receptor
qPCR	Quantitive polymerase chain reaction
RNA	Ribonucleic acid
SAA	Serym Amyloid A
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SGRM	Selective glucocorticoids receptor modulator
SHP2	SH2-domain-containing tyrosine phosphatase

SNS	Sympathetic nervous system
SOCS	Supressor of cytokine signalling
SPI-3	Serine protease inhibitor-3
STAT	Signal transducers and activators of transcription
TAT	Tyrosine aminotransferase
Tyk	Tyrosine kinase

To

My mother, Michelle Visser. Thank you for always believing in me

and for the countless sacrifices you made to make this possible.

TABLE OF CONTENT

CHAPTER 1 ..... **4**

LITERATURE REVIEW **4**

1.1 Introduction 4

1.2 Physiological mechanism of action of glucocorticoids and IL6. 7

1.2.1 Glucocorticoids 7

1.2.1.1 Glucocorticoid induced metabolic enzymes. 8

1.2.1.1.1 Tyrosine aminotransferase (TAT) 8

1.2.1.1.2 Phosphoenolpyruvate carboxykinase (PEPCK) 10

1.2.1.1.3 Gamma-glutamyltransferase (GGT) 11

1.2.2 Interleukin 6 12

1.2.2.2 Acute Phase Proteins 13

1.2.2.2.1 Corticosteroid Binding Globulin (CBG) 13

1.2.2.2.2 Serum Amyloid A (SAA) 14

1.2.2.2.3 C-Reactive Protein (CRP) 15

1.2.3 Crosstalk 15

1.3 Molecular mechanism of action of glucocorticoids and IL6 16

1.3.1 Glucocorticoids 16

1.3.2 Interleukin 6 20

1.3.3 Crosstalk 21

1.4 Clinical importance and current issues 23

1.4.1 Compound A, a nonsteroidal GR modulator 24

1.5 Hypothesis and aims 25

1.6 References 27

CHAPTER 2 37

EFFECT OF MODULATORS OF INFLAMMATION ON HEPATIC ACUTE PHASE PROTEINS AND METABOLIC ENZYMES 37

2.1 Abstract 38

2.2 Introduction 39

2.3 Material and methods 41

2.3.1 Test Compounds 41

2.3.2 Cell Culture 42

2.3.3 Whole Cell Binding Assay 42

2.3.4 Western Blot 43

2.3.5 Metabolic Enzyme Assays 44

2.3.5.1 TAT Assay 45

2.3.5.2 PEPCK Assay 46

2.3.5.3 GGT Assay 46

2.3.6 ELISA Assays 46

2.3.7 RT-PCR 47

2.3.7.1 RNA Isolation 47

2.3.7.2 cDNA synthesis 48

2.3.7.3 Quantitive Polymerase Chain Reaction (qPCR) 49

2.3.8 Data manipulation and statistical analysis. 51

2.4 Results 51

2.4.1 Modulation of GR concentration by Dex or CpdA in the presence or absence of IL6. 52

2.4.2 Modulation of metabolic enzymes by Dex or CpdA in the presence or absence of IL6. 54

2.4.2.1 TAT 54

2.4.2.2 PEPCK 55

2.4.2.3 GGT 56

2.4.3 Modulation of acute phase proteins by Dex or CpdA in the presence or absence of IL6. 57

2.4.3.1 CBG 57
2.4.3.2 CRP 59
2.4.3.3 SAA 61
2.5 Discussion 62
2.6 Reference list 70
2.8 Supplementary figures 75
CHAPTER 3 76
GENERAL CONCLUSION AND DISCUSSION 76
3.1 References 81
ADDENDUM A 85
ADDENDUM B 93
ADDENDUM C 99

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The body is under a constant onslaught of stressors. These stressors may be an intrinsic or an extrinsic threat to homeostasis and may be real or perceived. Homeostasis may be defined as the natural equilibrium that is maintained within the body. In response to stress, both physical and mental, reactions will be activated in order to return the body to a state of homeostasis. Stress is thus defined as a state of disharmony or threatened homeostasis [1].

The stress response may be acute and of short duration. Acute stressors are perceived as intensely stressful and can last from a few seconds to a few hours. The limited time span of the acute stress response means its catabolic, anti-anabolic and immunosuppressive effects are beneficial and without severe long-term consequences. However, chronic stress, which is less intense than acute stress, can last for a few days or even for months [1]. The chronic stress response may lead to a syndromal state that was described by H. Selye in 1936 as one that entails anorexia, loss of weight, depression, hypogonadism, peptic ulcers, and/or immunosuppression [2,3].

The function of the stress response is the re-establishment of homeostasis in the body by activating central and peripheral responses. The central responses mediate functions such as vigilance, alertness, cognition, focussed attention and appropriate aggression. The peripheral responses include increased cardiovascular tone, which leads to elevation in blood pressure and heart rate, respiratory rate, gluconeogenesis and lipolysis. The peripheral responses increase the availability of vital substrates such as oxygen, glucose, and nutrients which can be directed to the central nervous system and stressed body sites [1].

When the body is under threat two main pathways may be activated. The activation of the hypothalamic-pituitary-adrenal (HPA) axis represents one pathway and a stress response via the locus ceruleus - norepinephrine (LC/NE) system the other (Fig. 1.1).

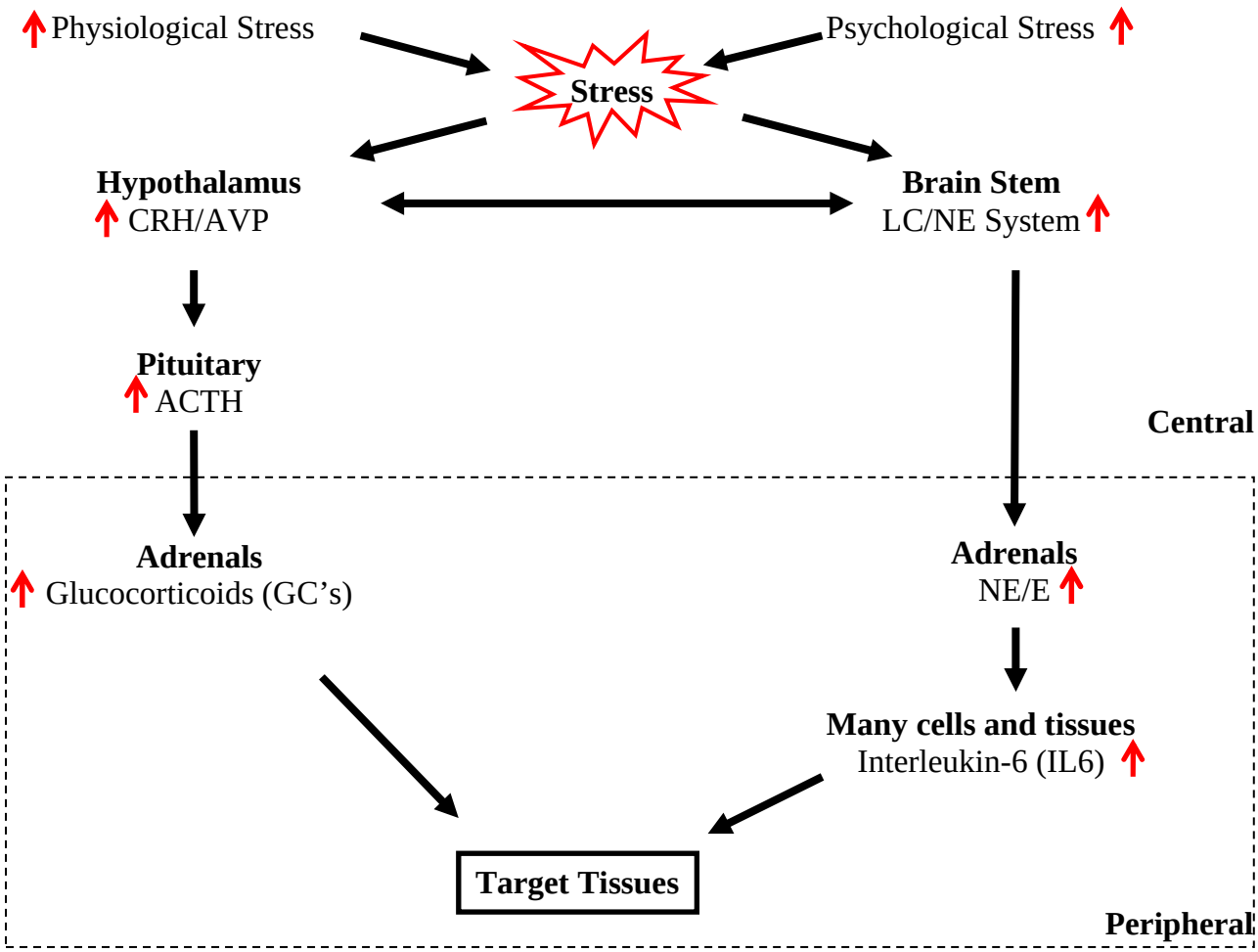


Figure 1.1 A simplified schematic representation of the central and peripheral components of the stress system. The stress response is mediated by the HPA axis and LC/NE system. The final effectors of these systems are glucocorticoids (GC's) and IL6. Adapted and simplified from Charmandari *et al*. [4] and Tsigos *et al* [3]. ACTH, adrenocorticotrophin releasing hormone; AVP, arginine vasopressin; CRH, corticotrophin releasing hormone; LC, locus ceruleus; NE, norepinephrine; E, epinephrine. Solid lines indicate stimulatory effects.

Upon activation of the HPA axis by stress the hypothalamus is stimulated to release corticotrophin releasing hormone (CRH), also known as corticotrophin releasing factor (CRF), and arginine vasopressin (AVP). AVP synergistically enhances the effect of CRH to stimulate the anterior pituitary to induce the release of adrenocorticotrophin releasing hormone (ACTH). CRH also

activates the sympathetic nervous system (SNS). ACTH acts on the adrenal cortex and stimulates the release of glucocorticoids (GCs) such as cortisol in humans and corticosterone in rodents [1,4,5]. Glucocorticoids are not only the final effectors of the stress response, they also play a role in the termination of the stress response by acting at extra-hypothalamic centres, the hypothalamus, and the pituitary. This negative feedback on the secretion of CRH and ACTH serves to limit the duration of exposure to glucocorticoids [4].

Activation of the LC/NE central sympathetic system in the brain stem stimulates the release of nor-epinephrine (NE) and epinephrine (E) from a dense network of neurons in the brain [1]. LC/NE activation can also stimulate peripheral responses through innervation by efferent preganglionic fibres that arise from the spinal cord. The preganglionic sympathetic fibres that end in the adrenal medulla mediate the sympatho-adrenal response in which E and NE is secreted from the adrenal gland. The E and NE secreted from the adrenals will stimulate a variety of cells and tissues, ranging from bone marrow to the liver, to release the pro-inflammatory cytokine interleukin 6 (IL6) [3,6]. IL6 plays important roles in immune function [7,8], hepatic acute phase protein synthesis [7,9], HPA axis activation [10,11], and emotional behaviour [12,13]. IL6 stimulates the HPA-axis at the level of the pituitary and the hypothalamus and causes increased secretion of ACTH and AVP. AVP enhances ACTH secretion which, in turn, will lead to elevated glucocorticoid secretion [4,14,15].

Crosstalk exists between the two main pathways of the stress response. This crosstalk has been extensively studied at the central level [3,4,11]. The crosstalk in the periphery has, however, not been as well studied, especially not in traditionally non-inflammatory cells and organs like the liver. This review, therefore, will focus on the physiological and molecular effects of glucocorticoids and IL6, the final effectors of the HPA-axis and LC/NE system, and how they modulate each other's action in a peripheral, non inflammatory organ, the liver.

1.2 Physiological mechanism of action of glucocorticoids and IL6.

Upon stimulation of the stress response system glucocorticoids and IL6 are released in the periphery. The following sections will discuss their transport to, and the physiological effects they elicit once they reach target organs. Where possible, the liver will be discussed as the target organ. The liver is chosen because it is the site synthesis of metabolic enzymes involved in gluconeogenesis as well as the site of synthesis of acute phases proteins, both of which are influenced by glucocorticoids and IL6.

1.2.1 Glucocorticoids

During stress the HPA axis is activated and this activation leads to the secretion of glucocorticoids by the adrenal cortex (Fig. 1.1) [5]. Within blood up to 90% of glucocorticoids are bound to corticosteroid binding globulin (CBG), with the remaining glucocorticoids either bound to albumin or free [16,17]. Currently two models exist explaining the functional significance of plasma proteins such as CBG. The first model, the free hormone model, suggests that the hormone bound to CBG is unavailable to cells for biological functions and that only the free hormone can cross the cell membrane to exert its function [17]. In this model the primary role of CBG is the regulation of free levels and clearance rate of hormones like glucocorticoids. More recent work attributes a more active role, in steroid-tissue interactions, to CBG. This bound hormone model, suggests that hormones can also exert their biological functions if they are bound to carrier proteins like CBG. The bound hormone model was formulated after the discovery of specific, high affinity CBG receptors on the plasma membranes of various cell types, including liver cells [17,18]. During inflammation, CBG, a member of the serine protease inhibitor (serpin) superfamily, is cleaved by serine protease elastase, which accumulates at sites of inflammation [19]. This results in the release of glucocorticoids at sites of inflammation where they will act as potent endogenous suppressors of the immune system by preventing the migration of leukocytes from circulation into extravascular

fluid spaces, reducing the accumulation of monocytes and granulocytes at sites of inflammation and inhibiting the production and/or action of cytokines [5,20,21]. Physiologically, glucocorticoids are also regulators of the catabolic response to stress, modulating changes in peripheral carbohydrate, amino acid and triglyceride metabolism [22].

Carbohydrate, amino acid and triglyceride metabolism produces substrates for hepatic gluconeogenesis, which is also stimulated by glucocorticoids [23]. In addition, glucocorticoids inhibit pituitary gonadotropin and growth hormone and make the target tissues of sex steroids and growth factors resistant to these substances, thus suppressing the reproductive and growth functions [3,23]. Glucocorticoids also alter cardiovascular tone and increase blood pressure, increase respiratory rate, alertness and cognition, and repress the immune or inflammatory reponse [4].

1.2.1.1 Glucocorticoid induced metabolic enzymes.

Glucocorticoids elicit a wide range of metabolic effects to increase blood glucose levels. These include stimulation of gluconeogenesis in the liver and the mobilization of both amino acids and free fatty acids, to provide substrates for gluconeogenesis [5]. These increased metabolic functions are the result of increased transcription of metabolic enzyme genes. This section will discuss three of these metabolic genes. These specific metabolic enzymes were chosen, because they have been shown to be regulated by glucocorticoids, are synthesized in the liver, and are all involved in gluconeogenesis in the liver (Fig. 1.2).

1.2.1.1.1 Tyrosine aminotransferase (TAT)

TAT is the first rate limiting enzyme in the conversion of the amino acid tyrosine to p-hydroxyphenylpyruvate (pHpp) in a transamination reaction during the catabolism of tyrosine [25]. The formed pHpp is converted to malate which is incorporated into the gluconeogenic pathway which leads to the formation of glucose (Fig. 1.2). The TAT gene is directly regulated via three

glucocorticoid response elements (GRE's) as well as four CCAAT/enhancer binding protein (C/EBP) binding sites in its promotor [26,27] (Fig. 1.3) and is generally used to study side effects associated with glucocorticoids.

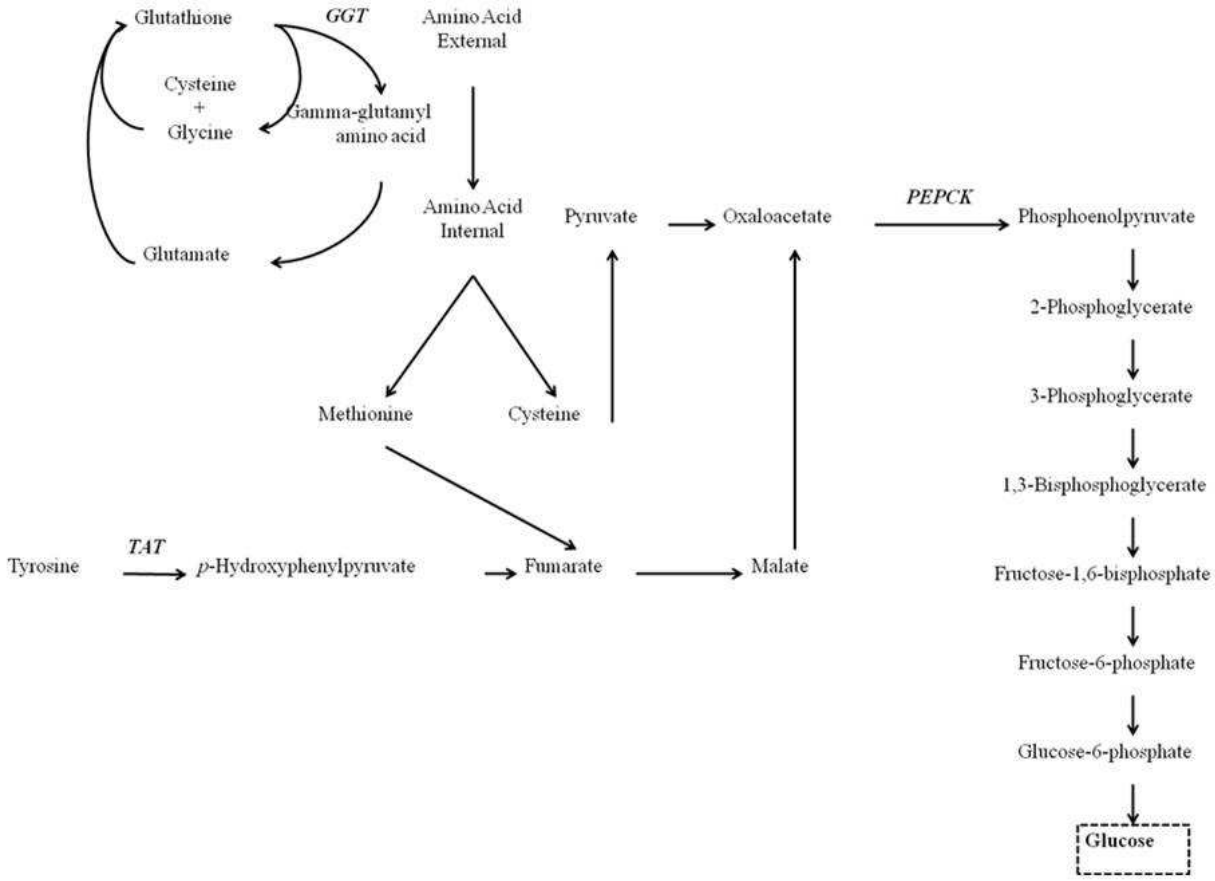


Figure 1.2 Simplified schematic representation of the conversion of amino acids to gluconeogenic inputs and the gluconeogenesis pathway that yields glucose as final product. Adapted and simplified from Voet *et al.* [24]. TAT, tyrosine aminotransferase; GGT, gamma-glutamyltransferase; PEPCK, phosphoenolpyruvate carboxykinase.

TAT is found in many organs in the body including the liver, thyroid, kidney, heart, muscle, cerebellum, cerebrum, skin, and adipose tissue [28].

Figure 1.3 Diagrammatical representation of the TAT promotor region indicating the presence of glucocorticoid receptor (GR) and C/EBP (CCAAT/enhancer binding protein) binding sites. Adapted from Bodwell *et a*l. [27]

The regulation of TAT activity by glucocorticoids is well studied, both on the protein as well as the mRNA level, both *in vivo* and *in vitro*, and it serves as a mature hepatocyte-specific marker of glucocorticoid action [29,30]. Studies show that glucocorticoids cause a increase in TAT mRNA levels [29,31,32] and TAT protein activity [27,29,33,34] and that an increase in TAT protein activity is associated with an increase in TAT mRNA levels [35].

1.2.1.1.2 Phosphoenolpyruvate carboxykinase (PEPCK)

PEPCK is a metabolic enzyme involved in gluconeogenesis in the liver and catalyses the regulatory and irreversible conversion of oxaloacetate to phosphoenolpyruvate (Fig. 1.2) [36,37]. PEPCK is expressed primarily in the liver, kidney, small intestine, and adipose tissue and its synthesis is under multi-hormonal control. Glucocorticoids exert their action through glucocorticoid response elements (GREs) in the PEPCK gene promoter sequence, but the sequence also contains three binding sites for C/EBP as well as a binding site for the cyclic AMP-response element (CRE) (Fig. 1.4) [36,38,39].

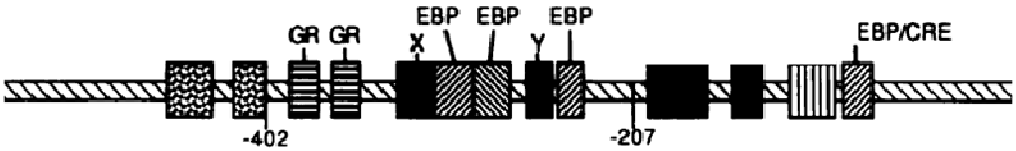


Figure 1.4 Diagrammatical representation of the PEPCK promotor region indicating the presence of GR, C/EBP (CCAAT/enhancer binding protein), and CRE (cyclic AMP-response element - shown to bind C/EBP [40]) binding sites. Adapted from Short *et al*. [41].

PEPCK is a marker of mature hepatocytes [30] and increased PEPCK expression is a marker of obesity and type II diabetes in animal models [38]. It has been shown that glucocorticoid treatment increases PEPCK mRNA levels [42-44] as well as PECK protein expression [42].

1.2.1.1.3 Gamma-glutamyltransferase (GGT)

Gamma-glutamyl transferase, also referred to as gamma-glutamyltranspeptidase, is situated on the external surface of cell membranes and is involved in the gamma-glutamyl cycle, which transports amino acids into the cell [24,45]. Specifically, it catalyzes glutathione (GSH) breakdown and accepts amino acids, like cysteine and methionine, for translocation into the cell (Fig. 1.2). GSH is transported to the external surface of the cell membrane, where the transfer of the gamma-glutamyl group from GSH to an external amino acid occurs, while the release glycine and cysteine will be used in regeneration of the GSH. The gamma-glutamyl amino acid is then transported back into the cell, the amino-acid is released and the formed 5-oxoproline is converted to glutamate [24]. GSH is finally reconstituted from the formed glutamate, glycine and cysteine. Intra-cellulary, methionine is converted to succinyl-CoA and enters gluconeogenesis via oxaloacetate while cysteine is converted to pyruvate thus also entering gluconeogenesis.

GGT is expressed in the kidney, pancreas, spleen, small intestine and liver of rats [46]. Regulation of GGT expression is complex and in mice and rats transcription is initiated from at least seven or five promoters, respectively [47,48]. A GRE [49] and NF-κB binding site [48] have been identified in the GGT promoter and GGT is also regulated by the Ras-PI3K (phophoinositide 3-kinase) – ERK (extracellular-regulated kinase)1/2 pathway [49].

GGT is a marker of liver function and increased GGT levels is associated with alcoholic liver disease, non-alcoholic fatty liver disease, cardiovascular disease and cholestasis [46,50,51]. Increased cellular GGT activity is found in various types of cancers [46,49]. Glucocorticoid

treatment increases GGT activity and GGT mRNA levels, both *in vitro* and *in vivo* [52,53]. The study by Chobert *et al*. [53] concludes that GGT is under positive control by glucocorticoids.

1.2.2 Interleukin 6

Interleukin 6 is a member of the pro-inflammatory cytokine family and plays an important role in the acute phase and immune responses of the organism [8]. IL6 is produced by a wide variety of cells such as hepatic Kupffer cells [54], cells of the immune system [55], and cells in neuronal and endocrine tissues such as the hypothalamus, pituitary, and the adrenal gland [56] in response to biological, chemical or physical stimulus [57].

Although IL6 is produced at the site of tissue damage or inflammation, the long distance systemic role of IL6 is dependent on the transport of IL6 in the blood [58]. IL6 can circulate in the body as part of a complex with other proteins (chaperone proteins), such as the soluble IL6 receptor as well as endogenous antibodies and in this form IL6 is kept in circulation and serves as a reservoir of potentially active IL6 [59,60].

IL6 is responsible for eliciting the acute phase reactions in the liver [6]. In addition, IL6 also elicits the development of specific cellular and hormonal immune responses. These include, end-stage B cell differentiation, immunoglobulin secretion and T-cell activation. IL6 is important for the transition between acute and chronic inflammation. This transition is charecterised by the recruitment of monocytes to the area of inflammation [61,62]. *In vivo* IL6 also plays a role in the recruitment of leucocytes [61]. The following section will review three acute phase proteins that are produced in the liver in response to inflammation.

1.2.2.2 Acute Phase Proteins

The acute phase response is important to restore homeostasis to the body of the organism and is an early and non-specific, primarily short term, response. Triggering factors, such as infection, trauma or malignant growth, signal disturbance of homeostatsis. The local reation, manifested as acute inflammation at the site of injury, is mediated by macrophages, fibroblasts and other cells, which also secrete inflammatory mediators, such pro-inflamatory cytokines. These inflammatory mediators mediate a secondary systemic reaction, which includes neurological, endocrine and metabolic alterations expressed as fever, aggregation of platelets, increased release of several hormones, the accumulation and activation of granulocytes and mono-nuclear cells, and changes in the concentration of acute phase proteins [6,62]. IL6 is the main mediator of the acute phase respose in the liver.

Binding of IL6 to the IL6 receptor on hepatocytes lead to the activation of signalling pathways which will regulate the synthesis of acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) [63]. CRP and SAA are markers of inflammation and their levels increase dramatically during times of inflammation and therefore they were chosen as proteins of interest. CBG as well as being the high affinity transport protein for GCs (discussed in Section 1.2.1) is also a negative acute phase protein that is down-regulated during inflammation and was thus chosen as the third APP to be investigated. The following sections will discuss these proteins of interest that are synthesised in the liver.

1.2.2.2.1 Corticosteroid Binding Globulin (CBG)

Corticosteroid binding globulin is a plasma protein, mainly produced in the liver, which binds glucocorticoids and regulates their availability to target cells [64,65]. In a human hepatoma cell line, HepG2, IL6 was found to downregulate CBG levels on the protein as well as the mRNA level

[64] and the downregulatory effect in this cell line was shown to be at the transcriptional level [66]. IL6 is capable of repressing CBG production via a C/EBP binding site in the CBG promoter region [67,68]. Low CBG levels serves as a marker of insulin resistance as well as low grade inflammation [69].

1.2.2.2.2 Serum Amyloid A (SAA)

Serum Amyloid A (SAA) (promotor region shown in Fig.1.5) is one of the major acute phase proteins produced in the liver and its regulation and synthesis is largely modulated by cytokines such as IL6 in response to tissue injury, infection and trauma [70,71].

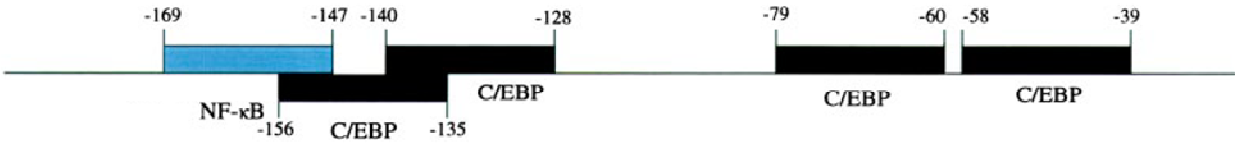


Figure 1.5 Diagrammatical representation of the SAA promotor region indicating the presence of C/EBP (CCAAT/enhancer binding protein) and NF-κB binding sites. Adapted from Uhlar *et al.* [71].

SAA induces the formation of extracellular matrix (ECM) enzymes, such as collagenase, which are important for repair processes after tissue damage and has been shown to be a chemoattractant for immune cells such as monocytes, leukocytes, mast cells, and T lymphocytes [71]. Increased levels of SAA are a marker of inflammation, insulin resistance, atherosclerosis, and cardiovascular disease [72,73]. The SAA promotor has been shown to contain binding sites for NF-κB as well C/EBP [74]. In a human hepatoma cell line, HepG2, IL6 is capable of inducing a SAA promotor containing promotor-reporter construct as well as the SAA protein [74,75] and induction by IL6 is also seen on the mRNA level [75].

1.2.2.2.3 C-Reactive Protein (CRP)

CRP is one of the major acute phase proteins produced in the liver and its concentrations increase rapidly in response to inflammation [70]. CRP is able to recognize pathogens and damaged cells and will aid in their removal by recruiting phagocytes and the complement system [76]. Increased levels of CRP are a sensitive marker of inflammation [77].

The CRP promoter contains binding sites for NF-κB, C/EBP as well as STAT3 [78,79]. IL6 has been shown to play a central role in the induction of CRP in human hepatoma cell lines both on the protein as well as the mRNA level [80,81].

1.2.3 Crosstalk

Glucocorticoids are hormones secreted by the adrenal glands and have long been known to have an effect on the immune system [82]. It has, however, become apparent that this effect is bi-directional and that the effectors of the immune system, such as IL6, are capable of modulating the action of glucocorticoids [83]. The next section will not only focus on the modulation of IL6 action by glucocorticoids, but also on the modulation of the effect of glucocorticoids by IL6. The latter modulation has not been well described and studies have mainly focused on this modulation in the central system and not in peripheral tissues. The focus of this section will therefore be on crosstalk in the liver as a peripheral organ.

The effect of glucocorticoids on the pro-inflammatory cytokines and acute phase proteins has been extensively studied, mainly due to the pharmacological use of glucocorticoids as anti-inflammatory drugs. The production and secretion of pro-inflammatory cytokines, such as IL6 and IL1β, is down-regulated by glucocorticoids [82]. In addition, it is proposed that for some APPs glucocorticoids can only enhance cytokine driven upregulation of APPs and that it does not have a significant effect on its own [71]. For example, glucocorticoids, in the absence of pro-inflammatory

cytokines, cause a moderate [84] to no induction [70] of SAA levels, but in the presence of these pro-inflammatory cytokines they are able to synergise with pro-inflammatory cytokines in up-regulating SAA [70,75,84]. This synergism in the presence of pro-inflammatory cytokines is also observed for CRP [70,76] and CBG [16,64]. Thus, although glucocorticoids generally down-regulate pro-inflammatory cytokine production and as such function as a negative feedback system antagonising the inflammatory response, in the case of APPs the effect appears to different in that glucocorticoids potentiate the effects of inflammatory cytokines thereby leading to a containment of inflammation.

In peripheral tissues IL6 antagonizes glucocorticoid action and is able to up-regulate glucocorticoid receptor (GR) levels in the absence of glucocorticoids and will reduce the down-regulation of GR in the presence of glucocorticoids [85]. IL6 also down-regulates glucocorticoid induced TAT levels in the liver *in vivo* [85] and basal TAT levels *in vitro* [86] and IL6 decreases PEPCK expression in mice [38] as well as in rats [87]. In contrast, serum GGT levels are elevated during times of inflammation [88]. Even though current studies on effects of IL6 on serum or cellular GGT levels are few [45], some studies have shown that GGT is up-regulated by IL6 [89], while in contrast others observe no significant effect [90,91].

1.3 Molecular mechanism of action of glucocorticoids and IL6

The following section will review the molecular sequence of events that can occur when glucocorticoids or IL6 reach their target organs and bind to their respective receptors. The molecular crosstalk between the stress and the inflammatory system will also be discussed.

1.3.1 Glucocorticoids

Glucocorticoids have a lipophyllic nature and this enables them to readily diffuse across cell membranes and exert their action through intracellular receptors called glucocorticoid receptors

(GR) [92]. The GR is a member of the superfamily of ligand regulated nuclear receptors, including the mineralocorticoid, androgen, progesterone, and estrogen receptors, and share characteristics with members of this family such as a modular structure whose principal functions are localised to specific domains (Fig. 1.6) [5]. Figure 1.6 depicts the human GR, but it is evolutionary conserved across a wide variety of species including rodents [93].

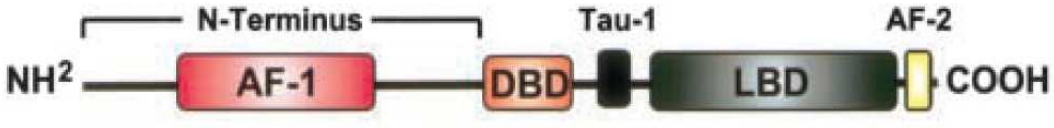


Figure 1.6 Diagram of the modular structure of the human glucocorticoid receptor. The GR consists of three domains namely, the N-terminus, DNA binding domain (DBD), and the C-terminal ligand-binding domain (LBD). NH^2 and COOH depict the amino and carboxy terminal respectively while AF-1, AF-2, depicts the binding sites for activation factors 1 and 2. Figure taken from Necela *et al*. [93]

Human GR (hGR) has two isoforms, GRα and GRβ. hGRα is expressed in almost all human tissues and although hGRβ is also expressed in a variety of human tissues and cells, it is expressed at a lower concentration than hGRα [94]. GRβ cannot bind to ligand and is transcriptionally inactive and acts as a dominant negative inhibitor of GRα [94,95]. The GRβ isofrom is not conserved across all species and is not present in mice [96]. Glucocorticoids down-regulate the concentration of GR via homologous down-regulation and this is a mechanism thought to protect the cell from constant signalling in the presence of ligand [97,98]. This downregulation is achieved by both a decrease in the transcription of GR as well as by a posttranslational increase in receptor turnover [97].

In the absence of ligand GR is maintained in the cytoplasm as an inactive multi-protein complex [5]. The unliganded receptor is bound to heat shock protein (hsp) 90 in the form of a heterohexamer containing the receptor, two molecules of hsp 90, and one molecule each of hsp 70, hsp 56, and hsp 26 [95]. Binding of glucocorticoids to the LBD of the GR will cause a conformational change in the receptor. This change leads to the dissociation of the multi-protein

complex and nuclear translocation will ensue. The GR can now bind to DNA sequences, called glucocorticoid response elements (GREs), as a homodimer and can either activate or repress the transcription of responsive genes [99].

GR homodimers bind to classical GRE sites (Fig. 1.7A) in the promotor regions of specific genes to activate transcription [100]. Examples of genes that are activated in this fashion are the metabolic enzyme genes PEPCK [101] and TAT [102]. Another transactivation model describes the interaction of a GR monomer with a second transcription factor (Fig. 1.7B) in a manner that involves DNA-binding of both factors. An example of this composite model is the ability of the GR monomer to act synergistically with activator protein-1 (AP-1) [103]. It is also possible for a GR monomer to interact with a second transcription factor to activate transcription in a manner that does not require DNA binding of the GR monomer (Fig. 1.7C). An example of this tethering GRE model is where the transcription factor STAT is bound by GR without requiring DNA-binding of the GR, with synergistic up-regulation of the gene concerned [5].

The Ligand bound GR can also repress genes. This repression may be due to the direct binding of the GR homodimer to a nGRE (Fig. 1.7D). Pro-opiomelancortin (POMC), an ACTH precursor protein, is an example of a gene that is repressed in this manner [104]. DNA binding of the GR homodimer to a nGRE may also block the binding of positive factors and thereby cause transcriptional repression (Fig. 1.7E). The transcription of the glycoprotein hormone α subunit gene is an example that illustrates the competitive binding model. This gene is positively regulated by the cAMP response element binding protein (CREB) and the promotor contains overlapping binding sites for both CREB and GR [105]. Binding of the GR homodimer to one of these overlapping sites inhibits transcriptional activation by preventing the binding of CREB [5]. Repression of a gene can also occur via the tethering of a GR monomer to transcription factors (Fig. 1.7F), for example, in the interaction of GR with the NF-κB transcription factor [106]. The tethering mechanism has been

proposed to be the reason for the glucocorticoid repression of genes that do not contain nGRE's in their promotor regions like, for example, the inflammatory genes [5]. Lastly, interaction of DNA bound GR and transcription factors may result in repression of a gene in a composite manner (Fig. 1.7G). An example of this mechanism is for the proliferin gene where a GRE and AP-1 binding site is found next to each other in the genes promotor region. The GR does not affect the binding of AP-1 and repression is thought to be due to the bound GR affecting the activation abilities of the bound transcription factor [5].

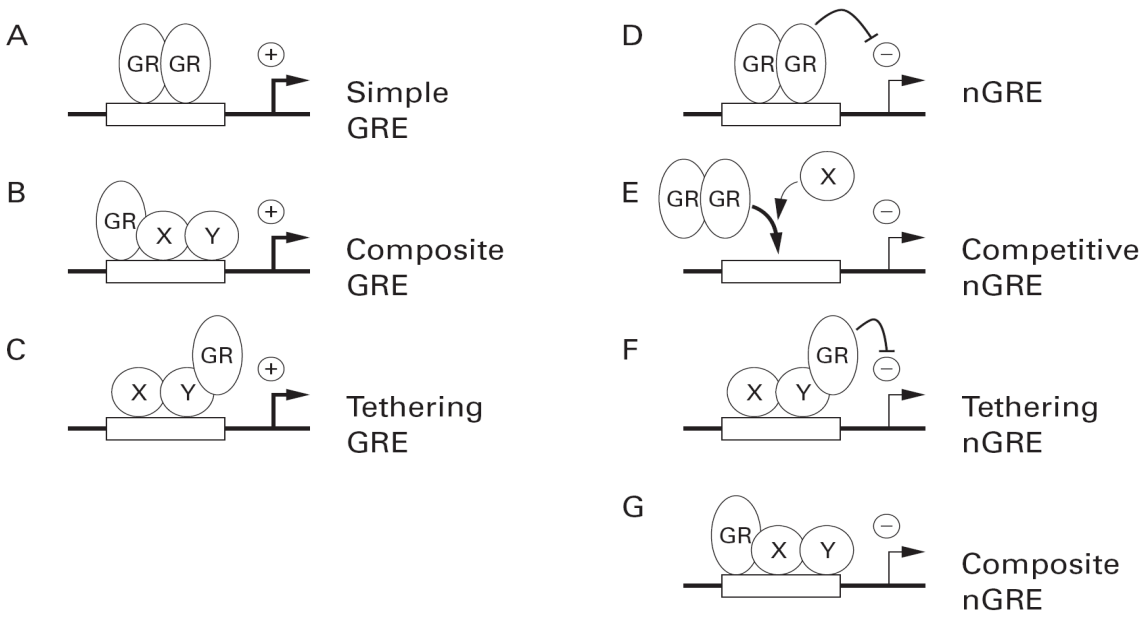


Figure 1.7 Models describing the molecular mechanism of activated GR. (A) GR homodimers bind cooperatively to classical GRE sites to activate transcription. (B) Interaction of GR with a second transcription factor can activate transcription from composite binding sites in a manner that involves DNA binding of both factors. (C) Interaction of GR with a second transcription factor in a manner that does not require the DNA binding of GR. (D) Homodimers of GR repress transcription from a simple negative GRE (nGRE). (E) Binding of GR to the GRE at a competitive nGRE prevents the binding of factors that are required for transcriptional activation thereby causing transcriptional transrepression. (F) Interaction of GR with a second transcription factor results in repression of transcription in a manner that does not require DNA binding by the GR. (G) Interaction of GR with a second transcription factor repress transcription from composite binding sites in a manner that involves DNA binding of both factors. X and Y represent transcription factors. Figure taken from Newton [5].

1.3.2 Interleukin 6

On target cells IL6 will bind to the IL6 receptor. These receptors may be divided into the non-signalling α-receptors (IL-6Rα, R refers to receptor) and the non-ligand binding signal transducing receptors (gp130) [8,107]. IL-6Rα exists in two forms, a transmembrane and a soluble form. The transmembrane form has a short intercytoplasmic region and is stimulated to associate with gp130 upon binding of IL6 [7]. Both the transmembrane form of IL6-Rα and the signal transducing gp130 are present in the liver [108,109]. The soluble form of IL-6Rα can also form a stimulatory complex with IL6 [107]. The stimulatory complex (IL6 plus either transmembrane or soluble IL-6R) associates with gp130 (Fig. 1.8) and triggers the homodimerization of gp130. Thereafter Janus kinases (JAKs), (JAK1, JAK2) are activated [110].

The activated JAK's phosphorylate gp130 at several residues. Phosphorylated gp130 serves as a docking site for SH2 domain-containing molecules, STAT1 or STAT3, members of the signal transduction activated transcription factors (STAT) family. Phosphorylated STAT's translocate as dimers to the nucleus where they bind to the promotor regions of their specific response genes (Fig. 1.8) [110]. One of these genes is the SOCS (suppressor of cytokine signalling) gene that is rapidly up-regulated by IL6 via the JAK/STAT pathway and subsequently inhibits STAT mediated signal transduction, thereby acting as a classical feedback inhibitor of this signalling pathway (Fig. 1.8) [8].

CAAT/enhancer binding protein (C/EBP) is a transcription factor that binds to the CAAT box of several mammalian promoters. Several C/EBP genes have been cloned and therefore many names for it exist, for example, in humans it is referred to as NF-IL6 and in mice as AGP/EBP [6]. It has been shown that phosporylation via a Ras dependent MAP kinase is essential for activating this transcription factor [111]. In response to IL6, Grb2-associated binder-1 (Gab1) is phosphorylated and interacts with SHP2 (SH2-domain-containing tyrosine phosphatise) and PI3K

(phosphoinositide 3-kinase). This association leads to the activation of several MAP kinases, including ERK (extracellular-regulated kinase) 2 [8]. ERK2 phosphorylates C/EPB whereafter it moves across the nuclear membrane and interacts with response elements in the promotor regions of target genes (Fig 1.8) [6].

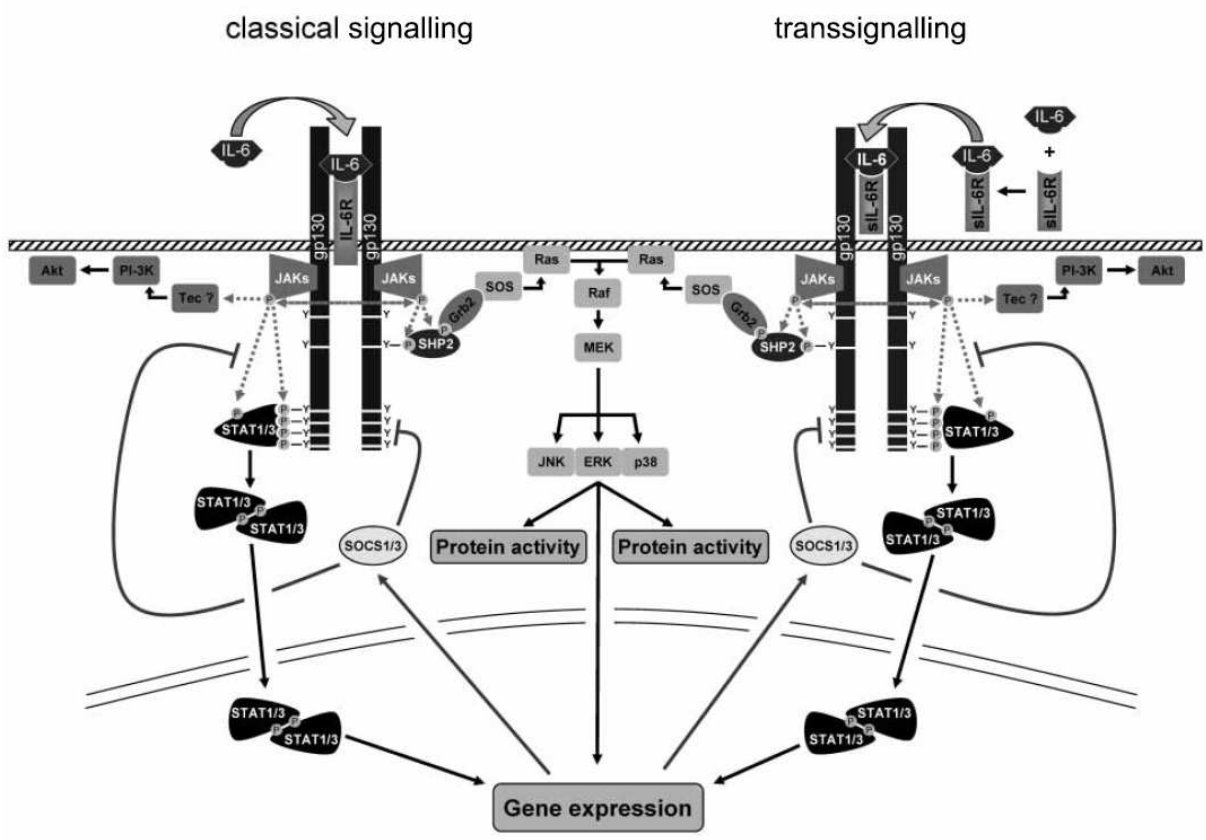


Figure 1.8. Schematic cascades of classical IL6 signalling and IL6 transsignalling showing the activation of the JAK/STAT as well as the Ras-ERK signalling pathway. Classical signalling is the binding of IL6 to the transmembrane IL6 receptor whereas, transsignalling is the binding of IL6 to the soluble IL6 receptor. JAK, Janus kinase; STAT, signal transduction activated transcription factors; ERK, extracellular-regulated kinase; Y, tyrosine; JNK, Jun N-terminal Kinase; PI3K, phosphoinositide 3-kinase; Grb2, growth factor receptor-bound protein; SHP2, SH2 domain-containing protein-tyrosine phosphatise; SOCS, suppressor of cytokine signaling. Figure taken from Schuett *et al.* [63].

1.3.3 Crosstalk

At a molecular level, crosstalk entails the interaction of GR with transcription factors such as NF-κB, the transcription factor responsible for the regulation of IL6 transcription, or STAT3 and

C/EBP, both of which are involved in the transcription of APPs. The level of expression of these transcription factors, both in inflammatory or non-inflammatory cell types, compared to the expression of GR may alter the net effect. For example, in inflammatory cells expressing high levels of GR, the GR may have the strongest effect and this will result in an anti-inflammatory action, however, in glucocorticoid resistant cells, the divergent effect will favour the pro-inflammatory transcription factors. Crosstalk, at a molecular level, is also dependent on the duration of each response and whether the pathways are activated at the same time. For example, in non-inflammatory cells, the cells may not be exposed to the influence of cytokines and glucocorticoids at the same time [93] and this could affect the respective responses.

NF-κB is an inducible transcription factor that activates a variety of cytokines and cytokine-inducecd genes in the immune system and GR inhibits many of these NF-κB activated genes [112]. These genes do not contain GREs and GR inhibition is believed to occur via a tethering mechanism (Fig. 7F). NF-κB is composed of two subunits, p50 and the transcriptionally active p65 unit [93]. These subunits translocate to the nucleus where they dimerize and bind to NF-κB response elements, activating proinflammatory genes. This induction is antagonised by the interaction of the GR to the p65 unit and likewise the p65 subunit can interact with the DNA bound GR homodimers and inhibit the transcription of GRE driven genes [93].

IL6 binds to the IL6 receptor and activates STAT3 throught the activation of the JAK/STAT pathway (Fig. 1.9). The GR can physically interact with DNA bound STAT3 and synergistically enhance STAT3 mediated gene expression [9]. STAT3 proteins are also capable of physically interacting with DNA bound GR and may either induce or repress GR gene activation (Fig. 1.9) [113] . In response to IL6, C/EBP is phosphorylated, moves across the nuclear membrane and interacts with response elements in the promotor regions of target genes (Fig. 1.8). This activation occurs through the Ras-ERK-MAPK pathway and GR can repress the MAPK family by inhibiting

the phosphorylation step required for their activation [93,114]. In addition, it is possible that GR directly interferes with ERK signalling modules upon hormone binding, possibly via tethering, however, this possibility has not yet been proven [114].

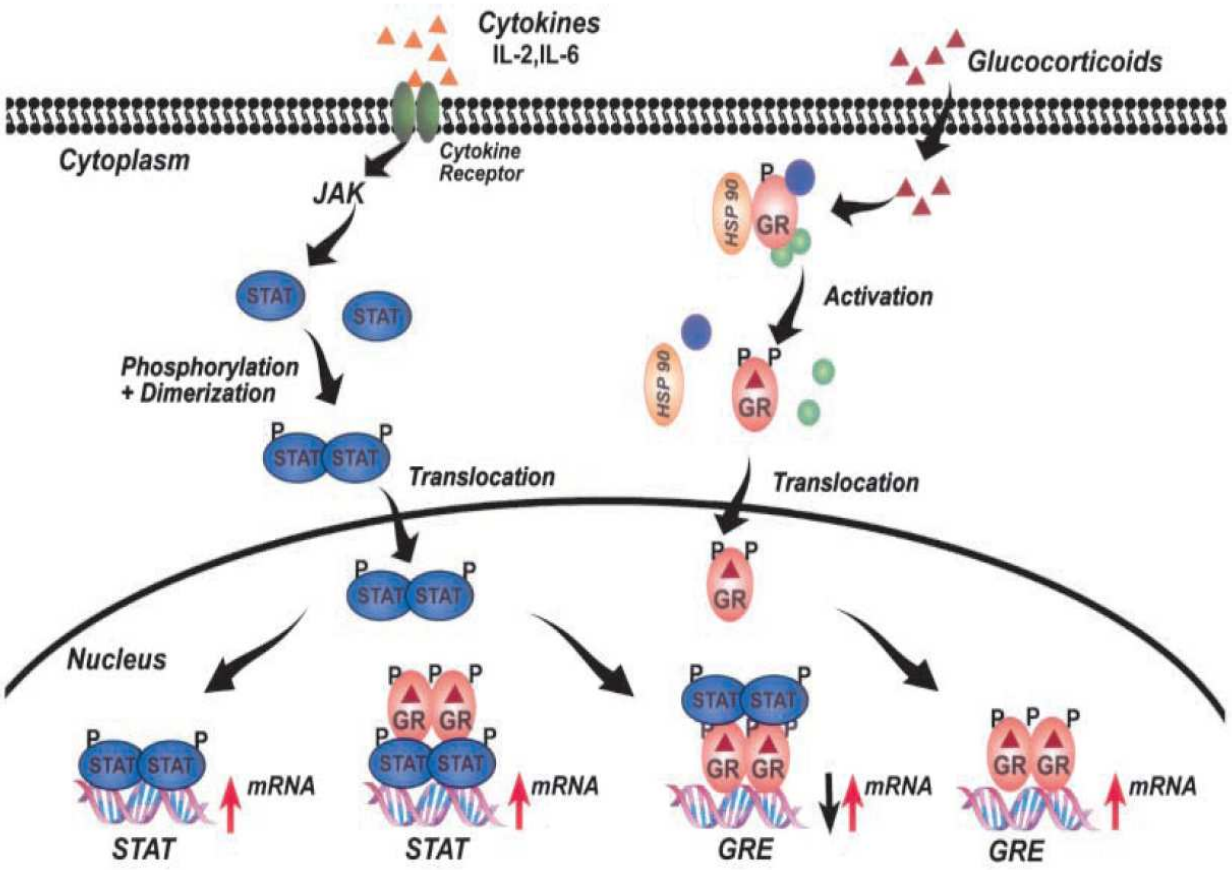


Figure 1.9 Mechanism of transcriptional regulation by STAT and the crosstalk between GR and STAT. Cytokines such as IL6 activate STAT proteins which then dimerizes and translocates to the nucles where it binds response elements and regulate the expression of genes. Physical interaction between GR and STAT can alter their respective mediated gene epression. STAT, signal transduction activated transcription factors; HSP90, heat shock protein 90; ↑, upregulation; ↓, downregulation. Figure taken from Necela *et al*. [93].

1.4 Clinical importance and current issues

Synthetic glucocorticoids have been developed for therapeutic use and are among the most widely prescribed drugs in the world for the treatment of immune and inflammatory diseases, including

asthma and rheumatoid arthritis [92]. Glucocorticoids induce apoptosis and therefore they can also be utilized as a component of the chemotherapeutic treatment of cancers of haematological origin like Hodgkin's lymphoma, acute lymphoblastic leukemia, and multiple myelomas [115].

The long term use of glucocorticoids for therapeutic purposes has, however, been limited. This limitation may be ascribed to the adverse side effects of long term glucocorticoid use, which include reduced muscle mass and repair, insulin resistance, fat deposition, growth failure, osteoporosis and the suppression of the HPA axis [92]. In addition, some patients, after initially responding well to glucocorticoid administration, develop glucocorticoid resistance after long term treatment [116]. This resistance has been proposed to be due to the down regulation of GR by its ligand in some patients [117] as cellular sensitivity to a ligand is directionally proportional to its receptor concentration.

The positive therapeutic qualities of glucocorticoids may be attributed to the repression of pro-inflammatory genes, whilst the side effects may be attributed to the activation of genes like the metabolic enzyme genes involved in gluconeogenesis. It is necessary to continue the study of GR and its positive, and negative transcriptional effects to identify and characterise new classes of glucocorticoids, or GR modulators, that maintain anti-inflammatory effects whilst minimising unwanted side effects. Recently several compounds displaying such dissociative properties have been identified: AL-438 [118], ZK 209614 [119], and CpdA [44,120].

1.4.1 Compound A, a nonsteroidal GR modulator

Compound A (CpdA), 2-(4-acetoxyphenol)-2chloro-*N*-methyl-ethylammonium chloride, is an analogue of a phenyl aziridine precursor that occurs in the shrub Salsola *tuberculatiformis Botch.* [121,121]. CpdA does not have a steroidal structure (Fig. 1.10) but it binds GR and downregulates NF-κB driven genes via the GR [120].

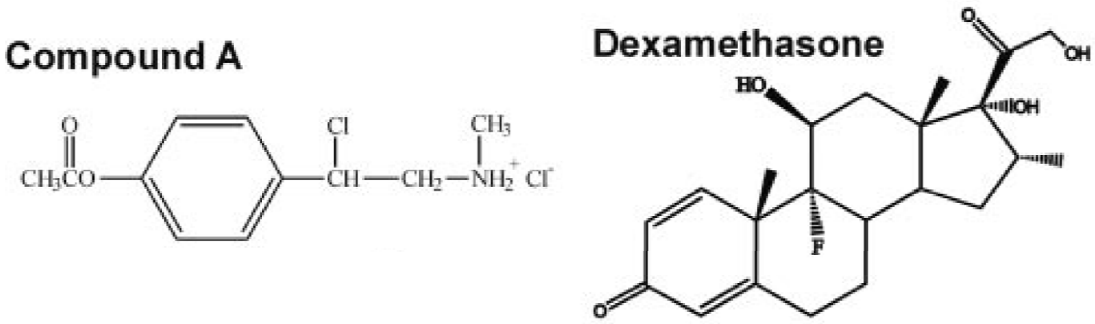


Fig 1.10 Chemical structures of Compound A and dexamethasone [120].

CpdA, unlike glucocorticoids, does not upregulate metabolic enzymes, like glucose-6-phosphatase and PEPCK [44] *in vivo* on the mRNA level in mice, nor does it induce a GRE driven promotor-reporter construct in a murine fibrosarcoma cell line [120]. In addition, CpdA also does not cause the homologous down regulation of GR, as seen with classical GR agonists [98]. However, CpdA is able to downregulate IL6, basal as well as induced, levels and other NF-κB-driven proinflammatory genes [120]. In animal models CpdA does not increase insulin [44] or blood glucose levels, unlike glucocorticoids, but it does reduce inflammation to a similar extent as glucocorticoids [44,98,120,122,123].

CpdA is suggested to have complete dissociated properties *in vivo* at the gene regulatory level and can be classified as a selective GR modulator (SGRM). This may be attributed to the inhibition of GR dimerization by CpdA [44]. These findings suggest that CpdA shows potential to be developed as an effective fully dissociated anti-inflammatory drug.

1.5 Hypothesis and aims

In response to stress the body will release glucocorticoids and IL6 into the circulation via the activation of the HPA-axis and the LC/NE system, respectively. Glucocorticoids and IL6 will be

transported to peripheral target organs, like the liver, where they will cause a variety of effects such as the regulation of metabolic enzymes and APPs. These effects may be due to stimulation or repression at the protein as well as the mRNA level. Based on previous work we hypothesize that in the liver IL6 will have a divergent effect to that of glucocorticoids on the transcription of GR and metabolic enzymes, while IL6 will have a convergent effect with glucocorticoids on acute phase proteins. CpdA, unlike glucocorticoids, is not expected to activate the metabolic enzymes or cause homologues downregulation of GR concentrations. In contrast, its effect on APPs is expected to be similar to that of the glucocorticoids.

Thus, the aim of the current study is to investigate the effect of Dex (a potent synthetic GR agonist) and CpdA (a selective GR modulator), in the presence and absence of IL6, on the GR, metabolic enzymes, and acute phase protein genes in BWTG3 cells (a mouse hepatome cell line). We will investigate effects on both mRNA and protein levels.

1.6 References

1. G.P. Chrousos, P.W. Gold, The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 267 (9) (1992) 1244-1252.

2. H. Selye, A syndrome produced by diverse nocuous agents. 1936. Reprinted in J. Neuropsychiatry Clin. Neurosci. 10 (2) (1998) 230-231.

3. C. Tsigos, G.P. Chrousos, Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53 (4) (2002) 865-871.

4. E. Charmandari, C. Tsigos, G. Chrousos, Endocrinology of the stress response. Annu. Rev. Physiol. 67 (2005) 259-284.

5. R. Newton, Molecular mechanisms of glucocorticoid action: what is important? Thorax 55 (7) (2000) 603-613.

6. A. Koj, Initiation of acute phase response and synthesis of cytokines. Biochim. Biophys. Acta 1317 (2) (1996) 84-94.

7. T. Kishimoto, Interleukin-6: discovery of a pleiotropic cytokine. Arthritis Res. Ther. 8 Suppl 2 (2006) S2.

8. P.C. Heinrich, I. Behrmann, S. Haan, H.M. Hermanns, G. Muller-Newen, F. Schaper, Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem. J. 374 (Pt 1) (2003) 1-20.

9. T. Takeda, H. Kurachi, T. Yamamoto, Y. Nishio, Y. Nakatsuji, K. Morishige, A. Miyake, Y. Murata, Crosstalk between the interleukin-6 (IL-6)-JAK-STAT and the glucocorticoid-nuclear receptor pathway: synergistic activation of IL-6 response element by IL-6 and glucocorticoid. J. Endocrinol. 159 (2) (1998) 323-330.

10. N.J. Rothwell, S.J. Hopkins, Cytokines and the nervous system II: Actions and mechanisms of action. Trends Neurosci. 18 (3) (1995) 130-136.

11. R.S. Perlstein, M.H. Whitnall, J.S. Abrams, E.H. Mougey, R. Neta, Synergistic roles of interleukin-6, interleukin-1, and tumor necrosis factor in the adrenocorticotropin response to bacterial lipopolysaccharide in vivo. Endocrinology 132 (3) (1993) 946-952.

12. V. Butterweck, S. Prinz, M. Schwaninger, The role of interleukin-6 in stress-induced hyperthermia and emotional behaviour in mice. Behav. Brain Res. 144 (1-2) (2003) 49-56.

13. M.B. Howren, D.M. Lamkin, J. Suls, Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. Psychosom. Med. 71 (2) (2009) 171-186.

14. G. Mastorakos, J.S. Weber, M.A. Magiakou, H. Gunn, G.P. Chrousos, Hypothalamic-pituitary-adrenal axis activation and stimulation of systemic vasopressin secretion by recombinant interleukin-6 in humans: potential implications for the syndrome of inappropriate vasopressin secretion. J. Clin. Endocrinol. Metab. 79 (4) (1994) 934-939.

15. G. Mastorakos, G.P. Chrousos, J.S. Weber, Recombinant interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans. J. Clin. Endocrinol. Metab. 77 (6) (1993) 1690-1694.

16. C.L. Smith, G.L. Hammond, Hormonal regulation of corticosteroid-binding globulin biosynthesis in the male rat. Endocrinology 130 (4) (1992) 2245-2251.

17. R. Mihrshahi, J.G. Lewis, S.O. Ali, Hormonal effects on the secretion and glycoform profile of corticosteroid-binding globulin. J. Steroid Biochem. Mol. Biol. 101 (4-5) (2006) 275-285.

18. O.A. Strel'chyonok, G.V. Avvakumov, Evidence for the presence of specific binding sites for transcortin in human liver plasma membranes. Biochim. Biophys. Acta 755 (3) (1983) 514-517.

19. U. Rescher, V. Goebeler, A. Wilbers, V. Gerke, Proteolytic cleavage of annexin 1 by human leukocyte elastase. Biochim. Biophys. Acta 1763 (11) (2006) 1320-1324.

20. P.A. Pemberton, P.E. Stein, M.B. Pepys, J.M. Potter, R.W. Carrell, Hormone binding globulins undergo serpin conformational change in inflammation. Nature 336 (6196) (1988) 257-258.

21. I.J. Elenkov, E.L. Webster, D.J. Torpy, G.P. Chrousos, Stress, corticotropin-releasing hormone, glucocorticoids, and the immune/inflammatory response: acute and chronic effects. Ann. N. Y. Acad. Sci. 876 (1999) 1-11; discussion 11-3.

22. S. Rashid, G.F. Lewis, The mechanisms of differential glucocorticoid and mineralocorticoid action in the brain and peripheral tissues. Clin. Biochem. 38 (5) (2005) 401-409.

23. G.P. Chrousos, The role of stress and the hypothalamic-pituitary-adrenal axis in the pathogenesis of the metabolic syndrome: neuro-endocrine and target tissue-related causes. Int. J. Obes. Relat. Metab. Disord. 24 Suppl 2 (2000) S50-5.

24. P.L. Pedersen, Biochemistry, 2nd edit., edited by Donald Voet and Judith G. Voet. New York: Wiley, 1995.

25. Z.N. Canellakis, P.P. Cohen, Purification studies of tyrosine-alpha-ketoglutaric acid transaminase. J. Biol. Chem. 222 (1) (1956) 53-62.

26. E. Schmid, W. Schmid, M. Jantzen, D. Mayer, B. Jastorff, G. Schutz, Transcription activation of the tyrosine aminotransferase gene by glucocorticoids and cAMP in primary hepatocytes. Eur. J. Biochem. 165 (3) (1987) 499-506.

27. J.E. Bodwell, L.A. Kingsley, J.W. Hamilton, Arsenic at very low concentrations alters glucocorticoid receptor (GR)-mediated gene activation but not GR-mediated gene repression: complex dose-response effects are closely correlated with levels of activated GR and require a functional GR DNA binding domain. Chem. Res. Toxicol. 17 (8) (2004) 1064-1076.

28. D.J. Prieur, J.R. Gorham, R.K. Wood, Distribution of tyrosine aminotransferase activity in mink (Mustela vison). Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 130 (2) (2001) 251-256.

29. H. Mitsuyoshi, T. Nakashima, K. Inaba, H. Ishikawa, Y. Nakajima, Y. Sakamoto, M. Matsumoto, T. Okanoue, K. Kashima, Ursodeoxycholic acid enhances glucocorticoid-induced

tyrosine aminotransferase-gene expression in cultured rat hepatocytes. Biochem. Biophys. Res. Commun. 240 (3) (1997) 732-736.

30. P. Kheolamai, A.J. Dickson, Liver-enriched transcription factors are critical for the expression of hepatocyte marker genes in mES-derived hepatocyte-lineage cells. BMC Mol. Biol. 10 (2009) 35.

31. M.S. Miller, G.S. Buzard, A.E. McDowell, In vivo inhibition of glucocorticoid-inducible gene expression by dimethylnitrosamine in rat liver. Biochem. Pharmacol. 45 (7) (1993) 1465-1470.

32. A. Courtois, L. Payen, A. Guillouzo, O. Fardel, Up-regulation of multidrug resistance-associated protein 2 (MRP2) expression in rat hepatocytes by dexamethasone. FEBS Lett. 459 (3) (1999) 381-385.

33. D.C. DuBois, Z.X. Xu, L. McKay, R.R. Almon, N. Pyszcznski, W.J. Jusko, Differential dynamics of receptor down-regulation and tyrosine aminotransferase induction following glucocorticoid treatment. J. Steroid Biochem. Mol. Biol. 54 (5-6) (1995) 237-243.

34. M. Einstein, M. Greenlee, G. Rouen, A. Sitlani, J. Santoro, C. Wang, S. Pandit, P. Mazur, I. Smalera, A.P. Weaver, Y.Y. Zeng, L. Ge, T. Kelly, T. Paiva, W. Geissler, R.T. Mosley, J. Williamson, A. Ali, J. Balkovec, G. Harris, Selective glucocorticoid receptor nonsteroidal ligands completely antagonize the dexamethasone mediated induction of enzymes involved in gluconeogenesis and glutamine metabolism. J. Steroid Biochem. Mol. Biol. 92 (5) (2004) 345-356.

35. D.C. DuBois, Z.X. Xu, L. McKay, R.R. Almon, N. Pyszcznski, W.J. Jusko, Differential dynamics of receptor down-regulation and tyrosine aminotransferase induction following glucocorticoid treatment. J. Steroid Biochem. Mol. Biol. 54 (5-6) (1995) 237-243.

36. M.J. Park, H.J. Kong, H.Y. Kim, H.H. Kim, J.H. Kim, J.H. Cheong, Transcriptional repression of the gluconeogenic gene PEPCK by the orphan nuclear receptor SHP through inhibitory interaction with C/EBPalpha. Biochem. J. 402 (3) (2007) 567-574.

37. E. Inoue, J. Yamauchi, AMP-activated protein kinase regulates PEPCK gene expression by direct phosphorylation of a novel zinc finger transcription factor. Biochem. Biophys. Res. Commun. 351 (4) (2006) 793-799.

38. E. Chichelnitskiy, A. Vegiopoulos, M.B. Diaz, A. Ziegler, A. Kleiman, A. Rauch, J. Tuckermann, S. Herzig, In vivo phosphoenolpyruvate carboxykinase promoter mapping identifies disrupted hormonal synergism as a target of inflammation during sepsis in mice. Hepatology 50 (6) (2009) 1963-1971.

39. T.A. Pedersen, O. Bereshchenko, S. Garcia-Silva, O. Ermakova, E. Kurz, S. Mandrup, B.T. Porse, C. Nerlov, Distinct C/EBPalpha motifs regulate lipogenic and gluconeogenic gene expression in vivo. EMBO J. 26 (4) (2007) 1081-1093.

40. E.A. Park, W.J. Roesler, J. Liu, D.J. Klemm, A.L. Gurney, J.D. Thatcher, J. Shuman, A. Friedman, R.W. Hanson, The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). Mol. Cell. Biol. 10 (12) (1990) 6264-6272.

41. M.K. Short, D.E. Clouthier, I.M. Schaefer, R.E. Hammer, M.A. Magnuson, E.G. Beale, Tissue-specific, developmental, hormonal, and dietary regulation of rat phosphoenolpyruvate carboxykinase-human growth hormone fusion genes in transgenic mice. Mol. Cell. Biol. 12 (3) (1992) 1007-1020.

42. X.L. Wang, B. Herzog, M. Waltner-Law, R.K. Hall, M. Shiota, D.K. Granner, The synergistic effect of dexamethasone and all-trans-retinoic acid on hepatic phosphoenolpyruvate carboxykinase gene expression involves the coactivator p300. J. Biol. Chem. 279 (33) (2004) 34191-34200.

43. R.K. Hall, X.L. Wang, L. George, S.R. Koch, D.K. Granner, Insulin represses phosphoenolpyruvate carboxykinase gene transcription by causing the rapid disruption of an active transcription complex: a potential epigenetic effect. Mol. Endocrinol. 21 (2) (2007) 550-563.

44. P. Dewint, V. Gossye, K. De Bosscher, W. Vanden Berghe, K. Van Beneden, D. Deforce, S. Van Calenbergh, U. Muller-Ladner, B. Vander Cruyssen, G. Verbruggen, G. Haegeman, D. Elewaut, A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. J. Immunol. 180 (4) (2008) 2608-2615.

45. D.H. Lee, D.R. Jacobs Jr, Association between serum gamma-glutamyltransferase and C-reactive protein. Atherosclerosis 178 (2) (2005) 327-330.

46. M. Irie, N. Suzuki, T. Sohda, A. Anan, K. Iwata, Y. Takeyama, H. Watanabe, P. Fischer, J.E. Scherberich, S. Sakisaka, Hepatic expression of gamma-glutamyltranspeptidase in the human liver of patients with alcoholic liver disease. Hepatology Research 37 (11) (2007) 966-973.

47. M.W. Lieberman, R. Barrios, B.Z. Carter, G.M. Habib, R.M. Lebovitz, S. Rajagopalan, A.R. Sepulveda, Z.Z. Shi, D.F. Wan, gamma-Glutamyl transpeptidase. What does the organization and expression of a multipromoter gene tell us about its functions? Am. J. Pathol. 147 (5) (1995) 1175-1185.

48. S. Reuter, M. Schnekenburger, S. Cristofanon, I. Buck, M.H. Teiten, S. Daubeuf, S. Eifes, M. Dicato, B.B. Aggarwal, A. Visvikis, M. Diederich, Tumor necrosis factor alpha induces gamma-glutamyltransferase expression via nuclear factor-kappaB in cooperation with Sp1. Biochem. Pharmacol. 77 (3) (2009) 397-411.

49. H. Zhang, H.J. Forman, Redox regulation of gamma-glutamyl transpeptidase. Am. J. Respir. Cell Mol. Biol. 41 (5) (2009) 509-515.

50. T. Bose, V.S. Voruganti, M.E. Tejero, J.M. Proffitt, L.A. Cox, J.L. VandeBerg, M.C. Mahaney, J. Rogers, J.H. Freeland-Graves, S.A. Cole, A.G. Comuzzie, Quantitative loci regulating plasma levels of gamma glutamyl transferase and albumin and their genetic correlations with cardiovascular risk factors. Exp. Biol. Med. (Maywood) 234 (12) (2009) 1519-1524.

51. E.H. Harris, Elevated Liver Function Tests in Type 2 Diabetes. Clinical Diabetes 23 (3) (2005) 115-119.

52. R. Barouki, M.N. Chobert, M.C. Billon, J. Finidori, R. Tsapis, J. Hanoune, Glucocorticoid hormones increase the activity of gamma-glutamyltransferase in a highly differentiated hepatoma cell line. Biochim. Biophys. Acta 721 (1) (1982) 11-21.

53. M.N. Chobert, G. Grondin, A. Brouillet, Y. Laperche, A.R. Beaudoin, Control of gamma-glutamyl transpeptidase expression by glucocorticoids in the rat pancreas. Correlation with granule formation. J. Biol. Chem. 271 (21) (1996) 12431-12437.

54. P.A. Knolle, E. Loser, U. Protzer, R. Duchmann, E. Schmitt, K.H. zum Buschenfelde, S. Rose-John, G. Gerken, Regulation of endotoxin-induced IL-6 production in liver sinusoidal endothelial cells and Kupffer cells by IL-10. Clin. Exp. Immunol. 107 (3) (1997) 555-561.

55. J. Van Snick, Interleukin-6: an overview. Annu. Rev. Immunol. 8 (1990) 253-278.

56. N. Muramami, J. Fukata, T. Tsukada, H. Kobayashi, O. Ebisui, H. Segawa, S. Muro, H. Imura, K. Nakao, Bacterial lipopolysaccharide-induced expression of interleukin-6 messenger ribonucleic acid in the rat hypothalamus, pituitary, adrenal gland, and spleen. Endocrinology 133 (6) (1993) 2574-2578.

57. D.R. Germolec, R.P. Frawley, E. Evans, Markers of inflammation. Methods Mol. Biol. 598 (2010) 53-73.

58. M.I. Ndubuisi, K. Patel, R.J. Rayanade, A. Mittelman, L.T. May, P.B. Sehgal, Distinct classes of chaperoned IL-6 in human blood: differential immunological and biological availability. J. Immunol. 160 (1) (1998) 494-501.

59. L.T. May, R. Neta, L.L. Moldawer, J.S. Kenney, K. Patel, P.B. Sehgal, Antibodies chaperone circulating IL-6. Paradoxical effects of anti-IL-6 "neutralizing" antibodies in vivo. J. Immunol. 151 (6) (1993) 3225-3236.

60. L.T. May, M.I. Ndubuisi, K. Patel, D. Garcia, Interleukin-6 chaperones in blood. Ann. N. Y. Acad. Sci. 762 (1995) 120-128.

61. C. Gabay, Interleukin-6 and chronic inflammation. Arthritis Res. Ther. 8 Suppl 2 (2006) S3.

62. P.C. Heinrich, J.V. Castell, T. Andus, Interleukin-6 and the acute phase response. Biochem. J. 265 (3) (1990) 621-636.

63. H. Schuett, M. Luchtefeld, C. Grothusen, K. Grote, B. Schieffer, How much is too much? Interleukin-6 and its signalling in atherosclerosis. Thromb. Haemost. 102 (2) (2009) 215-222.

64. A. Emptoz-Bonneton, J.C. Crave, H. LeJeune, C. Brebant, M. Pugeat, Corticosteroid-binding globulin synthesis regulation by cytokines and glucocorticoids in human hepatoblastoma-derived (HepG2) cells. J. Clin. Endocrinol. Metab. 82 (11) (1997) 3758-3762.

65. H.H. Petersen, T.K. Andreassen, T. Breiderhoff, J.H. Brasen, H. Schulz, V. Gross, H.J. Grone, A. Nykjaer, T.E. Willnow, Hyporesponsiveness to glucocorticoids in mice genetically deficient for the corticosteroid binding globulin. Mol. Cell. Biol. 26 (19) (2006) 7236-7245.

66. L. Bartalena, G.L. Hammond, A. Farsetti, I.L. Flink, J. Robbins, Interleukin-6 inhibits corticosteroid-binding globulin synthesis by human hepatoblastoma-derived (Hep G2) cells. Endocrinology 133 (1) (1993) 291-296.

67. D.A. Underhill, G.L. Hammond, Cis-Regulatory Elements within the Proximal Promoter of the Rat Gene Encoding Corticosteroid-Binding Globulin. Gene 162 (2) (1995) 205-211.

68. X.F. Zhao, D.A. Underhill, G.L. Hammond, Hepatic nuclear proteins that bind cis-regulatory elements in the proximal promoter of the rat corticosteroid-binding globulin gene. Mol. Cell. Endocrinol. 126 (2) (1997) 203-212.

69. J.M. Fernandez-Real, M. Pugeat, M. Grasa, M. Broch, J. Vendrell, J. Brun, W. Ricart, Serum corticosteroid-binding globulin concentration and insulin resistance syndrome: a population study. J. Clin. Endocrinol. Metab. 87 (10) (2002) 4686-4690.

70. M.K. Ganapathi, D. Rzewnicki, D. Samols, S.L. Jiang, I. Kushner, Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep 3B cells. J. Immunol. 147 (4) (1991) 1261-1265.

71. C.M. Uhlar, A.S. Whitehead, Serum amyloid A, the major vertebrate acute-phase reactant. Eur. J. Biochem. 265 (2) (1999) 501-523.

72. L. Scheja, B. Heese, H. Zitzer, M.D. Michael, A.M. Siesky, H. Pospisil, U. Beisiegel, K. Seedorf, Acute-phase serum amyloid A as a marker of insulin resistance in mice. Exp. Diabetes Res. 2008 (2008) 230837.

73. S. Hua, C. Song, C.L. Geczy, S.B. Freedman, P.K. Witting, A role for acute-phase serum amyloid A and high-density lipoprotein in oxidative stress, endothelial dysfunction and atherosclerosis. Redox Rep. 14 (5) (2009) 187-196.

74. J.C. Betts, J.K. Cheshire, S. Akira, T. Kishimoto, P. Woo, The role of NF-kappa B and NF-IL6 transactivating factors in the synergistic activation of human serum amyloid A gene expression by interleukin-1 and interleukin-6. J. Biol. Chem. 268 (34) (1993) 25624-25631.

75. C.F. Thorn, A.S. Whitehead, Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. J. Immunol. 169 (1) (2002) 399-406.

76. J.E. Volanakis, Human C-reactive protein: expression, structure, and function. Mol. Immunol. 38 (2-3) (2001) 189-197.

77. H.A. Kim, H.Y. Chun, S.H. Kim, H.S. Park, C.H. Suh, C-reactive protein gene polymorphisms in disease susceptibility and clinical manifestations of Korean systemic lupus erythematosus. J. Rheumatol. 36 (10) (2009) 2238-2243.

78. Y.S. Choi, J. Hur, S. Jeong, Beta-catenin binds to the downstream region and regulates the expression C-reactive protein gene. Nucleic Acids Res. 35 (16) (2007) 5511-5519.

79. P.P. Singh, B. Voleti, A. Agrawal, A novel RBP-J kappa-dependent switch from C/EBP beta to C/EBP zeta at the C/EBP binding site on the C-reactive protein promoter. J. Immunol. 178 (11) (2007) 7302-7309.

80. S. Zhang, Q. Liu, J. Wang, D.C. Harnish, Suppression of interleukin-6-induced C-reactive protein expression by FXR agonists. Biochem. Biophys. Res. Commun. 379 (2) (2009) 476-479.

81. M.K. Ganapathi, L.T. May, D. Schultz, A. Brabenec, J. Weinstein, P.B. Sehgal, I. Kushner, Role of interleukin-6 in regulating synthesis of C-reactive protein and serum amyloid A in human hepatoma cell lines. Biochem. Biophys. Res. Commun. 157 (1) (1988) 271-277.

82. A. Angeli, R.G. Masera, M.L. Sartori, N. Fortunati, S. Racca, A. Dovio, A. Staurenghi, R. Frairia, Modulation by cytokines of glucocorticoid action. Ann. N. Y. Acad. Sci. 876 (1999) 210-220.

83. A.V. Turnbull, C.L. Rivier, Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiol. Rev. 79 (1) (1999) 1-71.

84. J.V. Castell, M.J. Gomez-Lechon, M. David, T. Hirano, T. Kishimoto, P.C. Heinrich, Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. FEBS Lett. 232 (2) (1988) 347-350.

85. C. Smith, N.W. Wilson, A. Louw, K.H. Myburgh, Illuminating the interrelated immune and endocrine adaptations after multiple exposures to short immobilization stress by in vivo blocking of IL-6. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292 (4) (2007) R1439-47.

86. I. Zvibel, S. Brill, R. Kariv, A. Traister, T. Golan, J. Chebath, Z. Halpern, M. Revel, R. Oren, Chimeric molecule IL-6/soluble IL-6 receptor is a potent mitogen for fetal hepatocytes. J. Cell. Physiol. 200 (2) (2004) 245-252.

87. M. Hill, R. McCallum, Altered transcriptional regulation of phosphoenolpyruvate carboxykinase in rats following endotoxin treatment. J. Clin. Invest. 88 (3) (1991) 811-816.

88. D.H. Lee, D.R. Jacobs Jr, M. Gross, C.I. Kiefe, J. Roseman, C.E. Lewis, M. Steffes, Gamma-glutamyltransferase is a predictor of incident diabetes and hypertension: the Coronary Artery Risk Development in Young Adults (CARDIA) Study. Clin. Chem. 49 (8) (2003) 1358-1366.

89. T. Stojakovic, H. Scharnagl, M. Trauner, B. Pieske, B. Wellnitz, U. Seelhorst, D. Schilling, B.R. Winkelmann, B.O. Boehm, W. Marz, Serum gamma-glutamyl transferase and mortality in persons undergoing coronary angiography-The Ludwigshafen Risk and Cardiovascular Health Study. Atherosclerosis (2009).

90. F. Gude, J. Rey-Garcia, C. Fernandez-Merino, L. Meijide, L. Garcia-Ortiz, C. Zamarron, A. Gonzalez-Quintela, Serum levels of gamma-glutamyl transferase are associated with markers of nocturnal hypoxemia in a general adult population. Clin. Chim. Acta 407 (1-2) (2009) 67-71.

91. A. Fukumura, M. Tsutsumi, M. Tsuchishima, N. Hayashi, M. Fukura, H. Yano, K. Ozaki, S. Takase, Effect of the inducer of interleukin-6 (ME3738) on rat liver treated with ethanol. Alcohol. Clin. Exp. Res. 31 (1 Suppl) (2007) S49-53.

92. D. Duma, C.M. Jewell, J.A. Cidlowski, Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. J. Steroid Biochem. Mol. Biol. 102 (1-5) (2006) 11-21.

93. B.M. Necela, J.A. Cidlowski, Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells. Proc. Am. Thorac. Soc. 1 (3) (2004) 239-246.

94. R.H. Oakley, J.C. Webster, M. Sar, C.R. Parker Jr, J.A. Cidlowski, Expression and subcellular distribution of the beta-isoform of the human glucocorticoid receptor. Endocrinology 138 (11) (1997) 5028-5038.

95. C.M. Bamberger, A.M. Bamberger, M. de Castro, G.P. Chrousos, Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. J. Clin. Invest. 95 (6) (1995) 2435-2441.

96. C. Otto, H.M. Reichardt, G. Schutz, Absence of glucocorticoid receptor-beta in mice. J. Biol. Chem. 272 (42) (1997) 26665-26668.

97. R.H. Oakley, J.A. Cidlowski, Homologous down regulation of the glucocorticoid receptor: the molecular machinery. Crit. Rev. Eukaryot. Gene Expr. 3 (2) (1993) 63-88.

98. V. Gossye, D. Elewaut, K. Van Beneden, P. Dewint, G. Haegeman, K. De Bosscher, A plant-derived glucocorticoid receptor modulator attenuates inflammation without provoking ligand-induced resistance. Ann. Rheum. Dis. (2009).

99. M. Beato, M. Truss, S. Chavez, Control of transcription by steroid hormones. Ann. N. Y. Acad. Sci. 784 (1996) 93-123.

100. K. De Bosscher, W. Vanden Berghe, G. Haegeman, The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr. Rev. 24 (4) (2003) 488-522.

101. D.K. Scott, P.E. Stromstedt, J.C. Wang, D.K. Granner, Further characterization of the glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. The role of the glucocorticoid receptor-binding sites. Mol. Endocrinol. 12 (4) (1998) 482-491.

102. M.S. Bradshaw, M.J. Tsai, B.W. O'Malley, A steroid response element can function in the absence of a distal promoter. Mol. Endocrinol. 2 (12) (1988) 1286-1293.

103. D. Pearce, K.R. Yamamoto, Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. Science 259 (5098) (1993) 1161-1165.

104. J. Charron, J. Drouin, Glucocorticoid inhibition of transcription from episomal proopiomelanocortin gene promoter. Proc. Natl. Acad. Sci. U. S. A. 83 (23) (1986) 8903-8907.

105. I.E. Akerblom, E.P. Slater, M. Beato, J.D. Baxter, P.L. Mellon, Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. Science 241 (4863) (1988) 350-353.

106. J. Liden, I. Rafter, M. Truss, J.A. Gustafsson, S. Okret, Glucocorticoid effects on NF-kappaB binding in the transcription of the ICAM-1 gene. Biochem. Biophys. Res. Commun. 273 (3) (2000) 1008-1014.

107. T. Taga, M. Hibi, Y. Hirata, K. Yamasaki, K. Yasukawa, T. Matsuda, T. Hirano, T. Kishimoto, Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell 58 (3) (1989) 573-581.

108. T. Wuestefeld, C. Klein, K.L. Streetz, U. Betz, J. Lauber, J. Buer, M.P. Manns, W. Muller, C. Trautwein, Interleukin-6/glycoprotein 130-dependent pathways are protective during liver regeneration. J. Biol. Chem. 278 (13) (2003) 11281-11288.

109. G.H. Fey, M. Hattori, G. Hocke, T. Brechner, G. Baffet, M. Baumann, H. Baumann, W. Northemann, Gene regulation by interleukin 6. Biochimie 73 (1) (1991) 47-50.

110. E. Arzt, Gp130 Cytokine Signaling in the Pituitary Gland: a Paradigm for Cytokine-Neuro-Endocrine Pathways. J. Clin. Invest. 108 (12) (2001) 1729-1733.

111. T. Nakajima, S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, S. Akira, Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc. Natl. Acad. Sci. U. S. A. 90 (6) (1993) 2207-2211.

112. L.I. McKay, J.A. Cidlowski, Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr. Rev. 20 (4) (1999) 435-459.

113. Z. Zhang, S. Jones, J.S. Hagood, N.L. Fuentes, G.M. Fuller, STAT3 acts as a co-activator of glucocorticoid receptor signaling. J. Biol. Chem. 272 (49) (1997) 30607-30610.

114. M.V. Gonzalez, J.M. Gonzalez-Sancho, C. Caelles, A. Munoz, B. Jimenez, Hormone-activated nuclear receptors inhibit the stimulation of the JNK and ERK signalling pathways in endothelial cells. FEBS Lett. 459 (2) (1999) 272-276.

115. N.Z. Lu, J.A. Cidlowski, The origin and functions of multiple human glucocorticoid receptor isoforms. Ann. N. Y. Acad. Sci. 1024 (2004) 102-123.

116. J. Kirwan, L. Power, Glucocorticoids: action and new therapeutic insights in rheumatoid arthritis. Curr. Opin. Rheumatol. 19 (3) (2007) 233-237.

117. I.M. Adcock, P.J. Barnes, Molecular mechanisms of corticosteroid resistance. Chest 134 (2) (2008) 394-401.

118. M.J. Coghlan, P.B. Jacobson, B. Lane, M. Nakane, C.W. Lin, S.W. Elmore, P.R. Kym, J.R. Luly, G.W. Carter, R. Turner, C.M. Tyree, J. Hu, M. Elgort, J. Rosen, J.N. Miner, A novel antiinflammatory maintains glucocorticoid efficacy with reduced side effects. Mol. Endocrinol. 17 (5) (2003) 860-869.

119. H. Schacke, A. Schottelius, W.D. Docke, P. Strehlke, S. Jaroch, N. Schmees, H. Rehwinkel, H. Hennekes, K. Asadullah, Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proc. Natl. Acad. Sci. U. S. A. 101 (1) (2004) 227-232.

120. K. De Bosscher, W. Vanden Berghe, I.M. Beck, W. Van Molle, N. Hennuyer, J. Hapgood, C. Libert, B. Staels, A. Louw, G. Haegeman, A fully dissociated compound of plant origin for inflammatory gene repression. Proc. Natl. Acad. Sci. U. S. A. 102 (44) (2005) 15827-15832.

121. A. Louw, P. Swart, S.S. de Kock, K.J. van der Merwe, Mechanism for the stabilization *in vivo* of the aziridine precursor --(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride by serum proteins. Biochem. Pharmacol. 53 (2) (1997) 189-197.

122. S. Wust, D. Tischner, M. John, J.P. Tuckermann, C. Menzfeld, U.K. Hanisch, J. van den Brandt, F. Luhder, H.M. Reichardt, Therapeutic and adverse effects of a non-steroidal glucocorticoid receptor ligand in a mouse model of multiple sclerosis. PLoS One 4 (12) (2009) e8202.

123. Z. Zhang, Z.Y. Zhang, H.J. Schluesener, Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. J. Immunol. 183 (5) (2009) 3081-3091.

CHAPTER 2

EFFECT OF MODULATORS OF INFLAMMATION ON HEPATIC ACUTE PHASE PROTEINS AND METABOLIC ENZYMES

Koch Visser[1], Carine Smith[2] and Ann Louw[1]*

[1]Department of Biochemistry, Stellenbosch University, Stellenbosch, 7600, South Africa and
[2]Department of Physiological Sciences, Stellenbosch University, Stellenbosch, 7600, South Africa

*Corresponding author. Tel: +27 21 8085873; fax +27 21 8085863
E-mail address: al@sun.ac.za (A Louw)

[#]To be submitted to Journal of Steroid Biochemistry and Molecular Biology

For the purpose of this thesis the methods and discussion sections have been expanded. In addition, 2 addendums, Addendum A and B, have been added. As this section was written in manuscript form there will necessarily be some overlap and repetition with Chapters 1 and 3.

[#]Poster presentation of some of the findings of this study was presented at:

1. K. Visser, C. Smith, and A. Louw (2008) Modulation of Glucocrticoid Action by Interleukin 6 in the Liver. Multidisciplinary conference supported by the South African Society for Microbiology, Biotech SA and the South African Society for Biochemistry and Molecular Biology, Grahamstown.

2.1 Abstract

Crosstalk exists between the stress- and immune-system and this crosstalk has pharmacological importance in the use of glucocorticoids (GC's) as anti-inflammatory drugs for diseases such as asthma and arthritis. The focus of studies on this crosstalk has mainly been on the effects of GC's on immune function. The effect of modulators of the immune system on GC action, especially in the periphery, is not as well studied. The liver plays an important role in inflammation and stress in producing the acute phase proteins (APP's) required for resolution of inflammation as well as in producing systemic glucose, through gluconeogenesis, required to fuel the stress responses. Understanding the effects of stress and inflammation and their interplay in the liver is thus not only useful to expand our understanding of these systems but could also have clinical applications in understanding the side-effects associated with pharmacological use of GC's. The current study investigated effects of GC's, including a dissociated GC, Compound A (CpdA), and interleukin 6 (IL6) on the glucocorticoid receptor (GR) and also on three metabolic genes and three APP genes in a mouse hepatoma cell line, BWTG3. The metabolic proteins studied were tyrosine aminotransferase (TAT), phosphoenolpyruvate carboxykinase (PEPCK), and gamma glutamyltransferase (GGT) while the APP's studied were serum amyloid A (SAA), C-reactive protein (CRP), and corticosteroid binding globulin (CBG). The study investigated effects at protein level, with Western blotting and ELISA assays, at the level of protein activity, with enzyme activity assays and whole cell binding, and at the mRNA level, with quantitive PCR (qPCR). The study shows that the GC, dexamethasone (Dex), and IL6 generally have divergent effects on the GR and metabolic enzymes while their functions are convergent on the acute phase proteins. In contrast to Dex, CpdA was found to up-regulate GR and down-regulate the metabolic enzymes while, similarly to Dex, to up-regulate acute phase proteins.

2.2 Introduction

During stress two major stress response pathways are activated, the hypothalamic-pituitary-adrenal (HPA) axis and the locus ceruleus - norepinephrine (LC/NE) system [1]. Stimulation of the HPA-axis leads to the systemic secretion of GCs, such as cortisol in humans and corticosterone in rodents, by the adrenal cortex [2,3] while, activation of the LC/NE system will result in the release of pro-inflammatory cytokines, like interleukin-6 (IL6), from a variety of cells and tissues [1]. The secreted GCs and IL6 will move to target organs, like the liver, in the periphery, where they will exert their effect [3-6].

At their site of action the lipophyllic GCs move across the cell membrane and interact with cytoplasmic glucocorticoid receptors (GR) [7]. The ligand bound receptor translocates to the nucleus of the cell where it can either activate or repress the transcription of GC responsive genes [8]. Generally transactivation is mediated by a GR homodimer binding to specific glucocorticoid response elements (GREs). Transrepression, on the other hand, is generally mediated via a tethering mechanism where the GR does not directly bind to the DNA but binds as a monomer to other transcription factors, such as nuclear factor-κB (NF-κB), CAAT/enhancer binding protein (C/EBP), and STAT, thereby repressing transcription [3,9].

In contrast, IL6 binds to a membrane bound IL6 receptor (IL-6R), and associates with gp130, triggering the homodimerization of gp130 which activates the JAK-STAT-pathway (Janus kinase - signal transducers and activators of transcription – pathway) that results in phosphorylated STAT3 dimers moving across the nuclear membrane and binding to specific response elements in the promoters of IL6 responsive genes [10]. Activation of the IL6 receptor can also lead to phosphorylation of Grb2-associated binder-1 (Gab1), which through interaction with SH2-domin-containing tyrosine phosphatase (SHP2) and phosphoinositide 3-kinase (PI3K) activates extracellular-regulated kinase 2 (ERK2) [11]. ERK2 in turn phosphorylates C/EPB which

translocates to the nucleus and interacts with response elements in the promotor region of IL6 target genes [12].

GCs are known to have a profound effect on the immune system. It has, however, become apparent that this effect is bi-directional and that effectors of the immune system, such as IL6, are capable of modulating the actions of GCs [13]. In addition, the effect of GCs on IL6 production and action in cells and organs that are traditionally associated with inflammation like, T cells and B cells, are well studied [14]. The effect of GCs on IL6 actions in non inflammatory cells and organs, like the liver, where IL6 is produced by Kupfer cells and which is an important organ for the hepatic acute phase response [15], is, however, not as well known.

In the liver GCs elicit a wide range of metabolic effects which include increased transcription of metabolic genes [3], like tyrosine aminotransferase (TAT) [16-20], phosphoenolpyruvate carboxykinase (PEPCK) [21-23], and gamma-glutamyltransferase (GGT) [24,25], which are all involved in gluconeogenesis. IL6 antagonizes the action of GCs, not only at the level of the GR [26], but also on TAT [26,27], and PEPCK [28,29]. Furthermore, increased GGT levels have been shown to be associated with elevated IL6 levels [30,31]. In addition GC's down-regulate the production of IL6 [14].

IL6 will activate the positive acute phase response in the liver by stimulating the transcription of acute phase protein (APP) genes including serum amyloid A (SAA) [32,33] and C-reactive protein (CRP) [34,35]. However, CBG, a negative acute phase protein, is repressed by IL6 [36,37]. Like IL6, GC's up-regulate some APPs, such as SAA and CBG [38,39] and can also synergize with IL6 in upregulating SAA [33,39,40], CRP [40,41] as well as CBG [36].

Synthetic GCs developed for therapeutic use are among the most widely prescribed drugs in the world for the treatment of immune and inflammatory diseases, such as asthma and rheumatoid arthritis. The long term use of GCs for therapeutic purposes are, however, limited by adverse side

effects of long term GC use, such as reduced muscle mass and repair, insulin resistance, fat deposition, growth failure, osteoporosis and the suppression of the HPA axis, in addition to the development of GC resistance [42,43]. The positive therapeutic qualities of GCs may be attributed to the repression of pro-inflammatory genes, whilst the side effects have traditionally been attributed to the activation of genes, like the liver metabolic enzyme genes. Thus it has been suggested that compounds that transrepress, but do not transactivate, GC-inducible genes may retain anti-infammatory action while displaying less side effects [42]. Compound A (CpdA), 2-(4-acetoxyphenol)-2chloro-*N*-methyl-ethylammonium chloride [44], appears to be such a compound in that it retains anti-inflammatory action without inducing side effects such as hyperglycemia [45,46]. In addition, CpdA does not cause the homologues downregulation of GR [47] even though it binds to the GR [45]. Apart from CpdA, other compounds that display dissociative properties have also been identified: AL-438 [48] and ZK 209614 [49].

In a mouse hepatic cell line we investigated the effect of dexamethasone (Dex), a potent synthetic GR agonist, and CpdA, a selective GR modulator (SGRM), in the presence or absence of IL6, a pro-inflammatory cytokine. We evaluated the effects on the GR, the metabolic enzymes, TAT, PEPCK, and GGT, and acute phase proteins, SAA, CRP, and CBG, on a protein and protein activity level as well as mRNA level.

2.3 Material and methods

2.3.1 Test Compounds

Dex was obtained from Sigma, recombinant murine IL6, (cat# 216-16, 10µg) from Peprotech Inc. and Compound A (CpdA; 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride) was synthesized as described by Louw *et al.* [44]. Dex and CpdA stock solutions were prepared in

ethanol. Murine IL6 was reconstituted in sterile bovine serum albumin-PBS (0.2%) to a concentration of 100µg/ml.

2.3.2 Cell Culture

BWTG3 mouse hepatoma cells (LEGEST, University of Gent, Belgium), which naturally express GRα, were maintained in high glucose (4.5g/ml) Dulbecco's modified eagles's medium (DMEM) (Sigma) supplemented with 10% FCS (Highveld Biologicals, South Africa), 100 IU/ml penicillin and 100µg/ml streptomycin (Gibco), 2mM glutamine (Merck), 44mM sodium-bicarbonate (Gibco), 1mM sodiumpyruvate (Gibco), and 0.1mM non essential amino acids (Gibco). All cells were maintained in a humidified cell incubator, set at 97% relative humidity and 5% CO_2 at 37˚C.

Cells were seeded into tissue culture plates and incubated for 24 hours. The next day medium was changed to DMEM containing 10% charcoal treated FCS and 1% penicillin and streptomycin mixture and the cells were steroid starved for 24 hours. Cells were then induced for 24 hours with ethanol, 10^{-8}M Dex or 10^{-5}M CpdA. Designated cells were induced with 10 000pg/ml IL6 or bovine serum albumin-PBS (0.2%) as control, 21 hours after induction with ethanol, Dex or CpdA.

2.3.3 Whole Cell Binding Assay

BWTG3 cells were seeded into 24-well tissue culture plates at a density of 2.5 x 10^5 cells/well and treated as described in section 2.3.2. Medium was aspirated after the induction period and wells washed three times at room temperature for 15 minutes while shaking with pre-warmed bovine serum albumin-PBS (0.2%) to remove bound and free ligand. After the washout has been completed all wells were washed three times with pre-warmed PBS at room temperature to remove albumin.

After washing, DMEM (minus FCS or penicillin and streptomycin mixture) containing 20nM ^{3}H-Dex (specific activity of 89Ci/mmol; AEC-Amersham) plus (non specific binding) or minus (total binding) 1000 fold unlabelled Dex was added to wells and cells were incubated at 37˚C for 2 hours. Plates were then placed on ice, the medium aspirated, and all wells washed three times with ice cold bovine serum albumin-PBS (0.2%) for 15 minutes at 4˚C to remove free ligand. One hundred microliters of lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA and 1.44mM EDTA) was then added to each well, after which the plates were shaken at room temperature for 15 min and frozen overnight at -20˚C.

On thawing of samples, 5µl of lysate from each well was used for protein determination using the Bradford method [50]. The remaining lysate was transferred to scintillation vials to which 1.5ml of scintillation fluid (Quickszint FLOW 2, Zinsser Analytic, South Africa) had been added. Radioactivity was determined using a Beckman LS 3801 Beta-scintillation counter. Results were normalised for protein content and specific binding (total binding – non specific binding) expressed as fmol GR/g protein. All binding experiments included a control for ligand depletion which was less than 10% for all experiments. Counting efficiency was 37.52%.

2.3.4 Western Blot

BWTG3 cells were seeded into 6-well tissue culture plates at a density of 2.5 x 10^{5} cells/well and treated as described in section 2.3.2. After induction, cells were placed on ice and washed once with ice cold PBS. Two hundred microliters of lysis buffer A (10mM Hepes pH 7.5 (Gibco, Invitrogen Corporation), 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP-40 (Roche) and Complete Mini protease inhibitor cocktail (Roche) were then added to each well after which the plates were shaken on ice for 15 min and frozen overnight at -20˚C.

On thawing of samples the wells were scraped on ice and lysate transferred to 1.5ml Eppendorf tubes on ice. The lysate was then centrifuged for 10 min at 12 000 x g at 4˚C and the cleared lysate was transferred to 1.5ml Eppendorf tubes on ice, alliquoted and stored at -20˚C until assayed.

Lysates (20µl) were separated on a 10% SDS-PAGE gel. Following electrophoresis, proteins were electro-blotted and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences), which was probed for GR (sc-8992 antibody from Santa Cruz Biotechnology diluted 1:1000), CBG (S1000-76Z antibody from US Biological diluted 1:250), MR (sc-11412 antibody from Santa Cruz Biotechnology diluted 1:200), ERα (sc-542 antibody from Santa Cruz Biotechnology diluted 1:200), PR (sc-539 antibody from Santa Cruz Biotechnology diluted 1:1000), or AR (sc-13062 antibody from Santa Cruz Biotechnology diluted 1:1000) and actin (sc-1616-R (rabbit) or sc-1616 (goat) antibody from Santa Cruz Biotechnology diluted 1:250). Proteins were visualized using ECL Peroxidase labelled anti-rabbit antibody (Santa Cruz) for GR (1:10000), MR (1:2000), PR (1:10000), AR (1:10000), ERα (1:5000), and actin (1:10000), or anti-goat antibody (Jackson Immuno Research Laboratories) for CBG and actin (1:20 000), and ECL Western blotting detection reagents (GE Healthcare Amersham) on Hyperfilm (Amersham Biosciences). For determination of GR or CBG levels Hyperfilm bands of GR, CBG and actin were quantified using UN-SCAN-IT and results expressed as the intensity of the GR or CBG band relative to the actin band.

2.3.5 Metabolic Enzyme Assays

BWTG3 cells were seeded into 6-well tissue culture plates at a density of 2.5 x 10^5 cells/well and treated as described in section 2.3.2. After induction cells were placed on ice and washed once with ice cold PBS. Two hundred microliters of lysis buffer (10mM Tris-HCl, pH 7.5, 0.25M sucrose, 0.1mM phenylmethylsulfonyl fluoride (PMSF), 10µg/ml aprotinin, and Complete Mini protease

inhibitor cocktail (Roche)) was then added to each well after which the plates were shaken on ice for 15 min and frozen overnight at -20˚C.

On thawing of samples the wells were scraped on ice and lysate transferred to 1.5ml Epppendorf tubes on ice. The lysate was then centrifuged for 10 min at 12 000 x g at 4˚C and the cleared lysate was transferred to 1.5ml Eppendorf tubes on ice, alliquoted and stored at -20˚C until assayed.

2.3.5.1 TAT Assay

Determination of TAT activity was done with the method by Diamondstone [51] and spectrophotometrically measures the conversion of 4-hydroxyphenylpyruvate (pHpp) to p-hydroxybenzylaldehyde (pHBA). pHpp is formed when adding TAT to a solution of tyrosine, α-ketoglutarate, and L-glutamate. Stock solutions prepared for this assay were as follow: 10M KOH, 0.125M KH_2PO_4, pH 7.6, 7mM L-Tyrosine (Merck) in KH_2PO_4, pH 6.8, 0.5M α-ketoglutarate (α-KG) (Sigma), pH 7.0, and 5mM pyridoxal-5'-phosphate (PLP) (Sigma), pH 6.5. A reaction mixture is prepared for each sample containing 160µl 7mM L-Tyrosine, 4µl 0.5 M α-KG, 2µl 5 mM PLP, and 20µl sample. For each sample an additional blank is prepared by adding 14µl 10M KOH (stop solution) to the reaction mixture before adding the sample.

The sample mixtures and blanks were incubated at 37˚C for 15 minutes. At the end of the reaction time 14µl of 10M KOH was added to the reaction mixture to stop the reaction and the mixture was left at 37˚C for 30 minutes to allow for the conversion of pHPP to pHBA. The absorbance of the reaction mixtures and the blanks were then read at 330nm on a BioTek® PowerWave 340 spectrophotometer.

2.3.5.2 PEPCK Assay

The assay is based on the rate of NADH oxidation in the presence of malate dehydrogenase, which uses oxaloacetate, the product of PEPCK conversion of phosphoenolpyruvate, as substrate and may be determined spectrophotometrically with the method of Chang *et al.* [52]. A solution was made containing 50µl cell lysate, 10µl NADH (0.16mM), 5µl $NaHCO_3$ (1mM), 5µl malate dehydrogenase (5U/ml), 5µl glutathione (GSH) (0.5mM), 5µl guanosine diphosphate (GDP) (1mM), 15µl Hepes (3mM) containing 1mM $MnCl_2$, pH6.6. The reaction is allowed 10 minutes to stabilize and for NADH cycling to stop and then 5µl phosphoenolpyruvate (PEP) (1mM) is added to the mixture. NADH oxidation is spectrophotometrically followed for 10 minutes at 340 nm on a BioTek® PowerWave 340 spectrophotometer. The change in absorbance over time for the initial velocity is determined and plotted.

2.3.5.3 GGT Assay

The assay is based on the colour reaction of enzymatically formed glutamohydromaxic (GHA) acid with ferric chloride and is adapted from the method used by Lipmann *et al.* [53]. A solution of 500µl lysate, 500ul hydroxylamine solution (4M hydroxylamine hydrochloride (Sigma), 3.5M NaOH), pH 6.4, and 500µl acetate buffer (0.1M acetic acid, 0.1M Sodium Acetate), pH 5.4, is prepared and incubated at 37˚C for 10 min. After incubation, 500µl of each of the following solutions are added to the mixture: HCL (1:30 diltion), 12% trichloroacetic acid, and 5% ferric chloride (Sigma) in 0.1M HCl. Absorbance is measured after a 20 minute incubation at room temperature at 450 nm on a BioTek® PowerWave 340 spectrophotometer.

2.3.6 ELISA Assays

BWTG3 cells were seeded into 6-well tissue culture plates at a density of 2.5 x 10^5 cells/well and treated as described in section 2.3.2. After induction cells were placed on ice and DMEM is

transferred to sterile 1.5ml Eppendorf tubes on ice containing Complete Mini protease inhibitor cocktail (Roche) and frozen at -20˚C until assayed to determine extracellular protein levels. Cells then were washed once with ice cold PBS. Two hundred microliters of lysis buffer (as for metabolic enzyme assays) was then added to each well after which the plates were shaken on ice for 15 min and frozen overnight at -20˚C.

On thawing of samples the wells were scraped on ice and lysate transferred to 1.5ml Epppendorf tubes on ice. The lysate was then centrifuged for 10 min at 12 000 x g at 4˚C and the cleared lysate, representing intracellular protein, was transferred to 1.5ml Eppendorf tubes on ice, alliquoted and stored at -20˚C until assayed.

CRP (DRG International Inc., cat # EIA-4823) and SAA ELISA's (US Biological, cat # A2275-60V) were performed on extra- and intracellular protein, according to the manufacturer's directions with the only adaptation being the inclusion of additional lower concentrations of standards on the standard curves.

2.3.7 RT-PCR

BWTG3 cells were seeded into 12-well tissue culture plates at a density of 1 x 10^5 cells/well and treated as described in section 2.3.2. After induction cells were placed on ice and washed once with ice cold PBS.

2.3.7.1 RNA Isolation

After the PBS wash 400µl Tri reagent was added per well and the plates were frozen overnight at -80˚C. Upon thawing the wells were scraped and the content of the wells transferred to 1.5ml Eppendorf tubes and left at room temperature for 5 min. Chloroform, 80µl, was then added to each sample, vortexed for 15 seconds and centrifuged for 15 min at 14 000 rpm at 4˚C. The aqueous

phase was then transferred to a clean tube on ice and 200µl ice cold isopropanol was added. The tubes were vortexed immediately and left at room temperature for 15 minutes and then centrifuged for 10 minutes at 14 000 rpm at 4˚C. The supernatant was removed and the pellet washed in 500µl 75% ethanol (diluted in diethyl pyrocarbonate (DEPC) treated water) by vortexing for 1 minute and centrifuged for 5 minutes at 8000 rpm at 4˚C. The supernatant was removed and the samples centrifuged again, to remove the last vestiges of ethanol, after which the pellet was left to air dry for 5 to 10 minutes. The dry RNA pellet was then dissolved in 15 µl DEPC treated water. The optical density of the samples was measured at 260nm to determine the concentration of RNA per sample and the integrity of the RNA was anlaysed on a 1% agarose gel. Samples were stored at -80 ˚C.

2.3.7.2 cDNA synthesis

Synthesis of cDNA from 1µg BWTG3 RNA was performed using the ImProm-II™ reverse transcription system (Promega corporation, cat # A3800). The first reaction mixture per nuclease-free, thin-walled 0.5ml tube was as follows: 1µg experimental RNA and 1 µl Oligo$(dT)_{15}$ with the volume adjusted to 5µl with nuclease-free water. The reaction mixture was heated at 70˚C for 5 minutes and immediately chilled in ice-water for 10 minutes. The tubes were briefly centrifuged and 15µl of the second reaction mixture was added to each. The second reaction mixture consisted of: 4µl ImProm-II™ 5X reaction buffer, 4.8µl $MgCl_2$ (25mM), 1µl dNTP mix, 20 units (0.5µl) Recombinant RNasin® ribonuclease inhibitor, ImProm-II™ reverse transcriptase, with the volume adjusted to 15µl with nuclease-free water. Samples were then kept at 25˚C for 5 minutes, 42 ˚C for 60 minutes, and 70˚C for 15 minutes after which they were placed on ice. Optical density of each sample was then determined 280 nm.

2.3.7.3 Quantitive Polymerase Chain Reaction (qPCR)

Isolated RNA from BWTG3 cells were reverse transcribed to cDNA and amplified by means of PCR using primers designed to the target genes. The target genes and the primers used for their amplification is summarized in Table 1. The mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as reference gene as it is not under Dex regulation [54].

A cDNA dilution series, made of an ethanol induced sample, was used to generate a standard curve for all primer sets. Reaction mixtures were prepared in 0.1ml tubes (Corbett Life Science, Cat#3001-002). The reaction mixture is each tube were as follow: 0.5µl forward primer and 0.5µl reverse primer (both 400nM final concentration), 13µl SensiMix™*Plus*SYBR® (Quantace, cat# QT605-05), and 10µl PCR water. The final volume for each reaction was 25µl and each reaction was performed in duplicate (triplicate reactions were used for samples used to generate the standard curve). The tubes were then subjected to qPCR using the Rotor-Gene Q 5plex HRM protocol.

The Rotor-Gene Q 5plex HRM protocol is as follow: Samples are incubated at 95˚C for 10 minutes to activate the polymerase enzyme. Subsequent PCR amplification steps are as follow: Denaturation at 95˚C for 20 seconds, annealing at temperatures as given in Table 1 for 20 seconds, and extension at 72˚C for 30 seconds. Typically there would be 45 repeats of these three PCR steps. A melting curve was generated by varying the temperature between 72˚C and 95˚C, raising the temperature by one degree for each step, while the fluorescence is measured throughout the melting process.

Table 1. Primer sequences and annealing temperatures for genes of interest and reference gene

`	**Forward Primer**	**Reverse Primer**	**Annealing Temperature**
GR	5`-ACCAACGGAGGAGTGTGAAA-3`	5`-GGGGACCCAGCGGAAAAC-3` [55]	54 ˚C
TAT	5`-ACACCTGGAGTGTGGTTCTG-3`	5`-CCCAAGCTGACCTTGAACTCT-3`	54 ˚C
PEPCK	5`-GACAACTGTTGGCTGGCTCT-3`	5`-AGGGCGAGTCTGTCAGTTCA-3`	53 ˚C
GGT	5`-CTCAGAGATTGGACGGGATA-3`	5`-GCATTGATAACCTCAACTTTTCC-3`	50 ˚C
CBG	5`-GCTGGTGTCTTGCTGGTTA-3`	5`-TGACGCAACTCTAGCCACAC-3`	51 ˚C
SAA	5`-CCGTTTCATATAGAGTCGCATC-3`	5`-TCTCCACAAGTCCCAAGTCC-3`	50 ˚C
CRP	5`-ACTTCAGTGCCCGCCAGTTC-3`	5`-AAGCCTTCACTGTGTGTCTC-3` [56]	53 ˚C
GAPDH	5’-ACATGCCGCCTGGAGAAACCT-3’	5’-GCCCAGGATGCCCTTTAGTGG-3’[55]	54 ˚C

2.3.8 Data manipulation and statistical analysis.

The GraphPad Prism® version 5.10 for Windows (GraphPad Software) was used for graphical representations and statistical analysis. One-way ANOVA and Bonferroni's multiple comparison post-test were used for statistical analysis and significance is displayed on the graphs. If effects showed non-significant statistics with One-way ANOVA and multiple comparison post test we additionally did a one-tailed t-test after ANOVA to evaluate individual P-values. As this is a less stringent statistical analysis it was only utilised to discuss a trend towards significance in the text if $P<0.08$. For all experiments the error bars represent the SEM of at least two independent experiments.

2.4 Results

BWTG3 cells were characterised for presence of endogenous steroid receptors (Supplementary Fig. 1A) using Western blotting and the results suggest that these cells contain endogenous GR, estrogen receptor (ER), and progesterone receptor (PR), but no mineralocorticoid receptor (MR) or androgen receptor (AR).

Having shown the presence of endogenous GR, we wanted to determine effects of GC's and IL6 in BWTG3 cells under the same conditions for all experiments. Thus we set out to determine the optimum time of induction and concentration of test compounds. Using whole cell binding we determined that incubation with 10^{-8}M Dex or 10^{-5}M CpdA for 24 hours (Supplementary Fig. 1B) and 3 hours incubation (Supplemetary Fig. 1C) with 10 000pg/ml IL6 was optimal for eliciting significant effects on GR levels. Although it was accepted that the conditions established for GR activity levels may not be optimum to observe changes in all other proteins or mRNA assayed, it was decided to adhere to these conditions so that we could compare results obtained within one system under the same conditions at the same time.

2.4.1 Modulation of GR concentration by Dex or CpdA in the presence or absence of IL6.

The effect of Dex (10^{-8}M) and CpdA (10^{-5}M), in the presence or absence of IL6 (10 000 pg/ml), on GR concentrations was determined at protein activity level using whole cell binding assays (Fig. 1A), protein level using Western blotting (Fig. 1B) and mRNA level using qPCR (Fig. 1C). Dex significantly down-regulates the GR at activity ($P<0.001$) and protein level ($P<0.05$). At mRNA level the same trend is observed ($P=0.05$). IL6, in contrast to Dex, up-regulates the GR, significantly at activity ($P<0.01$) and protein level ($P<0.05$), but has no significant effect at mRNA level.

The IL6 ellicited signal in the presence of Dex is significantly lower than for IL6 alone at GR activity ($P<0.001$) and GR protein ($P<0.01$) level. Comparison of Dex effects in the presence and absence of IL6 suggests that in the presence of IL6, the Dex ellicited signal is not significantly altered for GR activity. For protein and mRNA levels, however, a trend to a higher signal in the presence of IL6 is observed. For example, GR protein levels increases from 3.42 to 4.91 GR/actin ($P=0.06$) and GR mRNA increases from 0.78 to 1.14 ($P=0.03$) relative GR expression, in the presence of IL6.

CpdA, in contrast to Dex, but similarly to IL6, significantly ($P<0.001$) increases GR activity levels but has no significant effect at protein and mRNA level. At GR activity level the increase in signal is significantly ($P<0.05$) higher for IL6 in the presence of CpdA than for IL6 alone. Comparison of the effect of CpdA on GR activity in the presence and absence of IL6 suggests that in the presence of IL6, the CpdA signal is slightly higher and increases from 46 x 10^3 to 52 x 10^3 fmol/g protein ($P=0.04$).

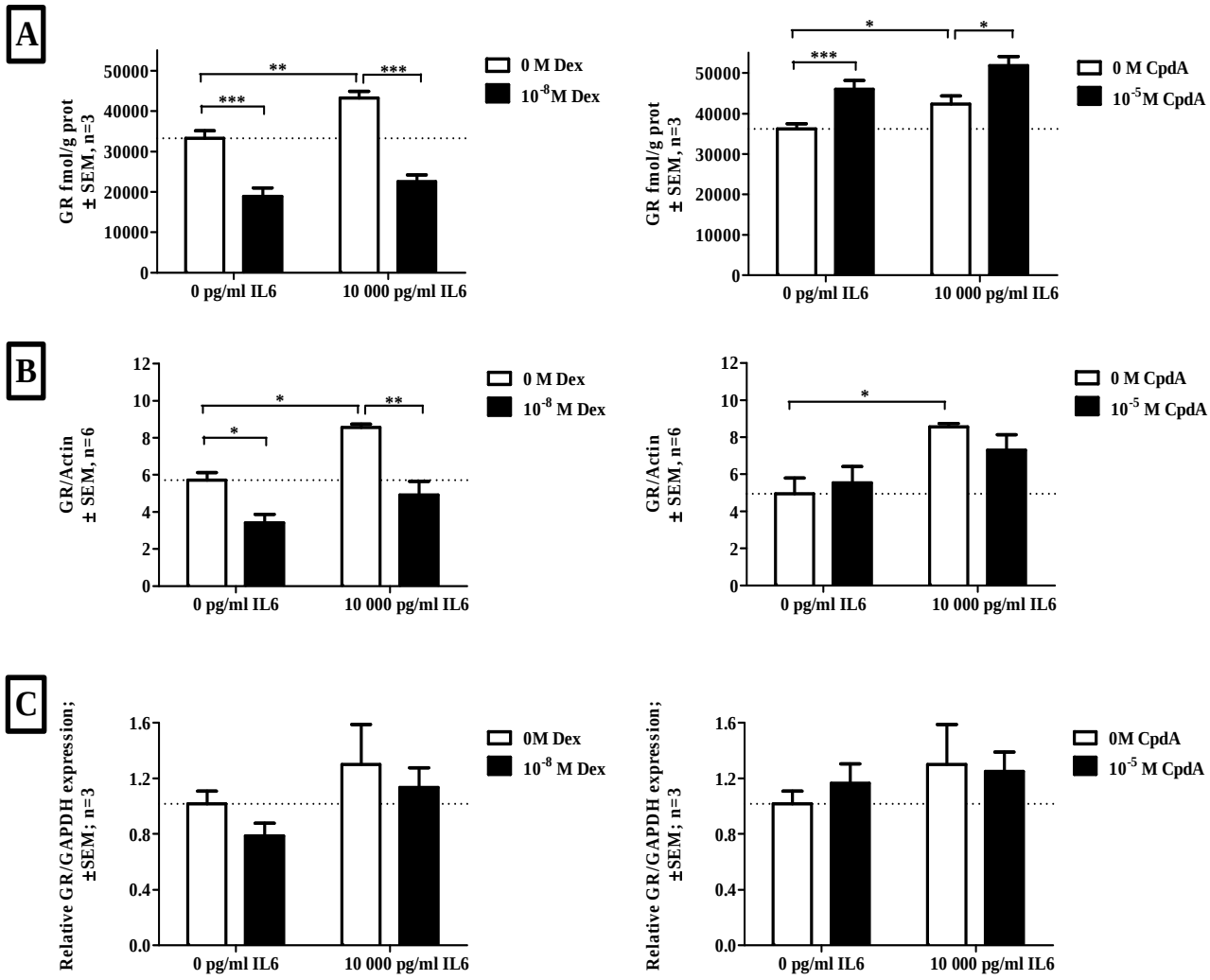


Figure 1. Modulation of GR levels by Dex or CpdA, in the presence or absence of IL6. (A) GR activity levels using whole cell GR-binding, (B) GR protein levels using Western Blot, and (C) GR mRNA levels using qPCR. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post tests (*P<0.05, **P<0.01, and ***P<0.001). If differences were non-significant with the multiple comparisons test we additionally did one-tailed t-tests after ANOVA to evaluate individual P values. These are then given as actual P values. The dotted line through the bars represents the value for solvent. (A) Represents the average ± SEM of three independent experiments done in triplicate, (B) represents the average ± SEM of six independent experiments, and (C) represents the average ± SEM of three independent experiments.

2.4.2 Modulation of metabolic enzymes by Dex or CpdA in the presence or absence of IL6.

2.4.2.1 TAT

The effect of Dex (10^{-8}M) and CpdA (10^{-5}M), in the presence or absence of IL6 (10 000 pg/ml), on TAT concentrations was determined at protein activity level using an enzyme activity assay (Fig. 2A), and mRNA level using qPCR (Fig. 2B).

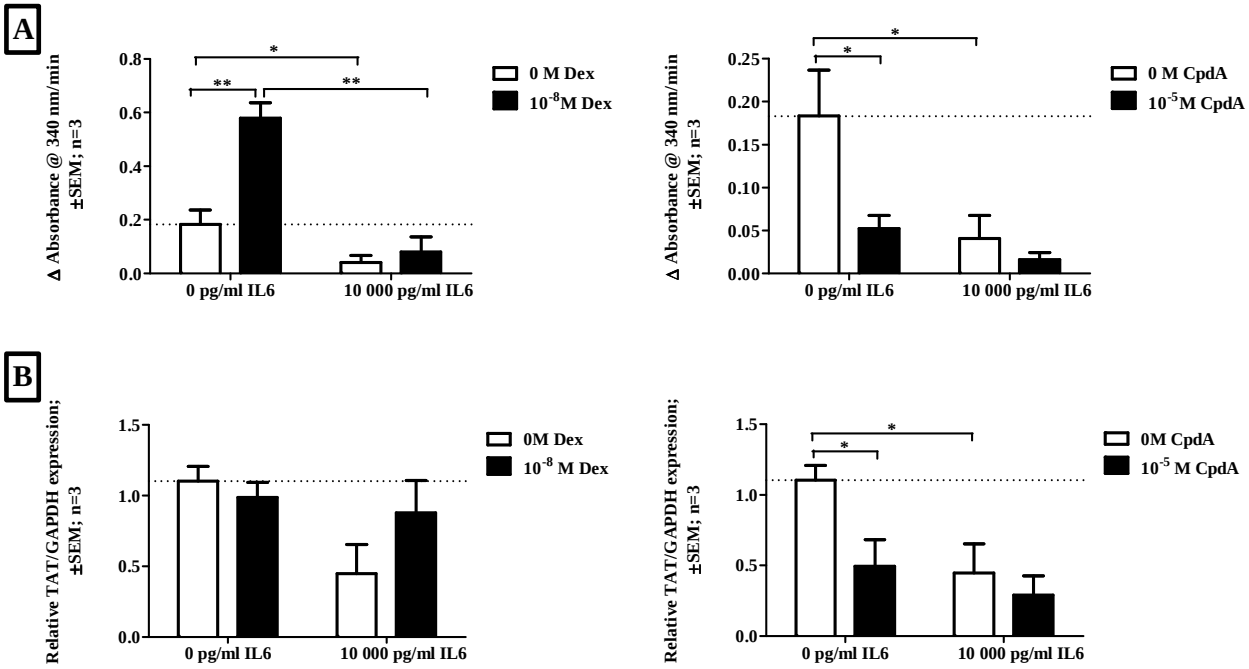


Figure 2. Modulation of TAT levels by Dex or CpdA, in the presence or absence of IL6. (A) TAT activity levels using enzyme activity assay and (B) TAT mRNA levels using qPCR. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post tests (*P<0.05, **P<0.01, and ***P<0.001). If differences were non-significant with the multiple comparisons test we additionally did one-tailed t-tests after ANOVA to evaluate individual P values. These are then given as actual P values. The dotted line through the bars represents the value for solvent. (A) Represents the average ± SEM of three independent experiments and (B) represents the average ± SEM of three independent experiments.

Dex significantly (P<0.01) up-regulates TAT at activity level, but has no effect at mRNA level. IL6, in contrast, significantly (P<0.05) down-regulates TAT at protein activity level and shows a trend (P=0.001) at down-regulating at mRNA level. Comparison of the effect of Dex on TAT activity in

the presence and absence of IL6 suggests that in the presence of IL6, the Dex signal is significantly ($P<0.01$) less than in the absence of IL6 with TAT activity decreasing from 0.58 to 0.08 absorbance/min.

CpdA, in contrast to Dex, but similarly to IL6, down-regulates TAT significantly at activity ($P<0.05$) and mRNA level ($P<0.05$). Comparison of the effect of CpdA, in the absence and presence of IL6, on TAT activity suggests that in the presence of IL6 the TAT activity signal has a trend ($P=0.05$) to be lower.

2.4.2.2 PEPCK

The effect of Dex (10^{-8}M) and CpdA (10^{-5}M), in the presence or absence of IL6 (10 000 pg/ml), on PEPCK concentrations was determined at protein activity level using an enzyme activity assay (Fig. 3A), and mRNA level using qPCR (Fig. 3B).

Dex shows a trend to up-regulate PEPCK at activity level ($P=0.006$). IL6, like Dex, shows a trend to up-regulate PEPCK activity ($P=0.03$) but significantly ($P<0.001$) down-regulates PEPCK mRNA levels. The PEPCK mRNA levels are significantly ($P<0.05$) lower in the presence of IL6 alone when compared to IL6 in the presence of Dex. The Dex signal in the absence and presence of IL6 is similar.

CpdA, similarly to IL6, significantly ($P<0.001$) down-regulates PEPCK mRNA levels. CpdA does not modulate the effect of IL6, nor does IL6 modulate the effect of CpdA, on PEPCK activity or mRNA levels.

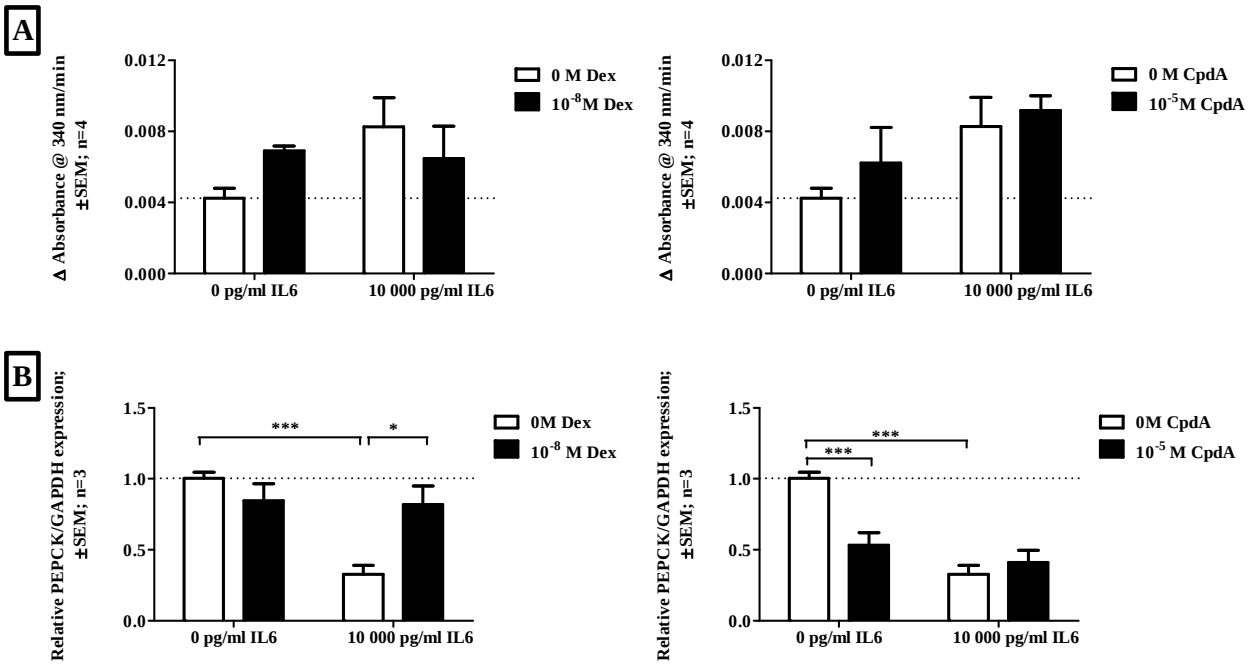


Figure 3. Modulation of PEPCK levels by Dex or CpdA, in the presence or absence of IL6. (A) PEPCK activity levels using enzyme activity assay and (B) PEPCK mRNA levels using qPCR. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post tests (*P<0.05, **P<0.01, and ***P<0.001). If differences were non-significant with the multiple comparisons test we additionally did one-tailed t-tests after ANOVA to evaluate individual P values. These are then given as actual P values. The dotted line through the bars represents the value for solvent. (A) Represents the average ± SEM of four independent experiments and (B) represents the average ± SEM of three independent experiments.

2.4.2.3 GGT

The effect of Dex (10^{-8}M) and CpdA (10^{-5}M), in the presence or absence of IL6 (10 000 pg/ml), on GGT concentrations was determined at protein activity level using an enzyme activity assay (Fig. 4A), and mRNA level using qPCR (Fig. 4B).

At GGT protein activity level no significant effects were seen with Dex, CpdA or IL6. At mRNA level the Dex signal shows a trend to be higher (P=0.008) while the IL6 signal shows a trend to be lower (P=0.03). The IL6 showed a trend (P=0.03) to ellicite a higher signal at GGT mRNA levels in the presence of Dex than in the absence of Dex. Comparison of Dex effects on GGT mRNA levels in the presence and absence of IL6 suggest that the Dex signal shows a trend (P=0.01) to be lower

in the presence of IL6. At mRNA level, CpdA, in contrast to Dex, showes a trend (P=0.06) to down-regulate GGT levels.

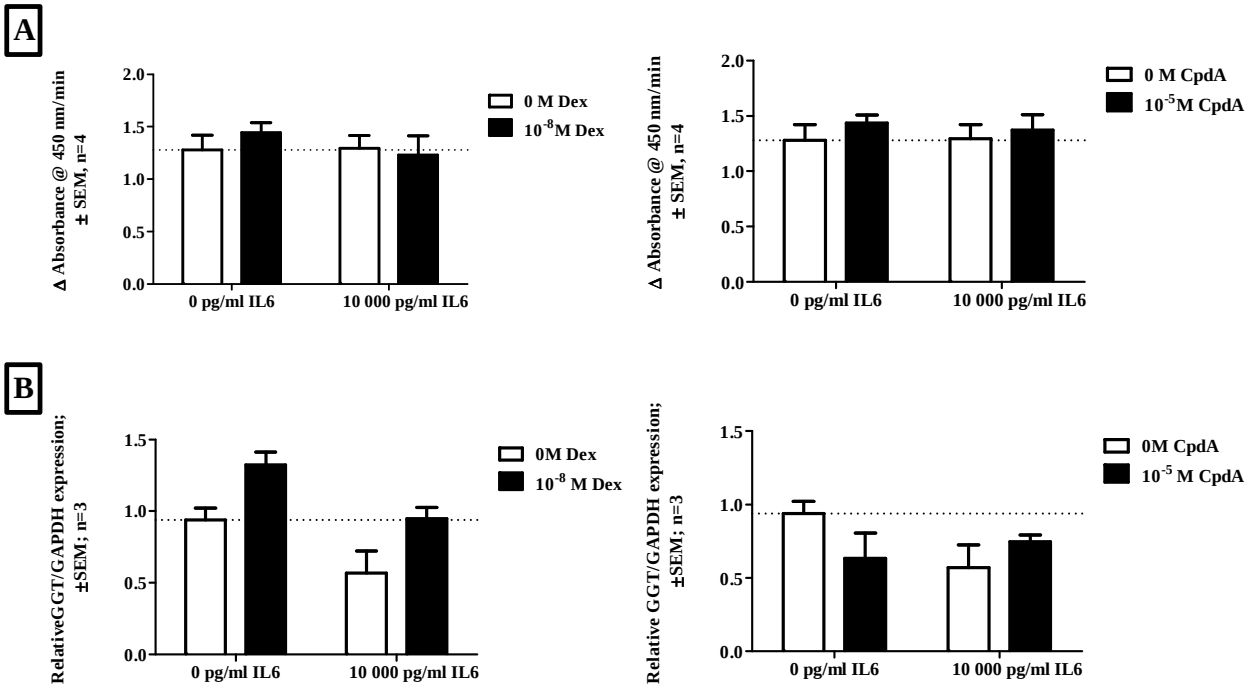


Figure 4. Modulation of GGT levels by Dex or CpdA, in the presence or absence of IL6. (A) GGT activity levels using enzyme activity assay and (B) GGT mRNA levels using qPCR. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post tests (*P<0.05, **P<0.01, and ***P<0.001). If differences were non-significant with the multiple comparisons test we additionally did one-tailed t-tests after ANOVA to evaluate individual P values. These are then given as actual P values. The dotted line through the bars represents the value for solvent. (A) Represents the average ± SEM of three independent experiments and (B) represents the average ± SEM of three independent experiments.

2.4.3 Modulation of acute phase proteins by Dex or CpdA in the presence or absence of IL6.

2.4.3.1 CBG

The effect of Dex (10^{-8}M) and CpdA (10^{-5}M), in the presence or absence of IL6 (10 000 pg/ml), on CBG concentrations was determined at protein level using Western blotting (Fig. 5A), and mRNA level using qPCR (Fig. 5B).

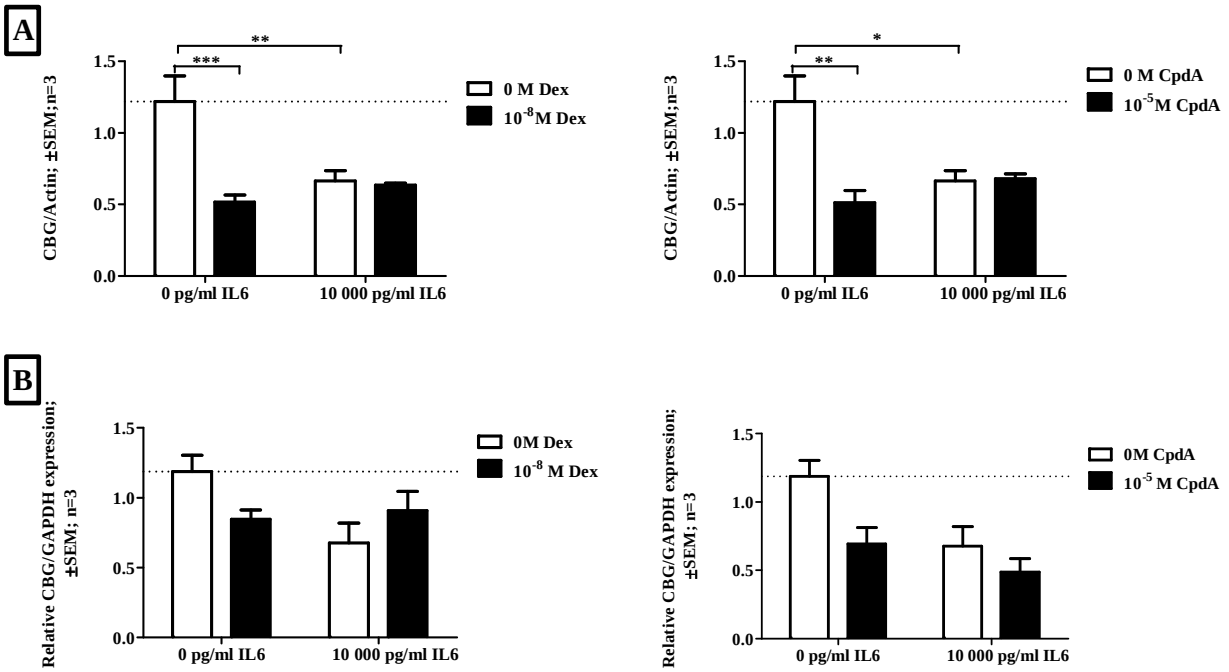


Figure 5. Modulation of CBG levels by Dex or CpdA, in the presence or absence of IL6. (A) CBG protein levels using Western blotting and (B) CBG mRNA levels using qPCR. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post tests (*P<0.05, **P<0.01, and ***P<0.001). The dotted line through the bars represents the value for solvent. (A) Represents the average ± SEM of three independent experiments and (B) represents the average ± SEM of three independent experiments.

Dex significantly (P<0.001) down-regulates CBG protein levels and shows a trend (P=0.02) to down-regulate CBG mRNA levels. IL6, similarly to Dex, significantly (P<0.01) down-regulates CBG protein levels and shows a trend (P=0.02) to down-regulate CBG mRNA. The signal elicited by IL6 at protein or mRNA levels is not significantly different in absence or presence of Dex. Comparison of the effect of Dex in the presence or absence of IL6 suggest that there is a trend to a higher signal in the presence of IL6 at the level of CBG protein (P=0.04) while at the level of mRNA no trend is observed.

CpdA, like Dex, down-regulates CBG at protein (P<0.01) and shows a trend (P=0.01) to down-regulate mRNA levels. The signal elicited by IL6 at protein or mRNA levels is not significantly different in absence or presence of CpdA. Comparison of CpdA effects in the absence and presence

of IL6 show that, like Dex, the CpdA signal shows a trend to a higher signal in the presence of IL6 at the level of CBG protein (P=0.05) while at the level of mRNA no trend is observed.

2.4.3.2 CRP

The effect of Dex (10^{-8}M) and CpdA (10^{-5}M), in the presence or absence of IL6 (10 000 pg/ml), on CRP concentrations was determined at intracellular protein level (Fig. 6A), extracellular protein level (Fig. 6B) using an ELISA assay, and mRNA level using qPCR (Fig. 6C).

Dex significantly (P<0.05) up-regulates intracellular CRP protein levels, shows a trend to up-regulate extra-cellular protein levels (P=0,03) but has no significant effect on CRP mRNA levels. IL6, similarly to Dex, significantly (P<0.001) up-regulates intracellular CRP protein levels, shows a trend to up-regulate extra-cellular protein levels (P=0,07) but has no effect on CRP mRNA levels. The signal elicited by IL6, is significantly (P<0.001) lower in the presence of Dex at intracellular CRP protein levels but shows no difference at extra-cellular protein levels or mRNA levels. Comparison of Dex effects in the presence and absence of IL6 suggests that intra-cellular protein levels show a trend (P=0,07) to be lower in the presence of IL6 while at extra-cellular CRP protein and mRNA levels there is no effect.

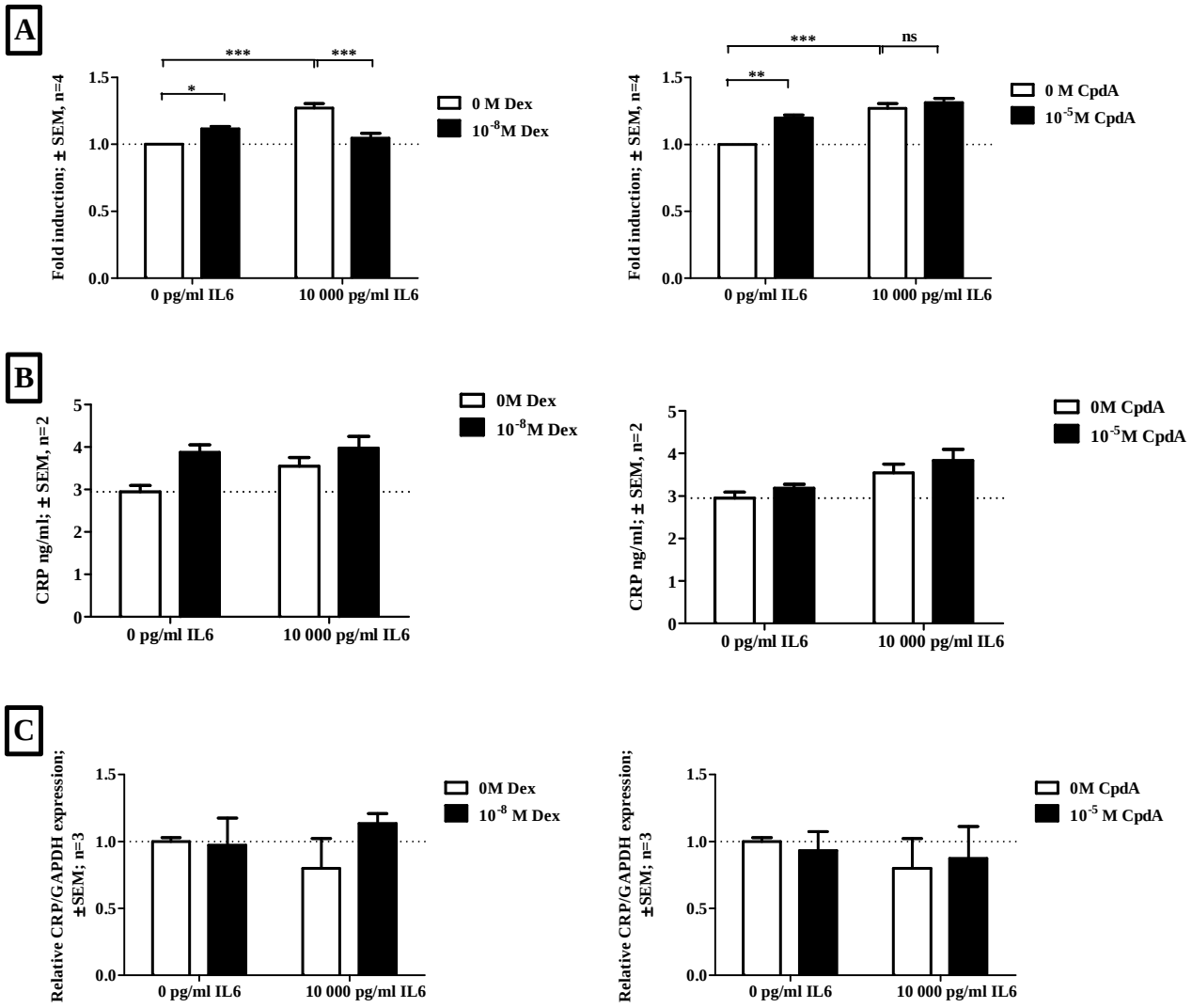


Figure 6. Modulation of CRP levels by Dex or CpdA, in the presence or absence of IL6. (A) Intracellular CRP protein levels, (B) extracellular CRP protein levels using an ELISA assay, and (C) CRP mRNA levels using qPCR. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post tests (*P<0.05, **P<0.01, and ***P<0.001). If differences were non-significant with the multiple comparisons test we additionally did one-tailed t-tests after ANOVA to evaluate individual P values. These are then given as actual P values. The dotted line through the bars represents the value for solvent. (A) Represents the average ± SEM of four independent experiments, (B) represents the average ± SEM of two independent experiments, and (C) represents the average ± SEM of three independent experiments.

CpdA, similarly to Dex and IL6, significantly (P<0.01) up-regulates intracellular CRP protein levels but has no effect on extra-cellular protein or mRNA levels. Comparison of CpdA effects in the presence and absence of IL6 show that there is a trend for the intra-cellular protein (P=0.05) and

extra-cellular protein levels (P=0.02) to be higher in the presence of IL6 while no effect is seen at mRNA level.

2.4.3.3 SAA

The effect of Dex (10^{-8}M) and CpdA (10^{-5}M), in the presence or absence of IL6 (10 000 pg/ml), on SAA concentrations was determined at intracellular protein level (Fig. 7A), extracellular protein level (Fig. 7B) using an ELISA assay, and mRNA level using qPCR (Fig. 7C).

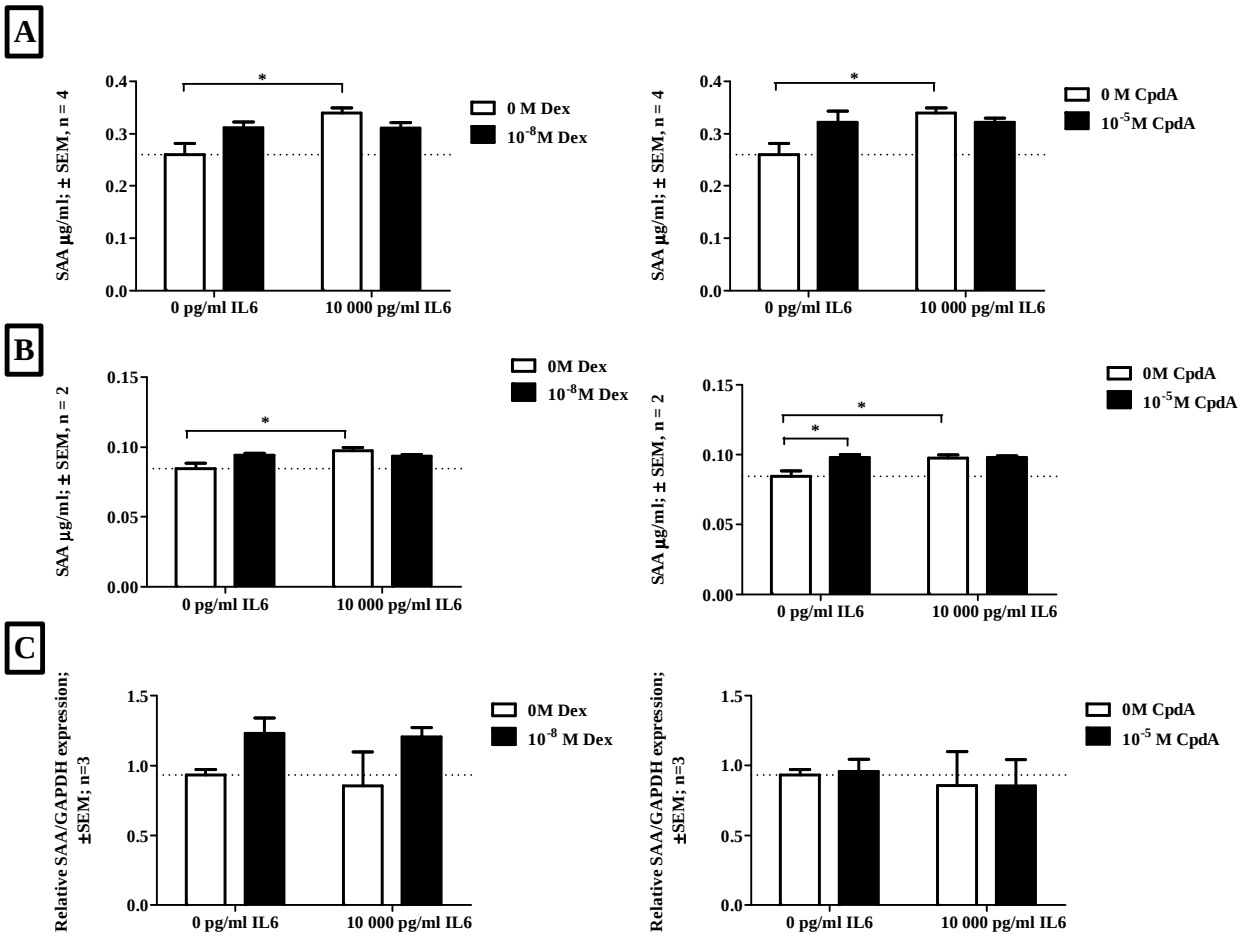


Figure 7. Modulation of SAA levels by Dex or CpdA, in the presence or absence of IL6. (A) Intracellular SAA protein levels , (B) extracellular SAA protein levels using an ELISA assay,and (C) SAA mRNA levels using qPCR. Statistical analysis was done using one-way ANOVA with Bonferroni multiple comparisons post tests (*P<0.05, **P<0.01, and ***P<0.001). If differences were non-significant with the multiple comparisons test we additionally did one-tailed t-tests after ANOVA to evaluate individual P values. These are then given as actual P values. The dotted line through the bars represents the value for solvent. (A) Represents the average ± SEM of six independent experiments, (B) represents the average ± SEM of two independent experiments, and (C) represents the average ± SEM of three independent experiments.

Dex shows a trend to up-regulate intracellular SAA protein levels (P=0.04), extra-cellular SAA protein levels (P=0.02) and mRNA levels (P=0.02). IL6, like Dex, up-regulates intracellular SAA protein levels (P<0.05) and extra-cellular SAA protein levels (P<0.05) but has no effect on mRNA levels. The signal elicited by IL6 shows a trend (P=0.04) to be lower in the presence of Dex for intracellular SAA protein levels but Dex has no effect on the signal elicited by IL6 for extra-cellular SAA protein levels or SAA mRNA levels.

Similarly to Dex, CpdA significantly (P<0.05) up-regulates extra-cellular SAA protein levels, shows a trend to up-regulate intracellular SAA protein levels (P=0.04) has no effect at mRNA levels.

2.5 Discussion

From previous results we hypothesized that, in the liver, IL6 will have a divergent effect to that of Dex on the transcription of GR and metabolic enzymes while it will have a convergent effect on acute phase proteins. We do not expect CpdA to activate metabolic genes [23,45] nor to down-regulate the GR [47]. To test our hypothesis we investigated the effect of Dex and CpdA, in the presence and absence of IL6, on GR levels and on the levels of three metabolic enzymes (TAT, GGT, PEPCK) and three acute phase proteins (SAA, CRP, CBG) in BWTG3 cells (Table 2).

The downregulation of GR by ligand serves as a protection mechanism to protect the cell or organism from continued signalling in the presence of ligand [47,57]. We show that GR protein activity (P<0.001) and protein levels (P<0.05) are significantly down-regulated by Dex and, in contrast to Dex, that GR protein activity (P<0.01) and protein (P<0.05) levels are significantly up-regulated by IL6, as was previously reported [19,26,47]. Although we do see a trend towards down-regulation of GR mRNA by Dex, it is not significant. CpdA, in contrast to Dex, caused a significant (P<0.001) increase in GR protein activity, but had no significant effect on GR protein

and mRNA levels. The failure of CpdA to increase mRNA levels was also observed by Gossye *et al.*[47]. In the presence of Dex, the up-regulation of GR protein activity and GR protein levels by Il6, are decreased significantly ($P<0.001$ and $P<0.01$, respectively) indicating a convergant effect. In contrast to Dex, CpdA, in the presence of IL6, shows a significant ($P<0.05$) convergant and additive effect towards the upregulation of GR protein activity, that is not significant at protein level or mRNA level. To conclude, Dex and IL6 appear to have a divergent effect on the GR while CpdA has a covergent effect and does not downregulate GR levels like classical GCs.

As expected from previous findings [16,19,20], Dex significantly up-regulated TAT protein activity ($P<0.01$). Previous work shows an increase in TAT mRNA [17,18] after Dex induction, which we did not observe. Courtois *et al.* [18] did show that TAT mRNA is only significantly upregulated upon induction with Dex at a concentration of 10^{-7}M and not at lower concentrations. After optimisation work done on GR levels (Supplementary Fig. 1B) it was decided to using 10^{-8}M Dex throughout the study to maintain comparability between different experiments. These findings by Courtois *et al.* [18], however, suggest that our Dex concentration may have been too low to elicit a significant increase in TAT mRNA levels. In addition, Miller *et al.* [17] also showed that maximal induction, of TAT mRNA levels, occurs 6 hours post stimulation, which suggests that the 24 hour stimulation period used during this study may be have been too long. Our findings that IL6 is capable of significantly down-regulating basal ($P<0.05$), as well as Dex induced ($P<0.01$) TAT activity correlates well with previous findings [26,27]. CpdA, contrary to Dex, significantly ($P<0.05$) down-regulated TAT activity, in contrast to work done by Robertson *et al.* (Addendum C) that has showed no influence of CpdA on TAT protein activity. An important difference between the current study and the one by Robertson *et al.* (Addendum C) is that during the current study cells were stimulated for 24 hours while cells were only stimulated for 4 hours in the Robertson *et al.* (Addendum C) study. This suggests that the down-regulatory effect of CpdA may be time dependent and that 4 hours is not adequate for a significant response, but that 24 hours is. We also

see a trend (P=0.05) towards a moderate, additive effect on TAT protein activity when CpdA and IL6 are added together. TAT mRNA levels are also significantly (P<0.05) decreased upon induction with CpdA or IL6. Previous studies had shown that CpdA is not able to significantly downregulate TAT mRNA levels [46].

In the current study, PEPCK protein activity levels were up-regulated (P=0.006) by Dex. However, Dex treatment had no significant effect on PEPCK mRNA levels in the current study despite the fact that other studies have found that GC treatment will increase PEPCK mRNA levels [21-23]. Wang *et al*. [21] showed an increase in PEPCK mRNA levels after treating rat hepatocytes and a rat hepatoma cell line with 0.5µM Dex for 4 hours. Hall *et al.* [22] treated rat hepatoma cells with 0.5µM Dex for 1 hour, and Dewint *et al.* [23] performed the study *in vivo* on mice that were treated with Dex 8 days prior to sacrifice. These studies provide a strong argument that we lost the effect of Dex on PEPCK mRNA due to stimulating the cells for too long a period (24 hours) with too low a concentration of Dex (10^{-8}M). In the current study IL6 has no significant effect at protein activity levels (P=0.40) but significantly (P<0.001) down-regulated PEPCK mRNA levels, as previously shown *in vivo* for mice [29] and rats [28]. Co-treatment of cells with Dex and IL6 showed that, even though it could not increase PEPCK mRNA levels, it was able to significantly (P<0.05) antgonise the downregulatory effect of IL6. In the current study, CpdA, in contrast to Dex, significantly (P<0.001) down-regulated PEPCK mRNA levels. The down-regulation of PEPCK mRNA levels by CpdA was also shown by others [23,46].

Stimulation with Dex, or CpdA, in the presence and absence of IL6 had no significant effect on GGT protein activity levels. Contrary to the findings of the current study, a 2 to 3 fold induction in GGT protein activity levels was previously obtained in a rat hepatoma cell line [24], however, induction was obtained after a 48-72 hour stimulation with10^{-6}M Dex. The stimulation period for the current study was 24 hours and the cells were stimulated with 10^{-8}M Dex. It is therefore

possible that our moderate induction is the result of stimulating for too short a period of time and with too low a concentration of Dex. This argument is supported by another study performed in a rat pancreatic cell line [25] showing that a maximal induction of GGT activity as well as GGT mRNA levels is achieved after 72 hours and that there is an initial 12 hour lag period before induction starts. In addition, it has been reported that ethanol is capable of inducing GGT levels 2 to 3 fold [58]. Dex and CpdA was prepared in ethanol and it is used as a solvent control. It is possible that basal levels were thus already induced by ethanol and therefore induction by Dex is moderate relative to basal levels. In the current study Dex also upregulated GGT mRNA levels moderately (P=0.008). Once again it is possible that a more significant increase could have been obtained if ethanol was not used as solvent, or if induction time was increased to 72 hours and Dex concentration was increased. A positive correlation between IL6 and GGT was previously shown in humans [30] by some, but others found no significant effect [59]. It has also been shown that in the liver of rats treated with an IL6 inducer, no significant effect was seen on GGT levels [60]. This is contrary to our findings that GGT mRNA levels are down-regulated by IL6 (P=0.03) but might be due to differences in tissue and species specificity. When cells were induced with Dex in the presence of IL6 it had no effect on GGT protein activity but GGT mRNA levels were lower (P=0.01) compared to Dex in the absence of IL6. CpdA downregulated the metabolic enzymes, TAT and PEPCK, either on protein level, mRNA level or both, but it had no significant effect (P=0.17) on GGT protein activity levels. CpdA did, howver, down-regulate GGT mRNA levels (P=0.06).

Treatment of BWTG3 cells with Dex lead to a significant (P<0.001) downregulation of CBG protein levels as shown by others [38]. The current study also showed down-regulation of CBG mRNA levels, but not to a similar extent (P=0.02). The fold down down-regulation found in the current study (1.4 fold) is much less than that found by Smith *et al.* [38] which showed a 14 fold decrease in CBG mRNA. This may be ascribed to differences in incubation time with the Smith

study incubating for 48 hours while in the current study cells were only treated for 24 hours. It must be mentioned that the down-regulatory effect of GC's on CBG levels is not always observed and varies between species, and that regulation of CBG can change depending on the of maturity of the organism [36]. IL6, similarly to Dex, significantly down-regulated CBG protein levels ($P<0.01$) and CBG mRNA levels ($P=0.02$). A difference can be seen on CBG protein, but not CBG mRNA levels when inducing with Dex in the presence or absence of IL6. In the current study CpdA, similarly to Dex, is shown to significantly down-regulate CBG protein ($P<0.01$) and CBG mRNA levels ($P=0.01$). The down-regulation of CBG protein by CpdA is also shown by Robertson *et al.* (Addendum C). The effect of Dex in the presence of IL6 was not additive in the current study, contrary to what has been found previously [36]. The reason for this might be that after a 24 hour period the CBG protein levels has been repressed to a maximum and no additive effect is possible.

Induction with Dex causes a moderate but significant up-regulation of intracellular ($P<0.05$) and extra-cellular ($P=0.03$) CRP protein levels. Induction with Dex, in the presence or absence of IL6, had no effect on CRP mRNA levels. It has been proposed that GC's, on their own, induce CRP to a moderate extent or not at all, but that glucocorticoids can have a synergistic effect in the presence of pro-inflammatory cytokines such as IL6 and IL1β [40,41]. The current study does not show such synergism, but it does show a convergent effect of Dex and IL6, as IL6 also significantly up-regulates CRP protein levels. The up-regulatory effect of IL6 on CRP protein and mRNA levels have been shown previously in a human hepatoma cell line [34,35]. The current study does, however, not show as significant effect by any of the inductions on CRP mRNA levels. Zhang *et al.* [35] tested the effect of IL6 on CRP protein and mRNA levels by inducing a human hepatoma cell line for 24 hours with 50 000 pg/ml IL6. The 5 times higher concentration of IL6, compared to the concentration used in the current study, could explain their observed effect on CRP mRNA levels as well as their high CRP protein inductions. Although CRP levels induced by Dex in the presence of IL6 was not significantly different compared to CRP protein levels in the absence of

IL6, the effect of IL6 was significantly ($P<0.001$) down-regulated in the presence of Dex. CpdA, similarly to Dex, significantly ($P<0.01$) up-regulated CRP protein levels, and had a moderate, not significant, additive effect when used in combination with IL6. This effect was not seen on the CRP mRNA level.

Induction with Dex caused a moderate up-regulation of SAA protein ($P=0.04$) and SAA mRNA ($P=0.02$) levels. This finding is supported by previous studies [39]. It is proposed that GC's, on their own, induce SAA to a moderate extent or not at all, but that Dex can synergise with pro-inflammatory cytokines such as IL6 and IL1β in inducing SAA[33,39,40]. The current study, however, does not show synergism, but rather shows a convergent effect of Dex and IL6, as IL6 significantly ($P<0.05$) up-regulates intracellular as well as extra-cellular SAA protein levels. The up-regulatory effect of IL6 on SAA protein levels have been shown in a human hepatoma cell line [33]. Thorn *et al.* [33] also shows upregulation of SAA mRNA levels after induction with IL6. The current study does, however, not show an effect by any of the inductions on SAA mRNA levels, except for Dex which up-regulated SAA mRNA levels ($P=0.02$). The conditions for Thorne *et al.*'s [33] study were similar to ours with the exception of using a human hepatoma cell line and it might be that the effect on SAA regulation is species specific. CpdA, like Dex, showed the ability to significantly up-regulate SAA protein levels, both extra-cellularly ($P<0.05$) and intra-cellularly ($P=0.04$). This effect was not seen on an mRNA level.

To summarise, we found that Dex was capable of down-regulating GR and generally up-regulating metabolic enzymes on a protein level and for select genes on the mRNA level, while IL6 had a divergent effect. GCs generally up-regulated positive acute phase proteins and down-regulated negative acute phase proteins on the protein level and for select genes on the mRNA level, and IL6 had a convergent effect. CpdA, in contrast to Dex and previous findings [23,46], was able to down-regulate metabolic enzymes on the protein and the mRNA level. Furthermore also in contrast to

Dex, CpdA was able to up-regulate GR levels while previous findings showed that CpdA had no effect on GR levels [47]. However, CpdA, like Dex, up-regulated the positive acute phase proteins and down-regulated negative acute phase proteins.

The current study was unique in that it compared a large panel of proteins, GR, three metabolic enzymes and three APPs, on both the protein as well as the mRNA level in one cell line under the same conditions. The study elucidates the crosstalk between the stress response system and the immune response system in the liver, providing insight into the effect of GCs and pro-inflammatory cytokines in the periphery in traditionally non-inflammatory tissues. The fact that CGs and IL6 effects converge for APPs suggest that both these peripheral mediators contribute to a speedy resolution of inflammation, while their divergent effects on metabolic genes involved in gluconeogenesis suggests opposing roles in maintaining blood glucose levels, with GCs involved in raising while IL6 is involved in lowering blodd glucose levels. The fact that CpdA is shown in this paper to have effects similar to that of Dex on APPs suggest that CpdA has a similar role in fighting inflammation. However, the fact that CpdA has opposing effcts to that of Dex on the GR and metabolic genes is novel and may have implications for the development of CpdA as a potential anti-inflammatory drug, specifically in the area of side-effects, such as hypoglycaemia and GC resistance.

Table 2. Comparison of the effect of Dex and CpdA, in the absence and presence of IL6, on protein levels, protein activity levels, and mRNA levels of GR, metabolic enzymes, and APP's.

	Protein Level								**mRNA Level**							
	Dex				**CpdA**				**Dex**				**CpdA**			
	Dex[a]	*IL6*[b]	*Dex + IL6*[c]	*Dex + IL6*[d]	*CpdA*[e]	*IL6*[b]	*CpdA + IL6*[f]	*CpdA + IL6*[g]	*Dex*[a]	*IL6*[b]	*Dex + IL6*[c]	*Dex + IL6*[d]	*CpdA*[e]	*IL6*[b]	*CpdA + IL6*[f]	*CpdA + IL6*[g]
GR: Whole cell binding	↓^{***h}	↑**	-	↓***	↑***	↑*	↑P=0.04	↑*	↓P=0.05	-	↑P=0.03	-	-	-	-	-
GR: Western blot	↓*	↑*	↑P=0.06	↓**	-	↑*	-	-								
Metabolic Enzymes																
TAT	↑**	↓*	↓**	-	↓*	↓*	↓P=0.05	-	-	↓P=0.001	-	-	↓*	↓*	-	-
PEPCK	↑P=0.006	↑P=0.03	-	-	-	↑P=0.03	-	-	-	↓***	-	↑*	↓***	↓***	-	-
GGT	-	-	-	-	-	-	-	-	↑P=0.008	↓P=0.03	↓P=0.01	↑P=0.03	↓P=0.06	↓P=0.03	-	-
Acute phase proteins																
CBG	↓***	↓**	↑P=0.04	-	↓**	↓*	↑P=0.05	-	↓P=0.02	↓P=0.02	-	-	↓P=0.01	↓P=0.02	-	-
CRP: Intra	↑*	↑***	↓P=0.07	↓***	↑**	↑***	↑P=0.05	-	-	-	-	-	-	↓P=0.07	-	-
CRP: Extra	↑P=0.03	↑P=0.07	-	-	-	↑P=0.07	↑P=0.02	-								
SAA: Intra	↑P=0.04	↑*	-	↓P=0.04	↑P=0.04	↑*	-	-	↑P=0.02	-	-	-	-	-	-	-
SAA: Extra	↑P=0.02	↑*	-	-	↑*	↑*	-	-								

[a]Effect of Dex relative to solvent

[b]Effect of IL6 relative to solvent

[c]Effect of Dex + IL6 relative to Dex

[d]Effect of Dex + IL6 relative to IL6

[e]Effect of CpdA relative to solvent

[f]Effect of CpdA + IL6 relative to CpdA

[g]Effect of CpdA + IL6 relative to IL6

[h]Statistical analysis was done using one-way ANOVA with a Bonferroni post test. ***P<0.001, **P<0.01, *P<0.05. If differences were non-significant with the multiple comparisons test we additionally did one-tailed t-tests after ANOVA to evaluate individual P values. These are then given as actual P values.

2.6 Reference list

1. C. Tsigos, G.P. Chrousos, Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53 (4) (2002) 865-871.

2. E. Charmandari, C. Tsigos, G. Chrousos, Endocrinology of the stress response. Annu. Rev. Physiol. 67 (2005) 259-284.

3. R. Newton, Molecular mechanisms of glucocorticoid action: what is important? Thorax 55 (7) (2000) 603-613.

4. M.I. Ndubuisi, K. Patel, R.J. Rayanade, A. Mittelman, L.T. May, P.B. Sehgal, Distinct classes of chaperoned IL-6 in human blood: differential immunological and biological availability. J. Immunol. 160 (1) (1998) 494-501.

5. L.T. May, M.I. Ndubuisi, K. Patel, D. Garcia, Interleukin-6 chaperones in blood. Ann. N. Y. Acad. Sci. 762 (1995) 120-128.

6. R. Mihrshahi, J.G. Lewis, S.O. Ali, Hormonal effects on the secretion and glycoform profile of corticosteroid-binding globulin. J. Steroid Biochem. Mol. Biol. 101 (4-5) (2006) 275-285.

7. D. Duma, C.M. Jewell, J.A. Cidlowski, Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. J. Steroid Biochem. Mol. Biol. 102 (1-5) (2006) 11-21.

8. M. Beato, M. Truss, S. Chavez, Control of transcription by steroid hormones. Ann. N. Y. Acad. Sci. 784 (1996) 93-123.

9. K. De Bosscher, W. Vanden Berghe, G. Haegeman, The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr. Rev. 24 (4) (2003) 488-522.

10. E. Arzt, Gp130 Cytokine Signaling in the Pituitary Gland: a Paradigm for Cytokine-Neuro-Endocrine Pathways. J. Clin. Invest. 108 (12) (2001) 1729-1733.

11. P.C. Heinrich, I. Behrmann, S. Haan, H.M. Hermanns, G. Muller-Newen, F. Schaper, Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem. J. 374 (Pt 1) (2003) 1-20.

12. A. Koj, Initiation of acute phase response and synthesis of cytokines. Biochim. Biophys. Acta 1317 (2) (1996) 84-94.

13. A.V. Turnbull, C.L. Rivier, Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiol. Rev. 79 (1) (1999) 1-71.

14. A. Angeli, R.G. Masera, M.L. Sartori, N. Fortunati, S. Racca, A. Dovio, A. Staurenghi, R. Frairia, Modulation by cytokines of glucocorticoid action. Ann. N. Y. Acad. Sci. 876 (1999) 210-220.

15. P.A. Knolle, E. Loser, U. Protzer, R. Duchmann, E. Schmitt, K.H. zum Buschenfelde, S. Rose-John, G. Gerken, Regulation of endotoxin-induced IL-6 production in liver sinusoidal endothelial cells and Kupffer cells by IL-10. Clin. Exp. Immunol. 107 (3) (1997) 555-561.

16. J.E. Bodwell, L.A. Kingsley, J.W. Hamilton, Arsenic at very low concentrations alters glucocorticoid receptor (GR)-mediated gene activation but not GR-mediated gene repression: complex dose-response

effects are closely correlated with levels of activated GR and require a functional GR DNA binding domain. Chem. Res. Toxicol. 17 (8) (2004) 1064-1076.

17. M.S. Miller, G.S. Buzard, A.E. McDowell, In vivo inhibition of glucocorticoid-inducible gene expression by dimethylnitrosamine in rat liver. Biochem. Pharmacol. 45 (7) (1993) 1465-1470.

18. A. Courtois, L. Payen, A. Guillouzo, O. Fardel, Up-regulation of multidrug resistance-associated protein 2 (MRP2) expression in rat hepatocytes by dexamethasone. FEBS Lett. 459 (3) (1999) 381-385.

19. D.C. DuBois, Z.X. Xu, L. McKay, R.R. Almon, N. Pyszcznski, W.J. Jusko, Differential dynamics of receptor down-regulation and tyrosine aminotransferase induction following glucocorticoid treatment. J. Steroid Biochem. Mol. Biol. 54 (5-6) (1995) 237-243.

20. M. Einstein, M. Greenlee, G. Rouen, A. Sitlani, J. Santoro, C. Wang, S. Pandit, P. Mazur, I. Smalera, A.P. Weaver, Y.Y. Zeng, L. Ge, T. Kelly, T. Paiva, W. Geissler, R.T. Mosley, J. Williamson, A. Ali, J. Balkovec, G. Harris, Selective glucocorticoid receptor nonsteroidal ligands completely antagonize the dexamethasone mediated induction of enzymes involved in gluconeogenesis and glutamine metabolism. J. Steroid Biochem. Mol. Biol. 92 (5) (2004) 345-356.

21. X.L. Wang, B. Herzog, M. Waltner-Law, R.K. Hall, M. Shiota, D.K. Granner, The synergistic effect of dexamethasone and all-trans-retinoic acid on hepatic phosphoenolpyruvate carboxykinase gene expression involves the coactivator p300. J. Biol. Chem. 279 (33) (2004) 34191-34200.

22. R.K. Hall, X.L. Wang, L. George, S.R. Koch, D.K. Granner, Insulin represses phosphoenolpyruvate carboxykinase gene transcription by causing the rapid disruption of an active transcription complex: a potential epigenetic effect. Mol. Endocrinol. 21 (2) (2007) 550-563.

23. P. Dewint, V. Gossye, K. De Bosscher, W. Vanden Berghe, K. Van Beneden, D. Deforce, S. Van Calenbergh, U. Muller-Ladner, B. Vander Cruyssen, G. Verbruggen, G. Haegeman, D. Elewaut, A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. J. Immunol. 180 (4) (2008) 2608-2615.

24. R. Barouki, M.N. Chobert, M.C. Billon, J. Finidori, R. Tsapis, J. Hanoune, Glucocorticoid hormones increase the activity of gamma-glutamyltransferase in a highly differentiated hepatoma cell line. Biochim. Biophys. Acta 721 (1) (1982) 11-21.

25. M.N. Chobert, G. Grondin, A. Brouillet, Y. Laperche, A.R. Beaudoin, Control of gamma-glutamyl transpeptidase expression by glucocorticoids in the rat pancreas. Correlation with granule formation. J. Biol. Chem. 271 (21) (1996) 12431-12437.

26. C. Smith, N.W. Wilson, A. Louw, K.H. Myburgh, Illuminating the interrelated immune and endocrine adaptations after multiple exposures to short immobilization stress by in vivo blocking of IL-6. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292 (4) (2007) R1439-47.

27. I. Zvibel, S. Brill, R. Kariv, A. Traister, T. Golan, J. Chebath, Z. Halpern, M. Revel, R. Oren, Chimeric molecule IL-6/soluble IL-6 receptor is a potent mitogen for fetal hepatocytes. J. Cell. Physiol. 200 (2) (2004) 245-252.

28. M. Hill, R. McCallum, Altered transcriptional regulation of phosphoenolpyruvate carboxykinase in rats following endotoxin treatment. J. Clin. Invest. 88 (3) (1991) 811-816.

29. E. Chichelnitskiy, A. Vegiopoulos, M.B. Diaz, A. Ziegler, A. Kleiman, A. Rauch, J. Tuckermann, S. Herzig, In vivo phosphoenolpyruvate carboxykinase promoter mapping identifies disrupted hormonal synergism as a target of inflammation during sepsis in mice. Hepatology 50 (6) (2009) 1963-1971.

30. T. Stojakovic, H. Scharnagl, M. Trauner, B. Pieske, B. Wellnitz, U. Seelhorst, D. Schilling, B.R. Winkelmann, B.O. Boehm, W. Marz, Serum gamma-glutamyl transferase and mortality in persons undergoing coronary angiography-The Ludwigshafen Risk and Cardiovascular Health Study. Atherosclerosis (2009).

31. S. Coban, F. Yildiz, A. Terzi, B. Al, D. Ozgor, C. Ara, A. Polat, M. Esrefoglu, The Effect of Caffeic Acid Phenethyl Ester (CAPE) Against Cholestatic Liver Injury in Rats. J. Surg. Res. (2008).

32. J.C. Betts, J.K. Cheshire, S. Akira, T. Kishimoto, P. Woo, The role of NF-kappa B and NF-IL6 transactivating factors in the synergistic activation of human serum amyloid A gene expression by interleukin-1 and interleukin-6. J. Biol. Chem. 268 (34) (1993) 25624-25631.

33. C.F. Thorn, A.S. Whitehead, Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. J. Immunol. 169 (1) (2002) 399-406.

34. M.K. Ganapathi, L.T. May, D. Schultz, A. Brabenec, J. Weinstein, P.B. Sehgal, I. Kushner, Role of interleukin-6 in regulating synthesis of C-reactive protein and serum amyloid A in human hepatoma cell lines. Biochem. Biophys. Res. Commun. 157 (1) (1988) 271-277.

35. S. Zhang, Q. Liu, J. Wang, D.C. Harnish, Suppression of interleukin-6-induced C-reactive protein expression by FXR agonists. Biochem. Biophys. Res. Commun. 379 (2) (2009) 476-479.

36. A. Emptoz-Bonneton, J.C. Crave, H. LeJeune, C. Brebant, M. Pugeat, Corticosteroid-binding globulin synthesis regulation by cytokines and glucocorticoids in human hepatoblastoma-derived (HepG2) cells. J. Clin. Endocrinol. Metab. 82 (11) (1997) 3758-3762.

37. L. Bartalena, G.L. Hammond, A. Farsetti, I.L. Flink, J. Robbins, Interleukin-6 inhibits corticosteroid-binding globulin synthesis by human hepatoblastoma-derived (Hep G2) cells. Endocrinology 133 (1) (1993) 291-296.

38. C.L. Smith, G.L. Hammond, Hormonal regulation of corticosteroid-binding globulin biosynthesis in the male rat. Endocrinology 130 (4) (1992) 2245-2251.

39. J.V. Castell, M.J. Gomez-Lechon, M. David, T. Hirano, T. Kishimoto, P.C. Heinrich, Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. FEBS Lett. 232 (2) (1988) 347-350.

40. M.K. Ganapathi, D. Rzewnicki, D. Samols, S.L. Jiang, I. Kushner, Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep 3B cells. J. Immunol. 147 (4) (1991) 1261-1265.

41. J.E. Volanakis, Human C-reactive protein: expression, structure, and function. Mol. Immunol. 38 (2-3) (2001) 189-197.

42. H. Schacke, W.D. Docke, K. Asadullah, Mechanisms involved in the side effects of glucocorticoids. Pharmacol. Ther. 96 (1) (2002) 23-43.

43. P.J. Barnes, Corticosteroids: the drugs to beat. Eur. J. Pharmacol. 533 (1-3) (2006) 2-14.

44. A. Louw, P. Swart, S.S. de Kock, K.J. van der Merwe, Mechanism for the stabilization *in vivo* of the aziridine precursor --(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride by serum proteins. Biochem. Pharmacol. 53 (2) (1997) 189-197.

45. K. De Bosscher, W. Vanden Berghe, I.M. Beck, W. Van Molle, N. Hennuyer, J. Hapgood, C. Libert, B. Staels, A. Louw, G. Haegeman, A fully dissociated compound of plant origin for inflammatory gene repression. Proc. Natl. Acad. Sci. U. S. A. 102 (44) (2005) 15827-15832.

46. Z. Zhang, Z.Y. Zhang, H.J. Schluesener, Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. J. Immunol. 183 (5) (2009) 3081-3091.

47. V. Gossye, D. Elewaut, K. Van Beneden, P. Dewint, G. Haegeman, K. De Bosscher, A plant-derived glucocorticoid receptor modulator attenuates inflammation without provoking ligand-induced resistance. Ann. Rheum. Dis. (2009).

48. M.J. Coghlan, P.B. Jacobson, B. Lane, M. Nakane, C.W. Lin, S.W. Elmore, P.R. Kym, J.R. Luly, G.W. Carter, R. Turner, C.M. Tyree, J. Hu, M. Elgort, J. Rosen, J.N. Miner, A novel antiinflammatory maintains glucocorticoid efficacy with reduced side effects. Mol. Endocrinol. 17 (5) (2003) 860-869.

49. H. Schacke, A. Schottelius, W.D. Docke, P. Strehlke, S. Jaroch, N. Schmees, H. Rehwinkel, H. Hennekes, K. Asadullah, Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proc. Natl. Acad. Sci. U. S. A. 101 (1) (2004) 227-232.

50. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248-254.

51. T.I. Diamondstone, Assay of tyrosine transaminase activity by conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde. Anal. Biochem. 16 (3) (1966) 395-401.

52. H.C. Chang, M.D. Lane, The enzymatic carboxylation of phosphoenolpyruvate. II. Purification and properties of liver mitochondrial phosphoenolpyruvate carboxykinase. J. Biol. Chem. 241 (10) (1966) 2413-2420.

53. F. Lipmann, L.C. Tuttle, A specific micromethod for the determination of acyl phosphates. J. Biol. Chem. 159 (1) (1945) 21-28.

54. Y. Engelbrecht, H. de Wet, K. Horsch, C.R. Langeveldt, F.S. Hough, P.A. Hulley, Glucocorticoids induce rapid up-regulation of mitogen-activated protein kinase phosphatase-1 and dephosphorylation of extracellular signal-regulated kinase and impair proliferation in human and mouse osteoblast cell lines. Endocrinology 144 (2) (2003) 412-422.

55. C. Pickering, L. Avesson, J. Lindblom, S. Liljequist, H.B. Schioth, Identification of neurotransmitter receptor genes involved in alcohol self-administration in the rat prefrontal cortex, hippocampus and amygdala. Prog. Neuropsychopharmacol. Biol. Psychiatry 31 (1) (2007) 53-64.

56. K. Fehsel, D. Plewe, V. Kolb-Bachofen, Nitric oxide-induced expression of C-reactive protein in islet cells as a very early marker for islet stress in the rat pancreas. Nitric Oxide 1 (3) (1997) 254-262.

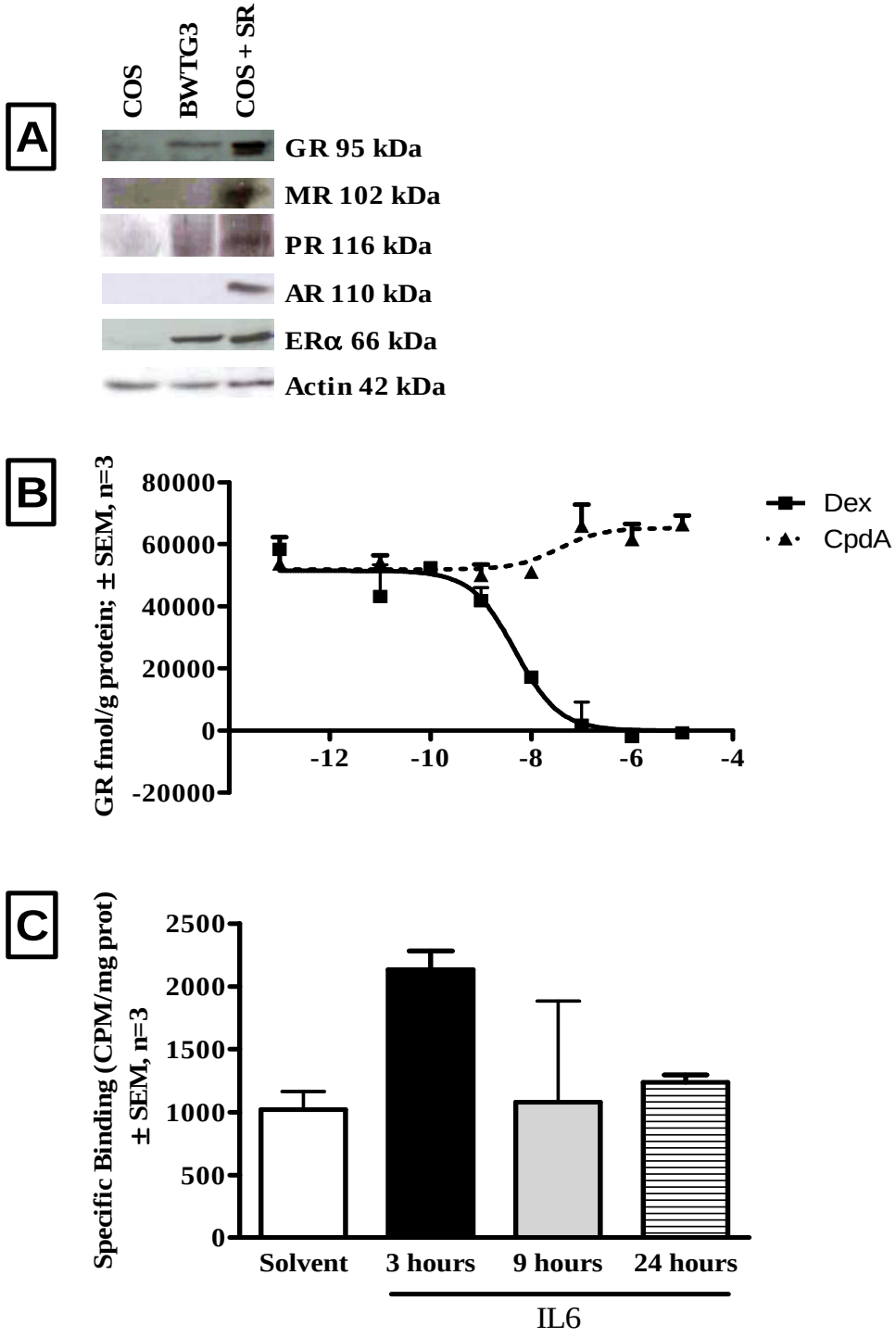
57. R.H. Oakley, J.A. Cidlowski, Homologous down regulation of the glucocorticoid receptor: the molecular machinery. Crit. Rev. Eukaryot. Gene Expr. 3 (2) (1993) 63-88.

58. M.W. Lieberman, R. Barrios, B.Z. Carter, G.M. Habib, R.M. Lebovitz, S. Rajagopalan, A.R. Sepulveda, Z.Z. Shi, D.F. Wan, gamma-Glutamyl transpeptidase. What does the organization and expression of a multipromoter gene tell us about its functions? Am. J. Pathol. 147 (5) (1995) 1175-1185.

59. F. Gude, J. Rey-Garcia, C. Fernandez-Merino, L. Meijide, L. Garcia-Ortiz, C. Zamarron, A. Gonzalez-Quintela, Serum levels of gamma-glutamyl transferase are associated with markers of nocturnal hypoxemia in a general adult population. Clin. Chim. Acta 407 (1-2) (2009) 67-71.

60. A. Fukumura, M. Tsutsumi, M. Tsuchishima, N. Hayashi, M. Fukura, H. Yano, K. Ozaki, S. Takase, Effect of the inducer of interleukin-6 (ME3738) on rat liver treated with ethanol. Alcohol. Clin. Exp. Res. 31 (1 Suppl) (2007) S49-53.

2.8 Supplementary figures



Supplementary figure 1. Characterization of BWTG3 cell line and determination of optimum concentrations of Dex and CpdA, as well as optimum incubation time for IL6. (A) Characterization by Western blotting and probing with antibody raised against GR, MR, PR, AR, ER, and Actin as loading control, (B)Whole cell binding assay to determine EC_{50} values for Dex and CpdA, and (C) Whole cell binding assay to determine optimum IL6 incubation time for maximal response. (B) and (C) represents average values ± SEM of experiment done in triplicate.

CHAPTER 3

GENERAL CONCLUSION AND DISCUSSION

In the liver, crosstalk exists between the stress response system and the immune response system. The effect of glucocorticoids on pro-inflammatory cytokines, such as IL6 and IL1β, and acute phase proteins (APPs), such as serum amyloid A (SAA), C-reactive protein (CRP), and corticosteroid binding globulin (CBG), has been extensively studied due to the pharmacological use of glucocorticoids as anti-inflammatory drugs. Specifically, glucocorticoids have been found to down-regulate the levels of pro-inflammatory cytokines [1] and to have a convergent effect with IL6 in up-regulating APPs, which may be additive [2-8]. The modulation of the stress system by the immune system has, however, not been as well studied, especially at the level of the periphery. It has been shown that IL6 generally has a divergent effect, compared to glucocorticoids, on the regulation of the glucocorticoid receptor (GR) [9] and metabolic enzymes [9-16]. This crosstalk serves as a protection mechanism against tissue damage and chronic inflammation due to the release of pro-inflammatory cytokines [1,17] but it also serves to curb the stress response. The stress response must be regulated to prevent the negative effects associated with excessive glucocorticoid signalling such as muscle wasting and hyperglycemia.

We investigated the effect of Dex, a synthetic glucocorticoid, and CpdA, a dissociated glucocorticoid, both in the presence and absence of IL6, on GR levels and on the levels of three metabolic enzymes, TAT, GGT, and PEPCK and three acute phase proteins, SAA, CRP, and CBG. We hypothesized that, in the liver, IL6 will have a divergent effect to that of Dex on the transcription of GR and metabolic enzymes while it will have a convergent effect to that of Dex on APPs. In addition, we hypothesised that CpdA, in contrast to Dex, will not activate metabolic genes [18] or down-regulate GR [19], but that CpdA would have similar effects to that of Dex on the APP's. The following section will elaborate on the discussion of results obtained from this

study (Chapter 2) by highlighting possible implications of current findings as well as by addressing future work.

GR concentrations are homologuesly down-regulated by glucocorticoids and this down-regulation serves to protect the cell from constant signalling in the presence of ligand [19,20]. In the current study GR protein levels were indeed found to be significantly ($P<0.05$) down-regulated by Dex but also to be significantly ($P<0.01$) up-regulated by IL6 as seen by others [9,21]. Dex was also shown to significantly ($P<0.001$) down-regulate GR up-regulation by IL6, correlating with previous findings [9]. In contrast, although CpdA was previously shown to not down-regulate GR [47], the current study shows that CpdA is in fact able to significantly ($P<0.001$) up-regulate GR levels and to significantly ($P<0.05$) add to IL6 up-regulation. Some patients, after initially responding well to glucocorticoid administration, develop glucocorticoid resistance after long term treatment [22]. This resistance has been proposed to be due to the down regulation of GR by its ligand in some patients [23] as cellular sensitivity to a ligand is directionally proportional to its receptor concentration. Gossye *et al.* [19] proposes that CpdA treatment would be beneficial in preventing glucocorticoid resistance as it does not lead to down regulation of GR levels, contrary to classical glucocorticoids. However, the current study showed that CpdA is actually able to up regulate GR concentrations and thus long term use of CpdA, although beneficial in preventing glucocorticoid resistance, might be detrimental to the organism as cells will not be protected against excessive ligand signalling. A further consideration is that *in vivo* CpdA would be administered against a background of endogenous glucocorticoids and thus the total (CpdA plus free endogenous glucocorticoids) level of GR ligand reaching the cells need to be taken into account. CpdA has previously been shown to inhibit adrenal synthesis of endogenous glucocorticoids [24], to increase the percentage free corticosterone in rats through increased displacement from CBG [24], and in this study to decrease CBG levels. Thus to venture a prediction of *in vivo* behaviour is not a trivial matter.

In times of stress the HPA-axis is stimulated to induce the release of glucocorticoids by the adrenals. Secreted glucocorticoids move to target organs, like the liver, where they induce the transcription of metabolic enzymes. These metabolic enzymes increase blood glucose levels by stimulating gluconeogenesis and by the mobilization of amino acids and free fatty acids, which serve as substrates for gluconeogenesis [25]. Increased blood glucose enables the organism to cope with the stressfull experience. The current study shows that Dex significantly up-regulates two of the three metabolic enzymes investigated, TAT and PEPCK. Although GGT levels were also up-regulated the effect was not significant. CpdA, in contrast, had variable effects in significantly down-regulating TAT on protein and mRNA levels, in significantly down-regulating PEPCK only at mRNA levels and in having no effect on GGT. Hyperglycaemia, a side effect of glucocorticoid treatment, is caused by increased blood glucose levels due to the activation of metabolic enzymes by glucocorticoids. De Bosscher *et al.* [26] and Zhang *et al.* [27] showed that CpdA treatment did not increase blood glucose levels in mice, in contrast to Dex, but rather they observed a decrease in blood glucose after a 5 to 10 day treatment with CpdA. This decrease in blood glucose levels might be due to the down-regulation of some of the metabolic enzymes by CpdA as showed by the current study and others [18,27]. The inability of CpdA to transactivate metabolic genes has always been seen in a positive light as limiting the side effects of glucocorticoid treatment. However, if CpdA transrepresses metabolic genes, long term CpdA treatment may lead to the development of hypoglycaemia, a shortage in blood glucose, which could have implications in septic shock [28,29]. On the other hand, the ability of CpdA to decrease blood glucose levels could find application in other diseases such as diabetes [30]. During inflammation blood glucose levels drop limiting glucose availability to pathogens. In the current study IL6 repressed the metabolic genes investigated, significantly at mRNA level for all genes and significantly only for TAT at protein level. CpdA, in the presence of IL6, showed additional transrepression of the TAT gene, which may exacerbate the glucose lowering effects of CpdA in inflammatory situations.

One of the earliest responses to inflammation is the increase in APPs produced in the liver, which is largely regulated by cytokines such as IL6 [5]. The function of APP's is to destroy pathogens, limit and repair tissue damage and drive the acquired immune response against the pathogen [31]. During inflammation glucocorticoids are also secreted and they also regulate acute phase proteins in a similar fashion as IL6. The combined effect of IL6 and glucocorticoids is thus to resolve inflammation as quickly as possible. However, the acute phase response should be of short duration [32], as an excessive response may lead to chronic inflammation and disorders associated with it, such as anorexia, depression, hypogonadism, peptic ulcers, and/or immunosuppression [33,34]. The current study shows that CpdA is able to regulate APP's, similarly to glucocorticoids and IL6, at either protein or mRNA level. CpdA has been shown to be an effective anti-inflammatory reagent, comparable to synthetic glucocorticoids [18,19,26,27,35,36] and eliciting the acute phase response may contribute to CpdA's efficiency.

Synthetic glucocorticoids have been developed for therapeutic use and are among the most widely prescribed and effective drugs for the treatment of immune and inflammatory diseases, including asthma and rheumatoid arthritis [37]. The long term use of glucocorticoids has, however, been associated with many side effects including hyperglycemia [26] and glucocorticoid resistance [19,38]. Traditionally, the positive therapeutic qualities of glucocorticoids have been attributed to the repression of pro-inflammatory genes, whilst the side effects have been attributed to the activation of genes like the metabolic enzyme genes [39]. On a molecular level transactivation via the GR is generally mediated by direct binding of the GR to GREs as homodimers [40] in genes such as TAT, PEPCK, and GGT. CpdA, however, does not cause dimerization of the GR and it is suggested that this may be the reason for its inability to transactivate genes [18] and (Addendum C). In contrast, GR repression of cytokines and cytokine induced genes is believed to occur via a tethering mechanism that does not require GR dimerization [41,42] and thus it has been suggested as the molecular mechanism of transrepression of CpdA (Addendum C). For example, it has also

been shown that CpdA can repress genes by tethering of GR monomers to DNA bound NF-κB [26]. In the current study CpdA, like Dex, was shown to repress negative APPs and upregulate positive APPs. IL6 regulate APPs via induction of STAT3 and C/EBP. The presence of C/EBP, STAT3 or NF-κB sites in the CBG, SAA, and CRP promoters suggest that these transcription factors may mediate the effect of CpdA. Positive tethering by GR via C/EBP and STAT3 has been shown [43,44] as has negative tethering by GR. In the current study, however, CpdA, unlike Dex, down-regulated metabolic enzymes. The up-regulation of these metabolic enzymes by Dex is thought to occur through GRE elements. CpdA has, however, been shown to be incapable of transactivation via GRE elements [26]. The presence of C/EBP or NF-κB binding sites in the promotor regions of metabolic enzymes [45,46] may provide insight into the action of CpdA. It is possible that CpdA might be repressing the transcription of these genes by tethering to DNA bound C/EBP or NF-κB. Future work is needed to clarify if tethering of CpdA induced GR monomers can regulate gene transcription via STAT3 or C/EBP elements.

The current study was unique in that it investigated a large panel of proteins, namely GR, three metabolic enzymes, and three acute phase proteins, in the same cell line under the same conditions both on the protein as well as the mRNA level. However, it has its limitations as it shows the importance of optimising conditions for all proteins at both protein and mRNA level. As optimisation in this study was only at the level of GR protein activity many of the mRNA results did not show significance.

The results obtained with CpdA provide valuable insight into the development of CpdA as an anti-inflammatory drug regarding possible side effects in long term use. Specifically, CpdA's effects on metabolic genes and blood glucose levels [26,27] merits further investigation. In addition, it is also one of the few studies showing that CpdA is capable of transactivating genes [27], probably through a tethering mechanism.

3.1 References

1. A. Angeli, R.G. Masera, M.L. Sartori, N. Fortunati, S. Racca, A. Dovio, A. Staurenghi, R. Frairia, Modulation by cytokines of glucocorticoid action. Ann. N. Y. Acad. Sci. 876 (1999) 210-220.

2. C.L. Smith, G.L. Hammond, Hormonal regulation of corticosteroid-binding globulin biosynthesis in the male rat. Endocrinology 130 (4) (1992) 2245-2251.

3. A. Emptoz-Bonneton, J.C. Crave, H. LeJeune, C. Brebant, M. Pugeat, Corticosteroid-binding globulin synthesis regulation by cytokines and glucocorticoids in human hepatoblastoma-derived (HepG2) cells. J. Clin. Endocrinol. Metab. 82 (11) (1997) 3758-3762.

4. C.M. Uhlar, A.S. Whitehead, Serum amyloid A, the major vertebrate acute-phase reactant. Eur. J. Biochem. 265 (2) (1999) 501-523.

5. M.K. Ganapathi, D. Rzewnicki, D. Samols, S.L. Jiang, I. Kushner, Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep 3B cells. J. Immunol. 147 (4) (1991) 1261-1265.

6. C.F. Thorn, A.S. Whitehead, Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. J. Immunol. 169 (1) (2002) 399-406.

7. J.E. Volanakis, Human C-reactive protein: expression, structure, and function. Mol. Immunol. 38 (2-3) (2001) 189-197.

8. J.V. Castell, M.J. Gomez-Lechon, M. David, T. Hirano, T. Kishimoto, P.C. Heinrich, Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. FEBS Lett. 232 (2) (1988) 347-350.

9. C. Smith, N.W. Wilson, A. Louw, K.H. Myburgh, Illuminating the interrelated immune and endocrine adaptations after multiple exposures to short immobilization stress by in vivo blocking of IL-6. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292 (4) (2007) R1439-47.

10. I. Zvibel, S. Brill, R. Kariv, A. Traister, T. Golan, J. Chebath, Z. Halpern, M. Revel, R. Oren, Chimeric molecule IL-6/soluble IL-6 receptor is a potent mitogen for fetal hepatocytes. J. Cell. Physiol. 200 (2) (2004) 245-252.

11. E. Chichelnitskiy, A. Vegiopoulos, M.B. Diaz, A. Ziegler, A. Kleiman, A. Rauch, J. Tuckermann, S. Herzig, In vivo phosphoenolpyruvate carboxykinase promoter mapping identifies disrupted hormonal synergism as a target of inflammation during sepsis in mice. Hepatology 50 (6) (2009) 1963-1971.

12. M. Hill, R. McCallum, Altered transcriptional regulation of phosphoenolpyruvate carboxykinase in rats following endotoxin treatment. J. Clin. Invest. 88 (3) (1991) 811-816.

13. D.H. Lee, D.R. Jacobs Jr, M. Gross, C.I. Kiefe, J. Roseman, C.E. Lewis, M. Steffes, Gamma-glutamyltransferase is a predictor of incident diabetes and hypertension: the Coronary Artery Risk Development in Young Adults (CARDIA) Study. Clin. Chem. 49 (8) (2003) 1358-1366.

14. T. Stojakovic, H. Scharnagl, M. Trauner, B. Pieske, B. Wellnitz, U. Seelhorst, D. Schilling, B.R. Winkelmann, B.O. Boehm, W. Marz, Serum gamma-glutamyl transferase and mortality in persons undergoing coronary angiography-The Ludwigshafen Risk and Cardiovascular Health Study. Atherosclerosis (2009).

15. F. Gude, J. Rey-Garcia, C. Fernandez-Merino, L. Meijide, L. Garcia-Ortiz, C. Zamarron, A. Gonzalez-Quintela, Serum levels of gamma-glutamyl transferase are associated with markers of nocturnal hypoxemia in a general adult population. Clin. Chim. Acta 407 (1-2) (2009) 67-71.

16. A. Fukumura, M. Tsutsumi, M. Tsuchishima, N. Hayashi, M. Fukura, H. Yano, K. Ozaki, S. Takase, Effect of the inducer of interleukin-6 (ME3738) on rat liver treated with ethanol. Alcohol. Clin. Exp. Res. 31 (1 Suppl) (2007) S49-53.

17. I.M. Beck, W. Vanden Berghe, L. Vermeulen, K.R. Yamamoto, G. Haegeman, K. De Bosscher, Crosstalk in Inflammation: The Interplay of Glucocorticoid Receptor-Based Mechanisms and Kinases and Phosphatases. Endocr. Rev. (2009).

18. P. Dewint, V. Gossye, K. De Bosscher, W. Vanden Berghe, K. Van Beneden, D. Deforce, S. Van Calenbergh, U. Muller-Ladner, B. Vander Cruyssen, G. Verbruggen, G. Haegeman, D. Elewaut, A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. J. Immunol. 180 (4) (2008) 2608-2615.

19. V. Gossye, D. Elewaut, K. Van Beneden, P. Dewint, G. Haegeman, K. De Bosscher, A plant-derived glucocorticoid receptor modulator attenuates inflammation without provoking ligand-induced resistance. Ann. Rheum. Dis. (2009).

20. R.H. Oakley, J.A. Cidlowski, Homologous down regulation of the glucocorticoid receptor: the molecular machinery. Crit. Rev. Eukaryot. Gene Expr. 3 (2) (1993) 63-88.

21. D.C. DuBois, Z.X. Xu, L. McKay, R.R. Almon, N. Pyszcznski, W.J. Jusko, Differential dynamics of receptor down-regulation and tyrosine aminotransferase induction following glucocorticoid treatment. J. Steroid Biochem. Mol. Biol. 54 (5-6) (1995) 237-243.

22. J. Kirwan, L. Power, Glucocorticoids: action and new therapeutic insights in rheumatoid arthritis. Curr. Opin. Rheumatol. 19 (3) (2007) 233-237.

23. I.M. Adcock, P.J. Barnes, Molecular mechanisms of corticosteroid resistance. Chest 134 (2) (2008) 394-401.

24. A. Louw, P. Swart, Salsola tuberculatiformis Botschantzev and an aziridine precursor analog mediate the in vivo increase in free corticosterone and decrease in corticosteroid-binding globulin in female Wistar rats. Endocrinology 140 (5) (1999) 2044-2053.

25. R. Newton, Molecular mechanisms of glucocorticoid action: what is important? Thorax 55 (7) (2000) 603-613.

26. K. De Bosscher, W. Vanden Berghe, I.M. Beck, W. Van Molle, N. Hennuyer, J. Hapgood, C. Libert, B. Staels, A. Louw, G. Haegeman, A fully dissociated compound of plant origin for inflammatory gene repression. Proc. Natl. Acad. Sci. U. S. A. 102 (44) (2005) 15827-15832.

27. Z. Zhang, Z.Y. Zhang, H.J. Schluesener, Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. J. Immunol. 183 (5) (2009) 3081-3091.

28. H. Hirasawa, S. Oda, M. Nakamura, Blood glucose control in patients with severe sepsis and septic shock. World J. Gastroenterol. 15 (33) (2009) 4132-4136.

29. J. Szrama, P. Smuszkiewicz, I. Trojanowska, Glycemic profile and effectiveness and safety of insulin therapy in septic patients: is the blood glucose level sufficient? Pol. Arch. Med. Wewn. 119 (10) (2009) 621-627.

30. B. Yang, R.P. Trump, Y. Shen, J.A. McNulty, L.G. Clifton, S.A. Stimpson, P. Lin, G.L. Pahel, RU486 did not exacerbate cytokine release in mice challenged with LPS nor in db/db mice. BMC Pharmacol. 8 (2008) 7.

31. P. Borghetti, R. Saleri, E. Mocchegiani, A. Corradi, P. Martelli, Infection, immunity and the neuroendocrine response. Vet. Immunol. Immunopathol. 130 (3-4) (2009) 141-162.

32. G.P. Chrousos, P.W. Gold, The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 267 (9) (1992) 1244-1252.

33. H. Selye, A syndrome produced by diverse nocuous agents. 1936. J. Neuropsychiatry Clin. Neurosci. 10 (2) (1998) 230-231.

34. C. Tsigos, G.P. Chrousos, Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53 (4) (2002) 865-871.

35. S. Wust, D. Tischner, M. John, J.P. Tuckermann, C. Menzfeld, U.K. Hanisch, J. van den Brandt, F. Luhder, H.M. Reichardt, Therapeutic and adverse effects of a non-steroidal glucocorticoid receptor ligand in a mouse model of multiple sclerosis. PLoS One 4 (12) (2009) e8202.

36. V. Gossye, D. Elewaut, N. Bougarne, D. Bracke, S. Van Calenbergh, G. Haegeman, K. De Bosscher, Differential mechanism of NF-kappaB inhibition by two glucocorticoid receptor modulators in rheumatoid arthritis synovial fibroblasts. Arthritis Rheum. 60 (11) (2009) 3241-3250.

37. D. Duma, C.M. Jewell, J.A. Cidlowski, Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. J. Steroid Biochem. Mol. Biol. 102 (1-5) (2006) 11-21.

38. H. Schacke, W.D. Docke, K. Asadullah, Mechanisms involved in the side effects of glucocorticoids. Pharmacol. Ther. 96 (1) (2002) 23-43.

39. H. Schacke, A. Schottelius, W.D. Docke, P. Strehlke, S. Jaroch, N. Schmees, H. Rehwinkel, H. Hennekes, K. Asadullah, Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proc. Natl. Acad. Sci. U. S. A. 101 (1) (2004) 227-232.

40. M. Beato, M. Truss, S. Chavez, Control of transcription by steroid hormones. Ann. N. Y. Acad. Sci. 784 (1996) 93-123.

41. K. De Bosscher, W. Vanden Berghe, G. Haegeman, The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr. Rev. 24 (4) (2003) 488-522.

42. H.M. Reichardt, J.P. Tuckermann, M. Gottlicher, M. Vujic, F. Weih, P. Angel, P. Herrlich, G. Schutz, Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. EMBO J. 20 (24) (2001) 7168-7173.

43. K. Johansson-Haque, E. Palanichamy, S. Okret, Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occurs through a tethering mechanism involving C/EBP. J. Mol. Endocrinol. 41 (4) (2008) 239-249.

44. B.M. Necela, J.A. Cidlowski, Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells. Proc. Am. Thorac. Soc. 1 (3) (2004) 239-246.

45. J.E. Bodwell, L.A. Kingsley, J.W. Hamilton, Arsenic at very low concentrations alters glucocorticoid receptor (GR)-mediated gene activation but not GR-mediated gene repression: complex dose-response effects are closely correlated with levels of activated GR and require a functional GR DNA binding domain. Chem. Res. Toxicol. 17 (8) (2004) 1064-1076.

46. M.K. Short, D.E. Clouthier, I.M. Schaefer, R.E. Hammer, M.A. Magnuson, E.G. Beale, Tissue-specific, developmental, hormonal, and dietary regulation of rat phosphoenolpyruvate carboxykinase-human growth hormone fusion genes in transgenic mice. Mol. Cell. Biol. 12 (3) (1992) 1007-1020.

ADDENDUM A

Whole Cell Binding Assay Optimisation for use in determining GR levels

A.1 Washout Test

The following test was performed to show that it is possible to remove unlabelled ligand, used during pre-incubation induction, before incubation with ^{3}H-Dex for the whole cell binding assay. BWTG3 cells were seeded into 24-welltissue culture plates at a density of 2.5 x 10^{5} cells/well and incubated for 24 hours. The next day medium was changed to DMEM containing 10% charcoal treated FCS and 1% penicillin and streptomycin mixture and the cells were steroid starved for 24 hours. Cells were induced for 1 hour with ethanol, 10^{-5}M dex or 10^{-5}M CpdA. After induction the medium is aspirated from the wells and either washed with bovine serum albumin-PBS (0.2%) three times at room temperature with shaking or just replaced with PBS (Fig. A1). After the washout has been completed all wells (washout and no-washout) were washed three times with PBS at room temperature.

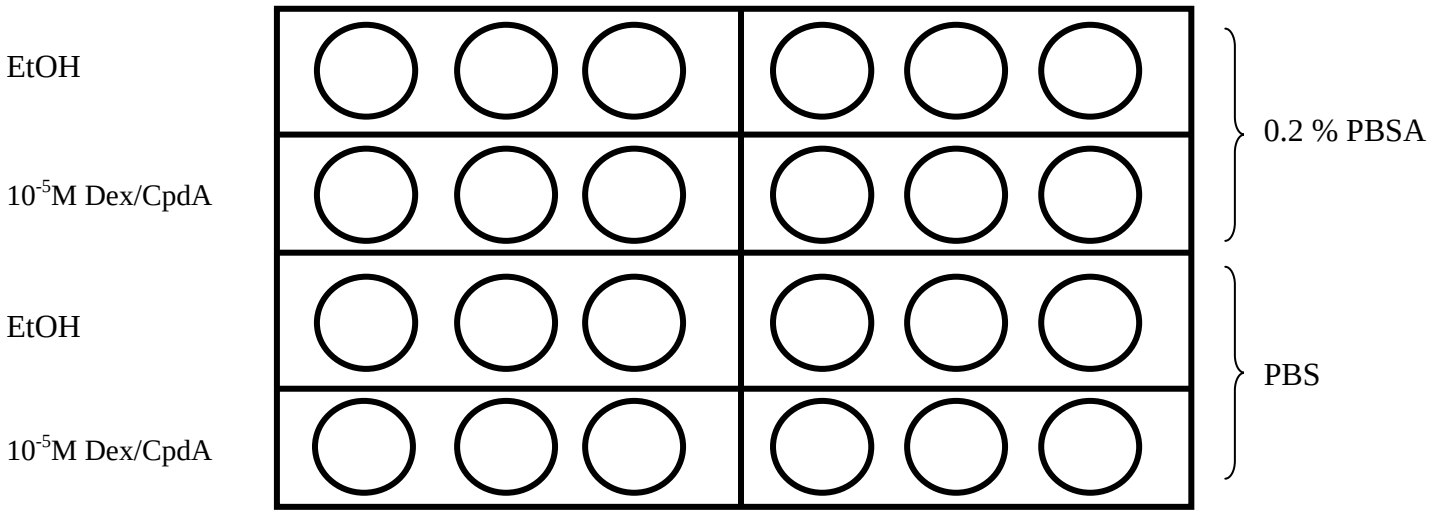


Figure A1. Schematic representation of plate layout for washout test. PBS, phosphate buffered saline; 0.2% PBSA, bovine serum albumin-PBS (0.2%).

After all wash steps had been completed DMEM containing 20 nM ^{3}H-Dex (Total Binding) or DMEM containing 20 nM ^{3}H-Dex + 1000 fold unlabelled Dex (Non Specific Binding) was added to wells and cells were incubated at 37 ˚C for 2 hours. After 2 hour incubation the plates were placed on ice, the medium aspirated and all wells were washed three times with ice cold bovine serum albumin-PBS (0.2%) for 15 minutes at 4˚C. Lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA and 1.44 mM EDTA) (100µl) was then added to each well after which the plates were shaken at room temperature for 15 min and frozen overnight at -20˚C.

On thawing of samples, 5 µl of lysate from each well was used for protein determination using the Bradford method [1]. The remaining lysate was transferred to scintillation vials to which 1.5 ml of scintillation fluid (Quickszint FLOW 2, Zinsser Analytic, South Africa) had been added. Radioactivity of the assay samples was determined using a Beckman LS 3801 Beta-scintillation counter. Results were normalised for protein content and expressed as CPM/mg protein.

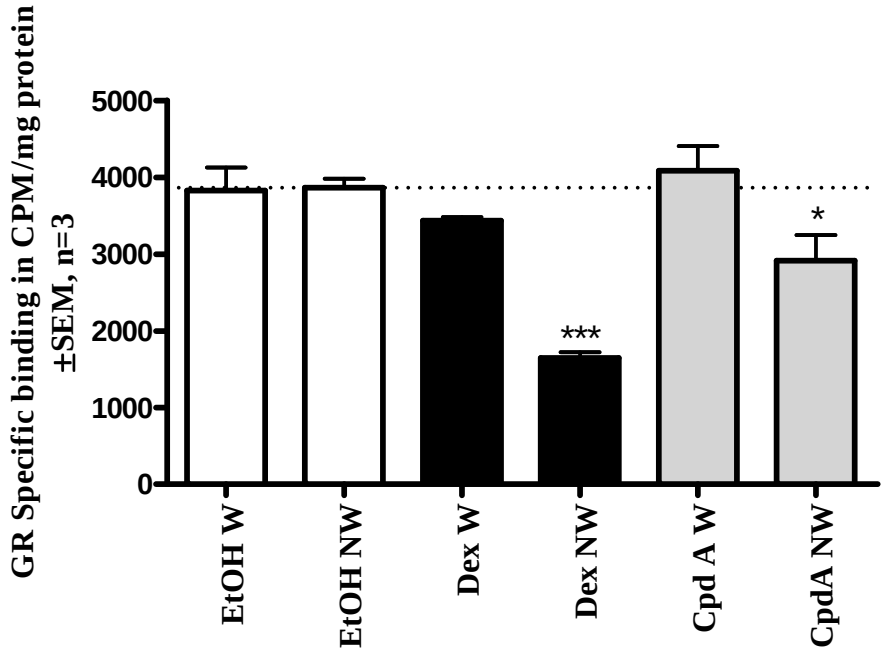


Figure A2. Recovery of GR binding activity after performing a washout with bovine serum albumin-PBS (0.2%) at room temperature. Statistical significance was determined by performing a one-way ANOVA with Dunnett's post test, comparing all columns to ethanol washout column. *P<0.05, **P<0.01, and ***P<0.001. Graph represents average values ± SEM for one experiment done in triplicate. W, washout performed; NW, no washout performed.

Performing a washout led to full recovery of GR binding activity (Fig. A2), compared to the significant loss of binding activity when no washout was performed. This confirms that it is possible to remove unlabelled ligand, used during the pre-incubation step with Dex or CpdA in the determination of GR activity levels, before incubation with ^{3}H-Dex for the whole cell binding assay.

A.2 Time to equilibrium

Whole cell binding assays assume that the binding of radio-ligand has reached equilibrium, thus to determine the time for equilibtium binding studies were done at different times. BWTG3 cells were seeded into 24-well tissue culture plates at a density of 2.5 x 10^{5} cells/well and incubated for 24 h. The next day the medium was changed to DMEM containing 10% charcoal treated FCS and 1% penstrep and the cells were steroid starved for 24 hours. Medium was aspirated after starvation and all wells were washed with bovine serum albumin-PBS (0.2%) three times at room temperature for 15 minutes with shaking. After the washout has been completed all wells were washed three times with PBS at room temperature.

After all wash steps had been completed DMEM containing 20 nM ^{3}H-Dex (Total Binding) or DMEM containing 20 nM ^{3}H-Dex + 1000 fold unlabelled Dex (Non Specific Binding) was added to wells and cells were incubated at 37 ˚C for 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 6 hours. After incubation, the plates were placed on ice, the medium aspirated and all wells were washed three times with ice cold bovine serum albumin-PBS (0.2%) for 15 minutes at 4 ˚C. Lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA and 1.44 mM EDTA) (100µl) was then added to each well after which the plates were shaken at room temperature for 15 min and frozen overnight at -20˚C.

On thawing of samples, 5 µl of lysate from each well was used for protein determination using Bradford method [1]. The remaining lysate was transferred to scintillation vials to which 1.5 ml of

scintillation fluid (Quickszint FLOW 2, Zinsser Analytic, South Africa) had been added. Radioactivity of the assay samples was determined using a Beckman LS 3801 Beta-scintillation counter. Results were normalised for protein content and expressed as CPM/mg protein.

Binding of labelled ligand equilibrated after two hours (Fig. A3) and it was decided to continue with experiments using this incubation period.

A.3. Unstripped FCS vs. Charcoal Treated FCS

The following experiment was performed to determine if the steroids present in untreated FCS would have an effect on the results obtained during whole cell binding assays.

BWTG3 cells were seeded into 24-well tissue culture plates at a density of 2.5 x 10^5 cells/well and cells and lysates were treated as in section A.2, using DMEM supplemented with 10% charcoal treated FCS and DMEM supplemented with 10% unstripped FCS.

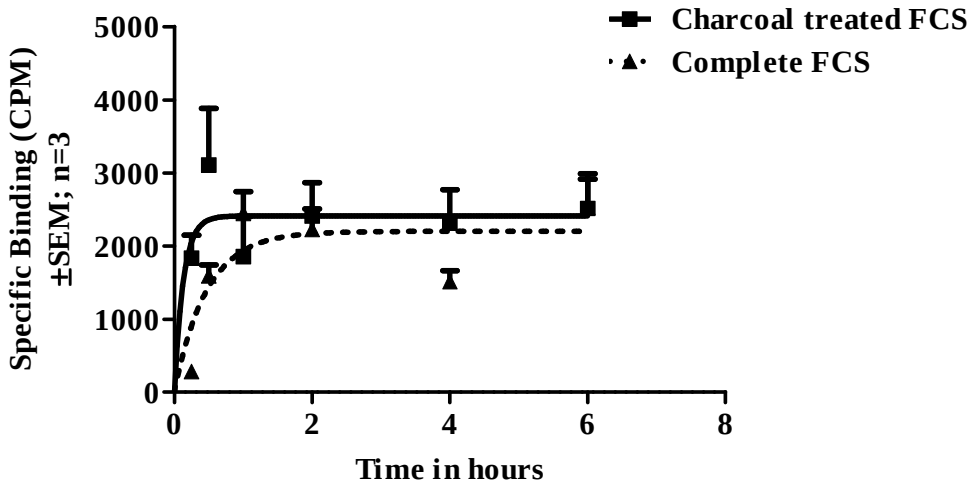


Figure A3. Time to equilibrium for ^{3}H-Dex and comparison between charcoal treated FCS and complete FCS binding. Graph represents average values ± SEM for one experiment done in triplicate. FCS, fetal calf serum.

Specific binding was higher when using DMEM containing charcoal treated FCS as supplement than when using DMEM supplemented with unstripped FCS. These additional steroids may have

an down-regulatory effect on GR activity levels and therefore it was decided to continue all experiments using DMEM containing charcoal treated FCS.

A.4. Determination of Kd value for Dexamethasone

The following experiment was done to determine the Kd value for Dex. It is necessary to determine the Kd value as it used in the equation to determine specific binding in fmol/gram protein (Equation 1).

BWTG3 cells were seeded into 24-well tissue culture plates at a density of 2.5 x 10^5 cells/well and treated as in section A.2 up until incubation with labelled ligand.

After all wash steps have been completed DMEM containing 20 nM ^{3}H-Dex or 10 nM ^{3}H-Dex are added to appropriate wells. Decreasing amounts of unlabelled Dex are then added to the plates containing 20 nM – and 10 nM [H]^3-Dex and the plates are incubated at 37 ˚C for 2 hours. After 2 hour incubation the plates are placed on ice, the medium is aspirated and all wells are washed three times with ice cold bovine serum albumin-PBS (0.2%) for 15 minutes at 4 ˚C. One hundred microliters of lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA and 1.44 mM EDTA) was then added to each well after which the plates were shaken at room temperature for 15 min and frozen overnight at -20˚C.

On thawing of samples, 5 µl of lysate from each well was used for protein determination using Bradford method [1]. The remaining lysate was transferred to scintillation vials to which 1.5 ml of scintillation fluids (Quickszint FLOW 2, Zinsser Analytic, South Africa) had been added. Radioactivity of the assay samples was determined using a Beckman LS 3801 Beta-scintillation counter. Results were normalised for protein content and expressed as CPM/mg protein.

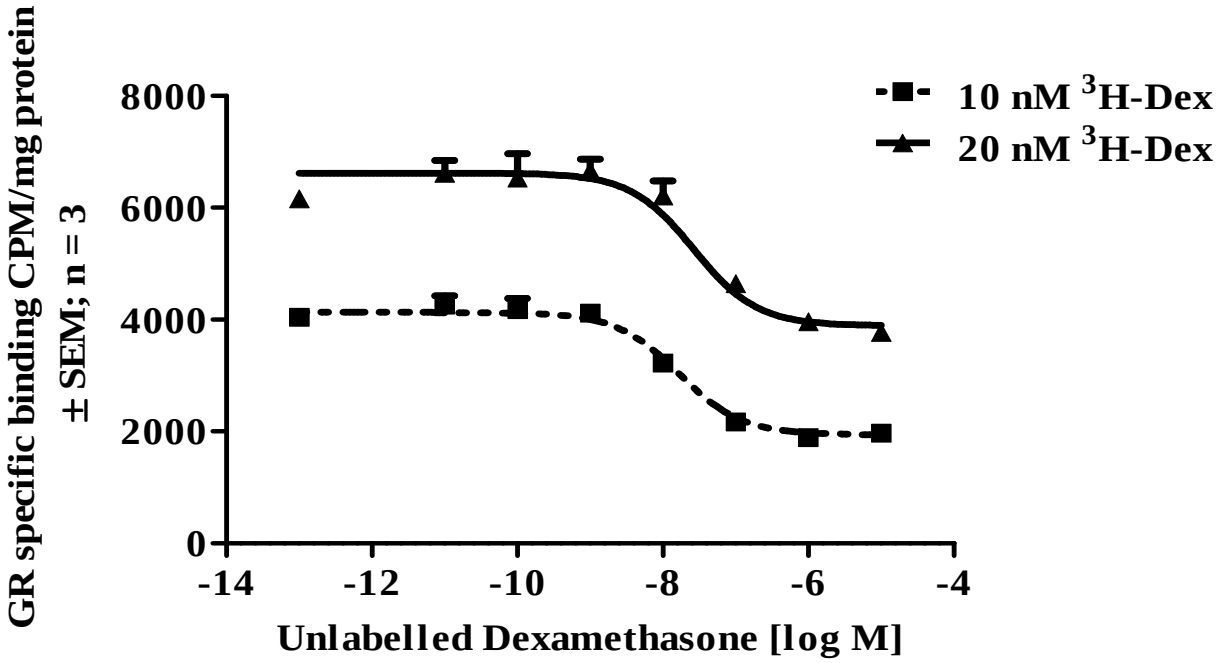


Figure A5. Competitive binding curves generated by the incubation of BWTG3 cells with two concentrations (10nM and 20nM) of ^{3}H-Dex and increasing amounts of unlabelled Dex to determine the Kd value using Global Fitting on GraphPad Prism®. Graph represents average values ± SEM for one experiment done in triplicate

Homologues competitive binding experiments measures the binding of a single labelled ligand in the presence of increasing amounts of the same, but, unlabelled ligand. In Fig.A5 a global fit model is created by the GraphPad Prism® program that defines a family of curves. A global fit model is useful as it allows us to share parameters between data sets and for each shared parameter the program will find one (global) best-fit value that is applicable to all data sets. To fit the data in Fig.A5 the program was instructed to fit one value for receptor number (Bmax) and one value for receptor affinity (Kd) that applies to both data sets. Once we had obtained the Kd value (6.6nM) for GR we could use it in the following equation to determine Bmax, the concentration of GR in fmol/g protein:

Y_{max} is defined as the specific binding (Total binding - Non-specific binding) and is measured in cpm/mg protein, which may be transformed to fmol/g protein by following the following procedure.

Convert CPM to DPM:

$$DPM = CPM \times (100/\text{Counting Efficiency}) \quad [1]$$

(CE was 37.2% in our system)

Convert DPM to Ci: (1 Ci = 2.22×10^{12} dpm):

$$Ci = DPM \text{ (Equation 1)} / 2.22 \times 10^{12} \quad [2]$$

Convert Ci value to fmol using specific activity (SA) of ligand in Ci/mmol:

SA for ^{3}H-Dex = 89 Ci/mmol

$$fmol = ((Ci \text{ (Equation 2)}/SA)) \cdot 10^{12} \quad [3]$$

And thus

$$fmol/g \text{ protein} = fmol \text{ (Equation 3)} / g \text{ protein} = Y_{max} \quad [4]$$

The Ymax (Equation 4) in fmol/g protein, Kd, and [Ligand] values in nM are now known and therefore Bmax may be calculated in fmol / g protein (Equation 5).

$$B_{max} = \frac{(Y_{max} ([Ligand] + Kd))}{[Ligand]} \quad [5]$$

A.5 Reference list

1. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248-254.

ADDENDUM B

Quantitive polymerase chain reaction (qPCR)

PCR efficiency is generally assumed to be two where the product is doubled with every PCR cycle. This efficiency is dependent on the efficiency of the primers and the PCR reaction and therefore it may vary. qPCR allows for these differences in primer efficiency to be taken into account and thereby an accurate analysis is obtained. The PCR product may be visualised in real time as the SYBR green is incorporated into the PCR product and the fluorescence is then measured at the completion of each cycle.

B.1 Determination of primer pair efficiency

Solvent treated RNA, transcribed to cDNA, from BWTG3 cells were used to generate standard curves for all primer pairs used, by making a serial dilution series of cDNA, and each dilution was analysed in triplicate. The PCR protocol is described in Material and Methods, Chapter 2. The cDNA standard triplicates (Fig. B1) have a similar PCR profiling indicating accurate pipetting, which is important for reducing experimental error. A no template control was added and confirmed that no primer self-amplification or contamination occurred. Similar dilution profiles were generated for all primer pairs, but are not shown.

The average cycle number, calculated from Fig. B1 by Rotor-Gene Q 5plex HRM software, was plotted against the sample concentration to generate a standard curve (Fig.B2). The slope obtained from this curve is used to calculate the exponential amplification value (E) for each primer (Table B1) set by using the following equation [1]:

$$(E) = 10^{[-1/\text{slope}]} \qquad [1]$$

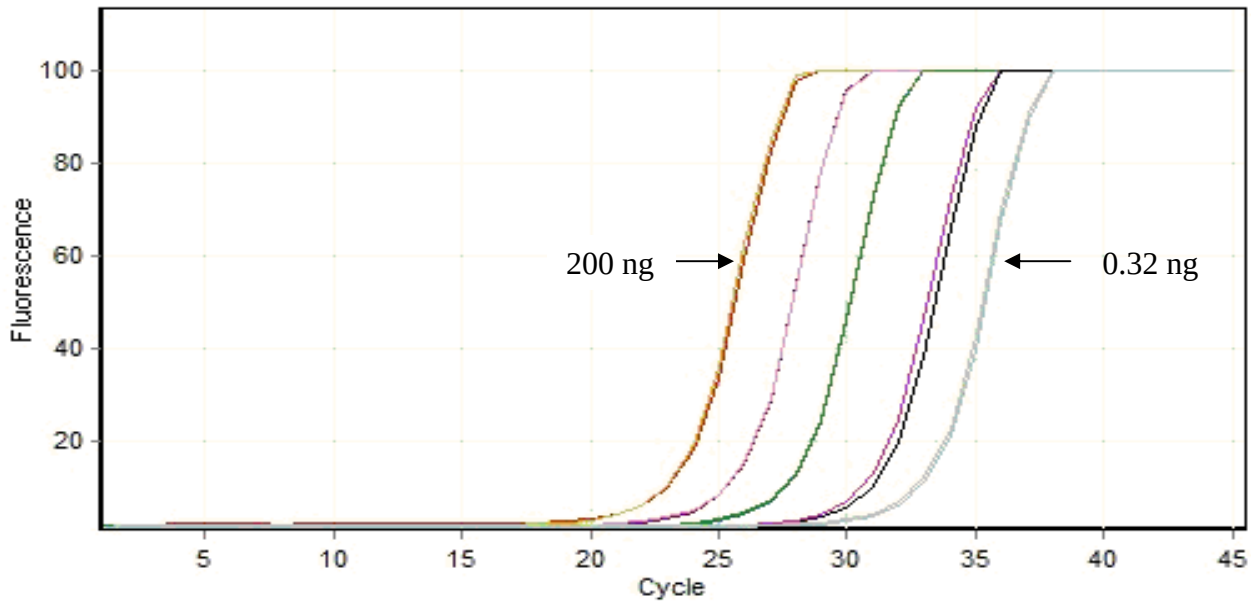


Figure B1. qPCR of a standard dilution series. Samples were analysed in triplicate. Relative expression was determined using the average cycle number (CT). The lines represent 200ng, 40ng, 8ng, 1.6ng, and 0.32ng. This specific curve was generated for the GR primers.

A new standard curve was generated, by Rotor-Gene Q 5plex HRM software, for each experimental repeat and as a result more than one E value was determined for each primer set and this allows for the elimination of experimental variations between subsequent repeats.

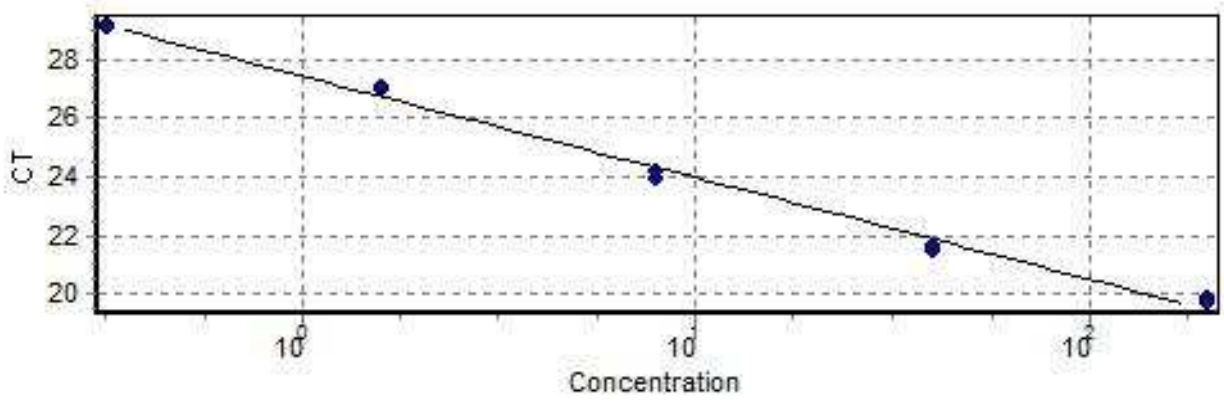


Figure B2. Standard curve showing cycle number (CT) versus the concentration of DNA amplified The slope obtained from this graph was used in equation 1: $E = 10^{[-1/slope]}$ [1] to calculate the exponential amplification value (E). This specific curve was generated for the GR primers.

Table B1. Exponential amplification (E) and R^2 values obtained, in triplicate, for each primer pair. R^2 values determined from standard curve by Rotor-gene software and $E = 10^{[-1/slope]}$.

Primer Set	Exponential Amplification Value (E)			R^2 value from standard curve		
	Repeat 1	**Repeat 2**	**Repeat 3**	**Repeat 1**	**Repeat 2**	**Repeat 3**
GAPDH	2.12	2.16	2.05	0.998	0.997	0.999
GR	1.95	2.07	1.82	0.993	0.993	0.977
TAT	2.40	2.42	2.36	0.987	0.991	0.989
PEPCK	2.92	2.4	2.58	0.930	0.961	0.989
GGT	2.65	2.02	2.07	0.976	0.963	0.935
CBG	2.82	2.25	2.21	0.950	0.933	0.985
CRP	2.49	2.37	2.35	0.980	0.989	0.985
SAA	2.81	2.68	2.53	0.904	0.969	0.966

B.2 Determination of relative expression values.

Analysis of the treated samples could begin once the primer efficiency was determined. The amount of product that is formed is dependent on the amount of the starting template being used. The formation of product can be measured in terms of fluorescence. A typical graph (Fig.B3) has a linear range where the amount of input template is proportional the PCR product. Similar curves were generated for all primer pairs, but are not shown. The crossing point (CP), determined by Rotor-Gene Q 5plex HRM software, of the sample fluorescence at this point is measured as a cycle number and is used for the quantification of the PCR product. When the formation of the PR product stops, due to the depletion of components needed for the PCR, a plateau phase is reached and is characterised by a flattening of the fluorescence with subsequent cycles.

Once linear range is reached, the cycle number at that particular fluorescence can be used in equation 1 [1] to determine the relative expression (R):

$$R = \frac{(E_{\text{target gene}})^{\Delta CP(\text{control} - \text{sample})}}{(E_{\text{reference gene}})^{\Delta CP(\text{control} - \text{sample})}} \qquad [2]$$

The value for ΔCP is obtained by subtracting the treated sample ΔCP (sample in equation 2) from the ethanol ΔCP (control in equation 2) and E is determined from equation 1. E is determined for every experimental repeat for which a standard curve dilution series was generated. The relative response (R) is therefore expressed as a ratio of target gene to reference gene (GAPDH). A ratio of one indicates no difference between the treated and solvent treated cells for the target gene primers normalised to the GAPDH reference gene.

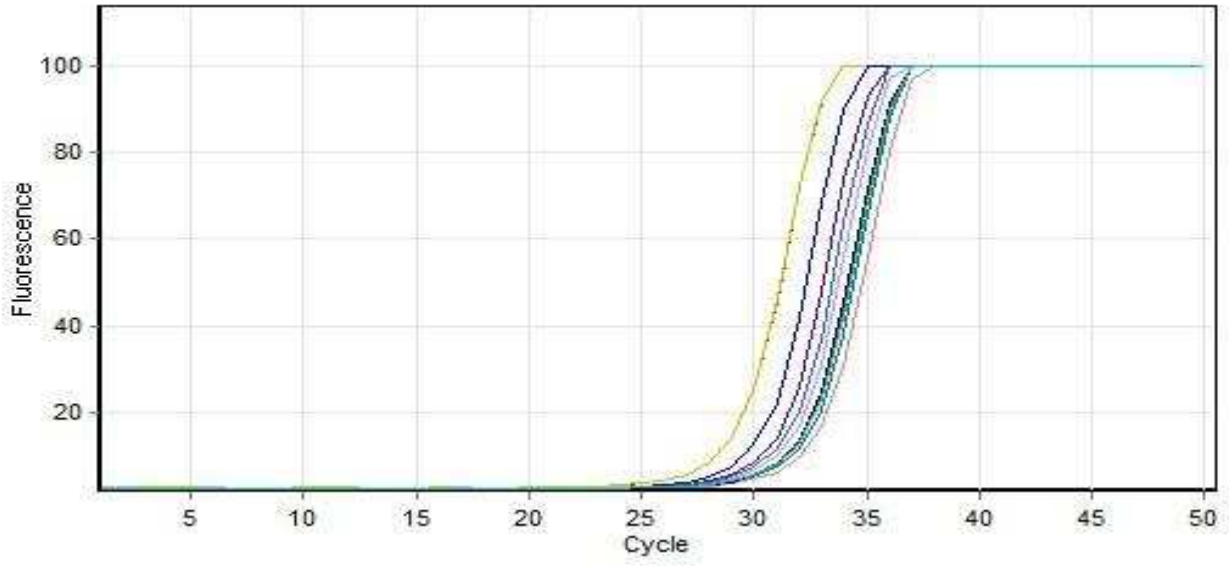


Figure B3. qPCR of cDNA samples. The increase in PCR product is indicated as an increase in fluorescence. The crossing point is chosen, by the Rotor-Gene Q 5plex HRM software, as the cycle number of the sample at a particular fluorescence where all the samples show linear amplification. This specific curve was generated for the TAT primers. For simplification none of the samples have been labelled.

A melting curve was generated by varying the temperature between 72˚C and 95˚C, raising the temperature by one degree for each step, while the fluorescence is measured throughout the melting process and the fluorescence is plotted as dF/dT (Fig.B4) against temperature by the Rotor-Gene Q

5plex HRM software and this generates a melting peak. The presence of only one peak per sample confirms that only one product was amplified in the PCR reaction and no primer dimers were formed. The melting temperature for the GAPDH is indicated in Fig. B4 as 83.5˚C. Similar curves were generated for all primer pairs (Table B2).

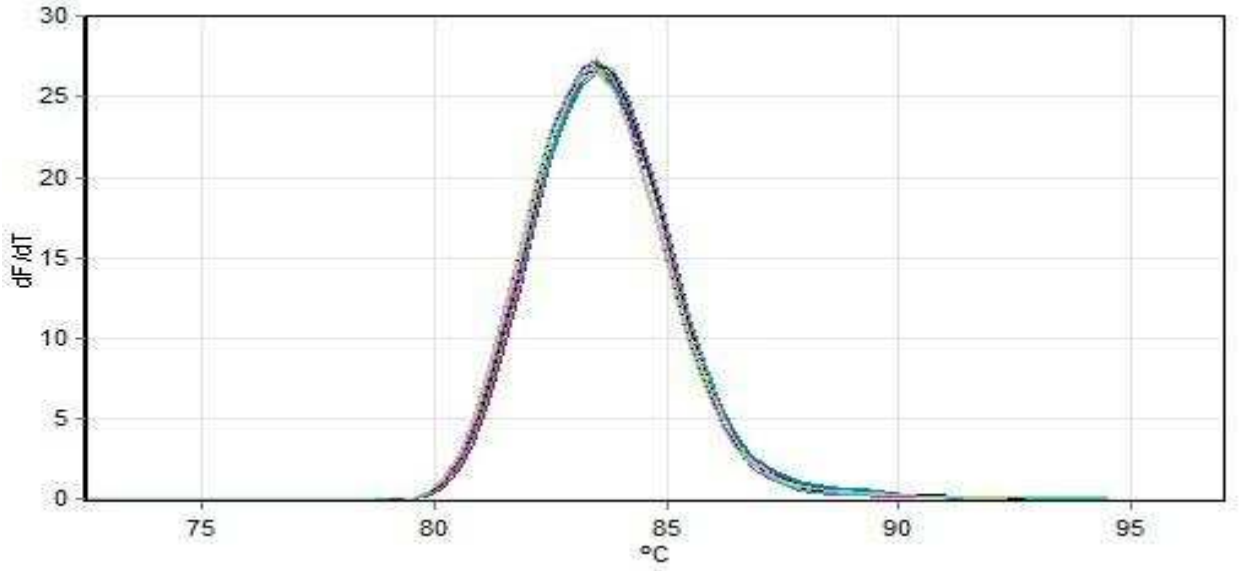


Figure B4. Melting peak of the PCR products. This specific curve was generated for the GAPDH. For simplification none of the samples have been labelled.

Tabel B2. Melting temperatures for primer sets. The data was obtained from melting curves, generated by the Rotor-Gene Q 5plex HRM software.

Primer set	Melting Temperature
GAPDH	83.5˚C.
GR	87.0˚C
TAT	86.3˚C
PEPCK	87.5˚C
GGT	85.7˚C
CBG	87.0˚C
CRP	88.0˚C
SAA	87.8˚C

B.3 Reference list

1. M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9) (2001) 2002-2007.

ADDENDUM C

ABROGATION OF GLUCOCORTICOID RECEPTOR DIMERIZATION CORRELATES WITH DISSOCIATED GLUCOCORTICOID BEHAVIOR OF COMPOUND A

Steven Robertson[1]*, Fatima Allie-Reid[1]*, Wim Vanden Berghe[2], Koch Visser[1], Anke Binder[1], Donita Africander[1], Michael Vismer[1], Karolien De Bosscher[2], Janet Hapgood[3], Guy Haegeman[2] and Ann Louw[1]

From Department of Biochemistry, University of Stellenbosch, Matieland 7602, Stellenbosch, Rep. of South Africa[1], Laboratory of Eukaryotic Gene Expression and Signal Transduction (LEGEST), Department of Physiology, Ghent University, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium[2] and Department of Molecular and Cell Biology, University of Cape Town, Private Bag X37701, Cape Town, Rep. of South Africa[3].

Running head: CpdA abrogates GR dimerization

Address correspondence to: Ann Louw, Department of Biochemistry, University of Stellenbosch, Matieland 7602, Stellenbosch, Rep. of South Africa. Telephone number +27-21-8085873, fax number +27-21-8085863 and email address al@sun.ac.za**.**

*****Steven Robertson and Fatima Allie-Reid contributed equally to the experimentation.

This article has been accepted for publication by the Journal of Biological Chemistry.

My contribution to the article was Figs. 3D, 6C, 7A, 8B, and 8C.

Compound A (CpdA), a dissociated glucocorticoid receptor modulator, decreases corticosteroid-binding globulin (CBG), adrenocorticotropic hormone (ACTH) and luteneinizing hormone (LH) levels in rats. Whether this is due to transcriptional regulation by CpdA is not known. Using promoter reporter assays we show that CpdA, like dexamethasone (Dex), directly transrepresses these genes. Results using a rat CBG proximal-promoter reporter construct in BWTG3 and HepG2 cell lines support a glucocorticoid receptor (GR)-dependent transrepression mechanism for CpdA. However, CpdA, unlike Dex, does not result in transactivation via glucocorticoid-responsive elements (GREs) within a promoter reporter construct even when GR is co-transfected. The inability of CpdA to result in transactivation via GREs is confirmed on the endogenous tyrosine aminotransferase (TAT) gene while transrepression ability is confirmed on the endogenous CBG gene. Consistent with a role for CpdA in modulating GR activity, whole-cell binding assays revealed that CpdA binds reversibly to the GR, but with lower affinity than Dex, and influences association of ^{3}H-Dex, but has no effect on dissociation. In addition, like Dex, CpdA causes nuclear translocation of the GR, albeit to a lesser degree. Several lines of evidence, including fluorescence resonance energy transfer (FRET), co-immunoprecipitation (CoIP) and nuclear immunofluorescence studies of nuclear localization-deficient GR show that CpdA, unlike Dex, does not elicit ligand-induced GR dimerization. Comparison of the behaviour of CpdA in the presence of wtGR to that of Dex with a dimerization-deficient GR mutant (GR^{dim}) strongly supports the conclusion that loss of dimerization is responsible for the dissociated behaviour of CpdA.

CpdA is a more stable analogue of the labile compound found in the Namibian plant, *Salsola tuberculatiformis* Botschantzev, which causes prolonged gestation in sheep and contraception in rats (1). CpdA, like the shrub, causes contraception in rats (2). Further investigation at a molecular level has shown that CpdA competitively inhibits sheep adrenal cytochrome P450-dependent steroid 11β-hydroxylase (P450c11), the enzyme responsible for the final step in the synthesis of glucocorticoids (GCs) (2), (3). In addition, CpdA binds to and displaces endogenous GCs from rat and sheep CBG, the plasma globulin that binds GCs with high affinity (4). Studies in Wistar rats suggest that the latter mechanism predominates *in vivo* with significantly increased free corticosterone levels due to displacement from CBG by CpdA and concomitant decreases in CBG, ACTH and LH levels (5). Although the significant decreases in CBG, ACTH, and LH levels may be ascribed solely to feedback regulation mediated by the increase in free, biologically active GC concentration, it was postulated that direct interaction of CpdA with the GR should not be discounted (5). The fact that CpdA interacts with the two GC-binding proteins, P450c11 and CBG, implies that interaction and signalling through the GR may be a distinct possibility. Indeed, our recent paper describes such an interaction within the context of anti-inflammatory action (6).

The GR is a ligand-dependent transcription factor mediating the effects of GCs (7). In the absence of ligand, the GR is predominantly cytoplasmic. Upon ligand binding the GR translocates to the nucleus, where the activated receptor can transactivate or transrepress specific genes (8). Several models for transcriptional modulation by the GR have been presented (9), (10), (11). Broadly speaking, transactivation is mediated by binding of a GR dimer to glucocorticoid response elements (GREs) in the promoter region of GC-responsive genes, followed by recruitment of coactivators, chromatin remodelling, and increased gene transcription (12). Although transrepression may also be mediated via direct binding to DNA, via negative GREs (nGREs) (9), it mostly proceeds, without direct DNA binding by the GR, via protein-protein interactions that require binding of the GR monomer to other transcription factors, such as NFκB, AP-1 and C/EBP (13), (14), (10), (15), (16). This last mechanism is often called tethering.

The current study establishes that CpdA, like Dex, directly transrepresses the three genes, CBG, proopiomelanocortin (POMC), and gonadotropin-releasing hormone (GnRH), shown to be involved in the *in vivo* response to CpdA and that these results are thus not only due to the increase in free corticosterone (5). Further investigation indicates that CpdA transrepression of CBG is GR-mediated. Despite its ability to transrepress GC-sensitive genes, CpdA is unable to transactivate GRE-containing promoters. Analysis of the molecular mechanism of action of CpdA via the GR indicates that CpdA binds reversibly to endogenous rat GR, influences association but not dissociation of ^{3}H-Dex, and causes nuclear translocation of liganded GR, although to a lesser extent than Dex. However, CpdA, unlike Dex, does not result in dimerization of the GR. The implications of loss of dimerization are explored further by comparing the activity of CpdA via the wtGR with that of Dex via a dimerization-deficient GR mutant (GR^{dim}) (16). Nuclear translocation behaviour and transrepression mediated by CpdA via the wtGR does not differ significantly from that observed with Dex via the GR^{dim} strongly supporting a mechanism whereby the inability of CpdA to elicit ligand-induced dimerization of the GR is responsible for its dissociated behaviour.

Experimental Procedures

Test compounds – Dex, phorbol 12-myristate 13-acetate (PMA), aldosterone, 4,5α-dihydrotestosterone (DHT), 17β-estradiol (E_2), spironolactone, and mifepristone (RU486) were obtained from Sigma, ICI 182,780 from Tocris, R5020 from Perkin Elmer and hydroxyflutamide was a kind gift from Dr. C. Tendler (Schering Plough Research Institute, USA). Compound A (CpdA; 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride), was synthesized as described previously (2).

Plasmids – G.L. Hammond kindly provided the rat CBG proximal promoter reporter construct (rCBG295Luc) (17). The GnRH promoter reporter construct (3446mGnRHluc) was donated by D. DeFranco (18). The rat POMC promoter reporter construct (JA300), and the expression vector for NUR77 (pCMX-Nur77) were gifts from J. Drouin (19). The β-galactosidase reporter plasmid (pPGKβGeopbA) was a gift from P. Soriano

(Fred Hutchinson Cancer Research Centre, Seattle, USA) and pGL2-basic empty vector was obtained from Promega. The rat GRα (pSVGR1) expression vector was a gift from R. Miesfield (20), while the human GR (pRS-hGRα) and human MR (pRS-hMR) were gifts from R.M. Evans (Howard Hughes Medical Institute, La Jolla, USA). CFP-tagged GR (pECFP-hGRα) and YFP-tagged GR (pEYFP-hGRα) were gifts from J. Cidlowski (21). Mouse wtGR (pcDNA3.1-GRWT) and mouse GR^{dim} (pcDNA3.1-GR^{dim}) mutant were gifts from H. Reichardt (22). Rat GR (pTC2-wtGRrat) and c-myc tagged nuclear translocation mutant GR (myGR_{NL1^-}) were gifts from R. Haché (23). Human ERα (pcDNA3-ERα) was a gift from D. Harnish (24). Human PRB (pSG5hPRB) was obtained from S. Simons Jr (25) and human AR (pSVARo) was obtained from A. Brinkmann (26). $(GRE)_2$50hIL6PLuc and the Flag-tagged GR pEFFlaghGRα (Mr 96 kDa) were constructed as previously described (27), (28), while the $(GRE)_2$tkLuc construct, and GFP-tagged GR (pEGFP-C2-GR, Mr 128.5 kDa) were provided by S. Okret (Dept. of Medical Nutrition, Karolinska Institute, Sweden). The GRE-containing promoter reporter construct (pTAT-GRE2-Elb-luc) was a gift from G. Jenster (29), the ERE-containing promoter reporter construct (pGL2-3x-ERE-TATA-luc) from D. McDonnell (30), whereas the IL6-luc promoter reporter construct ($p(IL6\kappa B)_3$50hu.IL6Pluc+) has been described previously (31).

Cell Culture - BWTG3 (LEGEST, University of Gent, Belgium), HepG2 (Highveld Biological Association, South Africa), and COS-1 cells (ATCC) were cultured in high glucose (4.5 g/ml) Dulbecco's modified eagle's medium (DMEM) (Sigma) supplemented with 10% FCS (Highveld Biologicals, South Africa), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco), 2mM glutamine (Merck), 44mM sodium-bicarbonate (Gibco), and 1 mM sodium-pyruvate (Gibco). In addition, BWTG3 and HepG2 cells had 0.1 mM non essential amino acids (Gibco) added to their medium. GT1-7 neuronal cells (a kind gift from P. Mellon, University of California, La Jolla, USA) were maintained in DMEM with 25 mM HEPES, 4500 mg/l glucose and pyroxidine, supplemented with 10% fetal bovine serum (FBS). AtT-20 (ATCC) pituitary tumour cells in suspension were cultured in Kaighn's modification of Ham's F12K medium (Invitrogen), supplemented with 15% horse serum and 2.5% FBS.

Competitive Whole-Cell Binding Assays - were performed essentially as described by (32) with the following modifications. Forty-eight h after plating BWTG3 cells ($2x10^5$ cells per well in 24-well tissue culture plates) were washed three times with pre-warmed phosphate-buffered saline (PBS) and then incubated in medium (minus FCS or Pen-Strep) with 20 nM ^{3}H-Dex (specific activity of 89 Ci/mmol; AEC-Amersham) and varying concentrations of unlabelled test compounds for one h at 37^{o}C. Cells were then placed on ice and after one h washed three times, for 15 minutes each, with ice-cold PBS containing 0.2% (w/v) bovine serum albumin (BSA). Cells were lysed with 100 µl lysis buffer (PE Biosystems) and binding was determined by scintillation counting. Total binding was normalized to protein concentration (Bradford assay) and expressed as percentage binding (top plateau for Dex binding designated as

100% binding and bottom plateau as 0% binding).

Kinetic Whole-Cell Binding Assays - were done in the presence of 1 nM ^{3}H-Dex in the absence or presence of CpdA. For association binding experiments, binding was measured for different times (0-240 min) after addition of radioligand in the absence (total binding) or presence of 10 μM unlabeled Dex (non-specific binding). The binding experiment then proceeded as described for competitive binding. Specific binding (total binding – non-specific binding) was plotted. For dissociation binding experiments, the cells were firstly incubated in the presence of 1 nM ^{3}H-Dex with or without CpdA for two h at 37^{o}C after which the medium was replaced with fresh medium without radioligand and CpdA. Binding at different time points (0-240 min) proceeded as described for competitive binding and was plotted as such.

Reversibility of Whole-Cell Binding Assay - was done by pre-incubating cells with 10μM CpdA or 10μM Dex for 1 h at 37^{o}C. Cells were then washed three times with pre-warmed PBS containing 0.2% (w/v) BSA and incubated for one h at 37^{o}C with 1 nM [^{3}H]-Dex in the presence or absence of 10 μM unlabeled Dex. The binding experiment then proceeded as described for competitive binding. Specific binding (total binding – non-specific binding) was plotted.

Whole-Cell Binding Assays to determine Steroid Receptor content of BWTG3 cells - was done using 20 nM ^{3}H-Dex (specific activity of 85 Ci/mmol; AEC-Amersham) for GR, 20 nM ^{3}H-E_2 (specific activity of 84 Ci/mmol; AEC-Amersham) for ER, 20 nM ^{3}H-R5020 (specific activity of 84.6 Ci/mmol; AEC-Amersham) for PR, 20 nM ^{3}H-mibolerone (specific activity of 76.8 Ci/mmol; AEC-Amersham) for AR and 9 nM ^{3}H-aldosterone (specific activity of 87.9 Ci/mmol; AEC-Amersham) for MR. Cognate unlabeled ligands were added in 1000-fold excess. The binding experiment proceeded essentially as described for competitive binding except that the incubation at 37^{o}C was done for 4 h. Specific binding (total binding – non-specific binding) was calculated in fmol/mg protein using a counting efficiency of 40%.

Promoter reporter construct studies **- Cells (BWTG3, HepG2, COS-1, GT1-7 and AtT-20 cells) were plated in relevant complete medium at densities indicated in the Figure legends. Cells were transfected 24h later with constructs (as indicated in Figure legends) in medium without FCS or, for GT1-7 and AtT-20 cells, medium with 10% dextran-coated charcoal-stripped FBS (Highveld Biologicals, South Africa) using Fugene^{TM}6 transfection reagent (Roche Molecular Biochemicals) or for GT1-7 cells, the lipofectamine method (Gibco), as described by the manufacturer. Cells plated into 10 cm tissue culture dishes were replated 24 h after transfection at densities indicated in the Figure legends. Test compounds were added to cells 24 or 4 h (GT1-7 cells) after transfection or replating and incubated for 24 or 20 h (GT1-7 cells). Induction in BWTG3, HepG2 and COS-1 cells occurred in medium without FCS and antibiotics except for assays investigating ER activity, where phenol-red free medium was used, while induction in GT1-7 and AtT-20 cells occurred in medium with 10% dextran-coated charcoal-stripped FBS. After induction cells were lysed with 100 μl lysis buffer (PE Biosystems), and frozen at –20°C. Luciferase activity was determined using the luciferase assay kit (Promega) and β-**

galactosidase activity was measured using the Galacto-star assay kit (PE Biosystems) according to the instructions of the manufacturer. Light emission was measured in a luminoskan plate reader (Labsystems). Luciferase relative light units (RLUs) were normalized with β-galactosidase values to correct for transfection efficiency. For replated cells, protein concentration was measured using the Bradford method and luciferase RLUs normalized with protein concentrations to correct for plating efficiency.

RNA isolation **- Twenty-four h after plating BWTG3 or HepG2 cells ($3x10^5$ cells per plate in 10 cm tissue culture dishes) medium was changed to Opti-MEM (Gibco) and cells were incubated for a further 24 h. Cells were then treated with test compounds for 72 h (HepG2 cells) or 24 h (BWTG3 cells), and total RNA was extracted according to the TRIzol method, as described by the manufacturer (Sigma). After extraction, the final RNA pellet was dissolved in 50 µl formazol (Molecular research centre, Inc), if used for Northern blotting, or 50 ml diethyl pyrocarbonate (DEPC) water, if used for RT-PCR, and kept at –70°C until used.**

Northern blotting **- was essentially preformed as previously described (33). Briefly, 20 µg of total RNA was loaded and run on a 1% formaldehyde-agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham). RNA was fixed on the membrane by using a UV crosslinker for 12 sec. The membrane was prehybridized in a hybridization oven at 50°C for 1 h with pre-warmed "Dig easy Hyb" solution (Roche). Plasmids carrying human CBG complementary DNA (cDNA kindly provided by G.L. Hammond) were amplified in a DH5α competent *E. coli* strain and digested with EcoRI in order to obtain the CBG cDNA insert of 1.2 kilobases. Hybridization was performed overnight at 50°C with [^{32}P]-CBG cDNA probes labelled with [α-^{32}P]-deoxycytidine triphosphate using the random priming technique (Amersham megaprime labelling kit). Membranes were washed twice for 5 min in 2xSSC, 0.1% SDS at room temperature, followed by two washes for 15 min in 0.1xSSC, 0.1% SDS at 50°C. Membranes were exposed for between 24 and 48 h at –70°C followed by densitometric scanning of the autoradiograms using the UN-SCAN-IT program. Membranes were stripped using a hot 0.5% SDS solution and reprobed with [^{32}P]-β–actin cDNA, provided by I. _Parker, UCT, South Africa. Autoradiography was for less than 24 h, followed by densitometric scanning and normalization of the CBG values with the corresponding β–actin values.**

RT-PCR - Total RNA (5µg) was reverse transcribed with oligo$(dT)_{15}$ primers (Promega) using the AMV reverse transcriptase enzyme (Promega) and followed by a PCR reaction on the obtained cDNA with Taq DNA polymerase (Promega) and primers, specific for mGAPDH, rCBG and rTAT (primer sequences available on request).

Tyrosine aminotransferase assay – Twenty-four h after plating BWTG3 cells (2.5×10^5 cells per well in a 6-well tissue culture plate) medium was changed to medium with stripped FCS for a further 24 h. Cells were then treated with test compounds for 4 h after which cells were washed twice with PBS and lysed with 250 µl lysis buffer (10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml aprotinin) and frozen at -20°C. On thawing, lysates were briefly sonicated and centrifuged (10000g, for 10 min at 4°C). TAT activity was determined according to the method of Diamondstone (34). TAT activity

was expressed as absorbance at 330 nm per minute and graphed as percentage of control.

Immunofluorescence analysis - **COS-1 cells were plated into 10 cm tissue culture dishes and transiently transfected 24 h later with constructs as indicated in the Figure legends using Fugene^{TM}6. Cells were replated 24-h later onto coverslips in 6-well plates in medium and at densities indicated in the Figure legends. BWTG3 cells were grown on coverslips in 6-well plates in complete medium at densities indicated in the Figure legends. Twenty-four h later cells were serum-starved for 24 h in medium indicated in Figure legends. Cells were treated with test compounds as indicated in Figure legends. After induction, cells were placed on ice, rinsed with 1 ml methanol and incubated at –20°C for 15 min with another 1 ml methanol. Cells were then washed three times with ice-cold PBS plus 0.2% BSA and transferred to new 6-well plates containing 2 ml blocking buffer (PBS with 3% (v/v) new born calf serum (NBCS) and 1% (v/v) BSA). Cells were incubated for 1 h at room temperature and then washed twice with ice-cold PBS plus 0.2% BSA. To visualize GR, cells were incubated with the primary rabbit anti-GR antibody, P-20 (Santa Cruz biotechnology) diluted 1:100 in blocking buffer for BWTG3 cells or H-300 (Santa Cruz biotechnology) diluted 1:1000 in blocking buffer for COS-1 cells. To visualize c-myc tagged GR cells were incubated with a mouse anti-c-myc antibody, 9E10 (Sigma), diluted 1:500. Cells were then washed three times with ice-cold PBS plus 0.2% BSA and incubated for 1 h at room temperature with secondary antibody, Alexa Fluor 488-tagged or 594-tagged anti-rabbit antibody (Molecular Probes) as indicated in the Figure legends, diluted 1:500 in blocking buffer. Nuclei were visualized by using either propidium iodide staining including RNase (30 min at 37°C), Hoechst 33258 stain (Sigma, according to manufacturer's instructions) or DAPI staining for 5 min at room temperature as indicated in Figure legends. Cells were then washed three times with ice-cold PBS, mounted on glass slides and analysed using the microscope indicated in the Figure legends. Cells were assessed for intracellular localization of protein signal in a double-blind fashion with 45-50 cells counted in each sample. Cells were either allocated to one of three groups, predominantly nuclear, predominately cytoplasmic or evenly distributed between cytoplasm and nucleus or allocated as nuclear or not.**

Co-immunoprecipitation – COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected 24 h after plating with 38.6 ng hGR (19.3 ng pEFFlaghGRα and 19.3 ng pEGFP-C2-GR) and 11.5 µg pGL2-basic using the DEAE-Dextran method. Cells were re-plated 24 h later into 6-well plates ($6x10^5$ cells/well) in medium with stripped FCS and after 24 h treated with ethanol, Dex (1 µM) or CpdA (10 µM) for 1 h. After induction cells were washed twice with PBS before extraction on ice in Buffer A (10mM Hepes pH 7.5 (Gibco, Invitrogen Corporation), 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP-40 (Roche) and Complete Mini protease inhibitor cocktail (Roche)). After two cycles of freeze thaw cells were collected, centrifuged at 14 000 rpm for 15 min, and supernatant collected. Protein concentrations were determined using Bradford and 20 µg protein/sample set aside for Western blots (input). Lysates (200 µg protein/sample) were precipitated with 30 µl EZview Red ANTI-FLAG M2 Affinity Gel beads (Sigma), pre-washed 4 times with Buffer A in presence of 0.5% BSA, in a total volume of 250 µl for 16 h at 4°C. Beads were washed four times with

200 µl Buffer A supplemented with 0.5% Triton X-100 (BDH) and 150mM NaCl. 20 µl of Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 1.25% (m/v) SDS, 0.00125% (m/v) bromophenol Blue, and 2.5% ß-mercaptoethanol) was then added to beads, which were boiled for 7.5 min at 95°C. For Western blotting immune precipitates (20 µl) were separated on a 10% SDS-PAGE gel, together with the inputs of total cell lysate (20 µg). Following electrophoresis, proteins were electro-blotted and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences), which were probed for GR (H-300 antibody from Santa Cruz Biotechnology diluted 1:3000) and visualized using ECL Peroxidase labelled anti-rabbit antibody (AEC Amersham) and ECL Western blotting detection reagents (GE Healthcare Amersham) on Hyperfilm (Amersham Biosciences).

Fluorescence resonance energy transfer - COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected with 5.8 µg pECFP-hGRα and 5.8 µg pEYFP-hGRα for FRET experiment and 5.8 µg pECFP-hGRα, or 5.8µg pEYFP-hGRα, plus 5.8 µg pGL2-basic for controls using the DEAE-Dextran method. Twenty-four h later cells were replated ($2x10^6$ cells per well) into 8-well Lab-Tek chambered coverglass plates (Nunc) in medium with stripped FCS. Twenty-four h after replating cells were analysed in the temperature-controlled chamber (37°C) of an Olympus Cells system attached to an IX 81 inverted fluorescence microscope (Olympus Corp.) equipped with an F-view-II cooled CCD camera (Soft Imaging Systems). The light source was a 150W Xenon lamp, part of the MT20 excitation source. Cells were observed with an X60 objective lens and the Cell® imaging software used for image acquisition and analysis. The YFP filter set excites at S500/20x (Chroma) and emission is detected at S535/30m, while the CFP filter set excites at S430/25x and emission is detected at S470/30m. Cells which express similar CFP-GR and YFP-GR levels were selected and treated with solvent, 10 µM CpdA or 10 µM Dex in DMEM containing no supplements. CFP, YFP and FRET images were taken every minute over a 30-minute period. FRET fluorescence was detected using a filter set with S430/25x excitation and S535/30m emission. An exposure time of 1500 ms at 100% light intensity was used. The signals measured in the FRET channel were corrected for crosstalk from the cyan and yellow channels using the following equation: NFRET = FRET signal – (*a**YFP signal) – (*b**CFP signal). N is normalized FRET and *a* and *b* were determined by measuring the crossover into the FRET channel of the YFP and CFP signals, respectively, in cells expressing each fusion protein on its own (35). In our system, approximately 59% of the CFP signal and 2.6% of the YFP signal was detected in the FRET channel. Background subtraction was carried out using an area where no cells were present.

RESULTS

CpdA causes transrepression of promoter reporter constructs of three genes involved in the in vivo response to CpdA administration in rats - CpdA administration was previously shown to significantly repress CBG, ACTH, and LH levels in Wistar rats, while concomitantly increasing free corticosterone

levels (5). Whereas this repression by CpdA might be attributed to the increase in biologically active corticosterone, the possibility of direct action of CpdA via the GR was not precluded. To test this assumption, we investigated the direct transcriptional effect of CpdA in cell lines on the promoter reporter constructs of CBG, POMC and GnRH. The latter two promoters were chosen as the POMC gene encodes the precursor to ACTH (36), while GnRH levels reflect circulating LH levels (37). It is clear (Fig. 1) that CpdA, like Dex, significantly ($P<0.05$) represses all three promoter reporter constructs.

CpdA transrepresses rat CBG via a GR-dependent mechanism in hepatic cell lines but does not transactivate GRE-containing promoters or genes **–** Having shown that CpdA transrepresses, we chose to focus on the CBG promoter, as little work has been done on GC responsiveness of this promoter in contrast to the POMC and GnRH promoters. Dose response curves of rat CBG promoter reporter transrepression in BWTG3 cells (Fig. 2A) revealed no significant ($P>0.05$) difference between the potency (logEC50) of Dex (-8.43) and CpdA (-8.93). However, analysis of the efficacy (maximal repression) indicates that Dex represses 34.5 ± 2.7%, which is significantly ($P<0.05$) more than CpdA (24.7 ± 3.8%). Both CpdA and Dex, also significantly repress CBG mRNA levels in HepG2 cells (Fig. 2B) and in BWTG3 cells (Fig. S1), which establishes that transcriptional regulation of the CBG promoter correlates with regulation at the mRNA level.

To test whether transrepression potential is dependent on the GR, BWTG3 cells were co-transfected with the rat GRα expression vector (rGRα) in the presence of the ratCBG295Luc reporter construct (Fig. 2C closed bars). Co-transfection with rGRα in BWTG3 cells significantly increased the transrepression response of Dex ($P<0.01$) and CpdA ($P<0.05$). To strengthen the case that the observed repression of the ratCBG295Luc promoter reporter construct by CpdA was mediated by the GR, the effect of the glucocorticoid antagonist, RU486, on transrepression in BWTG3 cells was also investigated (Fig. 2C, hatched bars). RU486 relieved the transrepression observed in the presence of both CpdA and Dex suggesting that repression of the ratCBG295Luc promoter reporter construct by Dex, as well as CpdA, is mediated by the GR.

Having shown that CpdA can result in transrepression of GC-responsive genes, such as CBG, we were interested in whether it could also result in transactivation of GC-responsive genes and thus evaluated the ability of CpdA to transactivate two GRE-containing promoters. BWTG3 cells were transiently transfected with a $(GRE)_2$tkLuc (Fig. 3A) or a $(GRE)_2$50hIL6PLuc promoter reporter construct (Fig. S2A). The result with both constructs shows that in the presence of endogenous GR (Figs. 3A and S2A, open bars), Dex significantly ($P<0.01$) transactivates the GRE-dependent promoter constructs while co-transfection of rat GRα (Figs. 3A and S2A, closed bars) almost doubled the transactivation response of Dex. However, even at ten times the concentration of Dex, CpdA did not result in transactivation, even when GR was co-transfected. The

glucocorticoid antagonist, RU486, abolishes Dex (Figs. 3B and S2B, hatched bars) transactivation but CpdA does not. Dex, but not CpdA, upregulates TAT mRNA (Fig. 3C) and protein activity levels (Fig. 3D) in BWTG3 cells, which confirms the inability of CpdA to transactivate an endogenous gene containing a GRE motif.

CpdA binds reversibly to the mouse GR, influences association of ^{3}H-Dex but has no effect on dissociation, and causes nuclear translocation of GR – Binding of CpdA to mGR was investigated, using whole-cell competitive binding, in BWTG3 cells that express endogenous GR (Fig. 4A). CpdA binds GR with a significantly ($P<0.05$) lower affinity (K_d = 81.8 nM) than Dex (K_d = 1.29 nM). In addition, CpdA displayed an atypical binding curve in displacing only 47% of the ^{3}H-Dex. CpdA can cyclise to an aziridine with alkylating potential (2) and thus to eliminate the possibility that CpdA is covalently modifying the GR and thus changing its affinity for Dex, BWTG3 cells were pre-incubated with CpdA or Dex, thoroughly washed and then tested for binding of ^{3}H-Dex (Fig. S3). Kinetic GR-binding studies (Fig. 4B) show that CpdA slows the association of Dex resulting in a significant ($P<0.01$) increase in the half-life (t½) of association (from 9.01 min in the absence of CpdA to 17.45 min in the presence of 10 μM CpdA), but that it does not significantly ($P>0.05$) affect the dissociation of Dex from GR.

Kinetic studies of the nuclear translocation of GR in COS-1 cells transiently transfected with rat GR (Fig. 4C) show that the nuclear import rate (t½) induced by CpdA is not significantly ($P>0.05$) slower than that induced by Dex. However, the nuclear localization plateaus at a significantly ($P<0.01$) lower level than seen with Dex (68.6 ± 3.4% nuclear for CpdA versus 95.8 ± 2.6% nuclear for Dex). These results are comparable to those found in BWTG3 cells where CpdA (1 μM), like Dex (1 μM), induces nuclear translocation of endogenous GR, albeit to a slightly lesser degree (72% nuclear) than Dex (100% nuclear) at 30 min (Fig. S4).

CpdA, unlike Dex, does not induce dimerization of the GR – It has been postulated that dissociated GC behaviour could result from differential interaction of the liganded GR with co-activators and co-repressors (38) or from loss of GR dimerization (39), (16). We investigated the latter mechanism using a co-immunoprecipitation assay of transiently transfected Flag- and GFP-tagged GR, immunoprecipitated with Flag antibody, which shows that CpdA, unlike Dex, does not result in enrichment of GFP-tagged GR and thus GR dimerization (Fig. 5A). In fact CpdA appears to decrease baseline GR dimerization as confirmed with FRET (Fig. 5B), where addition of CpdA results in a decrease in FRET indicating a decrease in GR dimerization while addition of Dex results in an increase in FRET indicating an increase in GR dimerization. The inability of CpdA to elicit GR dimerization is further confirmed with an elegant approach first used by Savory *et al.* (40). Briefly, a c-myc tagged, nuclear localization signal 1 (NL1)-deficient mutant GR ($myGR_{NL1^-}$) is co-transfected with wtGR in COS-1 cells (Fig. 5C). Co-transfection of the wtGR, with an intact NL1, enables the defective mutant GR ($myGR_{NL1^-}$) to translocate to the nucleus within the context of

a wtGR:myGR$_{NL1^-}$ dimer where the wtGR NL1 suffices for translocation of the dimer. Transfection of the wtGR alone was visualized by anti-GR antibody and confirms that Dex and CpdA can cause nuclear translocation of the wtGR (Fig. 5CI, closed bars). Transfection with the nuclear translocation mutant, myGR$_{NL1^-}$, alone (Fig. 5CII) was visualized using an anti-myc antibody and shows that neither Dex, nor CpdA, can cause substantial nuclear translocation of this mutant GR and thus the GR remains mainly cytoplasmic (Fig. 5CII, open bars). However, if the wtGR is cotransfected with the nuclear translocation mutant GR (Fig. 5CIII) and just the mutant receptor is visualized with an anti-myc antibody, nuclear translocation (Fig. 5CIII, closed bars) of the mutant GR is only observed with Dex (89% nuclear at 10 μM), but not with CpdA (5% nuclear at 10 μM).

Implications of loss of GR dimerization induced by CpdA – To test the implications of the loss of GR dimerization observed with CpdA we investigated the behaviour of Dex and CpdA via both wild type GR (wtGR) and a GR dimerization mutant (GR^{dim}) (16). Nuclear import (Fig. 6A) shows that, although there is no significant ($P>0.05$) difference in nuclear import rate (t½), the maximal import is significantly ($P<0.01$) higher for Dex via wtGR (93.28 ± 1.40%) than for Dex via GR^{dim} (75.76 ±3. 18%), which is not significantly different from that attained with CpdA via wtGR (65.83 ± 1.19%) or CpdA via GR^{dim} (64.60 ± 3.91%). Nuclear export (Fig. 6B) in contrast does show a significant ($P<0.05$) difference in nuclear export rate (t½) between Dex via wtGR (18.74 ± 1.56 h) and Dex via GR^{dim} (12.15 ± 0.32 h), CpdA via wtGR (12.56 ± 0.62 h), and CpdA via GR^{dim} (7.89 ± 0.95 h). Transactivation of a GRE-containing promoter construct (Fig. 6C) indicates that Dex, unlike CpdA, significantly ($P<0.001$) transactivates through wtGR, but not via GR^{dim}. This is in contrast to the ability of Dex, like CpdA, to repress the CBG (Fig. 6D) and IL6 (Fig. 6E) promoters via both wtGR and GR^{dim}.

Steroid receptor specificity of CpdA – Having established that CpdA repression of CBG is via the GR (Fig. 2), we set out to test the steroid receptor specificity of CpdA in COS-1 cells transiently transfected with GR, MR, PR, AR or ER (Fig. 7). To test for transactivation via GR, MR, PR and AR a GRE-containing promoter reporter was co-transfected as these receptors share a consensus response element (41), (42) while for transactivation via ER an ERE-containing promoter reporter was co-transfected. An NFκB-binding site-containing promoter reporter was cotransfected to test for transrepression. Both agonist and antagonist modes were tested and the results show that under these conditions CpdA acts as an AR antagonist in transactivation and as a GR, MR, PR and ER agonist in transrepression.

The promiscuous steroid receptor specificity of CpdA in transrepression via the NFκB-binding site, using COS-1 cells and PMA as a stimulus, was of concern, especially as previous data had shown transrepression of a NF-κB-driven promoter construct only via GR when using TNF as a pro-inflammatory stimulus in HEK293T cells (6). Therefore, further investigation focussed on transrepression in the context of the CBG proximal promoter and at CBG protein level. The CBG promoter is only significantly

(P<0.001) transrepressed by CpdA and the cognate agonist via the GR and not via the other steroid receptors (Fig.8A). As steroid receptor levels were quite low in this experiment we investigated transactivation of a GRE- or ERE-containing promoter reporter at the same levels of transfected steroid receptors and found that the receptors were active at these concentrations (Fig.S5). In addition, at CBG protein level only Dex and CpdA, but not the other steroid receptor ligands, show significant (P<0.01) transrepression via endogenous steroid receptors in BWTG3 cells (Fig.8B). Whole-cell binding assays indicate that BWGT3 cells contain mainly GR, ER and PR and little AR and MR (Fig.8B, table insert). Thus taken in isolation these results (Fig.8B) only support the findings that repression of CBG by CpdA in BWTG3 cells occurs through GR. However, if the results are considered together with that in Fig.8A it suggests increased steroid receptor specificity of CpdA in the context of the CBG promoter and at CBG protein levels. Interestingly, the antagonism of the AR by CpdA observed with a GRE-containing promoter reporter (Fig.7A) is confirmed at protein activity level, with CpdA, like hydroxyflutamide, significantly (P<0.001) antagonising DHT transactivation of TAT protein activity levels in BWTG3 cells transfected with AR (Fig.8C).

DISCUSSION

Glucocorticoids remain the most effective treatment for a variety of inflammatory diseases but prolonged treatment, especially at high doses, results in severe side-effects (43), (39). Selective GR agonists (SEGRAs) that retain the beneficial anti-inflammatory action of GCs but display fewer side-effects are actively sought. Specifically, it has been suggested that compounds that dissociate between transactivation, involved in many of the side-effects of GCs, and transrepression, the major mechanism whereby GCs mediate their anti-inflammatory action, may be useful (44), (12), (45). Recently, several compounds displaying such dissociation both *in vitro* and *in vivo* have been reported: AL-438 (38), ZK 209614 (46), and CpdA (6), (28).

CpdA was initially described within the context of contraception in rats (1) where *in vivo* studies in rats showed decreases in CBG, ACTH and LH levels (5). Our results suggest that the repression of these proteins is due to a direct transcriptional effect of CpdA.

CBG is a central player in GC disposition and of interest in inflammation as a negative acute-phase protein (47) and modulator of the acute phase response (48), (49). Our results strongly support a mechanism whereby CpdA results in GR-mediated repression of the CBG gene, as additional co-transfected GR increased transrepression, while addition of the GR antagonist, RU486, abrogated CpdA-induced transrepression.

CpdA, however, was unable to induce transactivation of GRE-containing-promoter reporter constructs or to induce an increase in TAT mRNA or activity. TAT, a liver enzyme, involved in gluconeogenesis, is upregulated by GCs (50) and is involved in the metabolic side-effects of GC therapy (39). Thus CpdA does not act as either a GR agonist or antagonist in transactivation of GRE-containing promoter reporters or endogenous genes despite acting as an agonist in repressing CBG. The ability of CpdA to

dissociate transrepression from transactivation shown previously in the context of inflammation (6), (28) and prostate cancer (51) is thus confirmed and strengthened by our biochemical results.

Our results also show that CpdA binds reversibly to the endogenous GR in BWTG3 cells with an affinity that is about 63-fold less than that for Dex, which is in contrast to previous results in L929sA cells (6) where CpdA was shown to have a 4-fold higher affinity for the GR than Dex. The results do, however, agree with those in DU145 cells (51) where CpdA was shown to bind the GR to a lesser extent than Dex. In addition, we also confirmed previous work showing that CpdA causes nuclear translocation of the GR (6), (28), (51). Interestingly, however, we show that although the nuclear import rate is similar, GR treated with CpdA does not result in full nuclear localization.

It has been suggested that GR ligands that dissociate transrepression from transactivation may be identified by selecting for compounds that prevent dimerization of the GR, as transgenic mice expressing a dimerization-deficient GR ($GR^{dim/dim}$) retain transrepression, but not transactivation, capacity (16). Our results, in agreement with a previous study (28), indeed show that CpdA prevents ligand-induced dimerization, while FRET studies show that CpdA in fact decreases basal GR dimerization.

Using a GR^{dim} mutant (22) we show that the behaviour of Dex via this mutant is comparable to that of CpdA via the wtGR. Dex-induced nuclear localization of the GR^{dim} mutant is similar to that induced by CpdA via the wtGR in that full nuclear localization is not attained in import studies, while in export studies the export rate (t½) is similar for CpdA via the wtGR and Dex via the GR^{dim} mutant. In addition, we show that Dex via the GR^{dim} mutant, like CpdA via the wtGR, cannot transactivate a GRE-containing promoter. Transactivation of GRE-containing genes is generally mediated through direct binding of the GR to DNA (9), (10) and is suggested to require GR dimerization (52), (16), which neither CpdA acting through wtGR nor Dex acting via the GR^{dim} mutant would elicit. Thus these results strongly support the idea that the lack of CpdA-induced dimerization of the GR may contribute to its inability to transactivate. Transrepression by the GR, in contrast, may be via nGREs, or via a tethering mechanism that does not require DNA binding by the GR, but rather protein-protein interactions with other transcription factors (53), (54), (10). The first mechanism is reported to require dimerization of the GR, while the latter mechanism may be mediated by monomeric GR (9), (55). We show that CpdA acting via the wtGR, like Dex via the GR^{dim} mutant, can repress an IL-6 promoter construct. This construct contains three NFκB-elements binding NFκB-proteins that have been shown to interact with GR via a tethering mechanism (10), (14) that does not require GR dimerization (16). In addition, we show that the CBG promoter is repressed as effectively by Dex acting via the GR^{dim} mutant as through the wtGR illustrating that dimerization of the GR is not required for transrepression. Although plasma levels of CBG are suppressed during prolonged administration of GCs, and physiological and physical stressors (56) and GCs decrease rat CBG hepatic transcription (57), promoter studies that

identify possible *cis*-acting sequence elements involved in GC regulation are lacking, nor have nGREs been identified in the CBG promoter (17). Our results thus suggest that CpdA and Dex repression of CBG gene expression does not require GR dimerization and therefore probably proceeds via a tethering mechanism. The fact that the POMC (58), (59) and GnRH (18) promoters, which we show to be repressed by CpdA, have also been reported to be repressed by the GR via a tethering mechanism strengthens this hypothesis.

Although our results suggest that CpdA acts via the GR in transrepression of CBG gene expression, investigation of CpdA action via other receptors yielded some unexpected results when promoters containing transcription binding sites, specifically for GRE and NFκB, were investigated in COS-1 cells. CpdA acts as an AR antagonist in transactivation of a GRE-binding site containing promoter reporter in our cell model. This agrees with previous work (51), (60), however, we did not find the antagonism of transactivation via the GR shown by Yemelyanov *et al*. (51) nor did we observe PR antagonism as shown by Tanner *et al.* (60). Whether the discrepancies related to GR and PR antagonism reflect differences in the ligand used or depend on clonal variation in posttranslational receptor modifications and/or receptor protein stability of cell lines used is not clear yet. Nevertheless, both our work and previous results (51), (60) indicate that CpdA does not act as an agonist in transactivation.

With respect to transrepression our results show that CpdA is not only a GR agonist but may also act via MR, PR and ER in transrepressing a NFκB-binding site containing promoter reporter in COS-1 cells induced with PMA. This is in contrast to previous work investigating transrepression, via the same construct, but in HEK293T cells, with TNF rather than PMA as the pro-inflammatory stimulus, that showed that CpdA acted only via GR (6).

Further examination of the steroid receptor specificity of CpdA in transrepression, however, indicates that within the context of the CBG minimal promoter and at CBG protein level CpdA displays a much greater steroid receptor specificity in only transrepressing CBG via the GR, and not via MR, PR, AR and ER. In contrast to this, we found that the AR antagonist activity displayed by CpdA on a GRE-containing synthetic reporter in COS-1 cells was mimicked in BWTG3 cells for expressed AR-mediated regulation of endogenous TAT activity. It has been suggested that the flanking sequences around GREs may play an important role in receptor specificity (61) and that individual GREs retain specific ‘architectural signatures’ that includes distinct GR-binding sites as well as binding motifs for other transcription factors (62). Thus by investigating only isolated transcription binding sites the role of these flanking sequences that may contribute to the composite elements (63) enriched for motives to GR as well as other transcription factors may be overlooked. We suggest then that the CBG promoter may contain such additional motifs that contribute to the steroid receptor specificity of CpdA. In addition, our results suggest that the steroid receptor specificity of the anti-inflammatory action of CpdA in transrepressing pro-inflammatory cytokines

may be codetermined by cell type, pro-inflammatory stimulus, promoter context, as well as cell-specific regulation of hormone receptor expression/activities.

In summary, our results suggest that the ability of CpdA to elicit a conformational change in the GR, which abrogates dimerization, is sufficient to explain the observed dissociation between transactivation and transrepression. However, our results do not exclude the possibility that additional mechanisms, such as an impaired ability of CpdA-liganded GR to bind to DNA, also play a role. Transrepression models shown to require DNA-binding but not dimerization, such as shown for the keratin genes (64), would be useful to investigate this possibility. Investigation of genes shown to transactivate via tethering mechanisms, where no DNA binding or dimerization is required, such as for the β-casein gene where GR was shown to transactivate through tethering to STAT5 (65), could also be useful to shed light on this aspect. It may be that CpdA elicits a conformational change in the GR that results in differential recruitment of cofactors as have been shown for the dissociated GC, AL-438 (38). In support of this hypothesis, it has previously been shown that, unlike Dex, CpdA bound to the GR does not recruit GRIP-1, SRC-1A, NCoR or SMRT, suggesting that CpdA, acting via the GR, may recruit different cofactors as compared to Dex (32).

Altogether, CpdA represents a novel, non-steroidal, dissociated GR modulator, which abrogates GR dimerization. This makes CpdA a very attractive candidate to investigate for future therapeutic applications (51), (28), (66). However, its use as a GR ligand that can shed light on the fundamental mechanisms underlying the regulation of gene expression by the GR, specifically the role of GR dimerization, should not be underestimated. Within this context the decrease in maximal nuclear import and increase in nuclear export rate for CpdA-bound wtGR, as for Dex-bound GR^{dim}, as compared to Dex-bound wtGR, shown in the current paper suggest that loss of GR dimerization results in a GR species that exits the nucleus at a significantly faster rate and that GR dimerization contributes to the amount of GR present in the nucleus upon ligand activation. Previous work has suggested that the hinge region of the GR contains a solution dimerization domain (23) involved in cytosolic dimerization of the liganded GR, a nuclear localization signal, NL1 (40), that mediates liganded and unliganded GR nuclear location, and a nuclear retention signal (NRS) that actively retains the GR in the nucleus (67). Loss of NL1 and NRS results in increased nuclear export and reduced nuclear occupancy, which closely resembles the effect found with CpdA and GR^{dim} in the current study. However, the regions involved do not overlap (hinge region: aa 505-547 and GR^{dim} mutation at aa 458 in the ligand binding domain) suggesting a potentially novel insight regarding additional factors that influence nuclear retention of the GR.

REFERENCES

1. Swart, P., Swart, A. C., Louw, A., and van der Merwe, K. J. (2003) *Bioessays* **25,** 612-619
2. Louw, A., Swart, P., de Kock, S. S., and van der Merwe, K. J. (1997) *Biochemical Pharmacology* **53,** 189-197
3. Louw, A., Allie, F., Swart, A. C., and Swart, P. (2000) *Endocr Res* **26,** 729-736
4. Louw, A., Swart, P., and Allie, F. (2000) *Biochemical Pharmacology* **59,** 167-175
5. Louw, A. and Swart, P. (1999) *Endocrinology* **140,** 2044-2053
6. De Bosscher, K., Vanden Berghe, W., Beck, I. M. E., Van Molle, W., Hennuyer, N., Hapgood, J., Libert, C., Staels, B., Louw, A., and Haegeman, G. (2005) *PNAS* **102,** 15827-15832
7. Beato, M., Truss, M., and Chavez, S. (1996) *Ann.N.Y.Acad.Sci.* **784,** 93-123
8. Beato, M. and Klug, J. (2000) *Hum.Reprod.Update.* **6,** 225-236
9. Newton, R. (2000) *Thorax* **55,** 603-613
10. De Bosscher, K., Vanden Berghe, W., and Haegeman, G. (2003) *Endocr Rev* **24,** 488-522
11. Zhou, J. and Cidlowski, J. A. (2005) *Steroids* **70,** 407-417
12. Adcock, I. M. (2000) *Pulm.Pharmacol.Ther.* **13,** 115-126
13. McEwan, I. J., Wright, A. P., and Gustafsson, J. A. (1997) *Bioessays* **19,** 153-160
14. Webster, J. C. and Cidlowski, J. A. (1999) *Trends Endocrinol Metab* **10,** 396-402
15. Hayashi, R., Wada, H., Ito, K., and Adcock, I. M. (2004) *European Journal of Pharmacology* **500,** 51-62
16. Reichardt, H. M., Tuckermann, J. P., Göttlicher, M., Vujic, M., Weih, F., Angel, P., Herrlich, P., and Schütz, G. (2001) *EMBO J* **20,** 7168-7173
17. Underhill, D. A. and Hammond, G. L. (1995) *Gene* **162,** 205-211
18. Chandran, U. R., Warren, B. S., Baumann, C. T., Hager, G. L., and DeFranco, D. B. (1999) *J Biol Chem* **274,** 2372-2378
19. Therrien, M. and Drouin, J. (1993) *Mol Cell Biol.* **13,** 2342-2353
20. Miesfeld, R., Rusconi, S., Godowski, P. J., Maler, B. A., Okret, S., Wikström, A. C., Gustafsson, J. A., and Yamamoto, K. R. (1986) *Cell* **46,** 389-399
21. Schaaf, M. J. and Cidlowski, J. A. (2003) *Mol Cell Biol* **23,** 1922-1934
22. Waddell, D. S., Baehr, L. M., van den Brandt, J., Johnsen, S. A., Reichardt, H. M., Furlow, J. D., and Bodine, S. C. (2008) *Am J Physiol Endocrinol Metab* **295,** E785-E797
23. Savory, J. G., Prefontaine, G. G., Lamprecht, C., Liao, M., Walther, R. F., Lefebvre, Y. A., and Haché, R. J. (2001) *Mol Cell Biol* **21,** 781-793
24. Harnish, D. C., Scicchitano, M. S., Adelman, S. J., Lyttle, C. R., and Karathanasis, S. K. (2000) *Endocrinology* **141,** 3403-3411

25. Kastner, P., Bocquel, M. T., Turcotte, B., Garnier, J. M., Horwitz, K. B., Chambon, P., and Gronemeyer, H. (1990) *J.Biol.Chem.* **265,** 12163-12167

26. Brinkmann, A. O., Faber, P. W., van Rooij, H. C., Kuiper, G. G., Ris, C., Klaassen, P., van der Korput, J. A., Voorhorst, M. M., van Laar, J. H., Mulder, E., and . (1989) *J Steroid Biochem.* **34,** 307-310

27. De Bosscher, K., Vanden Berghe, W., Vermeulen, L., Plaisance, S., Boone, E., and Haegeman, G. (2000) *Proc Natl Acad Sci U.S A* **97,** 3919-3924

28. Dewint, P., Gossye, V., De Bosscher, K., Vanden Berghe, W., Van Beneden, K., Deforce, D., Van Calenbergh, S., Müller-Ladner, U., Vander Cruyssen, B., Verbruggen, G., Haegeman, G., and Elewaut, D. (2008) *J Immunol* **180,** 2608-2615

29. Sui, X., Bramlett, K. S., Jorge, M. C., Swanson, D. A., von Eschenbach, A. C., and Jenster, G. (1999) *J.Biol.Chem.* **274,** 9449-9454

30. Weatherman, R. V., Chang, C. Y., Clegg, N. J., Carroll, D. C., Day, R. N., Baxter, J. D., McDonnell, D. P., Scanlan, T. S., and Schaufele, F. (2002) *Mol Endocrinol* **16,** 487-496

31. Vanden Berghe, W., Plaisance, S., Boone, E., De Bosscher, K., Schmitz, M. L., Fiers, W., and Haegeman, G. (1998) *J.Biol.Chem.* **273,** 3285-3290

32. Ronacher, K., Hadley, K., Avenant, C., Stubsrud, E., Simons Jr., S. S., Louw, A., and Hapgood, J. P. (2009) *Molecular and Cellular Endocrinology* **299,** 219-231

33. Sadie, H., Styger, G., and Hapgood, J. (2003) *Endocrinology* **144,** 1958-1971

34. Diamondstone, T. I. (1966) *Analytical Biochemistry* **16,** 395-401

35. Tron, L., Szöllősi, J., Damjanovich, S., Helliwell, S. H., Arndt-Jovin, D. J., and Jovin, T. M. (1984) *Biophysical Journal* **45,** 939-946

36. Drouin, J., Trifiro, M. A., Plante, R. K., Nemer, M., Eriksson, P., and Wrange, O. (1989) *Mol Cell Biol* **9,** 5305-5314

37. Adelman, J. P., Mason, A. J., Hayflick, J. S., and Seeburg, P. H. (1986) *PNAS* **83,** 179-183

38. Coghlan, M. J., Jacobson, P. B., Lane, B., Nakane, M., Lin, C. w., Elmore, S. W., Kym, P. R., Luly, J. R., Carter, G. W., Turner, R., Tyree, C. M., Hu, J., Elgort, M., Rosen, J., and Miner, J. N. (2003) *Mol Endocrinol* **17,** 860-869

39. Schäcke, H., Docke, W. D., and Asadullah, K. (2002) *Pharmacology & Therapeutics* **96,** 23-43

40. Savory, J. G., Hsu, B., Laquian, I. R., Giffin, W., Reich, T., Haché, R. J., and Lefebvre, Y. A. (1999) *Mol Cell Biol* **19,** 1025-1037

41. Neubig, R. R., Spedding, M., Kenakin, T., and Christopoulos, A. (2003) *Pharmacol Rev* **55,** 597-606

42. Verrijdt, G., Haelens, A., Schoenmakers, E., Rombauts, W., and Claessens, F. (2002) *Biochem.J* **361,** 97-103

43. Barnes, P. J. (2006) *European Journal of Pharmacology* **533,** 2-14

44. Schäcke, H. and Rehwinkel, H. (2004) *Curr.Opin.Investig.Drugs* **5,** 524-528

45. Barnes, P. J. (2006) *Eur Respir J* **27,** 413-426

46. Schäcke, H., Schottelius, A., Docke, W. D., Strehlke, P., Jaroch, S., Schmees, N., Rehwinkel, H., Hennekes, H., and Asadullah, K. (2004) *PNAS* **101,** 227-232

47. Black, P. H. and Garbutt, L. D. (2002) *J Psychosom Res* **52,** 1-23

48. Beishuizen, A., Thijs, L. G., and Vermes, I. (2001) *Intensive Care Med.* **27,** 1584-1591

49. Ingenbleek, Y. and Bernstein, L. (1999) *Nutrition* **15,** 305-320

50. Rigaud, G., Roux, J., Pictet, R., and Grange, T. (1991) *Cell* **67,** 977-986

51. Yemelyanov, A., Czwornog, J., Gera, L., Joshi, S., Chatterton, R. T., Jr., and Budunova, I. (2008) *Cancer Res* **68,** 4763-4773

52. Reichardt, H. M., Tuckermann, J. P., Bauer, A., and Schütz, G. (2000) *Z.Rheumatol.* **59 Suppl 2,** II/1-II/5

53. Schoneveld, O. J. L. M., Gaemers, I. C., and Lamers, W. H. (2004) *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1680,** 114-128

54. De Bosscher, K. and Haegeman, G. (2009) *Mol Endocrinol* **23,** 281-291

55. Schäcke, H., Hennekes, H., Schottelius, A., Jaroch, S., Lehmann, M., Schmees, N., Rehwinkel, H., and Asadullah, K. (2002) *Ernst.Schering.Res Found.Workshop* 357-371

56. Deak, T., Meriwether, J. L., Fleshner, M., Spencer, R. L., Abouhamze, A., Moldawer, L. L., Grahn, R. E., Watkins, L. R., and Maier, S. F. (1997) *Am J Physiol* **273,** R1998-R2004

57. Smith, C. L. and Hammond, G. L. (1992) *Endocrinology* **130,** 2245-2251

58. Martens, C., Bilodeau, S., Maira, M., Gauthier, Y., and Drouin, J. (2005) *Mol Endocrinol* **19,** 885-897

59. Bilodeau, S., Vallette-Kasic, S., Gauthier, Y., Figarella-Branger, D., Brue, T., Berthelet, F., LaCroix, A., Batista, D., Stratakis, C., Hanson, J., Meij, B., and Drouin, J. (2006) *Genes Dev.* **20,** 2871-2886

60. Tanner, T. M., Verrijdt, G., Rombauts, W., Louw, A., Hapgood, J. P., and Claessens, F. (2003) *Molecular and Cellular Endocrinology* **201,** 155-164

61. Jacobsen, B. M., Jambal, P., Schittone, S. A., and Horwitz, K. B. (2009) *Mol Endocrinol* **23,** 989-1000

62. So, A. Y., Cooper, S. B., Feldman, B. J., Manuchehri, M., and Yamamoto, K. R. (2008) *Proc.Natl.Acad.Sci.U.S.A* **105,** 5745-5749

63. So, A. Y., Chaivorapol, C., Bolton, E. C., Li, H., and Yamamoto, K. R. (2007) *PLoS.Genet.* **3,** e94

64. Radoja, N., Komine, M., Jho, S. H., Blumenberg, M., and Tomic-Canic, M. (2000) *Mol Cell Biol* **20,** 4328-4339

65. Stöcklin, E., Wissler, M., Gouilleux, F., and Groner, B. (1996) *Nature* **383,** 726-728

66. Zhang, Z., Zhang, Z. Y., and Schluesener, H. J. (2009) *J Immunol* **183,** 3081-3091

67. Carrigan, A., Walther, R. F., Salem, H. A., Wu, D., Atlas, E., Lefebvre, Y. A., and Haché, R. J. G. (2007) *J.Biol.Chem.* **282,** 10963-10971

68. Cheng, Y. and Prusoff, W. H. (1973) *Biochem Pharmacol* **22,** 3099-3108

FOOTNOTES

Research was supported by the Flemish-South African bilateral agreement (BIL99/39) and also for A.L. and J.H. in part by grants from the Medical Research Council, National Research Foundation (NRF), and Stellenbosch University (South Africa). KDB is a postdoctoral researcher at the FWO-Vlaanderen. We would like to thank Carmen Langeveldt for excellent technical assistance.

The abbreviations used are: adrenocorticotropic hormone (ACTH), co-immunoprecipitation (CoIP), corticosteroid-binding globulin (CBG), Compound A (CpdA; 2-(4acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride), dexamethasone (Dex), 4,5α-dihydrotestosterone (DHT), dimerization-deficient GR mutant (GR^{dim}), 17β-estradiol (E_2), fluorescence resonance energy transfer (FRET), gonadotropin-releasing hormone (GnRH), glucocorticoid (GC), glucocorticoid receptor (GR), glucocorticoid response elements (GREs), glucose-6-phosphatase (G-6-P), hypothalamic-pituitary-adrenal axis (HPA-axis), luteneinizing hormone (LH), mifepristone (RU486), nuclear localization signal 1 (NL1), nuclear retention signal (NRS), 12-myristate 13-acetate (PMA), P450-dependent steroid 11β-hydroxylase (P450c11), proopiomelanocortin (POMC), selective GR agonists (SEGRAs), tyrosine amino transferase (TAT).

FIGURE LEGENDS

Fig. 1. Transrepression of promoter reporter constructs of three genes involved in the *in vivo* response to CpdA administration in rats. *(A) rat CBG.* BWTG3 cells ($5x10^4$ cells per well in 24-well tissue culture plates) were transiently transfected with 360 ng rat CBG promoter reporter construct (ratCBG295Luc), 200 ng pGL2-basic and 40 ng β–galactosidase reporter plasmid (pPGKβGeopbA) ***(B) rat POMC.*** AtT-20 cells ($2.5x10^5$ cells per well in 24-well tissue culture plates) were transiently transfected with 240 ng rat POMC promoter reporter construct (JA300), 60 ng rat GR expression vector (pSVGR1) and 60 ng rat expression vector for Nur77 (CMX-Nur77), and ***(C) mouse GnRH.*** GT1-7 cells ($1.25x10^5$ cells per well in 24-well tissue culture plates) were transiently transfected with 600 ng mouse GnRH promoter reporter construct (3446mGnRHluc). Twenty-four (A & B) or 4 (C) h after transfection test compounds were added at a concentration of 1 μM. Control wells received an equal amount of ethanol. Cells were treated for 24 (A & B) or 20 (C) h, respectively. Luciferase values were normalized for β–galactosidase (A) or protein (B & C) values and plotted as a percentage of the average control. Statistical analysis was done to compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*$P<0.05$; **$P<0.01$).

Fig. 2. Transrepression of the rat CBG proximal promoter reporter construct and endogenous gene in liver cell lines. (A) Dose response curves of transrepression of the rat CBG295Luc promoter reporter construct by CpdA and Dex in the presence of endogenous GR. The ratCBG295Luc promoter reporter construct (360 ng) plus 200 ng pGL2-basic and 40 ng pPGKβGeopbA was transiently transfected into BWTG3 cells ($5x10^4$ cells per well in 24-well tissue culture plates). Twenty-four h after transfection cells were treated with increasing concentrations of test compounds, as indicated, and lysed after 24 h. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and plotted as a percentage of the average control. LogEC50 and percentage repression values were determined by fitting a sigmoidal dose response curve with variable slope. (B) Transrepression of human CBG mRNA. HepG2 cells were treated with Dex (1 μM), or CpdA (10 μM) for 72 h. Control wells received an equal amount of ethanol. Total RNA was analysed with Northern blot analysis, using hCBG cDNA, stripped and reprobed with β-actin to control for loading. CBG results are presented as normalized relative to β-actin and as % of average control. Statistical analysis was done to compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05; ***P<0.001). (C) Transrepression of the rat CBG295Luc promoter reporter construct by CpdA and Dex in the absence or presence of co-transfected rGRα and RU486. BWTG3 cells ($5x10^4$ cells per well in 24-well tissue culture plates) were transiently transfected with ratCBG295Luc (360 ng), 200 ng rGRα (pSVGR1) or pGL2-basic as indicated, and 40 ng pPGKβGeopbA. Twenty-four h after transfection, test compounds were added (CpdA at 10 μM; Dex at 1 μM; RU486 at 20 μM) and cells lysed after 24 h. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05; **P<0.01), and to (ii) compare values without GR (–rGRα) to values with co-transfected GR (+rGRα) and RU486 (+RU486) for each compound tested using one-way ANOVA followed by Dunnett's multiple comparison's posttest (^{a}P<0.05; ^{b}P<0.01; ^{c}P<0.001).

Fig. 3. Transactivation of a GRE-containing promoter reporter construct and endogenous gene in a liver cell line. (A) Transactivation of a GRE-containing promoter by CpdA and Dex in BWTG3 cells, in the absence or presence of co-transfected rGRα. BWTG3 cells ($5x10^4$ cells per well in 24-well tissue culture plates) were transiently transfected with 360 ng GRE-driven promoter reporter construct ($(GRE)_2$tkLuc), 200 ng rGRα (pSVGR1) or pGL2-basic as indicated, and 40 ng pPGKβGeopbA. Twenty-four h after transfection, test compounds were added (CpdA at 10 μM; Dex at 1 μM) and cells lysed after 24 h. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05; **P<0.01) and to (ii) compare values without GR (–rGRα) to values with co-transfected GR (+rGRα) for each compound tested using a two-tailed

unpaired t-test ($^{a}P<0.05$; $^{b}P<0.01$). (B) Transactivation of GRE-containing promoter by CpdA and Dex in BWTG3 cells, in the absence or presence of RU486. The $(GRE)_2$tkLuc promoter reporter construct (360 ng) was transiently transfected into BWTG3 cells ($5x10^4$ cells per well in 24-well tissue culture plates), together with 200 ng rGRα (pSVGR1) and 40 ng pPGKβGeopbA. Cells were treated with 10 μM test compounds as indicated. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and plotted as a percentage of the average transactivation by Dex alone. Statistical analysis was done to compare values in presence of test compounds relative to the corresponding controls using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05; **P<0.01; ***P<0.001). (C) Transactivation of TAT mRNA. BWTG3 cells were treated with Dex (1 μM) or CpdA (10 μM) for 72 h. Control wells received an equal amount of ethanol. Total RNA was reverse transcribed and the cDNA obtained subjected to PCR analysis with primers to detect mTAT and GAPDH (housekeeping gene used as an internal control) in separate reactions. PCR products were separated on agarose gel and visualized under UV light after EtBr staining. The figure is representative of three independent experiments. (D) Transactivation of TAT activity. BWTG3 cells were treated with Dex (1 μM), or CpdA (10 μM) for 4 h. Control wells received an equal amount of ethanol. Cell lysate was prepared and assayed for TAT activity. Statistical analysis was done to compare values in the presence of test compounds relative to control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05).

Fig. 4. Binding of CpdA to rodent GR and translocation to nucleus. (A) Competitive whole-cell binding in BWTG3 cells of 20nM ^{3}H-Dex in the presence of increasing concentrations of unlabeled Dex or CpdA. The results shown are from two independent experiments performed in quadruplicate. Curve fitting was performed using non-linear regression and one-site competition to obtain logEC50 and maximal displacement. Ki values were obtained using the method of Cheng and Prusoff (68). (B) Kinetics of binding in BWTG3 cells of 1nM ^{3}H-Dex in the absence (0 μM CpdA) and presence of CpdA (1 and 10 μM). The results shown are from three independent experiments performed in triplicate. To obtain t½ curve fitting was performed using non-linear regression and one phase exponential association and one phase exponential decay, respectively. (C) Nuclear translocation of transiently transfected rGR in COS-1 cells. pTC2-wtGRrat (11.6 μg) was transiently transfected into COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) and replated 24 h later ($3 x 10^5$ cells per well in a 6-well plate) into DMEM supplemented with 10% stripped FCS for 24 h before being treated with Dex (1 μM) or CpdA (10 μM) for 0, 2, 4, 6, 10, 15, 20, 40 or 60 min. After fixation, cells were subjected to immunostaining with rabbit anti-GR, followed by anti-rabbit-Alexa 488 as a secondary antibody. Hoechst 33258 stain was used to visualize nuclei. Cells were analysed using an IX 81 Olympus microscope. The percentage of cells showing nuclear localization of GR was quantified for three independent experiments. One-phase exponential association curve fitting was conducted to determine t½ and maximal localization.

Fig. 5. Dex, but not CpdA, results in ligand-induced dimerization of the GR in COS-1 cells. (A) Co-IP of differentially tagged GR. COS-1 cells were transiently transfected with Flag-tagged GR (pEFFlaghGRα, Mr 96 kDa), GFP-tagged GR (pEGFP-C2-GR, Mr 128.5 kDa) and pGL2-basic and treated with ethanol (control), 10^{-6} M Dex or 10^{-5} M CpdA for 1 h. Cellular extracts were immunoprecipitated with anti-Flag beads. Western blots were probed with anti-GR antibody. The figure is representative of three independent experiments. (B) FRET analysis of GR dimerization. COS-1 cells were transiently transfected with CFP-tagged GR (pECFP-hGRα) and YFP-tagged GR (pEYFP-hGRα) and treated with ethanol (control), 10^{-5} M Dex or 10^{-5} M CpdA for 30 min while FRET intensity was monitored on the Olympus IX 81 motorized inverted microscope at 37°C. Corrected FRET is plotted against time. The figure is representative of three independent experiments. (C) Dex, but not CpdA, induces nuclear localization of GR mutant when co-transfected with wtGR in COS-1 cells. COS-1 cells (3×10^6 per plate in 10 cm tissue culture dishes) were transfected (I) with 12μg wt rat GRα (pSVGR1) alone (wtGR), (II) with 12μg c-myc tagged nuclear translocation mutant GR alone ($myGR_{NL1^-}$), or (III) with 12μg DNA in an 8:1 ratio of wtGR:$myGR_{NL1^-}$ (wtGR:$myGR_{NL1^-}$) and replated 24 h later (5×10^5 cells per well in a 6-well plate) into Opti-MEM for 24 h before induction with Dex (1 or 10 μM) or CpdA (1 or 10 μM) for 60 min. Control wells received an equal amount of ethanol. Localization of constructs was visualized as follows: (I) wtGR alone by indirect immunofluorescence using a rabbit anti-GR antibody followed by an Alexa Flour 594-tagged anti-rabbit antibody (anti-GR; red); (II & III) $myGR_{NL1^-}$ alone or wtGR:$myGR_{NL1^-}$ by indirect immunofluorescence using a mouse anti-c-myc antibody followed by an Alexa Flour 488-tagged anti-mouse antibody (anti-myc; green). Nuclei were visualized with DAPI staining (blue). Cells were analysed using a Zeiss confocal LSM410 microscope. The percentage of cells showing either nuclear (closed bars), both (hatched bars) or cytoplasmic (open bars) localization of GR was quantified for three independent experiments and is presented graphically. Representative micrographs for control, Dex (10 μM) and CpdA (10 μM) are presented to the right of each graph.

Fig. 6. Implications of loss of GR dimerization induced by CpdA. (A) Nuclear import of transiently transfected mouse GR and mouse GR^{dim} mutant in COS-1 cells. COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected with 7.5 μg pcDNA3.1-GRWT or pcDNA3.1-GR^{dim} and replated 24 h later (3×10^5 cells per well in a 6-well plate) in DMEM supplemented with 10% stripped FCS. After 24 h cells were treated with Dex (1 μM) or CpdA (10 μM) for 0, 2, 4, 6, 10, 20, 30, 45 or 60 min. After fixation, cells were subjected to immunostaining with anti-GR, followed by anti-rabbit-Alexa 488 as a secondary antibody. Hoechst 33258 stain was used to visualize nuclei. Cells were analysed using an IX 81 Olympus microscope. The percentage of cells showing nuclear localization of GR was quantified for three independent experiments. One-phase exponential association curve fitting was conducted to determine t½ and maximal localization. (B) Nuclear export of transiently transfected mouse GR and mouse GR^{dim} mutant in COS-1 cells. COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected with 7.5 μg pcDNA3.1-GRWT or pcDNA3.1-GR^{dim} and replated 24 h later (3×10^5 cells per well in a 6-well plate) in DMEM supplemented with 10% stripped FCS for 24 h before being treated with Dex (1 μM) or CpdA (10 μM) for 45 min, washed and monitored over 0, 4, 8,

12, 20, 24, and 28 h. After fixation, cells were subjected to immunostaining with rabbit anti-GR, followed by anti-rabbit-Alexa 488 as a secondary antibody. Hoechst 33258 stain was used to visualize nuclei. Cells were analysed using an IX 81 Olympus microscope. The percentage of cells showing nuclear localization of GR was quantified for three independent experiments. One phase exponential decay curve fitting was conducted to determine t½. (C) Transactivation of the transiently transfected GRE-containing promoter reporter construct via mouse GR or mouse GR^{dim} mutant. COS-1 cells ($1x10^4$ cells per well in 96-well tissue culture plates) were transiently transfected with 100 ng pTAT-GRE2-Elb-luc, 10 ng pcDNA3.1-GRWT or pcDNA3.1-GR^{dim} as indicated, and 10 ng pPGKβGeopbA. Twenty-four h after transfection cells were induced with Dex (1 μM) or CpdA (10 μM) for 24 h. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values plotted as fold-induction relative to average control. (D) Transrepression of the transiently transfected rat CBG295Luc promoter reporter construct via mouse GR or mouse GR^{dim} mutant. COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected with 9 μg CBG295Luc and 3 ng pcDNA3.1-GRWT or pcDNA3.1-GR^{dim} as indicated. Twenty-four h after transfection cells were replated ($5x10^4$ cells per well in 24-well tissue culture plates). Cells were induced for 24 h with Dex (1 μM) or CpdA (10 μM) 24 h after replating. Control wells received an equal amount of ethanol. Luciferase values were normalized with protein concentration and values plotted as fold-induction relative to average control. (E) Transrepression of the transiently transfected IL6-luc promoter reporter construct via mouse GR or mouse GR^{dim} mutant. COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected with 9 μg $p(IL6\kappa B)_3$50hu.IL6Pluc+ and 0.9 μg pcDNA3.1-GRWT or pcDNA3.1-GR^{dim} as indicated. Twenty-four h after transfection cells were replated ($5x10^4$ cells per well in 24-well tissue culture plates). Cells were induced for 24 h with PMA (10ng/ml) alone or with Dex (1 μM) or CpdA (10 μM) 24 h after replating. Control wells received an equal amount of ethanol. Luciferase values were normalized with protein concentration and values plotted as percentage induction with PMA induction alone as 100%. Statistical analysis was done to compare values in the presence of test compounds relative to the corresponding control (C & D) or PMA induction alone (E) using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05; **P<0.01; ***P<0,001).

Fig. 7. Steroid receptor specificity of CpdA. (A) Transactivation of transiently transfected GRE-containing promoter reporter construct via GR, MR, PR, or AR, or ERE-containing promoter reporter construct via ER. COS-1 cells ($1x10^5$ cells per well in 24-well tissue culture plates) were transiently transfected with 300 ng pTAT-GRE2-Elb-luc, 30 ng pPGKβGeopbA, and 30 ng pRS-hGRα, pRS-hMR, pSG5hPRB or pSVARo as indicated, or 1200 ng pGL2-3x-ERE-TATA-luc, 30 ng pPGKβGeopbA, and 30 ng pcDNA3-ERα. Twenty-four h after transfection cells were induced for 24 h with solvent (ethanol), agonist (10^{-6} M) or CpdA (10^{-5}M) (agonist mode) or with agonist (10^{-6} M), agonist (10^{-6} M) plus antagonist (10^{-6} M), or agonist (10^{-6} M) plus CpdA (10^{-5} M) (antagonist mode). Luciferase values were normalized with β–galactosidase and values plotted as fold-induction relative to average solvent. Statistical analysis was done to compare values in the presence of test compounds relative to solvent (*P<0.05, **P<0.01, ***P<0.00) for agonist mode or corresponding agonist ($^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001) for antagonist mode using one-way ANOVA

with Dunnett's multiple comparisons test as posttest. (B) Transrepression of transiently transfected IL-6 promoter reporter construct via GR, MR, PR, AR or ER. COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected with 9 μg $p(IL6\kappa B)_3$50hu.IL6Pluc+ and 0.9 μg pRS-hGRα, pRS-hMR, pSG5hPRB, pSVARo, or pcDNA3-ERα as indicated. Twenty-four h after transfection cells were replated ($5x10^4$ cells per well in 24-well tissue culture plates). Cells were induced for 24 h with solvent (ethanol), PMA (10ng/ml), PMA plus agonist (10^{-6} M) or PMA plus CpdA (10^{-5} M) (agonist mode) or with PMA, PMA plus agonist (10^{-6} M), PMA plus agonist (10^{-6} M) and antagonist (10^{-6} M), or PMA plus agonist (10^{-6} M) and CpdA (10^{-5} M) (antagonist mode) 24 h after replating. Luciferase values were normalized with protein concentration and values plotted as fold-induction relative to average solvent (agonist mode) or as percentage of PMA induction (antagonist mode). Statistical analysis was done to compare values in the presence of test compounds relative to PMA induction (*P<0.05, **P<0.01, ***P<0.00) for agonist mode or PMA plus corresponding agonist induction ($^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001) for antagonist mode using one-Way ANOVA with Dunnett's multiple comparisons test as post test. Agonists used: Dex for GR; aldosterone for MR; R5020 for PR; DHT for AR; E_2 for ER. Antagonists used: RU486 for GR; spironolactone for MR; RU486 for PR; hydroxyflutamide for AR; ICI 182,780 for ER.

Fig. 8. CpdA shows greater steroid receptor specificity in context of the CBG promoter construct and at CBG, but not TAT, protein level. (A) Steroid receptor specificity of transrepression of the transiently transfected rat CBG295Luc promoter reporter construct via GR, MR, PR, AR or ER. COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected with 9 μg CBG295Luc and 3 ng pRS-hGRα, pRS-hMR, pSG5hPRB, pSVARo or pcDNA3-ERα as indicated. Twenty-four h after transfection cells were replated ($5x10^4$ cells per well in 24-well tissue culture plates) in medium with 10% dextran-coated charcoal-stripped FCS and 1% antibiotics, except for assays investigating ER activity, where phenol-red free medium with 10% dextran-coated charcoal-stripped FCS and 1% antibiotics was used. Cells were induced, in DMEM without supplements except in assays investigating ER activity, where phenol-red free medium with 10% dextran-coated charcoal-stripped FCS and 1% antibiotics was used, for 24 h with solvent (ethanol), agonist (10^{-6} M) or CpdA (10^{-5}M) 24 h after replating. Luciferase values were normalized with protein concentration and values plotted as fold-induction relative to average control. Statistical analysis was done for each steroid receptor to compare values in the presence of test compounds relative to the corresponding control using two-way ANOVA followed by Bonferroni posttests (*P<0.05; **P<0.01; ***P<0,001). (B) Transrepression of CBG protein levels by steroid receptor ligands via endogenous steroid receptors in BWTG3 cells. BWTG3 cells ($2.5x10^5$ cells per well in 6-well tissue culture plates) were induced 24 h after plating with solvent (ethanol), steroid receptor agonist (10^{-6} M) or CpdA (10^{-5}M) in medium with 10% dextran-coated charcoal-stripped FCS and antibiotics except for assays investigating ER activity, where phenol-red free medium with 10% dextran-coated charcoal-stripped FCS and antibiotics was used. After 24 h cells were lysed and lysates separated on a SDS-PAGE gel and transferred to Hybond-ECL nitrocellulose membrane, which was probed for CBG (S1000-76Z) and actin (sc-1616). Proteins were visualized using ECL Peroxidase labelled anti-goat antibody and ECL Western blotting detection reagents on Hyperfilm. For determination of CBG levels Hyperfilm bands of CBG and actin were quantified and results

expressed as intensity of CBG band relative to actin band. Statistical analysis was done to compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05; **P<0.01; ***P<0,001). Table insert: whole-cell binding was done in BWTG3 cells to determine endogenous steroid receptor levels by incubating BWTG3 cells with ^{3}H-Dex for GR, ^{3}H-E_2 for ER, ^{3}H-R5020 for PR, ^{3}H-mibolerone for AR or ^{3}H-aldosterone for MR. Cognate unlabeled ligands were added in 1000-fold excess. Specific binding is presented in fmol/mg protein. (C) Transactivation of TAT activity in BWTG3 cells transfected with AR. BWTG3 cells ($2.5x10^5$ cells per well in 6-well tissue culture plates) were transiently transfected with 150 ng pSVARo and induced 24 h after transfection with solvent (ethanol), DHT, AR agonist (10^{-6} M), DHT, AR agonist (10^{-6} M) plus hydroxyflutamide, AR antagonist (10^{-6} M), or DHT, AR agonist (10^{-6} M) plus CpdA (10^{-5} M) in medium with 10% dextran-coated charcoal-stripped FCS and antibiotics. Cell lysate was prepared and assayed for TAT activity 4 h after induction. Statistical analysis was done to compare values in the presence of test compounds relative to control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05). Agonists used: Dex for GR; aldosterone (Ald) for MR; R5020 for PR; DHT for AR; E_2 for ER. Antagonists used: hydroxyflutamide for AR.

FIGURES

Figure 1:

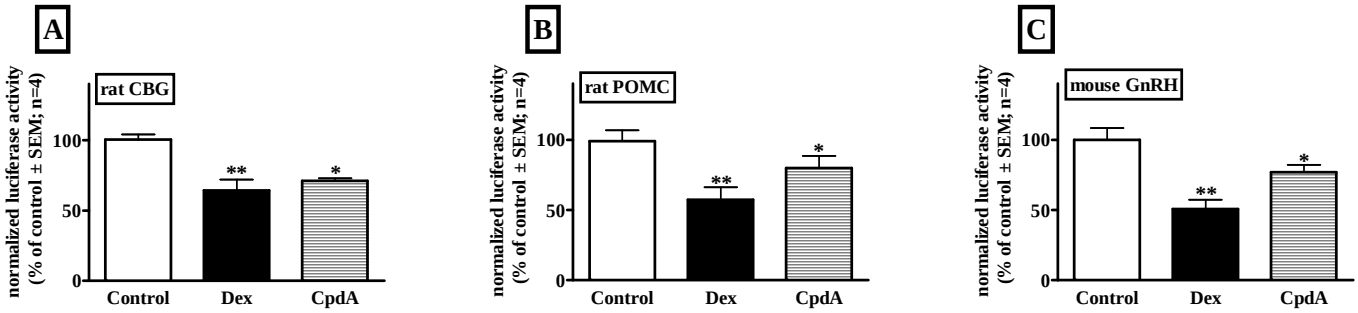


Figure 2:

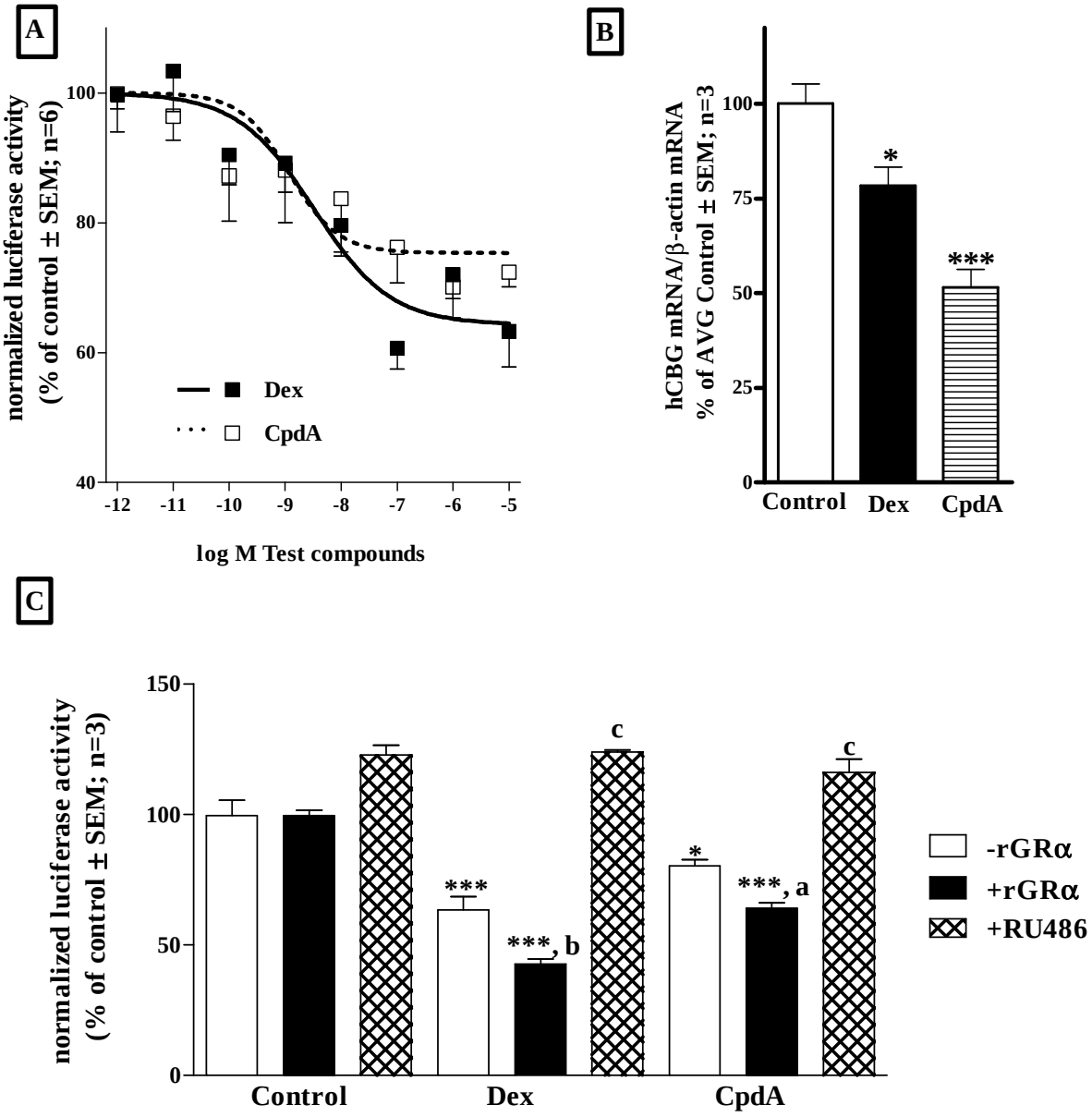

A
normalized luciferase activity
(% of control ± SEM; n=6)
100
80
60
40
Dex
CpdA
-12
-11
-10
-9
-8
-7
-6
-5
log M Test compounds
B
hCBG mRNA/β-actin mRNA
% of AVG Control ± SEM; n=3
100
75
50
25
0
*
***
Control
Dex
CpdA
C
normalized luciferase activity
(% of control ± SEM; n=3)
150
100
50
0
***
***, b
c
*
***, a
c
Control
Dex
CpdA
-rGRα
+rGRα
+RU486

Figure 3:

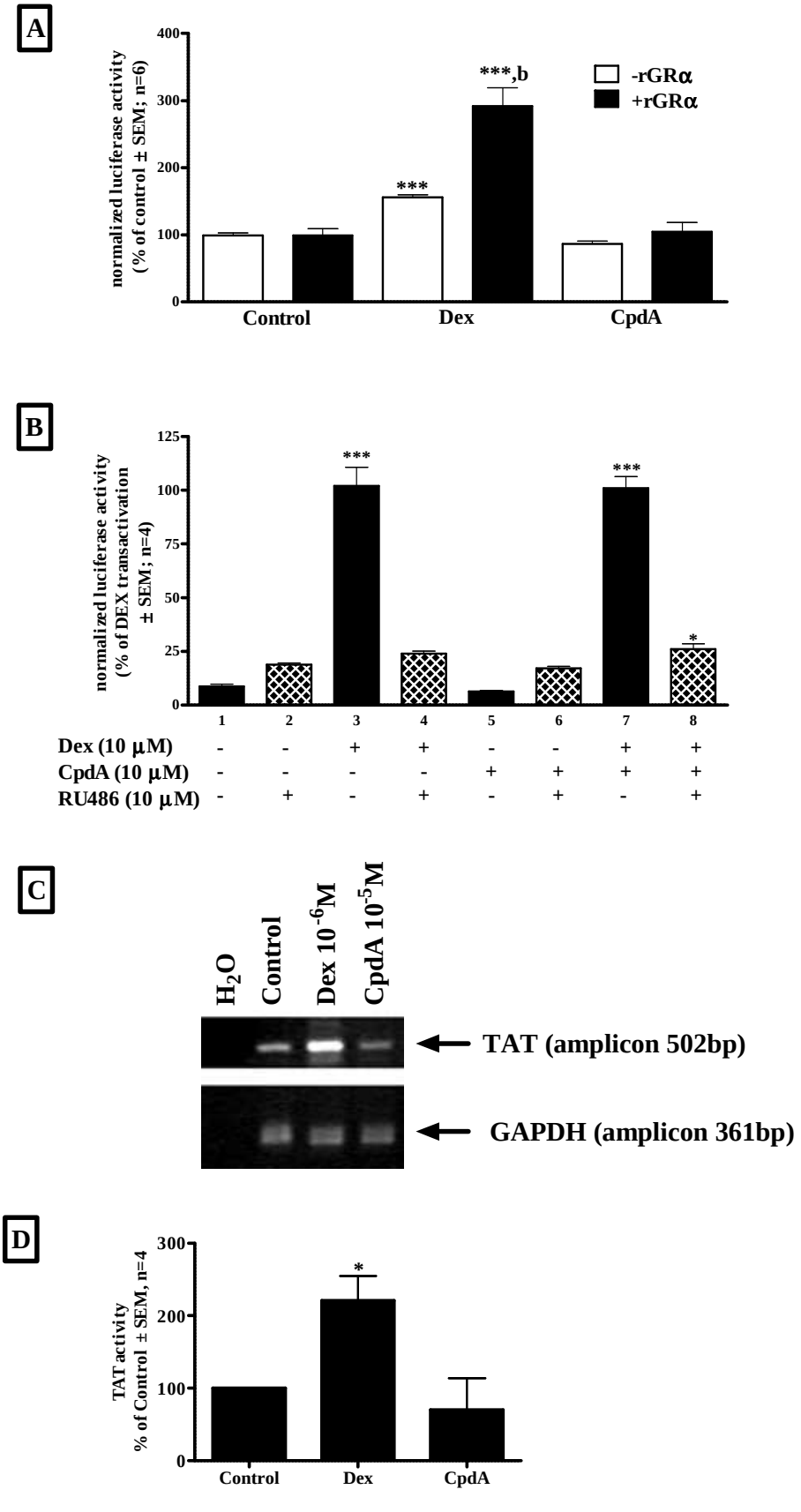

A
normalized luciferase activity
(% of control ± SEM; n=6)
400
300
200
100
0
***
***,b
-rGRα
+rGRα
Control
Dex
CpdA
B
normalized luciferase activity
(% of DEX transactivation
± SEM; n=4)
125
100
75
50
25
0
***
***
*
1 2 3 4 5 6 7 8
Dex (10 µM) - - + + - - + +
CpdA (10 µM) - - - - + + + +
RU486 (10 µM) - + - + - + - +
C
H2O
Control
Dex 10-6M
CpdA 10-5M
TAT (amplicon 502bp)
GAPDH (amplicon 361bp)
D
TAT activity
% of Control ± SEM, n=4
300
200
100
0
*
Control
Dex
CpdA

Figure 4:

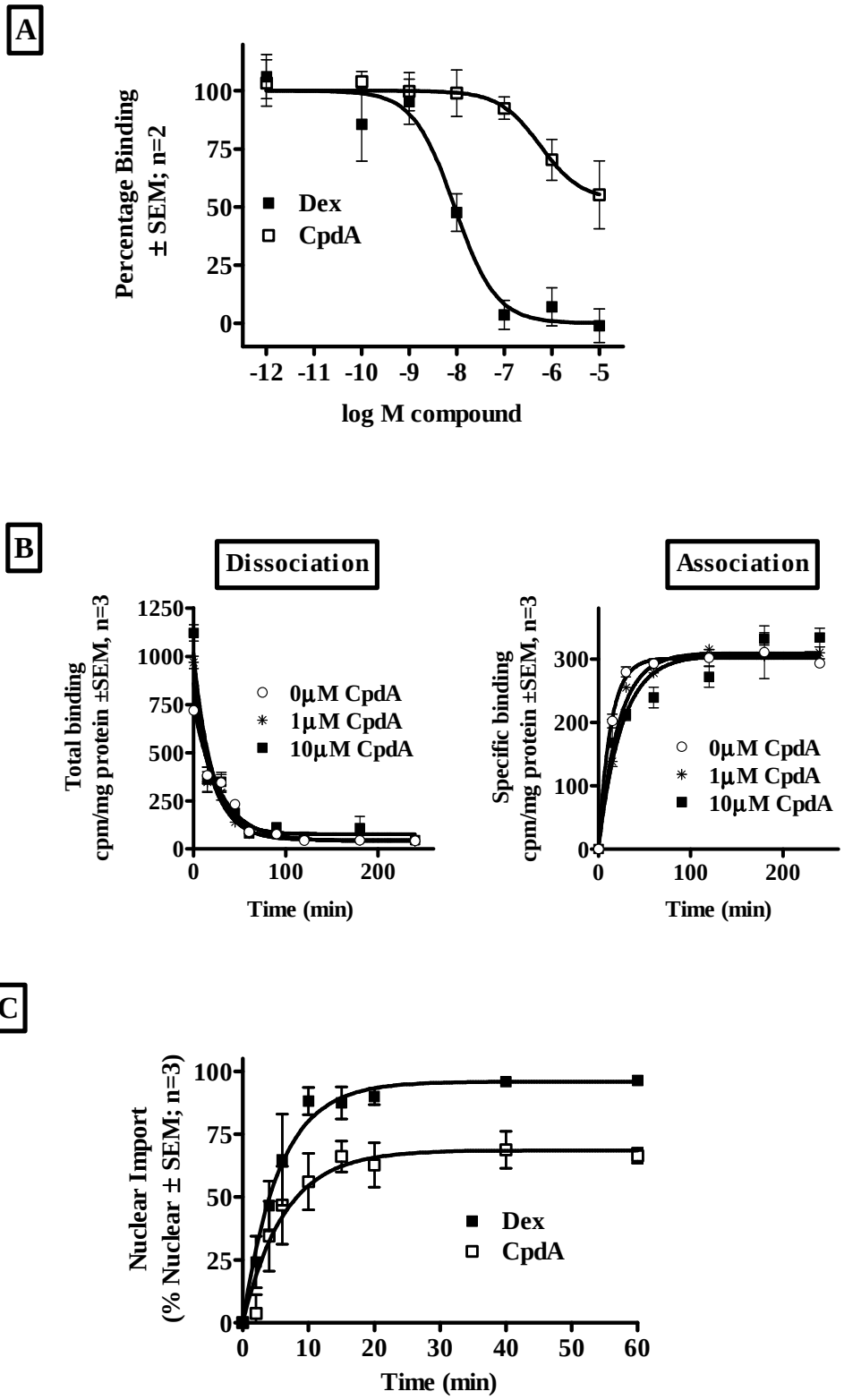

A
Percentage Binding
± SEM; n=2
100
75
50
25
0
Dex
CpdA
-12 -11 -10 -9 -8 -7 -6 -5
log M compound
B
Dissociation
Total binding
cpm/mg protein ±SEM, n=3
1250
1000
750
500
250
0
0µM CpdA
1µM CpdA
10µM CpdA
0 100 200
Time (min)
Association
Specific binding
cpm/mg protein ±SEM, n=3
300
200
100
0
0µM CpdA
1µM CpdA
10µM CpdA
0 100 200
Time (min)
C
Nuclear Import
(% Nuclear ± SEM; n=3)
100
75
50
25
0
Dex
CpdA
0 10 20 30 40 50 60
Time (min)

Figure 5:

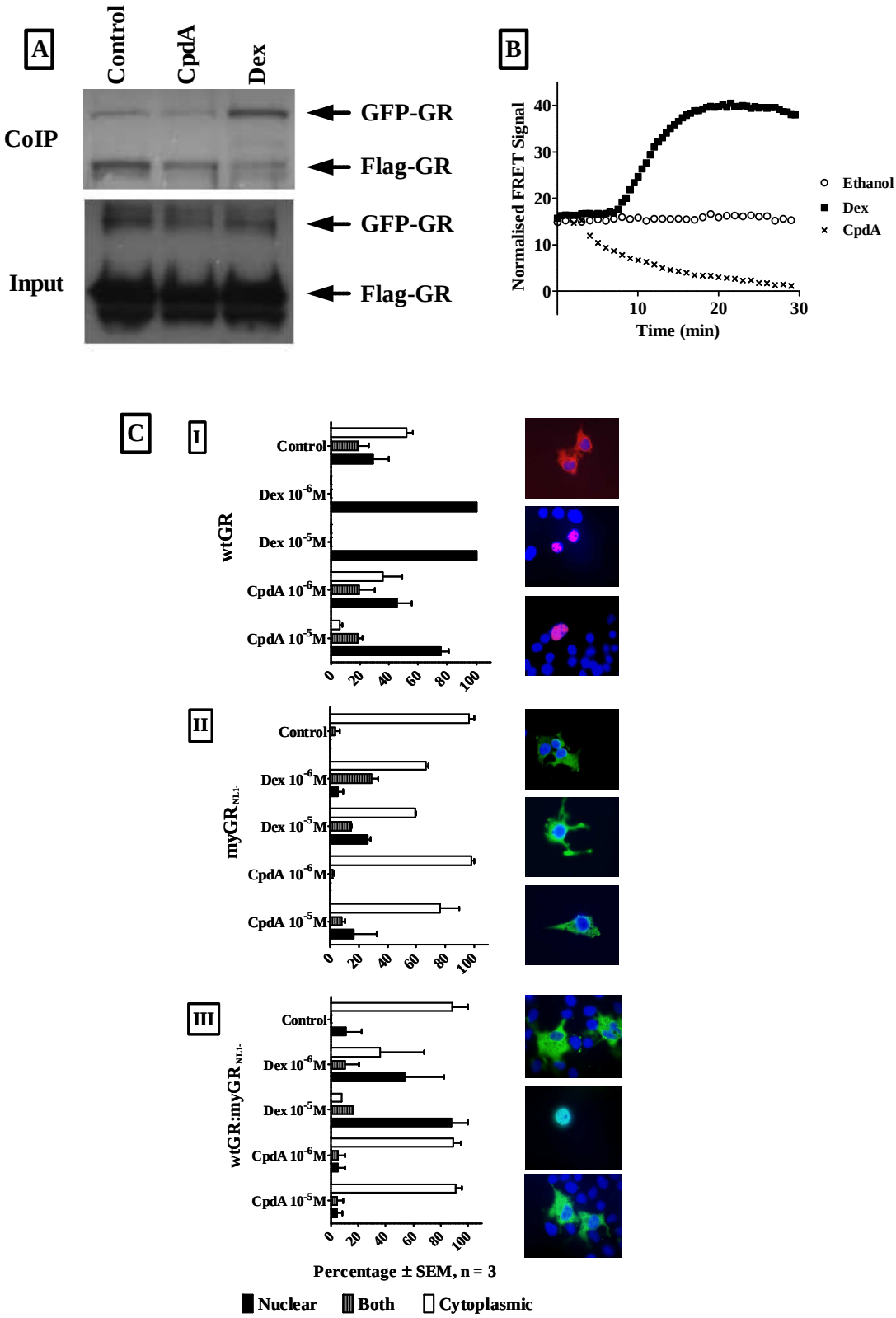

A
Control
CpdA
Dex
CoIP
Input
GFP-GR
Flag-GR
GFP-GR
Flag-GR
B
Normalised FRET Signal
Time (min)
Ethanol
Dex
CpdA
C
I
wtGR
II
myGR
III
wtGR:myGR
Control
Dex 10-6M
Dex 10-5M
CpdA 10-6M
CpdA 10-5M
Percentage ± SEM, n = 3
Nuclear
Both
Cytoplasmic

Figure 6:

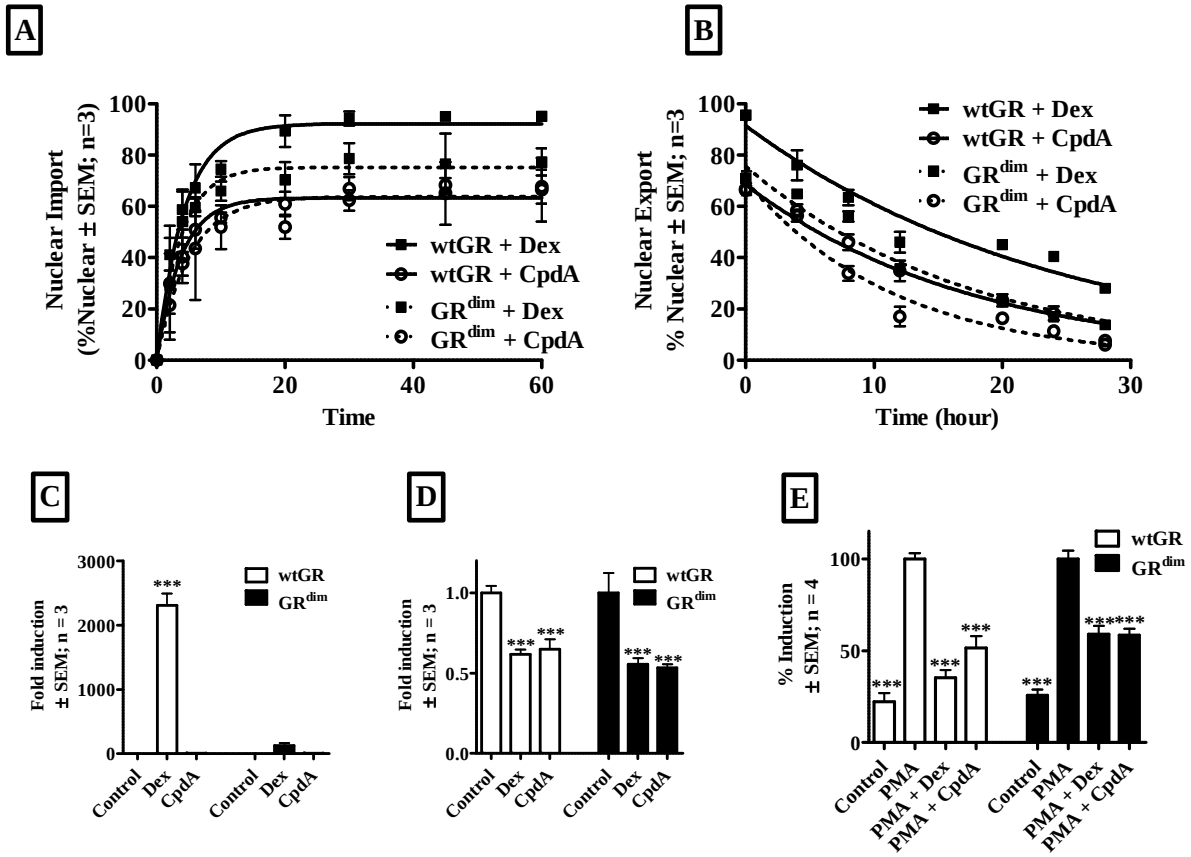

A
Nuclear Import
(%Nuclear ± SEM; n=3)
Time
wtGR + Dex
wtGR + CpdA
GRdim + Dex
GRdim + CpdA
B
Nuclear Export
% Nuclear ± SEM; n=3
Time (hour)
C
Fold induction
± SEM; n = 3
D
E
% Induction
± SEM; n = 4
Control
Dex
CpdA
PMA
PMA + Dex
PMA + CpdA

Figure 7:

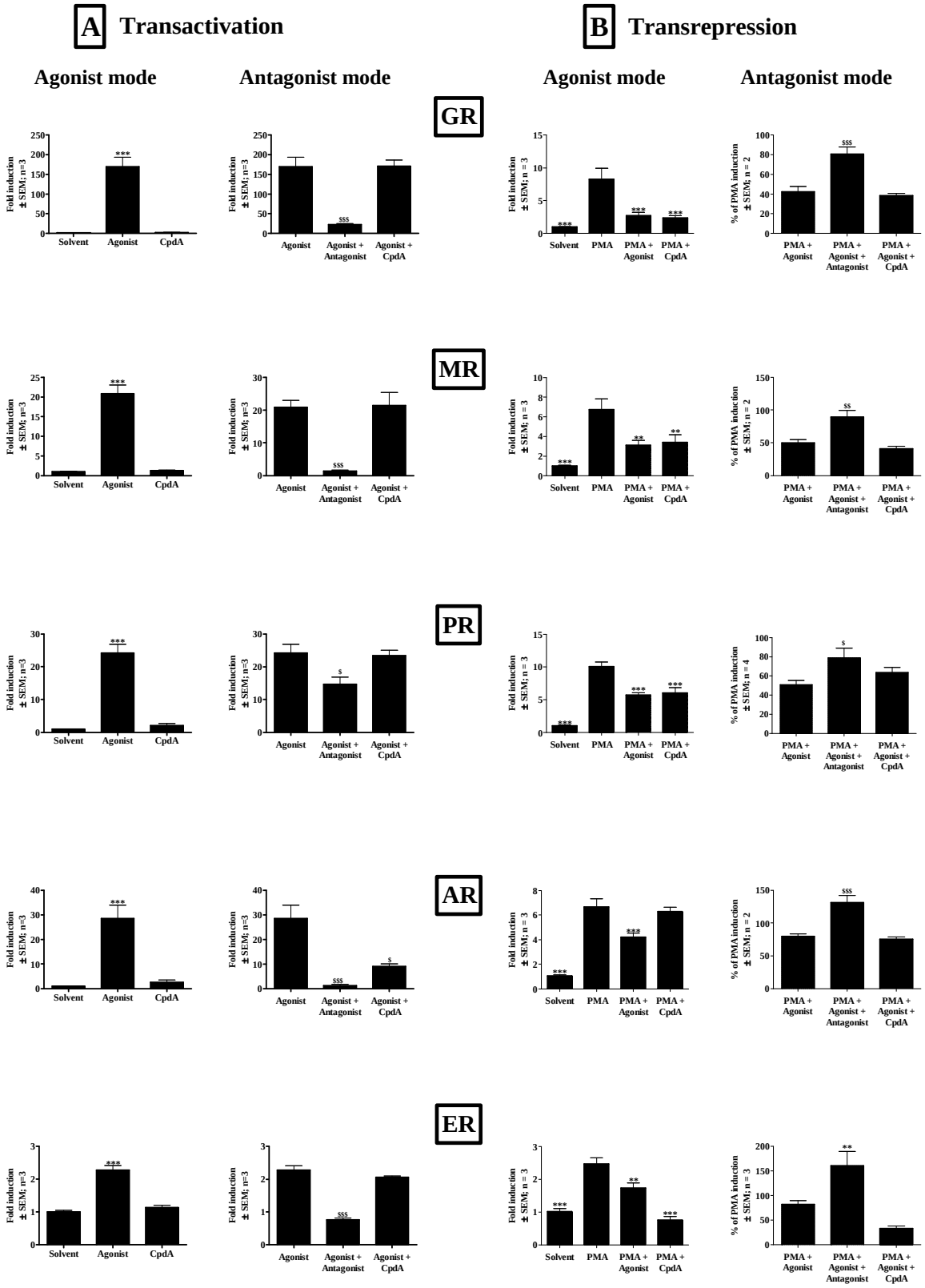
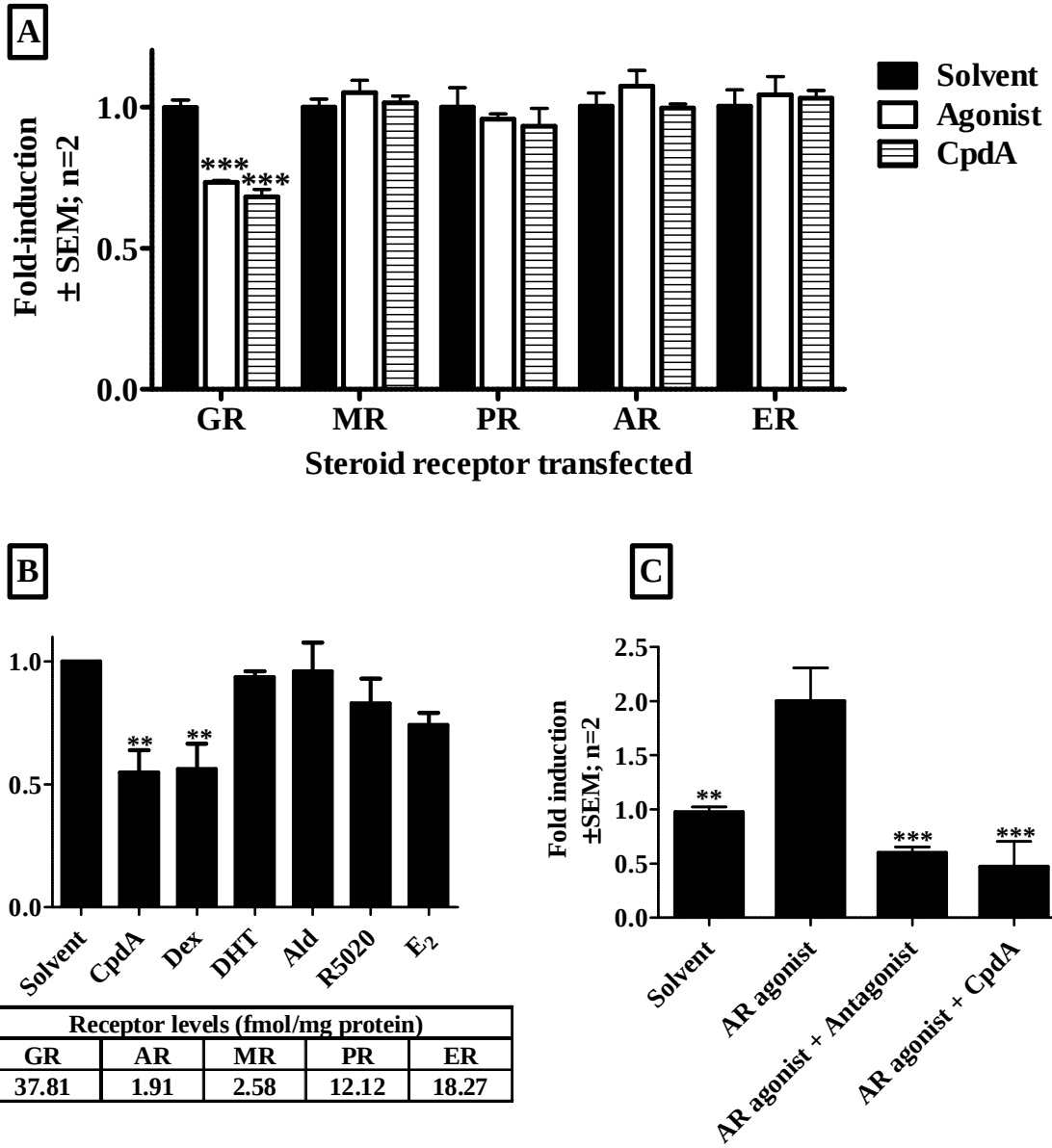

A Transactivation
B Transrepression
Agonist mode
Antagonist mode
Agonist mode
Antagonist mode
GR
MR
PR
AR
ER
Fold induction ± SEM; n=3
Fold induction ± SEM; n = 3
% of PMA induction ± SEM; n = 2
% of PMA induction ± SEM; n = 4
Solvent
Agonist
CpdA
Agonist + Antagonist
Agonist + CpdA
PMA
PMA + Agonist
PMA + CpdA
PMA + Agonist + Antagonist
PMA + Agonist + CpdA

Figure 8:



Receptor levels (fmol/mg protein)				
GR	AR	MR	PR	ER
37.81	1.91	2.58	12.12	18.27

Supplementary data not shown

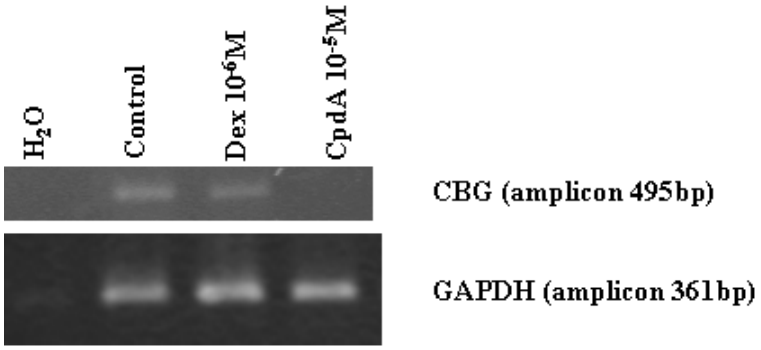


Fig. S1: RT-PCR of CpdA and Dex repression of mouse CBG: BWTG3 cells were induced with Dex (1 μM), or CpdA (10 μM) for 24 hr. Control wells received an equal amount of ethanol. Total RNA was reverse transcribed and the cDNA obtained subjected to PCR analysis with primers to detect mCBG and GAPDH (housekeeping gene used as loading control), in separate reactions. PCR products were analyzed on agarose gel and visualized under UV light. The figure is representative of three independent experiments.

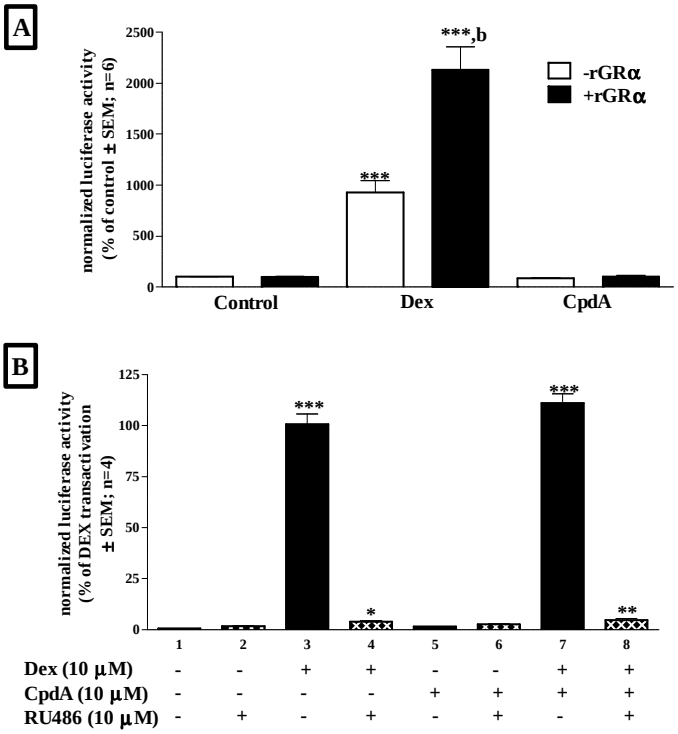


Fig. S2: Transactivation of GRE-containing promoter reporter construct. ***(A) Transactivation of GRE-containing promoter by CpdA and Dex in the absence or presence of co-transfected rGRα (pSVGR1).*** BWTG3 cells ($5x10^4$ cells per well in 24-well tissue culture plates) were transiently transfected with 360 ng GRE-containing promoter reporter construct ($(GRE)_2$50IL6PLuc), 200 ng rGRα (pSVGR1) or pGL2-basic as indicated, and 40 ng pPGKβGopbA. Twenty-four hours after transfection, test compounds were added (CpdA at 10 μM; Dex at 1 μM) for 24 hrs. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05; **P<0.01) and to (ii) compare values without GR (–rGRα) to values with co-transfected GR (+rGRα) for each compound tested using a two-tailed unpaired t-test (^{a}P<0.05; ^{b}P<0.01). ***(B) Transactivation of GRE-containing promoter by CpdA and Dex in HepG2 cells, in the absence or presence of RU486.*** The $(GRE)_2$tkLuc promoter reporter construct (360 ng) was transiently transfected into HepG2 cells ($5x10^4$ cells per well in 24-well tissue culture plates), together with 200 ng human GRα (pRS-hGRα) expression vector and 40 ng pPGKβGopbA. Twenty-four hours after transfection cells were treated for 24 hrs with 10 μM test compounds as indicated. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and plotted as a percentage of the average transactivation by Dex alone. Statistical analysis was done to compare values in presence of test compounds relative to the corresponding controls using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*P<0.05; **P<0.01).

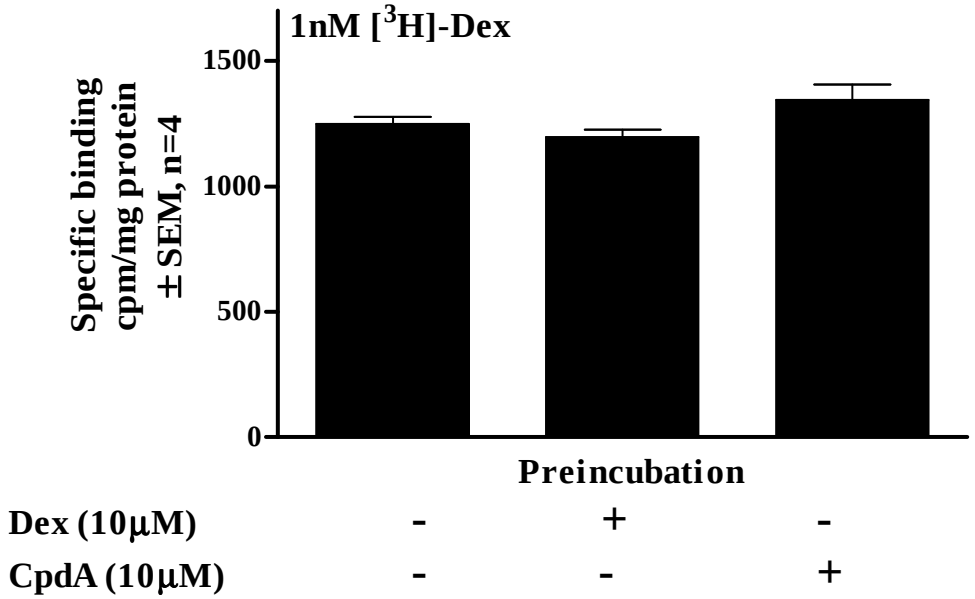


Fig. S3: Effect of pre-incubation. BWTG3 cells were incubated with 10μM CpdA and 10μM Dex for 1hr followed by washout and whole cell binding with 1nM ^{3}H-Dex. Results show specific binding. Statistical analysis was done to compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: P<0.05; **: P<0.01).

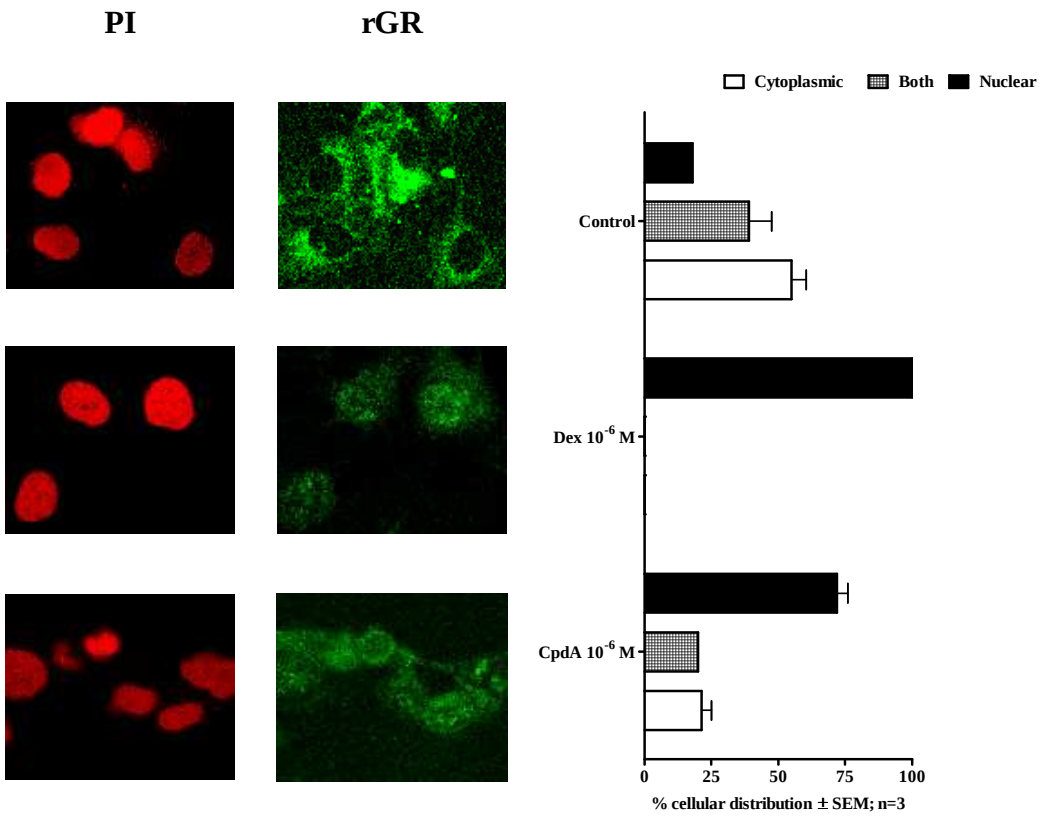


Fig. S4: Nuclear translocation of endogenous GR in BWTG3 cells. BWTG3 cells (5 x 10^5 cells per well in 6-well plates) were serum-starved in Opti-MEM for 24 h before induction with Dex (1 µM) or CpdA (1 µM) for 30 min. Control wells received an equal amount of ethanol. After fixation, cells were subjected to immunostaining with anti-GR, followed by anti-rabbit-Alexa 488 as a secondary Ab (GR; green). Propidium iodide staining (PI; red) was used to visualize nuclei. Cells were analysed using an Zeiss confocal LSM410 microscope. The percentage of cells showing GR in either the nuclear or cytoplasmic or both compartments was quantified for three independent experiments and is presented graphically.

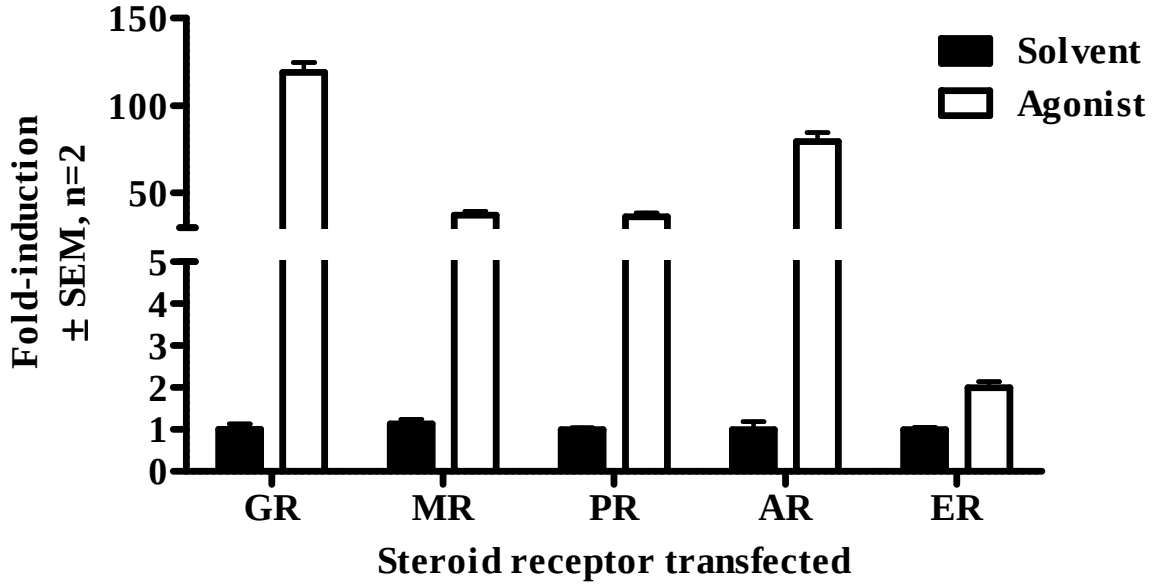


Fig. S5: Transactivation of transiently transfected GRE-containing promoter reporter construct via low levels of GR, MR, PR, or AR, or ERE-containing promoter reporter construct via low levels of ER. COS-1 cells ($2x10^6$ cells per plate in 10 cm tissue culture dishes) were transiently transfected with 9 μg pTAT-GRE2-Elb-luc, and 3 ng pRS-hGRα, pRS-hMR, pSG5hPRB or pSVARo as indicated, or 9 μg pGL2-3x-ERE-TATA-luc, and 3 ng pcDNA3-ERα. Twenty-four h after transfection cells were replated ($5x10^4$ cells per well in 24-well tissue culture plates). Cells were induced for 24 h with solvent (ethanol) or agonist (10^{-6} M) 24 h after replating). Luciferase values were normalized with protein concentration and values plotted as fold-induction relative to average solvent. Agonists used: Dex for GR; aldosterone for MR; R5020 for PR; DHT for AR; E_2 for ER.