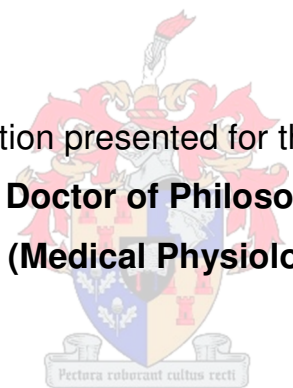


**THE ROLE OF MELATONIN IN
CARDIOPROTECTION:
AN INVESTIGATION INTO THE MECHANISMS
INVOLVED IN GLUCOSE HOMEOSTASIS,
MICROVASCULAR ENDOTHELIAL FUNCTION AND
MITOCHONDRIAL FUNCTION IN NORMAL AND
INSULIN RESISTANT STATES**

by

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April 2014

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Introduction:

The cardioprotective actions of the hormone melatonin against myocardial ischaemia-reperfusion injury (IRI) are well-established. It has recently been shown to prevent the harmful effects of hyperphagia-induced obesity on the susceptibility of the heart to IRI as well as many of the harmful effects of obesity and insulin resistance. However, the exact mechanism whereby it exerts its beneficial action is still unknown.

The aims of this study were to determine the effects of relatively short-term melatonin treatment in a rat model of diet-induced obesity on: (i) biometric and metabolic parameters, lipid peroxidation, myocardial IRI and intracellular signalling (ii) mitochondrial oxidative phosphorylation function (iii) cardiomyocyte glucose uptake and intracellular signalling. In addition, the effects of acute melatonin treatment of cardiac microvascular endothelial cells (CMEC) were determined on cell viability, nitric oxide production (NO), TNF- α -induced dysfunction and intracellular signalling.

Material and Methods:

Male Wistar rats were randomly allocated to two groups for 20 weeks feeding with either standard rat chow or a high calorie diet. Each group was subdivided into 3 groups receiving either water throughout or melatonin (4mg/kg/day, in the drinking water) for the last 6 or 3 weeks of the experimental programme. Hearts, perfused in the working mode, were subjected to ischaemia/reperfusion and infarct size determined. Mitochondria and cardiomyocytes were isolated according to standard techniques and oxidative function and glucose uptake respectively determined. CMEC NO production and cell viability were quantified by FACS analysis of the fluorescent probes, DAF-2/DA and propidium iodide/Annexin V respectively. Intracellular signalling was evaluated using Western blot and appropriate antibodies.

Results:

The high-calorie diet caused significant increases in body weight gain, visceral adiposity, fasting blood glucose, serum insulin, triglycerides, HOMA-IR index and a concomitant

reduction in serum adiponectin levels as well as larger myocardial infarct sizes after exposure to IRI compared to the control, indicating increased susceptibility to damage. Three as well as six weeks of melatonin administration to obese and insulin resistant rats reduced serum insulin levels and the HOMA-IR index. Myocardial infarct size was reduced in both control and diet groups. These effects were associated with increased activation of baseline myocardial STAT-3 and the RISK pathway during reperfusion.

The diet had no effect on the oxidative phosphorylation capacity of mitochondria, isolated from non-perfused hearts (baseline), but melatonin administration for 6 weeks induced a reduction in state 3 respiration rate; mitochondria isolated from diet hearts subjected to global ischaemia, exhibited an attenuated oxidative phosphorylation process which was improved by melatonin treatment.

Melatonin *in vitro* enhanced cardiomyocyte insulin stimulated glucose uptake of normal young rats but not of insulin resistant rats. *In vivo* melatonin treatment for 6 weeks increased basal (in diet group) and insulin stimulated glucose uptake in both control and diet groups.

Melatonin (1nM) *in vitro* caused a significant reduction in necrosis and apoptosis of cultured CMEC, associated with a decrease in nitric oxide availability and eNOS activation and a concomitant increase in PKB/Akt, p38MAPK and AMPK activation. The harmful effects of TNF- α treatment on signalling in CMEC could be prevented by co-treatment with melatonin.

Conclusions:

The results suggest that short-term melatonin treatment was able to significantly attenuate the diet-induced increased myocardial susceptibility to ischaemia/reperfusion damage. It may also improve cardiac glucose homeostasis and mitochondrial oxidative phosphorylation in an insulin resistant state. Melatonin *in vitro* protects CMEC against apoptosis and necrosis and reduces nitric oxide availability. These beneficial effects of melatonin may ultimately be due to its anti-oxidant capacity or receptor-mediated actions, but this remains to be established.

ABSTRAK

Inleiding:

Die vermoë van die hormoon, melatonien, om die hart teen iskemie/ herperfusiebesering (IHB) te beskerm, is welbekend. Onlangs is ook getoon dat melatonien IHB en verskeie van die nadelige effekte van vetsug en insulienweerstandigheid in hiperfagiegeïnduseerde vetsug kan voorkom. Die meganisme(s) betrokke by hierdie voordelige prosesse is egter grootliks onbekend.

Die doel van hierdie studie was om die gevolge van korttermyn melatonienbehandeling in 'n model van hiperfagiegeïnduseerde vetsug te ondersoek op (i) biometriese en metaboliese parameters, lipiedperoksidasie, miokardiale IHB en intrasellulêre seintransduksie, (ii) mitochondriale oksidatiewe fosforilasie, (iii) glukoseopname en intrasellulêre seintransduksie in kardiomiosiete en aanvullend, (iv) die invloed van akute melatonienbehandeling van kardiaal mikrovaskulêre endoteelselle op sellulêre oorlewing, stikstofoksiedproduksie, TNF- α -geïnduseerde disfunksie en seintransduksie.

Metodiek:

Manlike Wistarrotte is ewekansig in twee groepe verdeel en vir 20 weke met standaard-rotkos of 'n hoëkaloriedieet gevoer. Elke groep is in 3 subgroepe verdeel, wat deurgaans water of melatonien (4mg/kg/dag in die drinkwater) vir 3 of 6 weke voor die beëindiging van die eksperiment ontvang het. Harte is geperfuseer volgens die werkharttegniek, blootgestel aan iskemie/herperfusie en die infarkt grootte bepaal. Mitochondria en kardiomiosiete is volgens standaardtegnieke geïsoleer vir bepaling van oksidatiewe funksie en glukoseopname respektiewelik. NO produksie en sellewensvatbaarheid was gekwantifiseer deur vloeisitometrie analises (FACS) van die fluoresserende agense, DAF-2/DA en propidium jodied/Annexin V onderskeidelik. Intrasellulêre seintransduksie is evalueer met behulp van die Western kladtegniek en geskikte antiligggame.

Resultate:

Die hoëkaloriedieet het 'n beduidende toename in liggaamsgewig, visserale vet, vastende bloedglukose, seruminsulienvlakke, trigliseriede, HOMA-IR-indeks en 'n gepaardgaande verlaging in serumadiponektienvlakke tot gevolg gehad, sowel as groter miokardiale infarkte na iskemie/herperfusie. Laasgenoemde dui op 'n groter vatbaarheid vir iskemiese beskadiging in harte van vetsugtige diere.

Drie sowel as ses weke van melatonienbehandeling het die seruminsulienvlakke en HOMA-indeks in vetsugtige diere beduidend verlaag, vergeleke met die kontroles. Miokardiale infarktgroottes was verminder in beide kontrole- en vetsuggroepe. Hierdie effekte het met 'n verhoogde aktivering van basislyn STAT-3 en PKB/Akt en ERKp44/p42 tydens herperfusie gepaard gegaan.

Die dieet het geen invloed op die oksidatiewe fosforilasiekapasiteit van mitochondria, geïsoleer uit harte van ongeperfuseerde harte, gehad nie (basislyn), maar melatonienbehandeling vir 6 weke het Staat 3 respirasie verlaag. Mitochondria, geïsoleer uit harte van vetsugtige rotte wat aan globale iskemie onderwerp was, het 'n onderdrukte oksidatiewe fosforilasieproses gehad, wat egter deur melatonienbehandeling verbeter is.

Melatonien *in vitro* het insuliengestimuleerde glukoseopname deur kardiomyosiete van jong, maar nie vetsugtige rotte nie, verhoog. *In vivo* melatonientoediening vir 6 weke het egter basale (in die dieetgroep) en insuliengestimuleerde glukoseopname in beide kontrole- en vetsuggroepe verhoog.

Toediening van melatonien *in vitro* aan mikrovaskulêre endoteelselkulture het 'n beduidende afname in nekrose, apoptose, stikstofoksied- beskikbaarheid en eNOS aktivering teweeggebring, tesame met 'n verhoogde aktivering van PKB/Akt, p38MAPK en AMPK. Die nadelige effekte van TNF- α toediening op seintransduksie in die mikrovaskulêre endoteelselle is deur melatonien voorkom.

Gevogtrekkings:

Die resultate toon dat melatonien 'n merkwaardige beskermende effek op die toename in vatbaarheid vir iskemiese beskadiging in vetsugtige rotte gehad het. Dit mag ook miokardiale

glukose-homeostase en mitochondriale oksidatiewe funksie in insulienweerstandigheid verbeter. Melatonien *in vitro* beskerm mikrovaskulêre endoteelselle teen nekrose asook apoptose en verminder die beskikbaarheid van stikstofoksied. Hierdie voordelige effekte van melatonien mag aan sy anti-oksidantvermoëns of stimulasie van die melatonienreseptor toegeskryf word, maar bewyse daarvoor ontbreek nog.

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LIST OF ABBREVIATIONS

I. UNITS OF MEASUREMENT

%: percentage	min: minute
cm: centimeter	mM: millimolar
g: gram	mm: millimeter
Hg: mercury	mmol: millimol
IU: International unit	°C: degree celcius
kg: kilogram	v: volume
kJ: kilojoules	μ: micro
L: litre	μL: microlitre
M: molar	μm: micrometer
mg: milligram	

II. CHEMICAL COMPONENTS

AA-NAT: Arylalkylamine N-acetyltransferase	AT: Angiotensinogen
ACC: Acetyl-CoA carboxylase	AT1R: Angiotensin type 1 receptor
AFMK: N-acetyl-N-formyl-5-methoxykynuramine	ATP: Adenosine triphosphate
AGE: Advanced glycation end products	ATPase: Adenosine triphosphatase
AMK: N1-acetyl-5-methoxy-kynurenine	BHT: Butylated hydroxytoluene
AMP: Adenosine monophosphate	BSA: Bovine serum albumin
AMPK: AMP-activated protein kinase	Ca ²⁺ : Calcium
Ang II: Angiotensin	cAMP: Cyclic adenosine monophosphate
ANP : Atrial natriuretic peptide	CAT: Catalase
AP-1: Activator protein-1	CD: Conjugated dienes
apoB: Apolipoprotein B	cGMP: Cyclic guanosine monophosphate
	CO ₂ : Carbon dioxide

COX-2: Cyclooxygenase- 2	IR: Insulin resistance
CPK: Creatine phosphokinase	IRS: Insulin receptor substrate
CREBP: cAMP-response element binding protein	JNK : c-Jun N-terminal kinases
CRP: C-reactive protein	LCFA: Long chain fatty acid
DAF-2/DA: diaminofluorescein-2/diacetate	LDH: Lactate dehydrogenase;
DAG: Diacylglycerol	LDL: Low density lipoprotein
DHPR : Dihydropyridine receptor	MAPK: Mitogen activated protein kinase
DMSO: Dimethyl sulfoxide	MDA: Malondialdehyde
ERK 42/44: Extracellular signal regulated kinase p42/p44	MPO: Myeloperoxidase
ER: endoplasmic reticulum	MPTP: Mitochondrial permeability transition pore
8-epi-PGF2 α : 8-epi-prostaglandin F-2alpha	MT1/MT2: Melatonin receptor 1 and 2
FFA: Free fatty acids	NADPH: Nicotinamide adenine dinucleotide phosphate
FATP : Fatty acid transport protein	NF- κ B:Nuclear factor kappa-B
FABPpm : Plasma membrane fatty acid binding protein	NO: Nitric oxide
GLUT-1; 4: Glucose transporter 1 and 4	NOS: nitric oxide synthase
GPCR: G-protein coupled receptor	ONOO $^-$: Peroxynitrite
GPx: Glutathione peroxidase	PAI-1: Plasminogen activator inhibitor-1
GSH: Reduced glutathione;	PDE2: Phosphodiesterase-2
GSK-3: Glycogen synthase kinase-3	PPAR: Peroxisome proliferator-activated receptor- coactivator
HDL: High density lipoprotein	PGC: Peroxisome proliferator-activated receptor gamma coactivator
HRP: Horseradish peroxidase	PGE2: Prostaglandin-E2
IKK β : I-Kappa B kinase β	PI3-K: Phosphatidylinositol 3 kinase
IL-6: Interleukin-6	PKA: Protein kinase A
IP3: Inositol 1,4,5-trisphosphate	PKB/Akt: Protein kinase B

PKC: Protein kinase C	SOCS3: Suppressor of cytokine signalling 3
PLB: Phospholamban	SOD: Superoxide dismutase
RISK: Reperfusion injury salvage kinase	STAT-3: Signal transducer and activator of transcription 3
RNS: Reactive nitrogen species	TBA: Thiobarbituric acid
ROOH/LOOH: Lipid hydroperoxide	TBARS: Thiobarbituric acid reaction substance
ROS: Reactive oxygen species	TLRs: Toll-like receptors
RPB-4: Retinol binding protein-4	TMB: Tetramethylbenzidine
RT-PCR: Reverse transcription-polymerase chain reaction	TNF- α : Tumour necrosis factor alpha
RyR: Ryanodine receptor	TG: Triglyceride/triacylglycerol
SAA: Serum amyloid A	TTC: Triphenyl tetrazolium chloride
SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis	VLDL: Very low density lipoprotein
SERCA2a: Sarco-endoplasmic reticulum Ca ²⁺ -ATPase 2	ZAG: Zinc- α 2-glycoprotein

III. OTHERS

MI: myocardial infarction	CAL: Coronary artery ligation
ANOVA: Analysis of variance	CF: Coronary flow
AO: Aortic output	CMEC: Cardiac microvascular endothelial cells
AR: Area at risk	CO: Cardiac output
AUC: area under curve	CVD: Cardiovascular disease
BMI: Body mass index	D (DIO): Diet (diet-induced obesity)
bAEC: bovine aortic endothelial cells	DP: Diastolic blood pressure
BW: Body weight	ECL: Enhanced chemiluminescence
BP: Blood pressure	e.g.: for example (<i>exempli gratia</i>)
C: Control	EDRF: Endothelium-derived relaxing factor
CAD: Coronary artery disease	

EDCF: Endothelium-derived contractile factor	LAD: Left anterior descending (coronary artery)
EGM: endothelial cell growth medium	LD: Langendorff
ELISA: enzyme linked-immunosorbent assay	LV: Left ventricle
ER: Endoplasmic reticulum	LVDevP: Left ventricular developed pressure
ETC: Electron transport chain	LVEDp: Left ventricular end-diastolic pressure
FACS: Flow activated cell sorter	MAP: Mean arterial pressure
Fig.: Figure	MetS: Metabolic syndrome
GI: Global ischaemia	NCEP ATP III: National Cholesterol Education Program's Adult Panel III
GK: Goto Kakizaki	OSAS: Obstructive sleep apnoea syndrome
HGO: Hepatic glucose output	PBS: phosphate buffered saline
HOMA: Homeostasis model assessment	PPCI: primary percutaneous coronary intervention
HR: Heart rate	PVDF: Polyvinylidene fluoride
HUVEC: Human umbilical vein endothelial cells	RAS /RAAS: Renin angiotensin system or renin-angiotensin-aldosterone system
HW: Heart weight	RCI: Respiratory control index
i.e.: That is (<i>id est</i>)	RI: Regional ischaemia
i.p.: Intraperitoneal injection	RIA: Radioimmunoassay
IGF-1: Insulin-like growth factor-1	SAFE: Survivor activating factor enhancement
IFS: Infarct size	SABS/SANS: South african bureau of standards/South african national standards
IPC: Ischaemic preconditioning	SCN: Suprachiasmatic nucleus
IPGTT:Intraperitoneal glucose tolerance test	SD: Sprague Dawley
IPOC: Ischaemic postconditioning	SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
IR:Insulin resistance	
IRI: Ischaemia and reperfusion injury	
KHB: Krebs-Henseleit bicarbonate buffer	

SEM: Standard error of the mean

vs.: versus

SP: Systolic blood pressure

WH: Working heart

SRC: Standard rat chow

WHO: World Health Organization

T1D: Type 1 diabetes

Wtot: Work total or total work

T2D: Type 2 diabetes

ZO: Zucker obese (rat)

VF: Ventricular fibrillation

ZDF: Zucker diabetic fatty (rat)

VT: Ventricular tachycardia

CHAPTER ONE

GENERAL INTRODUCTION

Globally, cardiovascular disease (CVD) especially ischaemic heart disease, is the leading cause of death in the world (Lozano et al., 2013) and contributes to 29% of all deaths each year (Barry et al., 2008). Previously recognized in developed countries only, CVD has invaded developing countries including South Africa, where ischaemic heart disease is the fifth highest cause of mortality (Wyk et al., 2013) and the leading cause of mortality in the Western Cape Province (Bradshaw et al., 2004).

Although multiple causes may contribute to the prevalence of CVD, modern life style associated with excessive food intake and reduced physical activity as well as night shift work have led to increased risk factors for this condition (Murphy et al., 2006; Grundy, 2008; Pothiwala et al., 2009; Danaei et al., 2011), including obesity, insulin resistance, raised blood pressure, atherogenic dyslipidaemia, glucose intolerance and a pro-inflammatory state (Alberti et al., 2009). Among these factors, insulin resistance is a fundamental feature in the development of type 2 diabetes (T2D) and a common pathological link between obesity and CVD in general, and ischaemic heart disease in particular (Reaven, 2011; Abel et al., 2012).

Convincing epidemiological and experimental studies have shown a strong association between obesity/insulin resistance and cardiac diseases including coronary artery disease, atrial fibrillation and congestive heart failure (Yusuf et al., 2005; Poirier et al., 2006; Banerjee and Peterson, 2007; Reaven, 2012; Shah et al., 2013; Wensley et al., 2013). Obesity and insulin resistance have many effects on cardiac structure and function (Lee et al., 2010; Gray and Kim, 2011; Abel et al., 2012; Narumi et al., 2012; Wensley et al., 2013), correlating significantly with dysfunction of the heart (Ingelsson et al., 2005; Banerjee et al., 2013). Importantly, high fasting insulin levels are positively associated with adverse echocardiographic features and risk of subsequent heart failure (Banerjee et al., 2013).

The mechanism of the development of cardiac disease in obesity and insulin resistance is complex and not well understood. Various metabolic abnormalities occurring in the insulin

resistant state have been implicated as risk factors for cardiac diseases (Reaven,1988; Reaven , 2004). Visceral adipose tissue dysfunction and its associated oxidative stress have also been linked to increased cardiac disorders (Despres, 2006). Importantly, impairment of myocardial glucose uptake and metabolism may contribute to the mechanical dysfunction observed in T2D and obese patients (Chess and Stanley, 2008) and increase their susceptibility to ischaemic heart disease (Cubbon et al., 2013)

The concept of cardioprotection refers broadly to all strategies aimed at the reduction of infarct size and full restoration of myocardial function (Heusch, 2013). The most effective therapeutic intervention for reducing acute myocardial ischaemic injury and limiting infarct size is timely and effective myocardial reperfusion but this also is damaging (Heusch, 2013). Although numerous *in vivo* and *in vitro* animal investigations reported potential beneficial effects (Vander Heide and Steenbergen, 2013), most previous clinical trials have been disappointing (Heusch, 2013; Vander Heide and Steenbergen, 2013). Many experimental myocardial infarction (MI) models fail to represent the clinical setting in terms of comorbidities (such as obesity, diabetes, dyslipidaemia, age and hypertension), the presence of which may interfere with the therapeutic cardioprotective intervention (Ghaboura et al., 2011; Sack and Murphy, 2011; Whittington et al., 2013). Thus omission to consider these comorbidities has been one of the pitfalls of recent promising cardioprotective strategies when translated to the clinic (Vander Heide and Steenbergen, 2013).

In view of the lack of effective therapy, cardioprotection is still an active research area. However, with the rising prevalence of obesity and its multiple comorbidities, an ideal therapeutic strategy must retain its efficacy under pathological conditions, not only in lean subjects, but also in obese and insulin resistant conditions.

Melatonin or 5-methoxy-N-acetyltryptamine is the neurohormone produced mainly by the pineal gland upon the activation of the suprachiasmatic nucleus (SNC) of the hypothalamus during the night (Zawilska et al., 2009). It is a highly conserved indolamine found in most organisms, including mammals (Hardeland and Fuhrberg, 1996). Its chemical structure is

represented by the figure 1.1. Melatonin is a small molecule able to enter all cells (due to its lipophilic nature) and confer its pleiotropic effects including, amongst others, immunomodulatory, anti-inflammatory, oncostatic and vasomotor properties (Hardeland et al., 2006; Pandi-Perumal et al., 2006; Hardeland et al., 2011). In this regard, its classical role as a chronobiotic or endogenous synchronizer in the regulation of seasonal as well as circadian rhythms along with its sleep inducing effects is well-established (Zawilska et al., 2009). Importantly, acute myocardial infarction has been associated with reduced nocturnal serum melatonin levels (Dominguez-Rodriguez et al., 2002; Dominguez-Rodriguez et al., 2012). Cardioprotective properties of melatonin have been demonstrated in lean animals where melatonin (at either physiological or pharmacological doses), given before or after the ischaemic insult protected the heart against myocardial ischaemia/reperfusion damage (Lochner et al., 2013).

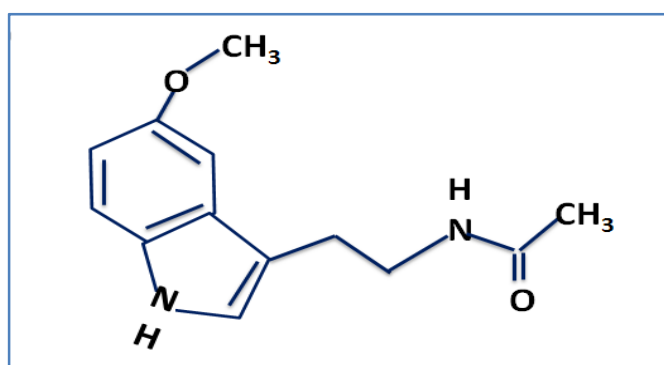


Figure 1.1 Chemical structure of melatonin

The role of melatonin in obesity has only recently become a subject of investigations. Its potential as a tool for effective therapy in obesity-induced metabolic abnormalities was recently reviewed (Nduhirabandi et al., 2012; Srinivasan et al., 2013), indicating a growing interest for a role for the hormone in obesity. Convincing evidence supports the role for melatonin in metabolic regulation (Prunet-Marcassus et al., 2003; Barrenetxe et al., 2004; Hussein et al., 2007; She et al., 2009; Shieh et al., 2009; Ríos-Lugo et al., 2010; Agil et al., 2011; Nduhirabandi et al., 2011) and in glucose homeostasis (Nishida, 2005; Peschke, 2008; Peschke and Muhlbauer, 2010). We previously showed that melatonin consumption starting before the establishment of obesity, prevented obesity-related abnormalities and protected the

isolated hearts subjected to ischaemia-reperfusion injury (Nduhirabandi et al., 2011). However the effects of melatonin in obesity are still not yet established, especially on the heart.

Several mechanisms, including antioxidant activities, anti-adrenergic, anti-inflammatory and anti-excitatory effects have been demonstrated (Genade et al., 2008; Dominguez-Rodriguez et al., 2009; Patel et al., 2010). Additionally, inhibition of mitochondrial permeability transition pore (MPTP) opening (Petrosillo et al., 2009) as an end-effector in cardioprotection has also been indicated. However, how melatonin affects these parameters has not been investigated in the context of obesity and insulin resistance. Furthermore, the effect of melatonin on microvascular endothelial function, which may play a crucial role in ischaemic damage, has not been investigated either. Moreover, the effect of melatonin treatment on the heart in established obesity remains unknown.

In view of the many outstanding issues regarding melatonin's effects, the present dissertation focussed on the role of melatonin in cardioprotection in obesity and insulin resistance.

To better understand the mechanisms of cardioprotection in obesity and insulin resistance, the current literature on insulin resistance and obesity and potential mechanisms linking these conditions to CVD (metabolic syndrome, adipose tissue dysfunction and oxidative stress) as well as their related cardiac alterations in normoxia and ischaemia-reperfusion injury are reviewed in the following chapter. Additionally, before the discussion on the role of melatonin and the heart, and the effects of melatonin in obesity and insulin resistance, cardioprotection in obesity is addressed.

CHAPTER TWO

LITERATURE REVIEW

2.1 OBESITY AND INSULIN RESISTANCE: A FOCUS ON THE HEART

2.1.1 INTRODUCTION

Excessive food intake and reduced physical activity associated with modern life style have led to the current dramatic increase in the prevalence of obesity (James, 2008a; de Onis et al., 2010; Finucane et al., 2011; Swinburn et al., 2011) which is paralleled by an elevated incidence of metabolic disorders, including, among others, metabolic syndrome (MetS), type 2 diabetes (T2D) and cardiovascular diseases (CVD) (Murphy et al., 2006; Grundy, 2008; Pothiwala et al., 2009; Danaei et al., 2011). The association of these disorders with obesity has been recognized for decades (Haslam, 2007); however it is only in recent years that obesity and its related disorders have attracted attention as a serious public health concern (James, 2008b). Indeed, apart from an increased risk for T2D and CVD, numerous other disorders including obstructive sleep apnoea syndrome (Wolk and Somers, 2007), chronic kidney disease (Ruan and Guan, 2009) and some cancers (Pothiwala et al., 2009) have been associated with obesity. A major basis for this association is the ability of obesity to induce insulin resistance (Johnson and Olefsky, 2013). This is a fundamental aspect in the development of T2D (Taylor, 2012) and a common pathological link between obesity and heart disease (Reaven, 2011a; Abel et al., 2012), the top leading cause of death in the world (Lozano et al., 2013).

In this section, insulin resistance and obesity and their specific related metabolic abnormalities are described to better understand how obesity and/or insulin resistance affect cardiac structure and function. In this regard, the roles of adipose tissue dysfunction and obesity-associated oxidative stress are discussed. Current literature on the morphology, metabolism and function of the heart in the insulin resistant state is also summarized. In the present dissertation the concept of “insulin resistant state” refers to any condition or disorder associated with impaired insulin action.

2.1.2 INSULIN RESISTANCE

2.1.2.1 Overview: definition, concepts and assessment

Insulin is a small peptide hormone produced by β -cells of the pancreatic islets and released into the circulation following an increase in blood glucose or amino acids. After its discovery and its first clinical use around 1920 by Banting and coworkers, when diabetes was a devastating disorder without any effective intervention, insulin has been welcomed as anti-diabetic treatment [for a review, see (Polonsky, 2012)]. In this regard, insulin's actions have since attracted the interest of several investigators (Polonsky, 2012). Indeed, insulin has been shown to play a crucial role in the regulation of carbohydrate and lipid metabolism in the body and as well as in appropriate tissue development and growth (Sesti, 2006). Moreover, it is involved in multiple other cellular processes in various tissues of the body such as the brain (Derakhshan and Toth, 2013), pancreatic β -cells (Leibiger et al., 2008), kidneys (Hale and Coward, 2013), and the heart and vascular endothelium (Muniyappa et al., 2007; Beale, 2013). Therefore, in view of these pleiotropic activities, defects in insulin actions could give rise to multiple disorders.

The concept of insulin resistance was proposed more than seven decades ago when it was noted that some insulin-treated diabetic patients required higher doses of insulin than usual (Himsworth, 1936). Classically, insulin resistance refers to the condition in which the body produces insulin but does not utilize it properly because of a decreased cellular sensitivity to its effects on glucose uptake, metabolism and storage (Formiguera and Canton, 2004). In this condition, normal insulin concentrations become inadequate to produce a normal insulin response in target tissues (Cefalu, 2001; Hardy et al., 2012). Insulin resistance can be measured as a reduced glucose disposal rate in rodents and humans in response to defined concentrations of insulin (Hardy et al., 2012, Reaven, 1988, Muniyappa et al., 2008). In addition to insulin resistance, other concepts such as insulin sensitivity and glucose tolerance (or glucose intolerance) have been introduced in the assessment of insulin action or resistance to indicate the concentration of insulin required for a half maximal response and the

measurement of how quickly glucose infusion is cleared from the blood, respectively (Muniyappa et al., 2008).

To assess insulin action and resistance various techniques are being used [for details, see (Muniyappa et al., 2008; Dube et al., 2013)]. Among the available techniques, although it is more laborious and complex, the glucose clamp method is regarded as the gold standard. The simple and more convenient technique to assess insulin resistance is the homeostasis model assessment-estimated insulin resistance (HOMA-IR) index. This is expressed as the product of fasting plasma insulin and fasting plasma glucose divided by 22.5 (Dube et al., 2013).

2.1.2.2 Insulin signalling and action: tissue-specific

Insulin signalling has been shown to be tissue-specific and varies depending on the type and function of the organ/tissue target (Benito, 2011; Rask-Madsen and Kahn, 2012). Accordingly, the actions of insulin manifest also differently in various organs such as in the liver, adipose tissue, skeletal muscle, heart and vessels (Benito, 2011; Rask-Madsen and Kahn, 2012). Insulin signalling pathways have been intensively reviewed (Saltiel and Kahn, 2001; Sesti, 2006; Mlinar et al., 2007; Bertrand et al., 2008; Benito, 2011; Rask-Madsen and Kahn, 2012). In this section, a brief summary of insulin signalling and actions in insulin sensitive and resistant tissues is given. Additionally, a simplified representation of insulin signalling and its actions is supplied in figure 2.1.

2.1.2.2.1 Insulin signalling in insulin-sensitive tissues

In insulin-sensitive tissues, insulin binds to its receptor and initiates a complex spectrum of biological effects including insulin receptor autophosphorylation creating docking sites for downstream interacting proteins such as insulin receptor substrate 1 to 4 (IRS1-4). After IRS interaction, three potential signal transduction pathways take place including: the phosphatidylinositol 3-kinase (PI3-K)/Akt, the Ras-mitogen-activated protein kinase (MAPK) and the CAP/Cbl/TC10 pathways (Saltiel and Kahn, 2001).

- **PI3-K/Akt pathway**

The PI3-K/Akt dependent pathway is responsible for most of the metabolic actions of insulin. It is initiated by tyrosine phosphorylation of the insulin receptor substrate (IRS-1-4) which, when phosphorylated, associates with p85, a subunit of PI3-K enabling formation and activation of the holo-enzyme to produce phosphatidylinositol-3,-4,-5-phosphate (PIP₃). Increased PIP₃ results in activation of protein kinase B (PKB/Akt) *via* activation of phosphoinositide-dependent kinase 1 (PDK1) and other down stream effector molecules, which mediate the metabolic effects of insulin, including translocation of the glucose transporter 4 (GLUT-4) to the membrane, and glycogen synthesis *via* PKB/Akt mediated inhibitory phosphorylation of glycogen synthase kinase 3 (GSK-3). Other actions such as lipid synthesis, protein synthesis as well as nitric oxide (NO) production are also mediated by this PKB/Akt pathway (see figure 2.1). In parallel with PKB/Akt, PDK1 is also able to activate atypical protein kinase C (PKC) ζ and λ . The role of PKC ζ and λ in insulin-induced activation of GLUT-4 translocation and long-chain fatty acid uptake has recently been demonstrated in cardiomyocytes (Luiken et al., 2009; Habets et al., 2012).

- **Ras- MAPK pathway**

The Ras-MAPK-dependent pathway cooperates with the PI3-K/Akt pathway to control cell proliferation. It is initiated by the successive activation of Shc, Grb₂, mSOS, and Ras. Ras activation triggers the cascade of Raf to MEK₁ to extracellular regulated kinase (ERK) 42/44. ERK 42/44 are MAPK subtypes that mediate the mitogenic and the pro-inflammatory effects of insulin (Sasaoka et al., 1994).

- **CAP/Cbl pathway**

The CAP/Cbl pathway has been suggested as an additional pathway for glucose uptake in order to be fully manifested (Saltiel and Kahn, 2001). In this pathway, after the phosphorylation of insulin receptor substrate (IRS), the adapter protein CAP recruits proto-oncogen Cbl to the phosphorylated IRS and the activation of Cbl results in a cascade reinforcing GLUT-4 translocation (Gupte and Mora, 2006). This translocation is also stimulated by the PI3-K

pathway and has recently been demonstrated in the cardiomyocytes (Contreras-Ferrat et al., 2010).

All of these insulin signalling pathways act in a concerted fashion to coordinate the regulation of GLUT-4 vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression, which result in the regulation of glucose, lipid and protein metabolism and cell survival (Sesti, 2006).

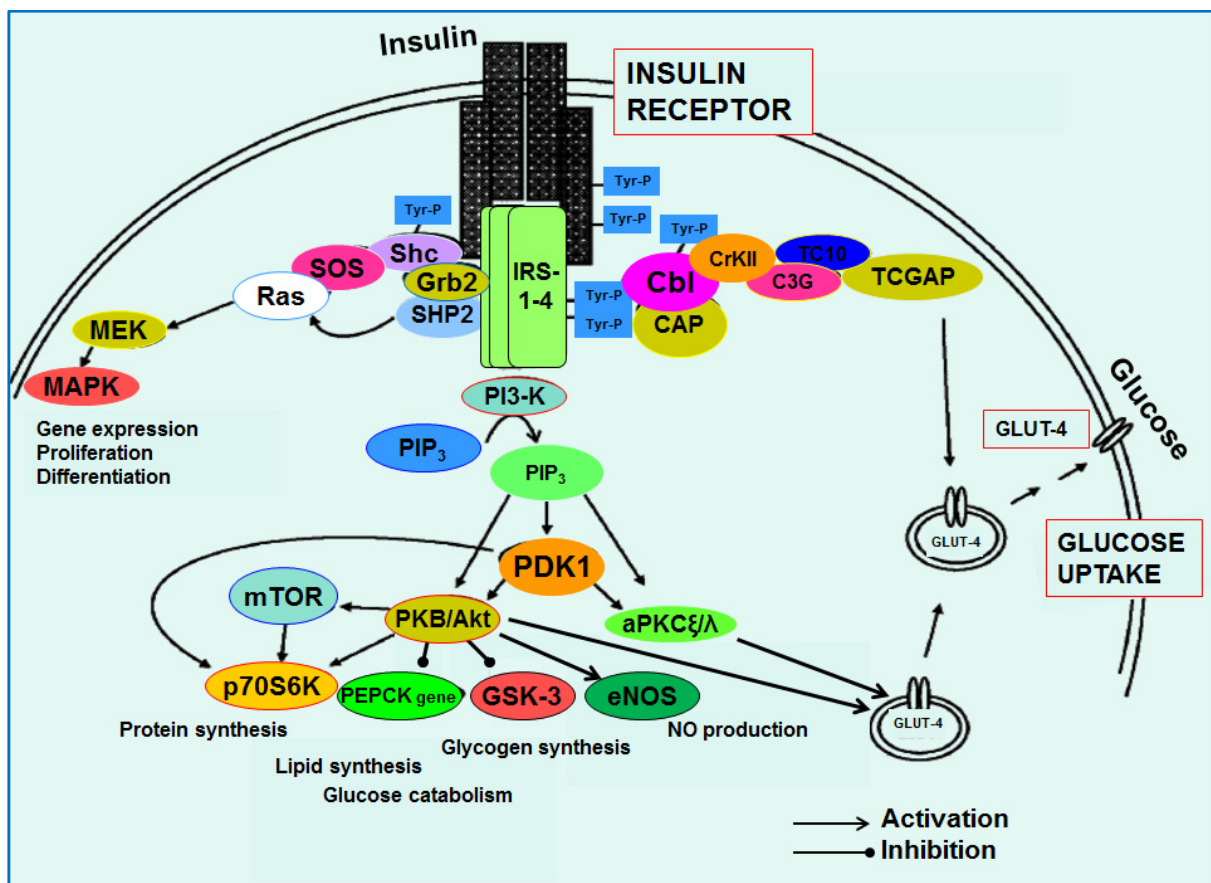


Figure 2.1 Insulin signalling and actions (simplified). Binding of insulin to the IR activates three different signal transductions: 1) PI3-K/PKB/Akt dependent pathway for metabolism (lipid synthesis, glucose catabolism, glycogen synthesis, and protein synthesis as well as nitric oxide (NO) production), 2) CAP/Cbl pathway (additional pathways required for glucose transporter-4 (GLUT4) translocation), and 3) the mitogen-activated protein kinase (MAPK) pathway (for gene expression, proliferation, differentiation). IRS: insulin receptor substrate; PI3-K: phosphoinositide-3-kinase; PDK-1: phosphoinositide-dependent kinase 1; PKB/Akt: protein kinase B; aPKC: atypical protein kinases C; mTOR: mammalian target of rapamycin; p70S6K: p70

ribosomal S6 kinase; PEPCK: phosphoenol pyruvate carboxykinase; GSK-3: glycogen synthase kinase 3 (adapted from Saltiel and Kahn, 2001; Sesti, 2006; Mlinar et al., 2007).

2.1.2.2.2 Compromised insulin signalling/action

Impaired insulin signalling/action may result from defects at several levels: 1) prereceptor (abnormal insulin), 2) receptor (reduced receptor number or affinity), 3) post-receptor such as abnormal signal transduction and phosphorylation [e.g., phosphorylation of IRS-1 at serine/threonine (Ser/Thr)] as well as defects in glucose transporters (e.g., decreased GLUT-4 expression or translocation) [for review, see (Sesti, 2006; Benito, 2011; Beale, 2013)]. Amongst these defects, post-receptor insulin signalling abnormalities have been indicated to contribute significantly to insulin resistance and are associated with various clinical disorders including, amongst others, hyperglycaemia-induced tissue damage, dyslipidaemia, inflammation, hypertension, and MetS (Griffin et al., 1999; Sesti, 2006; Mlinar et al., 2007; Chess and Stanley, 2008). This is discussed further below in the section on the mechanism of obesity-induced insulin resistance (section 2.1.4).

2.1.2.2.3 Manifestation of insulin resistance

In obesity and T2D, the manifestation of impaired insulin actions and signalling have been shown to be time-dependent and tissue-specific (Benito, 2011; Rask-Madsen and Kahn, 2012). For example, in the study conducted by Kleemann et al. (2010) on mice fed a high fat diet, peripheral tissues implicated in body metabolism were differently affected by insulin resistance. With time, mice fed a high fat diet became increasingly obese and showed a gradual increase in glucose intolerance (Kleemann et al., 2010). At a cellular level, impaired insulin action first manifested in the liver (week 6) and then in white adipose tissue (week 12), while skeletal muscle remained insulin-sensitive (Kleemann et al., 2010). Additionally, the development of insulin resistance was paralleled by tissue-specific gene expression changes, metabolic adjustments, changes in lipid composition and inflammatory responses (Kleemann et al., 2010)

In skeletal muscle, impaired insulin action is manifested by a decrease in glucose transport and a decline in muscle glycogen synthesis in response to normal circulating insulin (Hardy et al. 2012). In the liver, it refers to the inability of insulin to suppress gluconeogenesis while continuing to stimulate fatty acid synthesis (Hardy et al. 2012). This is believed to be the cause of enhanced fasting hyperglycaemia and hypertriglyceridaemia or lipoproteinaemia in MetS or T2D (Benito, 2011, Hardy et al. 2012, Rask-Madsen and Kahn, 2012). In adipose tissue, impaired insulin action is manifested as reduced insulin-stimulated glucose transport, as well as attenuated inhibition of lipolysis (Hardy et al. 2012). As described further below in the section on adipose tissue dysfunction (section 2.1.4.1), this is believed to promote lipolysis in adipocytes and secretion of pro-inflammatory cytokines by macrophages (Rask-Madsen and Kahn, 2012, Hardy et al. 2012).

Impaired insulin action is a prerequisite precursor for the development of T2DM and a feature of a number of clinical disorders such as hypertension, dyslipidaemia and MetS (DeFronzo and Ferrannini, 1991; Reaven, 1988). However, it can also be observed in non-clinical states such as human pregnancy (Beale, 2013). The present dissertation will focus exclusively on obesity. Not only this is the most prevalent insulin resistant state, but also many of the metabolic abnormalities associated with insulin resistance are closely related to obesity (Cornier et al., 2008). Accordingly, the following section focuses on obesity and its related metabolic alterations as well as the mechanism of obesity-induced insulin resistance.

2.1.3 OBESITY

2.1.3.1 Obesity: definition, cause and prevalence

Obesity is a chronic metabolic disorder characterized by increased body fat accumulation to the extent that adverse health consequences may occur. It is generally defined by a body mass index (BMI) of $\geq 30 \text{ kg/m}^2$. BMI (ratio of weight divided by height squared) is a simple screening tool to estimate total body fat (Stegger et al., 2011). Because of its poor sensitivity and specificity, other measurements such as waist circumference, waist-to-hip ratio, skinfold thickness, and bioelectrical impedance are commonly used additionally to assess the body fat

distribution and composition (Lau and Obesity Canada Clinical Practice Guidelines Steering Committee and Expert Panel, 2007; Stegger et al., 2011; Canoy et al., 2013; De Schutter et al., 2013). The causes of obesity are complex and multifactorial. Apart from the involvement of genetic susceptibility, gender and ethnicity factors, obesity results from imbalance between energy consumption and expenditure and it is closely associated with reduced physical activity (Haslam and James, 2005; Swinburn et al., 2009; Swinburn et al., 2011; Malik et al., 2012).

Previously only recognized in developed countries, recent observations show also an increase in obesity prevalence in developing countries including South Africa (Malik et al., 2012). A recent systematic review of health examination surveys and epidemiological studies has shown that the prevalence of global obesity increased from 4.8% in 1980 to 9.8% in 2008 for men, and from 7.9% in 1980 to 13.8% in 2008 for women, representing 205 million men and 297 million women with obesity and 1.46 billion people with overweight in 2008 (Finucane et al., 2011). If the current trend continues it is predicted that by 2030 up to 58% of the worldwide adult population (3.3 billion) could be either overweight or obese (Kelly et al., 2008). This alarming prevalence is not only a concern among adults, but overweight and obesity prevalence is also dramatically increasing in children. In 2010, 43 million children (35 million in developing countries) were estimated to be overweight and obese while 92 million were at risk of overweight (de Onis et al., 2010). Factors including excessive food intake and reduced physical activity associated with modern life style due to the current socio-economic and nutrition transition have been blamed for this phenomenon in developing countries (James, 2008a; Malik et al., 2012; Popkin et al., 2012).

2.1.3.2 Obesity and insulin resistance

Obesity is associated with defects in insulin action and signalling (Kahn and Flier, 2000). In addition, convincing evidence exists that increased body fat accumulation, particularly visceral obesity, is involved in the development of the insulin resistant state (Tchernof and Despres, 2013). It was established that lean individuals with no previous family history of obesity or diabetes became insulin resistant following experimental overnutrition (DeFronzo and

Ferrannini, 1991). In animal models of obesity, insulin resistance/hyperinsulinaemia induced by a high fat/calorie feeding was accompanied with (Lima-Leopoldo et al., 2008) or without (du Toit et al., 2008) glucose intolerance. Insulin resistance was also shown to be associated with obesity related metabolic abnormalities such as dyslipidaemia, endothelial dysfunction and hypertension as well as other features of the MetS (Cornier et al 2008; Beale, 2013, Reaven, 2004). However, apart from this evident association, the link between insulin resistance and obesity/overweight has been shown to be more complex (McLaughlin et al., 2004; McLaughlin et al., 2007; Hardy et al., 2012; Bradshaw et al., 2013; Tchernof and Despres, 2013).

Epidemiological investigations have shown that not all overweight/obese persons are insulin resistant or metabolically unhealthy (McLaughlin et al., 2004; Vikram et al., 2006; McLaughlin et al., 2007). Additionally, it has also been shown that not all people with a normal body weight or without insulin resistance are necessarily metabolically healthy (Vikram et al., 2006). This heterogeneity in the relationship between insulin resistance and obesity has attracted more recent investigations to better understand the mechanism of development of insulin resistance as well as MetS (Hardy et al., 2012; Bradshaw et al., 2013; Tchernof and Despres, 2013). A study conducted by McLaughlin et al. (2004) showed that among people who developed insulin resistance, 36% were obese ($BMI \geq 30.0 \text{ kg/m}^2$) and approximately 16% were of normal weight ($BMI \leq 25 \text{ kg/m}^2$) while 30% of insulin sensitive individuals were overweight or obese ($BMI \geq 25 \text{ kg/m}^2$). Another epidemiological study reported that 28.8% of Asian Indian adolescents with normal BMI and waist circumference presented with fasting hyperinsulinaemia (Vikram et al., 2006).

These heterogeneous phenotypes in insulin resistant and obese people could be explained by genetic predisposition or previous family history and ethnicity as well as environmental factors (Wang et al., 2009; Norris and Rich, 2012). Interestingly, genetic predisposition can also be modified by environmental factors such as physical exercise and dietary habits (Naukkarinen et al., 2011), supporting the actual association between an “obesogenic environment” and increased prevalence of insulin resistance and its related disorders (Ginter and Simko, 2012).

Furthermore, the crucial role of obesity in the actual rise in insulin resistance prevalence has strongly been supported by the observation that an increase in BMI is more prevalent in insulin resistant individuals (Stefan et al., 2008). More interestingly, although some overweight/obese individuals were found to have normal insulin sensitivity, it was recently observed that these obese metabolically healthy subjects were more susceptible to develop metabolic abnormalities at a later stage compared to normal weight individuals (Bradshaw et al., 2013). To better understand the mechanism of obesity-induced insulin resistance, its associated metabolic abnormalities are described in the section below, focusing on MetS .

2.1.3.3 Obesity-associated metabolic abnormalities

2.1.3.3.1 MetS

- **Concept and importance**

The concept of MetS defined as cluster of metabolic abnormalities associated with increased CVD was proposed and published by the WHO and other medical groups including the National Cholesterol Education Program's Adult Treatment Panel III (NCEP ATPIII) for an easy clinical diagnosis and treatment of an increased cardiometabolic risks (Cornier et al., 2008). The most accepted components of MetS include abdominal obesity, insulin resistance, raised blood pressure, atherogenic dyslipidaemia (raised triglycerides and lowered high-density lipoprotein cholesterol), glucose intolerance, and a pro-inflammatory state (Alberti et al., 2009).

Historically, the MetS concept has evolved from the concept of the "Syndrome X" introduced in 1988 by Reaven, to describe an aggregation of independent coronary heart disease risk factors in the same individual (Reaven, 1988). Concordantly, additional risk factors or biomarkers have been identified in patients with MetS (Khoshdel et al., 2012). As a consequence, many other terms including the "deadly quartet", the "cardiometabolic syndrome", and the "insulin resistance syndrome" and recently the "circulatory syndrome" have been introduced in the medical literature to describe the syndrome (Khoshdel et al., 2012). Despite ongoing debate and considerable controversies over its specific definition and related

clinical utility (Kahn et al., 2005; Woodward and Tunstall-Pedoe, 2009; Reaven, 2011b; Rachas et al., 2012; Ma and Zhu, 2013), MetS is widely recognised.

Although differing definitions might have affected its estimated prevalence, there is a continuing rise in MetS in most developed and developing countries with 20% to 30% of the adult population being characterized as having this condition (Grundy, 2008). Furthermore, to worsen the situation, apart from the increased risk for CVD and T2D, additional numerous co-morbidities have been observed in patients with MetS including non-alcoholic fatty liver disease (Uchil et al., 2009), reproductive disorders (Michalakis et al., 2013), obstructive sleep apnoea syndrome (Wolk and Somers, 2007), chronic kidney disease (Ruan and Guan, 2009), osteoarthritis (Katz et al., 2010), periodontal diseases (Pietropaoli et al., 2012), some cancers (Pothiwala et al., 2009), sleep/wake disturbances as well as other circadian alterations (Gomez-Abellan et al., 2012; Reiter et al., 2012). Moreover, patients with MetS were recently indicated to be at high risk for neurological disorders such as depression and Alzheimer's disease (Farooqui et al., 2012).

- **Pathophysiology of MetS: role of obesity and insulin resistance**

The pathological processes underlying the development of MetS in obesity are still complex and not well understood. In this regard, epidemiological evidence has suggested obesity to be the driving force behind MetS prevalence, while insulin resistance is regarded a key pathological mechanism (Eckel et al., 2010). In subjects with a first-degree relative with T2D, insulin resistance has been shown to be the best predictor of MetS as opposed to BMI and waist circumference (Utzschneider et al., 2010). However, not all MetS patients exhibit insulin resistance. As previously emphasized, this can be explained by the heterogeneity of insulin resistance and/or obesity phenotype(s), where some non-obese and obese people are found to be insulin resistant and insulin sensitive, respectively.

Insulin resistance frequently clusters with abnormal circulating lipids, glucose intolerance and high blood pressure in obese MetS patients (Steinberger et al., 2009). Since these abnormalities are strongly associated with an increase in visceral fat and/or ectopic lipid

deposition, mechanistically the hypothesis of adipose tissue dysfunction (see section 2.1.4.1) could explain the development of MetS and serves as key explanation for the actual increase in other metabolic disorders. As recently indicated, this entails the most accepted view of the MetS concept as an useful tool to identify the centrally obese patient with increased risk for CVD and T2D (Eckel et al., 2010). Although not all obese people present with MetS, obesity has been observed to be more prevalent in MetS patients (Grundy, 2008; Grundy, 2012). The pathological pathways of obesity-induced MetS are represented in figure 2.2.

Although many hypotheses have been suggested for the development of insulin resistance and MetS in obesity, the role of adipose tissue has recently attracted more attention. This is described in the following section.

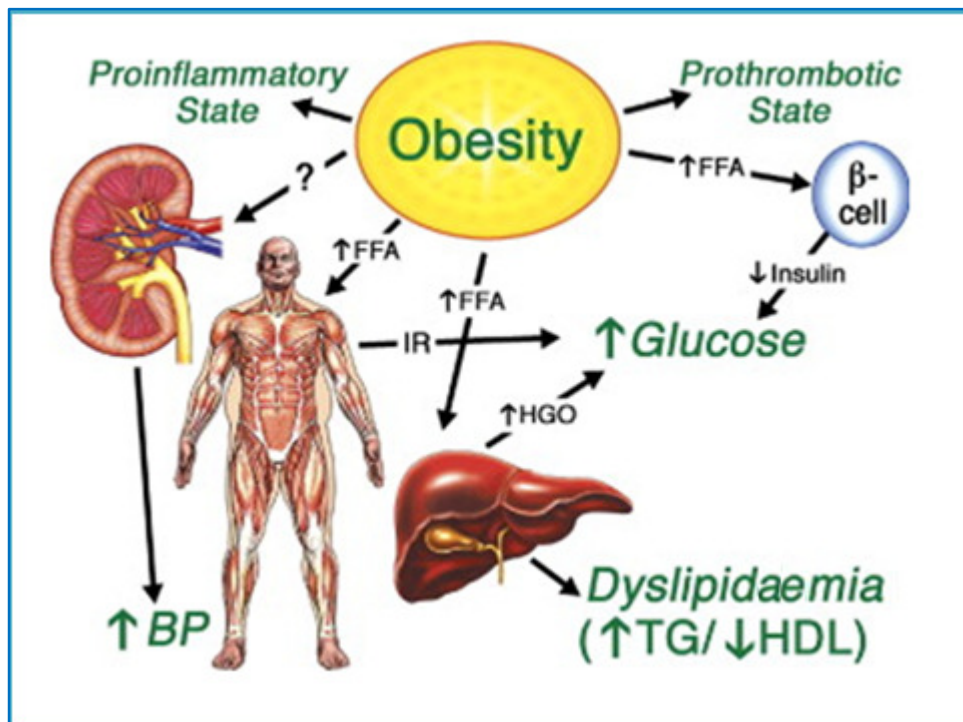


Figure 2.2 Simplified mechanisms of obesity-induced MetS. An increase in adipose tissue causes elevations of circulating free fatty acids (FFA) and altered adipose tissue derived substances which contribute to a proinflammatory state and a prothrombotic state. An increase in FFA induces insulin resistance (IR) (in muscle) which contributes to an elevation of plasma glucose. Over time, a high FFA may impair beta-cell function through “lipotoxicity”; this also contributes to elevated blood glucose levels. Elevated FFA probably contributes additionally to

an increase in hepatic glucose output (HGO) and worsening of hyperglycaemia. Furthermore, elevated circulating FFA underlies an increase in plasma triglycerides (TG), which in turn lowers high-density lipoprotein (HDL) cholesterol levels. Finally, although the mechanisms for increased blood pressure (BP) in obesity remain unknown, many investigators also believe that a proinflammatory state predisposes to CVD, as does a prothrombotic state (adapted from Grundy, 2012).

2.1.4 MECHANISM OF OBESITY- INDUCED INSULIN RESISTANCE

The mechanism underlying the development of insulin resistance in obesity and its related disorders is still not well-elucidated. Several pathophysiological mechanisms from increased fat accumulation to a low-grade inflammatory state have been proposed to explain the mechanisms of insulin resistance (Boden, 2011; Ye, 2013). They include essentially: 1) adipose tissue dysfunction (adipose tissue-derived hormone abnormalities), 2) generation of lipid metabolites, 3) lipotoxicity, 4) cellular stress (oxidative and endoplasmic reticulum stress) and 5) hypoxia [for review, see (Otani 2011; Boden, 2011; Qatanani et al 2007; Ye, 2013; Samuel and Shulman, 2012)]. Mitochondrial dysfunction, though controversial in being a consequence of insulin resistance, was also included as a cause due to its potential involvement in adipose tissue dysfunction (Ye, 2013). Similarly, some studies suggested that hyperinsulinaemia may lead to insulin resistance, especially in the presence of fatty acids (Ye, 2013).

The mechanism of insulin resistance has been recently reviewed focussing on ectopic lipid accumulation, the role of endoplasmic reticulum stress and activation of the unfolded protein response, and the contribution of systemic inflammation (Samuel and Shulman, 2012). All these factors taken together, it appears that the insulin resistant condition is a complex and multifactorial disorder resulting from an interaction of various factors including genetic predisposition, ethnicity as well as environmental factors (Beale, 2013).

The description of all of these factors involved in obesity-induced insulin resistance, though very important for a better understanding of the impact of obesity and insulin resistance on the

heart as well as the potential therapy in these conditions, do not fall within the scope of the present dissertation. In the following section, an overview on the role of adipose tissue in the development of insulin resistance is given.

2.1.4.1 Role of adipose tissue in obesity-induced insulin resistance

2.1.4.1.1 Adipose tissue: structure

Adipose tissue is traditionally known as the major storage site of surplus energy in the form of fat. In addition to adipocytes held together by a framework of collagen fibers, adipose tissue also consists of other cells including stromal-vascular cells, endothelial cells, leukocytes, macrophages and pre-adipocytes as well as nerve tissue (Fain et al., 2004; Schuster, 2009; Turer et al., 2012). Mammalian adipose tissue is essentially divided into brown and white adipose tissues while a third type “brite or beige fat” has recently been described (Mueller, 2013). In humans, fat exists mainly as white adipose tissue, brown adipose tissue being located mainly around the neck and large blood vessels of the thorax at birth and thereafter replaced by white adipose tissue (Sethi and Vidal-Puig, 2007). However, recent evidence revealed the presence of “brown adipose tissue-like” fat in adult humans (Virtanen et al., 2009; Mueller, 2013). In the present dissertation (except where it is specifically mentioned), the term adipose tissue generally refers to white adipose tissue.

2.1.4.1.2 Adipose tissue: active endocrine and immune organ

Adipose tissue plays a crucial role in the regulation of various metabolic and physiological processes (Trujillo and Scherer, 2006; Sethi and Vidal-Puig, 2007; Li et al., 2011; Blüher, 2013). Under normal conditions, adipose tissue stores surplus energy in the form of neutral triglycerides and releases it into circulation in the form of free fatty acids (FFA) during fasting or starvation. Unlike white adipose tissue, brown adipose tissue provides energy from non-oxidative phosphorylation in the form of heat, largely for cold adaptation (Saely et al., 2010). In addition to this traditional view, adipose tissue is also recognized as a highly dynamic endocrine and immune organ releasing a range of bioactive substances collectively called “adipokines” (Fain et al., 2004; Leal and Mafra, 2013). These adipose-tissue secreted

substances have both local (autocrine and/or paracrine) and systemic (endocrine) actions, playing an important role in the regulation of various metabolic and physiological processes including, amongst others, adipocyte differentiation, local and systemic inflammation, overall energy balance, glucose and lipid metabolism as well as blood pressure in normal and pathological conditions (Trujillo and Scherer, 2006; Sethi and Vidal-Puig, 2007; Li et al., 2011; Blüher, 2013)(see table 2.1).

The number of adipokines continues to grow day by day to such extent that actually there are more than 600 bioactive factors (Blüher et al., 2013), for example, leptin, adiponectin, resistin, zinc- α 2-glycoprotein (ZAG), apelin, visfatin, vaspin, retinol binding protein-4 (RBP-4), omentin, chemerin and endotrophin (Turer et al., 2012; Leal and Mafra, 2013). Adipokines that are also secreted by other tissues include tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), monocyte chemo-attractant protein 1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1), serum amyloid A (SAA), angiotensinogen (AT) and lipoprotein lipase (Vazquez-Vela et al., 2008; Leal and Mafra, 2013). In view of the role of these various adipokines, adipose tissue may indeed play an important role in the development of metabolic alterations in obesity (Vazquez-Vela et al., 2008; Blüher, 2013; Leal and Mafra, 2013; Xu et al., 2013).

Table 2.1 Adipokines in obesity

ADIPOKINE	MAIN FUNCTION	LEVELS IN OBESITY
ENERGY HOMEOSTASIS		
Leptin	Beneficial: ↑ Energy expenditure and ↓ Energy consumption	↑ (but neuronal resistance to its action)
GLUCOSE HOMEOSTASIS		
Adiponectin	Beneficial: ↑insulin sensitivity, ↑AMPK and ↓ gluconeogenesis	↓
Omentin	Beneficial: ↑ adipocytes glucose uptake	↓
Leptin	Harmful: ↑ SOCS-3 expression	↑
Resistin	Harmful: ↑ gluconeogenesis and ↓AMPK and IRS-2	↑
RBP-4	Harmful: ↑ hepatic gluconeogenesis and ↓ muscle insulin signalling	↑
TNF- α	Harmful: ↓ insulin signalling	↑
IL-6	Harmful: ↓ Insulin signalling	↑
CARDIOVASCULAR SYSTEM		
Adiponectin	Beneficial: ↓ adhesion molecules expression, ↑cardioprotection	↓
Apelin	Beneficial: ↑ cardiac contractility and ↓ blood pressure	↑
AT	Harmful: ↑ blood pressure	↑
PAI-1	Harmful: ↓ fibrinolysis	↑
TNF- α	Harmful: ↑ adhesion molecules	↑
IL-6	Harmful: ↑VLDL and CRP	↑
INFLAMMATION		
Adiponectin	Beneficial: inhibition of TNF- α , NF- κ B activation	↓
Omentin	Beneficial: NF- κ B inhibition	↓
Leptin	Harmful: cytokine-like structure	↑
TNF- α	Harmful: NF- κ B activation	↑
IL-6	Harmful: pro-inflammatory factor	↑

Only some adipokines have been included in the table. AMPK: AMP-activated protein kinase; SOCS: suppressor of cytokine signalling; RBP-4: retinol-binding protein-4; TNF- α : tumor necrosis factor-alpha; IL-6: interleukin-6; AT: angiotensinogen; PAI-1: plasminogen activator

inhibitor-1; VLDL, very low density lipoprotein; CRP, C-reactive protein; NF- κ B: nuclear factor kappa-B (adapted from Leal and Mafra, 2013).

2.1.4.1.3 Adipose tissue dysfunction-induced inflammation

Obesity is associated with low-grade inflammation (Lionetti et al., 2009; Bluher et al., 2013). In fact, in genetically and environmentally susceptible individuals, chronic overnutrition with an excessive energy intake and low energy expenditure results in adipocyte hypertrophy and visceral fat accumulation, due to saturation of the expansion capacity or inability of subcutaneous adipose tissue to expand (Lionetti et al., 2009; Bluher et al., 2013). This is accompanied by macrophage recruitment, hypoxia as well as oxidative stress, all leading to adipose tissue dysfunction (Lionetti et al., 2009, de Luca and Olefsky, 2008, Furukawa et al 2004) (see figure 2.3). As a consequence, circulating FFA increase and excessive fats accumulate inappropriately in non-adipose tissues such as liver, pancreas, kidneys and muscles as well as the heart (or around them) and negatively affect their normal metabolism and function (Christoffersen et al., 2003; Vazquez-Vela et al., 2008; Shimabukuro, 2009). This ectopic fat deposition in undesired sites leads to a lipotoxic state inducing local and systemic low-grade inflammation that gives rise to insulin resistance (Lionetti et al., 2009).

Dysfunctional and inflamed adipose tissue is accompanied by dramatic alterations in the release of adipokines collectively termed adipokines dysregulation (Leal and Mafra, 2013). For example, compared to normal subjects, circulating leptin levels are elevated in obesity (Lin et al., 2000) while surprisingly, circulating adiponectin levels are reduced in obese subjects despite being produced by adipose tissue (Arita et al., 1999). However, similar to leptin, resistin, chemerin, apelin, angiotensin, TNF- α and IL-6 are elevated in obesity while omentin, similar to adiponectin, is reduced in overweight or obese subjects compared to lean individuals (Leal and Mafra, 2013). Adipokine dysregulation may play a crucial role in the development of obesity-induced metabolic abnormalities, endothelial cell dysfunction and cardiovascular diseases (Turer et al., 2012; Blüher, 2013).

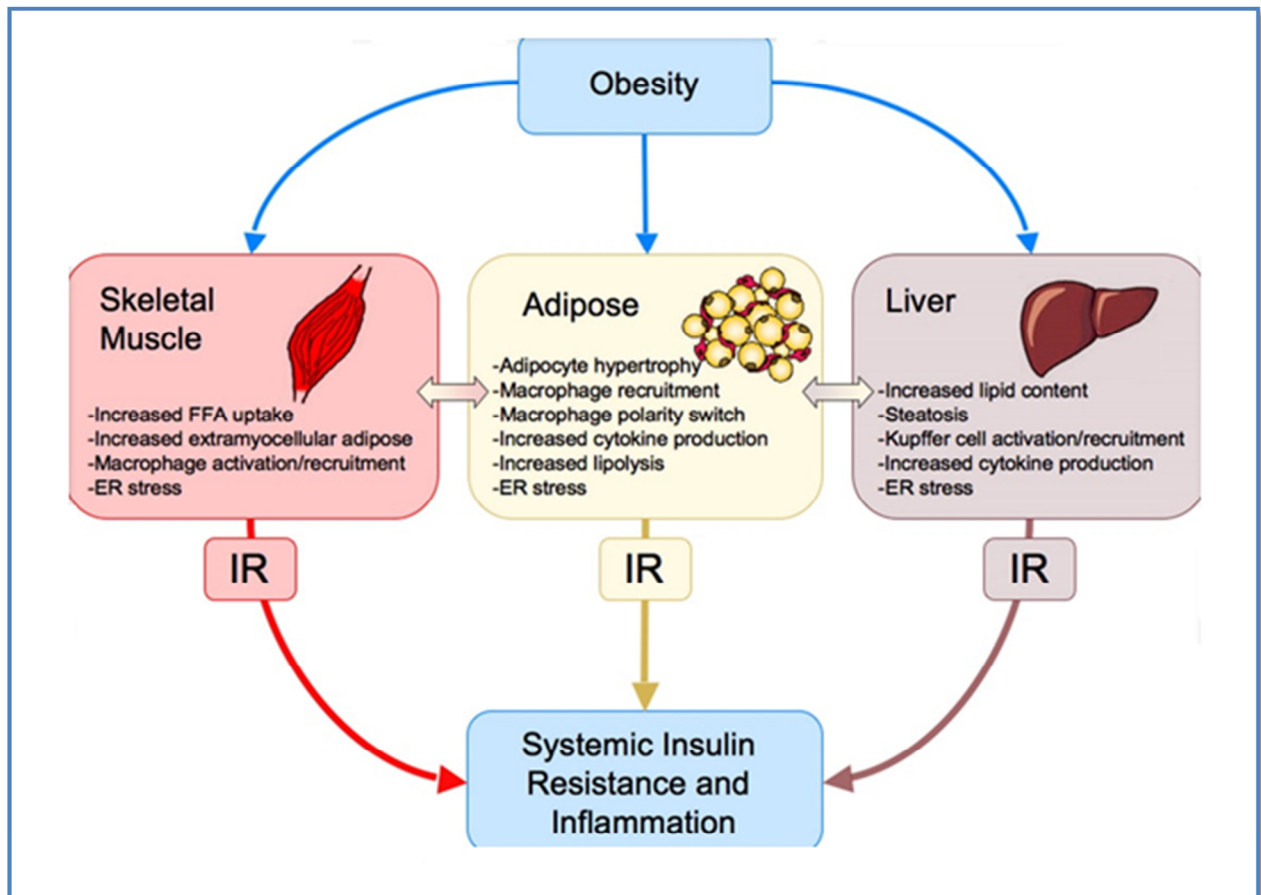


Figure 2.3 Simplified mechanisms for adipose tissue-induced inflammation and insulin resistance in obesity. Obesity-induced changes in skeletal muscle, adipose tissue and the liver result in localized inflammation and insulin resistance (IR) through autocrine and paracrine signalling. Endocrine-mediated cross-talk between insulin target tissues contributes to insulin resistance in distant tissues. Systemic inflammation and insulin resistance are the net effect of these changes. \longleftrightarrow : endocrine crosstalk, FFA: free fatty acids, ER: endoplasmic reticulum [adapted from de Luca and Olefsky (2008)].

In general, adipokines have been shown to affect central and peripheral organs implicated in metabolism (brain, liver, pancreas, muscle, adipose tissue) as well as the reproductive tract [for review, see (Trujillo and Scherer, 2006)]. Specifically, adipokines are actively implicated in various processes such as the regulation of energy homeostasis (e.g., leptin), insulin sensitivity (glucose homeostasis) and inflammation (e.g., TNF- α , IL-6, resistin, visfatin, adiponectin) as

well as cardiovascular function (e.g., adiponectin, AT, PAI-1, RPB-4) (Leal and Mafra, 2013). Some functions of adipokines are summarized in table 2.1.

In obesity, as well as in other related metabolic disorders, adipokines may induce insulin resistance either directly by affecting the insulin signalling pathway or indirectly *via* stimulation of inflammatory pathways (Piya et al., 2013). In this context, it has been shown that pro-inflammatory adipokines (e.g., TNF- α , leptin, IL-6, resistin, visfatin) may directly induce serine phosphorylation of insulin receptor substrate 1 and 2 (IRS-1,2) or indirectly disrupt the insulin signalling *via* inflammatory pathways including the c-Jun N-terminal kinases (JNK) and I-Kappa B kinase β (IKK β)/NF- κ B through a series of transcriptional events (de Luca and Olefsky, 2008) (see figure 2.4).

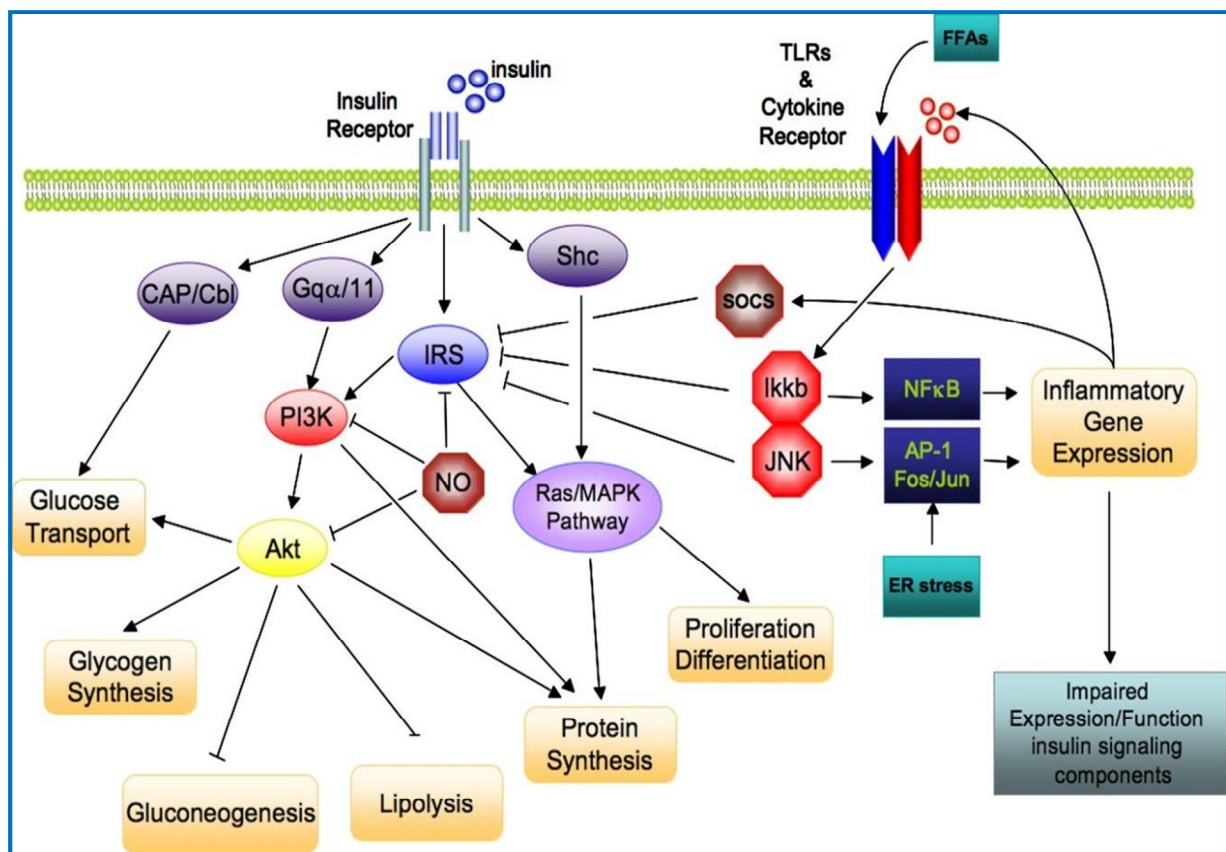


Figure 2.4 Interactions between insulin signalling and inflammatory pathways. The insulin signalling cascade branches into two main pathways. The PI3-K-PKB/Akt pathway mediates insulin action on nutrient metabolism including glucose uptake. The Ras/MAPK pathway mediates insulin's effect on gene expression, but also interacts with the PI3-K-PKB/Akt

pathway to control cell growth and differentiation. Activation of the insulin receptor leads to tyrosine phosphorylation of IRS-1 thereby initiating signal transduction. Stimulation of the NF κ B and AP-1 Fos/Jun inflammatory pathways results in the activation of the serine kinases, IKK β and JNK1, which reduce the signalling ability of IRS-1. Additional inflammation-related negative regulators of IRS proteins include the suppressors of cytokine signalling (SOCS) proteins and nitric oxide (NO), which are induced in inflammation, and promote IRS degradation. NO also reduces PI3-K/Akt activity by s-nitrosylation of PKB/Akt (de Luca and Olefsky, 2008).

2.1.4.1.4 The role of adipose dysfunction in insulin resistance

While adipose tissue volume or distribution has been traditionally considered as crucial in the development of metabolic disorders, mounting evidence also supports the role of adipose tissue dysfunction in the development of metabolic complications independent of adipose tissue volume or distribution [for review see (Patel and Abate, 2013)]. As described above, obesity-induced insulin resistance may result from complex cross-talks between the skeletal muscles; liver and adipose tissue (see figure 2.3). Therefore, a decreased capacity for adipocyte differentiation and angiogenesis along with adipocyte hypertrophy can trigger a vicious cycle of inflammation leading to subcutaneous adipose tissue dysfunction as indicated by increased visceral fat accumulation and ectopic fat deposition, all leading to systemic insulin resistance (Goossens, 2008; Lionetti et al., 2009).

In skeletal muscle, elevated FFA and pro-inflammatory cytokines (TNF- α , Il-6) inhibit insulin-mediated glucose uptake pathways which result in a reduced insulin sensitivity contributing to hyperglycaemia, which is associated with increased oxidative stress (Furukawa et al., 2004; Vincent and Taylor, 2006; Galili et al., 2007; Singh et al., 2011).

2.1.4.2 Role of oxidative stress in obesity and insulin resistance

2.1.4.2.1 An overview: concept and assessment

Except organisms specifically adapted to life under anaerobic conditions, all animals and plants on earth require oxygen for efficient production of energy. At cellular level, this occurs *via* various metabolic pathways localized in different cellular compartments and leads to the production of “free radical species” and normal molecules. Free radicals are defined as any chemical species capable of independent existence that contains one or more unpaired electrons. The concept of reactive oxygen species (ROS) refers to chemical species (free radicals or non-radicals) associated with the oxygen atom and having a higher reactivity with other molecules than molecular oxygen (O_2). ROS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen (1O_2), but there are also other species which are particularly associated with oxidation of membrane lipids (“reactive lipid species”) such as lipid peroxy radical ($\cdot ROO$), lipid alkoxy radical ($\cdot RO$) and lipid hydroperoxide (ROOH) (Toyokuni, 1999). Reactive nitrogen species (RNS) refers to the chemical species derived from nitric oxide (NO). They include nitric oxide (NO) and its derivatives such as nitrogen dioxide radical ($\cdot NO_2$) and other non-radicals like peroxynitrite ($ONOO^-$) and its protonated form (peroxynitrous acid, ONOOH), alkylperoxynitrite (ROONO), nitrosil (NO^+), nitronium cation (NO_2^+), and dinitrogen trioxide (N_2O_3). Other reactive species include carbonyl radical ($\cdot RC_3$), thiyl radical ($RS\cdot$) and hypochlorous acid (HOCl) as well as ozone (O_3). For more details on reactive species, see Pourova et al. (2010) and Valko et al. (2007).

Cells regulate the ROS/RNS levels by a common scavenging system composed of various antioxidants and the equilibrium between oxidative agents and the antioxidant defense is of crucial importance for homeostasis (Cadenas, 1997; Valko et al., 2007). Cellular antioxidant defense mechanisms include, amongst others, enzymatic antioxidants such as superoxide dismutase (SOD) [with cytosolic (Cu-ZnSOD) and mitochondrial (Mn-SOD) forms], glutathione peroxidase (GPx), catalase (CAT) and non-enzymatic antioxidants such as ascorbic acid

(Vitamin C), α -tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, α -lipoic acid, and trace minerals (zinc, selenium, and magnesium), melatonin and other antioxidants (Valko et al., 2007).

Under well controlled conditions, ROS/RNS play a role in the regulation of cell function and they may also act as signalling molecules [for review see (Valko et al., 2007; Pourova et al., 2010)]. On the other hand, if imbalanced, ROS/RNS can contribute to the progression of various human diseases. This condition is referred to as “oxidative stress” and is characterized by an elevated ROS/RNS production and insufficient antioxidant capacity causing accumulation of these species and eventual cell damage (DNA damage, lipid peroxidation and oxidized protein) (Singal et al., 1998; Hansel et al., 2004; Grattagliano et al., 2008) as well as disruption of redox balance and signalling (Jones, 2006).

Oxidative stress levels can be assessed in biological fluids or specific tissues using appropriate biomarkers including measurement of the levels of endogenous and dietary antioxidants, ROS or RNS (Tarpey and Fridovich, 2001; Gomes et al., 2005), lipid peroxidation products [thiobarbituric reactive acid substances (TBARS), malondialdehyde (MDA), lipid hydroperoxides (PEROX), F₂-isoprostanes (e.g., 8-epiPGF₂ α) and conjugated dienes (CD)], derivatives of reactive oxidative metabolites (DROM) as well as the ratio of oxidized to reduced glutathione (Eh-GSH) and cysteine (Eh-CySH) (Neuman et al., 2007).

2.1.4.2.2 Evidence for obesity- associated oxidative stress

Increased body fat accumulation and its related abnormalities have been associated with increased systemic and tissue oxidative stress (Furukawa et al., 2004; Vincent and Taylor, 2006; Galili et al., 2007; Singh et al., 2011). It is now generally accepted that obesity may be a state of chronic oxidative stress (Vincent et al., 2007; Codoner-Franch et al., 2009; Wu et al., 2009). For instance, in a meta-analysis of data published between 1975 and 2006 (Vincent et al., 2007), it was found that obesity elevates oxidative stress in young and old clinical populations as shown by elevation in lipid peroxidation or protein oxidation, associated with low systemic anti-oxidant defense. In a study evaluating the metabolic profile of 148 clinically

healthy middle-aged women, anthropometric measurements, leptin, high-sensitivity C-reactive protein (hs-CRP) and plasminogen activator inhibitor-1 (PAI-1) were higher in the overweight group compared to normal weight women (Wu et al., 2009). In addition, these parameters were positively associated with oxidative stress as measured by urinary excretion of 8-epi-PGF₂α (Wu et al., 2009). Similar observations have also been made in obese men with or without diabetes, supporting the role of increased adiposity in the development oxidative stress (D'Archivio et al., 2011). It has also been reported in animal studies that chronic consumption of high fat/high refined sugar diets results in elevation of oxidative stress (Roberts et al., 2006). Importantly, obesity is associated with coronary endothelial dysfunction (Galili et al., 2007), increased oxidative stress (Vincent et al., 1999) as well as apoptosis (Li et al., 2005) in the heart.

2.1.4.2.3 Oxidative stress in obesity and insulin resistance

The potential causes of increased oxidative stress in obesity include hyperglycaemia, increased muscle activity to carry excessive weight, increased tissue lipid levels, inadequate antioxidant defenses, chronic inflammation, endothelial ROS production and hyperleptinaemia [for reviews see (Vincent et al., 1999; Vincent and Taylor, 2006; de Ferranti and Mozaffarian, 2008)]. Briefly, at cellular and molecular levels, these free-radical generating conditions have been shown to be associated with elevated free fatty acids, cortisol, angiotensin II, deregulation of adipokines and increased inflammatory cytokines (Lastra and Manrique, 2007; Qatanani and Lazar, 2007; Monteiro and Azevedo, 2010). It has also been suggested that many stress signals that originate in adipose tissue result in increased ROS and RNS levels, alter the function of mitochondria and endoplasmic reticulum, and converge on common pathways, which in turn regulate one another (Codoñer-Franch et al., 2011).

A study performed on a mouse model of diet-induced insulin-resistance demonstrated that excessive ROS production in skeletal muscle and its subsequent mitochondrial dysfunction development resulted from hyperglycaemia and hyperlipidaemia, and normalization of glycaemia or antioxidant treatment decreased muscle ROS production and restored

mitochondrial integrity (Bonnard et al., 2008). Additionally, as described below, the coordinated regulation of the pathways affected by ROS and RNS appears to be a central feature of obesity and may result in the perpetuation of inflammation and oxidative stress (Codoñer-Franch et al., 2011).

However, the mechanisms linking obesity and its associated insulin resistance and overproduction of ROS/RNS are still to be clarified. In addition, the question of whether excessive formation of ROS/RNS is a primary cause or a downstream consequence of insulin resistance remains under debate. It was suggested that in response to energy substrate over-supply in obesity, in addition to an inflammatory state, increased oxidative stress in adipose tissue and then in remote tissues [*via* increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity], and a concomitant reduction in antioxidant enzymes are the early triggers of insulin resistance and associated cardiovascular complications (e.g., endothelial dysfunction) (Furukawa et al., 2004; Grattagliano et al., 2008; Otani, 2011).

In the pre-diabetic state, insulin resistance-induced hyperglycaemia is also known to contribute to increased ROS production exacerbating the insulin resistant state *via* glycation pathways (Grattagliano et al., 2008). In this regard, it is well established that elevated ROS levels negatively affect insulin signalling (PI3-K/Akt) *via* stress-activated kinases (e.g., p38MAPK, JNK, GSK-3 β , IKK β) (Houstis et al., 2006; Henriksen et al., 2011; Rains and Jain, 2011; Zhai et al., 2011) (see figure 2.5). However, it was shown that mice lacking one of the key enzymes involved in the elimination of physiological ROS, glutathione peroxidase 1 (GPx1), were protected from high-fat diet-induced insulin resistance confirming the causal evidence for the enhancement of insulin signalling by ROS *in vivo* (Loh et al., 2009). These controversial results clearly indicate that more studies are required to solve this problem.

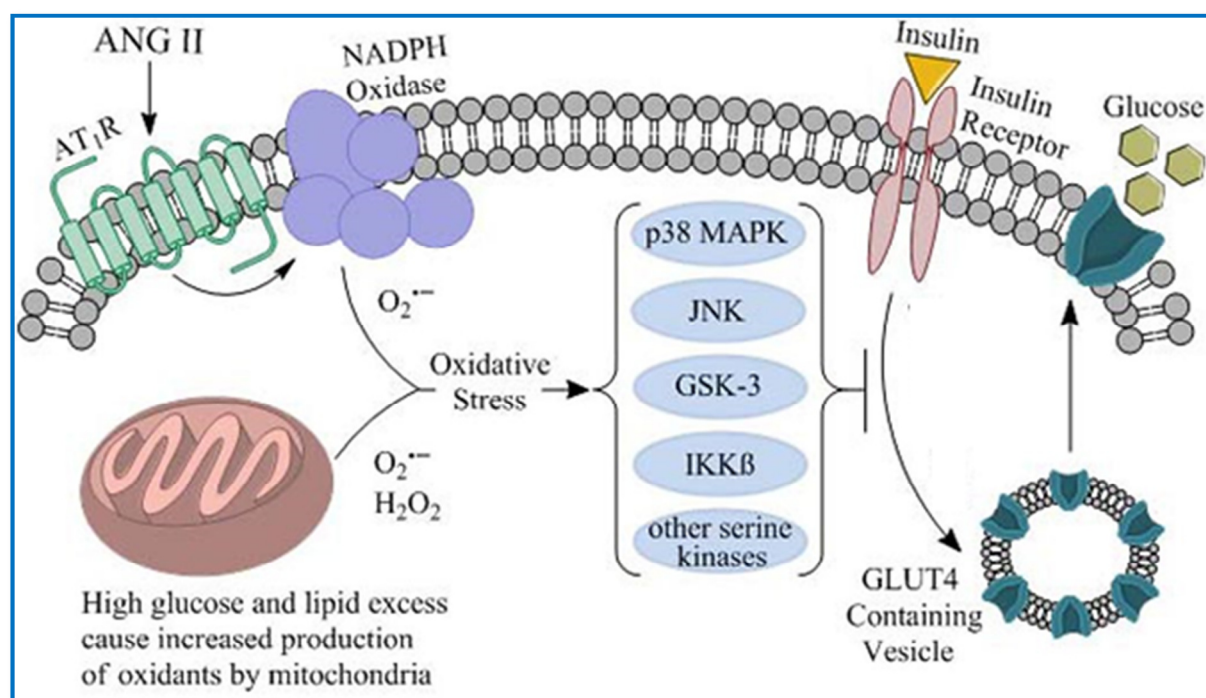


Figure 2.5 Schematic representation of the overproduction of oxidants from NADPH oxidase and mitochondrial sources in mammalian skeletal muscle, with subsequent enhanced engagement of p38 MAPK and other stress-activated kinases, including JNK, GSK-3, IKK β , and others, associated with diminished insulin signalling and reduced glucose transport activity. GLUT-4: glucose transport 4, ANG II: angiotensin II, AT₁R: angiotensin type 1 receptor [adapted from Henriksen et al. (2011)].

2.1.4.2.4 Clinical use of antioxidants in obesity and insulin resistance

In view of the above mentioned evidence for increased oxidative stress in obesity, the question arises whether antioxidant supplementation could reverse its detrimental actions. Antioxidant supplementation in obesity, the MetS, diabetes and CVD unfortunately yielded disappointing findings in large scale human studies with a number of reports even showing detrimental effects (Blot et al., 1993; Skrha et al., 1999; Yusuf et al., 2000; Kris-Etherton et al., 2004). These failures have been recently reviewed (Steinhubl, 2008; Sheikh-Ali et al., 2011). The type and dose of antioxidants used, patient cohorts included in the trials as well as the trial design itself have been pointed out as the main causes of the disappointment (Steinhubl, 2008; Sheikh-Ali et al., 2011). Additionally, a mechanistic explanation involving endoplasmic

reticulum stress as the cause of the failure has been suggested (Mooradian and Haas, 2011). However, the overall clinical relevance of conventional antioxidants in metabolic diseases is still a challenging field. Some authors even suggest that there is no clear evidence that a reduction in free radical generation will correct the imbalance causing obesity and insulin resistance (Tiganis, 2011). In fact, there are still divergences around the role of ROS and antioxidants (promoting and decreasing insulin sensitivity) (Tiganis, 2011). These findings led some authors to suggest that more caution should be exercised in the clinical use of antioxidants in insulin resistant conditions (Tiganis, 2011). Indeed, as argued in a recent review the point where ROS/RNS levels go from “good” to “bad” has not yet been delineated (Tiganis, 2011).

Nevertheless, surprisingly, small clinical studies using antioxidants did report beneficial effects (Vincent et al., 2006; Sutherland et al., 2007). A study by Vincent et al. (2009) showed that 8 weeks of antioxidants (combination of vitamin E, vitamin C, and β -carotene) supplementation to overweight young adults, moderately improved insulin sensitivity. This was associated with an increase in adiponectin levels and a reduction in plasma lipid hydroperoxide and endothelial adhesion molecules (Vincent et al., 2009). Here, additional long term and large scale studies are urgently required to validate these promising results. Furthermore, a meta-analysis of 50 studies and 534,906 individuals indicated that the non-classical antioxidant (Mediterranean diet) has exhaustively reduced abdominal obesity and other MetS components (Kastorini et al., 2011). The antioxidant melatonin, which is also present in some of the common ingredients of the traditional Mediterranean diet (Iriti et al., 2010), is reported to be more effective compared to other classical antioxidants (vitamins C and E) (Martin et al., 2000; Montilla-Lopez et al., 2002) and it does not have pro-oxidant actions (Korkmaz et al., 2009a). We have recently indicated its potential therapeutic use in obesity-related abnormalities (Nduhirabandi et al., 2012). This is described further in the section on melatonin and obesity (section 2.4).

2.1.5 SUMMARY

The association of obesity and insulin resistance is an area of much interest and enormous public health impact: more than 27068 articles are found in Pubmed with more than a half of them being published in the last 5 years (searched on 20/08/2013). The increased prevalence of obesity in insulin resistant patients is evident. However, insulin resistance *per se* has been viewed as an adaptative mechanism (to protect cells against acute lipid overload and overflow and inflammation) which becomes ineffective and leads to multiple metabolic abnormalities in the chronic state (Tsatsoulis et al., 2013). Although many of these abnormalities can also occur in non-insulin resistant subjects (Reaven, 2004), they are closely related to obesity (Cornier et al., 2008) and are strongly associated with increased risk for cardiac diseases (Reaven, 1988).

2.1.6 CARDIAC ALTERATIONS IN OBESITY AND INSULIN RESISTANCE

Convincing epidemiological and experimental studies have shown a strong association between obesity/insulin resistance and cardiac diseases, including coronary artery disease, atrial fibrillation, and congestive heart failure (Yusuf et al., 2005; Poirier et al., 2006; Banerjee and Peterson, 2007; Reaven, 2012; Shah et al., 2013; Wensley et al., 2013). Globally, the oversupply of substrates has been suggested to lead first to adaptive changes which over time become maladapted, causing contractile dysfunction of the heart (Harmancey et al., 2008).

This section focuses on the impact of obesity and insulin resistance on cardiac structure, metabolism and function in normoxia and ischaemia-reperfusion injury. Vascular endothelial dysfunction in obesity and insulin resistance is also summarized to better understand the contribution of vascular dysfunction in cardiac events. The description of hypertension as well as other related vascular abnormalities and their specific role in cardiac alterations associated with obesity and insulin resistance (Alpert, 2001), is unfortunately beyond the scope of the present dissertation.

2.1.6.1 Endothelial dysfunction in obesity and insulin resistance

2.1.6.1.1 Endothelial dysfunction: concept and assessment

The endothelium is the thin layer of cells that lines the interior of blood vessels. It acts as an endocrine organ, able to produce and release various metabolically active substances, playing a vital role in maintaining circulatory homeostasis (Davel et al., 2011). The endothelium mediates blood vessel tone, haemostasis, neutrophil recruitment, hormone trafficking, and fluid filtration (Hirase and Node, 2012). Vascular endothelial dysfunction is a pathological condition characterized by an imbalance between endothelium-derived relaxing (EDRFs) (e.g., nitric oxide (NO), prostacyclin) and contractile (EDCFs) (e.g., thromboxane A₂ or endothelin) factors (Davel et al., 2011). The definition of endothelial dysfunction has also been extended to include imbalance between growth promoting and inhibiting factors, pro-atherogenic and anti-atherogenic factors, and pro-coagulant and anti-coagulant factors (Caballero, 2003; Muniyappa and Sowers, 2013). Endothelial dysfunction can result from and/or contribute to a variety of disease states, including diabetes mellitus, coronary artery disease, hypertension, and hypercholesterolaemia (Davel et al., 2011). To date, endothelial dysfunction is the most clearly defined and well understood early precursor of atherosclerosis [for review see (Mudau et al., 2012)]. Various methods to determine endothelial function and dysfunction have been used in epidemiological and experimental studies. As recently reviewed, these include use of biochemical markers, circulating endothelial cells and endothelial microparticles, molecular genetic tests and invasive and non-invasive tools with and without pharmacological and physiological stimuli (e.g., peripheral arterial tonometry, flow-mediated dilation, forearm plethysmography, finger-pulse plethysmography, pulse curve analysis and quantitative coronary angiography) (Schnabel et al., 2011; Mudau et al., 2012; Bruyndonckx et al., 2013).

2.1.6.1.2 Endothelial dysfunction in obesity and insulin resistance

Obesity is associated with impaired systemic and coronary endothelial function (Galili et al., 2007). In this regard, the factors that could adversely affect endothelium function include changes in blood pressure (BP), glucose levels, lipid metabolism, systemic inflammation and

oxidative stress, sympathetic hyperactivity, adipokines and physical (in)activity (Kobayasi et al., 2010; Davel et al., 2011). Several studies have shown that endothelial dysfunction results from reduced NO bioavailability, increased oxidative stress, elevated expression of pro-inflammatory and pro-thrombotic factors, and leads to abnormal vasoreactivity (Muniyappa and Sowers, 2013). The role of endothelial dysfunction in obesity has been studied in several experimental studies. For example, Kobayasi et al. (2010) using obese mice fed a high-fat diet have shown that the expression of endothelial nitric oxide synthase (eNOS) and Cu/Zn-SOD was significantly decreased in the aortas from obese mice. Although total p65 NF- κ B subunit expression was not affected by obesity, the expression of the NF- κ B inhibitor I κ B- α was reduced with a concomitant impaired endothelium-dependent relaxation in aortas from obese mice (Kobayasi et al., 2010). In addition, the expression of TNF- α was increased in aortas from obese mice compared to the control group (Kobayasi et al., 2010). In the same study, the high-fat diet feeding was associated with an increase in body weight, fat mass, glucose blood levels and blood pressure, and increased lipid accumulation into macrovesicular droplets in hepatocytes, a feature of hepatic steatosis (Kobayasi et al., 2010). The obesity-induced impaired endothelial function was also demonstrated in other animal models of obesity (Villalba et al., 2009; Costa et al., 2011) as well as in humans (Jonk et al., 2011; Bagi et al., 2012). A study using venous occlusive plethysmography, reported a significant correlation between impaired endothelium-dependent vasodilatation and insulin resistance in subjects with uncomplicated obesity (Pasimeni et al., 2006).

Endothelial dysfunction may also contribute to insulin resistance (Caballero, 2003). In this regard, impaired endothelium function has been shown to alter the transcapillary passage of insulin to target tissues and reduce the insulin availability to metabolically active tissues (such as skeletal muscle, liver, heart) and subsequent impairment of insulin-stimulated glucose and lipid metabolism (Cersosimo and DeFronzo, 2006). For example, in obese pigs, coronary endothelium-dependent vasorelaxation was reduced in association with increased myocardial microvascular permeability compared to normal pigs (Galili et al., 2007). Interestingly, in this obesity model, endothelial dysfunction was associated with increased vascular oxidative stress

in association with increased levels of leptin and preceded the development of insulin resistance and systemic oxidative stress (Galili et al., 2007). Importantly, this study has demonstrated the potential contribution of endothelial dysfunction to impaired insulin action as previously suggested (Cersosimo and DeFronzo, 2006). In a rat model of diet-induced obesity, defective endothelium function was also identified in the absence of insulin resistance (Naderali et al., 2001).

However, the causal relationship between insulin resistance and endothelial dysfunction is more complex (see figure 2.6). Both disorders share causal factors such as dyslipidaemia, glucotoxicity, lipotoxicity, inflammation and impaired PI3-K-dependent insulin signalling pathways, leading to reciprocal relationships between insulin resistance and endothelial dysfunction (Muniyappa and Quon, 2007) (see figure 2.6). It has been suggested that during insulin-resistant conditions, pathway-specific impairment in PI3-K-dependent signalling may cause an imbalance between NO production (PI3-K/Akt-eNOS pathway) and secretion of endothelin-1 (MAPK pathway), leading to endothelial dysfunction (Muniyappa and Sowers, 2013). However, a preserved coronary endothelial function has been reported in insulin resistant animals (Sánchez et al., 2010; Contreras et al., 2011). This phenomenon could be explained by an association with other early adaptive mechanisms which, over time, may become maladaptive (Sánchez et al., 2010).

2.1.6.1.3 Endothelial dysfunction and adipose tissue

As presented in figure 2.6, visceral adiposity may also affect endothelial function (Bagi et al., 2012). For example, in a randomized controlled longitudinal study of normal-weight healthy young subjects, modest fat gain resulted in impaired endothelial function, even in the absence of changes in blood pressure; interestingly, this endothelial dysfunction recovered after weight loss (Romero-Corral et al., 2010). The role of visceral fat mass in endothelial dysfunction was also confirmed in an uncontrolled, cross-sectional study of severely obese subjects where the severity of endothelial dysfunction was shown to correlate with the degree of visceral adiposity independently of traditional risk factors or the degree of liver steatosis and plasma adiponectin

(Sturm et al., 2009). Although the mechanisms linking visceral adiposity to endothelial dysfunction are complex and still not yet clear, adipose tissue dysfunction and increased circulating fatty acids as well as deregulation of adipokines secretion (endocrine action) may play a crucial role in development of endothelial dysfunction in obesity (Caballero, 2003; Cersosimo and DeFronzo, 2006; Rega-Kaun et al., 2013).

In addition to visceral adiposity, increased pericardial adipose tissue in obese individuals may also contribute to endothelial dysfunction (Bagi et al., 2012; Gaborit et al., 2012; Payne et al., 2012). In this regard, a study by Gaborit et al. (2012) on 30 healthy volunteers with normal left ventricular function, has found a significant negative correlation between epicardial fat volume and myocardial blood flow which was also associated with reduced adiponectin levels, but not with obesity (BMI, waist circumference), C-reactive protein, lipid or glycemic parameters. Interestingly, while adiponectin levels and epicardial fat volume remained independently associated with myocardial blood flow, a high epicardial fat content was associated with a lower coronary microvascular response (Gaborit et al., 2012), suggesting that epicardial fat could influence endothelial function *via* its secreted adipokines (Gollasch, 2012; Payne et al., 2012). Recent evidence has shown that epicardial fat volume is an independent predictor of impaired diastolic function in apparently healthy overweight patients even after accounting for associated co-morbidities such as MetS, hypertension, and subclinical cardiac artery disease (Cavalcante et al., 2012).

Since it is important to maintain a balance between coronary blood flow and myocardial metabolism for normal cardiac function, an impairment in coronary blood flow due to endothelial dysfunction and its associated microvascular dysfunction, as demonstrated above, has been shown to play a crucial pathophysiological role in cardiac dysfunction in obesity and insulin resistance (Poirier et al., 2006). Importantly, a high prevalence of co-morbidities such as overweight/obesity, diabetes and chronic obstructive pulmonary disease drives myocardial dysfunction and remodelling *via* coronary microvascular endothelial inflammation, causing heart failure with preserved ejection fraction (Paulus and Tschöpe, 2013). Myocardial

remodelling in obesity and insulin resistance is described in the next section. It involves changes in myocardial metabolism, structure and function (Abel et al., 2008).

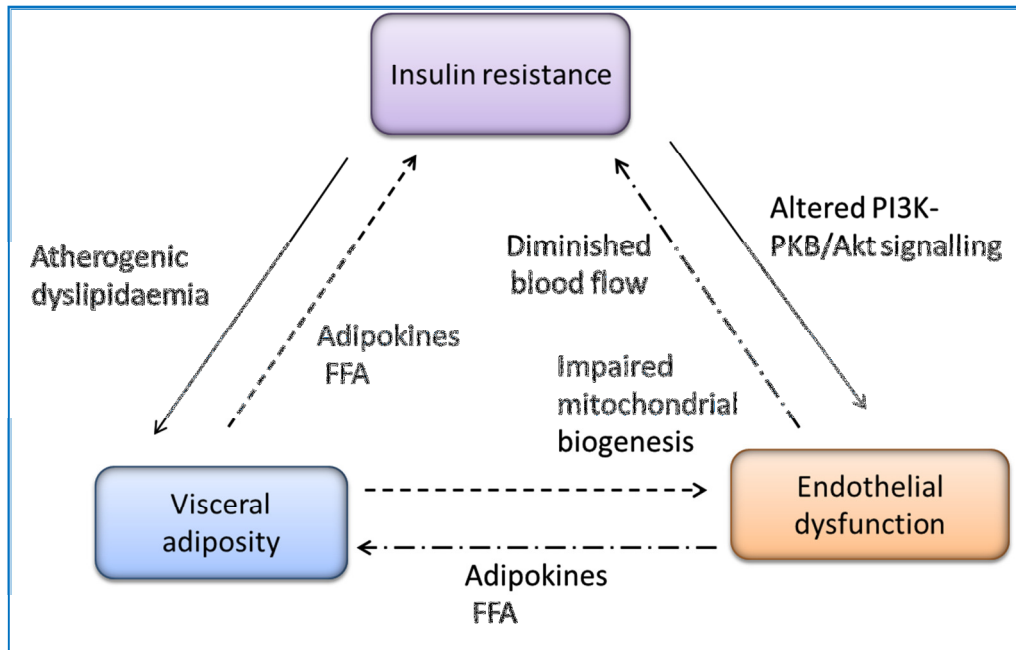


Figure 2.6 Relationships between insulin resistance, visceral adiposity and endothelial dysfunction. Insulin resistance can cause endothelial dysfunction through altered PI3K-PKB/Akt signalling. Endothelial dysfunction, in turn, can cause insulin resistance owing to diminished blood flow and capillary recruitment, leading to decreased substrate and insulin delivery. Endothelial dysfunction can lead to insulin resistance, as well as visceral adiposity by impaired mitochondrial biogenesis. Visceral adipose tissue can secrete adipokines and FFAs, which can cause endothelial dysfunction and peripheral insulin resistance. Finally, insulin resistance can lead to atherogenic dyslipidaemia, which can contribute to visceral obesity (Huang, 2009).

2.1.6.2 Myocardial metabolism in obesity and insulin resistance

Obesity and insulin resistance are associated with changes in myocardial metabolism (Abel et al., 2008; Harmancey et al., 2008). These changes have been observed in humans and in several animal models of diet-induced obesity and insulin resistance (Abel et al., 2008; Chess and Stanley, 2008). A reduced cardiac efficiency and altered substrate metabolism have been reported to precede the onset of hyperglycaemia and contractile dysfunction in obesity, insulin

resistance and diabetes (Buchanan et al., 2005). In this regard, the development of cardiac insulin resistance and decreased mitochondrial oxidative metabolism have been found to be the early metabolic changes in the development of cardiac hypertrophy, which create an energy deficit that may contribute to the progression from hypertrophy to heart failure (Zhang et al., 2013). Apart from an altered fuel (glucose and lipid) availability, various other mechanisms such as adipokine signalling and altered insulin signalling may also influence myocardial metabolism in obesity (Lopaschuk et al., 2007; Harmancey et al., 2008).

2.1.6.2.1 Myocardial metabolism under normoxic conditions

A normal healthy heart is able to display great flexibility in its choice of substrates, depending on their availability in the circulation, myocardial workload and relative oxygen supply (Taegtmeyer et al., 2004). Normally, a switch in cardiac fuel preference from glucose to fatty acids occurs just after birth, when oxygen availability and dietary fat content increase, rendering fatty acids the preferable substrate for the heart (Girard et al., 1992; Rider et al., 2013). This is related to substrate selection *via* the Randle cycle (inhibition of glucose uptake and catabolism by FFA oxidation) (Randle et al., 1963; Bertrand et al., 2008). Substrate uptake and metabolism in the normal heart is a highly regulated process. Several recent reviews have been published (Stanley et al., 2005; Coort et al., 2007; Lopaschuk et al., 2010; Rider et al., 2013) and only a short summary on FFA and glucose metabolism will be given below.

Under physiological conditions, cardiomyocytes oxidize predominantly long chain fatty acids (LCFA) (60-70%) while glucose (20%) and lactate (10%) are metabolized to lesser extent (Coort et al., 2007). Smaller contributions from other oxidizable substrates such as ketones and amino acids have also been indicated (Stanley et al., 2005). LCFA enter the sarcolemma by passive diffusion and a protein-mediated transport system (fatty acid transport protein (FATP)) or a plasma membrane fatty acid binding protein (FABPpm) (Coort et al., 2007). Once in the cytosol, the non-esterified FFAs bind to FABP and are then activated by esterification to fatty acyl-CoA. Fatty acid β -oxidation occurs primarily in the mitochondria and to a small extent

in peroxisomes (Stanley et al., 2005). Approximately 80% of the cytosolic long chain fatty acyl-CoA are transported across the inner mitochondrial membrane for further β -oxidation and eventual ATP generation, while the remaining (20%) is esterified into TG to constitute the intracardiac TG pool (Chess and Stanley, 2008).

Besides FFA β -oxidation, cardiomyocytes oxidize pyruvate derived in approximately equal amounts from glycolysis and lactate oxidation. Pyruvate can be converted to lactate in the cytosol or enter the mitochondria for either decarboxylation to acetyl-CoA by pyruvate dehydrogenase (PDH) or carboxylation to oxaloacetate or malate (in the citric acid cycle) (Sundqvist et al., 1989; Stanley et al., 2005). Glucose entry into the cardiomyocytes is passively regulated by the transmembrane glucose gradient and glucose transporters in the sarcolemma (mainly GLUT-4, and to a lesser extent GLUT-1) (Stanley et al., 2005). In response to insulin stimulation, glucose transporters translocate from intracellular vesicles to the sarcolemmal membrane which increases the membrane capacitance for glucose transport and the rate of glucose uptake. GLUT-4 translocation can also be stimulated by the activation of AMP-activated protein kinase (AMPK) following a cellular stress such as increased work demand or injury (Sambandam and Lopaschuk, 2003; Russell et al., 2004). It is well established that in the well-perfused heart, approximately 60–90% of the acetyl-CoA is derived from β -oxidation of fatty acids, with 10–40% produced by the oxidation of pyruvate (Stanley et al., 2005). However, a shift towards glucose, lactate and endogenous glycogen utilization assumes greater importance in conditions such as ischaemia, increased workload and pressure overload (Sambandam et al., 2002; Stanley and Chandler, 2002; Grossman et al., 2013).

2.1.6.2.2 Myocardial metabolism: contribution of obesity and insulin resistant conditions

Obesity results in elevated blood FFA levels and increased supply of FFA to the heart. This leads to an increase in FFA oxidation and a decrease in glucose utilization. With time,

continuous excessive FFA uptake overwhelms the FFA accumulation capacity of the heart causing contractile dysfunction (Young et al., 2002).

The role of elevated FFA in the development of myocardial insulin resistance and cardiac dysfunction involves various pathways related to excessive FFAs β -oxidation and excessive lipid accumulation (Lopaschuk et al., 2010).

Increased FFA β -oxidation is associated with increased oxygen consumption in animals and humans (Peterson et al., 2004; Banerjee and Peterson, 2007). This, initially adaptive, can over time cause an increased production of ROS and contribute to development of myocardial oxidative stress, ER stress, cell death and/or Ca^{2+} handling abnormalities (Mittendorfer and Peterson, 2008), which in turn may lead to heart failure (Chess & Stanley, 2008). Recently, in obese insulin resistant rats, myocardial dysfunction caused by obesity has been shown to be associated with reduced gene expression of proteins related to Ca^{2+} transport, impaired L-type Ca^{2+} channel activity without affecting SERCA2a expression and function as well as L-type Ca^{2+} protein levels (Lima-Leopoldo et al., 2008). Although the mechanism responsible for the alteration in genes responsible for myocardial Ca^{2+} handling is still unknown, it could reflect a compensatory mechanism to the impaired myocardial Ca^{2+} handling observed in obesity (Mittendorfer and Peterson, 2008). However, in another model of diet-induced obesity, a significant reduction of myocardial SERCA2a expression has been observed (Huisamen et al., 2012).

In addition to increased FFA β -oxidation, a continuous lipid supply may lead to excessive lipid storage associated with eventual intramyocellular FFA accumulation (steatosis) and lipotoxicity (Granér et al., 2013; Wang et al., 2013). Here, the excess FFA in the cardiomyocytes may contribute to lipotoxicity *via* increased diacylglycerol and ceramide pathways (Mittendorfer and Peterson, 2008; Zhang et al., 2011). This has been associated with mitochondrial dysfunction and impaired insulin-stimulated glucose oxidation (Zhang et al., 2011) and may lead to cell death and eventual cardiac dysfunction (Chess and Stanley, 2008; Mittendorfer and Peterson, 2008) (see figure 2.7).

Apart from the contribution of an excess supply of FFA, as above indicated, adipokine signalling may also affect the myocardial metabolism in obesity. For example, in patients with acute myocardial infarction as well as in animal models, obesity has been associated with a reduction in serum adiponectin levels (Piestrzeniewicz et al., 2007; Lee et al., 2010), which were associated with an atherogenic lipid profile and higher levels of inflammatory markers (Piestrzeniewicz et al., 2007). Additional important mechanisms which may directly contribute to the pathophysiology of cardiac insulin resistance and its complications include changes in AMP-activated protein kinase (AMPK) signalling, oxidative stress, inflammation, advanced glycation end products (AGE), endoplasmic reticulum stress and autophagy [for review, see (Abel et al., 2012; Mellor et al., 2013)].

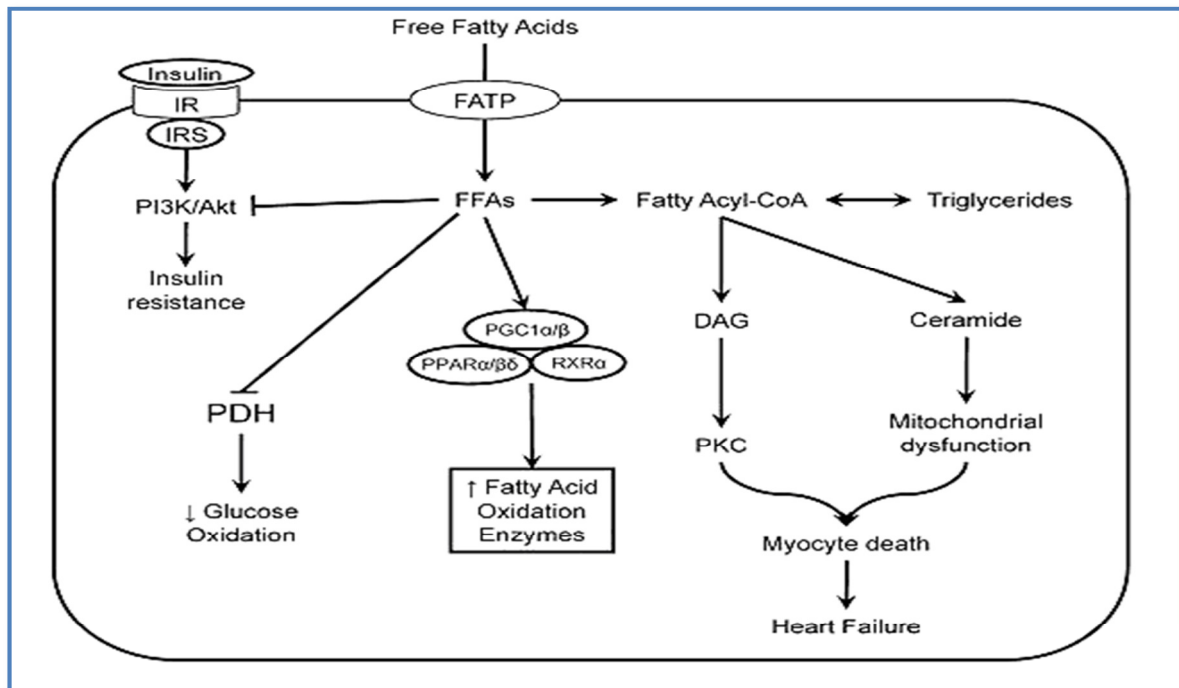


Figure 2.7 Myocardial metabolism under conditions of elevated fatty acids (details in the text).

FATP: fatty acid transport proteins; FFAs: free fatty acids; DAG: diacylglycerol; PKC: protein kinase C; PPAR: Peroxisome proliferator-activated receptor; PDH: pyruvate dehydrogenase; RXR: retinoid X receptor; PGC: peroxisome proliferator-activated receptor-gamma coactivator. IR: insulin receptor; IRS: insulin receptor substrate [adapted from Chess and Stanley (2008)].

2.1.6.2.3 Myocardial metabolism in obesity: contribution of impaired insulin action

As previously indicated, under physiological circumstances, insulin regulates substrate utilization in multiple tissues including the heart, skeletal muscle, liver and adipose tissue (Benito, 2011; Rask-Madsen and Kahn, 2012). In an *in vivo* setting, insulin by its antilipolytic effect reduces circulating FFA (Mazumder et al., 2004; Grossman et al., 2013), hence regulating FFA availability to the heart. In the perfused heart, insulin stimulates glucose uptake and oxidation as well as LCFA uptake but not LCFA oxidation, which may lead to an increased intracardiac TG pool (Dyck et al., 2001). Hence, due to the high and tightly regulated energy demands in the heart, changes in systemic insulin sensitivity or changes in myocardial insulin action can significantly impact on cardiac metabolism and function (Abel et al., 2012).

In insulin resistant states, because of the combined effects of elevated circulating FFA and insulin resistance, the myocardium uses fatty acids most exclusively to support its energy supply (Lopaschuk et al., 2010). In this regard, several studies have consistently reported a strong association between excess FFA accumulation in the myocardium and impaired insulin signalling and reduced glucose uptake (Lopaschuk et al., 2007; Martins et al., 2008). During the development of obesity and T2D, alterations in cardiac lipid homeostasis was found to precede and influence alterations in glucose homeostasis (Coort et al., 2007). For example, continuous exposure of cardiac myocytes to FFA was shown to reduce both insulin- and stress-stimulated glucose transport (Asrih et al., 2012).

Impairment of insulin-stimulated glucose transport has been considered as the most consistent change that develops early in the hearts in animal models in the evolution of insulin resistance (Wright et al., 2009). As recently demonstrated by Cook et al. (2010), this change manifests before or without any defect in insulin-stimulated increase in PI3-K and PKB/Akt signalling and occurs as a consequence of both reduced GLUT-4 protein and impaired GLUT-4 translocation (Cook et al., 2010; Abel et al., 2012). In a rat model of hyperphagia-induced obesity, early myocardial changes were characterized by elevated basal phosphorylation of PKB/Akt and severe downregulation of IRS-1 and SERCA2a expression (Huisamen et al.,

2012). These latter changes were also reported in rats fed a high fat diet (Ouwens et al., 2005). However, as expected over time, these rats exhibited impaired myocardial insulin signalling by reducing phosphorylation of PKB/Akt and associated PI3-K activity with a concomitant hypophosphorylation of phospholamban (Ouwens et al., 2005) and a reduced myocardial acetyl-CoA carboxylase (ACC) phosphorylation (Ouwens et al., 2007).

Convincing evidence in animal models of obesity and insulin resistance, including chronic high-fat feeding, db/db mice, and ob/ob mice, indicates that increased myocardial fatty acid oxidation rates are associated with depressed rates of glucose oxidation (Buchanan et al., 2005; Lopaschuk et al., 2007; Ussher and Lopaschuk, 2013). For example, high fat-induced obese mice had markedly reduced insulin-stimulated glucose oxidation compared with low fat-fed WT mice (Ussher et al., 2009). Concordantly, rats with heart failure fed a high-saturated fat diet exhibited peripheral and myocardial insulin resistance, decreased myocardial glucose utilization rates, alterations in cardiac insulin signalling (Christopher et al., 2010) and poor cardiac efficiency (Buchanan et al., 2005; Lopaschuk et al., 2007). As expected, the clinical relevance of these findings was confirmed in obese and insulin-resistant women, where elevated myocardial fatty acid oxidation rates were accompanied by a significant decrease in cardiac efficiency (Peterson et al., 2004).

However, surprisingly, despite impaired myocardial insulin responsiveness and profound metabolic changes associated with reduced rates of glucose uptake, hearts isolated from rats fed a high-sucrose diet exhibited increased rates of both glucose and oleate oxidation (Harmancey et al., 2012). This was associated with improved cardiac efficiency, indicating the initial role of insulin resistance as an adaptive mechanism to protect against fuel toxicity in the stressed heart of these rats (Harmancey et al., 2012). Recently an *in vivo* study in young and middle aged mice with a high-fat diet-induced hyperinsulinaemia reported an increased cardiac glucose uptake and mitochondrial function despite peripheral insulin resistance (Gupte et al., 2013). These findings were associated with elevation of myocardial fatty acid oxidation rates which can be considered as adaptive mechanism against the accumulation of lipid

intermediates in the heart (Gupte et al., 2013). In addition to this controversial metabolic effect (probably due to difference in animal models and experimental design), the role of myocardial insulin resistance on contractile function is also not consistent with studies reporting a depressed (Ouwens et al., 2005; Ouwens et al., 2007; Mellor et al., 2013) or preserved contractile function (Christopher et al., 2010). These controversial observations have also been reflected in the setting of myocardial ischaemia-reperfusion damage. This is discussed further below (see section 2.1.6.5).

2.1.6.3 Myocardial morphology in obesity and insulin resistance

2.1.6.3.1 Concepts

The morphology of the heart in obesity and insulin resistance has been intensively investigated (Alpert, 2001; Abel et al., 2008; Lavie et al., 2009; Aurigemma et al., 2013). Obesity, particularly morbid obesity, and insulin resistance have been strongly associated with cardiac remodelling including ventricular dilatation and hypertrophy which may result in heart failure (Luaces et al., 2012; Aurigemma et al., 2013). Myocardial remodelling is classically defined as genomic expression, molecular, cellular and interstitial changes that manifest clinically as changes in size (dimensions and mass), shape and function of the myocardium after cardiac injury, pressure or volume overload (Cohn et al., 2000). Cardiac hypertrophy is referred to as an increase in the size of the entire heart, but more commonly, alterations occur in specific cardiac chambers relative to body size (Rider et al., 2011). Two types of hypertrophy, namely concentric and eccentric hypertrophy, have been observed in obesity and insulin resistance (Sundström et al., 2000; Velagaleti et al., 2010; Ashrafian et al., 2011). Concentric hypertrophy indicates an increase in wall thickness relative to chamber size while eccentric hypertrophy is characterized by chamber enlargement that is more prominent than the increase in wall thickness (Abel et al., 2008).

The concept of “obesity cardiomyopathy” has been used to indicate cardiac functional and structural changes associated with obesity or adiposity, independently of other known cardiac disease such as hypertension and coronary artery disease (Alpert, 2001; Wong and Marwick,

2007). Although the existence of “obesity cardiomyopathy” has been questioned (Owan and Litwin, 2007), it has undoubtedly been proved in human and animal studies (Wong and Marwick, 2007). In diabetic conditions, this concept refers to the structural and functional abnormalities of the myocardium in diabetic patients without coronary artery disease or hypertension (Boudina and Abel, 2010; Miki et al., 2013). It is, however, difficult to separately assess the contribution of diabetic or obesity-related cardiomyopathy to the overall cardiac or ventricular dysfunction (Abel et al., 2008). Therefore, it appears that there is no single pathophysiologic mechanism which can account for the host of clinical expressions of obesity-related cardiomyopathies (Wong and Marwick, 2007). Metabolic as well as hemodynamic factors have been implicated in the development of obesity-related cardiac alterations (Ashrafian et al., 2011).

2.1.6.3.2 Evidence in humans

The adverse effect of obesity on cardiac morphology has been widely investigated in humans. Early post-mortem studies have focussed on the excessive epicardial fat accompanying the hypertrophied heart in severely obese subjects (Alpert and Chan, 2012). Echocardiographic and postmortem studies between 1980 and 1990 [for review see Alpert (2001)] have collectively reported that morbid obese patients had LV enlargement, increased LV wall thickness, increased LV radius to thickness ratio, increased LV mass, left atrial enlargement, right ventricular enlargement and hypertrophy (Alpert and Chan, 2012). From these studies it was concluded that an eccentric LV hypertrophy is commonly present in patients with severe obesity (Poirier et al, 2006). A recent study by Luaces et al. (2012) in morbid obese patients (average age of 40 years, 82.9% being female), has shown that the morbid obesity phenotype was not homogeneous: 29.3% of patients had normal LV geometric patterns, the remaining 70.7% had some form of ventricular remodelling, the most frequent being eccentric hypertrophy (which was present in 34.1% of patients) and concentric hypertrophy (in 19.5% of cases). These variations could be linked to the presence of other associated comorbidities

(87.8% of patients). Importantly, only patients with an eccentric hypertrophy pattern were found to have a lower LV ejection fraction (Luaces et al., 2012).

In contrast to the traditional concept that the hypertrophy associated with obesity is eccentric, most other recent studies (echocardiographic and cardiac magnetic resonance) reported a greater prevalence of concentric patterns (Woodiwiss et al., 2008; Turkbey et al., 2010; Rider et al., 2011; Shah et al., 2013). In a cohort of healthy female subjects with a wide BMI range, ventricular hypertrophy occurred without associated cavity dilatation in overweight individuals, while in obesity both cavity dilatation and ventricular hypertrophy were observed (Rider et al., 2011).

2.1.6.3.3 Animal evidence

Cardiac remodelling has also been demonstrated in animal models of obesity [for review, see (Abel et al., 2008)]. In spontaneously hypertensive rats, dietary-induced obesity has been shown to accelerate the progression from concentric cardiac hypertrophy to pump dysfunction, independent of blood pressure changes or alterations in glycaemia (Majane et al. 2009). Compared to controls, hearts isolated from diet-induced obese rats were hypertrophied as indicated by increased heart weight to body weight ratios (Du Toit et al., 2005; Nduhirabandi et al. 2011) and LV weight to tibial length ratios (du Toit et al., 2008). An echocardiographic study in obese dogs has reported asymmetric concentric hypertrophy without chamber dilatation (Mehlman et al., 2013).

2.1.6.3.4 Mechanism of myocardial hypertrophy/remodelling

The traditionally accepted mechanism underlying ventricular adaptation to obesity suggests that cavity dilatation in response to increased blood volume and elevated filling pressure results in ventricular hypertrophy as a compensatory mechanism (Rider et al., 2011, Alpert 2012). This has been supported by early studies in 1980s to 1990s and was associated with eccentric hypertrophy (Alpert 2012). It appears that obesity may influence cardiac structure both directly and *via* its closely associated comorbidities such as hypertension, diabetes, sleep-disordered breathing, kidney dysfunction, atherosclerosis, and endothelial dysfunction

(Abel et al., 2008; Aurigemma et al., 2013). Additional mechanisms other than haemodynamic adaptations may also play a crucial role in cardiac remodelling in obesity and insulin resistance (Abel et al., 2008; Ashrafian et al., 2011). These are represented in the figure 2.8 and include metabolic factors (fuel availability) as well as humoral factors (such as oxidative stress, adipokines and inflammation) (Abel et al., 2008). In this regard, visceral obesity was shown to correlate with LV mean wall thickness, concentric LV hypertrophy and remodelling, but not with LV end-diastolic diameter or eccentric LV hypertrophy (Woodiwiss et al., 2008). From these findings it was concluded that obesity may promote left ventricular concentric rather than eccentric geometric remodelling and hypertrophy independently of blood pressure (Woodiwiss et al., 2008). This effect may be mediated through leptin resistance and obesity-induced hypertrophy (Majane et al., 2009; Rider et al., 2012). Hyperglycaemia as well as insulin resistance may promote LV concentric remodelling (Sundström et al., 2000; Velagaleti et al., 2010).

2.1.6.4 Myocardial function in obesity and insulin resistance

Obesity and insulin resistance affect cardiac function in many ways (Gray and Kim, 2011; Abel et al., 2012; Narumi et al., 2012). Here, only studies under normoxic conditions are considered. Globally, studies have shown that in obese (BMI ≥ 30) subjects the degree of impairment of the heart function was parallel to the degree of obesity (de Divitiis et al., 1981; Sokmen et al., 2013). In addition, not only morbidly obese subjects develop obesity-related cardiac dysfunction (Luaces et al., 2012), but also overweight people (BMI > 25) were found to have impaired cardiac function (Marinou et al., 2010), independent of their age.

In humans, most of the studies performed on obesity and insulin resistant conditions have reported an association of LV hypertrophy with LV diastolic dysfunction, and occasionally LV systolic dysfunction in long standing obesity (Aurigemma et al., 2013). In addition, subclinical myocardial alterations were also observed in obese adults especially hypertension and hypertrophy, reflecting impaired regional LV relaxation, segmental atrial, and systolic dysfunction (Santos et al., 2011). In an elderly randomly-derived community cohort study,

overweight and obesity were both associated with an impairment of LV diastolic function independently of LV mass and obesity-associated risk factors such as hypertension, diabetes (Russo et al., 2011), indicating an additive effect of ageing.

Subclinical LV diastolic dysfunction was present in all grades of isolated obesity (i.e., without other pathological conditions such as hypertension) and correlated with increased systolic function in the early stages of obesity (Pascual et al., 2003). A strong positive correlation between insulin resistance (HOMA-IR) and indices of LV function in all grades of obesity at a preclinical stage was also reported (Zeybek et al., 2010; Kibar et al., 2013), confirming the implication of metabolic factors in myocardial dysfunction as previously indicated. However, similar to the metabolic and morphological changes in the heart of obese and insulin resistant subjects, the mechanisms leading to cardiac dysfunction are complex and still not fully understood.

Adaptative and maladaptative mechanisms associated with increased adiposity may be implicated in the development of LV dysfunction in obesity (Harmancey et al., 2008; Luaces et al., 2012; Aurigemma et al., 2013; Khan and Movahed, 2013; Lavie et al., 2013). This is linked to cardiac remodelling as discussed above. Conventionally, the total blood volume and cardiac output correlate positively and proportionately with the degree of excess body weight (Chan and Alpert, 2012). Therefore, to meet the increased metabolic (oxygen) demand associated with excess adipose tissue in obese patients a compensatory increase in cardiac output is required (Poirier et al., 2006). This is accomplished by increasing stroke volume and as consequence, the left ventricular chamber dilates to accommodate the increased venous return and LV hypertrophy may ensue (Vasan, 2003; Abel et al., 2008; Ashrafian et al., 2011). This is an adaptative mechanism for normalizing LV wall stress. Should normalization fail, it predisposes to an impaired LV diastolic relaxation (Alpert and Chan, 2012). If the wall stress remains high, a subsequent LV systolic dysfunction may develop (Alpert, 2001). However, as previously indicated in the section of cardiac morphology, this traditional haemodynamic hypothesis to explain the development of obesity-induced cardiac abnormalities, though

attractive, is under debate as it is not fully supported by many of the recent findings (Litwin, 2010; Ashrafian et al., 2011).

Similar to the LV, obesity may affect right ventricular (RV) function through increased cardiac output. The coexistence of high cardiac output, LV hypertrophy, and LV diastolic dysfunction (and in some cases systolic dysfunction) may lead to left heart failure with subsequent pulmonary venous hypertension, pulmonary arterial hypertension and right heart failure as obesity-related obstructive sleep complications (Alpert and Chan, 2012). A recent study in young normotensive healthy adults has found that isolated obesity was associated with subclinical impairment in RV diastolic and systolic function and subsequent dilatation of right cardiac chambers (Sokmen et al., 2013).

In animal models of diet-induced obesity, these alterations in cardiac function were also demonstrated *in vivo* and in isolated hearts as well as in cardiomyocytes (Abel et al., 2008). Increased body weight was associated with a reduced cardiac performance/efficiency in rats (du Toit et al., 2005; Akki and Seymour, 2009; Majane et al., 2009), mice (Christoffersen et al., 2003; Buchanan et al., 2005) and rabbits (Carroll et al., 1997; Carroll et al., 1999) as well as in dogs (Mehlman et al., 2013). After 16 weeks of feeding rats a high calorie diet, basal function of the isolated hearts from obese rats was significantly depressed compared to age-matched controls (du Toit et al., 2008; Nduhirabandi et al., 2011). Similarly, Akki and Seymour (2009) observed that isolated hearts from rats fed with a western diet for 9 weeks showed a decline in myocardial function and a concomitant increase in myocardial oxygen consumption. Conversely, obesity had no effect on *in vivo* basal cardiac function of obese rats (Wensley et al., 2013) and mice (Calligaris et al., 2013), fed an obesity-inducing diet for 30 weeks.

However, the pathophysiological mechanisms underlying the development of myocardial dysfunction in obesity are not well understood. The potential mechanisms involved in the development of cardiac dysfunction in obesity and insulin resistance are represented in figure 2.8. Briefly, obesity may affect the heart as follows: 1) volume overload leads to increased cardiac output and hypertension (haemodynamic factors). 2) Increased sympathetic and renin-

angiotensin-aldosterone activation causes hypertension. 3) Insulin resistance associated hyperinsulinaemia increases advanced glycation end-products and IGF-1 production and facilitates fatty acid uptake, leading to lipotoxicity and cell damage. 4) Visceral adipose tissue increases oxidative stress which causes adipokines dysregulation (leptin resistance and hypoadiponectaemia). These alterations lead to cellular changes and metabolic alterations. 5) Cellular alterations are due to neurohormonal and metabolic factors, oxidative stress and adipokines.

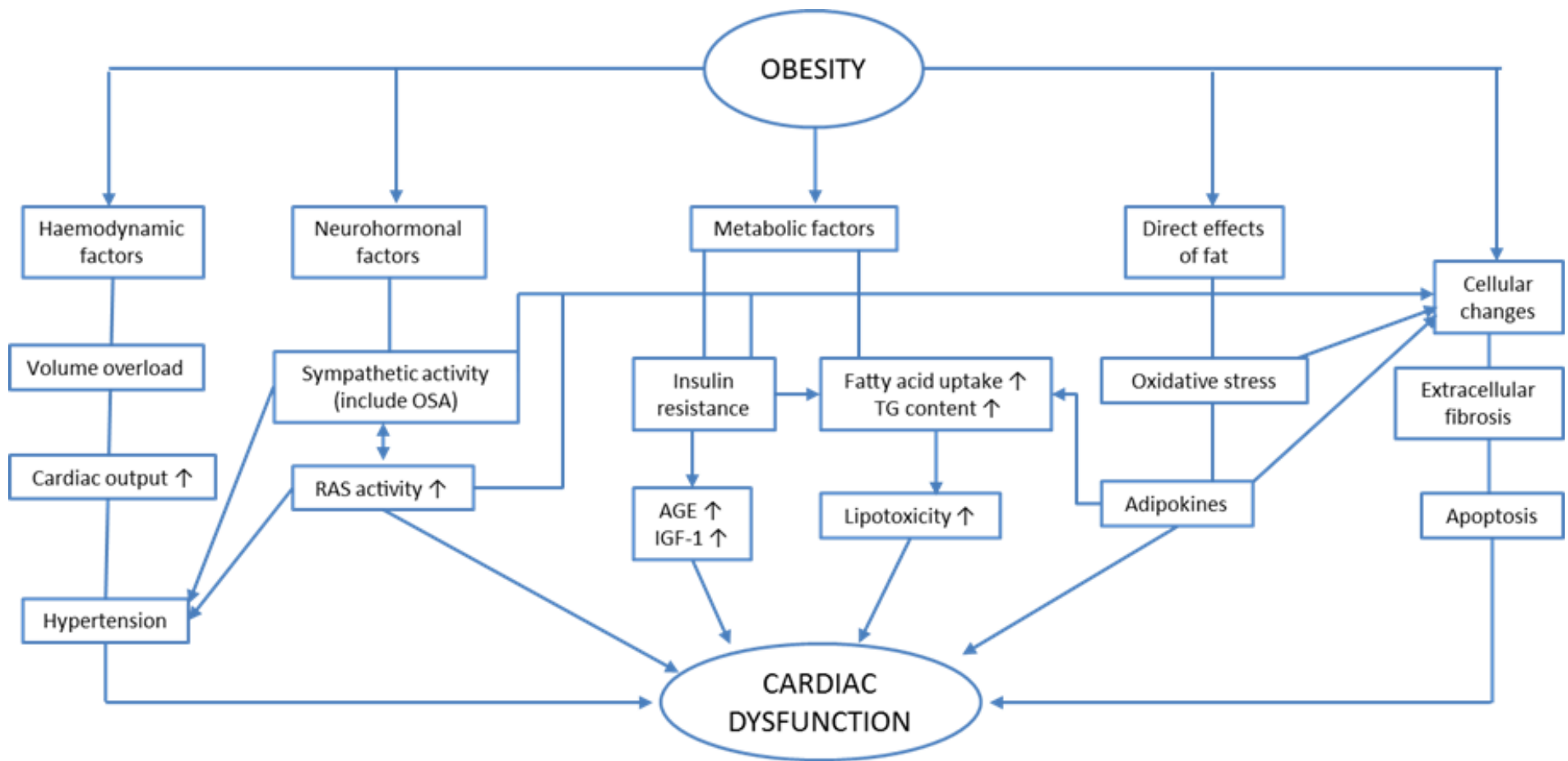


Figure 2.8 Potential mechanisms of the structural and functional changes of the heart in obesity (description in the text). OSA: obstructive sleep apnea, AGE: advanced glycation end-products, IGF-1: insulin-like growth factor-1, TG: triglyceride, RAS: renin angiotensin system [adapted from Ashrafian et al.(2008); Sung and Kim (2010)].

2.1.6.5 Myocardial ischaemia-reperfusion injury in obesity and insulin resistance

2.1.6.5.1 Myocardial ischaemia-reperfusion injury (IRI): concepts

Myocardial ischaemia refers to the condition in which the coronary blood supply to the heart muscle is diminished and thus the supply of oxygen and nutrients is inadequate or insufficient to maintain normal oxidative metabolism (Jennings and Yellon, 1992; Opie, 2004). Myocardial infarction (MI) is defined as a pathological condition characterized by cell death due to prolonged ischaemia (Thygesen et al., 2012). Clinically, myocardial ischaemia or ischaemic heart disease (IHD) generally results from the formation of atherosclerotic lesions in the coronary arteries and when this situation is not rectified, it leads to MI or cell death (Opie, 2004; Thygesen et al., 2012). The ischaemic myocardium can be salvaged by reperfusion even hours after the onset of the coronary occlusion (Skyschally et al., 2008). However, reperfusion itself induces severe and irreversible damage to the myocardium and coronary arteries, collectively called “reperfusion injury”. Therefore, the concept of myocardial IRI refers to myocardial damage induced by both sustained ischaemia and reperfusion (Skyschally et al., 2008).

The pathophysiology of myocardial IRI has been reviewed in great detail (Opie, 2004; Yellon and Hausenloy, 2007; Perrelli et al., 2011; Hausenloy and Yellon, 2013; Jennings, 2013) and is summarized in the section on obesity and cardioprotection (see section 2.2). The following section focuses on available evidence for myocardial infarction in human and animal studies. The so-called “obesity paradox” is also included.

2.1.6.5.2 Epidemiological evidence

Obesity, particularly visceral obesity, is widely recognised as a major risk factor for coronary artery disease (CAD) (Jensen et al., 2008) and MI (Yusuf et al., 2005; Zeller et al., 2008). For example, in a large prospective study of >54 500 Danish men and women aged 50 to 64 years at baseline (1993 to 1997) and free of coronary artery disease, overweight (BMI ≥ 25 and ≤ 29.9 kg/m²) and obese (BMI ≥ 30 kg/m²), were independently associated with a higher risk of acute

coronary syndrome (Jensen et al., 2008). In a cohort of 16 657 patients with MI recruited in 444 Polish cities, 42.8% of subjects were classified as overweight, 31.7% as obese and 39.8% had visceral obesity, indicating that obesity and overweight had a high prevalence in CAD patients, with women the most affected (Kaess et al., 2010). A similar association was also reported by Zeller et al. (2008) in a French population-based cohort of patients with acute MI where 75% were overweight or obese with 50% having visceral obesity and 15% being obese ($\text{BMI} \geq 30 \text{ kg/m}^2$). Using the concentration of total creatine phosphokinase (CPK) and CPK-MB isoenzyme as an estimate of myocardial infarct size, a strong association between visceral obesity and acute MI was confirmed in a cross sectional study of 40 men with acute MI (Iglesias et al., 2009). Importantly, compared with normal weight, overweight and obesity were associated with premature occurrence of acute MI (Suwaidi et al., 2001). In addition, the association of visceral adiposity with acute MI was reported to be independent of other risk factors like HDL-c, IL-6 levels, hypertension, and diabetes (Nicklas et al., 2004). Obese women have been reported to have a higher risk for CAD than men (Nicklas et al., 2004; Kaess et al., 2010), indicating the impact of the gender on the incidence of MI.

Apart from the gender influence, other factors such as obesity-related comorbidities may also affect the risk for acute MI in obese patients. Studies have consistently shown that insulin resistance and other components of the MetS such as dyslipidaemia, fasting hyperglycaemia and elevated blood pressure were associated with an increased incidence of CAD (Ranjith et al., 2007; Fedorowski et al., 2009; Prasad et al., 2010). A recent study by Karrowni et al. (2013) in nondiabetic post-MI patients found that insulin resistance, as measured by HOMA-IR, was strongly associated with multivessel CAD. As expected, the incidence of MetS was elevated in patients with MI (54%) with obesity as the essential feature (Prasad et al., 2010). This association has also been confirmed in other studies (Ranjith et al., 2009; Al-Aqeedi et al., 2013). In these studies, the most common abnormalities were reduced HDL-cholesterol, elevated fasting blood glucose, hypercholesterolaemia, raised non-HDL-cholesterol, and elevated TG, followed by increased waist circumference and raised blood pressure (Ranjith et

al., 2009; Al-Aqeedi et al., 2013). As a consequence, it has been suggested that the combination of established risk factors and additional metabolic derangements associated with excess adiposity may predispose to early plaque rupture and MI in obese patients (Madala et al., 2008).

- **Obesity paradox and MI**

Despite the above evidence for a higher prevalence of obesity in MI patients, obesity has been consistently reported to have an unexpected 'protective effect' on the outcome of percutaneous coronary intervention (PCI), referred to as the obesity paradox (Diercks et al., 2006; Romero-Corral et al., 2006). This was described with a U-shape relationship where overweight and moderate obesity were beneficial while underweight and severely obese patients showed an increased mortality (Azimi et al., 2013). The mechanism underlying the obesity paradox is complex and remains unclear. Importantly, additional studies have also reported that in the long term the obesity paradox is no longer significant and the adverse health effects of obesity may predominate (Das et al., 2011; Kadakia et al., 2011).

In this regard, it is indicated that excess weight at time of presentation of myocardial infarction is associated with lower initial mortality risks but with higher long-term risks including recurrent re-infarction and cardiac death (Nigam et al., 2006). In these patients, the obesity paradox could be affected by a later occurrence of type 2 diabetes (Adamopoulos et al., 2011) or by age (Maggioni et al., 1993; Shih et al., 2011). It has been reported that hyperinsulinaemia, age and estimates of left ventricular systolic function were independently associated with 7-year all-cause of mortality following acute MI in non-diabetic subjects (Kragelund et al., 2004).

2.1.6.5.3 Animal evidence

Despite the evidence of an association between obesity and ischaemic heart disease (Ranjith et al., 2007; Fedorowski et al., 2009; Prasad et al., 2010) and a high sensitivity of the diabetic heart to myocardial IRI in humans (Cubbon et al., 2013), experimental data on the impact of obesity and insulin resistance on myocardial IRI are still controversial (Miki et al., 2012;

Whittington et al., 2012). In this regard, several investigations using diet-induced obesity and insulin resistance as well as obese or non-obese diabetic models have been conducted (Miki et al., 2012; Whittington et al., 2012), however, only a few studies will be referred to in this section. Compared to non-obese animals, myocardial infarct size in animal models of obesity and insulin resistance was larger or smaller or similar (Miki et al., 2012; Whittington et al., 2012). The reason for this inconsistency remains unclear.

A number of studies have reported that diet-induced obesity and insulin resistance decreased myocardial tolerance to ischaemia-reperfusion damage. For example, *in vivo* (Clark et al., 2011; Wensley et al., 2013) and *in vitro* (du Toit et al., 2008; Essop et al., 2009; Huisamen et al., 2011; Nduhirabandi et al., 2011) studies using obese and insulin resistant male rats reported increased susceptibility to IRI in the obese hearts compared with their control counterparts. Similar findings were reported in other studies using mice fed a high-fat diet (Thakker et al., 2008), ob/ob mice (*in vivo*) (Bouhidel et al., 2008), db/db mice (*in vivo*) (Jones et al., 1999; Lefer et al., 2001), Zucker obese (ZO) rats (*in vitro*) (Katakam et al., 2007), Zucker diabetic fatty (ZDF) rats (Sidell et al., 2002) as well as type 2 diabetic BBZ rats (*in vivo*) (Li et al., 2008). Collectively, these studies have shown increased infarct size associated with concomitant poor functional recovery in obese hearts compared to the controls. Interestingly, the changes in obese rat hearts have been associated with a reduction in PKB/Akt and GSK-3 β expression and phosphorylation as well as eNOS expression (Wensley et al., 2013).

A recent study in a porcine dietary model of MetS showed profound contractile dysfunction and reduced mechanical/metabolic efficiency after relatively brief low-flow ischaemia compared to their lean controls (Huang et al., 2013). With a similar protocol (global low-flow ischaemia), hearts from obese male Sprague Dawley (SD) rats fed a high-fat/low-carbohydrate diet also had impaired post-ischaemic LV functional recovery associated with increased lactate dehydrogenase release (Liu and Lloyd, 2013) and reduced insulin signalling as assessed by PKB/Akt phosphorylation (Wang et al., 2008). Impaired cardiac functional recovery was also

observed in male SD rats fed a high sucrose diet (total global ischaemia) (Harmancey et al., 2013) and C57BL/6J mice fed a high-fat diet (Aasum et al., 2008).

Paradoxically, in other studies (both *in vivo* and *in vitro*), diet-induced obesity and insulin resistance (Donner et al., 2013) and NaCl-induced hypertensive-glucose intolerant (HGI) rats (Mozaffari and Schaffer, 2003; Mozaffari and Schaffer, 2008) have shown to improve myocardial ischaemic tolerance. For example, Donner et al. (2013) after feeding rats a high carbohydrate obesogenic diet for 32-weeks found that *in vivo* (45 min left anterior descending artery occlusion and 120 min reperfusion) or *ex vivo* (25 min ischaemia and 60 min reperfusion) hearts from obese rats had small myocardial infarct size and a concomitant increase in functional recovery. These hearts also exhibited post-ischaemic increases in PKB/Akt expression and phosphorylation of eNOS, GSK3 β and ERK42/44 (Donner et al., 2013). Interestingly, isolated hearts from young rats (fed a high-calorie diet for 8 weeks) exhibited smaller infarct size despite their poor post-ischaemic recovery (Huisamen et al., 2012). This “cardioprotective” feature of obesity and insulin resistance has previously been reported in male Goto Kakizaki (GK) rats and ZDF rats compared to their controls (*in vitro*) (Kristiansen et al., 2004; Wang and Chatham, 2004; Kristiansen et al., 2011).

To complicate matters further, a high caloric diet (Lacerda et al., 2012) or a high fat diet (induced MetS) (Thim et al., 2006; Mozaffari and Schaffer, 2008) had no effect on myocardial infarct size. Similar findings were also reported in *in vitro* studies using hearts from ob/ob mice (Przyklenk et al., 2011) and *in vivo* GK rats (Matsumoto et al., 2009).

2.1.6.5.4 Why are different responses to IRI observed?

The differences in responses to IRI observed in obese, insulin resistant animals are difficult to explain. In studies demonstrating obesity-induced exacerbation of myocardial IRI, various reasons have been proposed such as: (1) increased inflammation (Thakker et al., 2008), (2) increased oxidative stress (Katakam et al., 2007; Ansley and Wang, 2013) and (3) a cascade of maladaptative metabolic changes in FFA metabolism (Harmancey et al., 2013). Indeed, the development of microinfarctions associated with an exaggerated inflammatory response was

found in hearts from DIO animals (Thakker et al., 2008). This was accompanied by an increase in inflammatory markers such as chemokines (e.g, MIP-1 α and β) and cytokines (e.g. TNF- α , IL-6, IL-10, TGF- β 1) (Thakker et al., 2006), elevated circulating ANG II, TNF- α and FFA (du Toit et al., 2005; du Toit et al., 2008; Thakker et al., 2008). Although it is known that moderate ROS production is a crucial factor in cardioprotection (Perrelli et al., 2011; Braunersreuther et al., 2012), chronic oxidative stress has been shown to contribute to increased myocardial susceptibility to IRI following high-fat feeding (Ballal et al., 2010; Ansley and Wang, 2013; Liu and Lloyd, 2013).

Concerning adaptative changes in substrate metabolism in IRI, it was shown that in an early pre-diabetic state, the tolerance of the myocardium to ischaemia is reduced by decreasing glycolytic flux adaptation (Morel et al., 2005). Additionally, metabolic alterations in T2D were associated with protection against IRI at the onset, but in the late stages progressive dysfunction of glucose oxidation occurred (Povlsen et al., 2013). A recent study on rats fed a high sucrose diet has suggested that myocardial insulin resistance induced metabolic derangements that negatively impact on contractile recovery during reperfusion (Harmancey et al., 2013). These metabolic derangements were accompanied by a decreased capacity for mitochondrial oxidation of long chain fatty acids, rather than a change in glucose oxidation (Harmancey et al., 2013).

Thus, obesity and insulin resistance induced-cardioprotection (Povlsen et al., 2013) or exacerbation of post-ischaemic injury (Harmancey et al., 2013; Povlsen et al., 2013), depends on the duration of adaptation and appears to be the overall result of adaptative changes in FFA and glucose metabolism (Lopaschuk et al., 2007). However, as substrate availability, severity of ischaemia, and duration of diabetes (hyperglycaemia, hyperinsulinaemia) could not justify the improved post-ischaemic recovery (low-flow ischaemia) in the hearts from ZDF rats (Wang and Chatham, 2004), other mechanisms or factors may be implicated. The susceptibility or sensitivity of the diabetic heart to acute IRI may be dependent on the animal models and experimental conditions used (Paulson, 1997; Whittington et al., 2012). In this regard, as

pointed out by Whittington et al. (2012), the diabetic heart was shown to be “protective” or “less sensitive” to acute IRI in studies which used (1) a short duration of diabetes (<6 weeks); (2) glucose as the only substrate; (3) global ischaemia (Paulson, 1997; Whittington et al., 2012). On the other hand, hearts obtained from rats with more severe and prolonged diabetes, perfused with fatty acids as substrates using a low-flow IRI protocol (i.e, a better simulation of the clinical scenario), were found to be more susceptible or sensitive to IRI (Paulson, 1997; Whittington et al., 2012).

2.1.6.5.5 The role of mitochondria and myocardial IRI in obesity and insulin resistance

Mitochondria are the primary subcellular organelles for oxidation of fatty acid and glucose. Mitochondrial dysfunction is defined as reduction in mitochondrial number, density or function (Ansley and Wang, 2013) and may contribute to myocardial FFA and lipid accumulation (Ansley and Wang, 2013). Evidence supports excess ROS generation in parallel with mitochondrial dysfunction in the obese or diabetic heart (Boudina et al., 2009). Excessive ROS production is proposed to contribute to both functional perturbations and susceptibility to ischaemic injury in the obese or diabetic heart (Ansley and Wang, 2013) which display fewer and more widely dispersed mitochondria than images from lean hearts (Katakam et al., 2007). The increase in myocardial infarct size in obese rats was associated with diminished mitochondrial ADP phosphorylation rates (Essop et al., 2009), but without differences in mtDNA levels and the degree of oxidative stress-induced damage (Essop et al., 2009). Thus, it was suggested that a decreased mitochondrial bioenergetic capacity in pre-diabetic rat hearts may impair the respiratory capacity and thus reduce basal contractile function and tolerance to acute oxygen deprivation (Essop et al., 2009).

Furthermore, mitochondria isolated from ZO hearts exhibit enhanced ROS production and morphological features suggestive of metabolic and oxidative stress (Katakam et al., 2007; Boudina et al., 2009). Similar observations were made in Zucker diabetic fatty rats, which are genetically identical to the ZO rats except that they develop severe hyperglycaemia at an early age (Kristiansen et al., 2004).

2.2 CARDIOPROTECTION AND OBESITY

2.2.1 CARDIOPROTECTION: ACTIVE AND CHALLENGING FIELD

The concept of cardioprotection refers broadly to all strategies aimed at the reduction of infarct size and full restoration of myocardial function (Heusch, 2013). The most effective therapeutic intervention for reducing acute myocardial-ischaemic injury and limiting infarct size (MI) is timely and effective myocardial reperfusion using either thrombolytic therapy or primary percutaneous coronary intervention (PPCI) (Heusch, 2013). Since reperfusion induces damage, there is a continuous need to prevent or reduce the damage resulting from myocardial IRI. This has recently been a subject of focused reviews in *Circulation Research* (Jennings, 2013; Kloner, 2013; Ovize et al., 2013; Vander Heide and Steenbergen, 2013). Only a summary is given below.

Despite a tremendous number of *in vivo* and *in vitro* animal investigations reporting potential beneficial effects (Vander Heide and Steenbergen, 2013), previous clinical trials have been disappointing (Heusch, 2013; Vander Heide and Steenbergen, 2013). Various reasons have been suggested. They include among others mainly inappropriate use of experimental animal models, the clinical testing of inconclusive therapies, and poor clinical trial design (Heusch, 2013). Many experimental MI models fail to represent the clinical setting in terms of comorbidities (such as age, diabetes, dyslipidaemia, and hypertension) and concomitant medication, the presence of which may interfere with the therapeutic cardioprotective intervention (Ghaboura et al., 2011; Sack and Murphy, 2011; Whittington et al., 2013).

Many reviews on the pathophysiology of myocardial IRI have appeared in recent years (Opie, 2004; Yellon and Hausenloy, 2007; Perrelli et al., 2011; Hausenloy and Yellon, 2013; Jennings, 2013). In this section, to better understand the contribution of obesity and insulin resistance to the IRI and potential cardioprotective strategies, particularly in the context of melatonin's effects, only events associated with IRI are summarized focusing on the role of mitochondria and ROS.

2.2.2 ALTERATIONS AND MECHANISM OF ISCHAEMIC INJURY

The pivotal feature of ischaemia is that oxygen supply to the mitochondria is inadequate to support oxidative phosphorylation (Solaini and Harris, 2005). It is well established that the major outcomes of ischaemia are poor oxygen delivery (hypoxia) and poor washout of metabolites including lactate and protons associated with a severe cellular acidosis (low pH) (Hausenloy and Yellon, 2013; Jennings, 2013; Vander Heide and Steenbergen, 2013). Functional changes due to ischaemic injury are associated with metabolic and ultrastructural alterations (Jennings, 2013). It is also well established that the gravity (e.g., reversibility or irreversibility) of the injury depends on severity of coronary flow reduction, the length of the ischaemic insult as well as the location being affected (e.g., possibility of collateral flow) [for review see (Skyschally et al., 2008; Jennings, 2013)]. The most likely mechanism causing cardiomyocyte death during severe ischaemia is the disruption of the sarcolemma (Jennings, 2013).

2.2.3 ALTERATIONS DUE TO REPERFUSION INJURY

It is technically difficult to clearly identify and distinguish between damage induced by ischaemia from that caused by reperfusion (Skyschally et al., 2008). Nevertheless, it is well known that despite its beneficial effect in limiting the progression of ischaemic injury, reperfusion itself exacerbates the ischaemic alterations as it can be responsible for 40–50% of the infarct (Kloner, 1993). While the myocardium can tolerate 15 to 20 min of ischaemia (Skyschally et al., 2008), the restoration of blood flow may result in arrhythmias, contractile dysfunction (myocardial stunning), microvascular impairment (endothelial dysfunction) as well as irreversible myocardial damage (infarction) within the first minutes of reperfusion (Kloner, 1993). Thus, the concept of “lethal reperfusion injury” refers to the damage caused by reperfusion, resulting in death and loss of cells that were viable or reversibly injured during ischaemic episode (Kloner, 1993). Although microvascular impairment/injury is also important (Prasad et al., 2009), only the mechanism of lethal reperfusion injury is summarized below focusing on the role of mitochondria and ROS.

2.2.4 MITOCHONDRIA AND ROS: CRUCIAL ROLE IN REPERFUSION INJURY

Although reperfusion injury is complex and multifactorial, the mitochondria and ROS have been suggested to play a crucial role in this phenomenon (Perrelli et al., 2011; Hausenloy and Yellon, 2013). A recent review on the potential mechanisms of reperfusion injury has revealed four pathways including the role of pH, Ca^{2+} , cell swelling and oxidative stress as the hub of this phenomenon (Vander Heide and Steenbergen, 2013). With regard to oxidative stress, ROS are produced by several extracellular and intracellular processes with the mitochondria representing the most relevant site for ROS formation in the cardiomyocytes (Perrelli et al., 2011), indicating the central role of mitochondria in exacerbation of cardiomyocyte injury (Perrelli et al., 2011).

The role of excessive ROS generation at early reperfusion has consistently attracted much attention (Dhalla et al., 2000; Opie, 2004; Misra et al., 2009). The rapid restoration of oxygen supply and physiological pH causes excessive ROS generation by xanthine oxidase (mainly from endothelial cells), the re-energized mitochondrial electron transport chain (ETC) and by NADPH oxidase (mainly from neutrophils) (Dhalla et al., 2000). As described by Opie (2004), ROS mediate myocardial injury by: 1) inducing mitochondrial permeability transition pore (MPTP) opening, 2) acting as neutrophil chemo-attractants, 3) mediating dysfunction of the sarcoplasmic reticulum, 4) contributing to intracellular Ca^{2+} overload and 5) damaging essential molecules (lipid, DNA, protein). Despite the detrimental actions of ROS in acute IRI, experimental and clinical studies using antioxidants yielded inconsistent and disappointing outcomes probably due to inability of the antioxidants to enter the cell (Hausenloy and Yellon 2013). Therefore an effective antioxidant with elevated ability to enter the cell would be beneficial in this context.

Recent studies have revealed that mitochondrial dysfunction and damage, particularly the MPTP opening, may play a critical role in the reperfusion injury (Yellon and Hausenloy, 2007; Perrelli et al., 2011; Hausenloy and Yellon, 2013). The MPTP is a non selective large conductance pore in the inner mitochondrial membrane connecting the mitochondrial matrix

and the cytosol (Halestrap and Pasdois, 2009). In the setting of acute IRI, the MPTP has been shown to remain closed during ischaemia and open at reperfusion only in response to mitochondrial Ca^{2+} and phosphate overload, oxidative stress, relative ATP depletion and rapid elevation of pH (Halestrap and Pasdois, 2009). Massive MPTP opening results in complete mitochondrial depolarisation, uncoupling of oxidative phosphorylation leading to ATP depletion, mitochondrial swelling and rupture of external mitochondrial membranes (Halestrap and Pasdois, 2009).

Collectively, at early reperfusion, MPTP opening, mitochondrial damage, the rapid washout of lactic acid and metabolites together with the re-energized ETC in the setting of increased intracellular Ca^{2+} lead to cardiomyocyte hypercontracture, apoptosis and necrosis (Hausenloy and Yellon, 2013). Thus, a combined cardioprotective strategy correcting and/or preventing altered myocardial metabolism and mitochondrial damage would be of great therapeutic relevance in the energy-depleted heart (Grossman et al., 2013). However, omission to consider the comorbidities has been one of the pitfalls of recent promising cardioprotective strategies when translated to the clinic (Vander Heide and Steenbergen, 2013). This is the focus of the following section.

2.2.5 CARDIOPROTECTION IN OBESITY AND INSULIN RESISTANT CONDITIONS: UNRESOLVED ISSUE

Despite the disappointing outcomes of previous clinical trials, the discovery of the endogenous cardioprotective mechanisms such as ischaemic preconditioning (IPC) and postconditioning (IPOC) evoked a new interest in the possibility of effective cardioprotection (Heusch, 2013). In the laboratory, using animals IPC and IPOC elicit significant cardioprotection by applying short periods of ischaemia separated by short reperfusion intervals either before ischaemia or at the beginning of reperfusion, respectively (Zhao and Vinten-Johansen, 2006).

Unfortunately, IPC and IPOC are impaired in experimental obesity and insulin resistance as well as in ageing and diabetes (Katakam et al., 2007; Yin et al., 2012). Katakam and co-

workers (2007) were the first to show the failure for IPC to confer cardioprotection in ZO rats compared with Zucker lean rats. Their study showed that inhibition of mitochondrial K_{ATP} channels abolished IPC in lean hearts with no effect on ZO hearts (Katakam et al., 2007). They further demonstrated a significantly diminished membrane depolarization and ROS generation in isolated mitochondria from ZO hearts compared with lean hearts. Similarly, in ZDF rats, IPC was also not effective (Kristiansen et al., 2004).

Furthermore, IPOC was found ineffective in obese ob/ob mice (Bouhidel et al., 2008). Hearts from these obese animals when exposed to IRI, exhibited increased infarct sizes and a concomitant reduction in activation of PKB/Akt, ERK42/44 and AMPK during reperfusion (Bouhidel et al., 2008). Figure 2.9 represents the major signalling pathways of IPC- and IPOC-mediated cardioprotection. It was suggested that obesity and diabetes may inhibit cardio protective pathways and exacerbate ischaemia-reperfusion-induced MI (Yin et al., 2012).

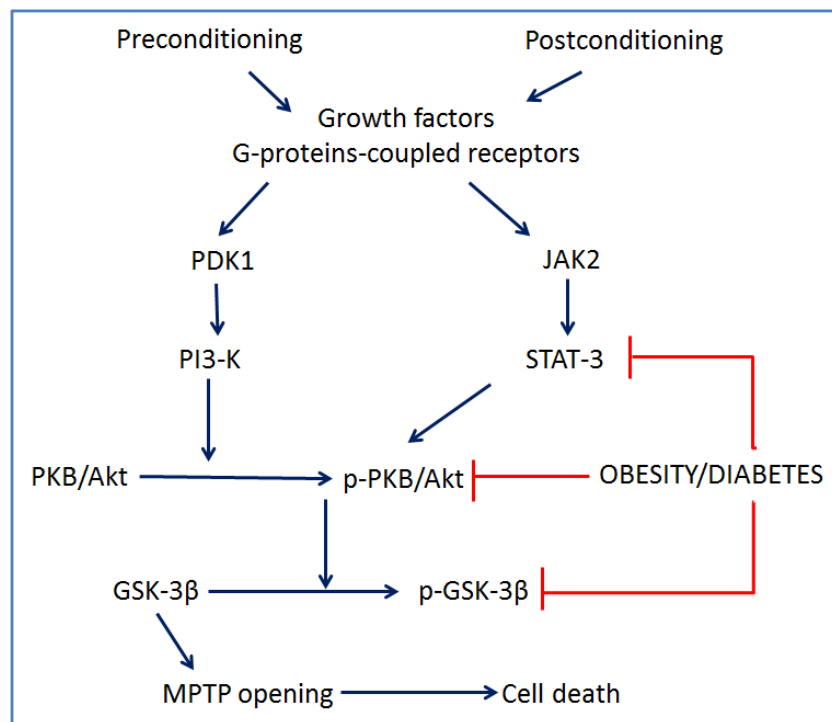


Figure 2.9 Major signalling pathways of IPC- and IPOC-mediated protection against cardiac cell death [adapted from Yin et al. (2012)]. Myocardial protection of IPC and IPOC were proposed to be mediated by stimulation of the prosurvival signalling pathway PI3-K/Akt pathway to inhibit the GSK-3 β activation either *via* PI3-K pathway or JAK2/STAT-3 pathway.

Obesity and diabetes can inhibit the activation of STAT-3 or PKB/Akt to consequently activate GSK-3 β that in turn induces mitochondrial cell death which is the critical cellular event for ischemia/reperfusion-induced MI (Yin et al., 2012).

In view of the current lack of effective therapy as discussed above, cardioprotection is still an active research area. However, with the rising prevalence of obesity and its multiple comorbidities, an ideal therapeutic strategy must retain its efficacy under pathological conditions, not only in lean subjects, but also in obese and insulin resistant conditions.

As described in the following section, the pineal gland hormone melatonin is a multifunctional molecule having anti-excitatory, antioxidant, immunomodulatory, anti-inflammatory, oncostatic and vasomotor properties (Hardeland et al., 2006). Its potential cardioprotective properties have been established in lean animals (Lochner et al., 2013). Additionally, melatonin has been shown to be involved in body weight regulation in seasonal animals. We previously showed that melatonin consumption starting before the establishment of obesity, prevented obesity-related abnormalities and protected the isolated hearts subjected to IRI (Nduhirabandi et al., 2011). The effects of melatonin in lean and obese conditions are summarized in the following sections.

2.3 MELATONIN AND THE HEART

2.3.1 MELATONIN: A MULTIFUNCTIONAL MOLECULE

As indicated above, melatonin is the neurohormone mainly produced by the pineal gland upon the activation of the suprachiasmatic nucleus (SNC) of the hypothalamus during the night [for details on biosynthesis and metabolism of melatonin, see Zawilska et al. (2009)]. It is a highly conserved indolamine found in most organisms including bacteria, algae, plants, fungi, insects, nematodes and vertebrates including mammals (Hardeland and Fuhrberg, 1996). In humans, the normal circulating melatonin levels vary between 1-10pM and 43-400pM, during the day and the night, respectively (Barrenetxe et al., 2004; Bonnefont-Rousselot and Collin, 2010).

Viewed as a multifunctional molecule with very versatile biological signalling properties (Hardeland et al., 2011), melatonin is a small compound able to cross all morphological barriers and acts within every subcellular compartment due to its highly lipophilic and hydrophilic properties (Venegas et al., 2012). Since its isolation by Lerner and co-workers (Lerner et al., 1958), melatonin has been shown to play a role in most physiological functions in animals and humans (Hardeland et al., 2011; Hardeland, 2012). Indeed, apart from its classical role as a chronobiotic or endogenous synchronizer in the regulation of seasonal as well as circadian rhythms along with its sleep inducing effects (Zawilska et al., 2009), melatonin has anti-excitatory, antioxidant, immunomodulatory, anti-inflammatory, oncostatic and vasomotor properties (Hardeland et al., 2006; Pandi-Perumal et al., 2006; Hardeland et al., 2011). However, besides its universal availability and, presumably, its presence in our daily food consumption (Tan et al., 2012), its effects and underlying mechanisms are vast and not fully explored (Hardeland et al., 2012).

Melatonin has powerful antioxidant properties with strong cytoprotective activities [for review, see (Reiter et al., 2008; Korkmaz et al., 2009a)]. It has been proved to be more effective than other classical antioxidants (Montilla-Lopez et al., 2002; Sener et al., 2003) due to its multiple free radical scavenger cascades and its ability to stimulate the natural antioxidant capacity (Tan et al., 2007a). In addition to this, its metabolites also have free radical scavenging activities and, thereby enhancing the actions of melatonin (Tan et al., 2007b; Korkmaz et al., 2009a). Recently, the efficacy of melatonin to improve oxidative stress-mediated metabolic disorders *via* its gene regulation has been shown to be linked to epigenetic mechanisms (Korkmaz et al., 2012). The antioxidant actions of melatonin are summarized in table 2.2.

Besides its pineal production, melatonin is also secreted by a wide variety of tissues including the retina, thymus, spleen, heart, muscle, liver, stomach, intestine, placenta, testis, cerebral cortex, and striatum (Stefulj et al., 2001; Sanchez-Hidalgo et al., 2009; Sanchez-Hidalgo et al., 2009; Venegas et al., 2012). The melatonin content in these tissues varies and decreases with age to a similar extent as the pineal melatonin production (Sanchez-Hidalgo et al., 2009;

Sanchez-Hidalgo et al., 2009). Commonly, nocturnal melatonin production as shown by its circulating levels as well as its primary urinary metabolite, 6-sulfatoxymelatonin (aMT6s), is lowered in various pathologies characterized by increased oxidative stress, such as neurological disorders, CVD and T2D as well as obstructive sleep apnoea syndrome (Hernandez et al., 2007; Hardeland, 2012). Interestingly, circulating melatonin levels can be influenced by the diet (Sae-Teaw et al., 2012; Johns et al., 2013) and the minute amounts of melatonin present in red wine have been shown to protect the heart against myocardial ischaemia-reperfusion damage (Lamont et al., 2011), indicating its therapeutic potential in CVD.

This section summarizes the available evidence for the effects of melatonin on the heart, focusing on its cardioprotective properties.

2.3.2 THE LINK BETWEEN THE HEART AND MELATONIN

The link between the heart and melatonin and the influence of endogenous melatonin on cardiovascular function in general are well established (Dominguez-Rodriguez et al., 2010). Diurnal variations can be seen in blood pressure, heart rate, cardiac output and endothelial dilatory capacity of peripheral and coronary arteries, sympathetic activity, cardiac electrical stability and platelet aggregation (Dominguez-Rodriguez et al., 2010; Takeda and Maemura, 2011). Pathological conditions, for example adverse cardiac events, including MI (Dominguez-Rodriguez et al., 2002), sudden cardiac death (Muller et al., 1987) and arrhythmias (Siegel et al., 1992) have also been linked to the circadian rhythm in humans, having a higher incidence in the early morning, when circulating melatonin levels are considerably lower (Altun et al., 2002).

The most important evidence linking melatonin to cardiac function was the identification of membrane MT1 and MT2 receptors along with other intracellular binding sites such as the cytosolic quinone reductase 2 enzyme [also called the putative melatonin receptor 3 (QR2/MT3) and melatonin nuclear receptors (e.g., retinoic acid subfamily of orphan receptors

(RORx)] in the heart and arteries (Ekmekcioglu et al., 2003; Peliciari-Garcia et al., 2011; Schepelmann et al., 2011). These receptors offer possibilities for melatonin signalling to interact with the heart function. Melatonin may indirectly affect cardiovascular function *via* MT receptors in the SCN which are known to modulate the cardiovascular function *via* multisynaptic autonomic neurons (Scheer et al., 2003). Melatonin may also influence the physiology of the heart *via* its direct actions on the peripheral intrinsic circadian clocks identified in both cardiomyocytes (Durgan and Young, 2010; Peliciari-Garcia et al., 2011) and vessels (Reilly et al., 2007). Surprisingly, melatonin has also been shown to be secreted within the heart (Sanchez-Hidalgo et al., 2009; Sanchez-Hidalgo et al., 2009). However, the exact role for cardiac melatonin remains unknown.

2.3.3 EFFECTS OF MELATONIN ON THE HEART

2.3.3.1 Animal studies

Under normal conditions melatonin treatment has no effect on heart function. Animal studies showed that prolonged melatonin consumption under normal conditions affected cardiac metabolism, reduced the absolute and relative heart weights (Bojkova et al., 2008; Nduhirabandi et al., 2011) and increased its glycogen content (Kassayova et al., 2006) but had no effects on heart function *in vivo* (Patel et al., 2010) or *ex vivo* (Nduhirabandi et al., 2011).

Although raising circulating melatonin concentrations by administration of exogenous melatonin does not appear to be harmful to the heart, the presence of very low circulating concentrations (as occurring during daytime) is essential and pinelectomy has profound effects on the heart. Surgical removal of pineal gland followed by 2 months of stabilization caused an increase in serum cholesterol and cardiac malondialdehyde (MDA) levels as well as increased heart weight (Mizrak et al., 2004). Other morphological changes such as increased myocardial fibrosis, myxomatous degeneration of the valves and thickening of left atrial endocardium were also observed in the hearts isolated from these pinelectomized rats (Mizrak et al., 2004). Importantly, despite failure to improve morphological alterations (presumably due to the short treatment time) melatonin administration (4 mg/kg/day) for 2 days

reversed the changes in circulating cholesterol and cardiac MDA levels in these animals (Mizrak et al., 2004).

2.3.3.2 Human studies

Similar to experimental animals, administration of 3 mg of melatonin in healthy male volunteers had no effect on their heart rate (Kitajima et al., 2001). In pathological conditions, patients with CAD have impaired nocturnal melatonin secretion (Brugger et al., 1995; Altun et al., 2002) and subjects with MI were reported to have reduced circulating melatonin levels (Dominguez-Rodriguez et al., 2002). Concordantly, a significant association between single nucleotide polymorphisms (SNPs) (rs28383653) of melatonin receptor type 1A (MT1A) and coronary artery disease has been demonstrated in a recent case-control study (SamimiFard et al., 2011). In a cohort of survivors of acute MI, reduction of circulating melatonin was also associated with greater adverse remodeling (Dominguez-Rodriguez et al., 2012). As a consequence, reduced serum melatonin concentrations measured at admission are now considered as an independent predictor of LV remodeling (Dominguez-Rodriguez et al., 2012). Importantly, in a double blind randomized clinical trial, melatonin supplementation was able to improve LV ejection fraction in patients with heart failure (Garakyaraghi et al., 2012). Thus, melatonin treatment could play a clinically relevant role in the pharmacotherapy of cardiac diseases.

The beneficial effects of exogenous melatonin on the heart have been documented in physiological conditions such as ageing (Petrosillo et al., 2010) and in pathophysiological conditions such as hyperthyroidism (Ghosh et al., 2007), cadmium-induced oxidative damage (Mukherjee et al., 2011) and myocardial hypertrophy (Reiter et al., 2010b). Detailed description of these effects, although important, is beyond the scope of the present dissertation. The effect of melatonin on the heart in obesity is discussed in the section on melatonin and obesity (see, section 2.4).

2.3.4 MELATONIN AND MYOCARDIAL IRI

The role of oxidative stress and excessive free radical production in the pathogenesis of myocardial infarction as well as experimental ischaemia-reperfusion damage is well established (Opie, 2004) (see also section 2.2., on cardioprotection in obesity). The ability of exogenous melatonin to attenuate ischaemia-reperfusion damage in rodent hearts has been demonstrated in isolated hearts (*ex vivo*) (Tan et al., 1998; Kaneko et al., 2000; Lagneux et al., 2000; Szarszoi et al., 2001; Lamont et al., 2011), *in vitro* in isolated cardiomyocytes (Salie et al., 2001) as well as *in vivo* (Lee et al., 2002; Sahna et al., 2002; Sahna et al., 2005). Table 2.3 summarizes the effects of melatonin in experimental MI.

The potential beneficial actions of endogenous melatonin were uncovered by the observation that hearts from pinealectomized animals exhibited a larger post-ischaemic MI than those from non-pinealectomized rats (Sahna et al., 2002). Melatonin administration was able to attenuate ventricular fibrillation and reduce infarct size and mortality rate in these pinealectomized rats (Sahna et al., 2002). Investigations in our own laboratory have demonstrated the long-term effects of melatonin: hearts perfused after 1 to 7 days after oral melatonin administration (20 or 40 µg/mL) were found to be protected against IRI damage and this cardioprotection persisted for 2-4 days after withdrawal of treatment (Lochner et al., 2006). Importantly, melatonin at either physiological or pharmacological doses, given before or after the ischaemic period was able to protect the heart against myocardial ischaemia/reperfusion damage (Lochner et al., 2013). Interestingly, the study done by Lamont and co-workers (2011) also found melatonin in the picomolar range to be protective in retrogradely perfused rat hearts. However, using the isolated perfused working rat heart, it was found that melatonin was effective only in the micromolar range (Lochner et al., 2006). Whether melatonin has a biphasic effect in these *ex vivo* perfused hearts (Lochner et al., 2013), remains to be further investigated.

2.3.4.1 Mechanisms of melatonin-induced cardioprotection

The mechanisms underlying the beneficial effects of melatonin on the ischaemic heart has not yet been fully elucidated. Several mechanisms including its antioxidant, anti-adrenergic, anti-inflammatory and anti-excitatory actions have been proposed. Importantly, melatonin receptors have been shown to be involved in its myocardial effects (Lochner et al., 2006).

2.3.4.1.1 Antioxidant mechanisms

The antioxidant actions of melatonin are well established (Reiter et al., 2008; Korkmaz et al., 2009a; Bonnefont-Rousselot and Collin, 2010). In the setting of myocardial IRI, several reports indicated that melatonin protects the heart directly *via* its free radical scavenging actions and indirectly *via* its stimulatory effects on antioxidant capacity (Patel et al., 2010; Reiter et al., 2010a) (see table 2.2). Indeed, melatonin was able to neutralize a number of toxic reactants including reactive oxygen species and free radicals induced by myocardial IRI (Reiter and Tan, 2003; Reiter et al., 2010a). For example, in *an in vivo* rat model of myocardial IRI, melatonin significantly increased glutathione (GSH) levels and reduced the lipid peroxidation (MDA) of the heart tissues after reperfusion (Sahna et al., 2005). Similar findings were observed in other *in vivo* and *in vitro* studies where it was shown that melatonin also suppressed superoxidase ($\cdot\text{O}_2$) production, reduced myeloperoxidase (MPO) activity and increased superoxide dismutase (SOD) (Lee et al., 2002; Ceyran et al., 2008; Sahna et al., 2008), reduced hydroxyl radical ($\text{OH}\cdot$) (Kaneko et al., 2000) as well as total ROS generation (Salie et al., 2001). Interestingly, these antioxidant properties were also confirmed in rat models of coronary artery ligation-induced ischaemic heart failure (Sehirli et al., 2013) and isopretrenol-induced MI (Mukherjee et al., 2010).

Table 2.2. Antioxidant effects of melatonin

ACTIONS	TARGET/SITE OF ACTION		OUTCOME
Scavenging	ROS	Hydrogen peroxide	↓
		Hydroxyl radical	↓
		Singlet oxygen	↓
		Superoxide anion	↓
	RNS	Nitric oxide	↓
		Peroxynitrite anion	↓
Stimulation	Antioxidant enzymes	Superoxide dismutase	↑
		Catalase	↑
		Glutathione peroxidase	↑
		Glutathione reductase	↑
		Glucose-6-phosphatase dehydrogenase	↑
		Gamma-glutamylcysteine synthase	↑
Inhibition	Pro-oxidant enzyme	Nitric oxide synthase	↓
		Lipo-oxygenase	↓
		Myeloperoxidase	↓

ROS: reactive oxygen species, RNS: reactive nitrogen species, ↑: increase, ↓: decrease

(adapted from Tengattini et al., 2008; Reiter et al., 2003).

Although the efficacy of the classic antioxidants such as vitamin E, vitamin C and glutathione (GSH) is increased when used in combination with melatonin (Gitto et al., 2001), melatonin was shown to be more potent than vitamin C in protecting against arrhythmias (Tan et al., 1998). In fact, several melatonin metabolites (e.g., N1-acetyl-N2-formyl-5-methoxy kynuramine (AFMK) which are formed when melatonin neutralizes damaging reactants are themselves free radical scavengers (Tan et al., 2007a; Reiter et al., 2008). This would increase the efficacy of melatonin in pathological conditions associated with increased oxidative stress (Korkmaz et al., 2009a; Bonnefont-Rousselot and Collin, 2010).

Furthermore, melatonin treatment has been shown to protect against mitochondrial dysfunction, the major source of reactive oxygen and nitrogen species (ROS/RNS) (Lopez et al., 2009; Paradies et al., 2010). Concordantly, the antioxidant properties of melatonin were

also associated with a reduction in intracellular Ca^{2+} (Tan et al., 1998; Salie et al., 2001) and apoptosis (Dobsak et al., 2003; Ceyran et al., 2008) as well as increases in SERCA and Na^+, K^+ -ATPase activity (Sehirli et al., 2013) in the cardiac tissue.

2.3.4.1.2 Other mechanisms

In addition to its antioxidant activities, the anti-adrenergic, anti-inflammatory and anti-excitatory effects of melatonin are also involved in cardioprotection (Genade et al., 2008; Dominguez-Rodriguez et al., 2009; Patel et al., 2010). In this regard, the significance of the MT receptors has been consistently demonstrated (Lochner et al., 2006; Sallinen et al., 2007; Genade et al., 2008). As mentioned above, the most important link between melatonin and cardiac function was the identification of membrane MT1 and MT2 receptors along with other intracellular binding sites in the heart (Ekmekcioglu et al., 2003; Peliciari-Garcia et al., 2011; Schepelmann et al., 2011). The role of these receptors in the cardiovascular system has been intensively reviewed (Paulis et al., 2012; Slominski et al., 2012; Lochner et al., 2013) and therefore in this section only their contribution to cardioprotection is discussed.

Despite the finding that circulating melatonin levels are reduced in patients with myocardial infarction (Dominguez-Rodriguez et al., 2002), experimental myocardial infarction was shown to increase circulating melatonin levels, followed by enhancement of MT receptors expression (Sallinen et al., 2007). The importance of these receptors in cardioprotection was further supported by the observation that luzindole, a melatonin receptor antagonist, was able to suppress the cardioprotection induced by melatonin (Lochner et al., 2006). These events may eventually affect the probability of the MPTP opening (Petrosillo et al., 2009). Petrosillo and co-workers (2009) reported that melatonin protected hearts against reperfusion injury by inhibiting the opening of the MPTP probably *via* prevention of mitochondrial cardiolipin peroxidation. Melatonin protected against the alterations in the various mitochondrial bioenergetic parameters associated with ischaemia-reperfusion (Petrosillo et al., 2006; Petrosillo et al., 2009). It significantly lowered the degree of mitochondrial lipid peroxidation, counteracted the

reduction in state 3 respiration and respiratory control ratio and prevented the loss of complex I and complex III activities (Petrosillo et al., 2009).

Using the rat model of isoproterenol (beta-adrenergic)-induced myocardial infarction, it was shown that melatonin pretreatment not only improved the antioxidant profile, but also reduced myocardial total cholesterol levels and increased phospholipids (Patel et al., 2010). Whether these effects are maintained in the scenario of ischaemia-reperfusion remains to be established.

Although the involvement of the MT receptor in melatonin-induced cardioprotection is known, to the best of our knowledge only one study investigated the effects of a melatonin agonist on myocardial IRI in mice (Chen et al., 2003). In this study, unexpectedly, the melatonin receptor agonist 8-Methoxy-2-propionamidotetralin which has no antioxidant activity was not found to be cardioprotective (Chen et al., 2003).

To date, cardioprotective actions of melatonin have been associated with downstream signalling events including activation of the reperfusion injury salvage kinase (RISK) pathway (PI3-K, PKB/Akt, ERK42/44) and the protective survivor activating factor enhancement (SAFE) pathway (JAK/STAT-3) [for more details on signalling pathways, see (Lochner et al., 2013)]. Lamont and coworkers (2011) showed that hearts pretreated with picomolar quantities of melatonin (followed by washout before freeze-clamping) had increased STAT-3 activation before the onset of sustained ischaemia and this was associated with a reduction in infarct size during reperfusion. Involvement of TNF- α and STAT-3 in melatonin-induced cardio protection was further demonstrated by the fact that hearts from TNF- α receptor 2 knockout and cardiac STAT-3-deficient mice could not be protected against IRI by melatonin (Lamont et al., 2011). This observation lends a new perspective to the ability of melatonin to protect the heart, suggesting that activation of the SAFE pathway before the onset of ischaemia may also play a role in subsequent cardioprotection. However, whether this holds true for PKB/Akt and ERK42/44 activation before ischaemia is not known.

Table 2.3 The effects of melatonin on myocardial ischaemia-reperfusion injury

STUDY	TYPE	MODEL	IR DURATION	MELATONIN EFFECTS
Tan et al., 1998	<i>Ex vivo</i>	isolated rat heart (Langendorf)	10/10 (min/min)	↓ premature ventricular contraction and fibrillation
Kaneko et al., 2000	<i>Ex vivo</i>	isolated rat heart (Langendorf)	30/30 (min/min)	↓ ventricular tachycardia and fibrillation; restored ventricular function; ↓ lipid peroxidation; lowered OH [•] generation
Lagneux et al., 2000	<i>Ex vivo</i>	isolated rat heart (Langendorf)	5/30 (min/min)	↓ reperfusion arrhythmias; ↓ infarct size
Szarszoi et al., 2001	<i>Ex vivo</i>	isolated rat heart (Langendorf)	20/40 (min/min)	↓ fibrillation; low concentration had no effect on IR of the isolated perfused heart rat
Salie et al., 2001	<i>Ex vivo</i>	Cardiomyocyte in culture (rats)	12.5 or 27.5/1.5	↓ morphological damage, lowered oxygen free radical generation, ↓ intracellular [Ca ²⁺]
Dobsak et al., 2003	<i>Ex vivo</i>	isolated and perfused working rat heart	30/45 (min/min)	Improved hemodynamic parameters and ↓ post-ischemic arrhythmias during reperfusion; ↓ incidence of apoptotic cells
Genade et al., 2006	<i>Ex vivo</i>	isolated rat heart / working heart	20/30 (min/min)	Global ischemia (GI): protective effects during reperfusion, abolishes cardioprotection before and during ischaemia preconditioning protocol
Lochner et al., 2006	<i>Ex vivo</i>	Isolated rat heart/ working heart	20/30 (GI) or 35/30(RI) (min/min)	Before and during reperfusion: improved cardiac output and work performance; ↓ infarct size; long term ↓ IFS (RI). Luzindole abolishes these effects. Melatonin receptor involved in cardioprotection.

STUDY	TYPE	MODEL	IR DURATION	MELATONIN EFFECTS
Petrosillo et al., 2006	<i>Ex vivo</i>	Langendorff	30(GI)/120 or 15 (min/min)	↑ content of cardiolipin with ↓ peroxidized cardiolipin, ↓ rates of mitochondrial oxygen consumption, complex I and complex III activity, and H ₂ O ₂ production; ↑post-ischaemic LVDp and ↓ LVED p
Genade et al., 2008	<i>Ex vivo</i>	Langendorff and working heart	35 RI/30 or 120 (min/min)	Anti β-adrenergic effects: ↓ IFS, ↓NOS & cAMP; ↑post-ischaemic PKB/Akt, ↓p38 MAPK activation.
Yeung et al., 2008	<i>Ex vivo</i>	Isolated heart rat (Langendorff) (chronic hypoxic rats)	30/120 min/min	infarct size reduction, improves Ca ²⁺ handling by preserving SERCA expression, ↓MDA levels, ↓LDH
Petrosillo et al., 2009	<i>Ex vivo</i>	Langendorff	30 GI /120 or 15 (min/min)	Inhibition of MPTP opening. Before and during reperfusion: ↓ IFS, improves function recovery (↑ LVDp and ↓ LVED p), ↓necrosis (↓LDH); reduction MPTP opening sensitivity by increasing resistance to Ca ²⁺ , prevention of NAD ⁺ release and mitochondrial cytochrome-c release
Lamont et al., 2011	<i>Ex vivo</i>	Langendorff	30 RI/120 (min/min)	↓ IFS, ↑ pre-ischaemic STAT3 phosphorylation
Nduhirabandi et al., 2011	<i>Ex vivo</i>	Working heart	40RI/120(min/min)	↓ IFS, ↑ post-ischaemic PKB/Akt, ERK42/44, ↓p38MAPK activation
Yang et al., 2013	<i>Ex vivo</i>	Langendorff,	45RI/60 (min/min)	↓IFS, ↑ STAT-3 activation, ↑Bcl2, ↓Bax, improved cardiac function

STUDY	TYPE	MODEL	IR DURATION	MELATONIN EFFECTS
Chen et al., 2009	<i>In vivo & ex vivo</i>	In situ mice & isolated mice heart (Langendorff)	50min/4 h 40/45(min/min)	↑LVEDp and ↓ LDH level , ↓IFS ; GPx not involved
Lee et al., 2002	<i>In vivo</i>	in situ rat heart	45/60 (min/min)	↓ ventricular tachycardia and fibrillation; lowered total ventricular contractions, suppression of superoxidase production (\dot{O}_2) and ↓MPO activity, ↓ IFS, ↓ survival
Sahna et al., 2002b	<i>In vivo</i>	in situ rat heart	7/7 (min/min)	↓ ventricular fibrillation, ↓mortality
Sahna et al., 2002a	<i>In vivo</i>	in situ rat heart	3/120 (min/min)	↓ IFS
Dave et al., 1998	<i>In vivo</i>	in situ rabbit heart	30/180 (min/min)	No improvement in cardiac function, no reduction in IFS
Chen et al.,2003	<i>In vivo</i>	In situ mice heart	60 /4 (min/h)	Treatment 30 min before ischemia: ↓ IFS/risk area. Treatment after ischaemia: no effect
Sahna et al., 2005	<i>In vivo</i>	in situ heart rat	30/120 (RI) (min/min)	↓ infarct size, ↓ MDA values, ↑GSH levels
Deniz et al., 2006	<i>In vivo</i>	in situ heart rat (chronic nitric oxide synthase inhibited rats)	30/120 (RI) (min/min)	↓ IFS & BP

STUDY	TYPE	MODEL	IR DURATION	MELATONIN EFFECTS
Bertuglia et al., 2007	<i>In vivo</i>	In situ heart cardiomyopathic hamsters	30/30 (min/min)	↓ post ischaemic VT and VF, lowered % lethality ; ↓ oxidative stress (lipid peroxidation: TBARS) and nitrosative stress (nitrite/nitrate), leukocyte adhesion, vascular permeability, increased capillary perfusion, reduced MAP.
Sahna et al., 2008	<i>In vivo</i>	in situ heart rat (chronic nitric oxide synthase inhibited rats)	30/120 (RI) (min/min)	↓ IFS & BP; ↓ MDA values, ↓MPO levels
Ceyran et al., 2008	<i>In vivo</i>	In situ heart rat	20/20 (min/min)	↓cardiac troponin (cTn-T), ↓MDA, ↓MPO levels ↑ SOD levels, ↑Bcl-2 expression ↓Fas expression, ↓ oedema myofibrils, vascularity, inflammatory infiltration

Legend: ↓: decrease, ↑: increase, GI: global ischaemia, RI: regional ischaemia, IR: ischaemia-reperfusion, IFS: infarct size, MPTP: mitochondrial permeability transition pore, SERCA2a,: sarco-(endo)-plasmic reticulum Ca²⁺-ATPase2, LDH: lactate dehydrogenase, VT: ventricular tachycardia; VF:ventricular fibrillation, MPO:myeloperoxidase, SOD: superoxide dismutase, MAP: mean arterial pressure, HR: heart rate, BP: blood pressure; GSH: reduced glutathione, LVDp:left ventricular developed pressure, LVEDp: left ventricular end-diastolic pressure, TBARS: thiobarbituric acid reaction substance, MDA: malondialdehyde.

2.4 MELATONIN AND OBESITY

2.4.1 LINK BETWEEN OBESITY AND MELATONIN: CIRCADIAN RHYTHM

“Circadian rhythm” classically refers to any biological process that displays an endogenous 24-hour cycle (e.g., neurohormone secretion)(Martino and Sole, 2009), while the term “diurnal” refers to conditions in which there are both an endogenously generated circadian cycle and one modulated by external cues (zeitgebers) from the environment, predominantly light(Martino and Sole, 2009). Light is the main zeitgeber for the master clock, which is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Martino and Sole, 2009). The pineal gland hormone melatonin is secreted during the dark and classically acts as a chronobiotic factor or endogenous synchronizer participating in regulation of seasonal as well as circadian rhythm (Zawilska et al., 2009).

There is a strong link between the development of obesity and a disrupted circadian system (chronodisruption) (Boer-Martins et al., 2011; Bray and Young, 2012; Gomez-Abellan et al., 2012; Reiter et al., 2012). Epidemiological and experimental studies have shown that alterations in circadian rhythm in obesity [as shown by the decrease in the amplitude of daily pineal melatonin rhythm in shift workers (Lund et al., 2001), T2D patients (Peschke et al., 2006) as well as in rats fed a high-fat diet (Cano et al., 2008)], is accompanied by changes in other circulating metabolic factors including, amongst others, glucose, insulin, leptin, corticosterone, thyroid-stimulating hormone, prolactin, luteinizing hormone and testosterone (Lund et al., 2001; Cano et al., 2008). Although the causal relationship between chronodisruption and obesity can be considered as bidirectional (Bray and Young, 2012), the supplementation of melatonin as well as melatonin agonists have been beneficial in resetting the circadian rhythm (Zawilska et al., 2009) and ameliorating the obesity-related abnormalities (She et al., 2009; Oxenkrug and Summergrad, 2010; Cardinali et al., 2011b; Hardeland, 2012).

The following section is limited to the role of melatonin in obesity and insulin resistance focusing on recent findings in animal and clinical studies. The potential underlying mechanisms are also included.

2.4.2 MELATONIN AND OBESITY: ANIMAL STUDIES

2.4.2.1 Body weight and fat mass regulation: from seasonal to non-seasonal animals

Melatonin plays an important role in body weight regulation and energy metabolism. The involvement of melatonin in the regulation of body fat mass and energy metabolism was first observed in seasonal animals (Bartness and Wade, 1985) and attributed to its role as regulator of seasonal and circadian rhythms (Arendt, 2006). In these seasonal animals, any increase in circulating melatonin levels due to photoperiodic changes or exogenous melatonin administration, depending on the animal species, was eventually associated with a reduction or an increase in body fat mass (Bartness and Wade, 1985; Bartness et al., 2002). Interestingly, non-seasonal animals like the obese Zucker rats exposed to long photoperiod conditions (characterized by a low nocturnal melatonin production) had an increased body mass gain compared to those exposed to short photoperiods (Larkin et al., 1991). In line with this, surgical removal of the pineal gland caused a reduction in circulating melatonin levels and an increase in body weight after 3 weeks in obese but not in normal rats (Prunet-Marcassus et al., 2003). When the postoperative period was extended to 2 months, the normal rats had also increased their body and heart weights (Kurcer et al., 2006). These pinealectomy-induced changes could be prevented by melatonin (30 mg/kg/day, i.p. at 1 hour before lights-out) for 3 weeks (Prunet-Marcassus et al., 2003). The same workers also showed that melatonin administered in the same manner, was able to reduce high-fat diet-induced body weight gain without affecting the total food intake (Prunet-Marcassus et al., 2003). In young normal rats a decrease in body weight and visceral fat mass was also noticed following 3 or 6 months of prolonged melatonin consumption in drinking water (4µg/mL) (Bojkova et al., 2006; Kassayova et al., 2006). Similar effects have also been reported in normal middle-aged rats (Rasmussen et al., 1999; Rasmussen et al., 2001) without altering food consumption (Wolden-Hanson et al., 2000). These metabolic effects were independent of gonadal function (Puchalski et al., 2003b).

2.4.2.2 Melatonin and obesity: effect on diet-induced obesity

Several animal investigations have consistently demonstrated the efficacy of melatonin to prevent the development of obesity or reduce obesity-related metabolic features (Prunet-Marcassus et al., 2003; Barrenetxe et al., 2004; Hussein et al., 2007; She et al., 2009; Shieh et al., 2009; Ríos-Lugo et al., 2010; Agil et al., 2011; Nduhirabandi et al., 2011) and the potential therapeutic value of melatonin treatment in obesity and MetS has recently been summarized (Nduhirabandi et al., 2012). Collectively, it was shown that long-term melatonin administration significantly reduced body weight and visceral fat mass as well as circulating glucose, insulin, leptin, TG, free fatty acid (FFA) and total cholesterol levels in young Zucker diabetic fatty rats (Agil et al., 2011; Agil et al., 2012b), in middle-aged rats fed a high-fat diet (Puchalski et al., 2003a; Ríos-Lugo et al., 2010), in young rats on a high-fat/high-sucrose diet (She et al., 2009) as well as rats drinking 10% fructose solution (Cardinali et al., 2013). In these studies, daily melatonin was administered in drinking water at 0.2-4µg/mL (Puchalski et al., 2003a), 25µg/mL (Ríos-Lugo et al., 2010; Cardinali et al., 2013) or *via* intraperitoneal injection at 4mg/kg (She et al., 2009) for a period of 8 to 12 weeks without affecting the total food intake. A study in rabbits fed a high fat diet has, however, reported a reduction in food intake after 4 weeks of melatonin treatment (1mg/kg/day subcutaneously) (Hussein et al., 2007). This weight loss inducing effect of melatonin was also confirmed in other animal models of obesity; for example, a rat model of ovariectomized-induced obesity (Sanchez-Mateos et al., 2007; Baxi et al., 2012) and female rats treated with olanzapine (Raskind et al., 2007).

2.4.2.3 Effect of melatonin on obesity-induced dyslipidaemia

Melatonin has been shown to improve obesity-induced dyslipidaemia. This was first documented in non-obese hypercholesterolaemic rats (Hoyos et al., 2000; Hussain, 2007) and thereafter confirmed in various rat models of obesity (Prunet-Marcassus et al., 2003; Agil et al., 2011; Nduhirabandi et al., 2011; Nduhirabandi et al., 2012; Zhang et al., 2012). For example, oral melatonin (4-10mg/kg/day for 6 to 12 weeks) raised the high-density-lipoprotein cholesterol (HDL-c) in both obese and lean Zucker rats (Agil et al., 2011) or in high fat/high-

sucrose diet-induced obese rats (She et al., 2009) . This has also been confirmed in obese rabbits (Hussein et al., 2007), associated with a concomitant reduction in circulating TG, FFA, low-density-lipoprotein cholesterol (LDL-c) with (Hussein et al., 2007; She et al., 2009) or without (Ríos-Lugo et al., 2010; Agil et al., 2011) effect on total cholesterol levels. Similar results were recently confirmed in ApoE-knockout C57BL/6J male mice fed a high-fat diet (Zhang et al., 2012) and in rats fed a high-fructose diet for 4 weeks (Kitagawa et al., 2012). Although the latter model did not gain weight, two weeks of co-administration of oral melatonin (10mg/kg/day) reduced the intra-abdominal fat mass and circulating FFA levels as well as hepatic TG and cholesterol contents (Kitagawa et al., 2012). These studies strongly support the suggestion that melatonin supplementation may ameliorate overweight and normalize lipid metabolism in humans.

2.4.2.4 Effect of melatonin on obesity-associated oxidative stress and inflammation

Obesity is associated with elevated oxidative stress and low antioxidant status (Vincent and Taylor, 2006; D'Archivio et al., 2011). The antioxidant as well as anti-inflammatory activities of melatonin are well established (Korkmaz et al., 2009a; Korkmaz et al., 2012) and a daily melatonin administration to obese rabbits (1mg/kg subcutaneously for 4 weeks)(Hussein et al., 2007) or rats (4mg/kg, i.p for 8 weeks) (She et al., 2009) increased GSH-Px, SOD activity and HDL-c levels and reduced oxidative stress as indicated by low plasma MDA levels. Furthermore, in young obese Zucker rats, chronic oral melatonin administration (10mg/kg/day) for 6 weeks attenuated circulating biomarkers of systemic oxidative stress [basal plasma lipid peroxidation and Fe²⁺/H₂O₂-induced lipid oxidation) and low-grade inflammation [plasma interleukin 6 (IL-6), tumor necrosis factor- α (TNF- α) and C-reactive protein (CRP) values] without affecting their profile in non-obese animals (Agil et al., 2012a). It is well established that low-grade chronic inflammation contributes to the pathogenesis of insulin resistance and diabetes as well as cardiovascular complications (de Rooij et al., 2009). The antioxidant properties of melatonin have been reported along with the improvement in the metabolic profile in diabetes (Nishida, 2005; Agil et al., 2011; Agil et al., 2012a) and diet-induced obesity

(Hussein et al., 2007; She et al., 2009). However, whether the improvement in insulin resistance by melatonin precedes or follows its suppressive effects on oxidative stress and inflammation is still not yet known.

2.4.2.5 Effects of melatonin on obesity-related cardiovascular alterations

Melatonin supplementation is able to improve cardiovascular function in lean or obese animals with insulin resistance (Leibowitz et al., 2008; Kozirog et al., 2011). A decrease in melatonin secretion was associated with hypertension in fructose fed rats (Leibowitz et al., 2008). Administration of melatonin to these fructose-induced MetS rats attenuated the increase in blood pressure and other metabolic abnormalities (Leibowitz et al., 2008; Cardinali et al., 2013). Similarly, in rabbits receiving a high-fat diet, intake of melatonin was associated with a lowering of BP, heart rate and sciatic nerve activity (Hussein et al., 2007). Melatonin prevented the appearance of fatty streaks produced by a mass of foam cells covered by the endothelium and a thin layer of mononucleated cells in the carotid artery intima of hypercholesterolemic rats (Pita et al., 2002). It was also able to prevent deposition of fat in the subintimal layer in the blood vessels and heart (Hussein et al., 2007), indicating melatonin's anti-atherosclerotic potential. In spontaneously hypertensive Wistar-Kyoto male rats, chronic (but not acute) administration of the selective melatonin receptor agonist, Ramelteon, in drinking water (8mg/kg/day, from 4 to 12 weeks of age) attenuated the age-associated increase of systolic BP by 45% (Oxenkrug and Summergrad, 2010), indicating the involvement of melatonin receptors in melatonin's effects in obesity.

Melatonin administration protected the hearts from rats with MetS (Nduhirabandi et al., 2011; Diez et al., 2013). Chronic oral melatonin consumption (4mg/kg/day for 16 weeks) starting before the establishment of obesity prevented the increase in the heart weight and protected the hearts against obesity-induced increased susceptibility to myocardial IRI (Nduhirabandi et al., 2011). As expected, it also prevented the development of obesity-induced metabolic alterations (elevated visceral fat, serum insulin, leptin, TG and reduced HDL-c) (Nduhirabandi et al., 2011). This finding was of particular significance since to date there is no effective

cardioprotective strategy available in obesity, diabetes as well as ageing conditions (Boengler et al., 2009; Downey and Cohen, 2009; Kloner and Schwartz Longacre, 2011).

How melatonin protects the heart in obesity is still unknown. From our previous findings (Nduhirabandi et al., 2011), we hypothesized that the direct effects of melatonin on the heart *via* its receptor mediated-effects may be involved but this requires further investigation. A recent study by Diez et al. (2013) using hearts isolated from insulin resistant rats fed a high fructose diet (model of MetS) with elevated oxidative stress and reduced HDL-c, reported that acute melatonin administration initiated at reperfusion (after 15 min of regional ischaemia) protected the heart against myocardial IRI as shown by a reduction in duration and amplitude of ventricular arrhythmias (Diez et al., 2013).

Globally, the melatonin-induced improvement of the myocardial structure and function in obesity and insulin resistance could be linked to the body weight loss and improvement of dyslipidaemia with eventual reduction of oxidative stress, inflammation, insulin resistance and hyperglycaemia (Hussein et al., 2007; Oxenkrug and Summergrad, 2010; Nduhirabandi et al., 2011; Cardinali et al., 2013).

2.4.3 MELATONIN AND OBESITY: HUMAN STUDIES

The overall circulating melatonin levels in obese humans are not consistent. For example, the mean nocturnal serum melatonin levels were reported to be reduced in patients with severe obesity (Shafii et al., 1997) as well as with T2D (Peschke et al., 2006), autonomic neuropathy (Tutuncu et al., 2005), retinopathy (Hikichi et al., 2011), and coronary heart disease (Cardinali et al., 2011a) as well as obese craniopharyngioma (Lipton et al., 2009). Surprisingly, despite a lack of difference in BMI or waist circumference between the obese non-diabetic and T2D subjects, nocturnal plasma melatonin levels were significantly higher in obese non-diabetic subjects compared to weight-matched T2D subjects (Mantele et al., 2012) who failed to produce any detectable melatonin (Mantele et al., 2012). However, in young male and female obese MetS patients (Robeva et al., 2006; Robeva et al., 2008) and in obese girls (Lee et al., 2012), circulating melatonin or urinary 6-sulfatoxymelatonin levels were not different from

control subjects, confirming the early observation that obesity had no effect on melatonin secretion and excretion (Rojdmark et al., 1991). Furthermore, it has been noticed that in MetS patients, the levels of melatonin *per se* are not as important as the melatonin/insulin ratio which correlates negatively with the lipid profile (Robeva et al., 2008).

The reasons for these inconsistencies in melatonin secretion in obese subjects remain complex and not clear. It is possible that the increased sympathetic tone in obesity and a consecutive alteration in sympathetic innervation of the pineal gland increased melatonin concentration in obese non-diabetic subjects (Mantele et al., 2012). Furthermore, low circulating melatonin levels have been linked to many factors including elevated oxidative stress and inflammation (Ozguner et al., 2005), but not to low testosterone levels in young men with MetS (Robeva et al., 2006). It appears therefore that circulating melatonin levels could vary depending on the age of patients and the severity of obesity (Shafii et al., 1997).

Few clinical investigations on melatonin or related drugs have considered body weight change and adiposity mass in their aims. However, as discussed above in animal studies, these parameters have been affected by the long-term melatonin treatment (Nduhirabandi et al., 2011).

2.4.4 MECHANISM OF ACTIONS OF MELATONIN IN OBESITY

As mentioned previously, the mechanism of action of melatonin in obesity is not well understood. Melatonin is a small pleiotropic molecule able to cross each membrane layer and enter each cellular compartment to exert its various activities with or without receptor-mediated pathways (Hardeland et al., 2011; Venegas et al., 2012). Melatonin receptors as well as adipose tissue function and plasticity may be involved.

2.4.4.1 Role of melatonin receptors

Involvement of the melatonin receptor in body fat mass regulation has been known for many years (LeGouic et al., 1996). Administration of a melatonin receptor agonist or antagonist to seasonal animals (before night) affected the body weight and adiposity mass regulation as well

as the onset of seasonal obesity: a melatonin agonist and the short day/light exposure had a same effect whereas an antagonist and long day/light exposure had similar effects (LeGouic et al., 1996). This involvement of MT receptors in the body weight and fat mass regulation was recently demonstrated in spontaneously hypertensive rats, using Ramelteon, a potent selective MT1/MT2 receptor agonist (Oxenkrug and Summergrad, 2010) and in obese rats, using the melatonin agonist NEU-P11 (She et al., 2009).

MT receptors have been identified in the major organs involved in metabolism: liver, pancreas and skeletal muscle (Muhlbauer et al., 2009) as well as adipose tissue (Brydon et al., 2001). They are involved in the regulation of insulin secretion and may play an active role in the regulation of glucose metabolism (Muhlbauer et al., 2009). It was recently reported that a variation in MTNR1B, the gene encoding for MT2, was associated with increased risk of T2D, increased fasting plasma glucose and impaired insulin secretion in populations of European ancestry (Bouatia-Naji et al., 2009; Prokopenko et al., 2009). Similar observations were also made in Chinese (Ling et al., 2011) as well as in Japanese (Tabara et al., 2011) populations. Thus, the overall effects of melatonin in obesity appear to be partly mediated through these receptors, in addition to the activation of the sympathetic nervous system *via* hypothalamic receptors and subsequent effects on lipolysis and adipose tissue plasticity (Song and Bartness, 2001; Bartness et al., 2002).

2.4.4.2 Role of adipose tissue

The exact mechanisms whereby melatonin reduces body fat mass are not clear. *In vivo* melatonin treatment prevents the increase in circulating TG and eventually body fat accumulation and weight gain in overweight and obese animals (Nduhirabandi et al., 2011; Agil et al., 2012b). *In vitro* melatonin treatment of adipocytes inhibits differentiation and limits adipose tissue hypertrophy (Alonso-Vale et al., 2009) by inhibiting fatty acid-induced TG accumulation in cells exposed to physiological levels of oleic acid (Sanchez-Hidalgo et al., 2007). The reduction in body weight gain might be due to a significant decrease in fat content as opposed to lean body mass (Wolden-Hanson et al., 2000) and could be related to

melatonin-induced improvements in the compromised insulin and leptin signalling associated with obesity (Sartori et al., 2009), accompanied by modulation of plasma levels of insulin, glucose, TG, cholesterol, leptin (Ríos-Lugo et al., 2010) .

The involvement of brown adipose tissue has also been suggested (Tan et al., 2011). While white adipose tissue is specialized in energy storage, brown adipose tissue has a high concentration of mitochondria and uniquely expresses uncoupling protein 1 (UCP-1), enabling it to specialize in energy expenditure and thermogenesis (Townsend and Tseng, 2012). Brown adipose tissue has been suggested to be the factor whereby animals lose weight in response to melatonin administration (and gain weight when there is a deficiency of melatonin), independently of food intake (Tan et al., 2011). Importantly, for the first time, oral melatonin supplementation has recently been shown to induce browning of the inguinal white adipose tissue in ZDF rats without affecting their locomotor activity (Jiménez-Aranda et al., 2013). Thus, the exploitation of the functional role of brown adipose tissue could be of great interest in obesity management.

2.4.4.3 Leptin resistance

Leptin resistance is an essential feature of human obesity and refers to the inability of elevated circulating leptin levels to reduce common obesity (Scarpace and Zhang, 2009). The adipokine leptin plays a central role in the regulation of food intake, body weight and energy expenditure (Benoit et al., 2004). Resistance to leptin action is associated with insulin resistance and an increased pro-inflammatory state (Lago et al., 2008). The effects of melatonin on leptin resistance have recently been reviewed (Nduhirabandi et al., 2012). At molecular levels, it has been found that melatonin, leptin and insulin activated the same intracellular signalling pathways namely PI3-K and STAT-3 (Carvalho et al., 2001; Anhe et al., 2004; Picinato et al., 2008). Therefore, melatonin may affect obesity by mimicking the actions of insulin and leptin signalling *via* cross-talk between these pathways. In this regard, insulin has been shown to modulate leptin-induced STAT-3 activation in rat hypothalamus (Carvalho et al., 2001). Thus, melatonin may act initially on the hypothalamic insulin and

leptin receptor sensitivity (as these hormones do under normal conditions) and eventually relay information about peripheral fat stores to central effectors in the hypothalamus to modify food intake and energy expenditure (Song and Bartness, 2001; Benoit et al., 2004).

2.5 MELATONIN AND INSULIN RESISTANCE

Resistance to insulin action results in increased postprandial and fasting circulating insulin levels in order to normalize glycaemia in pre-diabetic subjects and is closely associated with dyslipidaemia and other metabolic abnormalities (Benito, 2011; Tesouro and Cardillo, 2011). This section focuses on the effect of melatonin on insulin sensitivity and resistance, glucose uptake and tolerance and insulin secretion.

2.5.1 EVIDENCE FOR THE ROLE OF MELATONIN IN INSULIN RESISTANCE

Endogenous melatonin has been shown to play a role in the regulation of insulin secretion and glucose/lipid metabolism (Nishida, 2005; Peschke, 2008; Peschke and Muhlbauer, 2010). In normal rats, pinealectomy induced insulin resistance, glucose intolerance (Lima et al., 1998; Zanquetta et al., 2003) and increased serum cholesterol (Mizrak et al., 2004). To demonstrate the role of endogenous melatonin on insulin secretion, Nishida *et al.* (Nishida et al., 2003) using T2D rats, showed that after 21 weeks of pinealectomy, a significant increase in plasma insulin and accumulation of TG occurred. The same study found also that when the post-pinealectomy period was extended to 35 weeks, circulating insulin levels were significantly decreased. This decrease is a clear indicator of impairment of insulin release from pancreatic β -cells as seen in patients at an advanced stage of T2D (Kuzuya et al., 2002). Additionally, it was found that pineal gland melatonin synthesis is decreased in T2D Goto-Kakizaki rats (Frese et al., 2009).

In view of insulin resistance preceding the establishment of T2D, the question whether melatonin replacement could be beneficial in reversing insulin resistance has been a subject of numerous investigations in the field. In this regard, it was found that long term melatonin consumption (2.5 mg/kg/day for 9 weeks) increased plasma melatonin levels with a concomitant reduction in insulin levels in type 2 diabetic Goto-Kakizaki rats (Peschke et al.,

2010). In mice fed a high-fat diet, 8 weeks oral melatonin (100mg/kg/day) markedly improved insulin sensitivity and glucose tolerance (Sartori et al., 2009). In the same model of high-fat diet-induced diabetic mice, following 2 weeks of melatonin administration (10mg/kg/day, i.p), amelioration of insulin resistance and glucose intolerance were associated with an increase in hepatic glycogen and improvement in liver steatosis (Shieh et al., 2009). Consistently, in high fat/high sucrose-fed rats, 8 weeks treatment with melatonin or its agonist NEU-P11 increased insulin sensitivity (She et al., 2009). In rats with T2D, 30 weeks of melatonin treatment (1.1 mg/kg/day, subcutaneously *via* implanted melatonin-releasing pellets) reduced circulating insulin, leptin and TG levels (Nishida et al., 2002). These findings were also confirmed by additional studies in young Zucker diabetic fatty rats (Agil et al., 2011; Agil et al., 2012b) and fructose-fed rats (Kitagawa et al., 2012; Cardinali et al., 2013). In the latter model administration of melatonin (1 or 10mg/kg/day for 2 weeks) improved the abnormal serum insulin response curve in oral glucose tolerance tests (Kitagawa et al., 2012), indicating potential insulin sensitizing effects of melatonin.

Interestingly, in a recent study of more than 1000 young women without hypertension, obesity and T2D, elevated nocturnal melatonin secretion was independently associated with greater insulin sensitivity and a lower prevalence of insulin resistance (McMullan et al., 2013). In adults with T2D daily melatonin treatment diminished oxidative stress and enhanced glycemic control (Grieco et al., 2013).

2.5.2 MECHANISM OF ACTIONS OF MELATONIN IN INSULIN RESISTANCE

The melatonin-induced reduction in circulating insulin levels in obese animals may be linked to a reduced body weight and improved lipid metabolism as was recently demonstrated in young ZDF rats (Agil et al., 2011) or rats drinking 10% fructose solution (Cardinali et al., 2013). In these rat models, the amelioration of insulin resistance was also characterized by improvement in glucose tolerance (Agil et al., 2012b; Cardinali et al., 2013). In addition, in young ZDF, melatonin treatment reduced fasting blood glucose, plasma insulin, haemoglobin A1c (HbA1c), HOMA-IR, FFA levels and increased index of beta-cell function (Agil et al., 2012b).

In the insulin resistant state, the improvement of lipid and glucose regulation could be also linked to amelioration of the pro-inflammatory state and oxidative stress (Agil et al., 2012a). It is well established that oxidative stress and pro-inflammatory states are important pathological features that underlie the development of insulin resistance, MetS, diabetes, and CVD. Therefore, in insulin resistant conditions, the reduction in oxidative stress and the pro-inflammatory state may lead to lowering of lipid peroxidation resulting from free radicals generation, due to the continuous hyperglycaemia and hyperlipidaemia. Consistently, melatonin administration for 2 or 6 weeks (1 or 10mg/kg per day) attenuated the levels of circulating IL-6 and TNF- α (Agil et al., 2012a; Kitagawa et al., 2012). This amelioration of the pro-inflammatory state was also accompanied by a reduction in serum and hepatic lipid peroxidation and an increase in hepatic GSH concentration (Kitagawa et al., 2012). Interestingly, melatonin treatment was associated with increase in serum adiponectin levels and reduction in leptin levels with (Agil et al., 2012b) or without (Kitagawa et al., 2012) effects on body weight. Adiponectin (which is reduced in obese subjects) has been shown to have insulin sensitizing actions in the liver and peripheral tissues and other beneficial properties associated with cardiovascular protection (anti-apoptotic, anti-inflammatory and anti-atherogenic properties) (Hui et al., 2012). Therefore, increased circulating adiponectin levels may play an important role in melatonin's effects.

On a molecular level, insulin resistance is associated with an abnormal or compromised intracellular insulin signalling cascade in peripheral tissues/organs involved in the regulation of glucose metabolism (skeletal muscle, liver and adipose tissue). As mentioned above (see also section 2.1.2.2), this cascade principally includes binding of insulin to insulin receptor (IR), Tyr phosphorylation of IRS proteins and activation of phosphatidylinositol-3-kinase (PI3-K), PKB/Akt and PKC ζ or λ [for details see (Benito, 2011)]. Melatonin (1nM) treatment has been shown to stimulate glucose transport in skeletal muscle *via* the phosphorylation and activation of IRS-I and PI3-K respectively (Ha et al., 2006). It was further demonstrated that melatonin improves glucose homeostasis by restoring the vascular actions of insulin which were characterized by increased phosphorylation of PKB/Akt and eNOS in aortic tissue (Sartori et

al., 2009). In addition to the phosphorylation of PKB/Akt and PKC- ζ , melatonin (1nM) stimulated glycogen synthesis and increased the phosphorylation of glycogen synthase kinase 3 β (GSK3- β) in hepatic cells (Shieh et al., 2009). More interestingly, these effects of melatonin could be blocked by the non-selective MT1/MT2 antagonist, luzindole, or the MT2 selective antagonist, 4-phenyl-2-propionamido tetraline (4P-PDOT) (Ha et al., 2006; Shieh et al., 2009), suggesting possible MT receptor involvement. However, it is not clear how activation of the high affinity MT receptors, which are G-protein linked, leads to stimulation of the IRS-1/PI3-K pathway and the role of PKC- ζ in this regard.

Using adipose tissue from female fruit bat, *Cynopterus sphinx* (seasonal animal), it was shown that melatonin treatment (100ng/mL and 500pg/mL) enhanced the insulin-stimulated glucose uptake compared to untreated cells (Banerjee et al., 2011). There was however no correlation between glucose uptake and the expression of GLUT-4 in these cells (Banerjee et al., 2011). Pinealectomy was shown to reduce the expression of GLUT-4 translocation (to the plasma membrane) in adipose tissue (Lima et al., 1998; Zanquetta et al., 2003). Although a decrease in GLUT-4 gene expression was reported following melatonin treatment (1 μ M for 14 days) in human brown adipocyte cells lines (PAZ6) (Brydon et al., 2001), Zanquetta et al. (2003) found that 30 days of calorie restriction or melatonin replacement (50 μ g/100g/day, i.p.) to pinealectomized rats was accompanied by an improvement in insulin resistance and increased plasma membrane GLUT-4 protein content in adipose tissue. Importantly, in the hyperthyroid rat heart, melatonin administration was able to protect the heart against oxidative damage and restored expression of GLUT-4 gene, establishing the ability of antioxidants to reverse oxidative stress-mediated metabolic alterations (Ghosh et al., 2007). However, whether melatonin affects glucose metabolism in the normal or obese heart is still not yet explored.

Melatonin receptors may play an important role in the regulation of glucose metabolism. Removal of the MT1 receptor significantly impairs the ability of mice to metabolize glucose and probably induces insulin resistance in these animals (Contreras-Alcantara et al., 2010). Epidemiological studies have also revealed that variants near/in the MTNR1B (or MT2)

receptor are associated with impaired pancreatic beta-cell function as shown by impaired insulin secretion and concomitant elevated plasma fasting glucose levels (Kan et al., 2010; Tam et al., 2010). Indeed, MT1/MT2 receptors are expressed in pancreatic islets (Peschke et al., 2000) and since insulin levels exhibit a nocturnal drop, its production has been suggested to be controlled, at least in part, by melatonin (Mulder et al., 2009). Melatonin reduced the fasting insulin levels (Puchalski et al., 2003a; She et al., 2009) probably *via* its inhibitory effects on insulin secretion in rat pancreatic islets (Picinato et al., 2002; Peschke, 2008). On other hand, melatonin has been shown to act through MT1/MT2 receptors to activate hypothalamic PKB/Akt and suppress hepatic gluconeogenesis (Faria et al., 2013) while increasing peripheral glucose utilization (Sartori et al., 2009; Shieh et al., 2009).

2.5.3 INTERACTION OF CATECHOLAMINES, GLUCAGON, INSULIN AND MELATONIN IN GLUCOSE REGULATION

The interaction between catecholamines, glucagon, insulin and melatonin in glucose regulation has recently been reviewed by Peschke et al. (2013). Although the mechanism underlying the interactions between these hormones is complex and not yet clear, catecholamines have been indicated as a key feature to understand the biological relevance of insulin-melatonin antagonism in T2D as well as T1D (Peschke et al., 2012). It was found that catecholamines (noradrenaline and adrenaline) and melatonin levels were reduced in T2D GK rats (characterized by high insulin levels) and elevated in T1D rats (associated with reduced insulin levels) (Peschke et al., 2012), assuming that elevated catecholamines decrease insulin secretion *via* stimulation of melatonin synthesis (Peschke et al., 2012).

Glucagon is the hormone secreted from pancreatic α -cells under hypoglycaemic conditions. It plays an important role in blood glucose homeostasis *in vivo*: it acts as an antagonistic hormone to insulin and stimulates hepatic glucose output (Peschke et al., 2013). Melatonin stimulates glucagon secretion in pancreatic α -cells and its action in the liver (Peschke et al., 2013). It has been shown that oral melatonin treatment of T2D GK rats for nine weeks, increased hepatic expression of glucagon receptor mRNA, while this was reduced in healthy

wistar rats (Bahr et al., 2011), indicating the regulatory effects of melatonin on hepatic glucose and disturbances of these effects in T2D.

2.6 CONCLUDING REMARKS

Convincing experimental evidence supports the potential use of melatonin in cardioprotection. Considering that it is cheaper and sold over the counter, extensive clinical trials are required to establish the clinical potential of melatonin treatment in a number of pathologies. Currently only one prospective trial (Melatonin Adjunct in the acute myocaRdial Infarction treated with Angioplasty [MARIA]) is underway investigating whether pharmacological doses of melatonin in patients with ST-segment elevation MI will confer cardioprotection (Dominguez-Rodriguez et al., 2007). The outcome of this trial is very important as to date there is no effective strategy to save lives of people dying from MI, the top leading killer in the world. In the context of obesity and insulin resistance, the effect of melatonin is also still lacking clinical evidence. In this regard, the few clinical studies that used melatonin did not consider its potential actions in obesity and insulin resistance. Fortunately, a randomized controlled trial (n^o 01038921) of melatonin supplementation in men and women with the MetS has recently been designed (Terry et al., 2013). Collectively, current literature suggests that melatonin treatment may influence and improve metabolic abnormalities present in obese patients and prevent the development of CVD. Besides its strong antioxidant properties, the overall metabolic actions of melatonin in obesity and insulin resistant conditions appear to be a combined result from its various pleiotropic activities associated with multiple signalling in areas of the central nervous system and in peripheral organs (Hardeland et al., 2011).

Table 2.4 Potential effect of melatonin treatment on obesity and insulin resistance

Parameters/organ		Before treatment	Treatment effects	References
Body weight		↑	↓	(Rasmussen et al., 1999; Wolden-Hanson et al., 2000; Prunet-Marcassus et al., 2003; Puchalski et al., 2003a; Hussein et al., 2007)
Visceral fat		↑	↓	(Rasmussen et al., 1999; Prunet-Marcassus et al., 2003; Puchalski et al., 2003a; She et al., 2009)
Blood	Fasting glucose	↑	↓	Prunet-Marcassus et al., 2003; Shieh et al., 2009; Peschke et al., 2010; Rios-Lugo et al., 2010)
	Insulin	↑	↓	(Nishida et al., 2002; Puchalski et al., 2003a; Sartori et al., 2009; Shieh et al., 2009; Peschke et al., 2010)
	HDL-C	↓	↑	(Hussain, 2007; Hussein et al., 2007; She et al., 2009; Agil et al., 2011)
	LDL-C	↑	↓	(Hussein et al., 2007; Agil et al., 2011; Kozirog et al., 2011)
	TG	↑	↓	(Nishida et al., 2002; Hussain, 2007; Rios-Lugo et al., 2010; Agil et al., 2011; Kozirog et al., 2011)
	VLDL	↑	↓	(Hoyos et al., 2000)
	Leptin	↑	↓ or ↑	↓ (Wolden-Hanson et al., 2000; Nishida et al., 2002; Prunet-Marcassus et al., 2003; Puchalski et al., 2003a) or ↑ (Baltaci and Mogulkoc, 2007; Song and Chen, 2009)
	Adiponectin	↓ or ↑ (?)	↑ or ↓ (?)	↑ (Agil et al. 2011b, Kitagawa et al. 2011) or ↓ (Rios-Lugo et al., 2010) (?)
	TNF- α	↑	↓	(Nishida, 2005; Oztekin et al., 2006)
Muscle	Insulin sensitivity (glucose uptake)	↓	↑	(Sartori et al., 2009; Srivastava and Krishna, 2010)
	FFA /TG uptake	↑	↓	(Dauchy et al., 2003)
	Glycogen content	↓	↑	(Mazepa et al., 2000)

Table 2.4 Potential effect of melatonin in obesity and insulin resistance (continued)

Parameters/organ		Before treatment	Treatment effects	References
Liver	Fibrinogen secretion	↑	↓	(Bekyarova et al., 2010)
	TG & VLDL release	↑	↓	(Hussain, 2007)
	Glucose release	↑	↓	(Kaya et al., 2010)
	C-reactive protein	↑	↓	(Bekyarova et al., 2010)
	Glycogen content	↓	↑	(Shieh et al., 2009)
	Liver weight	↑	↓	(Pan et al., 2006)
Heart & Vessels	Blood pressure	↑	↓	(Paulis and Simko, 2007; Kozirog et al., 2011)
	Heart rate	↑	↓	(Hussein et al., 2007)
	Endothelial dysfunction	↑	↓	(Sartori et al., 2009)
	Cardiomyopathy /Hypertrophy	↑	↓	(Bertuglia and Reiter, 2007; Ghosh et al., 2007)

Table 2.4 Only some references have been indicated in this table as evidence of the potential effects of melatonin in obesity and insulin resistance.

↓: decreases or attenuates; ↑: increases or elevates; (?): needs more studies/unusual model of obesity; FFA: free fatty acids; TNF-α: tumor necrosis factor-alpha; VLDL: very low density lipoprotein; HDL-C: high density lipoprotein-cholesterol; LDL-C: Low density lipoprotein-cholesterol; TG: triglyceride (Nduhirabandi et al., 2012).

2.7. MOTIVATION FOR THE INVESTIGATION

2.7.1. PROBLEM STATEMENT

As reviewed above, current literature considers obesity as the driving force behind the rising prevalence of various metabolic disorders including amongst others T2D and CVD (Cornier et al., 2008). Insulin resistance is suggested to be important mechanism linking obesity to its cardiovascular disorders such as heart failure, coronary heart diseases, atherosclerosis as well as hypertension (Reaven et al., 2004; Poirier et al., 2006; Reaven, 2011). Importantly, impairment of myocardial glucose uptake and metabolism may contribute to the mechanical dysfunction observed in T2D and obese patients (Chess and Stanley, 2008) and increases their susceptibility to ischaemic heart disease (Cubbon et al., 2013), the worldwide leading cause of death (Lozano et al., 2013).

In view of the multiple abnormalities associated with obesity and insulin resistant states, the question has arisen whether anti-oxidant supplementation would attenuate or prevent their detrimental actions. Experimental studies provided a compelling evidence for melatonin as potential tool for effective therapy in obesity and related metabolic abnormalities (for review, see (Cardinali et al., 2011a; Nduhirabandi et al., 2012). Additionally, melatonin administration has a strong cardioprotective effect in normal healthy and hypertensive animals (Reiter et al., 2010a). A previous study from our laboratory showed that chronic melatonin treatment starting before the establishment of obesity prevented the harmful effects of diet-induced obesity and protected the hearts against IRI (Nduhirabandi et al., 2011). However, the exact mechanism whereby melatonin exerts its beneficial effects on the ischaemic heart in obesity and insulin resistant states remains to be established. In addition, it is not known whether short-term melatonin treatment in established obesity is able to confer cardioprotection.

Obesity and insulin resistance are associated with changes in cardiac mitochondrial function including reduced oxidative activity as well as depressed ATP synthesis (Boudina and Abel, 2006;

Boudina and Abel, 2007; Essop et al., 2009). Melatonin has been shown to protect against mitochondrial dysfunction [for review, (Acuna-Castroviejo et al., 2007; Srinivasan et al., 2011)]. Additionally, its cardioprotective action has recently been associated with inhibition of the opening of the MPTP (Petrosillo et al., 2009). However, the effect of melatonin on the mitochondria has not yet been studied in obesity.

Recent findings support the role of melatonin in glucose homeostasis. Melatonin stimulated glucose transport in skeletal muscle cells *via* the IRS-1/PI3-K pathway (Ha et al., 2006; Sartori et al., 2009). However, nothing is known about the effects of melatonin on myocardial glucose metabolism in healthy controls or in obese animals. In view of the significant role of anaerobic glycolysis in the survival of the ischaemic myocardium (Opie, 2004), it is possible that some of the beneficial actions of melatonin may be attributed to its actions on myocardial glucose uptake and metabolism.

In addition to the above, one of the features of obesity and insulin resistance is the development of a pro-inflammatory state (Furukawa et al., 2004; Martins et al., 2012). This is characterized by elevated pro-inflammatory cytokine levels including, amongst others, TNF- α and has been linked to endothelial dysfunction (Hotamisligil, 1999; Picchi et al., 2006). Melatonin treatment has been shown to ameliorate inflammation (Nishida, 2005; Agil et al., 2012a) and to improve endothelial dysfunction (Sartori et al., 2009; Hu et al., 2013; Rodella et al., 2013) in obesity and insulin resistance. However, the effect of melatonin on endothelial function has not yet been investigated in the context of the heart. Endothelial dysfunction plays an important role in MI (Libby, 2013). However, most of the studies done on the effect of melatonin on endothelial cells have been using cells from other vascular beds than the heart (Silva et al., 2007). In particular, the effect of melatonin on cardiac microvascular endothelial cells has not yet been investigated.

2.7.2. GENERAL AIM

In view of the above, we aimed to further investigate the manner in which and the mechanism whereby melatonin exerts its cardioprotective effects with particular emphasis on its effects on myocardial glucose homeostasis, mitochondrial function and cardiac microvascular endothelial function in normal and insulin resistant states. In this regard, signalling pathways associated with cardioprotection were also investigated.

2.7.3. HYPOTHESES

We hypothesized that obesity and insulin resistance as well as other related metabolic abnormalities would contribute to increased myocardial susceptibility to IRI and exacerbate infarct size *via* oxidative stress. By virtue of its pleiotropic actions and its antioxidant activity in particular, administration of melatonin would:

- (i) reverse the metabolic abnormalities associated with obesity and insulin resistance;
- (ii) protect cardiac mitochondrial function against adverse effects of obesity and insulin resistance;
- (iii) protect the heart against myocardial ischaemia-reperfusion damage;
- (iv) stimulate glucose uptake and metabolism in normal and insulin resistant cardiomyocytes;
- (v) prevent or reverse TNF- α -induced endothelial dysfunction

2.7.4. SPECIFIC AIMS AND OBJECTIVES (fig.2.10)

1. Investigation of the effects of melatonin treatment in a rat model of diet-induced obesity and insulin resistance by determining the effects of varying periods of melatonin administration (3 weeks and 6 weeks) on biometric and metabolic parameters, lipid peroxidation, myocardial IRI and myocardial signalling including activation of PKB/Akt, ERK42/44, GSK-3 β , PI3-K, STAT-3 and GLUT-4 expression (see chapter 4, Study I)

2. Investigation of the effects of melatonin treatment (6 weeks) on myocardial mitochondrial function in a rat model of diet-induced obesity and insulin resistance by determining basal and post-anoxic mitochondrial oxidative phosphorylation capacity (see chapter 5, Study II)
3. Investigation of the effects of melatonin treatment on myocardial glucose homeostasis (see chapter 6, Study III) with regard to:
 - (i) Acute effects of melatonin treatment on glucose uptake by cardiomyocytes isolated from young untreated rats
 - (ii) Acute effects of melatonin on glucose uptake by cardiomyocytes isolated from untreated control and obese rats
 - (iii) Effects of long-term (6 weeks) melatonin treatment on glucose uptake by cardiomyocytes isolated from control and obese rats
4. Investigation of the effects of acute melatonin treatment on cardiac microvascular endothelial cells (see chapter 7, Study IV) with regard to:
 - (i) Melatonin's effects on cell viability and nitric oxide production
 - (ii) Effects of melatonin on TNF- α -induced endothelial dysfunction by determining expression and phosphorylation of eNOS, PKB/Akt, ERK42/44, STAT-3 and AMPK, expression of iNOS and I κ B- α and the levels of nitrotyrosine.

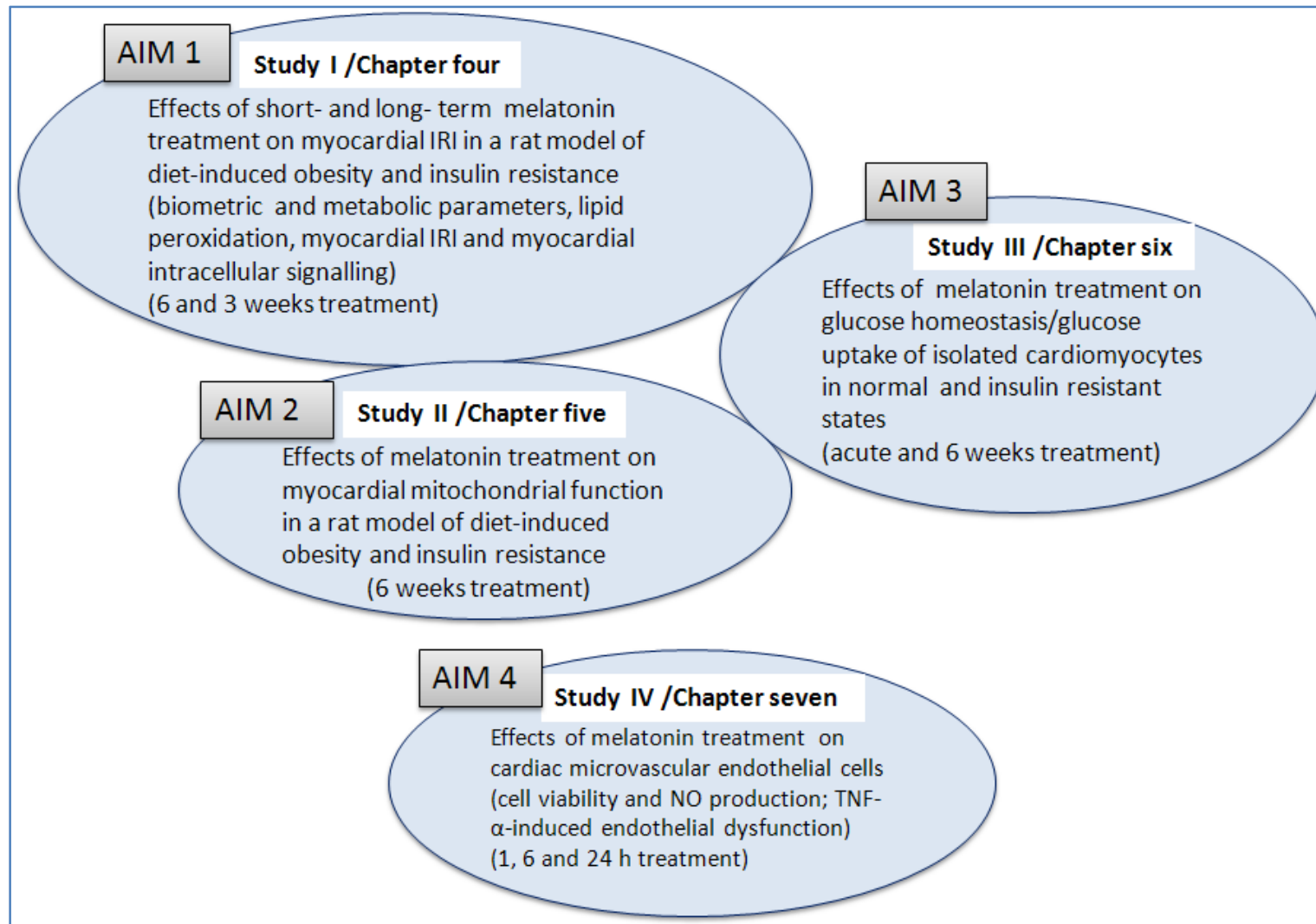


Figure 2.10 Synthetic diagram of the investigation with specific aims. IRI: ischaemia-reperfusion injury. Studies I, II and III used normal and insulin resistant rats; study IV used cultured cardiac microvascular endothelial cells.

CHAPTER THREE

MATERIALS AND METHODS: GENERAL

3.1. STUDY DESIGN

The present investigation was divided in four sub-studies according to their specific aim (fig. 2.10). Only general materials and methods including animals, feeding, treatment, western blotting technique and statistical analysis or any method used in more than two sub-studies will be described. Additional materials and methods are described along with the specific aims within their respective sub-study.

3.2. ANIMALS

All animals used in the present investigation were obtained from the University of Stellenbosch Central Research Facility. They were housed with free access to water and food, a 12-h dark/light cycle (light from 6:00 a.m. to 6:00 p.m.) with temperature and humidity kept constant at 22°C and 40%, respectively. The experimental procedure was assessed and approved by the Committee for Ethical Animal Research of the Faculty of Health Sciences, University of Stellenbosch (Ethical clearance no P08/05/008). Animals were treated according to the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

3.2.1. GROUPING AND FEEDING

To determine the effects of melatonin on the impact of obesity and insulin resistance on the heart, the feeding regime of our existing model of diet-induced obesity (Du Toit et al., 2005) was extended from 16 to 20 weeks to exacerbate the effects of obesity. This was first tested in our preliminary study on isolated cardiomyocytes from rats fed for 16 to 19 weeks and 20 to 23 weeks. Figure 3.1 represents how animals were grouped and their respective feeding and treatment programmes. Briefly, at the age of four weeks, male Wistar rats weighing 200 ± 20 g were

randomly allocated to the following groups: control rats receiving a standard commercial rat chow (SRC) and drinking water (group C); diet-induced obesity rats, receiving a high calorie diet (HCD) and drinking water (group D). After 14 weeks of feeding, melatonin was added to drinking water of a number of rats from group C or D for 6 weeks (groups CM6 and DM6). After 17 weeks of feeding, melatonin was added to the drinking water of a number of rats from group C or D for 3 weeks (groups CM3 and DM3). The rats remaining in groups C and D continued their respective diets with water without melatonin. For all animals, the duration of feeding was 20 weeks. The food was replaced daily.

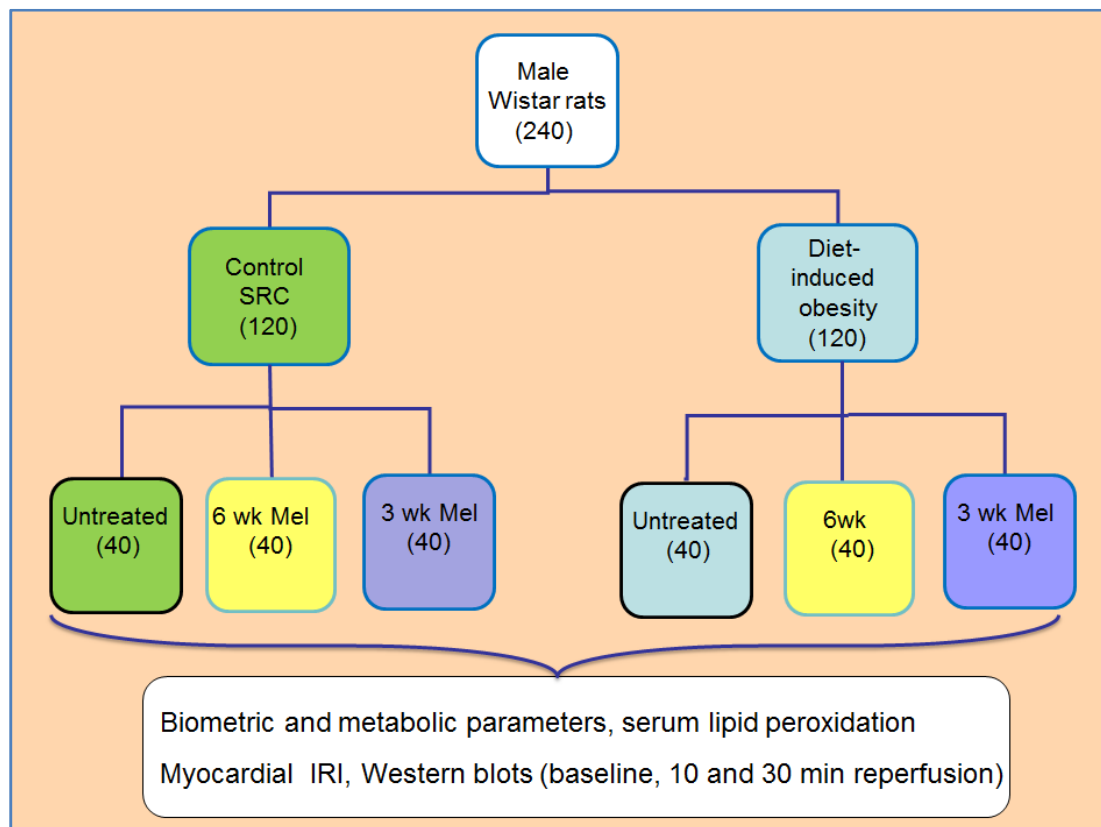


Figure 3.1 Simplified diagram for animal grouping, feeding and treatment, SRC: standard rat chow, Mel: melatonin, wk: week, IRI: ischaemia-reperfusion injury. Only the number of animals used for IRI and western blots (study I) (240) are indicated for each group. The numbers of animals used for mitochondrial function (study II) (48) and glucose uptake (study III) (60) are not

included in the diagram. Diagrams for these two studies are presented in chapters 5 and 6, respectively.

Diet-induced obesity groups (D, DM6 and DM3) were fed a high calorie diet consisting of 65% carbohydrates, 19% protein and 16% fat. Control groups (C, CM6 and CM3) were fed a SRC consisting of 60% carbohydrate, 30% protein and 10% fat. The high calorie diet was prepared containing 33% SRC and 33 % sweetened full cream condensed milk (Clover®), 7% sucrose and 27% water. Rats receiving a standard rat chow and a high calorie diet consumed approximately 371 ± 18 and 570 ± 23 kJ of energy per day, respectively (Du Toit et al., 2008).

3.2.2. MELATONIN TREATMENT

Melatonin (Sigma Aldrich, St Louis, MO, USA) was orally administered as previously reported (Nduhirabandi et al., 2011). It was first dissolved in a small amount of absolute ethanol and then added to the drinking water at a final concentration of 0.05% (v/v) ethanol with melatonin (60µg/mL or 40µg/mL). Bottles containing melatonin solution were covered with aluminium foil as melatonin is light sensitive. Animals drank water and melatonin (4mg/kg/day) *ad libitum*. Drinking water with or without melatonin was replaced every day one hour before light off as previously indicated (Wolden-Hanson et al., 2000; Prunet-Marcassus et al., 2003). The melatonin solution was prepared every second day and stored at -4°C until used. The dosage of 4mg/kg/day was regularly adjusted according to the body weight gain and water consumption for the entire period of treatment.

Procedures employed in the studies on acute effects of melatonin on cardiomyocytes and endothelial cells are described in chapters 6 and 7, respectively.

3.2.3. WESTERN BLOT TECHNIQUE

3.2.3.1. Heart tissue collection

Heart tissues were collected either at baseline or at 10 and 30 min of reperfusion after ischaemia (see experimental procedure in chapter 4. To obtain baseline values, the hearts were rapidly rinsed in ice-cold buffer and the surrounding tissues removed before freeze-clamping. These “baseline hearts” are also referred to as “non-perfused hearts”. In all conditions, the hearts were freeze-clamped with pre-cooled Wollenberger tongs and plunged into liquid nitrogen for subsequent biochemical analyses.

3.2.3.2. Preparation of lysates

Lysates were prepared differently depending on the type of the sample: heart tissues, cardiomyocytes or endothelial cells. Only the preparation of lysates from the heart tissue will be summarized here. Where applicable, modified lysate preparations are described in their specific sub-study. Lysates were prepared from whole heart tissue, cytosolic or nuclear fraction where applicable. The tissue extract was performed as follows: the frozen tissue was pulverized and homogenized in 600 to 900 μ L of lysis buffer by a Polytron PT10 homogenizer. The lysis buffer contained (in mM) Tris-HCl 20, *p*-nitrophenyl phosphate 20, EGTA 1.0, EDTA 1.0, NaCl 150, tetra-sodium-pyrophosphate 2.5, β -glycerophosphate 1.0, sodium orthovanadate 1.0, phenyl methyl sulphonyl fluoride (PMSF) 1.0, aprotinin 10 μ g/mL and leupeptin 10 μ g/mL, Triton-X100 1%, pH 7.4. Samples were then centrifuged at 1000 \times g for 10 min to obtain the supernatant, which was subsequently used for western blotting. Preparation of lysates from the cytosolic and nuclear fractions procedure are described in chapter 4.

To obtain equal loading during the separation process, the protein content of each sample was determined using the Bradford technique (Bradford, 1976). Thereafter, the lysates were adjusted accordingly by dilution in Laemmli sample buffer and boiled for 5min. The Bradford reagent

composition includes: (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol, and 8.5% (v/v) phosphoric acid.

3.2.3.3. Protein separation and transfer

Before separation, each sample was boiled for 5 min. Depending on the type of protein and available volume (lysates), 30 to 60 µg of protein was loaded and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the standard BIO-RAD Mini-Protean III system. The gel was made up of a 10 or 12% polyacrylamide gel (resolving gel) and 4% stacking gel on the top of resolving gel (see table 3.1).

The running buffer contained (in mM) Tris 250, glycine 192 and 1% sodium dodecyl sulphate (SDS). The electrophoresis separation technique is based on the mobility of proteins in an electric field. Samples, including the commercial molecular weights marker, were loaded into wells in the gel. The marker helped to localize exactly the level of each protein separated, according to its molecular weight. After separation, the proteins were transferred to a PVDF membrane (Immobilon™ P, Millipore) using a wet electrotransfer set up. The transfer buffer contained (in mM) Tris-HCl 25, glycine 192, and methanol (20% v/v). These membranes were routinely stained with Ponceau red reversible stain for visualization of proteins and to confirm an adequate transfer.

3.2.3.4. Blocking of membrane and incubation with antibodies

Depending on the protein, non-specific binding sites on the membranes were blocked with 5% fat-free milk in TBST (Tris-buffered saline + 0.1% Tween 20) for 1 to 2h at room temperature. It was then washed with TBST (5x5 min) and incubated overnight at 4°C with the primary antibodies that recognize total or phospho-specific proteins. After overnight incubation, membranes were washed with TBST (5x5 min) and then incubated for 1h at room temperature, with a diluted horseradish peroxidase-labelled secondary antibody (Cell Signalling Technology®). The secondary antibody was either diluted in TBST or in TBST containing 5% fat free milk.

Table 3.1. Content of SDS-polyacrylamide gel

Reagent	Stock	7% gel	10% gel	12% gel	4% stack gel
dH ₂ O	distilled	4.65mL	4.9 mL	3.35 mL	3.05 mL
Tris-HCl (pH 8.8)	1.5 M	2.50mL	2.50 mL	2.50 mL	_____
Tris-HCl (pH 8.8)	0.5 M	_____	_____	_____	1.25 mL
SDS	10%	90µL	100 µL	100 µL	50 µL
Acrylamide	40%	1.7mL	2.50 mL	3.0 mL	0.5 mL
APS	10%	50µL	50 µL	50 µL	50 µL
TEMED	99%	20µL	20 µL	20 µL	10 µL

Primary antibodies used: total STAT-3 and phospho-STAT-3 (Ser-727), phospho-STAT-3 (Tyr-705), total eNOS and phospho-eNOS (Ser-1177), total ERK p42/p44 and phospho-ERK p42/p44 (Thr-202/Tyr-204); total PKB/Akt and phospho-PKB/Akt (Ser-473); total GSK-3 β and phospho-GSK-3 β (Ser 9), total p38 MAPK and dual phospho-p38 MAPK (Thr-180/Tyr-182); total AMPK α and phospho-AMPK (Thr-172), Glut-4, I κ B- α , iNOS (NOS2) and nitrotyrosine. Except iNOS and Nitrotyrosine which were purchased from Santa Cruz Biotechnology, Inc, all other antibodies were from Cell Signalling Technology®. The primary antibodies were diluted in TBST solution or in 5% milk solution.

3.2.3.5. Immunodetection or visualisation

After thorough washing with TBST, membranes were covered with ECL (enhanced chemiluminescence) detection reagents (Amersham, LIFE SCIENCE) and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103-Amersham, LIFE SCIENCE) to detect light emission through a non-radioactive method (ECL western blotting). Films were densitometrically

analysed (UN-SCAN-IT, Silk Scientific Inc., Orem, Utah, USA) and protein activation was expressed as a ratio of phospho- to total protein while protein expression or phosphorylation was expressed as arbitrary densitometric units.

3.3. DATA ANALYSIS

Unless stated otherwise, all data were expressed as mean \pm standard error of the mean (SEM). For comparative studies, Student's t-test (unpaired), one- or two-way ANOVA analyses (with Bonferroni post-test, if $p < 0.05$) where applicable were used for statistical analyses. A p-value of <0.05 was considered as significant.

STUDY I

**THE EFFECTS OF SHORT- AND LONG-TERM MELATONIN TREATMENT ON
MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY IN A RAT MODEL OF OBESITY
AND INSULIN RESISTANCE**

CHAPTER FOUR

STUDY I

THE EFFECTS OF SHORT- AND LONG-TERM MELATONIN TREATMENT ON MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY IN A RAT MODEL OF OBESITY AND INSULIN RESISTANCE

4.1. INTRODUCTION

Recent evidence supports the role of melatonin in obesity and the potential beneficial effect of melatonin treatment in obesity-related disorders (Nduhirabandi et al., 2012; Reiter et al., 2012). In this regard, chronic melatonin treatment starting before the establishment of obesity prevented the development of obesity-related abnormalities and protected the hearts against myocardial ischaemia-reperfusion damage (Nduhirabandi et al., 2011). However, the effect of melatonin treatment on the heart in established obesity remains unknown. Additionally, the mechanism of cardioprotection by melatonin in obesity is not yet established. Previous studies have consistently reported that PI3-K/Akt and STAT-3 pro-survival signalling pathways were associated with cardioprotection (Suleman et al., 2008; Lecour, 2009; Tamareille et al., 2011; Yin et al., 2012). Interestingly, Genade et al. (2008) and Lamont et al. (2011) showed that PI3-K/Akt and STAT-3 signalling were associated with melatonin's cardioprotective properties in non-obese animals. Importantly, these signalling pathways have recently been shown to closely interact to promote maximal cardioprotection (Suleman et al., 2008; Tamareille et al., 2011). However, this has not yet been investigated focusing on melatonin's effect in obesity and insulin resistance.

In order to expand our knowledge of some of these outstanding issues, we investigated the effects of melatonin (administered after establishment of obesity) in a rat model of diet-induced obesity and insulin resistance. The specific objectives were to determine the effect of varying periods of oral melatonin treatment (3 and 6 weeks) on: (i) biometric and metabolic parameters: food and water consumption, body weight, heart weight, visceral fat, fasting blood glucose and glucose

tolerance, serum lipid profile, insulin and adiponectin levels; (ii) serum lipid peroxidation; (iii) post-ischaemic myocardial function and infarct size; (iv) basal and post-ischaemic activation of myocardial PKB/Akt, ERK42/44, GSK-3 β , STAT-3, PI3-K and GLUT-4 expression.

4.2. MATERIALS AND METHODS

4.2.1. STUDY DESIGN

Experimental groups, feeding and treatment have been previously described in the general methodology (see chapter 3). Here, only specific methods are included.

4.2.2. EXPERIMENTAL PROCEDURE

Major phases of this study included animal feeding, treatment and the experimental procedure (at the end of feeding). The treatment was continuously evaluated during the study by determining the daily food and water consumption and weekly body weight change. At the end of the treatment period or at the end of 20 weeks of feeding, non-fasting or overnight fasting animals were sacrificed. As previously described (Nduhirabandi et al., 2011), the animals were anaesthetized by intraperitoneal injection of sodium pentobarbitone (160mg/kg), and body weights as well as blood glucose levels were determined. The hearts were rapidly removed and blood collected immediately from the thoracic cavity of the rat and the visceral fat (intraperitoneal and gonadal fat) collected and weighed.

The heart weights (HW) were determined at baseline (immediately after isolation without perfusion, “non-perfused hearts”) or at the end of the reperfusion period of the isolated hearts. As it is described further below (section 4.2.2.1-4), in the case of infarct size studies, the hearts were subsequently frozen at -20°C while in the case of biochemical analyses experiments, the hearts were stored in liquid nitrogen. The weight of the infused suspension (i.e., Evans blue solution for infarct size determination) was subtracted from the measured HW to obtain the final HW. In separate experiments we found that the weights of non-perfused and perfused hearts (after 2h reperfusion) were not significantly different. The HW to BW ratios were calculated and expressed

in mg/g. The tibial length (TL) was measured and the HW to TL ratio calculated and expressed in mg/mm.

4.2.2.1. Heart perfusion

Hearts were perfused with the modified Krebs-Henseleit bicarbonate buffer (KHB) containing (in mM): NaCl 119, NaHCO₃ 24.9, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 0.59, Na₂SO₄ 0.59, CaCl₂·H₂O 1.25 and glucose 10. KHB was oxygenated and kept at pH 7.4 by gassing with 95% O₂ and 5% CO₂ at 37° C.

4.2.2.1.1. Perfusion technique

The heart was rapidly excised from the anaesthetized rat and arrested in ice-cold (4°C) KHB. Thereafter, it was mounted *via* the aorta onto the aortic cannula. The left atrium was also cannulated *via* the pulmonary vein.

After cannulation, hearts were first retrogradely perfused (Langendorff mode), in a non-recirculating manner at constant hydrostatic pressure (100cmH₂O) for 10min. This allows the washout of blood and stabilises the heart. After 10min of the retrograde perfusion, the perfusion mode was switched to the working heart mode (see figures 4.1 and 4.2) maintaining a preload of 15cmH₂O and afterload of 100cmH₂O) for 20min. The hearts were not electrically paced. Myocardial temperature was thermostatically controlled and monitored at regular intervals (constant at 37°C during reperfusion and 36.5°C during ischaemia). In our laboratory, maintenance of temperature at 36.5°C during ischaemia was found to be easier than 37°C. For this, a temperature probe was inserted into the pulmonary artery and the water bath adjusted accordingly.

4.2.2.1.2. Perfusion protocol

Two perfusion protocols were used: regional ischaemia (to determine myocardial infarct size) and global ischaemia (to evaluate biochemical changes).

- **Regional ischaemia protocol**

The regional ischaemia protocol was used for the evaluation of myocardial function and infarct size (see figure 4.1). Briefly, at the end of the stabilisation period, after measuring the coronary flow (CF), ischaemia was induced by ligating the left anterior descending (LAD) coronary artery, approximately 3mm from the coronary sinus, with a Ethicon silk suture (3/0, 26mm ½C Taper, Johnson & Johnson Medical (PTY) LTD, South Africa). The LAD was occluded for 35 min at 36.5°C. Adequate regional ischaemia was indicated by a 33% reduction in CF compared to the pre-ischaemic CF. Regional ischaemia was also confirmed by cyanosis of the myocardial surface. After the ischaemic period, the silk suture was released, and the heart reperfused for 120 min (see figure 4.1). Myocardial mechanical function was documented before induction of ischaemia and during reperfusion.

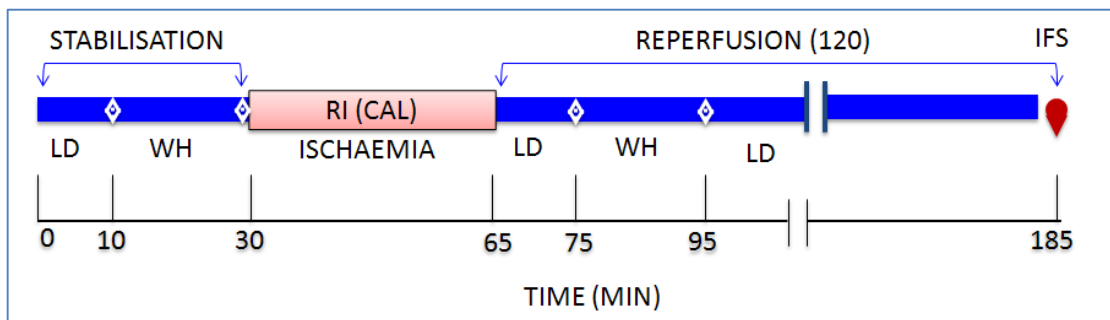


Figure 4.1 Diagram showing the perfusion protocol for determination of infarct size; LD: Langendorff, WH: working heart, RI: regional ischaemia, CAL: Coronary artery ligation, IFS: infarct size.

- **Global ischaemia protocol**

The global ischaemia protocol was used to evaluate post-ischaemic biochemical changes. Briefly, after stabilisation, the aortic and atrial cannulas were occluded resulting in a total cessation of coronary perfusion to the heart (no-flow ischaemia). During the ischaemia, myocardial temperature was maintained at 36.5°C, and after 15 min, the aortic cannula was opened for

reperfusion of the heart for 10 or 30 min (see figure 4.2). At the end of reperfusion, hearts were freeze-clamped and stored in liquid nitrogen for further analyses.

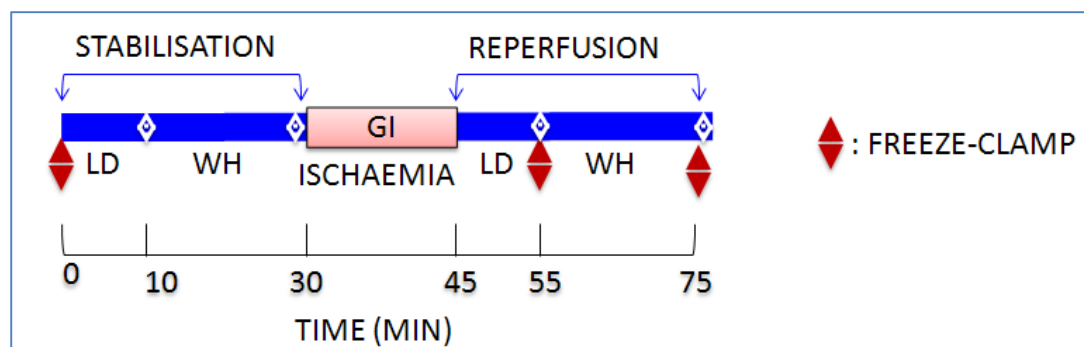


Figure 4.2 Diagram showing the perfusion protocol for biochemical analyses; GI: global ischaemia, LD: Langendorff, WH: working heart.

4.2.2.1.3. Determination of myocardial function

The coronary and aortic flow rates were measured manually. Cardiac output (CO) was calculated as the sum of aortic output (AO) and coronary flow (CF). The aortic systolic and diastolic pressure (mmHg) and heart rate (HR) [beats per minute (bpm)] were monitored and recorded on a computerized system through a side-arm of the aortic cannula connected to a Viggo-Spectramed pressure transducer coupled to the computer system. Total work performance (pressure power + kinetic power) developed by the heart was determined according to the formula described by (Kannengiesser et al., 1979). To determine functional recovery, the post-ischaemic value was expressed as the percentage of the pre-ischaemic value. All data were collected and analysed using a PhysiTutor® data acquisition system.

4.2.2.1.4. Determination of myocardial infarct size and area at risk

Myocardial infarct size was determined as previously described (Nduhirabandi et al., 2011). At the end of 2 h reperfusion, the silk suture around the coronary artery was securely tied and 1mL of Evans blue suspension (0.5%) was slowly injected *via* the aorta cannula. The heart was then frozen overnight before being cut into 2 mm thick slices. After defrosting, the slices were stained

with 1% w/v triphenyl tetrazolium chloride (TTC) in phosphate buffer (pH 7.4 at 37°C) for 15 min. To enhance the contrast between stained viable tissue and unstained necrotic tissue slices were fixed in 10% v/v formaldehyde solution (at room temperature). At this stage, the damage in each slice was visible, the blue area indicating viable tissue, the white or unstained area indicating the infarcted area which was surrounded by a red area. These two areas constituted the area at risk (AR). All slices were drawn and scanned. For each slice, the AR and the area of infarcted tissue were determined using computerized planimetry (UTHCSA Image Tool Programme, University of Texas Health Science Center at San Antonio, TX, USA). For each heart, the areas of the tissue slices were summated. The infarct size was expressed as the percentage of the area at risk (I/AR %).

4.2.2.2. Blood analysis

4.2.2.2.1. Blood collection

Blood samples were collected as previously described (Du Toit et al., 2008). Following the removal of the heart, blood was immediately collected from the thoracic cavity using serum separation tubes (BD Vacutainer, Lasec SA, Cape Town, South Africa). These tubes were centrifuged at 3000 rpm at 4°C for 10 min, within 30 min of collection. Separated serum was transferred to eppendorf tubes and stored at -80°C for further analyses.

4.2.2.2.2. Blood glucose determination

Fasting blood glucose was measured using the glucose meter (Gluco Plus™, distributed by CIPLA DIBCARE, Bellville, South Africa). A small drop of fresh blood, obtained by pricking the distal tail with a lancet was placed on a disposable test strip and after 5 seconds, the meter displayed the blood glucose level expressed in mmol/L.

4.2.2.2.3. Intraperitoneal glucose tolerance test (IPGTT)

This test reflects the efficiency of the body to dispose of glucose after an intraperitoneal injection of glucose (Muniyappa et al., 2008). Animals were fasted 18 h but with access to drinking water.

The animals were anaesthetized by intraperitoneal injection of sodium pentobarbitone (15 mg/kg), body weights were determined and blood glucose measured as described above. These glucose levels were considered as basal. A solution of glucose (50%) was then injected intraperitoneally at 1g/kg and blood glucose levels monitored using a drop of blood collected by tail prick and the glucose meter at various time intervals (5, 10, 15, 20, 25, 30, 45, 60, 90 and 120). Following the IPGTT, the animals were allowed to recover for a period of 1 week before other experiments.

4.2.2.2.4. Insulin assay

Serum insulin levels were determined using a competitive radioimmunoassay (RIA) (Coat-A-Count[®] Insulin, Siemens Healthcare Diagnostic Products Inc., LA, USA) according to the manufacturer's instructions. The homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated as the product of fasting glucose concentration (mmol/L) multiplied by the insulin concentration (μ IU/mL) divided by 22.5.

4.2.2.2.5. Adiponectin assay

Fasting serum was collected as mentioned above. Serum adiponectin levels were determined using an enzyme linked-immunosorbent assay (ELISA) kit (AdipoGen[™] Inc., Incheon, Korea) according to the manufacturer's instructions.

- **Assay procedure**

For quantitative determination of adiponectin in rat serum samples, a polyclonal antibody is precoated onto the 96 wells microtiter plate. After appropriate dilution, at room temperature, samples and standards (100 μ l) were added into their respective wells (in duplicate) in order to bind to the precoated antibody. Following 1h incubation at 37°C and extensive washing to stop competition and separate the free and bound antibodies, a specific monoclonal antibody (detection antibody) was added and incubated at 37°C for 1h. Excess of adiponectin detector antibody was washed out and HRP conjugated anti-mouse IgG added and incubated at 37°C for 1h again. After a final washing, peroxidase activity was quantified using the substrate 3, 3', 5, 5'-

tetramethylbenzidine (TMB). Within 30 min following incubation at room temperature in the dark for 20 min, the intensity of the resultant colour reaction was read at 450 nm using a microtiter plate reader. A standard curve was generated and adiponectin concentration calculated accordingly.

4.2.2.2.6. Lipid assay

Fasting and non-fasting serum samples were assayed for lipid peroxidation, total cholesterol (TC), triglycerides (TG) and phospholipids (PL) as previously described (Nduhirabandi et al., 2011). Serum TC, PL and TG concentrations were determined using enzymatic colorimetric kits (KAT Medicals, Calicom Trading (PTY), South Africa) and PL (WAKO Chemicals, Germany) by a Labsystems Multiskan MS analyzer (AEC Amersham Co., South Africa).

Serum levels of conjugate dienes (CD), lipid hydroperoxide (LOOH) and thiobarbituric acid reactive substances (TBARS) were measured spectrophotometrically as follows:

(1) CDs were measured at 234 nm by a GBC UV/VIS analyser (Wirsam Scientific and Precision Equipment, South Africa) after mixing the serum samples with cyclohexane (Spectrosol) (Pryor and Castle, 1984; Esterbauer et al., 1989). Separation was enhanced by centrifugation (14,000 × g for 10 min at 10°C).

(2) To determine LOOH, serum was assayed in the presence of xylenol orange (3,3'-bis[N,N-di(carboxymethyl)-aminomethyl]-o-cresolsulfonephthalein) (sodium salt) (Sigma Chemical Co., St Louis, MI, USA) and Fe²⁺ in the ferrous oxidation/xylenol orange (FOX) assay that was adapted to enhance the solubility of non-polar compounds by including chloroform. Absorbance of the resulting Fe³⁺-xylenol orange complex was measured at 560 nm (Jiang et al., 1991; Jiang et al., 1992) using Labsystems Multiskan MS Analyser (AEC Amersham Co., South Africa).

(3) TBARS were measured according to the method of Jentzsch et al. (1996). The samples (200 µL) were mixed with 25 µL butylated hydroxytoluene (BHT) (Fluka Chemie, Switzerland) in ethanol (Merck Chemicals, South Africa) and orthophosphoric acid (Sigma) buffer at pH 3.6 and vortexed for 10 seconds. 25 µL thiobarbituric acid (TBA) (Sigma) reagent was added and vortexed again.

After incubation at 90°C for 45 min in water bath, the reaction was stopped by putting tubes on ice. TBARS were extracted with n-butanol. Saturated NaCl was added and the mixture was then centrifuged at 12,000 rpm for 1 min. Absorbance was read at 532 nm using Labsystems Multiskan MS Analyser (AEC Amersham Co., South Africa).

The final concentrations were calculated using the appropriate molar extinction coefficients and standards.

4.2.2.3. Western blotting

Total PKB/Akt, phospho-PKB/Akt (Ser-473), total GSK-3 β , phospho-GSK-3 β (Ser-9), total STAT-3, phospho-STAT-3 (Ser-727), phospho-STAT-3 (Tyr-705), total ERK p42/p44, phospho-ERK p42/p44 (Thr-202/Tyr-204), total PI3-K (p85), phospho-PI3-K (p85) and β -tubulin were assessed in the isolated hearts at baseline (before perfusion) referred to as “ non-perfused hearts” and after 10 or 30 min of reperfusion using western blotting techniques as described above (see chapter 3). The specific protocol for each protein used in the present study is summarized in the table 4.1. Membrane was overnight incubated with primary antibody and exposed to the secondary antibody for 1h (see chapter 3). β -tubulin expression was used to assess equal loading. To determine activation, phospho-/total ratios were also calculated.

For all proteins except for STAT-3 where we used cytosolic and nuclear fractions, the whole-cell-extract was used as described in chapter 3. To obtain a cytosolic fraction, we used the lysis buffer described above but without 1% Triton-X100. The homogenates were centrifuged at 2500rpm for 10 min at 4°C to obtain the cytosolic fraction. The lysis buffer containing 1% triton was added to the resultant pellet and homogenized. The resultant homogeneous solution was then transferred into ultracentrifuge tubes and kept on ice for 10 min, before centrifugation at 13000rpm. The resultant supernatant was considered as the nuclear fraction. The protein content of cytosolic and nuclear fractions was determined using the Bradford method (chapter 3) and the rest of the lysates were kept frozen until analyses.

Table 4.1 Specificity of the protein

Protein	MW	Quantity	SDS-PAGE	Antibody dilution	
				Primary	Secondary
PKB/Akt	60kDa	50µg	12%	1:1000 TBST	1:4000 TBST(5% milk)
ERK 42/44	42/44kDa	30µg	12%	1:1000 TBST	1:4000 TBST
STAT-3	79/86 kDa	50µg	10%	1:500TBST	1:4000 TBST
PI3-K(p85)	85kDa	50µg	12%	1:1000 TBST	1:4000 TBST
GSK-3β	46kDa	50µg	10%	1:1000TBST	1:4000 TBST
GLUT-4	45kDa	40 µg	10%	1:1000 TBST	1:4000 TBST
β-tubulin	55kDa	30-50µg	10-12%	1:1000 TBST	1:4000 TBST

4.2.3. DATA ANALYSIS

All data were expressed as mean \pm SEM. Infarct size was expressed as a percentage of the area at risk. Post-ischaemic functional recovery (aortic output and work total) was expressed as a percentage of the pre-ischaemic value. When comparisons between two groups (e.g., treated and untreated groups) were made, an unpaired Student t-test was performed. For multiple comparisons, the ANOVA (two-way when appropriate) followed by the Bonferroni correction was applied. Statistical significance was considered for a p-value of <0.05 .

4.3. RESULTS

4.3.1. BIOMETRIC AND METABOLIC PARAMETERS

4.3.1.1. Animal model

Table 4.2 summarizes the characteristics of the animal model of high calorie diet-induced obesity and insulin resistance. After 20 weeks of feeding, the body weight of control (C) rats averaged 470 ± 8 g. The high calorie diet (D) increased the body weight by 14.4 % ($p < 0.001$) (fig.4.3A). It also increased the visceral fat content by 70.3 % ($p < 0.001$) (fig.4.3B).

Fasting blood glucose levels were also increased by 20.4% ($p < 0.01$) (fig.4.3C). Fasted serum insulin and TG levels were elevated by 55.8% ($p < 0.01$) and 82.4 % ($p < 0.05$) respectively in the diet fed (D) rats (fig.4.3E; 4.3D). The HOMA-IR index was also increased in rats fed a high calorie diet (92.3%, $p < 0.001$). Compared to control rats, the high calorie diet caused a significant decrease in adiponectin levels (16.0 ± 0.9 vs $3.7 \pm 1.4 \mu\text{g/mL}$, $p < 0.001$) (fig.4.5F).

The heart weight of control rats averaged 1.14 ± 0.03 mg. The high calorie diet increased the heart weight by 12.3 % ($p < 0.05$). While the heart weight to body weight (HW/BW) ratio was not affected by the diet [2.44 ± 0.05 (C) vs 2.43 ± 0.05 (D)], the heart weight to tibial length (HW/TL) ratio was increased in high calorie diet groups compared to control (13.3%, $p < 0.05$).

Table 4.2 Characteristics of animal model of obesity and insulin resistance

Parameters	C	D	n/group
Body weight (g)	470±8	538±15 (***)	14
Visceral fat (g)	19.71 ± 1.1	33.57 ± 1.5 (***)	14
Fasting blood glucose (mmol/L)	5.29 ± 0.1	6.37 ± 0.4 (**)	8-10
Serum insulin ($\mu\text{IU/mL}$)	37.6 ± 3.9	58.6 ± 6.4 (**)	8-10
Serum adiponectin ($\mu\text{g/mL}$)	16.0 ± 0.9	3.8 ± 1.4 (***)	6-7
Serum TG (mmol/L)	0.57 ± 0.07	1.04 ± 0.17(**)	7
HOMA-IR index	8.87 ± 0.9	17.06 ± 2.8 (**)	8-10
Heart weight (g)	1.14 ± 0.03	1.28 ± 0.04 (**)	7-10
HW/BW (mg/g)	2.44 ± 0.05	2.43 ± 0.05	7-10
HW/TL(mg/mm)	26.8 ± 0.8	30.38 ± 1.0 (*)	7-10

Blood and serum values were obtained from overnight fasting animals, C: control; D: High calorie diet; TG: triglycerides; HW: heart weight; BW: body weight; TL: tibial length; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (D vs C).

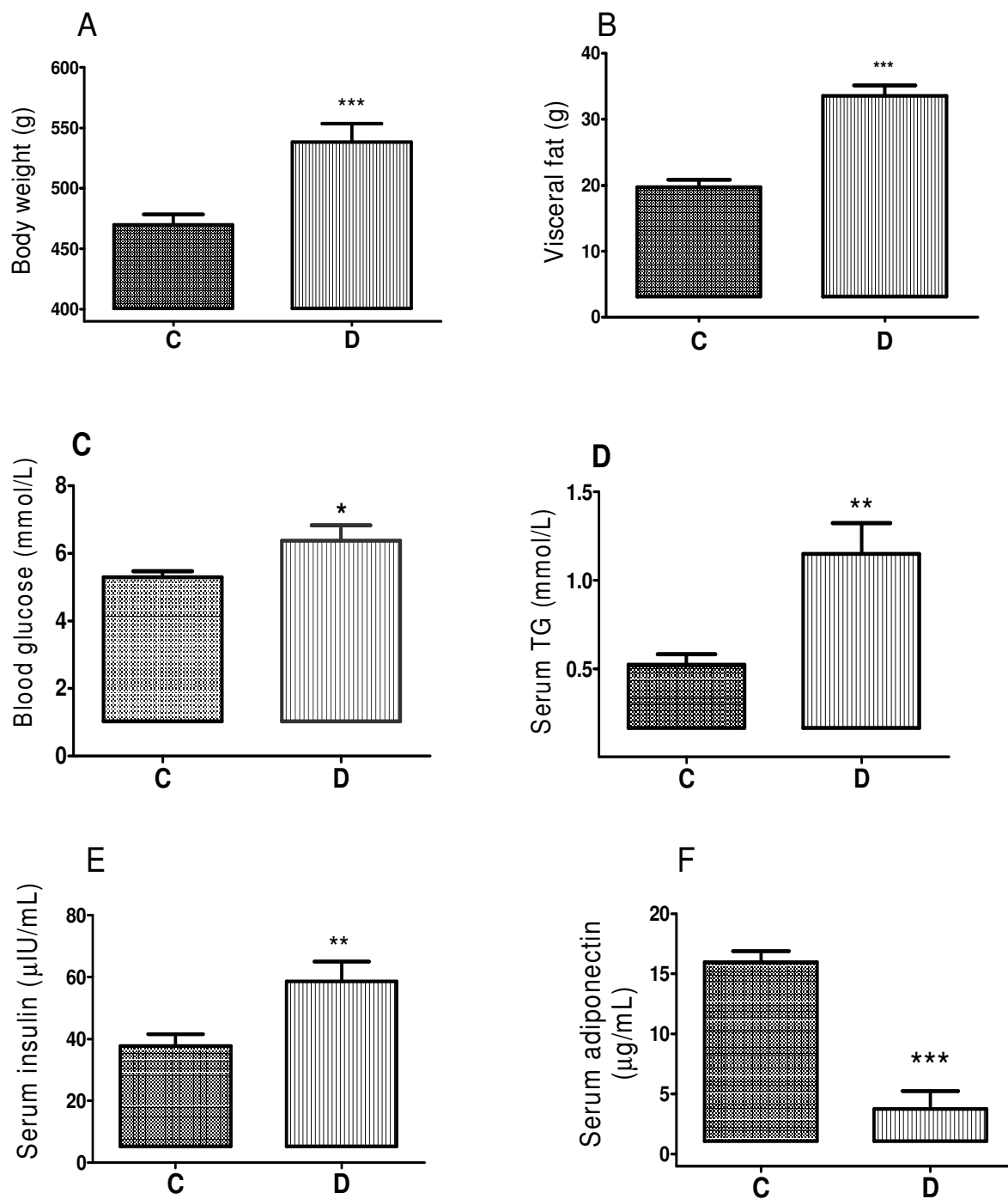


Figure 4.3 Characteristics of animal model after overnight fasting, body weights (A, n=14), visceral fat (B, n=14), blood glucose (C, n=8-10), serum triglycerides (TG) (D, n=6-7), serum insulin (E, n=8-10), serum adiponectin (F, n= 6-7), *p<0.05, **p<0.01, ***p<0.001 (D vs C).

4.3.1.2. Effects of melatonin treatment on biometric parameters

4.3.1.2.1. Body weight

To investigate the effect of melatonin on body weight during the treatment, for each group, body weight was determined before and at end of the treatment and the percentage body weight gain calculated (table 4.3).

Before the treatment (at the end of 14 and 17 weeks of feeding), the body weight of rats fed a high calorie diet had increased significantly compared to control ($p < 0.05$) (table 4.3). A similar increase was also observed after 20 weeks of feeding (538 ± 15 vs 470 ± 8 g, $p < 0.001$) (table 4.3).

After 6 weeks treatment, although melatonin did not prevent the weight gain, it lowered significantly the percentage weight gain from $13.8 \pm 0.9\%$ to $7.5 \pm 2.1\%$ in the C group, and from $18.1 \pm 1.5\%$ to $8.9 \pm 2.1\%$ in the diet group (table 4.3 and fig.4.4).

At the end of 3 weeks treatment, melatonin significantly reduced the body weight in the control group by 12.2% ($p < 0.05$) but not in the D group (538 ± 15 vs 505 ± 9 g, $p = 0.07$) (fig.4.6). The percentage body weight gain was also reduced in the C ($5.6 \pm 0.7\%$ vs $0 \pm 0.7\%$, $p < 0.05$) but not in the D group (fig.4.4).

Table 4.3 Effects of melatonin treatment on body weight variation

Treatment period	Group	BW (before)	BW (after)	% Gain	N/group
6 weeks (after 14 weeks)	C	413± 8	470± 8	13.8 ± 0.9	10
	CM6 (a)	407±10	436± 8*	7.1 ± 2.1(*)	14
	D	455 ± 15(*)	538 ± 15(***)	18.1 ± 1.5	14
	DM6 (a)	460±15(*)	500 ± 13(#)	8.7 ± 2.1(*)	14
3 weeks (after 17 weeks)	C (b)	445±8.7	470±8	5.6±0.7	10
	CM3 (a)	420±15	419±15(*)	-0.2 ± 0.7(**)	14
	D (b)	508±15(*)	538 ± 15(***)	5.9 ± 0.8	14
	DM3(a)	481± 10(*)	505 ± 9(##)	4.9 ± 0.9	14

BW: body weight (g), (a): rats receiving melatonin during the treatment; (b): same rats as in 6 weeks treatment but their body weights were determined after 17 weeks of feeding, *p<0.05 (vs C), **p<0.01 (vs C), ***p<0.001(vs C), #p=0.06 (DM6 vs D), ##p=0.07 (DM3 vs D), n=10-14/group.

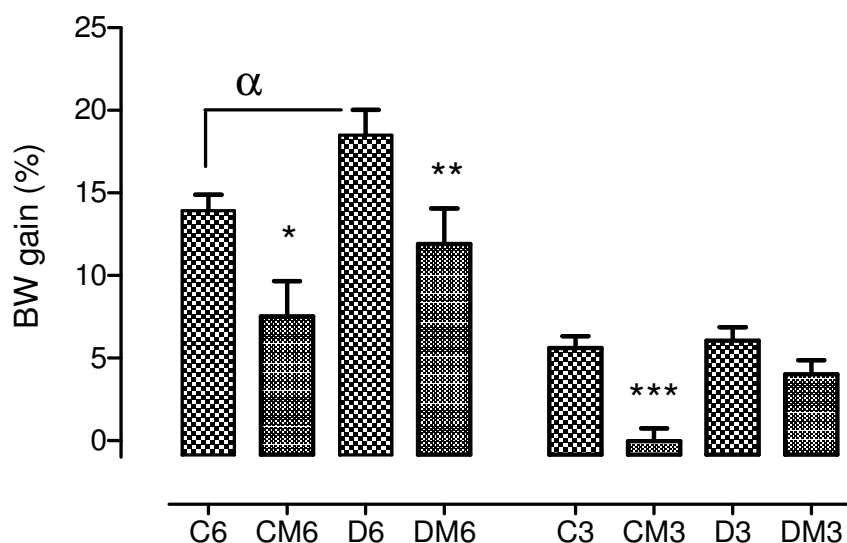


Figure 4.4 Body weight gain (percentage), BW: body weight; C6, D6 and C3, D3: weight gain of C and D rats during 6 and 3 weeks treatment, respectively. CM6, DM6 and CM3, DM3: weight gain of rats receiving melatonin for 6 and 3 weeks, respectively, * $p < 0.05$ (CM6 vs C6), ** $p < 0.05$ (DM6 vs D6), *** $p < 0.05$ (CM3 vs C3), α $p < 0.01$ (C6 vs D6), $n = 10-14$ /group.

4.3.1.2.2. Other biometric parameters

The high calorie diet significantly increased visceral adiposity, absolute heart weight and the heart weight to tibial length ratio, but not the heart weight to body weight ratio (table 4.4 and fig.4.7-9). Melatonin treatment for 6 weeks did not affect any of these parameters in either control or D groups. 3 weeks melatonin treatment significantly reduced the visceral fat in control (18.1%, $p < 0.01$), but not in D group (fig.4.8). In addition, it increased the heart weight to body weight ratio in control (15.9%, $p < 0.05$), but not in D group probably due to their reduced body weight (419.9 ± 15.3 g).

Table 4.4 Effects of melatonin on other biometric parameters

Groups	Visceral fat (g)	HW (g)	TL (mm)	HW/BW ratio (mg/g)	HW/TL ratio (mg/mm)
C	19.71 ± 1.1	1.14 ± 0.03	42.4±0.03	2.44 ± 0.05	26.8 ± 0.8
CM6	18.1± 1.1	1.13 ± 0.12	42.1±0.03	2.52± 0.26	27.03 ± 2.5
CM3	16.14 ± 0.7(*)	1.14 ± 0.04	42.2±0.04	2.83 ± 0.08(*)	27.8 ± 0.9
D	33.57 ± 1.5(**)	1.28 ± 0.04(*)	42.3±0.05	2.43 ± 0.05	30.38 ± 1.0(*)
DM6	31.47± 2.1	1.25 ± 0.03	42.5±0.04	2.49 ± 0.07	29.8± 0.5
DM3	32.1± 1.6	1.19 ± 0.03	41.1±0.03	2.46 ± 0.05	28.2± 0.8
n/group	14	7-10	10	7-10	7-10

HW: heart weight, BW: body weight, TL: tibial length, *p<0.05 (vs C), **p<0.01 (vs C)

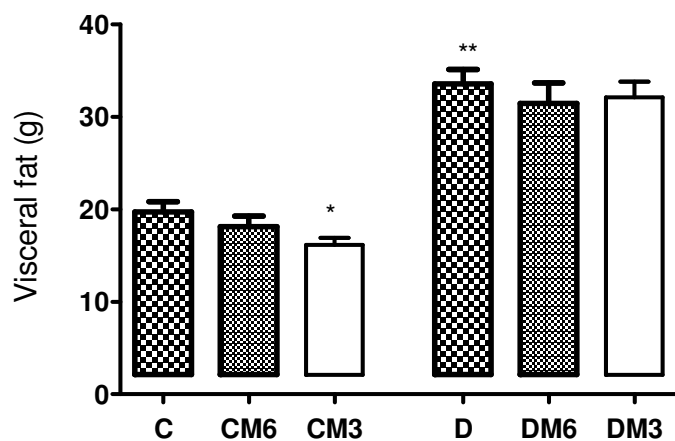


Figure 4.5 Visceral fat, * p<0.05 (C vs CM3), **p<0.001(C vs D), n=14/group

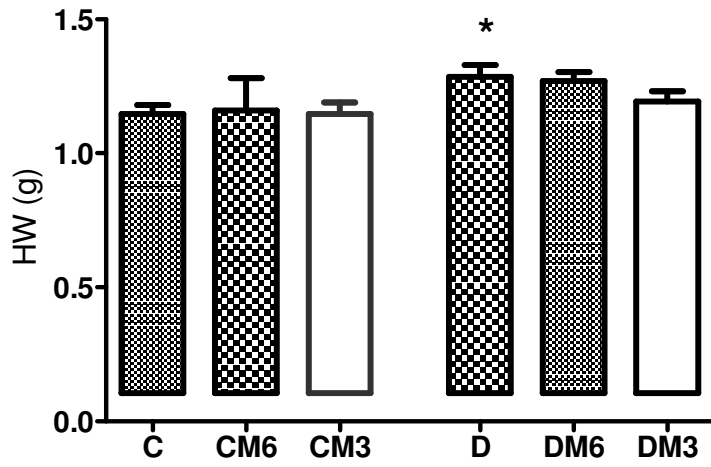


Figure 4.6 Absolute heart weight, *p<0.05 (C vs D), n=7-10/group

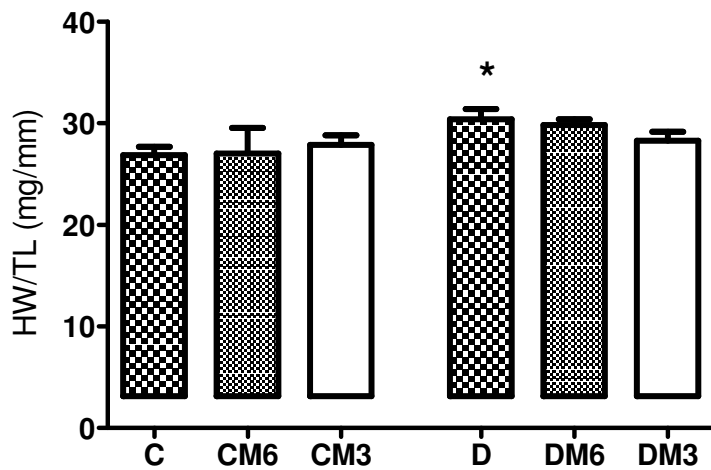


Figure 4.7 Heart weight to tibial length (HW/TL) ratios; *p<0.05 (C vs D), n= 7-10/group.

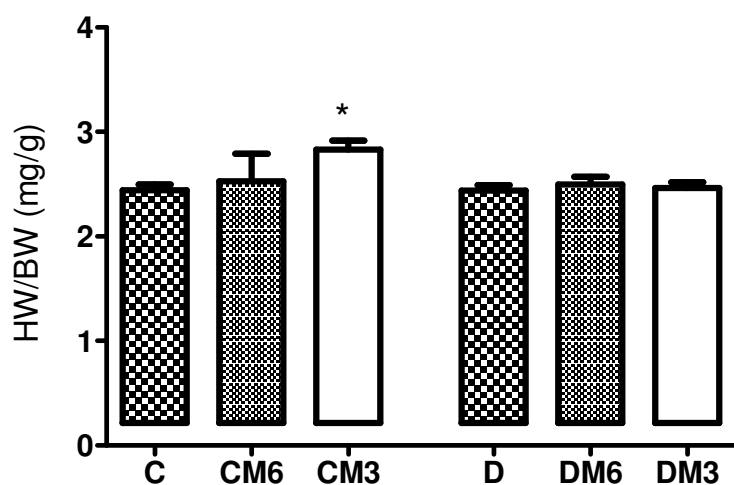


Figure 4.8 Heart weight to body weight (HW/BW) ratios, * $p < 0.05$ (CM3 vs C), $n = 7-10$ /group.

4.3.1.3. Effect of melatonin treatment on food and water consumption

In order to investigate the mechanism involved in body weight variation, water and food intake were determined. Before melatonin treatment, measurements of food and water consumption were recorded for 15 days. Animals were divided in two C and D groups. Each group was housed in two cages of which one received melatonin and the other received drinking water only (table 4.5). During the treatment, daily measurements were conducted for both water and food consumption. Results are presented in figures 4.9-10 and summarized in the table 4.5.

Rats fed a high calorie diet consumed more food than the control rats receiving the standard rat chow (30.9 ± 1.4 vs 21.07 ± 0.2 or 30.2 ± 1.3 vs 21.03 ± 0.2 g, $p < 0.001$), with a reduced intake of drinking water compared to the control group (17.63 ± 0.4 vs 30.6 ± 0.6 ; 18.1 ± 0.3 vs 32.7 ± 0.9 mL/day) (table 4.5; fig.4.9-10). Administration of melatonin did not affect the daily water and food consumption in either C or D groups (table 4.5; fig. 4.9-10).

Table 4.5 Water and food consumption before and during melatonin treatment

Parameters and treatment		C1	C 2	D1	D2
Water	A. Before (mL/rat/day)	30.6±0.6	32.7±0.9	17.63±0.4*	18.1±0.3*
	B. Treatment melatonin (mL/rat/day)	(-)	(+)	(-)	(+)
		32.4±0.9	34.7±1.03	18.10±0.7*	19.57±1.2*
Food	A. Before (g/rat/day)	21.07±0.2	21.03±0.2	30.9±1.4*	30.2±1.3*
	B. Treatment melatonin (g/rat/day)	(-)	(+)	(-)	(+)
		22.6±0.7	21.6±0.9	35.8±1.2*	34.7±0.9*

A: before treatment, B: during treatment, (-): no treatment (C 1 and D1), (+): treatment (C2 and D2), *p<0.001(D vs C), n=6/group.

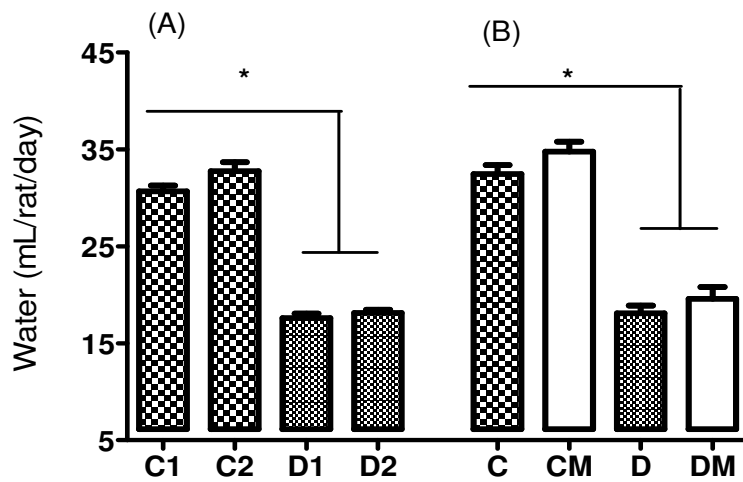


Figure 4.9 Water consumption before (A) and during (B) treatment, *p<0.001 (D vs C or DM vs CM); n=6/group.

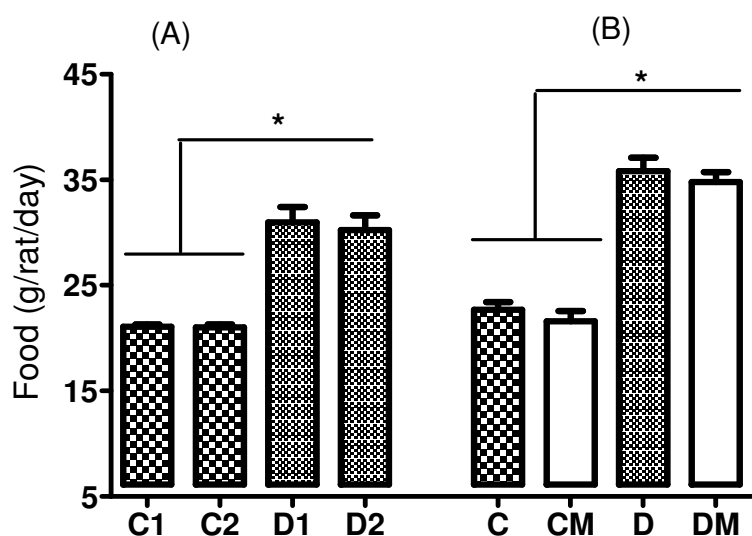


Figure 4.10 Food consumption, before treatment (A) and during treatment (B) * $p < 0.001$ (D vs C or CM vs DM); $n=6$ /group.

4.3.1.4. Effects of melatonin on metabolic parameters

Only the effects on fasting blood glucose, serum insulin and adiponectin levels and HOMA-IR index are presented in this section (fig.4.13-15; table 4.6). The effect on glucose tolerance is described separately in section 4.3.1.5 (fig.4.15-16). The effects on serum lipids are included in the section on lipid peroxidation (section 4.3.2) (tables 4.7-8).

Fasting blood glucose levels of diet (D) rats were increased compared to controls (6.37 ± 0.4 vs 5.29 ± 0.1 mmol/L, $p < 0.05$). Six and 3 weeks melatonin treatments had no significant effects on fasting blood glucose levels in either control or D groups (fig.4.11).

Following 6 weeks treatment, fasting serum insulin levels were significantly reduced in the treated D rats (58.6 ± 6.41 vs 36.12 ± 4.75 μ U/mL, $p < 0.01$) (fig.4.12). A similar reduction was also found after three weeks treatment in the D group but not in the control group (fig.4.12; table 4.6). Accordingly, HOMA-IR index was reduced by 49.9% ($p < 0.01$) and 47.2 % in the D group, after

both 6 and 3 weeks treatments, respectively (fig.4.13). There was, however, no significant difference between the HOMA index of treated and untreated control rats (fig.4.13).

The high calorie diet reduced the levels of serum adiponectin (16.0 ± 0.9 vs 3.8 ± 1.4 $\mu\text{g/mL}$, $p < 0.001$). In this group, 6 and 3 weeks melatonin treatment increased adiponectin by 184.8 % and 115.7%, respectively (fig.4.14). Melatonin treatment had no effect on adiponectin levels in the control group (fig.4.14).

Table 4.6 Effects of melatonin on metabolic parameters

Groups	Serum insulin ($\mu\text{IU/mL}$)	Fasting blood glucose (mmol/L)	HOMA-IR index	Serum adiponectin ($\mu\text{g/mL}$)
C	37.66 ± 3.9	5.29 ± 0.1	8.87 ± 0.9	16.0 ± 0.9
CM6	$27.3 \pm 4.11##$	5.3 ± 0.09	6.62 ± 0.06	10.5 ± 2.8
CM3	31.72 ± 3.406	5.18 ± 0.13	7.5 ± 0.85	10.4 ± 2.1
D	$58.6 \pm 6.41^{**}$	$6.37 \pm 0.4^*$	$17.06 \pm 2.8^{***}$	$3.8 \pm 1.4^{***}$
DM6	$36.12 \pm 4.75^{**}$	6.01 ± 0.13	$10.09 \pm 1.3^{**}$	$10.7 \pm 2.7^*$
DM3	$35.95 \pm 4.42^{**}$	$5.79 \pm 0.11\#$	$9.01 \pm 1.2^{**}$	8.1 ± 2.1
n/group	8-10	8-10	8-10	6-7

Triglycerides (TG), total cholesterol (TC), and phospholipid (PL) are presented in the section 4.3.2 (behind lipid peroxidation). * $p < 0.05$ (DM6 or DM3 vs D) or (D vs C); ** $p < 0.01$ (D vs C); *** $p < 0.001$ (D vs C), # $p = 0.07$ (DM6 vs D), ## $p = 0.08$ (CM6 vs C).

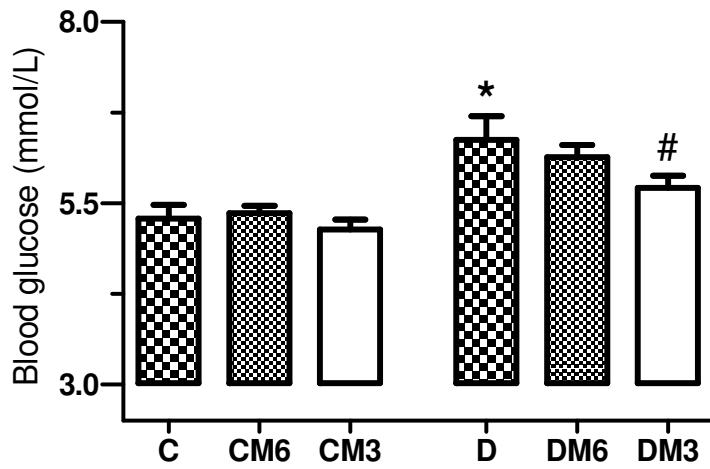


Figure 4.11 Fasting blood glucose, * $p < 0.05$ (D vs C), # $p = 0.07$ (DM3 vs D), $n = 8-10$ /group.

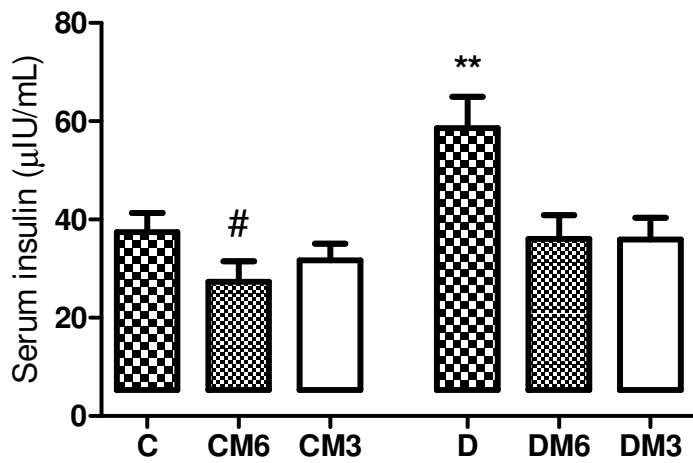


Figure 4.12 Fasting serum insulin, ** $p < 0.01$ (D vs C, DM6, DM3), # $p = 0.08$ (CM6 vs C), $n = 8-10$ /group.

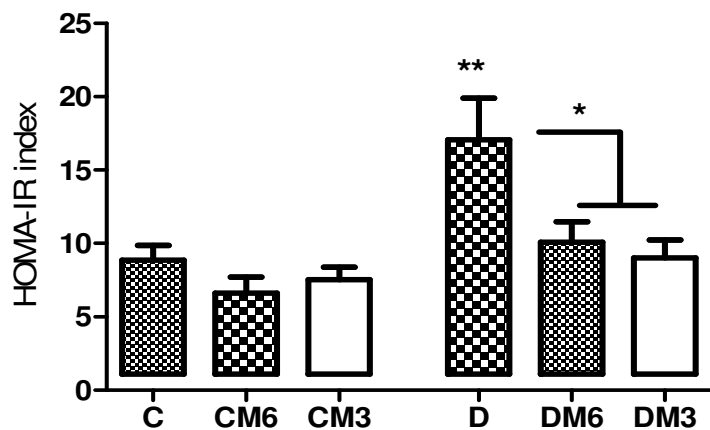


Figure 4.13 Homeostasis model assessment for insulin resistance (HOMA-IR) index; * $p < 0.05$ (D vs DM6 or DM3), ** $p < 0.01$ (D vs C), $n = 8-10$ /group.

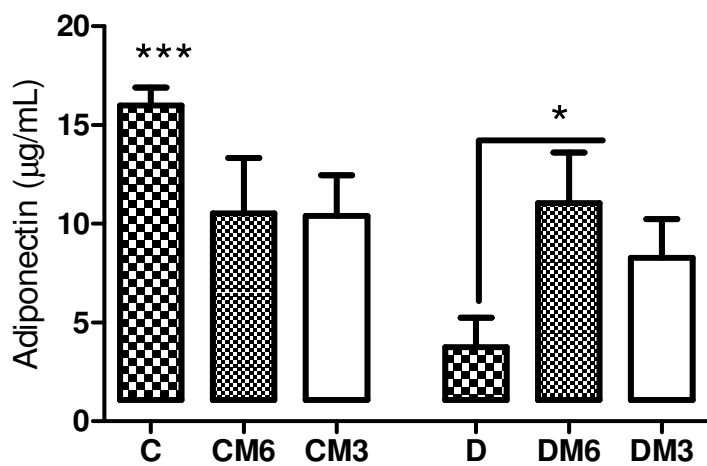


Figure 4.14 Fasting serum adiponectin, * $p < 0.05$ (D vs DM6), *** $p < 0.001$ (C vs D), $n = 6-7$ /group.

4.3.1.5. Intraperitoneal glucose tolerance test (IPGTT)

Following overnight fasting (18h), rats fed a high calorie diet had elevated basal blood glucose levels compared to the controls (5.2 ± 0.28 vs 6.4 ± 0.17 mmol/L, $p < 0.05$). Similarly, at the end of the test, these rats continued to have elevated glucose levels (4.5 ± 0.2 vs 5.2 ± 0.1 mmol/L, $p < 0.05$) compared to the control (fig.4.15). Melatonin treatment had no significant effect on the basal glucose levels in both groups (fig.4.16).

Rats fed a high calorie diet had an elevated area under curve (AUC) compared to the control (870.7 ± 25.6 vs 761.8 ± 27.7 , $p < 0.05$) (fig.4.15). Six week melatonin administration did not affect the AUC in either group (fig 4.16 A, B). Three weeks melatonin treatment significantly reduced the AUC in D but not in control rats (D: 870.7 ± 25.6 vs 756.9 ± 23.7 , $p < 0.05$; C: 761.8 ± 27.7 , vs 734.85 ± 41.3 , $p > 0$) (fig.4.16 A and B). In D group, blood glucose levels of 3 weeks melatonin treated rats were significantly reduced compared to untreated rats ($p < 0.05$) at 60 min, (fig.4.60 B). However at the end of the test there was no difference on blood glucose levels of treated and untreated rats.

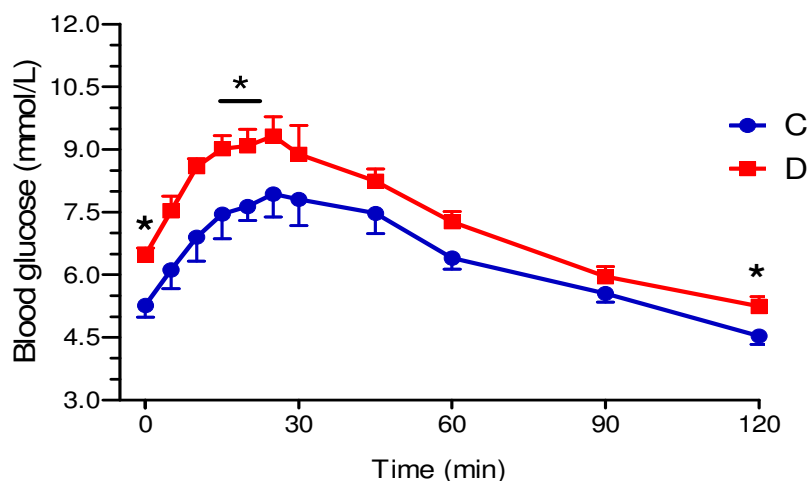


Figure 4.15 Intraperitoneal glucose tolerance test, C: control, D: high calorie diet, * $p < 0.05$ (C vs D), $n = 6$ /group.

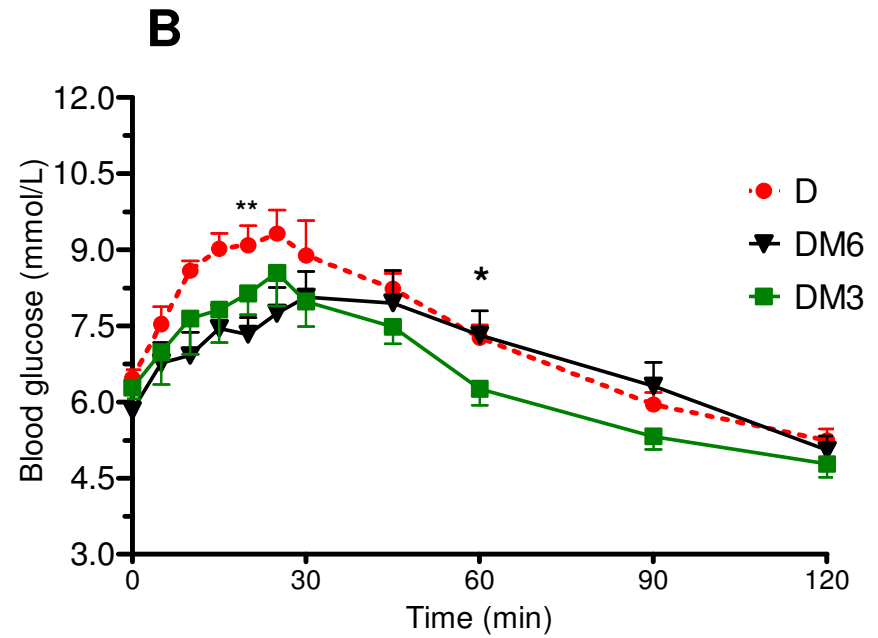
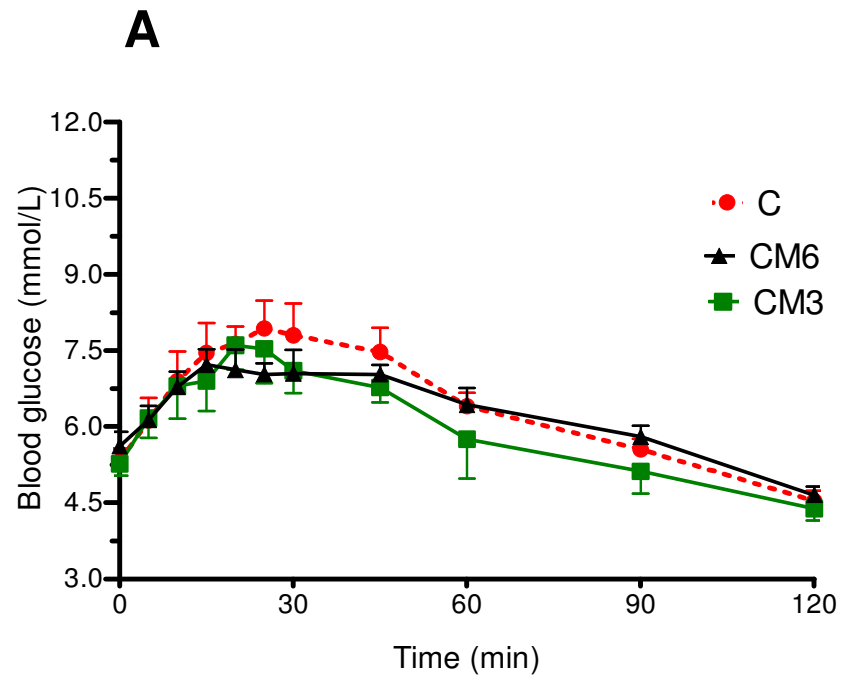


Figure 4.16 Intraperitoneal glucose tolerance test in control (A) and diet (B) groups, C; control, CM3 or CM6: control with melatonin for 3 or 6 weeks, D: high calorie diet, DM3 or DM6: diet with melatonin for 3 or 6 weeks treatment, * $p < 0.05$ (D vs DM3), ** $p < 0.05$ (D vs DM6), $n = 6$ group.

4.3.2. EFFECT OF MELATONIN TREATMENT ON LIPID PEROXIDATION

A separate series of experiments was included to investigate the effects of six or three weeks melatonin treatment on systemic lipid peroxidation as indicated by the levels of serum conjugated dienes (CD), lipid hydroperoxide (LOOH), and thiobarbituric acid reactive substances (TBARS). Serum lipids including total cholesterol (TC), triacylglycerol or triglycerides (TG), phospholipids (PL) were also measured. The CD, TBARS and LOOH values were additionally normalized by dividing their absolute concentrations by the sum of TG and PL. To explore all possible effects, we used sera from both fasting and non-fasting control and diet fed rats (collected during the day, from 10:00-12:00 A.M.). The period of fasting was 18h (overnight). The results are presented in tables 4.8-9 and figures 4.17-18.

In the non-fasting condition, rats fed a high calorie diet had increased absolute concentrations of CD with no effect on absolute values of TBARS and LOOH levels (fig.4.17; table 4.7). This was associated with an increase in TG while PL and TC concentrations were unchanged (table 4.7). In the fasting state, the increase in serum TG levels in the rats fed a high calorie diet was associated with an increase in PL and TBARS levels (table 4.8; fig.4.18) while TC, CD and LOOH levels remained at control levels (table 4.8).

Three or six melatonin treatment had no effect on serum TG, TC and PL in non-fasting condition (table 4.7). In fasting state, although the high calorie diet caused a significant increase TG and PL levels, these were not affected by melatonin after either 3 or 6 weeks treatment (fig. 4.17 and table 4. 8-9). Additionally, TC levels remained unchanged. However, three weeks treatment significantly reduced TC levels in D rats but not in controls (table 4.8).

In both control and diet groups, six weeks melatonin treatment had no effect on TBARS regardless of the feeding state (fig. 4.18B; fig.4.18B). However, it reduced the non-fasting serum CD and LOOH levels in the control but not in the diet groups (fig.4.17C, D). In fasting condition, 6

weeks melatonin treatment had no effect on absolute value of LOOH (fig.4.18C) while it significantly reduced CD levels in the diet but not in the control rats ($p < 0.05$) (fig.4.18D).

In non-fasting rats, three weeks melatonin treatment had no effect on TBARS, LOOH levels (fig.4.17). Interestingly, it increased CD levels in the controls but not in the diet rats (fig.4.17D). In fasting rats, 3 weeks treatment increased TBARS levels in the control group only (fig 4.18B) while having no effect on LOOH and CD levels in both groups (fig.4.18C, D).

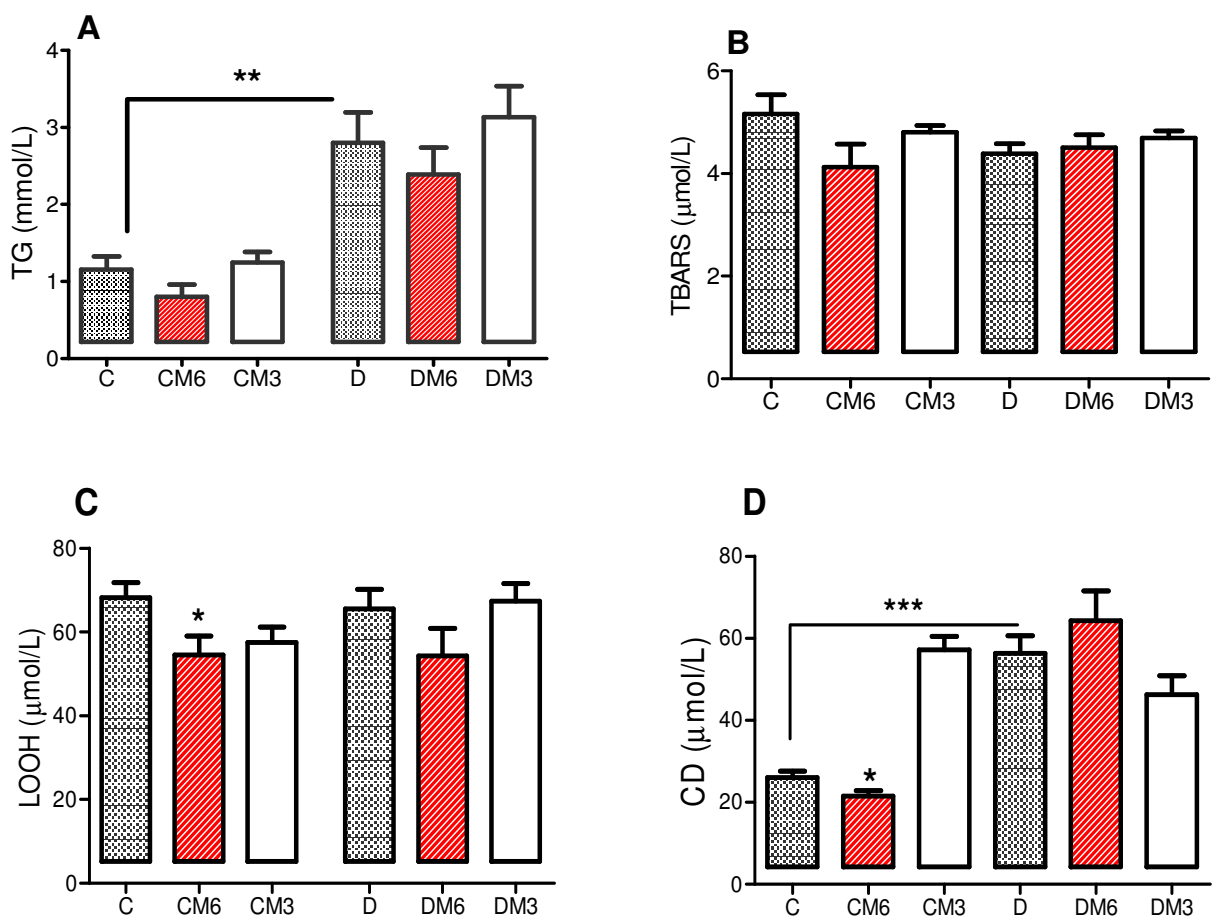


Figure 4.17 Non-fasting serum triglycerides (TG) (A), thiobarbituric acid reactive substances (TBARS) (B), lipid hydroperoxides (LOOH) (C) and conjugated dienes (D), A: $**p < 0.01$ (C vs CM6), C: $*p < 0.05$ (CM6 vs C), D: $*p < 0.05$ (CM6 vs C), $***p < 0.001$ (C vs CM3 or D), $n = 6/\text{group}$.

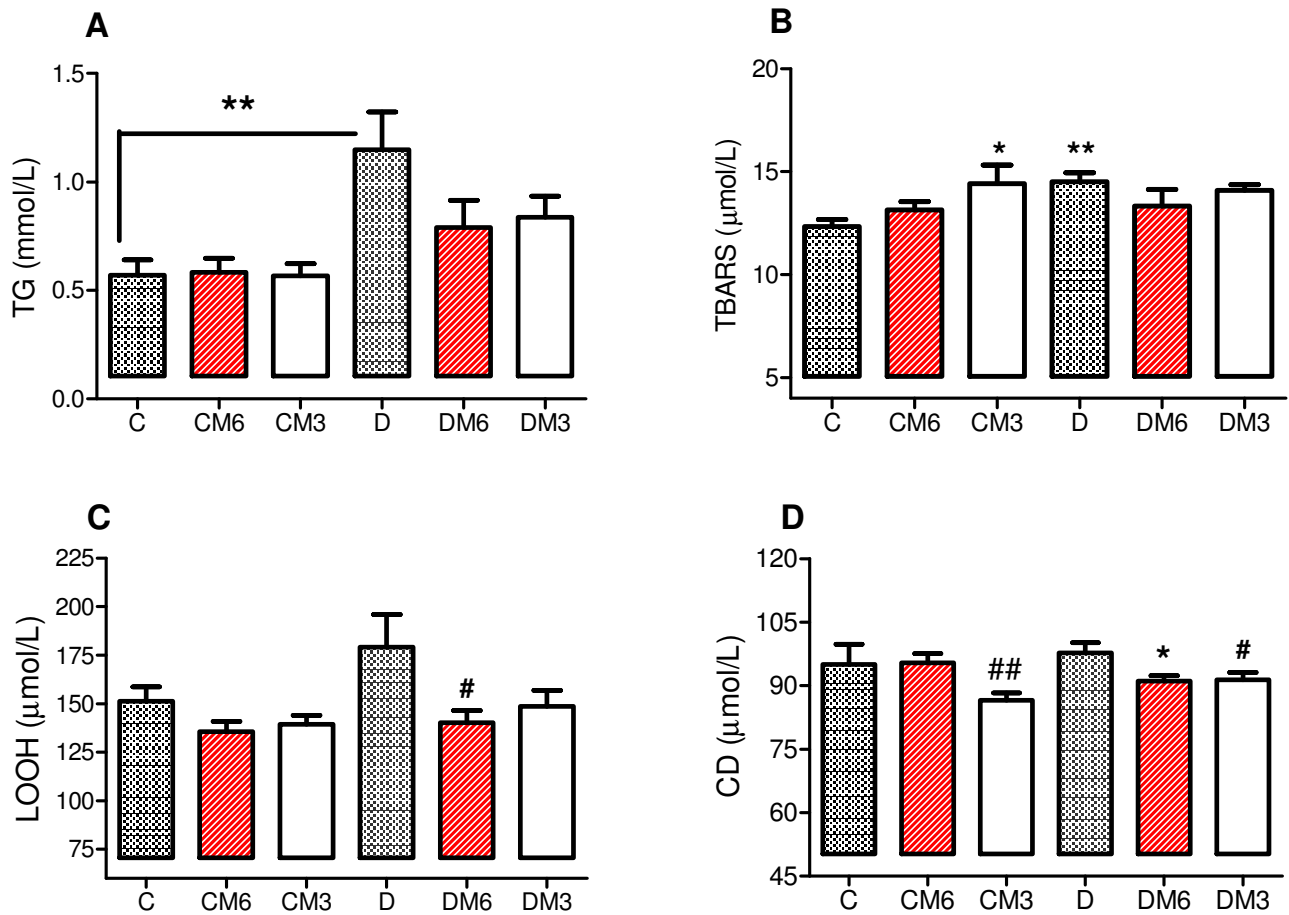


Figure 4.18 Fasting serum triglycerides (TG) (A), thiobarbituric acid reactive substances (TBARS) (B), lipid hydroperoxides (LOOH) (C) and conjugated dienes (D). A: ** $p < 0.01$ (D vs C), B: * $p < 0.05$ (CM3 vs C), ** $p < 0.01$ (D vs C), C: # $p = 0.06$ (DM6 vs D), D: * $p < 0.05$ (DM6 vs D), # $p = 0.06$ (DM3 vs D), ## $p = 0.1$ (CM3 vs C), $n = 6-7$ /group.

Table 4.7 Serum lipid peroxidation from non-fasting rats

Parameters	CONTROL GROUP			DIET GROUP		
	C	CM6	CM3	D	DM6	DM3
TBARS	5.16±0.36	4.13±0.45	4.8±0.13	4.38±0.19	4.5±0.24	4.69±0.13
CD	26.04±1.62	<u>21.49±1.36*</u>	<u>57.11±3.35***</u>	<u>56.33±4.2***</u>	64.27±7.73	46.24±4.59
LOOH	68.24±3.55	<u>54.53±4.47*</u>	57.48±3.63	65.50±4.66	54.23±6.64	67.33±4.23
TBARS (n)	1.78±0.12	1.73±0.20	1.66±0.08	<u>0.93±0.07*</u>	1.03±0.10	0.92±0.07
CD (n)	9.03±0.61	9.24±1.00	<u>19.82±1.46*</u>	<u>11.73±0.42**</u>	14.25±1.65	<u>8.96±0.83#</u>
LOOH (n)	24.12±2.71	<u>22.83±1.70*</u>	20.32±2.53	<u>13.85±1.19*</u>	12.50±1.81	13.55±1.74
TG+PL	2.92±0.20	2.42±0.20	2.92±0.17	<u>4.85±0.48*</u>	4.49±0.35	5.21±0.41
PL	1.77±0.07	1.62±0.06	1.24±0.13	2.05±0.13	2.10±0.08	2.08±0.03
TG	1.15±0.1	0.80±0.15	1.2±0.13	<u>2.80±0.3**</u>	2.39±0.3	3.13±0.4
T C	2.96±0.13	2.79±0.08	2.83±0.05	3.06±0.19	3.43±0.33	2.92±0.07
n/group	6	6	6	6	6	6

CD: conjugated dienes, LOOH: lipid hydroperoxides, TBARS: thiobarbituric acid reactive substances, TC: total cholesterol, TG: triglycerides, PL: phospholipid; TBARS, LOOH and CD ($\mu\text{mol/L}$), (n): normalized value (absolute concentration divided by TG+PL) ($\mu\text{mol/mmol}$); TC, TG and PL (mmol/L), * $p < 0.05$ vs C, ** $p < 0.01$ vs C, *** $p < 0.001$ vs C, # $p < 0.05$ vs D, n=6/group.

Table 4.8 Serum lipid peroxidation from fasting rats

Parameters	CONTROL GROUP			DIET GROUP		
	C	CM6	CM3	D	DM6	DM3
TBARS	12.35±0.34	13.15±0.40	<u>14.42±0.9*</u>	<u>14.52±0.42*</u>	14.55±1.86	14.11±0.26
CD	94.27±4.08	95.42±2.16	86.50±1.71	96.44±2.39	<u>91.07±1.27#</u>	91.38±1.73@
LOOH	151.3 ±7.5	135.6 ±5.3	139.3 ±4.6	176.4 ±17.1	140.3 ±6.1@	148.7 ±8.2
TBARS(n)	6.54±0.26	6.79±0.36	<u>7.49±0.30*</u>	5.76±0.44	6.49±0.64	6.19±0.17
CD(n)	50.08±2.80	49.24±2.33	45.48±2.39	38.58±3.39	41.38±2.49	40.06±0.79
LOOH(n)	79.47±1.74	<u>69.42±1.39*</u>	73.09±4.01	<u>67.34±2.25**</u>	64.09±5.31	65.15±3.58
TG+PL	1.90±0.07	1.95±0.09	1.92±0.08	<u>2.64±0.27*</u>	2.24±0.13	2.28±0.05
TG	0.57± 0.07	0.58± 0.06	0.56± 0.05	<u>1.05± 0.17**</u>	0.79 ±0.12	0.83 ±0.09
PL	1.33± 0.03	1.37± 0.05	1.35± 0.03	<u>1.59± 0.10*</u>	1.45± 0.02	1.44± 0.04
TC	1.47± 0.08	1.49± 0.07	1.42± 0.03	1.54± 0.07	1.33± 0.06@	<u>1.30 ±0.06#</u>
n/group	7	6	6	7	6	6

CD: conjugated diene, LOOH: lipid hydroperoxide, TBARS: thiobarbituric acid reactive substance; TC: total cholesterol, TG: triglycerides; PL: phospholipid; TBARS, LOOH and CD ($\mu\text{mol/L}$); TC, TG and PL (mmol/L), (n): normalized value (absolute concentration divided by TG+PL) ($\mu\text{mol/mmol}$), * $p < 0.05$ vs C; ** $p < 0.01$ vs. C; # $p < 0.05$ vs D, @ $p = 0.06-0.07$ vs D, $n = 6-7/\text{group}$.

4.3.3. EFFECTS OF MELATONIN ON MYOCARDIAL FUNCTION

The diet had no effect on basal (pre-ischaemic) myocardial function (table 4.9). Melatonin administered for six or three weeks had no significant effect on pre-ischaemic functional parameters (coronary flow, aortic output, work total, systolic pressure) of the isolated rat hearts from the control as well as the diet animals (table 4.9). During 30 min of reperfusion following 35 min of regional ischaemia, the hearts from D had reduced aortic output percentage recovery compared to C group. Six weeks melatonin treated rats increased aortic output and cardiac output percentage recovery in D rats ($p < 0.05$) while the coronary flow and systolic pressure percentage recovery remained unaffected (fig.4.19-23).

The aortic output, cardiac output and work total percentage recovery were increased significantly in the hearts isolated from D rats treated with melatonin for 3 weeks (fig.4.20-22). The systolic pressure and coronary flow was not affected by 3 and 6 weeks melatonin treatment (fig.4.19,23)

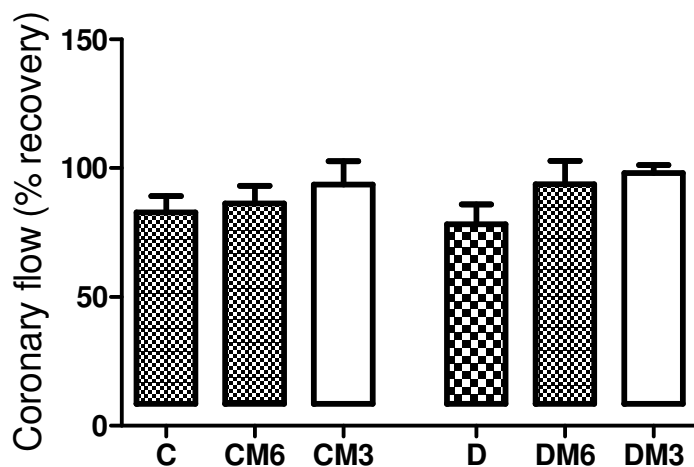


Figure 4.19 Percentage coronary flow recovery after 30 min reperfusion (n=6/group)

Table 4.9 Averaged values of pre-and post-ischaeamic myocardial function

Time	Parameters	C	CM6	CM3	D	DM6	DM3
Stabilization After 30 min	CF	16.20±0.56	16.83±0.52	15.00±1.0	16.32±0.88	15.33±0.62	14.83±0.61
	AO	36.20±1.4	39.63±2.14	38.33±3.65	29.43±2.18	30.83±2.15	35.33±2.61
Before ischaemia (Working heart)	CO	52.40±1.41	56.46±1.65	53.30±6.62	45.76±2.73	46.17±2.71	50.17±3.08
	SP	90.33±1.49	90.57±0.92	90.50±2.07	88.14±2.65	89.17±2.77	90.83±1.85
	Wtot	9.39±0.60	11.37±0.39	10.73±1.23	8.70±1.12	9.22±0.84	10.38±0.91
Reperfusion After 30 min	CF	13.41±1.31	14.35±1.29	14.20±2.33	12.0±1.11	14.50±1.16	14.4±1.25
	AO	<u>9.22±3.61(*)</u>	11.35±3.04	12.20±4.43	4.14±1.42	<u>8.04±1.02(*)</u>	<u>11.42±2.36(*)</u>
Post-ischaemia (Working heart)	CO	<u>22.63±4.78(#)</u>	26.6±3.43	26.40±6.98	16.14±1.9	<u>22.54±1.63(*)</u>	<u>25.67±2.88(*)</u>
	SP	79.25±4.64	80.83±3.68	85.34±2.35	72.14±3.64	77.50±2.97	82.17±3.01
	Wtot	3.19±0.81	3.38±0.39	5.41±1.42	2.640±0.36	3.02±0.65	<u>5.13±0.80(*)</u>

CF: coronary flow (mL/min); AO: aortic output (mL/min); CO: cardiac output (mL/min); SP: systolic pressure (mmHg); Wtot: work total (mWatts); *p<0.05 (vs D), #p=0.06 (vs D), n=6/group.

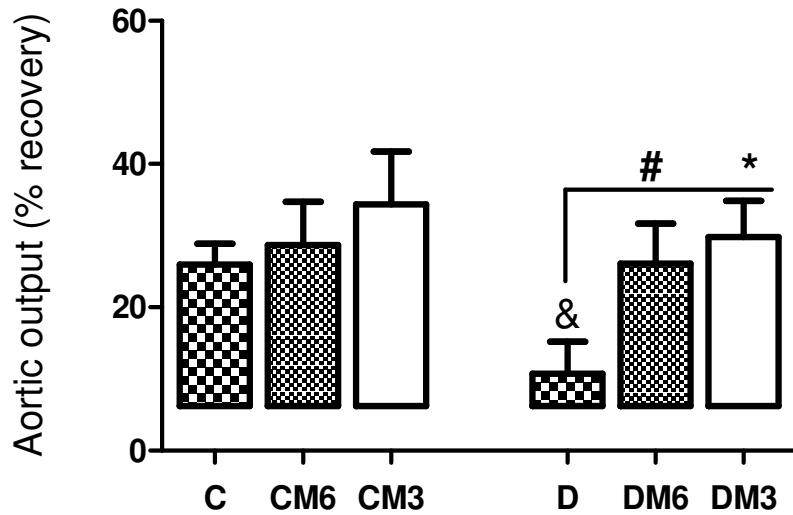


Figure 4.20 Aortic output percentage recovery after 30 min reperfusion, * $p < 0.05$ (DM3 vs D); # $p < 0.05$ (DM6 vs D), & $p < 0.05$ (C vs D), $n = 6$ /group

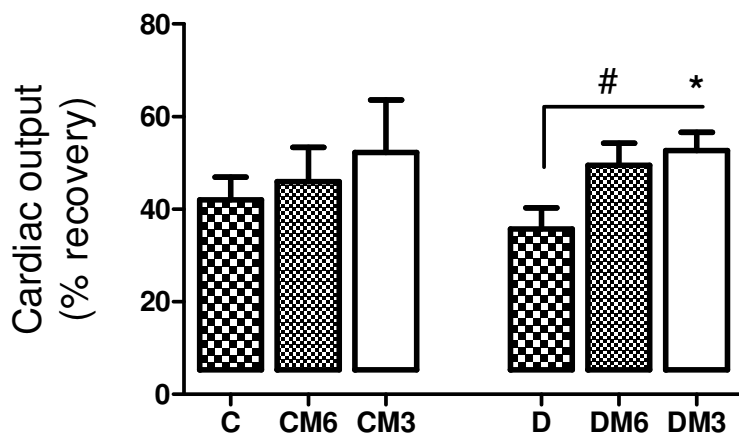


Figure 4.21 Cardiac output percentage recovery after 30 min reperfusion, * $p < 0.05$ (D vs DM3); # $p = 0.08$ (D vs DM6), $n = 6$ /group

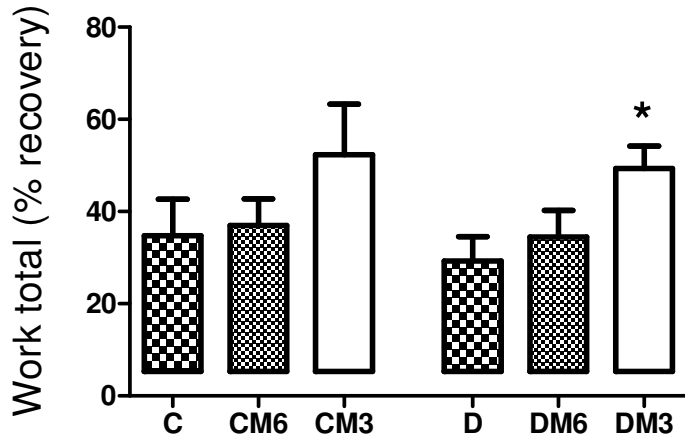


Figure 4.22 Work total percentage recovery after 30 min reperfusion, * $p < 0.05$ (DM3 vs D), $n = 6$ /group.

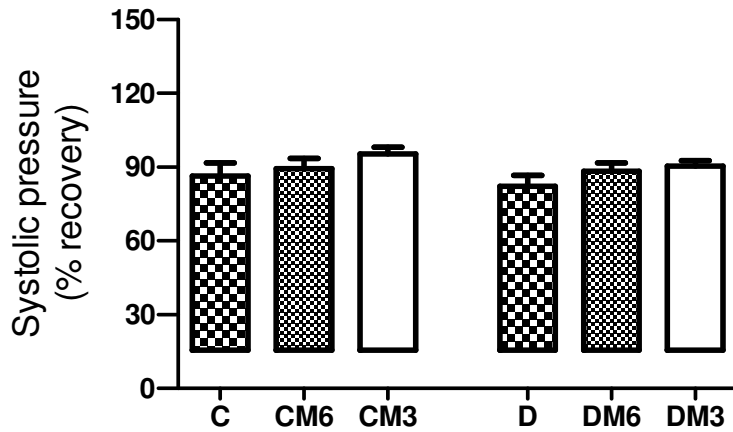


Figure 4.23 Systolic pressure percentage recovery after 30 min reperfusion, $n = 6$ /group

4.3.4. EFFECT OF MELATONIN TREATMENT ON MYOCARDIAL INFARCT SIZE

Figure 4.24 represents the effect of high calorie diet and melatonin administration on myocardial infarct size after exposure of the hearts to 35 min coronary ligation. Hearts from the untreated high calorie diet (D) group had larger myocardial infarct sizes compared to control hearts ($59.35 \pm 4.63\%$ (D) vs. $42.03 \pm 6.27\%$ (C), $p < 0.05$).

Myocardial infarct size was significantly reduced in six or three weeks treated rats compared to untreated ($23.04 \pm 3.30\%$ for CM6 or $24.19 \pm 4.56\%$ for CM3 vs $42.03 \pm 6.27\%$ for C, $p < 0.01$), and $30.31 \pm 2.97\%$ for DM6 or $22.74 \pm 2.87\%$ for DM3 vs $59.35 \pm 4.63\%$ for D, $p < 0.001$).

Six weeks treatment reduced infarct size by 45.6% and 48.9% in C and D groups, respectively. In the three weeks melatonin treated C and D groups the reduction in infarct size averaged 42.4% and 61.6%, respectively.

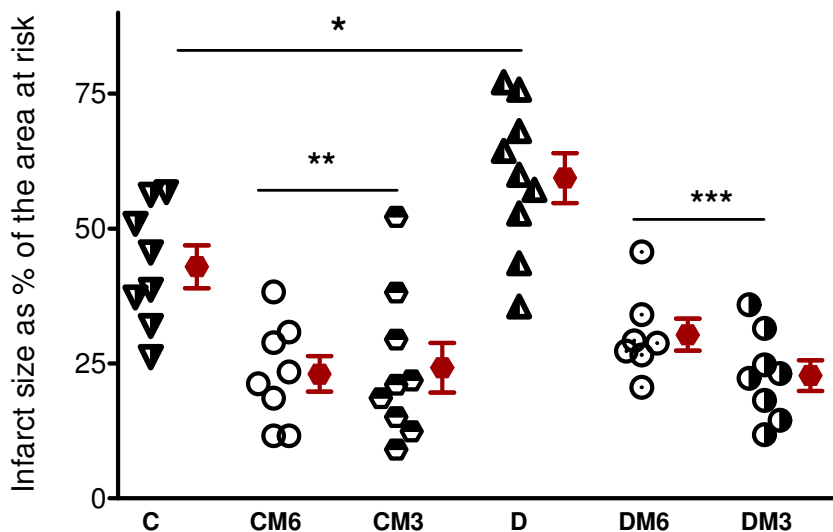


Figure 4.24 Effect of 3 and 6 weeks melatonin treatment on myocardial infarct size, * $p < 0.05$ (C vs D); ** $p < 0.01$ (CM3 or CM6 vs C); *** $p < 0.001$ (DM3 or DM6 vs D), $n = 7-9$ /group.

4.3.5. EFFECT OF MELATONIN TREATMENT ON MYOCARDIAL SIGNALLING

To determine by which signalling mechanism melatonin confers cardioprotection in our animal model, we investigated the effect of melatonin on the reperfusion injury salvage kinases (RISK) (PKB/Akt, ERK (p42/p44)), glycogen synthase kinase-3 beta (GSK-3 β) and signal transducer and activator of transcription 3 (STAT-3) (a member of the survivor activating factor enhancement (SAFE) pathway) using western blotting. To avoid contamination of ischaemic and non-ischaemic tissues which may occur with regional ischaemia we preferred to use a model of global ischaemia.

We assessed baseline (non-perfused hearts) and post- ischaemic (after 10 and 30 min reperfusion) phosphorylation of the kinases. Phosphorylation of STAT-3 was also evaluated in cytosolic and nuclear fractions at baseline and after 10 min reperfusion. PI3-K (p85) and GLUT-4 were also studied at baseline (non-perfused heart tissues). Results are presented in figures 4.25-54 and summarized in tables 4.10-11. Blots shown are representative. Each graph is accompanied by representative blots of total and phospho-proteins. In the case of GLUT-4 expression, a representantive blot of β -tubulin is included.

4.3.5.1. PKB/Akt, ERK 42/44 and GSK3- β

4.3.5.1.1. Baseline tissues

- **PKB/Akt**

In non-perfused tissues, the high calorie diet did not affect total PKB expression and phosphorylation in both groups (fig. 4.25-26).

Melatonin administration for six and three weeks did not affect the total expression of PKB (fig. 4.25-26). Hearts from 6 weeks melatonin treated groups exhibited a significant increase in PKB phosphorylation (Ser-473) ($p < 0.05$) in both control and D groups. Three weeks treatment increased PKB phosphorylation in control but not in D group (fig.4.25).

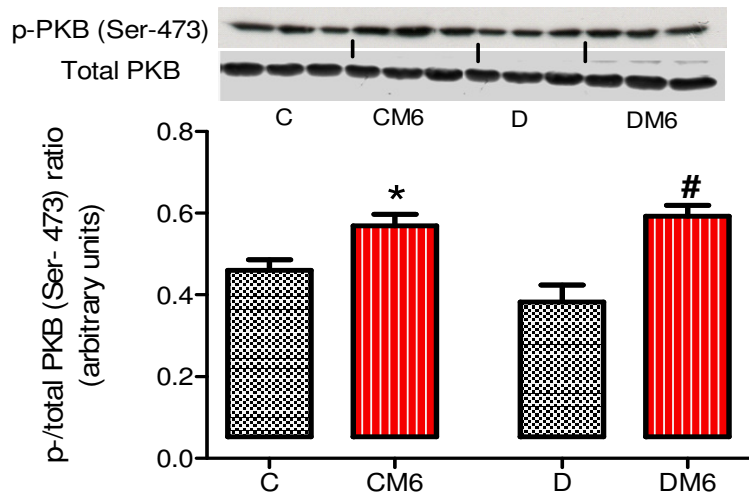


Figure 4.25 Effect of six weeks melatonin treatment on baseline PKB phosphorylation, * $p < 0.05$ (C vs CM3), # $p < 0.05$ (D vs DM6), $n = 6$ hearts/group.

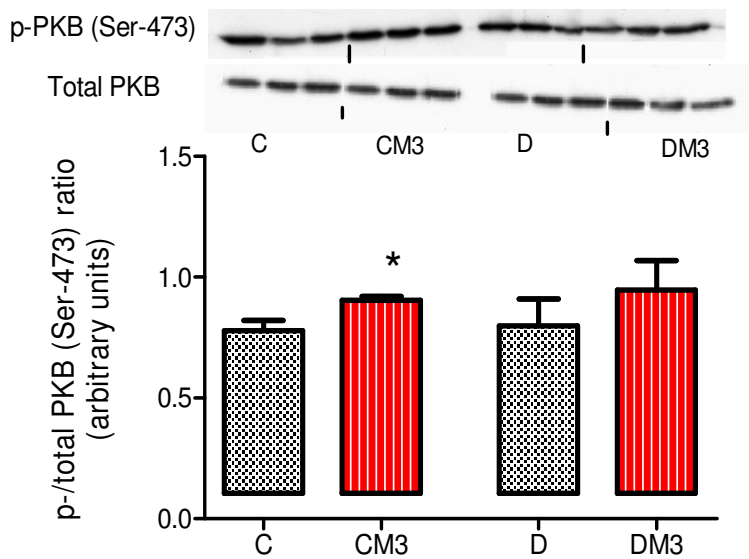


Figure 4.26 Effect of 3 weeks treatment on baseline PKB/Akt (Ser-473) phosphorylation, * $p < 0.05$ (C vs CM3), representative of blots $n = 3$ hearts/group.

- **ERK 42/44**

Feeding a high calorie diet did not affect the baseline phosphorylation pattern of ERK 42/44 (fig.4.27). Administration of melatonin had no significant effect on ERK 42/44 phosphorylation in either control or D groups (fig.4.27).

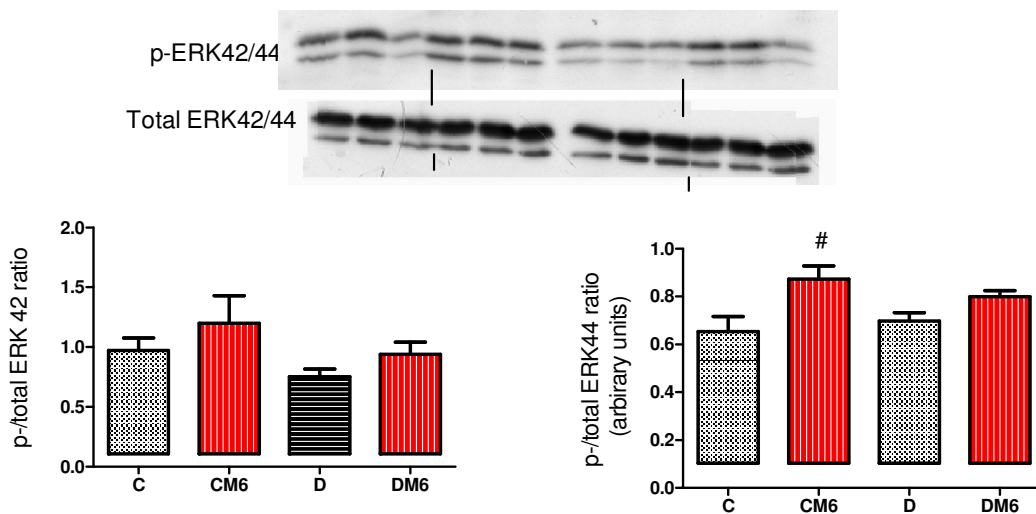


Figure 4.27 Effect of 6 weeks melatonin treatment on baseline ERK 42/44 phosphorylation, #p=0.06 (C vs CM6), n=6 hearts/group.

- **GSK-3 β**

The high calorie diet had no effect on the expression and phosphorylation of GSK-3 β when compared to those of controls. Administration of melatonin for six weeks increased GSK-3 β phosphorylation in D but not in control groups (fig.4.28), while three weeks treatment increased GSK-3 β phosphorylation in control group only (fig.4.29).

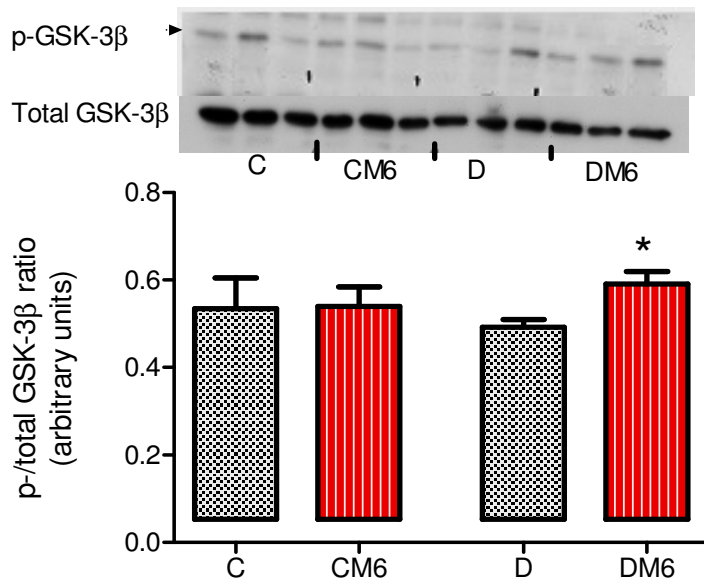


Figure 4.28 Effect of 6 weeks melatonin treatment on baseline GSK-3β phosphorylation, * $p < 0.05$ (D vs DM6), $n = 6$ hearts/group.

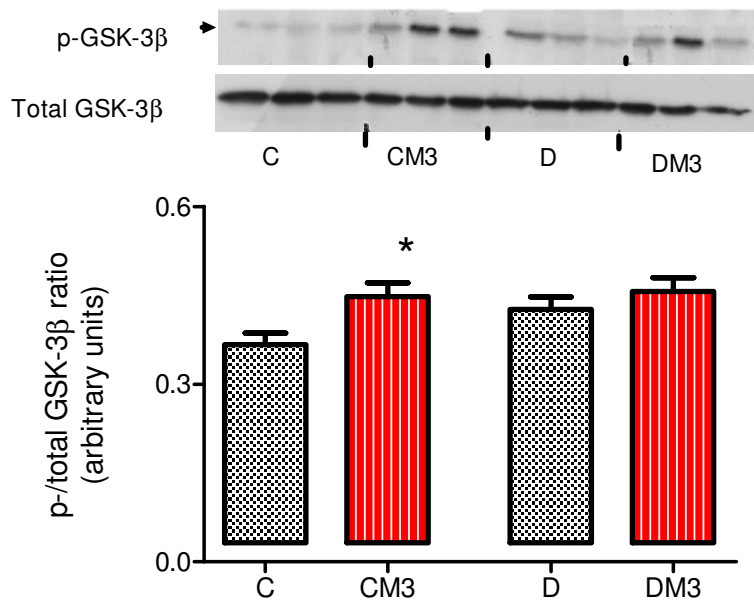


Figure 4.29 Effect of 3 weeks melatonin administration on baseline GSK3-β phosphorylation, * $p < 0.05$ (C vs CM3), $n = 3$ hearts/group.

4.3.5.1.2. The effects of ischaemia-reperfusion on kinases

4.3.5.1.2.1. After 10 min reperfusion

- **Effects of six weeks treatment**

Similar to baseline, the high calorie diet did not affect the post-ischaemic PKB and ERK42/44 expression and phosphorylation. Melatonin increased PKB and ERK42/44 activation in both control and D group (fig.4.30-31). Hearts from the six weeks treated group had increased phosphorylation and activation of GSK-3 β after 10 min reperfusion (fig.4.32)

- **PKB/Akt**

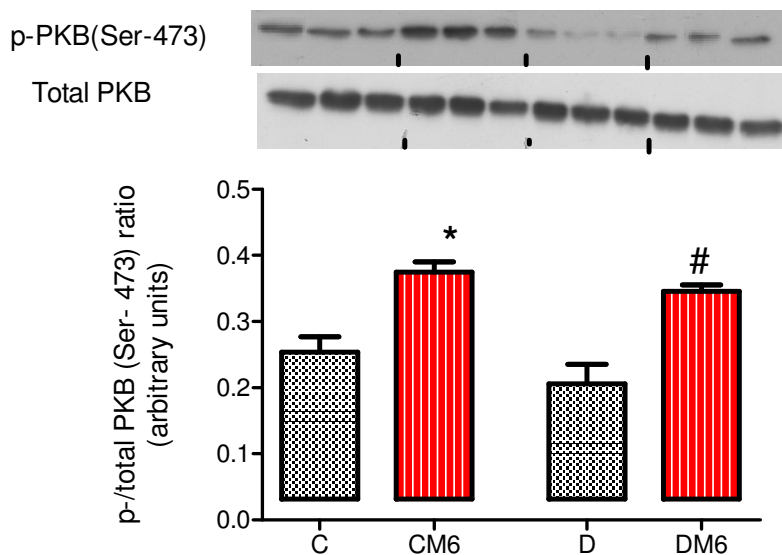


Figure 4.30 Effect of 6 weeks melatonin treatment on PKB phosphorylation (10 min reperfusion), * $p < 0.05$ (C vs CM6), # $p < 0.05$ (D vs DM6), $n = 6$ hearts/group.

○ ERK 42/44

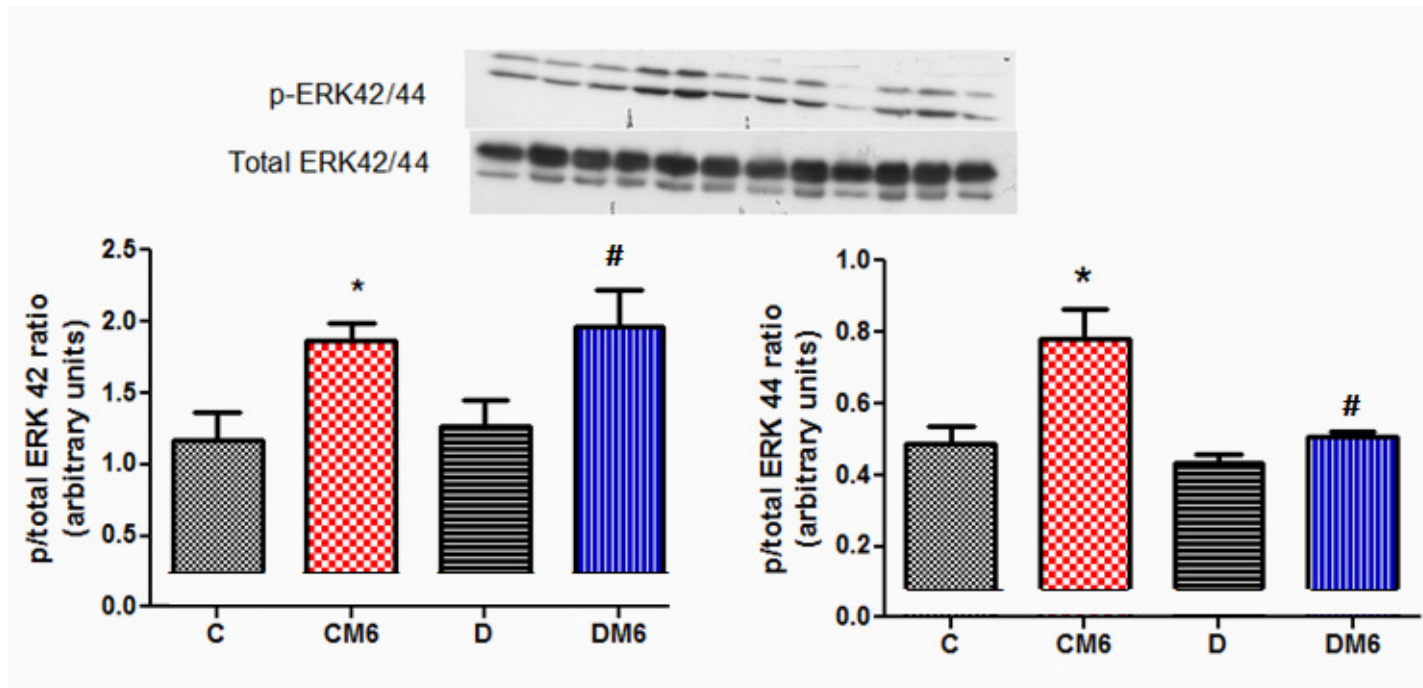


Figure 4.31 Effect of 6 weeks melatonin treatment on ERK42/44 phosphorylation (10 min reperfusion), * $p < 0.05$ (C vs CM6), # $p < 0.05$ (D vs DM6), $n = 6$ hearts/group.

○ GSK3- β

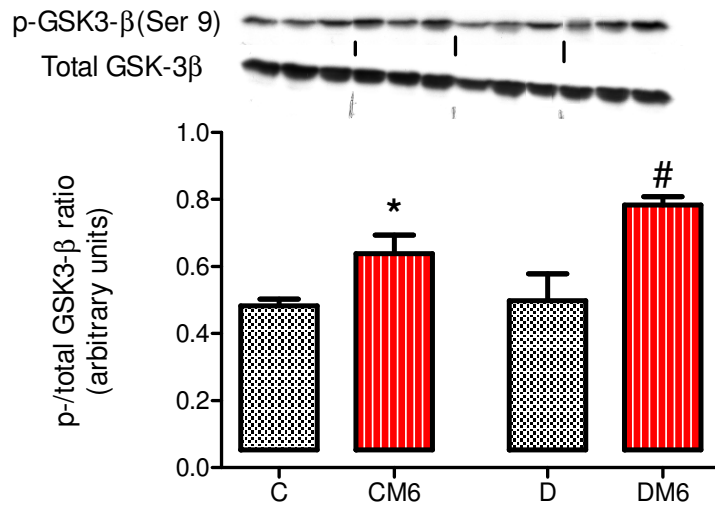


Figure 4.32 Effect of 6 weeks melatonin treatment on GSK-3 β phosphorylation (10 min reperfusion), * $p < 0.05$ (C vs CM6), # $p < 0.05$ (D vs DM6), $n = 6$ hearts/group.

- **Effect of three weeks treatment**

Following 15 min global ischaemia, hearts from three weeks melatonin treated group had increased the phosphorylation of PKB and GSK3- β ($p < 0.05$) in both control and D groups (fig.4.33, 35). ERK42 was also activated in both groups while ERK44 was activated in control group only (fig.4.34).

- **PKB/Akt**

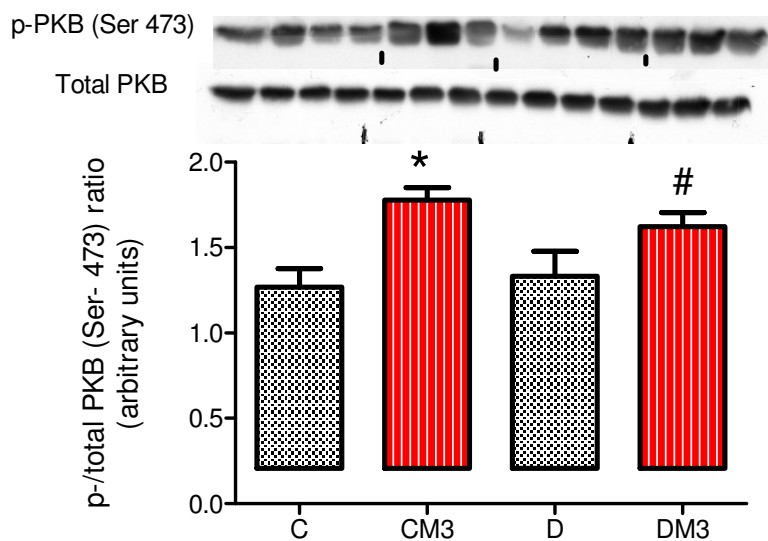


Figure 4.33 Effect of 3 weeks melatonin treatment on PKB phosphorylation (10 min reperfusion), * $p < 0.05$ (C vs CM3), # $p < 0.05$ (D vs DM3), $n = 3-4$ hearts/group

○ ERK 42/44

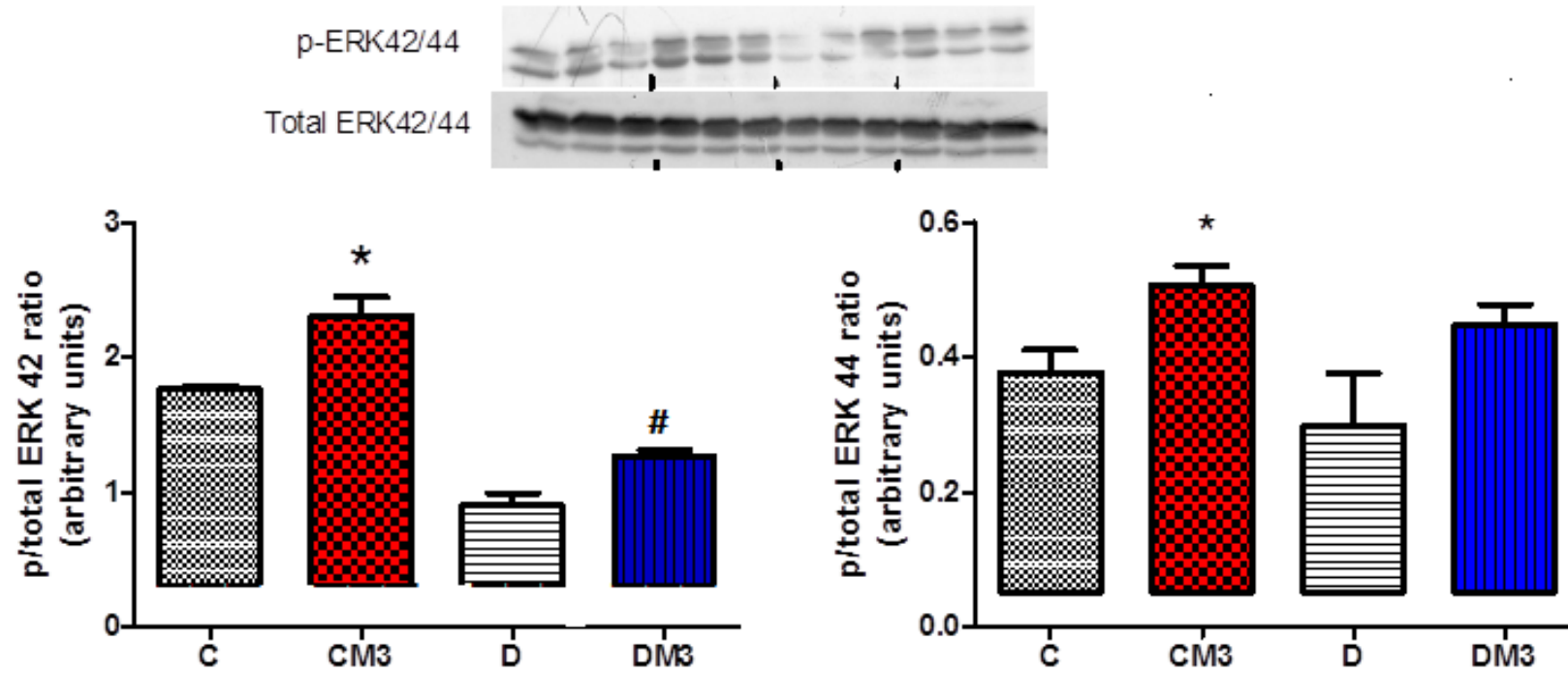


Figure 4.34 Effect of 3 weeks melatonin treatment on ERK42/44 phosphorylation (10 min reperfusion), * $p < 0.05$ (C vs CM3), # $p < 0.05$ (D vs DM3), $n = 6$ hearts/group.

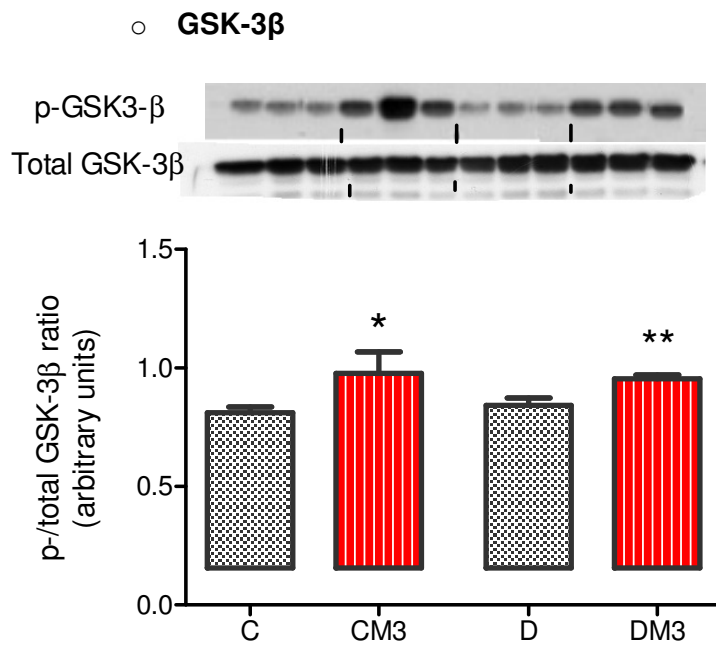


Figure 4.35 Effect of 6 weeks melatonin treatment on GSK-3 β phosphorylation (10 min reperfusion), * $p < 0.05$ (C vs CM3), ** $p < 0.05$ (D vs DM3), $n = 6$ hearts/group.

4.3.5.1.2.2. After 30 min reperfusion

In this series, only the effects of six weeks treatment were evaluated. Hearts from the melatonin treated group showed increased PKB phosphorylation (Ser-473) in both groups ($p < 0.05$) (fig. 4.36) without any effect on ERK 42/44 activation (fig.4.35). GSK3- β was phosphorylated in treated D group only (fig.4.36).

○ PKB/Akt

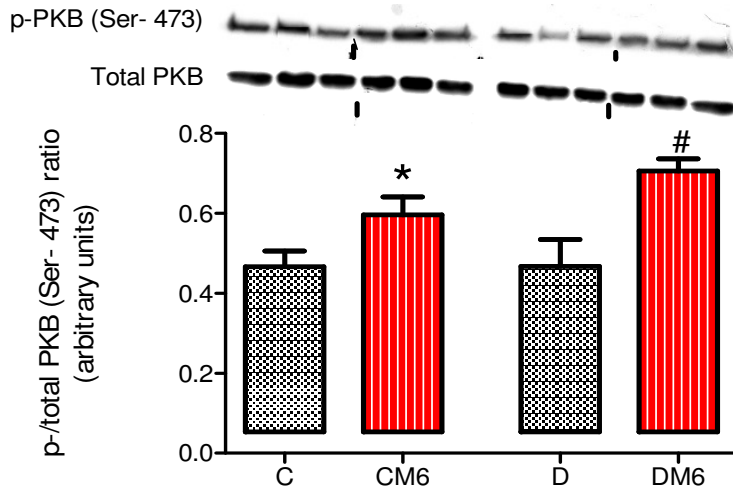


Figure 4.36 Effect of 6 weeks melatonin treatment on PKB phosphorylation (30 min reperfusion), * $p < 0.05$ (C vs CM6), # $p < 0.05$ (D vs DM6), $n = 3$ hearts/group.

○ ERK 42/44

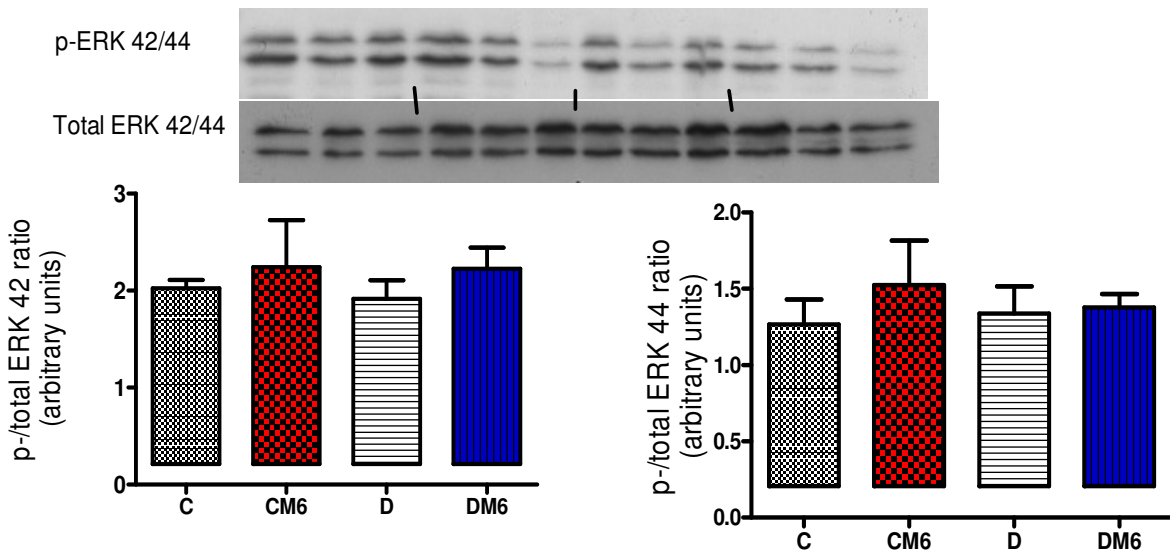


Figure 4.37 Effect of 6 weeks melatonin treatment on ERK42/44 phosphorylation (30 min reperfusion), $n = 3$ hearts/group.

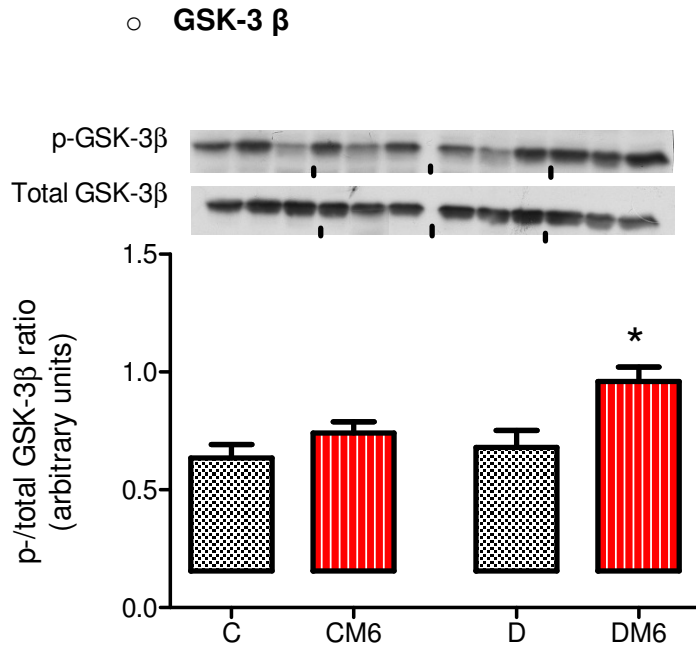


Figure 4.38 Effect of 6 weeks melatonin treatment on GSK-3 β phosphorylation (30 min reperfusion), * $p < 0.05$ (D vs DM6), $n = 3$ hearts/group.

4.3.5.2. Baseline PI3-K (p85)

Oral administration of melatonin for six or three weeks had no effect on baseline PI3-K phosphorylation and activation in both control and D groups (fig.4.39).

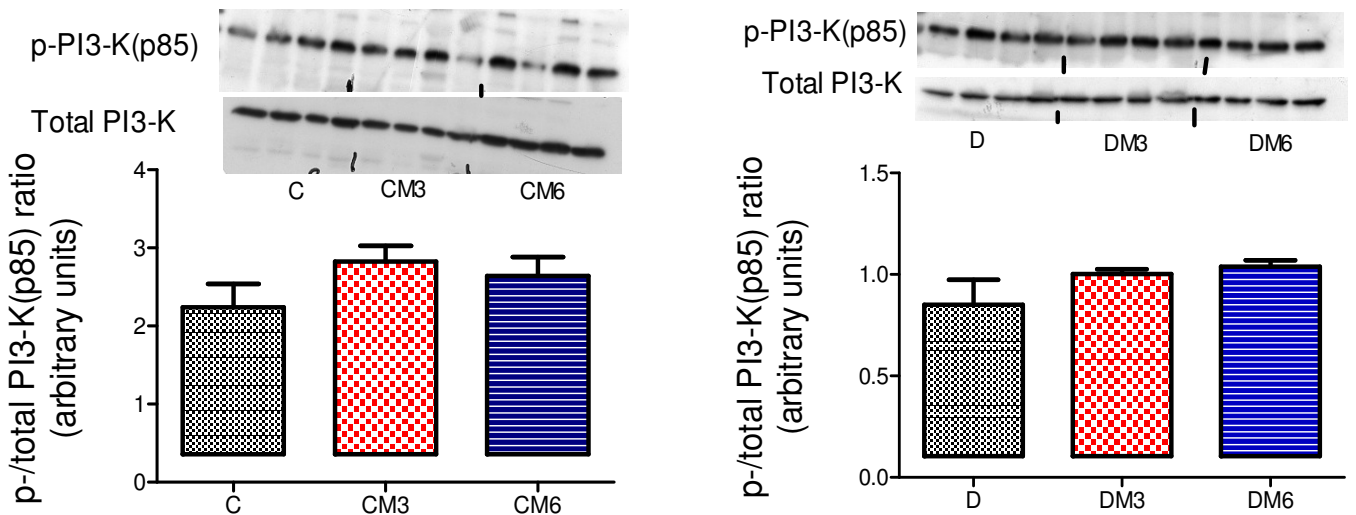


Figure 4.39 Effect of 3 and 6 weeks melatonin on baseline PI3-K (p85) phosphorylation, $n = 4$ hearts/group.

4.3.5.3. Baseline GLUT-4 expression

High calorie diet had no significant effect on GLUT-4 expression. However, 6 weeks but not 3 weeks melatonin treatment increased the levels of expression in both diet and control (fig.4.40).

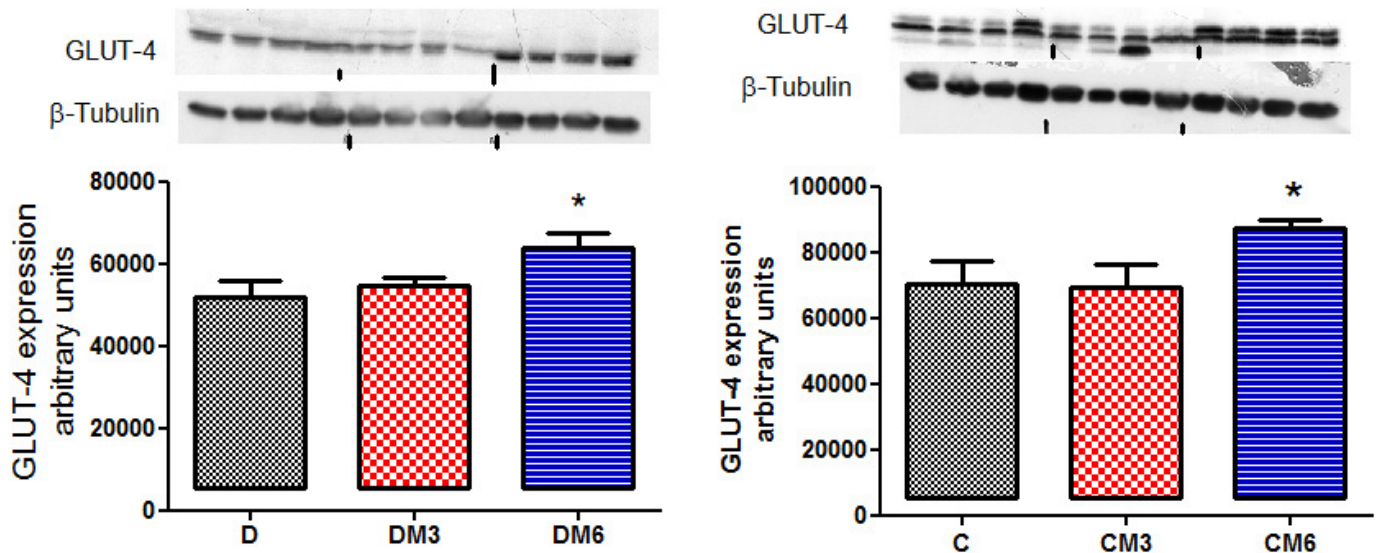


Figure 4.40 Effect of melatonin on baseline GLUT-4 expression, * $p < 0.05$ (D vs DM6 or C vs CM6), $n = 4$ hearts/group.

4.3.5.4. STAT-3

4.3.5.4.1. Baseline tissues

4.3.5.4.1.1. Effect of six weeks treatment

The high calorie diet did not affect nuclear phosphorylated STAT-3 (Tyr-705 and Ser-727). However hearts from the D group had a decreased cytosolic STAT-3 phosphorylation (Tyr-705) compared to control (not shown).

Six weeks melatonin did not affect baseline cytosolic STAT-3 (Tyr-705) in any of the groups, Ser-727 was however increased ($p < 0.05$) in control but not D group (fig.4.41-42). While cardiac nuclear fractions from melatonin treated groups had increased phosphorylation at Tyr-705, Ser-727 was not affected (fig.4.43-44). Six weeks treatment did not affect cytosolic or nuclear expression of total STAT-3.

- Cytosolic fraction

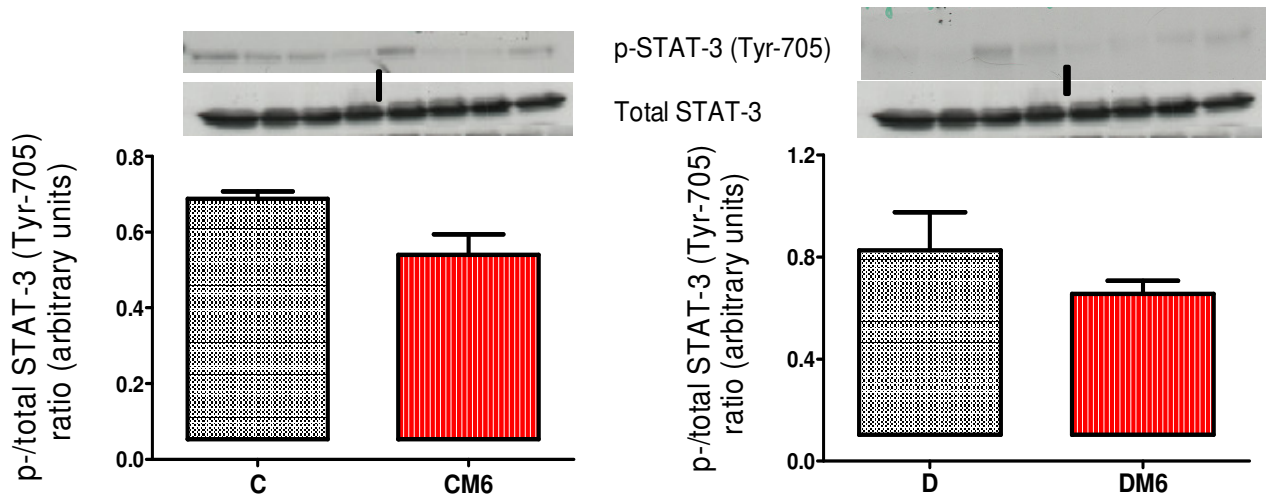


Figure 4.41 Baseline STAT-3 phosphorylation (Tyr-705), cytosolic fraction, n=4 hearts /group.

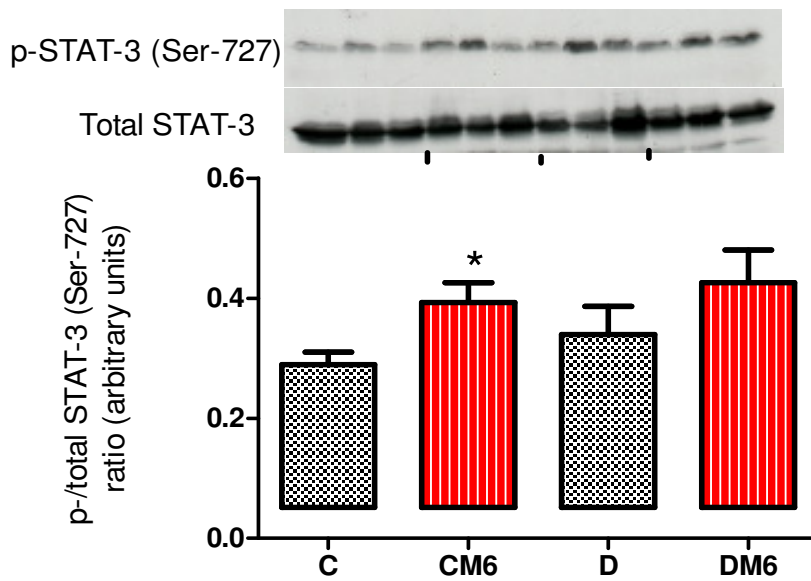


Figure 4.42 Baseline STAT-3 phosphorylation (Ser -727), cytosolic fraction, *p<0.05 (C vs CM6), n=3 hearts/group.

- Nuclear fraction

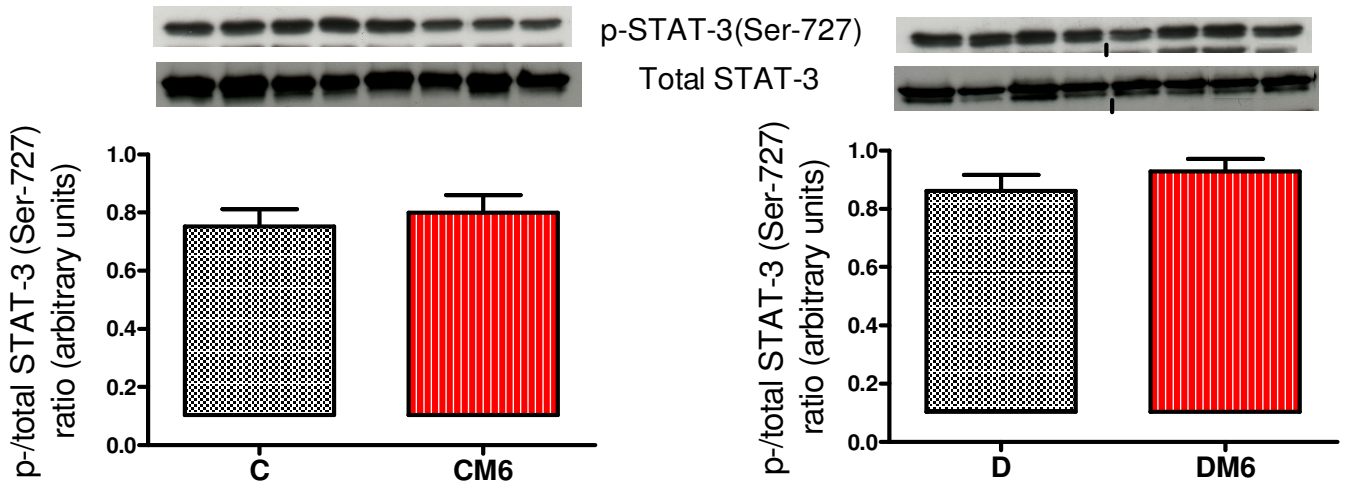


Figure 4.43 Baseline STAT-3 phosphorylation (Ser-727), nuclear fraction, n=4hearts/group.

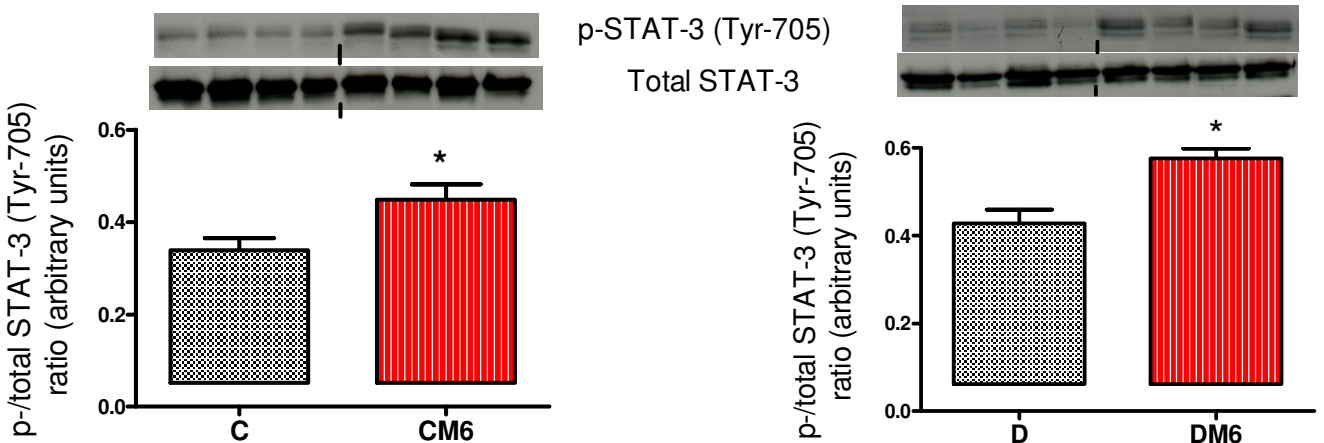


Figure 4.44 Baseline STAT-3 phosphorylation (Tyr-705), nuclear fraction, *p<0.05 (C vs CM6 or D vs DM6), n=4 hearts/group.

4.3.5.4.1.2. Effect of three weeks treatment

Three weeks melatonin treatment did not affect the expression of STAT-3 in any of the groups. It, however, did increase cytosolic STAT-3 phosphorylation at Ser-727 (p<0.05) in D but not in control group (fig.4.45). There was no effect of melatonin treatment on cytosolic Tyr-705 in both control and D groups (not shown). However, it increased the nuclear phosphorylated

STAT-3 (Tyr-705) in D group only (fig.4.46). Nuclear Ser-727 was not affected in both groups (fig.4.47).

- **Cytosolic**

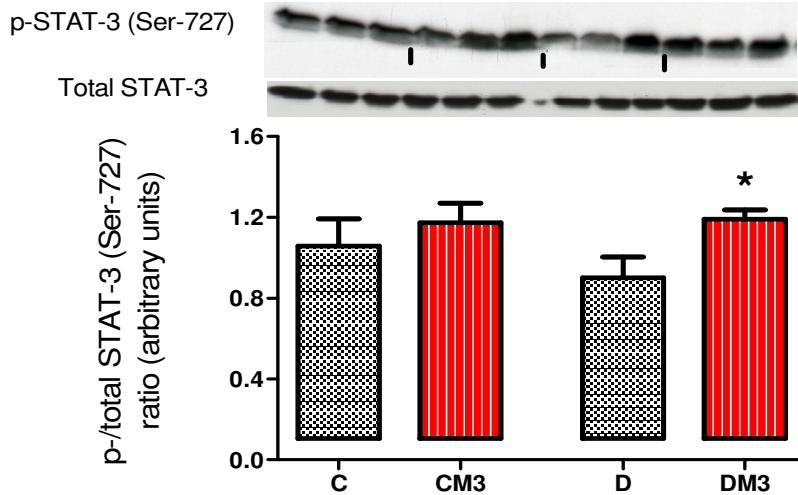


Figure 4.45 Baseline STAT-3 phosphorylation (Ser -727), cytosolic fraction, *p<0.05 (D vs DM3), n=3 hearts /group.

- **Nuclear fraction**

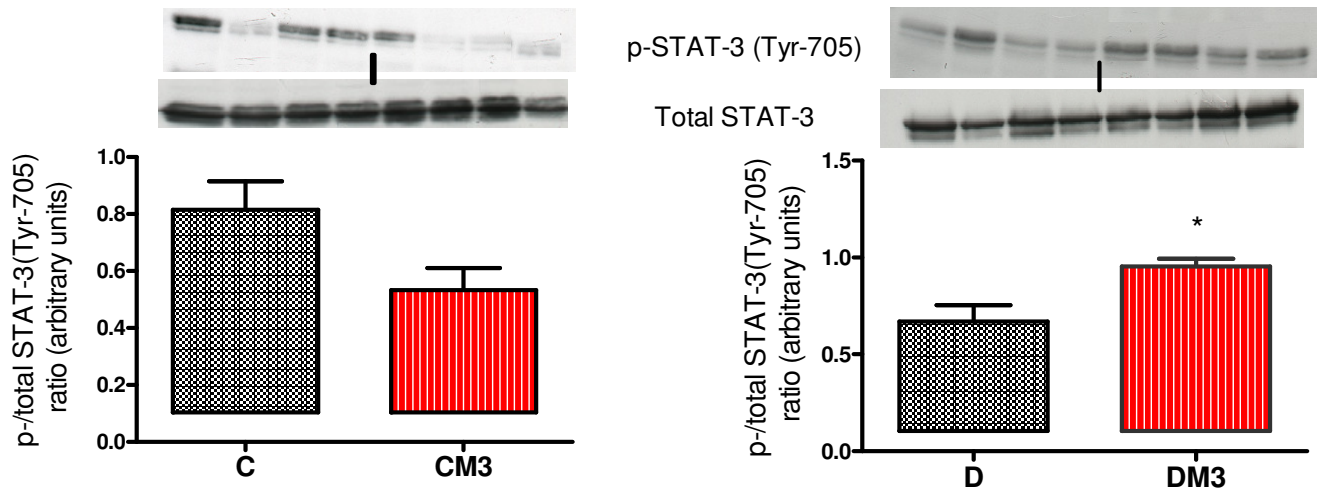


Figure 4.46 Baseline STAT-3 phosphorylation (Tyr-705), nuclear fraction, *p<0.05 (D vs DM3), n=4 hearts/group.

4.3.5.4.2. Effect of ischaemia-reperfusion on STAT-3

4.3.5.4.2.1. Effect of six weeks treatment

The diet had no effect on post-ischaemic STAT-3 expression and phosphorylation at 10 min reperfusion. Six weeks melatonin treatment increased post-ischaemic nuclear and cytosolic fractions of phosphorylated STAT-3 (Tyr-705) in both control and diet groups ($p < 0.05$) (fig.4.47, 50). However, nuclear fraction of phosphorylated STAT-3 Ser-727 was not affected by the treatment (fig.4.48) while the cytosolic fraction of STAT-3 Ser-727 showed increased phosphorylation in the control but not in diet group (fig.4.49).

- **Nuclear fraction**

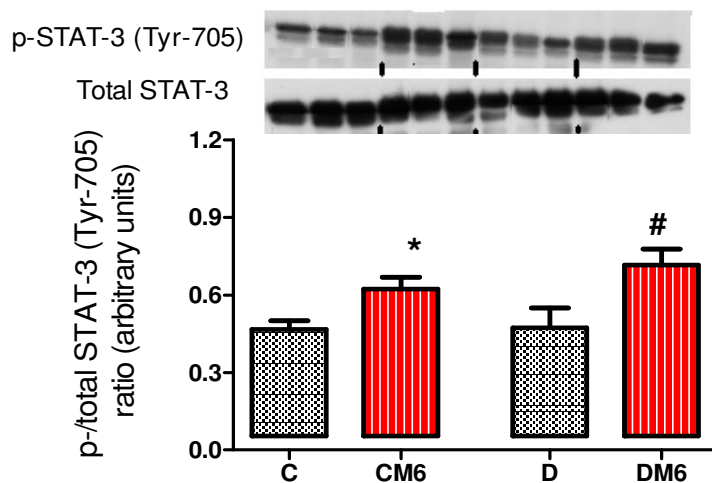


Figure 4.47 STAT-3 phosphorylation (Tyr-705), nuclear fraction (10 min reperfusion), * $p < 0.05$ (C vs CM6), # $p < 0.05$ (D vs DM6), $n = 3$ hearts/group

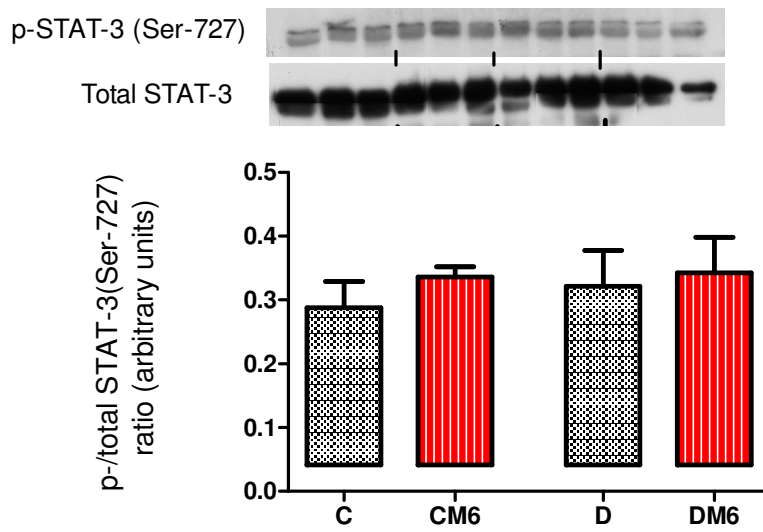


Figure 4.48 STAT-3 phosphorylation (Ser-727), nuclear fraction (10 min reperfusion), n=3 hearts/group.

- **Cytosolic fraction**

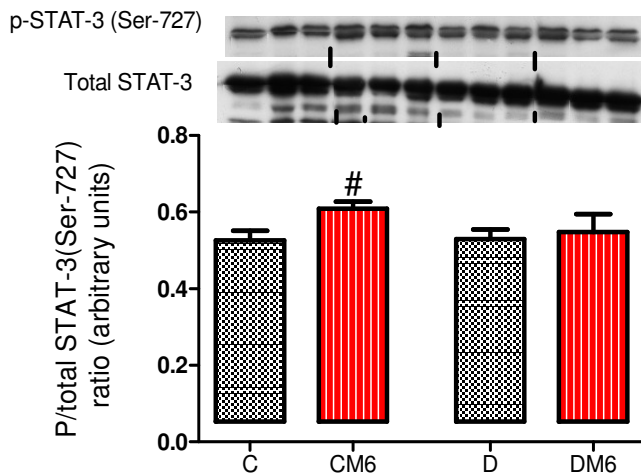


Figure 4.49 STAT-3 phosphorylation (Ser-727), cytosolic fraction (10 min reperfusion), #p=0.06 (C vs CM6), n=3 hearts/group

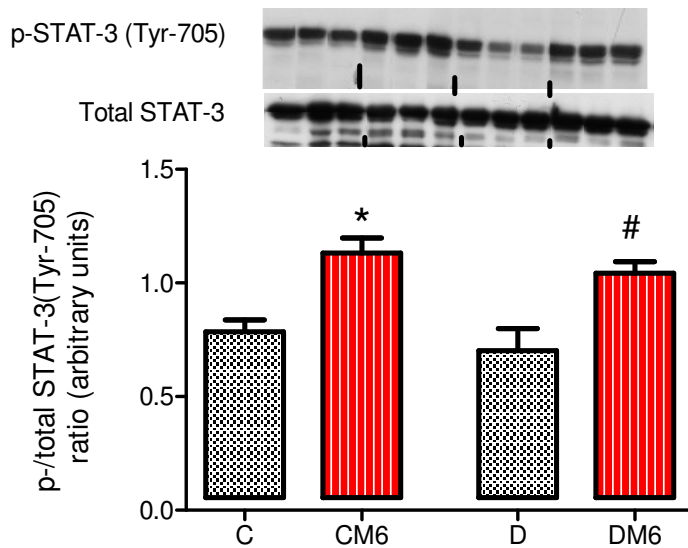


Figure 4.50 STAT-3 phosphorylation (Tyr 705), cytosolic fraction (10 min reperfusion), * $p < 0.05$ (C vs CM6), # $p < 0.05$ (D vs DM6), $n = 3$ hearts/group.

4.3.5.4.2.2. Effects of three weeks treatment

Three weeks melatonin treatment increased cytosolic STAT-3 (Ser-727) phosphorylation in the control but not in the diet group ($p < 0.05$) while the nuclear STAT-3 (Ser-727) was not affected in any of the groups (fig.4.51, 53). Nuclear phosphorylated STAT-3 (Tyr-705) was increased by melatonin treatment in control but not in D groups (fig.4.53). Cytosolic STAT-3 (Tyr-705) was not affected by three weeks melatonin treatment (fig.4.52).

- Cytosolic fraction

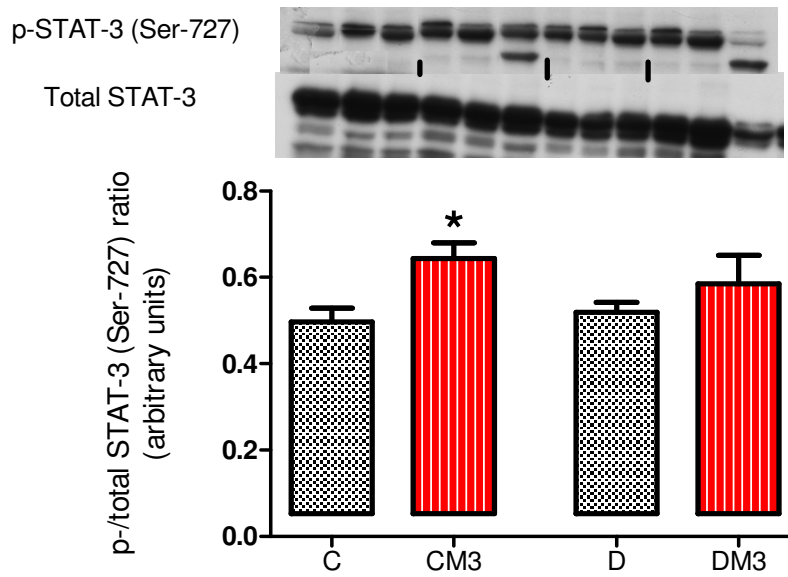


Figure 4.51 STAT-3 phosphorylation (Ser-727), cytosolic fraction (10 min reperfusion),

*p<0.05 (C vs CM3), n=3 hearts/group.

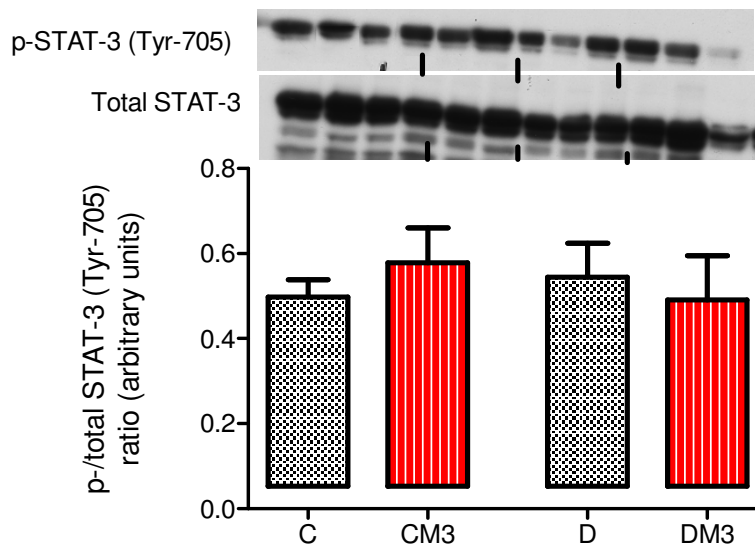


Figure 4.52 STAT-3 phosphorylation (Tyr-705), cytosolic fraction (10 min reperfusion), n=3

hearts/group.

- **Nuclear fraction**

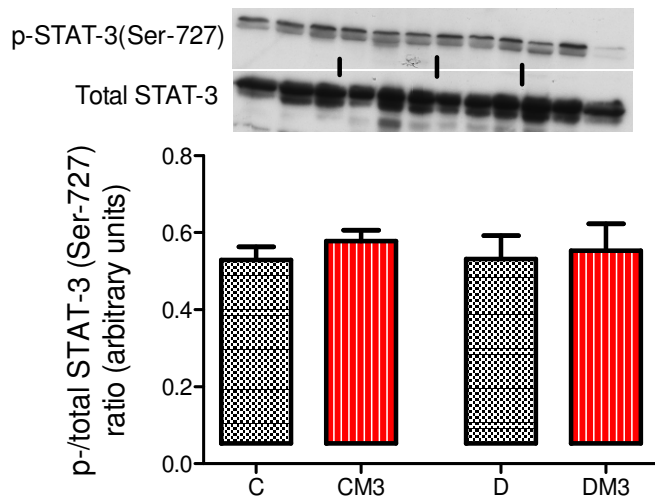


Figure 4.53 STAT-3 phosphorylation (Ser-727), nuclear fraction (10 min reperfusion), n=3 hearts/group.

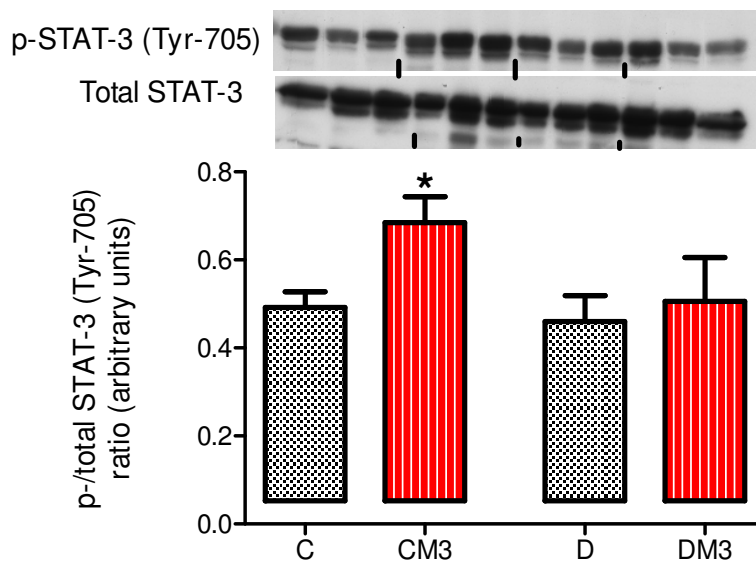


Figure 4.54 STAT-3 phosphorylation (Tyr-705), nuclear fraction (10 min reperfusion), *p<0.05 (C vs M3), n=3 hearts/group.

Table 4.10 Summary of melatonin's effect on PKB/Akt, ERK42/44, GSK-3 β , PI3-K and GLUT-4

Proteins		Baseline				10 min reperfusion				30 min reperfusion			
		Control group		Diet group		Control group		Diet group		Control group		Diet group	
		6WK	3WK	6WK	3WK	6WK	3WK	6WK	3WK	6WK	3WK	6WK	3WK
PKB/Akt	p (Ser-473)	↑	↑	↑	↔	↑	↑	↑	↑	↑	ND	↑	ND
ERK42/44	p42	↔	ND	↔	ND	↑	↑	↑	↑	↔	ND	↔	ND
	p44	↗	ND	↔	ND	↑	↑	↑	↔	↔	ND	↔	ND
GSK3- β	p (Ser-9)	↔	↑	↑	↔	↑	↑	↑	↑	↔	ND	↑	ND
PI3-K	p (p85)	↔	↔	↔	↔	ND	ND	ND	ND	ND	ND	ND	ND
GLUT-4	Expression	↑	↔	↑	↔	ND	ND	ND	ND	ND	ND	ND	ND

Activity or expression: ↔: no significant effect, ↑: increase, ↗: a trend increase (melatonin vs drinking water), (ND): not determined, p: phospho-, WK: week, C: control, D: diet. Total expression of PKB/Akt, ERK42/44, GSK-3 β and PI3-K were not affected by the treatment.

Table 4.11 Summary of melatonin's effect on STAT-3 activation

Samples /collection		Group	STAT-3	Nuclear fraction	Cytosolic fraction
Baseline (non-perfused hearts)	6 weeks	Control	Tyr- 705	act ↑	↔
			Ser-727	↔	act ↑
		Diet	Tyr -705	act ↑	↔
			Ser- 727	↔	↔
	3 weeks	Control	Tyr- 705	↔	↔
			Ser-727	↔	↔
		Diet	Tyr -705	act ↑	
			Ser-727	↔	act ↑
Post-ischaemic hearts (at 10 min reperfusion)	6 weeks	Control	Tyr- 705	act ↑	act ↑
			Ser-727	↔	a trend↗
		Diet	Tyr- 705	act ↑	act ↑
			Ser-727	↔	↔
	3 weeks	Control	Tyr- 705	act ↑	↔
			Ser -727	↔	act ↑
		Diet	Tyr -705	↔	↔
			Ser -727	↔	↔

Tyr: tyrosine, Ser: serine, act: activation (phospho-/total ratio), ↔: no significant effect,

↑: increase, ↗: (p=0.06) a trend increase (melatonin vs drinking water).

4.4. DISCUSSION

In the present study we investigated the effects of 3 and 6 weeks melatonin treatment on myocardial IRI in a rat model of diet-induced obesity and insulin resistance.

The key findings are as follows:

1. The high-calorie diet caused significant increases in body weight gain, visceral adiposity, fasting blood glucose, serum insulin, triglycerides, HOMA-IR index and a concomitant reduction in serum adiponectin levels. These changes were associated with increases in serum TBARS levels, cardiac hypertrophy as well as larger myocardial infarct size after exposure to IRI.
2. Three weeks melatonin administration to obese and insulin resistant rats reduced insulin resistance and protected the heart against *ex vivo* myocardial IRI independently of body weight gain and visceral adiposity. These effects were associated with increased activation of baseline myocardial STAT-3 and the RISK pathway during reperfusion.
3. Six weeks melatonin treatment also protected the heart against IRI with similar signalling effects as those of 3 weeks treatment but with additional phosphorylation of PKB/Akt and GSK-3 β at baseline and STAT-3 during reperfusion. The baseline expression of myocardial GLUT-4 was also increased while PI3-K (p85) phosphorylation was not affected.

4.4.1 DIET-INDUCED OBESITY AND INSULIN RESISTANCE

Long-term imbalance between energy consumption and expenditure induces obesity (Swinburn et al., 2009). Consistently, rats fed a high calorie diet consumed more food than their age matched controls (fig.4.10) and became obese due to hyperphagia (du Toit et al., 2008; Nduhirabandi et al., 2011; Flepisi et al., 2013; Huisamen et al., 2013). Increased body weight gain was associated with excess visceral fat accumulation and a concomitant increase in circulating TG as well as systemic insulin resistance as indicated by increases in the

HOMA-IR index, fasting blood glucose and serum insulin levels (fig.4.3) as previously reported (Flepisi et al., 2013; Huisamen et al., 2013). Because the obesity-inducing diet was essentially liquid compared to the standard rat chow, water consumption of obese rats was consequently reduced compared to control animals (fig.4.9).

Diet-induced visceral fat accumulation is an important factor in the development of insulin resistance and MetS (Grundy, 2012). In genetically and environmentally susceptible individuals, chronic overnutrition with an excessive energy intake and low energy expenditure results in adipocyte hypertrophy and visceral fat accumulation, due to saturation of the expansion capacity or inability of subcutaneous adipose tissue to expand (Lionetti et al., 2009; Bluher et al., 2013). An increase in adipose tissue causes elevation of circulating free fatty acids (FFA), altered adipose tissue derived factors and eventual ectopic fat accumulation which contribute to a proinflammatory (elevated TNF- α , IL-6) and a prothrombotic state (Grundy, 2012), the hallmarks of insulin resistance. In this regard, an increase in FFA may contribute to elevated glucose levels as follows: induction of insulin resistance (inhibition of insulin-mediated glucose uptake pathways) in skeletal muscle, impairment of beta-cell function (lipotoxicity) as well as an increase in hepatic glucose output (Grundy, 2012). In addition to lipotoxicity, hyperglycaemia may, over time, contribute to oxidative stress (Furukawa et al., 2004; Vincent and Taylor, 2006; Galili et al., 2007; Chess and Stanley, 2008; Singh et al., 2011).

In addition to the above, diet-induced obesity and insulin resistance are associated with alterations in adipose-tissue derived hormones (adipokines dysregulation) including, amongst others, reduced levels of circulating adiponectin (hypoadiponectinaemia) (Piya et al., 2013). Adiponectin is one of the most abundant adipose tissue-secreted hormones and has been shown to have many physiological effects including enhancing insulin sensitivity in various tissues, vasodilator, anti-apoptotic, anti-inflammatory and anti-oxidative activities in both cardiac and vascular cells [for review, see (Hui et al., 2012)]. Additionally, adiponectin may act directly or indirectly on the brain (*via* potentiation of the central actions of leptin) and mediate weight loss by increasing energy expenditure *via* thermogenesis without altering food

intake (Qi et al., 2004). A decrease in serum adiponectin levels (fig.4.3) was also observed in the diet animals in the present study, as consistently reported in obese animals and humans (Arita et al., 1999; Weyer et al., 2001; Kadowaki et al., 2008)

Furthermore, diet-induced obesity was associated with glucose intolerance (fig.4.15). The development of glucose intolerance is a complex and multifactorial process involving central and peripheral regulation (e.g., hypothalamus, liver, adipose tissue, pancreas, muscles) [for review, see (Norris and Rich, 2012)]. The present study showed that reduced glucose tolerance is associated with insulin resistance and low circulating levels of adiponectin (fig.4.3) as repeatedly indicated in humans and animal studies [for reviews, see (Kadowaki et al., 2008; Ye and Scherer, 2013)].

Indeed, as it will be further discussed below, improvement in circulating adiponectin may be beneficial in obesity and in cardiovascular health (Agil et al., 2012b; Hui et al., 2012). In view of its pleiotropic actions, although further research is required, circulating adiponectin levels is suggested as a potential clinical marker of metabolic diseases in general (Mather and Goldberg, 2013) and myocardial infarction in particular (Persson et al., 2010).

4.4.2 MELATONIN'S EFFECTS ON OBESITY AND INSULIN RESISTANCE

4.4.2.1 Effects of six weeks treatment

- **Obesity**

Convincing evidence exists for a role of melatonin in body weight regulation (Korkmaz et al., 2009b; Tan et al., 2011; Reiter et al., 2012). In the present study, melatonin administered for six weeks reduced body weight gain in both control and diet rats with no effect on food or water consumption (fig.4.9-10). This is in agreement with previous studies in obese [for review, see (Nduhirabandi et al., 2012)] and non-obese (Bojkova et al., 2006; Kassayova et al., 2006) animals. However, melatonin treatment has also been shown to affect the food intake in obese rabbits (Hussein et al., 2007) and a rat model of ovariectomized-induced obesity (Sanchez-Mateos et al., 2007; Baxi et al., 2012). These controversies may be due to differences in animal models (Nduhirabandi et al., 2012).

The reduction of body weight gain following long-term melatonin treatment is associated with a decrease in visceral fat mass and concomitant improvement in lipid profile (Nduhirabandi et al., 2011; Cardinali et al., 2013). Surprisingly, in the present study, body weight reduction by six weeks melatonin treatment was not associated with significant changes in visceral fat mass and lipid profile as indicated by circulating TG and total cholesterol levels (fig.4.4; table 4.7). It is well established that elevated circulating TG correlates with visceral adiposity (Tchernof and Despres, 2013). The dissociation between body weight gain and visceral adiposity could be due to the dosage and duration of the treatment as previously demonstrated (Rasmussen et al., 2001). For example, a reduction in body weight gain and visceral fat mass following six weeks melatonin treatment has been observed in studies using a higher concentration of 10mg/kg/day (Agil et al., 2011; Agil et al., 2012b), 2.5 times the dosage used in the present study. Other examples include studies where melatonin was administered for longer periods than 6 weeks such as 12 weeks (0.4µg/mL, oral) (Wolden-Hanson et al., 2000), 12 weeks (0.5 mg/kg, oral) (Zanuto et al., 2013), 16 weeks (4mg/kg/day, oral) (Nduhirabandi et al., 2011) and 24 weeks (4µg/mL, oral) (Kassayova et al., 2006). In a number of studies, melatonin was administered by injection for 4 weeks (1mg/kg/day, subcutaneous) in obese rabbits (Hussein et al., 2007), 8 weeks (10mg/kg/day, intraperitoneal) (She et al., 2009) or 10 weeks (5mg/kg/day, intraperitoneal) (Wan et al., 2013) in obese rats. Collectively, in these studies, weight gain reduction was associated with visceral adiposity reduction.

However, in agreement with the present study, 8 weeks oral melatonin administration to obese rats at 0.5mg/kg/day (Zanuto et al., 2013) or 5% fructose-fed rats at 20µg/mL (Cardinali et al., 2013) or obese mice (100mg/kg/day)(Sartori et al., 2009) had no effect on body weight gain, visceral adiposity and TG levels. Furthermore, although three weeks melatonin treatment was able to reduce the body weight gain at the high dose of 30mg/kg/day (twice per day, gavage), it had no significant effect on visceral adiposity (Prunet-Marcassus et al., 2003), supporting the dissociation of body weight gain and visceral adiposity as observed in the present study. Thus, it appears that the effects of melatonin may vary depending on dosage, duration of

treatment and type of animal model used as well as the mode of administration (Nduhirabandi et al., 2012).

The mechanism of body weight reduction by melatonin is complex and still not clear. Several studies have indicated that melatonin-induced body weight reduction may result from the lowering of circulating TG and subsequent decrease in body fat content (Nduhirabandi et al., 2011; Agil et al., 2012b). This is supported by *in vitro* studies where melatonin treatment of adipocytes inhibited their differentiation, TG accumulation and hypertrophy (Sanchez-Hidalgo et al., 2007; Alonso-Vale et al., 2009)(Alonso-Vale et al., 2009). The effect of melatonin on lipids is discussed below in the section on lipid peroxidation. Other mechanisms of weight loss would include the melatonin-induced loss of skeletal muscle since visceral fat mass remained unchanged. However, as far as we know, there is no available report on this topic.

Furthermore, the observed body weight loss could be related to melatonin-induced improvements in the compromised insulin and leptin signalling associated with obesity (Nishida et al., 2003; Sartori et al., 2009), accompanied by modulation of plasma levels of insulin, glucose, TG, cholesterol, leptin and adiponectin (Ríos-Lugo et al., 2010; Agil et al., 2011). It has been reported that melatonin, leptin and insulin activated the same intracellular signalling pathways namely PI3-K and STAT-3 (Carvalho et al., 2001; Anhe et al., 2004; Picinato et al., 2008). Thus, melatonin may reduce body weight gain by mimicking the actions of insulin and leptin signalling *via* cross-talk between these pathways.

- **Insulin resistance**

Insulin resistance refers to the condition associated with an impaired or decreased cellular sensitivity to insulin actions. In the present study, the body weight gain reduction by melatonin was associated with lowering insulin resistance as shown by the reduction in fasting insulin levels and HOMA-IR index (fig.4.6; fig.4.12-13). This is in agreement with previous observations in our laboratory (Nduhirabandi et al., 2011) and elsewhere (Wolden-Hanson et al., 2000; She et al., 2009; Wan et al., 2013).

However, how melatonin improves insulin sensitivity or reduced insulin levels in obesity is also not clear. Although the causal role between visceral adiposity and insulin resistance is uncertain (Knudsen et al., 2012), it is widely accepted that weight loss decreases visceral fat and improves insulin sensitivity (Tchernof and Despres, 2013). Since the observed body weight reduction and improved insulin sensitivity were not associated with visceral fat reduction and improvement in lipid profile (fig.4.6-7; fig.4.13) in the present study, the possibility exists that other mechanisms may be involved, for example, increased plasma levels of adiponectin (Yang et al., 2001). Interestingly, the improvement in insulin sensitivity associated with increased adiponectin levels (fig.4.12-14) has also been reported by others (Agil et al., 2012b; Kitagawa et al., 2012). As indicated above, adiponectin plays an important role in glucose homeostasis (Ye and Scherer, 2013). Apart from its insulin sensitizing actions in the liver and peripheral tissues, adiponectin has other beneficial effects associated with cardiovascular protection [for reviews, see (Kadowaki et al., 2008; Hui et al., 2012)]. Melatonin has also been shown to restore insulin-induced vasodilation to skeletal muscle, a major site of glucose utilization (Sartori et al., 2009).

Melatonin has also been shown to inhibit insulin secretion, thereby contributing to the reduced circulating insulin levels (Picinato et al., 2002; Peschke, 2008; Peschke et al., 2012). In these events MT1/MT2 receptors are highly involved. These receptors are expressed in pancreatic islets (Peschke et al., 2000) and since insulin levels exhibit a nocturnal drop, its production has been suggested to be controlled, at least in part, by melatonin (Mulder et al., 2009). Other contributing hormonal interactions include catecholamines as well as glucagon [for review, see (Peschke et al., 2013)]. While catecholamines regulate the interaction between melatonin and insulin levels, melatonin also stimulates glucagon secretion by the pancreatic α -cells and its actions in the liver (Bahr et al., 2011; Peschke et al., 2013).

In addition to the improvement of insulin sensitivity, the brown adipose tissue has recently emerged as important role player in the effects of melatonin on body weight gain [for review see (Tan et al., 2011)]. In this regard, oral melatonin supplementation induced browning of the inguinal white adipose tissue in ZDF rats (Jiménez-Aranda et al., 2013). Melatonin-

induced brown adipose tissue activation is thought to reduce body weight through increased thermogenesis (Tan et al., 2011) without affecting locomotor activity (Jiménez-Aranda et al., 2013). Concerning the latter, other reports have indicated that body weight reduction by melatonin treatment was associated with an increase in physical activity (Wolden- Hanson et al., 2000; Terron et al., 2013).

Furthermore, there is strong evidence for the involvement of MT receptors in melatonin's effects. Indeed, MT receptors have been identified in the central and peripheral organs involved in body metabolism including brain, liver, pancreas, skeletal muscle and adipose tissue (Brydon et al., 2001; Muhlbauer et al., 2009). Importantly, removal of the MT1 receptor significantly impairs the ability of mice to metabolize glucose and probably induces insulin resistance in these animals (Contreras-Alcantara et al., 2010). It is suggested that the overall effects of melatonin on obesity and insulin resistance may be partly mediated through MT receptors, in addition to the activation of the sympathetic nervous system *via* hypothalamic receptors and subsequent effects on lipolysis and adipose tissue plasticity (Song and Bartness, 2001; Bartness et al., 2002; Nduhirabandi et al., 2012).

Moreover, as noticed by others (Karamitri et al., 2013), melatonin's potential weight-lowering effects and the positive impact of melatonin in ameliorating metabolic-associated complications have never been tested in humans.

4.4.2.2 Effect of three weeks melatonin treatment

As discussed above, long-term melatonin treatment has been widely shown to reduce body weight and improve metabolic abnormalities associated with obesity (reviewed by Nduhirabandi et al., 2012). In control rats, three weeks melatonin treatment was able to reduce the body weight and visceral fat (fig.4.6) in contrast to six weeks treatment which had no effect on visceral fat. In this context, the starting point of treatment may be crucial. For example, administration of melatonin (oral, 0.2 µg /mL, drinking water) for 10 weeks, starting at 3 months of age, in contrast to 6 months, failed to suppress intra-abdominal fat deposition as well as insulin levels (Rasmussen et al., 2001), indicating age-dependent effects of

melatonin. Interestingly, body weight loss was associated with visceral fat mass reduction (fig.4.6).

However, in obese rats, the body weight gain and visceral fat mass were not affected by 3 weeks treatment (fig.4.4; fig.4.5). Since melatonin effects are dose and time-dependent, this may be due to the short period of treatment as previously indicated. In this regard, we found that 6 weeks reduced body weight gain (fig.4.4). Previous studies have also indicated that body weight loss was only observed from the third week of melatonin treatment in control rats (20µg/mL, oral) (Terrón et al., 2013) and rats fed a high fat diet (25µg/mL, oral) (Rios-Lugo et al., 2010). Furthermore, 3 weeks melatonin administration at high dose (30mg/kg/day, gavage) has been shown to reduce body weight gain but not visceral adiposity of rats fed a high-fat diet (Prunet-Marcassus et al., 2003). Importantly, even at high dose (100mg/kg/day), oral melatonin treatment for 8 weeks was not able to reduce the body weight of obese mice (Sartori et al., 2009).

Following melatonin treatment, enhancement of insulin sensitivity, as shown by decreased insulin levels and HOMA-IR index, was not associated with body weight and visceral fat loss in obese animals (fig.4.5,12-13) as previously reported in obese mice (Sartori et al., 2009) and rats (Zanuto et al., 2013). This may also indicate that amelioration of insulin actions preceded the significant reduction in body weight gain or visceral adiposity (Sartori et al., 2009; Zanuto et al., 2013). In this regard, various mechanisms such as the inhibitory actions of melatonin on insulin secretion (Peschke et al., 2013) as well as its insulin-mimicking/sensitizing activities (Carvalho et al., 2001; Anhe et al., 2004; Picinato et al., 2008; Zanuto et al., 2013) may have been involved as discussed above.

4.4.3 MELATONIN AND GLUCOSE TOLERANCE

To further characterize the effect of melatonin on insulin sensitivity, we performed an intraperitoneal glucose tolerance test. This reflects the efficiency of the body to dispose of glucose after a glucose load or meal and is closely associated with insulin sensitivity and secretion (Norris and Rich, 2012). Experimental evidence in pinealectomized, obese, insulin

resistant, diabetic animals has supported the role of melatonin in glucose homeostasis (for review, see (Peschke and Muhlbauer, 2010; Nduhirabandi et al., 2012; Karamitri et al., 2013; Peschke et al., 2013). This has also been confirmed in epidemiological studies (Bouatia-Naji et al., 2009; Liu et al., 2010; Mussig et al., 2010; Tam et al., 2010; Dietrich et al., 2011; Reinehr et al., 2011; Song et al., 2011; Nagorny and Lyssenko, 2012).

The effects of melatonin on glucose homeostasis are further discussed in chapter 6. Only blood glucose levels will be discussed here.

As discussed above, consistent with previous studies, melatonin treatment reduced the levels of insulin and HOMA-IR index in the insulin resistant rats to control (Bertuglia and Reiter, 2009; Sartori et al., 2009; Baxi et al., 2012; Kitagawa et al., 2012; Cardinali et al., 2013) without affecting blood glucose levels (Sartori et al., 2009; Nduhirabandi et al., 2011; Zanuto et al., 2013). Accordingly, six week melatonin administration did not affect the glucose tolerance (fig.4.16A, B) while three weeks improved glucose tolerance in the diet group (fig.4.16A, B). This difference is difficult to explain without further investigation. However, when comparing three weeks to 6 weeks treatment there was no significant difference between these two treatments. This may also reflect the fasting blood glucose levels where a slight diminution of blood glucose was observed after 3 weeks treatment (fig.4.11). At high dose (10 mg/kg/day), intraperitoneal injection of melatonin for 2 weeks ameliorated glucose tolerance and insulin sensitivity in obese diabetic mice with an increase in hepatic glycogen (Shieh et al., 2009). In control animals, 6 or 3 weeks melatonin treatment did not affect the blood glucose levels as well as glucose tolerance as previously reported (Sartori et al., 2009; Kitagawa et al., 2012; Cardinali et al., 2013).

4.4.4 MELATONIN AND LIPID PEROXIDATION

Increased body fat accumulation and related dyslipidaemia are associated with increased systemic and tissue oxidative stress (Furukawa et al., 2004; Vincent and Taylor, 2006; Galili et al., 2007; Singh et al., 2011) and it is generally accepted that obesity may be a state of chronic oxidative stress (Vincent et al., 2007; Codoner-Franch et al., 2009; Wu et al., 2009). Lipid

peroxidation is an important marker of oxidative stress, a condition where cell damage occurs as result of accumulation of reactive oxygen/nitrogen species (ROS/RNS) due to an imbalance between their production and their removal by antioxidants (Singal et al., 1998; Hansel et al., 2004; Grattagliano et al., 2008). In the present study, we evaluated serum lipid peroxidation parameters including conjugated dienes (CD), lipid hydroperoxide (LOOH), and thiobarbituric acid reactive substances (TBARS) as well as serum lipids including total cholesterol (TC), triacylglycerol or triglycerides (TG), and phospholipids (PL). Although fasting lipids are strongly recommended to assess the cardiovascular risks, (NCEP, 2002; Nigam, 2011), non-fasting measurements appear to be clinically more convenient than fasting and may also be used (Nigam, 2011).

4.4.4.1 Effect on blood lipids

Regardless of the feeding state of the animals, the high calorie diet elevated TG levels while having no effect on TC levels (tables 4.8-9). Melatonin treatment had no significant effect on these parameters except a reduction in TC levels observed in the fasting diet rats after 3 weeks treatment. The absence of effects on TG levels may be due to the duration of treatment as indicated above. Previous long-term studies have consistently demonstrated that melatonin reversed or attenuated alterations in circulating lipids in non-obese hypercholesterolemic rats (Hoyos et al., 2000; Hussain, 2007) and this was confirmed in various rat models of obesity (Prunet-Marcassus et al., 2003; Agil et al., 2011; Nduhirabandi et al., 2011; Nduhirabandi et al., 2012; Zhang et al., 2012). This has also been demonstrated in obese rabbits (Hussein et al., 2007) and has been associated with a reduction in TG and increased high-density-lipoprotein (HDL-c) as well as lowering of low-density-lipoprotein (LDL-c) cholesterol, with (Hussein et al., 2007; She et al., 2009) or without (Ríos-Lugo et al., 2010; Agil et al., 2011) effect on total cholesterol levels. Collectively, these studies strongly supported the potential beneficial effect of melatonin supplement in improving lipid metabolism.

4.4.4.2 Effect on lipid peroxidation parameters

The results obtained highlight the differences between serum samples from fasting and non-fasting animals, which in turn, could have a profound effect on the interpretation of the data. We will limit our discussion to the absolute (and not normalized) values obtained.

- **Fasting state**

In the fasting condition, the high calorie diet increased the serum TBARS compared to control while other lipid peroxidation parameters (CD and LOOH) were not affected (fig 4.18). This was associated with increases in TG levels and, because of short term period, six weeks melatonin treatment had no effect on serum TBARS (fig.4.18B). However, it reduced the CD in the diet but not in the control rats (fig.4.18D). This CD reduction may be indicative of the antioxidant activity of melatonin, or due to an effect of melatonin on unsaturated fatty acid metabolism (Hoyos et al., 2000; Nishida et al., 2002).

Although three weeks melatonin treatment had no effect on CD and LOOH (fig.4.18C,D), surprisingly, it increased TBARS in control but not in diet rats (fig.4.18.B). This increase is difficult to explain and needs further investigation. Whether it is a feature of transient effects as previously reported during the first weeks of melatonin treatment and considered as stabilization time (Puchalski et al., 2003a), will need to be elucidated.

Results reported in the literature regarding the effect of melatonin on lipid peroxidation are controversial and should be interpreted with care due to multiple activities of melatonin. In this regard, melatonin has been shown to elicit pro-free radical effects on a set of normal or tumor leukocytes but with no effect on oxidative stress, as shown by absence of protein carbonylation, preservation of viability and regular proliferation rate (Radogna et al., 2009). Additionally, melatonin treatment was recently shown to decrease and increase H₂O₂ levels of the liver in the aged and young rats, respectively (Güney et al., 2013). These controversial effects were further supported by other studies in which it was shown that prolonged melatonin administration for 3 but not 6 months increased lipid peroxidation (malondialdehyde) levels in the liver of male and female young (fasted) rats (Kasayova et al., 2006).

- **Non-fasting state**

In fed conditions, a high calorie diet was associated with a marked increase in CD levels (fig.4.17D) while six weeks melatonin treatment had no effect on serum TBARS (fig.4.17B). However, this was associated with reduction in CD and LOOH levels in control rats (fig.4.17.C, D). As mentioned above, these decreases in lipid peroxidation products could be indication of antioxidant activities of melatonin or due to its effect on lipid metabolism (Nishida et al., 2002). For example, melatonin has been shown to delay the onset of the propagation phase for conjugated dienes (Bonnetfont-Rousselot et al., 2003).

Three weeks melatonin treatment did not affect TBARS and LOOH levels (fig.4.17A, fig.4.17C) but, interestingly, caused a marked increase in CD in control animals (fig.4.17D). These observations are difficult to explain and further investigation is warranted. Importantly, 3 weeks melatonin treatment increased CD in the non-fasting state while increasing TBARS in the fasting state in controls. This may be indicative of the pro-oxidant activity of melatonin as previously indicated (Osseni et al., 2000; Guney et al., 2013).

Collectively, as shown by the reduction in CD and LOOH levels, melatonin manifested some features of its antioxidant activities (Korkmaz et al., 2009a; Bonnetfont-Rousselot and Collin, 2010). Probably due to the short-term treatment, TG levels were not affected. However, it appears that the cardioprotective effects observed in the present study are unlikely to be related to melatonin's effects on systemic lipid peroxidation. Therefore, an evaluation of lipid peroxidation at tissue level is further needed. Similar results were also reported in non-fasted serum following chronic melatonin treatment (16 weeks) which had no effect on myocardial and serum glutathione (Nduhirabandi et al., 2011).

4.4.5 MELATONIN, THE HEART AND OBESITY

This section includes the effects of melatonin on cardiac morphology and function (in normoxic conditions) and myocardial IRI.

4.4.5.1 Melatonin and cardiac morphology in obesity

Obesity and insulin resistance are associated with cardiac remodelling and hypertrophy [for review, see (Abel et al., 2008; Ashrafian et al., 2011)]. Cardiac hypertrophy refers to an increase in the size of the entire heart, but more commonly, alterations occur in specific cardiac chambers relative to body size (Rider et al., 2011). It is traditionally evaluated by the determination of absolute heart weight (HW), relative heart weight [i.e., heart weight to body weight (HW/BW) ratio] or heart weight to tibial length (HW/TL) ratio. HW/TL ratio appears to be a more sensitive indicator of cardiac hypertrophy compared to HW/BW which did not show any difference between control and obese groups (fig.4.6-8). Consistently with a previous study (Flepisi et al., 2013), in the present study, hearts from rats fed a high calorie diet exhibited increases in the absolute HW and HW/TL ratio compared to their age matched controls (fig.4.7).

The development of cardiac hypertrophy in obesity and insulin resistance involves numerous factors including hemodynamic changes as well as metabolic (fuel availability) and humoral (such as oxidative stress, adipokines and inflammation) factors (Abel et al., 2008). In this regard, it has been demonstrated that cardiac hypertrophy was associated with increased oxidative stress in hyperthyroidism (Ghosh et al., 2007) and leptin resistance in obesity (Majane et al., 2009; Rider et al., 2012). In addition to increased serum TBARS (in the fasting condition), low circulating adiponectin and elevated fasting blood glucose levels as well as insulin resistance observed in the present study may have contributed to development of cardiac hypertrophy (Sundström et al., 2000; Velagaleti et al., 2010). However, since cardiac hypertrophy is caused by multiple factors including the observed metabolic abnormalities, three or six weeks of melatonin treatment was seemingly not sufficient to confer a significant effect *via* the improvement of these metabolic factors. Conversely, 16 weeks melatonin

treatment prevented obesity related metabolic abnormalities and reduced the absolute HW as well as HW/BW ratio in both control and diet groups (Nduhirabandi et al., 2011).

Despite the absence of melatonin's effects on other hypertrophic parameters in both groups (fig.4.6-8), three weeks treatment increased the HW/BW ratio (fig.4.8) in agreement with a previous study (Bojkova et al., 2006). However, this increase could be due to the reduction in body weight. In agreement with our findings, 4 days of melatonin treatment (4mg/kg/day, intraperitoneal injection) to pinealectomized rats did not affect their heart weights due to the short period of treatment (Mizrak et al., 2004).

4.4.5.2 Melatonin and myocardial function in obesity

Several epidemiological studies have consistently indicated that obesity and insulin resistance negatively affect cardiac function in various degrees from subclinical to clinical dysfunction (Gray and Kim, 2011; Abel et al., 2012; Narumi et al., 2012). Globally, the extent of impairment of heart function paralleled the degree of obesity (de Divitiis et al., 1981; Sokmen et al., 2013). This has been demonstrated in animal models of diet-induced obesity *in vivo*, in isolated hearts as well as in cardiomyocytes (Abel et al., 2008). However, controversial results have also been obtained in this regard. This can possibly be due to the differences in animal models. Our laboratory has previously reported that rats fed a high calorie diet had a depressed *ex vivo* basal function compared to their age-matched controls (du Toit et al., 2008; Nduhirabandi et al., 2011). However, data obtained in this study indicate that a high calorie diet had no effect on *ex vivo* myocardial function in agreement with other previous findings in obese rats (Wensley et al., 2013) and mice (Calligaris et al., 2013). An explanation for these variable responses is not readily available.

Both six and three weeks melatonin treatments had no significant effects on *ex vivo* basal myocardial function of hearts from both control and obese rats (table 4.9). This is in agreement with previous studies *ex vivo* (Lochner et al., 2006; Nduhirabandi et al., 2011, Lamont et al., 2011) and *in vivo* (Sahna et al., 2005) in non-obese rats. The lack of effect of

melatonin treatment (compared with basal function after 16 weeks melatonin treatment (Nduhirabandi et al., 2011), is probably due to the short administration period.

4.4.5.3 Melatonin and myocardial IRI in obesity

Reperfusion injury is characterized by (i) myocardial dysfunction, (ii) arrhythmias and (iii) cell death or myocardial infarction [for review, see (Opie, 2004)]. Here, only myocardial functional recovery and infarct size are discussed.

4.4.5.3.1 Myocardial IRI , obesity and insulin resistance

As discussed before, convincing evidence exists for the association between obesity and ischaemic heart disease (Ranjith et al., 2007; Fedorowski et al., 2009; Prasad et al., 2010). In addition, the human diabetic heart is highly susceptible to myocardial IRI (Cubbon et al., 2013). However, experimental data on the impact of obesity and insulin resistance on myocardial IRI are controversial, reporting larger, smaller or similar myocardial infarct sizes in animal models of obesity and insulin resistance when compared to their age matched controls [for review, see (Miki et al., 2012; Whittington et al., 2012)]. Thus, the contribution of obesity and insulin resistance to IRI appears complex and subject to many confounding factors. It may reflect the clinical complexity of obesity (Diercks et al., 2006; Romero-Corral et al., 2006).

Hearts from rats fed a high calorie diet showed a significant increase in myocardial infarct size after coronary artery ligation *ex vivo* (fig 4.24) confirming previous *in vivo* (Clark et al., 2011; Wensley et al., 2013) and *ex vivo* (du Toit et al., 2008; Essop et al., 2009; Huisamen et al., 2011; Nduhirabandi et al., 2011) data. Similar observations were made in mice fed a high-fat diet (Thakker et al., 2008), ob/ob mice (*in vivo*) (Bouhidel et al., 2008), db/db mice (*in vivo*) (Jones et al., 1999; Lefer et al., 2001), Zucker obese (ZO) rats (*ex vivo*) (Katakam et al., 2007), Zucker diabetic fatty (ZDF) rats (Sidell et al., 2002) as well as type 2 diabetic BBZ rats (*in vivo*) (Li et al., 2008). The exacerbation of myocardial IRI in obesity and insulin resistance may be due to (amongst others): (1) increased inflammation (Thakker et al., 2008), (2) increased oxidative stress (Katakam et al., 2007; Ansley and Wang, 2013) and (3) a cascade of maladaptative metabolic changes in FFA metabolism (Harmancey et al., 2013) [for review,

see (Miki et al., 2012; Whittington et al., 2012)]. Adipokine dysregulation such as leptin resistance and hypoadiponectinaemia may also contribute to these observations (Lopaschuk et al., 2007).

In contrast to the exacerbation of infarct size observed in the present study, a part from a reduction in aortic output recovery, the high calorie diet had no significant effect on functional recovery compared to control (fig. 4.19-23). The dissociation between reduction in infarct size and myocardial function has been also reported by previous studies in control rats *in vivo* (Lee et al., 2002; Sahna et al., 2005) and *ex vivo* (Lochner et al., 2006; Genade et al., 2008; Lamont et al., 2011) confirming that a reduction in infarct size is not always accompanied by an improvement in functional recovery during reperfusion, due to concomitant stunning (Lochner et al., 2003).

4.4.5.3.2 Effect of melatonin on myocardial IRI

It is well established that melatonin treatment before the onset of ischaemia or during reperfusion confers cardioprotection in non-obese animals (for review, Reiter et al., 2003; Lochner et al., 2013). Recent investigations in insulin resistant animal models have confirmed melatonin's cardioprotective actions (Nduhirabandi et al., 2011; Diez et al., 2013). Consistent with the previous study on chronic melatonin treatment (16 weeks) in obese rats (Nduhirabandi et al., 2011), hearts isolated from rats treated with melatonin for three or six weeks also showed a significant reduction in myocardial infarct size after coronary artery ligation (fig.4.24). This was associated with an improvement in myocardial function in the diet but not in the control group (fig.4.19-23 and table 4.9), confirming previous findings obtained after chronic melatonin treatment (Nduhirabandi et al., 2011). The dissociation of reduced infarct size and myocardial function observed in the the latter groups was also reported in the previous studies (Lochner et al 2006; Nduhirabandi et al., 2011, Lamont et al., 2011) and suggest that the beneficial effects of melatonin have overridden the effect of stunning in the diet group.

How melatonin treatment confers cardioprotection in obesity is not yet established. Melatonin has been shown to protect the heart against IRI *via* its direct and indirect antioxidant properties involving free radical scavenging and stimulation of other cellular antioxidants (Reiter et al., 2003; Tengattini et al., 2008). In the present investigation, additional mechanisms may have also contributed to the observed cardioprotection. For example, in the obese group, 6 weeks melatonin treatment was associated with amelioration of insulin sensitivity and increased serum adiponectin levels. These factors could play a crucial role in the observed cardioprotection (Hui et al., 2012). Another factor to be considered is the attenuation of leptin resistance in obesity (Nduhirabandi et al., 2011, Prunet-Marcassus et al., 2003).

Additionally, for all melatonin-treated hearts, the anti-adrenergic, anti-inflammatory and anti-excitatory effects of melatonin may also have contributed to the observed cardioprotection (Genade et al., 2008; Dominguez-Rodriguez et al., 2009; Patel et al., 2010). In this regard, the significance of the MT receptors has been consistently demonstrated (Lochner et al., 2006; Sallinen et al., 2007; Genade et al., 2008). On other hand, melatonin is a small molecule able to enter in the cardiomyocyte and exert direct or indirect nuclear transcriptional activities of cardioprotective genes (Carlberg, 2000; Korkmaz et al., 2012). The following section discusses melatonin's effect on intracellular signalling which may also play an important role in the cardioprotection of the hearts isolated from melatonin-treated control and diet groups.

4.4.5.4 Melatonin and myocardial intracellular signalling

In addition to its non-receptor mediated actions (i.e., free radicals scavenging), melatonin has been shown to exert its effects *via* receptor-mediated activities. Melatonin membrane receptors 1 and 2 (MT1/2) and nuclear receptors have been identified in the heart (Ekmekcioglu et al., 2003). Melatonin has been shown to act *via* various signalling pathways (Lochner et al., 2013). To determine by which signalling pathways melatonin confers cardioprotection, we investigated the effects of 3 and 6 weeks melatonin treatment on the proteins implicated in two survival pathways namely PKB/Akt-ERK42/44 and GSK-3 β (Ser-9)

(reperfusion injury salvage kinases or RISK pathway) and STAT-3 (survivor activating factor enhancement or SAFE pathway). Normoxic hearts after removal from the body (“non-perfused hearts”) and post-ischaemic hearts at 10 or 30 min reperfusion following global ischaemia were used. GLUT-4 and PI3-K were additionally evaluated at baseline as members of the insulin signalling pathway.

4.4.5.4.1 Melatonin, PKB/Akt, ERK42/44 and cardioprotection

- **Baseline**

Under baseline conditions, isolated hearts from rats fed a high calorie diet did not show any significant differences in the phosphorylation or activation of PKB/Akt and ERK42/44 compared to their age-matched controls (fig.4.25-27). In contrast to our findings, elevated basal phosphorylation of PKB/Akt was reported as a feature of early myocardial changes in a rat model of hyperphagia-induced obesity (Huisamen et al., 2012) while over time, a depressed phosphorylation of PKB/Akt was observed in insulin resistant rats indicating impaired myocardial insulin signalling (Ouwens et al., 2005; Huisamen et al., 2013).

In the present study, 3 weeks melatonin treatment increased basal PKB/Akt phosphorylation without affecting ERK42/44 in both control and diet rats (fig.4.30-31). This has been also reported by previous studies in a number of tissues, for example, aorta of obese mice (Sartori et al., 2009), the hypothalamus, liver, skeletal muscle and peri-epididymal adipose tissue of old obese rats (Zanuto et al., 2013) after long-term melatonin treatment. In contrast to our findings, PKB/Akt phosphorylation was associated with an increase in the phosphorylation of ERK42/44 (Zanuto et al., 2013). Similar effects were also observed in hepatic cells (Shieh et al., 2009) and isolated pancreatic islets (Picinato et al., 2008), while ERK42/44 phosphorylation in C₂C₁₂ murine skeletal muscle cells was unchanged (Ha et al., 2006). The effect of melatonin on PKB/Akt and ERK42/44 activation is further discussed in chapter 7, focusing on endothelial cells.

The effects of melatonin on the PKB/Akt pathway have recently been reviewed as controversial or varying according to the type of cells and actions: inhibition (pro-apoptotic and

pro-oxidant effects in cancer cells) or activation (anti-apoptotic or anti-oxidant effects in non-tumor cells) or simply without effect (Hardeland, 2013).

- **Reperfusion**

After 10 min reperfusion following global ischaemia, six weeks melatonin treatment increased PKB/Akt and ERK42/44 phosphorylation in both control and diet groups and PKB/Akt phosphorylation remained significantly elevated after 30 min reperfusion (fig.4.33-37). This is consistent with previous studies in our laboratory where acute melatonin treatment protected the isolated heart by activating the PKB/Akt and ERK42/44 pathway (Genade et al., 2008; Lochner et al., 2013). Although this was associated with increased PKB/Akt phosphorylation at baseline, this is not a prerequisite for cardioprotection since three weeks treatment conferred cardioprotection without having PKB/Akt activation at baseline (fig.4.26). In addition, although a depression in PKB/Akt phosphorylation is generally associated with increased susceptibility to myocardial IRI (Huisamen, 2003; Huisamen et al., 2013), the role of PKB/Akt, particularly in diabetic state, is still under debate as blockade of PKB/Akt may also confer cardioprotection (Linares-Palomino et al., 2010).

Similar to six weeks, three weeks melatonin treatment also increased PKB/Akt and ERK42/44 activation in the control and the diet groups (fig.4.33-34). Thus the results obtained in the present study suggest that the cardioprotective actions of short-term melatonin-treatment are also associated with activation of the RISK pathway. It has been shown by several workers that activation of the RISK pathway is associated with anti-apoptotic activities and is closely linked to inhibition of mitochondrial permeability transition pore (MPTP)(Hausenloy et al., 2005). The effects of melatonin on PKB/Akt are further discussed in chapters 6 and 7 focusing on glucose homeostasis and endothelial cells, respectively.

4.4.5.4.2 Melatonin, GSK-3 β and cardioprotection

- **Baseline**

GSK-3 β has recently emerged as a multifunctional kinase, widely distributed in many cellular compartments including the cytosol, mitochondria, and nucleus (Juhaszova et al., 2009).

Originally identified as an enzyme that phosphorylates and down-regulates glycogen synthase (the rate-limiting enzyme of glycogen metabolism), GSK-3 β is phosphorylated (inhibition) by PKB/Akt at Ser-9. This enzyme has also emerged as an integration point of several survival pathways where it plays a central role in transferring protective signals downstream to target the MPTP [for review, see (Juhaszova et al., 2009)].

The high calorie diet did not affect the expression and phosphorylation of GSK-3 β (fig.4.28-29) under baseline conditions, in contrast to the study by Wensley et al. (2013). This could be due difference in animal models and protocols as they used a feeding period of 30 weeks feeding as opposed to 20 weeks in the present study. Melatonin treatment for 6 weeks increased baseline GSK-3 β phosphorylation in the diet but not in the control group while 3 weeks treatment increased this phosphorylation in the control but not in the diet group (fig.4.28-29). The reason for these discrepancies between the effects of 6 and 3 weeks treatment in the control and diet groups is not known. Recent results from our lab showed that long-term GSK-3 β inhibition (i.e., phosphorylation) had detrimental effects in control while promoting insulin sensitivity in obese rats (Flepisi et al., 2013). These opposing effects observed in control and obese rats need to be further investigated. As far we know, this is the first study evaluating the effect of melatonin on myocardial GSK-3 β . In HepG2 cells, melatonin increased the phosphorylation of GSK-3 β in a PKB/Akt-dependent pathway (Shieh et al., 2009).

- **Reperfusion**

As discussed above, the high calorie diet did not affect the expression and phosphorylation of GSK-3 β (fig.4.32, 35). Melatonin treatment for 3 or 6 weeks increased phosphorylation of GSK-3 β at 10 min reperfusion in both control and diet groups and this phosphorylation was observed until 30 min of reperfusion (fig.4.38). However, the exact mechanism whereby melatonin increased GSK-3 β in the heart remains unknown. Previous studies have shown that melatonin improved neurodegenerative disorders *via* indirect inhibition of the activity of GSK-3 β by activating and/or enhancing the activity of PKC or by inducing PKB/Akt phosphorylation, as both inactivate GSK-3 β through phosphorylation (Rosales-Corral et al., 2011).

A strong possibility is that the enhanced activation of RISK pathway observed during reperfusion causes GSK-3 β phosphorylation. Furthermore, melatonin has been shown to protect the heart by inhibition of MPTP opening (Petrosillo et al., 2009). However, whether GSK-3 β inactivation was involved in melatonin-induced cardioprotection *via* MPTP has not been investigated. In the present study, it appears that pre-ischaemic inhibition of GSK-3 β is not a prerequisite for the observed cardioprotection since baseline GSK-3 β phosphorylation was not affected by melatonin (fig.4.28-26). However, GSK-3 β phosphorylation during reperfusion is known to play an important role in cardioprotection (Juhaszova et al., 2009). For example, it has been found that level of cardioprotection afforded by pharmacological preconditioning (erythropoietin infusion) was closely correlated with the level of GSK-3 β (Ser-9) phosphorylation upon reperfusion in the heart (Nishihara et al., 2006). The results obtained in the present study also suggest that melatonin exerts its protective actions during reperfusion *via* this kinase.

4.4.5.4.3 Melatonin, STAT-3 and cardioprotection

Signal transducer and activator of transcription 3 (STAT-3) is a family of cytoplasmic proteins with roles as signal messengers and transcription factors that participate in normal cellular responses to cytokines, growth factors and hormones and also play a crucial role in cardioprotection (Zgheib et al., 2012; Zouein et al., 2013).

- **Baseline**

Under baseline conditions, the diet had no significant effect on the localization of phosphorylated nuclear STAT-3 (Tyr-705 and Ser-727) (fig.4.41-42). Six weeks melatonin treatment increased nuclear localization of phosphorylated STAT-3 t (Tyr-705) in both control and diet groups while the cytosolic STAT-3 phosphorylation (Ser-727) was increased in control only (fig.4.41-42). The increased nuclear STAT-3 phosphorylated at Tyr-705 may reflect translocation of STAT-3 to the nucleus, leading to increased transcriptional activities of cardioprotective genes (Zgheib et al., 2012; Zouein et al., 2013). Thus, melatonin treatment may have conferred cardioprotection by increasing nuclear phosphorylated STAT-3 (Tyr-705)

before the ischaemic insult consistent with a previous study in non-obese animals (Lamont et al., 2011). In agreement with a recent study by Yang et al. (2013) in control rats, we found increased STAT-3 (Ser-727) phosphorylation in the cytosol which may function as a switch favouring the mitochondrial actions of STAT-3 over its canonical nuclear actions (Zgheib et al., 2012).

Three weeks melatonin treatment increased baseline cytosolic phosphorylation of STAT-3 (Ser-727) and nuclear phosphorylated STAT-3 (Tyr-705) in the diet group (fig.4.45-46). Similar findings were also reported in non-obese rats (Lamont et al., 2011; Yang et al., 2013). However, the baseline phosphorylation of cytosolic or nuclear STAT-3 (Ser 727 or Tyr-705) in the control group was not affected (results not shown), indicating specificity of the effect of melatonin.

- **Reperfusion**

Similar to baseline, the diet had no effect on STAT-3 expression and phosphorylation at 10 min reperfusion of control and diet groups (fig.4.47-54). In both groups, six weeks melatonin treatment increased the phosphorylation of nuclear and cytosolic STAT-3 (Tyr-705) without affecting STAT-3 (Ser-727) (fig.4.47-50). Three weeks melatonin treatment increased phosphorylation of cytosolic STAT-3 (Ser-727) and nuclear phosphorylated STAT-3 (Tyr-705) in the control with no effect in the diet group (fig.4.51-4.54). This lack of phosphorylation at reperfusion in the diet group following 3 weeks treatment is difficult to explain without additional investigations but may be due to the short treatment period.

STAT-3 activation at reperfusion is in agreement with previous studies in non-obese animals (Lochner et al., 2013). Indeed, STAT-3 activation has been consistently reported as cardioprotective in the setting of early preconditioning as well as postconditioning (Lecour, 2009; Zgheib et al., 2012). As the present findings showed, STAT-3 can be phosphorylated at both its Ser-727 and Tyr-705 residues.

Theoretically, it is indicated that following JAK2 stimulation, phosphorylated Tyr-705 leads to STAT-3 dimerization and translocation to the nucleus where, with an additional Ser-727

phosphorylation, it is involved in gene expression (Lecour and James, 2011; Zgheib et al., 2012). However, recent evidence suggests that STAT-3 has additional direct non-transcriptional activities at the level of the mitochondria where it is involved in limiting ROS generation (Lecour, 2009; Zgheib et al., 2012; Yang et al., 2013). This was confirmed in a recent study where *ex vivo* melatonin pre-treatment conferred cardioprotection *via* activation of STAT-3 associated with a reduced apoptotic index, diminished lactate dehydrogenase release, up-regulation of the anti-apoptotic protein Bcl2 and down-regulation of the pro-apoptotic protein Bax (Yang et al., 2013). Interestingly, melatonin exposure preserved the mitochondrial redox potential and increased mitochondrial superoxide dismutase activity with concomitant reduction in hydrogen peroxide and malondialdehyde formation (Yang et al., 2013).

Taken together, it appears that STAT-3 is involved in cardioprotection of both control and diet groups with 6 weeks melatonin treatment being more effective (particularly at Tyr-705 in both nucleus and cytosol). In addition, it has been also demonstrated that STAT-3 phosphorylation was maximal between 2 and 5 min of reperfusion, probably preceding the phosphorylation of PKB/Akt, ERK42/44 and GSK-3 β (Smith et al., 2010) which was maximal at 10 min (Smith et al., 2010). Whether this time point may have affected our results in the 3 weeks melatonin treated group needs further investigation. The effect of melatonin on STAT-3 is further discussed in chapter 7 focusing on endothelial cells.

4.4.5.4.4 Interaction between STAT-3 and PKB/Akt-ERK42/44

Concomitant activation of STAT-3 and PKB/Akt-ERK42/44 as observed in the present study at reperfusion has been also demonstrated by others (Suleman et al., 2008; Lecour, 2009; Tamarelle et al., 2011). Briefly, both PI3 K/Akt and STAT-3 pro-survival signalling has been shown to closely interact to promote maximal protection and this was confirmed by the fact that inhibition of either STAT-3 or PKB/Akt activation abolished the protection induced by pre- or post-conditioning (Suleman et al., 2008; Tamarelle et al., 2011). In addition, it was found that inhibition of phosphorylation of STAT-3 inhibited activation of PKB/Akt and *vice versa*

(Suleman et al., 2008; Lecour, 2009). On other hand, in the anoxic-reoxygenated embryonic heart, STAT-3 was shown to interact with GSK-3 β in the nucleus and PKB/ERK42/44 in the cytosol (Pedretti and Raddatz, 2011). In addition, it was also shown that both STAT-3 and PKB/Akt pathways convey their signals to the mitochondria (where many of the pro-survival and death signals appear also to converge), leading to the inhibition of MPTP opening (Lecour, 2009; Smith et al., 2010). Hypothetical interaction between PKB/Akt and STAT-3 pathways as observed in the present study is represented in figure 4.55.

4.4.5.4.5 Melatonin, GLUT-4 expression and PI3-K (p85)

Transport of glucose across the cell membrane is the first step of glucose metabolism. It is triggered by various stimuli including amongst others increased workload, ischaemia, insulin and catecholamines [for review, see (Montessuit and Lerch, 2013)]. Glucose transporters (GLUT) are important integral membrane proteins, which are involved in the regulation of glucose transport between extracellular and intracellular compartments, maintaining a constant supply of glucose for the organism's metabolism (Montessuit and Lerch, 2013). Although the basal glucose transporter in many cell types is GLUT-1, GLUT-4 is the most prominently expressed in differentiated cardiomyocytes, adipocytes and skeletal muscle (Montessuit and Lerch, 2013; Mueckler and Thorens, 2013). Insulin stimulates glucose transport *via* phosphorylation of PI3-K (p85) which phosphorylates PKB/Akt. This in turn increases GLUT-4 expression and translocation to the membrane for eventual glucose uptake. In agreement with a previous study, the high calorie diet had no effect on GLUT-expression (Huisamen et al., 2013). However, although a depression in GLUT-4 expression may indicate impairment in glucose uptake and hence insulin resistance, expression of membrane GLUT-4 protein is a better indicator for glucose uptake (Huisamen et al., 2001).

The role of melatonin on glucose transporters has been supported by previous observations that pinealectomized animals (without circulating melatonin) had reduced GLUT-4 expression in adipose tissue (Lima et al., 1998; Zanquetta et al., 2003) and four weeks melatonin administration (after pinealectomy) restored the GLUT-4 protein expression to control values

in adipose tissue (Zanquetta et al., 2003). Additionally, melatonin administration was shown to restore the expression of the myocardial GLUT-4 protein as well as the GLUT-4 gene which were reduced in response to oxidative stress associated with cardiac hypertrophy (Ghosh et al., 2007). Consistently, administration of melatonin for 6 weeks increased the baseline levels of GLUT-4 expression in both diet and control hearts (fig.4.40). In other studies, as discussed in chapter 6, melatonin has also been shown to affect GLUT4-expression. However, the mechanism whereby melatonin increased myocardial GLUT-4 expression in control and obese animals remains unknown. In the present study, some factors including lowering of insulin resistance, increased adiponectin levels in obese animals and potential indirect effects of melatonin mimicking or enhancing insulin action in all melatonin treated animals, could contribute to increased GLUT-4 expression. The effect of melatonin on glucose uptake is further discussed in chapter 6 focusing on isolated cardiomyocytes.

Similar to GLUT-4, we showed that the high calorie diet had no effect on expression and phosphorylation of PI3-K (p85). Furthermore, in contrast to GLUT-4 and PKB/Akt, melatonin treatment had no effect on expression and phosphorylation of this kinase (fig.39). An association between PI3-K and increased PKB/Akt phosphorylation has been demonstrated in adipose tissue, skeletal muscle, liver and hypothalamus following melatonin treatment in obese rats (Zanuto et al., 2013). The significance of PI3-K in melatonin's effects was further demonstrated using a PI3-K inhibitor. For example, while melatonin administration induced PKB/Akt phosphorylation, co-administration of melatonin with a PI3-K inhibitor abolished PKB/Akt phosphorylation in hepatic tissues (Faria et al., 2013) and in primary astrocytes (Kong et al., 2008).

In view of the melatonin-induced PKB/Akt phosphorylation and increased GLUT-4 expression in the present study, we expected to observe an increase in PI3-K phosphorylation in the context of insulin signalling pathway. However, as indicated above, PKB/Akt activation is not always directly correlated with insulin-stimulated glucose uptake (Huisamen, 2003). In addition, a reduction in PI3-K with normal PKB/Akt (Nadler et al., 2001) and abnormal activation of PKB/Akt (Cook et al., 2010) were reported in T2D. Importantly, an increase in

PI3-K expression was recently been shown to be associated with normal PKB/Akt expression after melatonin treatment (Mendes et al., 2013), supporting the observed dissociation between PKB/Akt and PI3-K. Obviously, further investigation into this phenomenon is warranted.

In view of the increased GLUT-4 expression by 6, but not 3 weeks melatonin treatment, the contribution of improved glucose transport to the observed cardioprotection is still unclear. In addition, whether the elevated glucose transporter expression was associated with improved glucose metabolism needs to be determined. The role of melatonin in glucose homeostasis is further discussed in chapter 6.

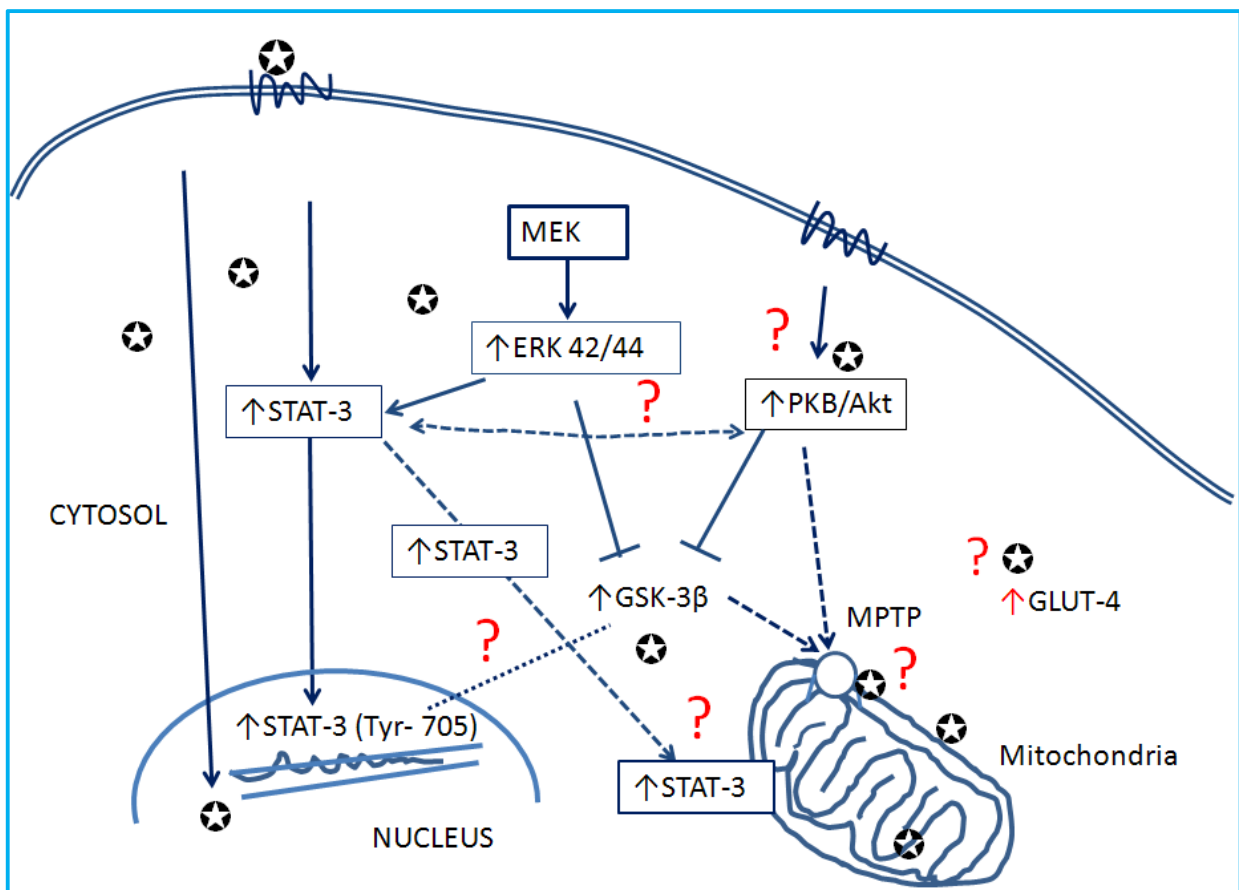


Figure 4.55 Hypothetical representation of the effect of melatonin on myocardial intracellular signalling. Melatonin treatment phosphorylated cytosolic STAT-3 at baseline which triggers its translocation to the nucleus and subsequent transcriptional activities for cardioprotective genes. This is associated with activation of the RISK pathway (PKB/Akt-ERK42/44) and inhibition of GSK-3 β at reperfusion. Both PKB/Akt and STAT-3 pathways are interconnected

and may convey their signals to the mitochondria. Whether these events are associated with increased glucose uptake/metabolism activities remains unknown. ☼ : Melatonin

4.5. STRENGTHS AND LIMITATIONS OF THE STUDY

The rat model of obesity and insulin resistance was successfully induced. In contrast to chronic melatonin treatment or parenteral administration the short-term oral treatment did not affect body weight, visceral adiposity, circulating lipids and cardiac morphology. Although there are inevitable physiological interactions in the *in vivo* setting, we were able to study the potential mechanisms implicated in cardioprotection induced by melatonin in obese and non-obese rats without indirect effects associated with body weight and visceral fat loss and heart weight reduction. Three weeks melatonin treatment appears to be the minimum period where metabolic changes become noticeable (Prunet-Marcassus et al., 2003; Rios-Lugo et al., 2010; Terrón et al., 2013), for example, body weight and visceral fat loss, increased serum CD and TBARS in control animals, improved glucose tolerance in diet animals. Interestingly, six weeks melatonin treatment, where several of its effects are visible (Agil et al., 2011; Agil et al., 2012b), had no effect on visceral fat. However, 6 weeks treatment contributed to our insight into myocardial signalling, body weight gain, adiponectin levels and lack of effect on glucose tolerance.

The present study did not investigate the cardioprotective effect (functional recovery) of melatonin using the global ischaemia protocol. This has previously been demonstrated in isolated hearts from non-obese rats (Lochner et al., 2006; Petrosillo et al., 2006; Genade et al., 2008). In this regard, additional studies using specific inhibitors or knockout mice are needed to confirm the association between the protein activation as indicated by the intracellular signalling pathways and cardioprotection.

4.6. CONCLUSION

Melatonin is a multifunctional molecule. In the present study, we have demonstrated that, independent of body weight and visceral fat loss, short-term (3-6 weeks) melatonin treatment was as effective in eliciting cardioprotection as the long term (16 weeks) treatment in obese

insulin-resistant animals (Nduhirabandi et al, 2011). This protection was associated with a reduction in insulin resistance with no effect on blood glucose and lipids. On a molecular level, melatonin treatment induced baseline activation of STAT-3 and activation of the RISK pathway and STAT-3 at reperfusion.

STUDY II

**THE EFFECTS OF MELATONIN TREATMENT ON MYOCARDIAL
MITOCHONDRIAL FUNCTION IN A RAT MODEL OF DIET-INDUCED
OBESITY AND INSULIN RESISTANCE**

CHAPTER FIVE

STUDY II

THE EFFECTS OF MELATONIN TREATMENT ON MYOCARDIAL MITOCHONDRIAL FUNCTION IN A RAT MODEL OF DIET-INDUCED OBESITY AND INSULIN RESISTANCE

5.1. INTRODUCTION

Obesity and insulin resistance are associated with metabolic, structural and functional cardiac alterations (Abel, 2008). These are accompanied by changes in mitochondrial function which are characterized by a reduction in oxidative activity and ATP synthesis (Boudina et al., 2007; Qatanani and Lazar, 2007). It is well established that mitochondria are the primary cellular site for energy supply to the heart and for both FFA oxidation and utilization (Stanley et al., 2005). In normal conditions the heart relies more on mitochondrial activity to obtain the energy required for contraction (Stanley et al., 2005). Therefore, defects in mitochondrial function are likely to have detrimental effects on heart function.

In the insulin resistant state, cardiac mitochondrial function is overwhelmed by excess free fatty acids and eventually subsequent ROS generation which have been shown to exacerbate IRI damage (Boudina et al., 2007, 2009). The contribution of mitochondrial dysfunction and oxidative stress to cardiomyocyte death in this regard is well established (Opie, 2004), with the opening of the MPTP playing an important role (Yellon and Hausenloy, 2007). Melatonin has been shown to protect the mitochondria in several ways; for example by its ability to scavenge free radicals and by increasing their antioxidant capacity (e.g., SOD, GSH, and catalase) (for review, Srinivasan et al., 2011; Reiter et al., 2008). Melatonin-induced cardioprotection has been shown to be associated with inhibition of the opening of the MPTP (Petrosillo et al., 2009). However, the role of melatonin has not yet been studied in the setting of myocardial mitochondrial function in obesity.

In view of the above, we aimed to investigate the effects of oral melatonin treatment on myocardial mitochondrial function in a rat model of diet-induced obesity. We tested the

hypothesis that melatonin treatment protects cardiac mitochondria against the adverse effects of obesity and insulin resistance and improves basal and post-anoxic mitochondrial function.

5.2. MATERIALS AND METHODS

5.2.2. Study design

Animals, feeding and treatment are described in chapter 3. Figure 5.1 represents animal grouping and feeding for the present investigation. Animals were fed for 20 weeks. Four groups were used in this study: (i) untreated control (C), (ii) treated control (CM), (iii) untreated diet (D) and (iv) treated diet (DM). The treated animals received melatonin in drinking water for 6 weeks starting after 14 weeks of feeding. Melatonin administration is described chapter 3.

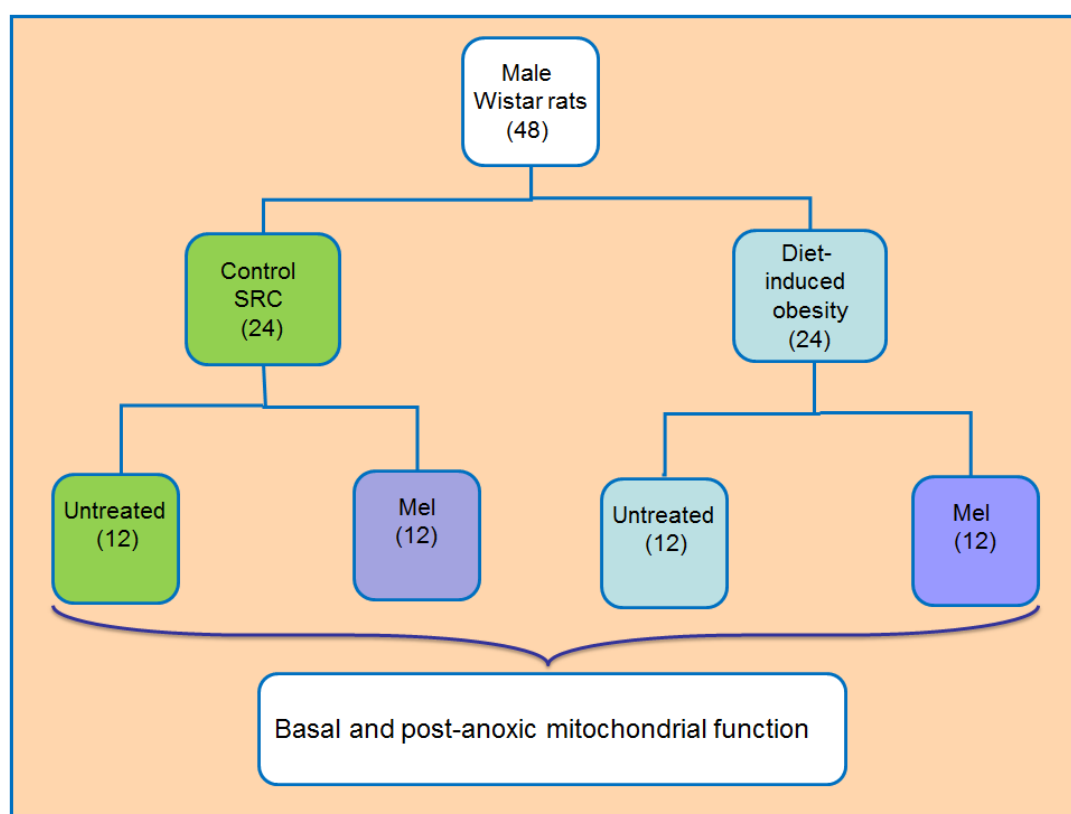


Figure 5.1 Animal grouping, feeding and treatment. SRC: standard rat chow, Mel: 6 weeks melatonin treatment, 48 animals were used with 12 animals for each group. For each group, 6 rats were used for the baseline studies (isolated hearts without perfusion referred to as “non-perfused hearts”) (see chapter 4) and 6 for perfusion studies (isolated hearts subjected to 20 min global ischaemia, further referred to as “perfused or ischaemic hearts”).

5.2.3. Mitochondrial isolation

Mitochondria were isolated from the hearts either at baseline (immediately after removal; referred to as “non-perfused hearts”) or after 20 min global ischaemia (referred to as “perfused or ischaemic hearts”). Briefly, after removal, hearts were immediately arrested in ice cold mitochondrial isolation medium (pH 7.4) containing: KCl (0.18M), EDTA (0.01M). All procedures for mitochondrial isolation were carried out on ice. Arrested hearts were rapidly cut into small pieces and washed repeatedly with the same isolation medium in order to remove all traces of blood. The tissue was then homogenized with a Polytron (PT10) homogenizer for 2 cycles of 4 seconds each (at setting 4) before centrifugation at 2500rpm (755xg) for 10min at 4°C. After centrifugation, the pellet was discarded and the supernatant re-centrifuged at 12500rpm (18800xg) to obtain a mitochondrial pellet. This was resuspended and homogenized in 0.5mL isolation medium using a Potter Elvehjem homogenizer and kept on ice until further use. 50µL of the mitochondrial suspension was used for determination of protein content using the Lowry method (Lowry et al. 1951).

5.2.4. Determination of mitochondrial function

Mitochondrial respiration was measured polarographically at 26°C using an oxygraph (Hansatech Instruments Bannan, UK) containing a Clarke-type electrode as previously described (Boudina et al., 2005)

Figure 5.2 represents the steps followed. After calibration, the mitochondrial incubation medium (250mM sucrose, 10mM Tris-HCl, 8.5mM K₂HPO₄·2H₂O, pH 7.4) containing 2mM malate and either 5mM glutamate or 0.45mM palmitoyl-L-carnitine as respiratory substrates was added to the oxygraph chamber (equilibrated with ambient oxygen). When the chamber temperature reached 26°C, the recording was started and 50µL of mitochondrial suspension added to the incubation buffer in the chamber. At this point, the baseline respiration (State 2), which is substrate-dependent, was measured. Thereafter, 50µL of 7.5mM adenosine diphosphate (ADP) solution was added and the chamber sealed. The addition of ADP caused rapid oxygen consumption by the mitochondria to produce ATP (State 3 respiration). After

phosphorylation of all added ADP, state 4 respiration ensued (see figure 5.2). After this period, the seal of the chamber was removed and an additional amount of ADP (100 μ L of 75mM ADP) was added to the mitochondrial suspension and the chamber resealed. This event caused the mitochondria to utilize all available oxygen in the chamber and thereby inducing anoxia. Following 20 min of anoxia, using a plastic Pasteur pipette for reoxygenation, the oxygen concentration was restored to approximately half of that present in the chamber prior to the addition of the mitochondrial suspension. This re-oxygenation in the presence of excess ADP induced another cycle of State 3 respiration. All these steps were repeated for each substrate (glutamate or palmitoyl-L-Carnitine).

- **Parameters investigated:**

Figure 5.2 represents a typical graph generated by an oxygraph. From this figure the following parameters were calculated: 1) State 2 respiration (slope A): mitochondrial respiration in the presence of the specific substrates in the incubation solution before addition of ADP. 2) State 3 respiration (slope B): mitochondrial respiration in the presence of ADP (at this stage substrates were oxidized in the presence of ADP, and as oxidative phosphorylation takes place, ADP was converted to ATP). 3) State 4 respiration (Slope C): mitochondrial respiration after all ADP was converted to ATP.

Mitochondrial respiratory rates (states 2, 3 and 4) were expressed as nAtoms oxygen uptake /mg mitochondrial protein/min. The respiratory control index (RCI), which is a good index of the coupling of oxidation and phosphorylation, was obtained according to the following formula: state 3 respiration/state 4 respiration. The amount of ADP added to the incubation system was obtained spectrophotometrically (the molar extinction coefficient of ADP: 15.4 at 259nm). The ADP/O ratio is calculated as follows: nmoles ADP phosphorylated/nAtoms oxygen uptake during state 3. The percentage recovery of the state 3 following reoxygenation was also determined to indicate the mitochondrial susceptibility to anoxia/reoxygenation.

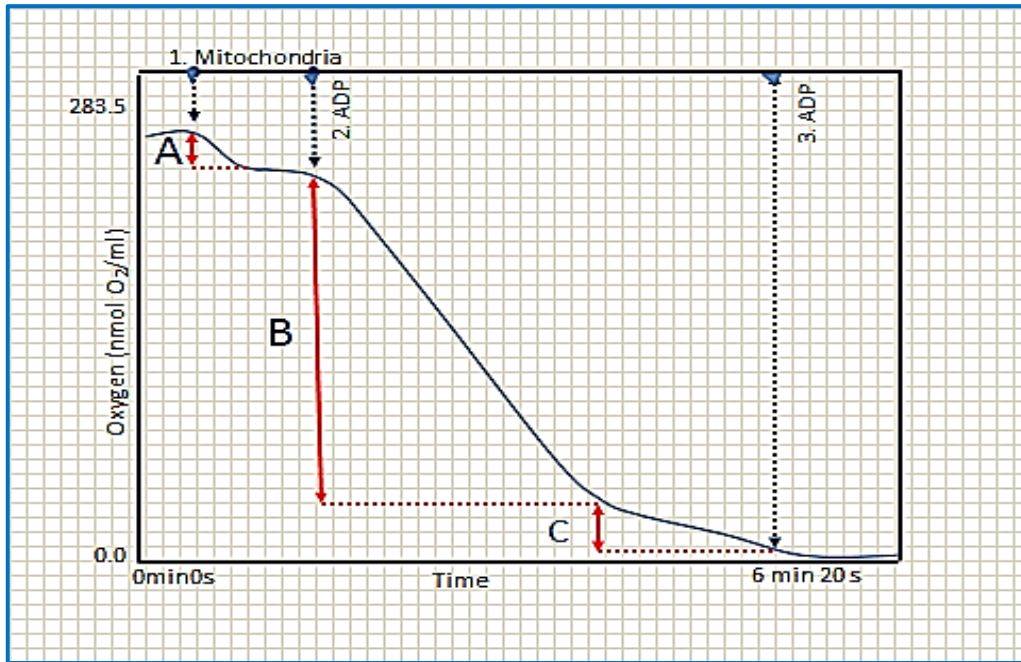


Figure 5.2 Diagram for a typical respiration graph generated by oxygraph, A: state 2 respiration, B: state 3 respiration, C: state 4 respiration.

5.2.5. DATA ANALYSIS

All statistical analyses were performed as indicated in chapter 3.

5.3. RESULTS

5.3.1. Biometric parameters

As in previous experiments, the high calorie diet caused a significant increase in body weight gain and administration of melatonin for 6 weeks reduced the body weight gain in both control and diet groups (table 5.1).

Table 5.1 Body weight

Groups	C	CM6	D	DM6
Body weight (g)	488±12	457±9	575±10 ^{***}	509±11 ^{**}
n/group	13	12	12	11

C: control, CM6: control receiving melatonin treatment, D: high calorie diet, DM: high calorie diet with melatonin treatment; * $p < 0.05$ (C vs CM6), ** $p < 0.01$ (D vs DM6), *** $p < 0.001$ (C vs D)

5.3.2. Mitochondrial function

To investigate the effect of melatonin and the high calorie diet as well as anoxia/reoxygenation on mitochondrial function, mitochondria were isolated from the hearts of treated and untreated rats at either baseline (i.e., without perfusion) or after 20min global ischaemia (referred to as perfused or ischaemic hearts). After polarographic evaluation of mitochondrial respiration and phosphorylation the mitochondria were subjected to 20min of anoxia followed by reoxygenation to elicit state 3 respiration, to assess the susceptibility of isolated mitochondria to anoxia/reoxygenation injury. This was expressed as the percentage recovery of state 3 respiration rate. Glutamate and palmitoyl-L-carnitine were used as respiratory substrates. Results are presented in tables 5.2-3.

- **Non-perfused hearts (baseline)**

Table 5.2 represents the results of the respiratory function of mitochondria isolated from non-perfused hearts. The rate of state 3 reoxygenation was significantly elevated in the diet group (compared to the control) with palmitoyl-L-carnitine as substrate. However, there was no significant difference between the respiratory function of mitochondria isolated from non-perfused control and diet hearts.

In the control group, melatonin treatment for 6 weeks reduced the rate of state 4 respiration with palmitoyl-L-carnitine as substrate (22.19 ± 0.72 vs 15.91 ± 2.69 nAtoms oxygen/min/mg, $p < 0.05$) while the other parameters of mitochondrial respiratory function were not affected regardless of substrate used (fig.5.3A,B; table 5.2).

In the diet group, melatonin treatment reduced the oxygen consumption rate (state 3) (121.1 ± 3.94 vs 87.48 ± 4.24 nAtoms oxygen/min/mg, $p < 0.05$) with glutamate as substrate, while the reduction seen with palmitoyl-L-carnitine was not significant. When palmitoyl-L-carnitine was used as respiratory substrate, melatonin also significantly reduced the rate of state 3 reoxygenation after exposure to anoxia (99.75 ± 6.62 vs 73.95 ± 3.46 nAtoms oxygen/min/mg, $p < 0.05$) with no effect on state 3 percentage recovery, due to the differences

in state 3 respiratory rates before induction of anoxia (table 5.2; fig.5.3B). Other parameters were not affected by melatonin treatment (fig. 5.3A, B; table 5.2).

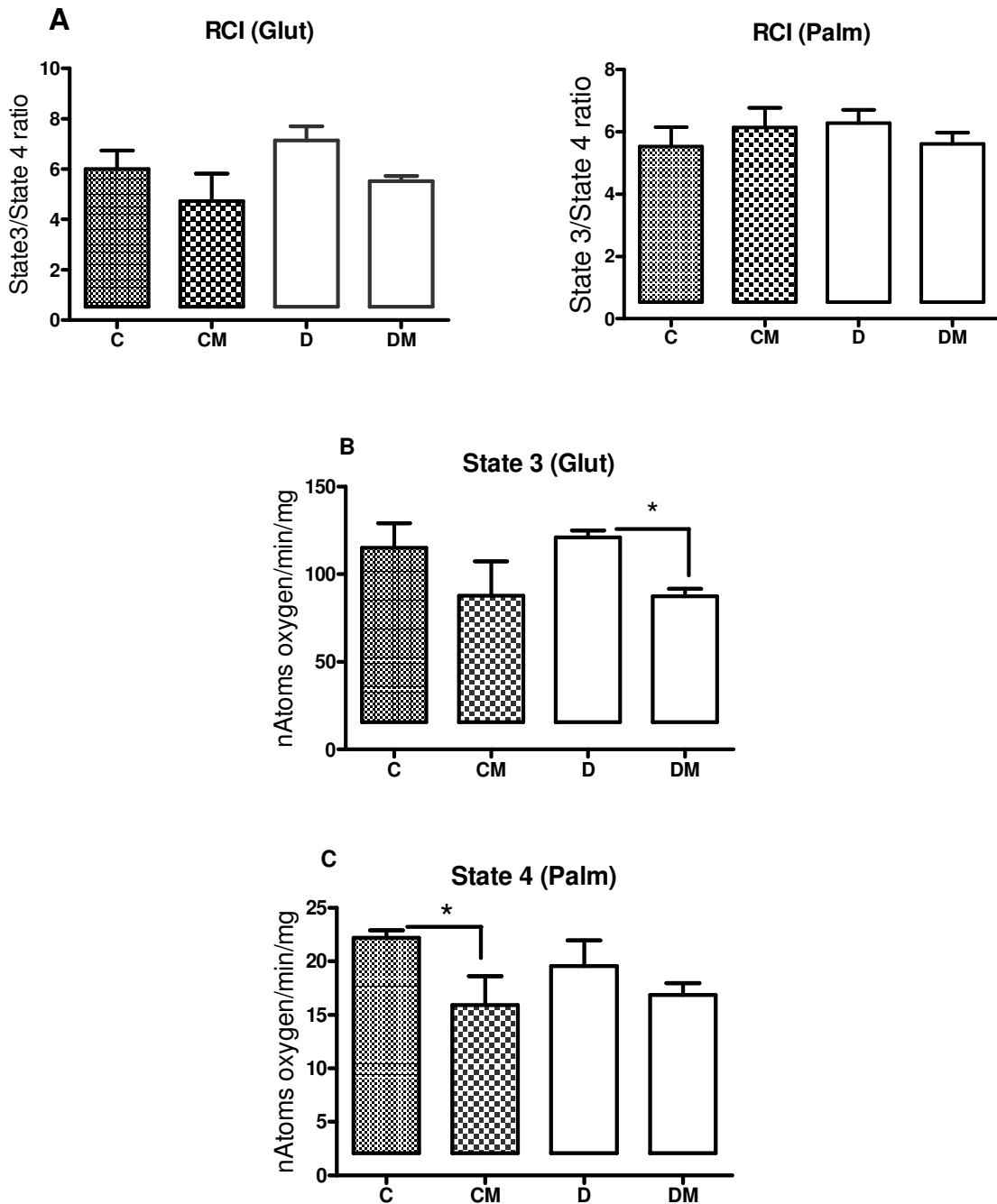


Figure 5.3 Non-perfused hearts, basal respiratory control index (RCI) (A), state 3 respiration rate (B), state 4 respiration rate (C), Glut; glutamate, Palm: palmitoyl-L-carnitine, * $p < 0.05$ (D vs DM or C vs CM), $n = 5-6$ /group

Table 5.2 Respiratory function of mitochondria isolated from non-perfused hearts (baseline)

Groups	C (n=6)		CM (n=6)		D (n=6)		DM (n=5)	
	Glut	Palm	Glut	Palm	Glut	Palm	Glut	Palm
State 2	22.07±2.03	24.64±1.79	20.70 ± 3.61	18.63±3.32	19.50±1.13	23.52±2.65	22.96±2.28	25.97±2.39
State 3	115.2±14.06	121.3±10.9	87.87±19.57	107.7±24.35	121.1±3.94	119.4±9.38	<u>87.48±4.24</u> (*)	94.20±7.64
State 4	19.55±1.39	22.19±0.72	17.43 ±3.92	<u>15.91±2.69</u> (#)	17.28± 1.26	19.52±2.41	15.90 ±1.10	16.84±1.10
State 3 reox	91.21±8.51	75.68±5.42	71.93±14.15	66.86±8.93	97.99±7.44	<u>99.75±6.62</u> (#)	<u>74.79±2.79</u> (p=0.06)	<u>73.95±3.46</u> (*)
RCI (State3/state4)	6.01±0.72	5.53±0.61	4.73±1.08	6.13±0.62	7.15±0.54	6.27±0.42	<u>5.52±0.21</u> (p=0.07)	5.61±0.36
ADP/O	2.52±0.25	2.28±0.15	2.60±0.13	2.55±0.17	2.38±0.22	2.14±0.22	2.24±0.10	2.18±0.13
Oxphos rate	285.4±36.57	277.9±34.9	187.3±37.24	230.5±57.8	289.1±31.43	289.6±26.8	<u>197.0±15.1</u> (p=0.07)	208.5±27.8
State 3 (%) recovery	80.72±3.19	63.95 ±5.40	84.39 ±6.03	67.95 ±7.17	81.14 ±6.44	66.53 ±11.69	85.59 ±1.14	79.21 ±3.07

Glut: glutamate, Palm: palmitoyl-L-carnitine, States 2, 3, 4 (nAtoms oxygen/min/mg), State 3 reox: state 3 after anoxia-reoxygenation (nAtoms oxygen/min/mg), Oxphos: oxidative phosphorylation rate (nmolADP/min/ mg), ADP/O: adenosine diphosphate /oxygen uptake (nmol ADP/ nAtoms oxygen), #p<0.05 vs C, *p<0.05 vs D, n=5-6/group.

- **Perfused hearts (after ischaemia)**

Table 5.3 represents results of respiratory function of mitochondria isolated from perfused hearts (after 20min of global ischaemia). The high calorie diet did not significantly affect the respiratory function with both substrates. In the control group, melatonin treatment did not affect the respiratory function regardless of substrate used. In the diet group, isolated mitochondria from melatonin treated rats had significantly increased the rates of state 2 respiration (both substrates) (table 5.3) and oxidative phosphorylation (149.8 ±21.6 vs 255.4 ±19.1 nmolADP/min/ mg, $p < 0.05$) (glutamate as substrate) (fig.5.4B). However, ADP/O and RCI were not significantly affected by melatonin treatment with both glutamate and palmitoyl-L-carnitine as substrates (fig.5.4A; table 5.3).

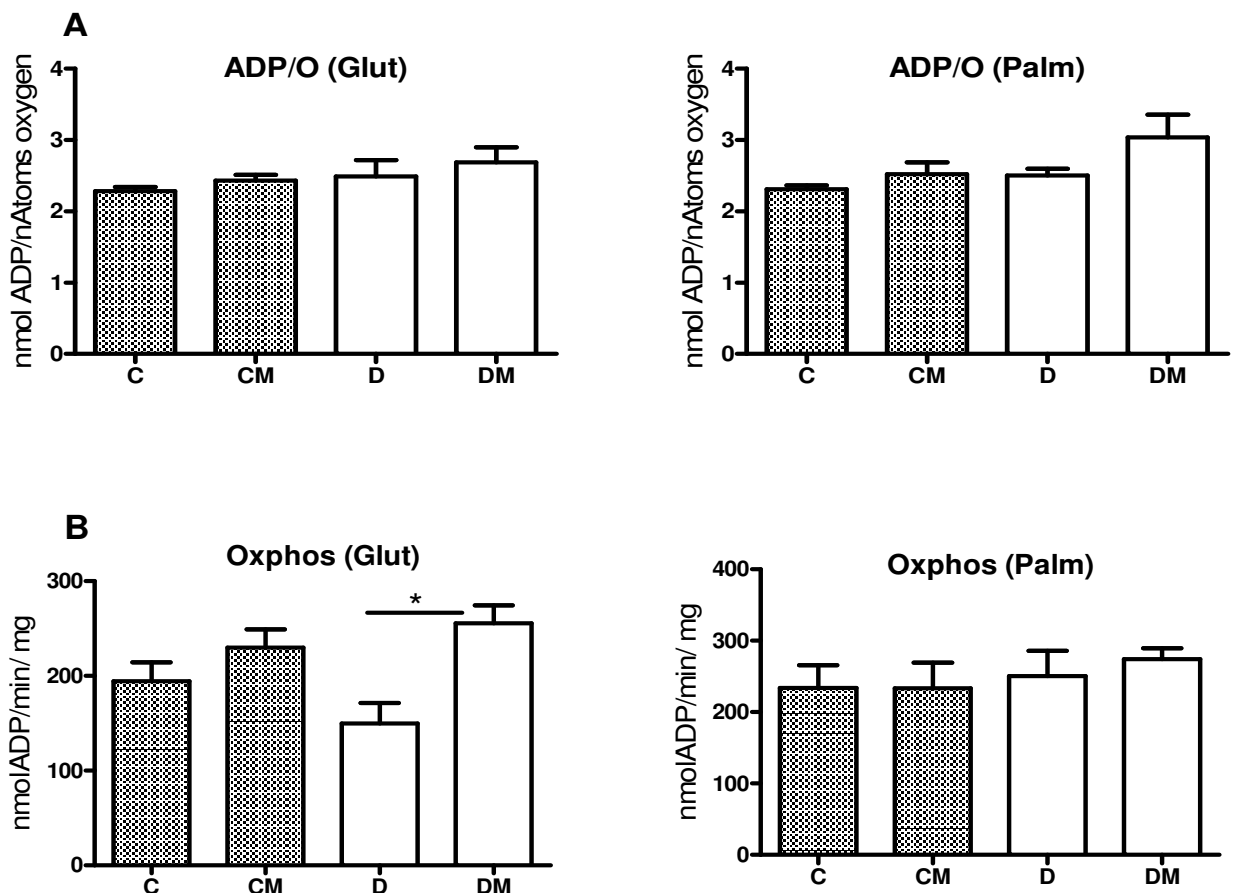


Figure 5.4 Perfused hearts, ADP/O ratio (A) and oxidative phosphorylation rate (Oxphos)

(B), Glut: glutamate, Palm: palmitoyl-L-carnitine, * $p < 0.05$ (D vs DM), $n = 6$ /group.

Table 5.3 Respiratory function of mitochondria isolated from the hearts subjed to 20min global ischaemia

Groups	C (n=6)		CM (n=6)		D (n=6)		DM (n=6)	
	Glut	Palm	Glut	Palm	Glut	Palm	Glut	Palm
State 2	19.14±1.37	20.10± 1.73	17.11±1.63	21.61 ±2.04	15.71±1.61	19.45±1.42	<u>24.03±3.35</u> (*)	<u>27.44±3.62</u> (*)
State 3	84.97 ±8.10	102.1±14.2	94.30 ±6.11	91.79±12.6	72.18± 10.5	98.70±11.75	97.69 ±10.38	113.7±24.29
State 4	14.65± 1.06	19.05 ±1.09	14.61 ±0.49	21.59± 2.63	14.87±0.95	17.77± .94	<u>19.08± 1.94</u> (p=0.07)	22.24 ±3.08
State 3 reox	71.38 ±8.51	65.51±8.78	79.72 ±10.86	59.45±16.84	66.42 ±8.21	77.88±9.71	66.42 ±8.21	68.14±4.90
RCI (State3/state4)	5.40±0.37	5.24± 0.42	6.44±0.32	4.52± 0.76	4.76±0.48	5.32±0.40	5.52 ±0.22	5.08 ±0.64
ADP/O	2.28±0.05	2.30±0.05	2.431±0.079	2.52 ±0.16	2.48±0.23	2.5±0.09	2.68 ±0.21	<u>3.03 ±0.32</u> (p=0.09)
Oxphos	194.4±19.9	206.0±19.36	229.8 ±19.2	233.3 ±35.8	149.8 ±21.6	250.6±35.2	<u>255.4 ±19.1</u> (**)	274.0±15.5
State 3 recovery (%)	84.07±8.45	63.61 ±11.2	84.04±7.67	64.36 ±17.05	91.41±4.70	78.98 ±4.02	68.66±18.77	59.90±18.61

Glut: glutamate, Palm: palmitoyl-L-carnitine, States 2, 3 and 4 (nAtoms oxygen/min/mg), State 3 reox: state 3 after reoxygenation (nAtoms oxygen/min/mg), Oxphos: oxidative phosphorylation rate (nmolADP/min/mg), ADP/O: adenosine diphosphate /oxygen uptake (nmol ADP/ nAtoms oxygen), * p<0.05 vs D, **p<0.01 vs D.

- **The effect of ischaemia on mitochondrial function**

To determine the effect of ischaemia *per se* on mitochondrial function, we compared the data obtained from mitochondria isolated from non-perfused hearts (baseline) with those obtained from mitochondria isolated from perfused hearts (from both untreated control and diet groups) subjected to 20 min global ischaemia. Results are presented in table 5.4

Global ischaemia had a depressing effect on mitochondrial function: the oxygen uptake rate was lower (with both substrates), but significance was obtained only in a few instances. For example, state 4 respiration rate (both substrates) was reduced in the control ($p < 0.05$) but not in the diet group (table 5.4). State 3 respiration rate, RCI and state 3 reoxygenation rate were reduced in the diet group only, with glutamate as substrate (table 5.4).

Importantly, the oxidative phosphorylation rate was decreased in both control and D groups, with glutamate as substrate ($p < 0.05$) (table 5.4). Other parameters including the ADP/O ratio and state 3 percentage recovery were not affected by ischaemia in both groups.

Table 5.4 Comparison of mitochondrial oxidative phosphorylation in perfused (ischaemic) and non-perfused hearts

Groups	C (non- perfused) (n=6)		C (perfused)(n=6)		D (non-perfused) (n=6)		D (perfused) (n=6)	
	Glut	Palm	Glut	Palm	Glut	Palm	Glut	Palm
State 2	22.07±2.03	24.64±1.79	19.14±1.37	20.10± 1.73	19.50±1.13	23.52±2.65	15.71±1.61	19.45±1.42
State 3	115.2±14.06	121.3±10.9	84.97 ±8.10	102.1±14.2	121.1±3.94	119.4±9.38	<u>72.18± 10.5*</u>	98.70±11.75
State 4	19.55±1.39	22.19±0.72	<u>14.65± 1.06#</u>	<u>19.05 ±1.09#</u>	17.28± 1.26	19.52±2.41	14.87±0.95	17.77± .94
State 3 reox	91.21±8.51	75.68±5.42	71.38 ±8.51	65.51±8.78	97.99±7.44	99.75±6.62	<u>66.42 ±8.21*</u>	77.88±9.71
RCI (State3/state4)	6.01±0.72	5.53±0.61	5.40±0.37	5.24± 0.42	7.15±0.54	6.27±0.42	<u>4.76±0.48*</u>	5.32±0.40
ADP/O	2.52±0.25	2.28±0.15	2.28±0.05	2.30±0.05	2.38±0.22	2.14±0.22	2.48±0.23	2.5±0.09
Oxphos rate	285.4±36.57	277.9±34.9	<u>194.4±19.9#</u>	206.0±19.36	289.1±31.43	289.6±26.8	<u>149.8 ±21.6*</u>	250.6±35.2
State 3 (%) recovery	80.72±3.19	63.95 ±5.40	84.07±8.45	63.61 ±11.2	81.14 ±6.44	66.53±11.69	91.41±4.70	78.98 ±4.02

Glut: glutamate, Palm: palmitoyl-L-carnitine, States 2, 3 and 4 (nAtoms oxygen/min/mg), State 3 reox: state 3 after reoxygenation (nAtoms oxygen/min/mg), Oxphos: oxidative phosphorylation rate (nmolADP/min/mg), ADP/O: adenosine diphosphate /oxygen uptake (nmol ADP/ nAtoms oxygen), RCI: respiratory control index, #p<0.05 vs C (perfused vs non-perfused hearts), *p<0.05 vs D (perfused vs non-perfused hearts), n=6/group.

5.4. DISCUSSION

In the present study the effects of six weeks melatonin treatment were evaluated on myocardial mitochondrial function in obesity and insulin resistance.

The main findings were as follows: (i) the high calorie diet caused a significant increase in body weight and insulin resistance without having any effect on the oxidative phosphorylation capacity of the mitochondria isolated from non-perfused (baseline) hearts; (ii) when respiratory function of mitochondria isolated from non-perfused hearts was compared to that of mitochondria isolated from perfused hearts subjected to global ischaemia, the function of the diet group showed a greater reduction than that of the controls, depending on the substrates; (iii) in mitochondria isolated from non-perfused hearts, melatonin administration for 6 weeks induced a reduction in oxygen consumption (state 3 respiration rate), particularly in the diet group, while other parameters were unchanged; (iv) in mitochondria isolated from perfused hearts (after global ischaemia), melatonin induced an increase in state 2 respiration and oxidative phosphorylation rate of mitochondria isolated from the diet group, while having no significant effect on the control group.

5.4.1. Non-perfused hearts (baseline)

Under normal conditions the heart relies mainly on mitochondrial activity for its energy requirements (Stanley et al., 2005). Results on the effects of obesity and insulin resistance on mitochondrial function are controversial: a reduction in oxidative activity and ATP synthesis has been reported (Boudina et al., 2005; Boudina and Abel, 2006). However, it has also been shown that despite peripheral insulin resistance, obesity is not always associated with defects in myocardial mitochondrial function (Ussher et al., 2009; Samocha-Bonet et al., 2012; Gupte et al., 2013). The latter may occur in the setting of pre-diabetes and diabetes due to hyperinsulinaemia which over time may cause a progressive loss of insulin actions and mitochondrial dysfunction as previously reported (Boudina and Abel, 2007; Boudina et al., 2009; Liu and Lloyd, 2013).

Interestingly, in the present study, mitochondria isolated from non-perfused hearts (baseline study) from rats fed a high calorie diet, despite an increase in body weight, exhibited no significant change in their respiratory parameters (RCI, ADP/O) and state 3 percentage recovery after anoxia/reoxygenation when compared to controls (fig.5.3A,B). Therefore, since the mitochondrial respiratory function was not significantly affected in the obese group, it was unlikely that melatonin would have any significant effects in this regard. It is known that mitochondria take up melatonin *in vitro* in a concentration-dependent manner (Lopez et al., 2009) while *in vivo* administration of melatonin also results in its accumulation in mitochondria (Reyes-Toso et al., 2006). Thus, long-term melatonin treatment in the present study is expected to affect the mitochondrial function.

Consistent with a previous study on isolated rat liver mitochondria following 40 days of melatonin treatment (Reyes-Toso et al., 2006), we observed that melatonin reduced the rate of state 4 respiration in control and state 3 reoxygenation in diet group respectively, with palmitoyl-L-carnitine as substrate (table 5.2). The inhibitory effect of melatonin on mitochondrial oxygen uptake was also previously observed in rat liver mitochondria and occurred in the presence of excess substrates and subsequent overstimulation of oxygen uptake (Reyes-Toso et al., 2006; Srinivasan et al., 2011). This could also be the case in mitochondria isolated from the hearts of diet rats during the reoxygenation phase when ADP and substrates are present in excess.

Furthermore, melatonin treatment also induced a reduction in the rate of oxygen consumption (state 3 respiration) in the diet group when glutamate was used as substrate (table 5.2). Similar findings were also previously reported in an *in vitro* study where melatonin administered to isolated rat liver mitochondria induced, in a dose-dependent manner, a reduction in oxygen consumption and membrane potential and inhibited the production of superoxide and H₂O₂ while maintaining the efficiency of oxidative phosphorylation (Lopez et al., 2009).

However, further research is needed to determine the mechanism whereby six weeks melatonin exhibited these effects in both control and diet groups. Long-term melatonin treatment has been suggested to prevent damage to the respiratory chain by induction of antioxidant enzymes such as SOD and GSH (Leon et al., 2005; Reyes-Toso et al., 2006). This is supported by a number of *in vitro* studies as indicated above and additionally, may involve the induction of antioxidant enzyme gene expression [for review, see (Rodriguez et al., 2004; Leon et al., 2005; Hardeland, 2009; Korkmaz et al., 2012)]. As stated above, recent observations indicate that melatonin is highly concentrated in the mitochondria upon melatonin treatment (Venegas et al., 2012). Hence, melatonin accumulated in the mitochondria during the treatment could also contribute directly to the observed effects in the present study.

On other hand, other studies have reported melatonin-induced increases in respiratory function [for details, see (Leon et al., 2005; Acuna Castroviejo et al., 2011; Srinivasan et al., 2011)]. However, this is not regarded as controversial because it reflects the multiple regulatory activities of melatonin: being able to increase or decrease or maintain mitochondrial function depending on the type of malfunction or experimental protocol (Hardeland, 2009; Paradies et al., 2010). This view was recently supported by a study using liver mitochondria from diabetic rats where melatonin administration (10mg/kg/day, 30 days, intraperitoneal) showed a considerable protective effect by reversing the decreased state 3 respiration rate and the diminished RCI to the control values (Cheshchevik et al., 2011).

5.4.2. Perfused hearts (after global ischaemia)

In addition to baseline data, we further investigated whether hearts from obese rats are more susceptible to ischaemic injury and to what extent long-term melatonin treatment affected these hearts. Hearts isolated from both control and diet groups were subjected to 20 min global ischaemia and mitochondria subsequently isolated. After polarographic evaluation of respiration and phosphorylation, the mitochondria were subjected to 20 min of anoxia followed by reoxygenation to elicit state 3 respiration, to assess the susceptibility of isolated mitochondria to anoxia-reoxygenation injury. Several studies have shown that myocardial

ischaemia leads to progressive injury to the mitochondrial function affecting the electron transport chain, oxidative phosphorylation, Krebs cycle enzymes and ATP production (Veitch et al., 1992; Lesnefsky et al., 2004; Kurian et al., 2012). Several of these studies have investigated the effects of reperfusion on mitochondrial function but the extent of mitochondrial injury that occurs during reperfusion compared to the effects of ischaemia alone remains unclear (Lesnefsky et al., 2004; Kurian et al., 2012) as reperfusion does not cause additional defects in the distal electron transport chain of intact subsarcolemmal mitochondria (Veitch et al., 1992; Lesnefsky et al., 2004).

Two subpopulations of mitochondria have been identified namely subsarcolemmal mitochondria which are located beneath the plasma membrane and interfibrillar mitochondria which are located between the myofibrils, the former being more susceptible to ischaemic injury than the latter (Lesnefsky et al., 2004; Kurian et al., 2012). In the present study the subsarcolemmal mitochondrial fraction was studied.

Interestingly, mitochondria isolated from hearts from untreated diet rats had similar mitochondrial respiratory function parameters to those isolated from the control rats regardless of respiratory substrates used (table 5.3). This is in contrast to previous studies where age-dependent defects in mitochondrial oxygen consumption and ATP synthesis were observed in mice with impaired insulin signalling (Boudina and Abel, 2006) and reduced ADP phosphorylation rates in obese rats (Boudina and Abel, 2006; Essop et al., 2009).

However, although there was no significant difference between the respiratory function of untreated control and diet groups, melatonin treatment exhibited different actions on the respiratory function of the mitochondria isolated from these hearts exposed to 20 min global ischaemia. Indeed, while it maintained the respiratory function of the mitochondria isolated from the control hearts, it caused a significant increase in a number of parameters of mitochondrial respiratory function in the diet group including the rate of state 2 respiration (with both substrates) and oxidative phosphorylation rate (glutamate as substrate) (fig.5.4B). This may be indicative of increased coupling efficiency (ATP production) as previously reported

(Petrosillo et al., 2006). Why this coupling efficiency was increased only in the diet group and not in the control group is not clear because the parameters of mitochondrial function in both untreated diet and control groups were similar (fig.5.4B). Additionally, the increase in state 2 respiration may be related to high endogenous mitochondrial ADP induced by melatonin treatment as previously demonstrated (Martin et al., 2002). The increased mitochondrial oxidative phosphorylation rate observed with glutamate as substrate is in agreement with the reduction in infarct size observed in hearts from melatonin-treated obese rats after coronary artery ligation (see chapter 4).

As far as we know, this is the first investigation on the effect of relatively long-term melatonin treatment on myocardial mitochondrial function in obesity and insulin resistance. In this regard, relatively few investigations have focussed on the effect of melatonin on cardiac mitochondrial function. Using normal rats, a study by Petrosillo et al. (2006) has shown that, while the rate of state 3 respiration *in vitro* was markedly decreased in mitochondria isolated from reperfused hearts after ischaemia, melatonin treatment significantly reversed these effects. Additionally, these effects were accompanied by a reduction in the degree of the mitochondrial lipid peroxidation (Petrosillo et al., 2006; Petrosillo et al., 2009b), inhibition of the MPTP opening (Petrosillo et al., 2009a) as well as mitochondrial STAT-3 phosphorylation (Yang et al., 2013).

5.4.3. Effect of ischaemia on mitochondrial function

As indicated above, a pivotal feature of ischaemia is that the supply of oxygen and nutrients to the mitochondria is inadequate to support oxidative phosphorylation (Solaini and Harris, 2005). Previously, it was shown that compared to baseline, mitochondria isolated from ischaemic hearts (Veitch et al., 1992) and rat liver (Okatani et al., 2003) were more prone to severe morphological and functional defects

To determine whether mitochondria isolated from hearts from obese rats were more susceptible to ischaemic damage than those from the control group, a comparison was made of the data obtained from mitochondria isolated from non-perfused hearts (baseline) and those obtained from mitochondria isolated from perfused hearts and exposed to 20 min global

ischaemia. Interestingly, we found that ischaemia reduced the state 3 respiration rate, RCI and the rate of oxidative phosphorylation and reoxygenation in the diet group with glutamate as substrate (table 5.4), suggesting that mitochondria isolated from obese hearts are indeed more prone to ischaemic injury. Although this was not observed with palmitoyl-L-carnitine as substrate, it may be indicative of a reduction in coupling efficiency in the diet group and may be associated with potential depletion of ATP synthesis (Leon et al., 2005).

The absence of any effect with palmitoyl-L-carnitine as substrate in mitochondria from hearts isolated from the diet group may be due to the metabolic changes occurring in the hearts in obesity. The increased supply of FFA to the heart is associated with increases in FFA uptake, malonyl-CoA levels and FFA oxidation [for review, see (Lopaschuk et al., 2010)]. Thus, it may be that mitochondria isolated from hearts from the diet animals are geared towards fatty acid metabolism and less susceptible to anoxia/reoxygenation damage than mitochondria when incubated with glutamate as substrate (table 5.4). Interestingly, as previously indicated, melatonin treatment was able to prevent the reduction in oxidative phosphorylation occurring in the diet group (fig.5.4B).

5.5. CONCLUDING REMARKS

“To understand melatonin’s mitochondrial role, it is necessary to interpret the results in a contextual and non-simplistic way” (Hardeland, 2009). To our knowledge, this is the first study on the effect of melatonin on myocardial mitochondrial function in obesity. We have shown that compared to control, obesity did not affect the respiratory function of mitochondria isolated from non-perfused hearts (baseline).

On the other hand, we also demonstrated that when the respiratory function of mitochondria isolated from non-perfused hearts is compared to that of mitochondria isolated from perfused hearts which were subjected to global ischaemia, the diet group was more susceptible to ischaemic damage. Additionally, the effects of six weeks melatonin treatment depended on whether mitochondria were isolated from non-perfused hearts or hearts previously exposed to ischaemia.

However, the present study is a preliminary investigation which does not allow definite conclusions regarding the effects of either obesity or ischaemia on mitochondrial behaviour. This will require additional parameters such as evaluation of ATPase activity, antioxidant activities, mitochondrial membrane potential and MPTP opening as well as morphological changes are warranted. However, the results obtained could form the basis for further studies to better understand the effects of melatonin treatment on myocardial mitochondrial function in obesity.

STUDY III

**EFFECTS OF MELATONIN TREATMENT ON GLUCOSE
HOMEOSTASIS IN NORMAL AND INSULIN RESISTANT
STATES**

CHAPTER SIX

STUDY III

EFFECTS OF MELATONIN TREATMENT ON GLUCOSE HOMEOSTASIS IN NORMAL AND INSULIN RESISTANT STATES

6.1. INTRODUCTION

Obesity is frequently associated with insulin resistance and defects in glucose homeostasis (Grundy, 2012). Glucose homeostasis encompasses various and complex physiological mechanisms for the maintenance of blood glucose concentrations within very narrow physiological limits (Norris and Rich, 2012). The normal glucose homeostasis in the fed and fasted states has recently been reviewed (Nolan et al., 2011). It is well established that the fasting blood glucose concentration is the net result of the balance between endogenous glucose production (mainly through hepatic glycogenolysis and gluconeogenesis) and utilization by insulin-independent tissues, such as the brain (Nolan et al., 2011). Additionally, in the non-fasting condition, insulin (produced by pancreatic β -cells) plays a crucial role in the regulation of blood glucose levels (following absorption of carbohydrates in the gut) by inhibiting endogenous glucose production and stimulating glucose uptake into peripheral tissues, including the heart, skeletal muscle and adipose tissue (Nolan et al., 2011). By definition, insulin resistance refers to the condition in which the body produces insulin but does not use it properly due to decreased cellular sensitivity to its effects on glucose uptake, metabolism and storage (Benito, 2011; Hardy et al., 2012; Rask-Madsen and Kahn, 2012). As a consequence, normal circulating insulin concentrations become inadequate to produce a normal insulin response in target tissues (Cefalu, 2001; Hardy et al., 2012).

In obesity, defects in glucose homeostasis lead to compensatory hyperinsulinaemia which, in the case of β -cell failure, leads to hyperglycaemia and eventually T2D (Reaven, 2004; Grundy, 2012). Recent animal and epidemiological studies support the involvement of melatonin in the regulation of glucose metabolism (Ha et al., 2006; Sartori et al., 2009; Xia et al., 2012). Removal of the MT1 receptor significantly impaired the ability of mice to metabolize glucose

and induced insulin resistance in these animals (Contreras-Alcantara et al., 2010). Interestingly, melatonin treatment has been shown to improve glucose homeostasis (insulin sensitivity and glucose tolerance) in obesity and insulin resistance (Sartori et al., 2009; Agil et al., 2012b; Wan et al., 2013). However, the mechanism underlying the improvement of glucose homeostasis is complex and not well understood (Peschke, 2008; Peschke et al., 2012).

In the evolution of insulin resistance, impairment of insulin-stimulated glucose transport has been considered as the most consistent change that develops early, in the hearts of animal models (Wright et al., 2009). As recently demonstrated by Cook et al. (2010), this change occurs as a consequence of both reduced GLUT-4 protein expression and impaired translocation (Cook et al., 2010; Abel et al., 2012). Melatonin replacement in pinealectomized rats improved pinealectomy-induced insulin resistance and increased plasma membrane GLUT-4 protein content in adipose tissue (Zanquetta et al., 2003). Importantly, in hyperthyroid rats melatonin treatment was able to protect the heart against oxidative damage and restore the expression of the cardiac GLUT-4 gene, supporting the ability of melatonin to improve alterations in glucose uptake (Ghosh et al., 2007). However, its effect on myocardial glucose uptake and metabolism has not yet been investigated.

In the present study, we aimed to investigate the effects of melatonin treatment on glucose homeostasis focusing on glucose uptake by isolated cardiomyocytes in normal and insulin resistant conditions. We tested the hypothesis that acute or long-term (6 weeks) melatonin treatment stimulates glucose uptake of normal and insulin resistant cardiomyocytes *via* melatonin receptors.

Our specific objectives were to determine:

1. The acute effects of melatonin on glucose uptake by cardiomyocytes isolated from young untreated rats.
2. The acute effects of melatonin on cardiomyocytes isolated from age-matched untreated control and obese rats by determining the glucose uptake, insulin sensitivity, effect of

the melatonin receptor antagonist (luzindole) and associated signalling events (PKB/Akt, AMPK and GLUT-4 expression).

3. The effects of long-term (6 weeks) melatonin on glucose uptake by cardiomyocytes isolated from control and obese rats.

6.2. MATERIALS AND METHODS

6.2.1. Animals

All animals used were obtained from the University of Stellenbosch Central Research Facility. They were housed in the same facility and kept under the same conditions as previously indicated in chapter 3.

6.2.2. Study design: grouping, feeding and treatment

Animal feeding and treatment are presented in figure 6.1. Animals were divided in five groups: normal young rats plus four groups of diet and control rats: untreated (C) and melatonin treated (CM) control rats, untreated (D) and melatonin treated (DM) obese rats.

Normal young rats (225–250g) fed a normal rat chow were used to determine the acute effects of melatonin on insulin-stimulated glucose uptake by isolated cardiomyocytes

Control (C) and diet (D) rats were fed as previously in chapter 3. To evaluate the progressive changes in insulin sensitivity, the feeding period was varied from 16 to 19 and 20 to 23 weeks. Similar to normal young rats, cardiomyocytes prepared from these rats, were used for the determination of the acute effect of melatonin treatment on insulin-stimulated glucose uptake. In addition to glucose uptake, these cardiomyocytes were also used for western blot analysis.

To evaluate the effect of six weeks melatonin treatment on glucose uptake, a number of rats were fed for 20 weeks. Four groups were used: (i) untreated control (C), (ii) treated control (CM), (iii) untreated diet (D) and (iv) treated diet (DM). The treated animals received melatonin in drinking water for 6 weeks (starting after 14 or 17 weeks after initiation of the diet) as described in chapter 3.

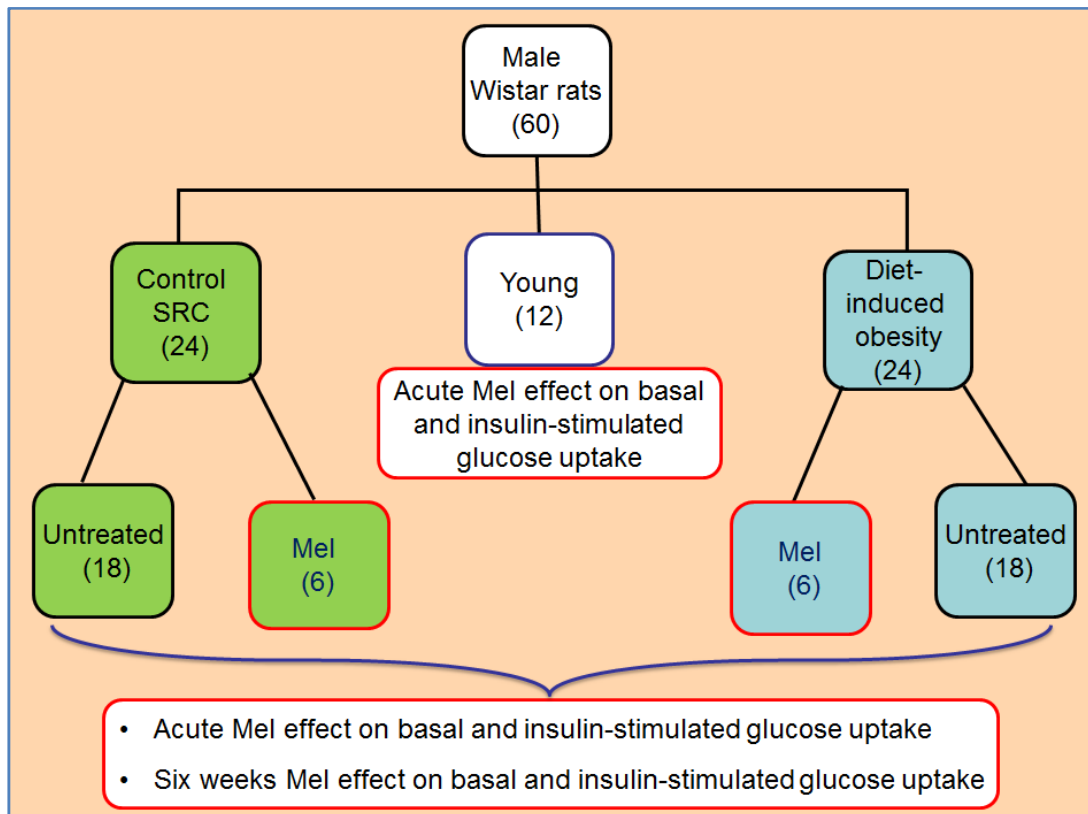


Figure 6.1. Simplified protocol for animal feeding and treatment. An additional group of normal young rats (12) was added to four groups of rats including untreated (18) and melatonin treated (6) control, untreated (18) and melatonin treated (6) obese rats. Mel: melatonin, SRC; standard rat chow.

For the administration of melatonin to isolated cardiomyocytes, the compound was first dissolved in ethanol and then in medium buffer (see below “solution E” without substrate) to yield a final concentration of 1nM, 10nM, 100nM, 1µM or 10µM. Luzindole and phloretin (Sigma-Aldrich , St Louis, MO) were dissolved in dimethyl sulfoxide (DMSO), stored at –80°C as stocks and diluted with medium “solution E” immediately before use.

6.2.3. Experimental procedure

6.2.3.1. Cardiomyocytes preparation

Cardiomyocytes from the different animal groups (fig.6.1) were prepared as previously reported (Huisamen et al., 2001; Strijdom et al., 2004). Briefly, after removal, the heart was retrogradely perfused with a calcium free medium or “solution A” (HEPES buffer: 6mM KC1, 1mM Na₂HPO₄, 0.2mM NaH₂PO₄, 1.4mM MgSO₄, 128mM NaCl, 10mM HEPES (Sigma-

Aldrich, St Louis, MO), 5.5mM glucose, 2mM pyruvate, pH 7.4, 37°C, equilibrated with oxygen) for 5 min to remove all blood present in the heart. Thereafter, the perfusion was switched to the second medium or “solution B” [50 mL; “Solution A” containing 0.7% bovine serum albumin (BSA) (fraction V, fatty acid free) (Roche, Cape Town), 1.1mg/mL collagenase (Worthington Type 2 Biochemical Corporation, Lakewood, NJ) and 15mM 2,3-butanedione monoxime (BDM) (Sigma-Aldrich , St Louis, MO)] for 15 min (fig. 6.2). At this stage, for adequate enzyme activity, the calcium concentration was gradually increased to 200µM over an additional 5min (2X50µL of 100mM CaCl₂) and the perfusion continued to a total time of 35 min.

After perfusion, the ventricles were carefully separated, minced with tweezers and suspended in the medium buffer “solution C” (50mL; 25mL of “solution B” and 25mL of “solution A” containing 1% BSA and 200µM CaCl₂). The suspension was placed in a flat-bottomed flask in a shaking water bath (37°C, 180 strokes/min) and digested for a further 15min, whereafter the calcium concentration was gradually raised to 1.25mM over an additional 5min (4 x 100µL followed by 1x125µL of 100mM CaCl₂ at intervals of 1min) (fig. 6.2).

The isolated cells were filtered through a nylon mesh (pore-size 200×200µm) and gently centrifuged (3min at 100rpm). The pellet obtained was re-suspended in HEPES buffer containing 1.25mM CaCl₂, 2% BSA (fraction V, fatty acid free) or solution “D” and the cells allowed to settle for 3 to 4min. The supernatant was carefully aspirated and cells resuspended in the same buffer and allowed to recover from the trauma of isolation for 90min before the glucose uptake assay. After recovery, the viability of the isolated cardiomyocytes was evaluated by trypan blue exclusion. Viability of > 80% was a prerequisite.

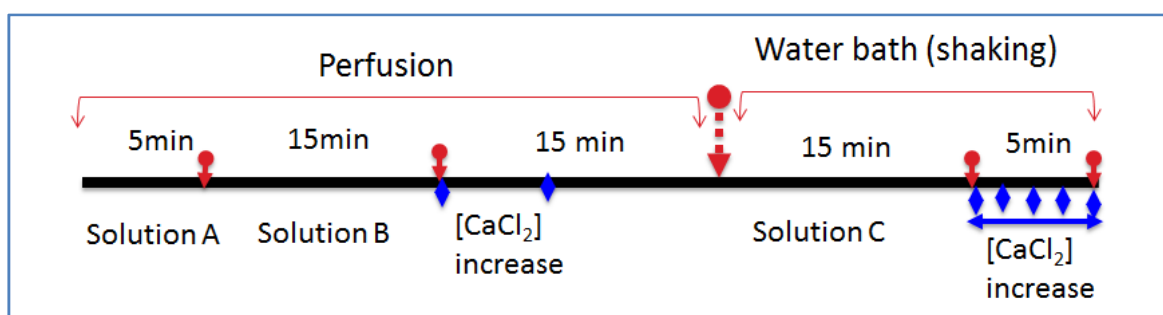


Figure 6.2 Simplified protocol for cardiomyocyte isolation: heart perfusion and digestion.

6.2.3.2. Determination of 2-deoxy-D-glucose uptake

- **Assay procedure**

2-deoxy-D-glucose (2DG) uptake was measured as previously reported (Huisamen et al., 2001). In brief, 500 μ L of suspension of cardiomyocytes (approximately 0.5 mg protein) were assayed in a total volume of 750 μ L of oxygenated medium buffer "solution E" containing (in mmol/L): KCl 6; Na₂HPO₄ 1; NaH₂PO₄ 0.2; MgSO₄ 1.4; NaCl 128; HEPES 10; CaCl₂ 1.25 plus 2% BSA (fraction V, fatty acid free); pH 7.4. Samples were assayed in duplicate.

Figure 6.3 represents the protocol followed for the determination of glucose uptake with different treatments. For insulin-stimulated glucose uptake experiments, the cells were pre-incubated for 15min in a shaking water bath (37°C, 180 strokes/min) with or without 50 μ L of phloretin (final concentration: 400 μ M) for measurement of non-carrier mediated glucose uptake (such as GLUT-4, GLUT-1). Each experimental group was then incubated with or without 75 μ L of insulin (final concentration: 1nM, 10nM, 100nM) or 75 μ L melatonin (final concentration: 1nM, 10nM, 100nM, 1 μ M and 10 μ M) under the same conditions for 30min before glucose uptake was initiated. When both hormones were added to the same cell population, melatonin was administered 30 min before addition of insulin (fig.6.3B). In another series of experiments, 7.5 μ L of luzindole (final concentration: 5 μ M) and/or melatonin were administered separately after 15min of stabilization. When both were added to the same cell population, luzindole was administered 30min before melatonin was added (fig 6.3B).

Glucose uptake was initiated by addition of 25 μ L of 2-deoxy-D- [³H] glucose (2DG) (1.5 μ Ci/mL; final concentration: 1.8 μ M) (Perkin Elmer, Boston). Glucose uptake was allowed to progress for 30 min before stopping the reaction by adding 50 μ L phloretin (final concentration: 400 μ M).

After this step, the cells were centrifuged at 2500 rpm for 1 min and the supernatant containing radio-labeled 2DG aspirated. Thereafter, the pellet was washed twice with HEPES buffer and then dissolved in 0.5mL 1N NaOH at 70 °C in a water bath for 30 to 40min. The final volume of the solution was brought to 1mL by adding 0.5mL of dH₂O. 50 μ L of this solution was used for

the determination of the protein content by the method of Lowry et al. (1951) while the remainder was counted for radioactivity.

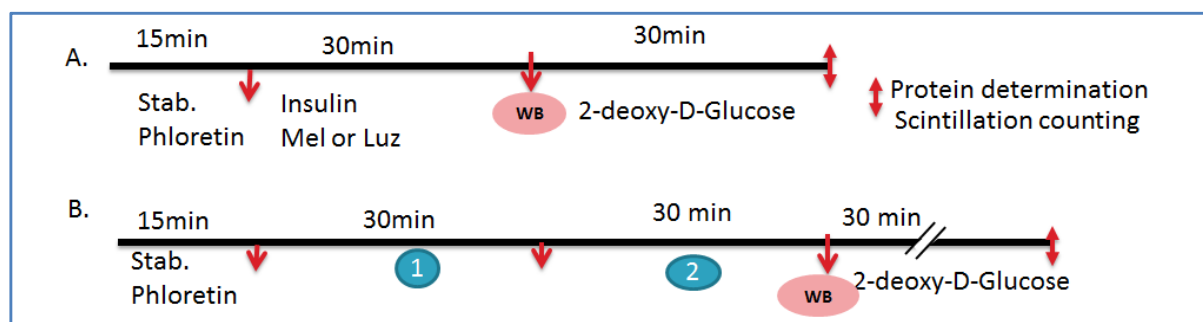


Figure 6.3 Simplified protocol for glucose uptake assay. A: When insulin, melatonin (Mel) or luzindole (Luz) are used separately, B: when melatonin (1) and insulin (2) or luzindole (1) and melatonin (2) are combined, Stab: stabilisation period. WB: Western blotting

- **Glucose uptake quantification**

Glucose uptake quantification was done in duplicate using 20mL-scintillation vials. Scintillation liquid (2mL) was added to 450 μ L of each sample and mixed before incubation. Additionally, two vials containing the scintillation liquid only or the scintillation liquid plus radio-labeled 2DG were included for determination of the blank and the total counts, respectively.

Counting of the radioactivity was done using a scintillation counter (Beckman) after overnight stabilization of the samples in the dark at room temperature. Glucose uptake was determined using the following formula: “**Counts-Blank /Specific activity of 2DG x protein content**”

Glucose uptake was expressed as pmol 2DG/mg protein/30min. The glucose uptake value of phloretin was subtracted from each sample to get the final glucose uptake value and the fold stimulation calculated.

- **Western blotting**

Cell lysates were made after 30min incubation with or without insulin or melatonin before the addition of 2-deoxy- D-[3 H] glucose (see figure 6.3). At the end of the incubation period, cells were put on ice, transferred to eppendorf tubes, quickly centrifuged and washed 3 times with ice-cold HEPES buffer without BSA. The cells were then lysed in 100 μ L of lysis buffer

(described in chapter 3). In this regard, cells were sonicated on ice 3 times (intervals of 3 seconds pulse and 1 second break). Thereafter, they were centrifuged for 20min and the subsequent supernatant used for western blotting (as described in chapter 3). We investigated the effect of melatonin on expression and phosphorylation of PKB/Akt (Ser-473); AMPK and GLUT-4 expression in cardiomyocytes after incubation with or without insulin or melatonin.

6.2.4. Data analysis

All statistical analyses were done as indicated in chapter 3.

6.3. RESULTS

6.3.1. Acute effect of melatonin on glucose uptake by cardiomyocytes

6.3.1.1. Normal young rats

The effect of melatonin administration on glucose uptake by cardiomyocytes isolated from normal young rats is represented in figures 6.4-5. Melatonin at 10 nM (Mel1), 100nM (Mel2), 10 μ M (Mel3) and 50 μ M (Mel4) had no significant effect on glucose uptake, compared to the basal levels (fig.6.4).

Insulin, on the other hand caused a 2.3 fold increase in glucose uptake, compared to the basal levels. Insulin (1nM) added to cells pre-treated with melatonin (100nM) caused a further stimulation of glucose uptake (3.36 ± 0.48 vs 2.48 ± 0.20 fold increase, $p<0.05$) (fig.6.5). However, melatonin at other concentrations did not influence the levels of insulin stimulated glucose uptake (fig.6.5).

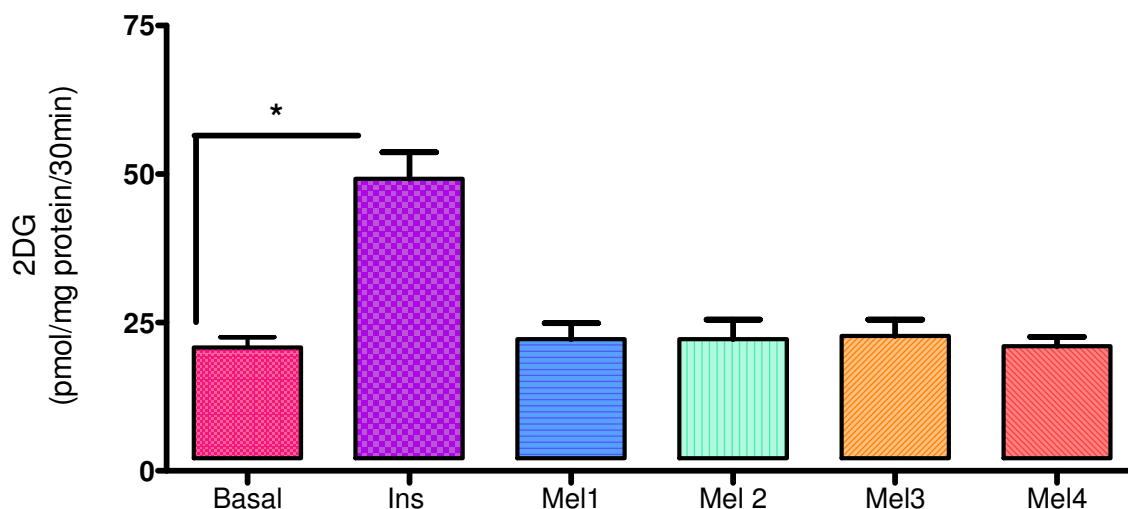


Figure 6.4 Effect of melatonin on glucose uptake of adult cardiomyocytes, Ins: insulin, Mel: melatonin, Mel1:10nM, Mel2:100nM, Mel3:10µM, Mel4: 50µM, *p<0.01 (Insulin vs basal), n= individual preparations: 12 (basal), 12 (Ins), 3 (Mel1), 8 (Mel2), 4 (Mel3), 3 (Mel4).

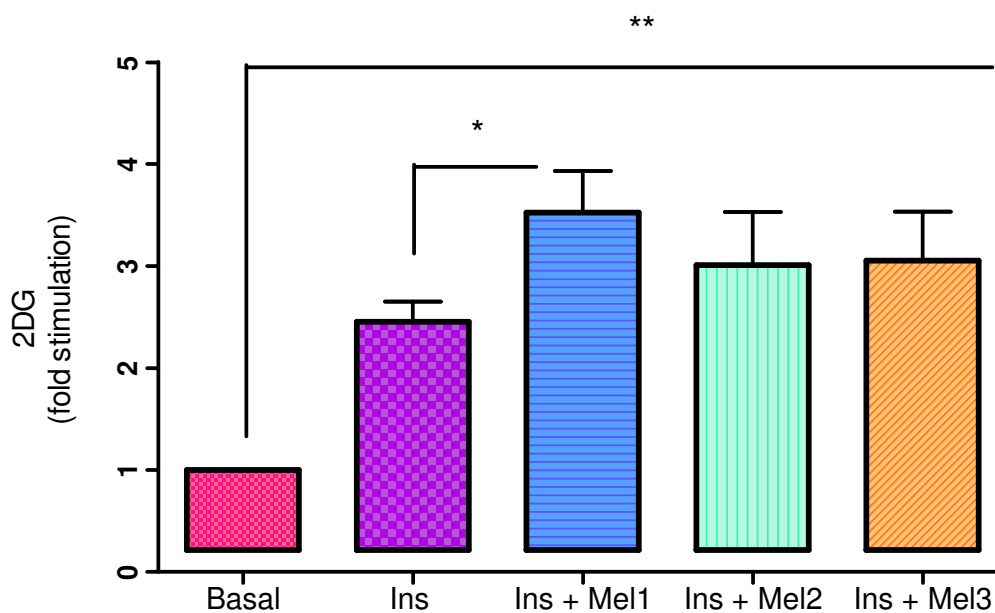


Figure 6.5 Effect of melatonin on insulin stimulated glucose uptake (fold stimulation), Ins: insulin, Mel: melatonin, Ins: 1nM; Mel1:100nM, Mel2:10nM, Mel3:10mM; *p<0.05 (Ins vs Ins+Mel1); **p<0.05 (basal vs Ins or Ins +Mel); n=5-10 individual preparations /group

6.3.1.2. Rats fed a high calorie diet for 16 to 19 weeks

Results obtained from the acute effect of melatonin treatment on insulin-stimulated glucose uptake are presented in figures 6.6-8 and summarized in table 6.2. Basal glucose uptake did not differ significantly between cardiomyocytes isolated from C and D groups (table 6.2).

In the control group, melatonin or/and luzindole alone did not affect the basal glucose uptake. However, melatonin (100nM) enhanced the insulin (1nM) stimulated glucose uptake as a trend (73.9 ± 4.1 vs 49.3 ± 5.6 pmol/mg protein, $p=0.05$) (fig.6.6).

Insulin-stimulated glucose uptake was similar in cardiomyocytes isolated from the C and D group (fig.6.8; table 6.2). Melatonin pre-treatment (100nM) caused an increase in insulin-stimulated glucose uptake as a trend in control ($p=0.053$) (fig 6.6-7). This was significantly elevated when compared to D group (fig.6.8B).

Table 6.2 Glucose uptake by cardiomyocytes from rats fed for 16 to 19 weeks of feeding

A: absolute values							
Group	Basal	Insulin	Ins+Mel	Mel	Mel+Luz	BW(g)	Vfat (g)
C	$25.6 \pm 2.8^*$	$49.3 \pm 5.6@$	$73.9 \pm 4.1^{**\text{‡}}$	25.5 ± 4.4	19.5 ± 3.5	435 ± 21	17.0 ± 1.4
D	$20.8 \pm 3.1^*$	40.8 ± 3.8	47.5 ± 4.9	20.0 ± 3.4	14.3 ± 3.05	$517 \pm 11\#$	$33.3 \pm 1.3\&$
B: Fold stimulation							
C	1	1.92 ± 0.12	$2.8 \pm 0.16^{**\text{‡}}$	0.99 ± 0.2	0.76 ± 0.07	-	-
D	1	1.96 ± 0.22	2.3 ± 0.07	0.96 ± 0.1	0.68 ± 0.06	-	-
n	6	6	4	6	5	6	6

C: control group, D: diet group, BW: body weight, Vfat: visceral fat, Mel: melatonin, Luz: luzindole, * $p < 0.01$ vs Ins, ** $p < 0.001$ vs basal, @ $p = 0.05$ vs Ins+Mel, ‡ $p < 0.05$ vs D, n=4-6 individual preparations /group; # $p < 0.001$ vs C, & $p < 0.001$ vs C, n=6/group. Glucose uptake was expressed as pmol 2DG/mg protein/30 min (A) or as fold stimulation (B).

Control group

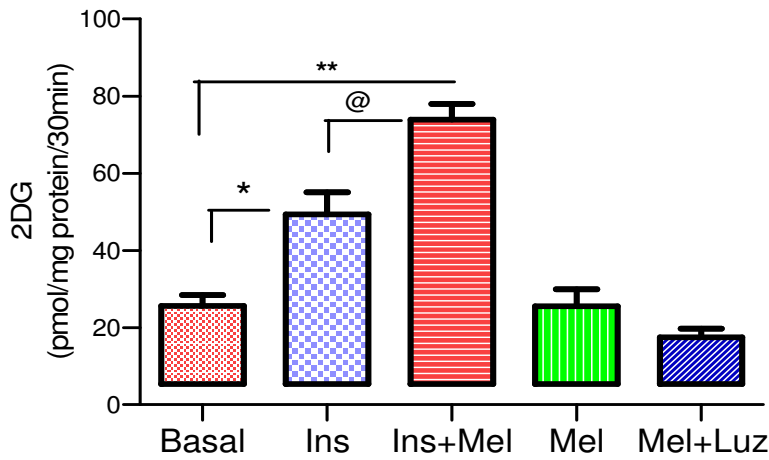


Figure 6.6 Effect of melatonin and luzindole on glucose uptake by cardiomyocytes isolated from control groups, Ins: insulin (1nM), Mel: melatonin (100nM), Luz: luzindole (5µM), *p<0.01 (basal vs Ins), **p<0.001 (basal vs Ins+Mel), @p=0.053 (Ins vs Ins+Mel), n=4-6 individual preparation/group.

Diet group

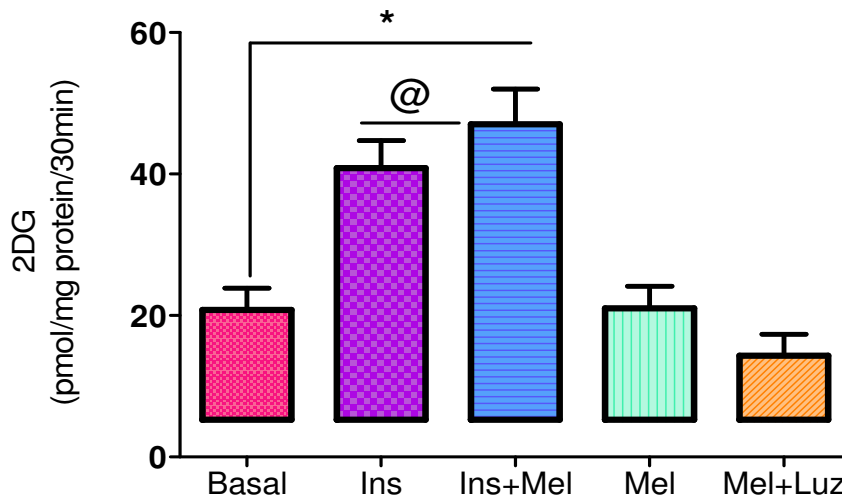


Figure 6.7 Effect of melatonin and luzindole on glucose uptake by cardiomyocytes isolated from diet groups, after 16-19 weeks, Ins: insulin (1nM), Mel: melatonin (100nM), Luz: luzindole (5µM), *p<0.01 (basal vs Ins or Ins+Mel), @p=0.3 (Ins vs Ins+Mel), n=4-6 individual preparations /group.

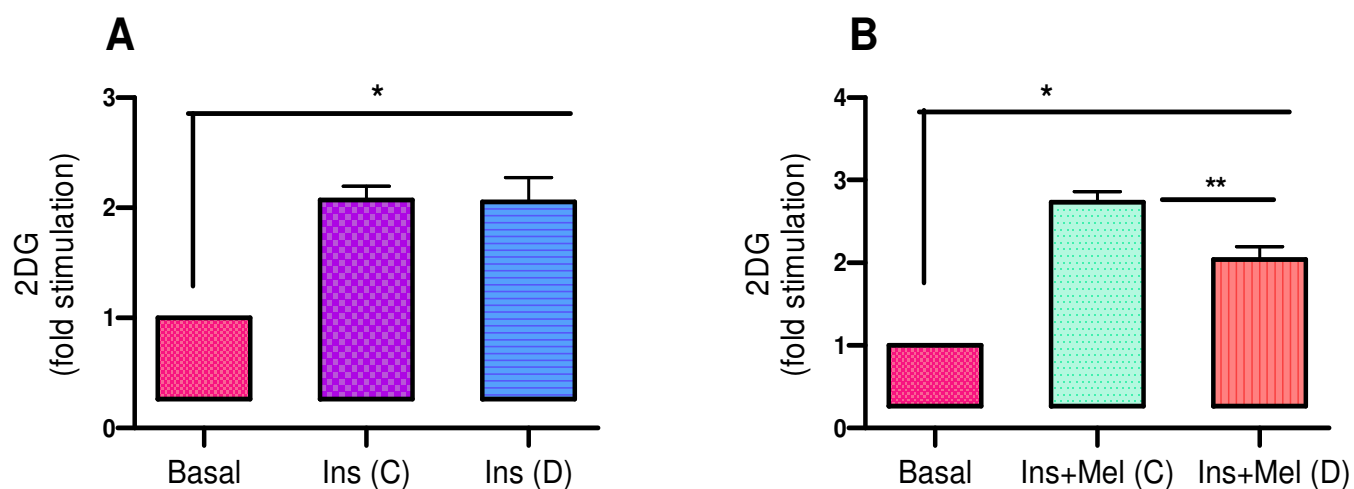
Insulin stimulation: C vs D

Figure 6.8 Insulin-stimulated glucose uptake in control (C) and diet (D) groups, after 16-19 weeks, A: Insulin alone, B: Insulin with melatonin (Ins+Mel), Ins: insulin (1nM), Mel: 100nM, * $p < 0.01$ (basal vs Ins or Ins+Mel), ** $p < 0.05$ (C vs D), $n = 4-6$ individual preparations/group.

6.3.1.3. Rats fed a high calorie diet for 20 to 23 weeks of feeding

Results for the acute effect of melatonin treatment and insulin-stimulated glucose uptake are presented in figures 6.9-11 and summarized in the table 6.3.

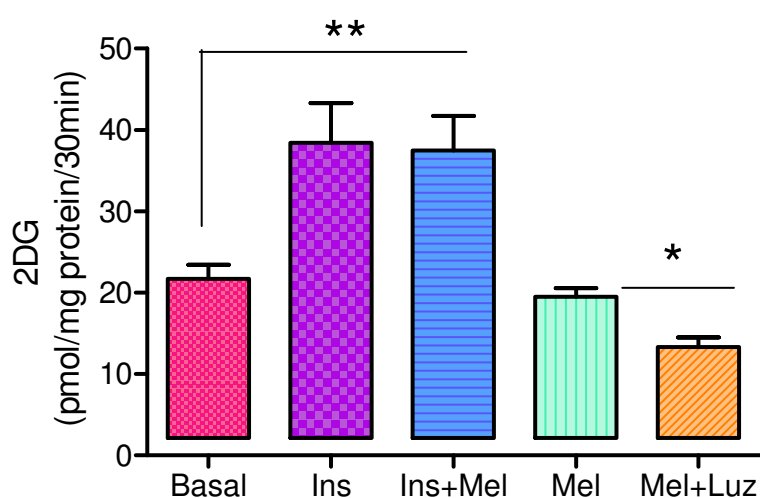
The high calorie diet fed for 20-23 weeks had no effect on basal glucose uptake by cardiomyocytes (table 6.3). While insulin-stimulated glucose uptake was low in the D group, compared with the control group (fig.6.11), insulin-stimulated glucose uptake with melatonin pre-treatment was similar in both groups (fig.6.11).

Table 6.3 Glucose uptake by cardiomyocytes isolated from rats fed for 20 to 23 weeks

A: Absolute values							
Group	Basal	Ins	Ins+ Mel	Mel	Mel+Luz	BW	Vfat
C	19.9±2.6***	35.3±6.3 F	33.5±5.9	19.2±1.7	12.5±2.1*	457±14	18.4±10.9
D	18.1±1.6 **	25.9±1.6	27.8±1.1	18.4±2.3	12.1±0.4*	575±61#	38.7±2.6&
B: Fold stimulation							
C	1	1.75±0.09F	1.68±0.07	0.96±0.03	0.62±0.13*	-	-
D	1	1.41±0.10	1.55±0.13	1.02±0.06	0.66±0.05*	-	-
n	6	6	5	6	4	6	6

C: control, D: diet, BW: body weight, Vfat: visceral fat, ND: not determined, Ins: insulin (1nM), Mel: melatonin (100nM), Luz: luzindole (5µM), *p<0.05 (Mel vs Mel+Luz), **p<0.01 vs Ins or Ins+Mel, ***p<0.01 vs Ins or Ins+Mel, Fp<0.05 vs D, n=4-6 individual preparations/group, #p<0.001 vs C, &p<0.001 vs C, n=6/group. Glucose uptake was expressed as 2DG pmol/mg protein/30min (A), and fold stimulation (B).

Control group

**Figure 6.9** Glucose uptake by cardiomyocytes isolated from control rats after 20-23 weeks of

feeding. Ins: 1nM; Mel: 100nM, Luz: luzindole (5 μ M), * $p < 0.05$ (Basal vs Ins or Ins +Mel);
 ** $p < 0.05$ (Mel vs Mel+Luz), n=4-6 individual preparations/group

Diet group

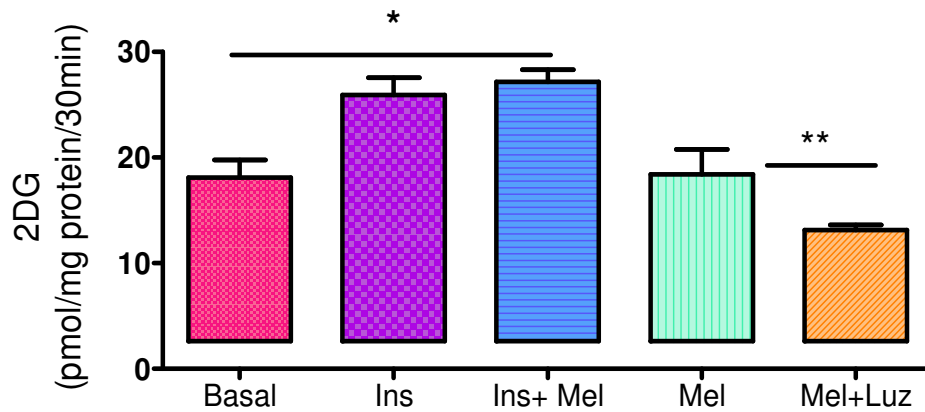


Figure 6.10 Glucose uptake by cardiomyocytes isolated from diet rats after 20-23 weeks of feeding, Ins (1nM); Mel (100nM), Luz: luzindole (5 μ M), ** $p < 0.05$ (Mel vs Mel+Luz), * $p < 0.01$ (Basal vs Ins or Ins+Mel), n=4-6 individual preparations/group.

Comparison between C and D group (20 to 23 weeks)

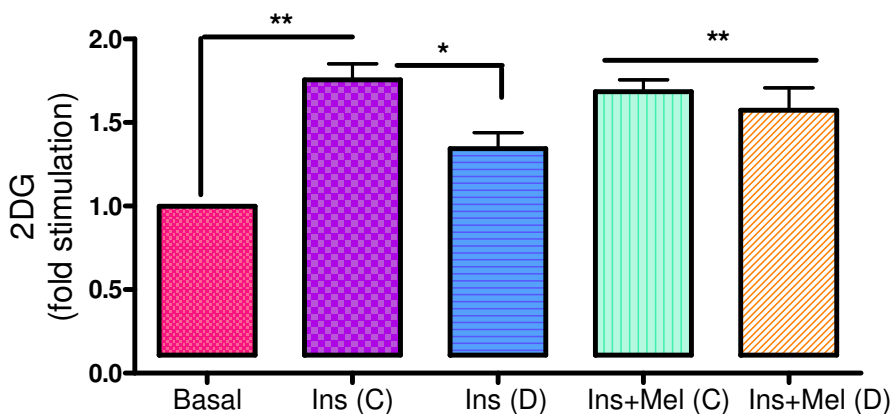


Figure 6.11 Insulin-stimulated glucose uptake by cardiomyocytes from control (C) and diet (D) groups. Ins: insulin (1nM); Mel: melatonin (100nM), * $p < 0.05$ [Ins (C) vs Ins (D) or Ins (D) vs basal], ** $p < 0.01$ [Ins +Mel (C) or Ins+Mel (D) vs basal or Ins (C) vs basal], n=5-6 individual preparations/group.

6.3.2. Effect of oral melatonin treatment on glucose uptake by cardiomyocytes isolated from rats fed a high-calorie diet.

- **Biometric parameters**

Similar to previous experiments, the high calorie diet significantly increased the body weight and visceral fat mass of the rats (table 6.4). Melatonin treatment attenuated body weight and the visceral fat mass in the D group (table 6.4).

Table 6.4 Body weight and visceral fat mass

Parameters	C	CM	D	DM	n
Body weight (g)	433±25	411± 17	538±43**	488±21#	6
Visceral fat (g)	17.7±1.8	14.33±1.9*	37.50±7.5**	28.0±4.0#	6

C: control, D: diet, CM and DM: control and diet receiving melatonin for 6 weeks, * $p < 0.05$ vs C, # $p < 0.05$ vs D, ** $p < 0.001$ vs C, $n = 6$ /group.

- **Glucose uptake**

A high calorie diet alone did not affect the basal glucose uptake by cardiomyocytes isolated from control and diet groups. It reduced however insulin-stimulated glucose uptake with 100nM insulin (fig.6.12).

In the control group, melatonin treatment did not affect the basal glucose uptake (22.6 ± 3.7 vs 21.1 ± 3.5 pmol/mg protein/30min, $p > 0.05$) (fig.6.12). However, administration of 1nM or 100nM insulin to cardiomyocytes isolated from the control treated group (CM) resulted in elevated insulin-stimulated glucose uptake compared to their respective untreated groups ($p < 0.05$).

In the D group, the basal glucose uptake by cardiomyocytes isolated from melatonin treated rats was higher than that of untreated rats (26.4 ± 2.1 vs 19.8 ± 3.4 pmol/mg protein/30min, $p < 0.05$) (fig.6.12). With administration of 100nM insulin, cardiomyocytes from the treated group (DM) had elevated stimulated glucose uptake when compared to their respective untreated group (50.1 ± 1.7 vs 32.1 ± 5.1 pmol/mg protein, $p < 0.01$) (fig.6.12).

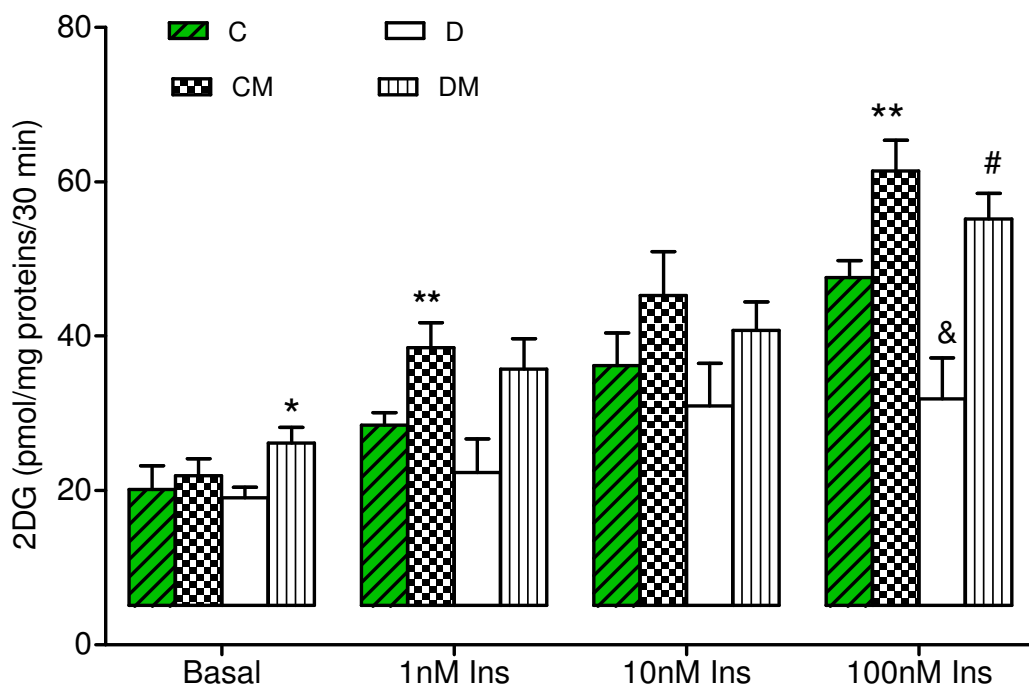


Figure 6.12 Insulin-stimulated glucose uptake by cardiomyocytes isolated from rats fed a high calorie diet with or without melatonin treatment, Ins: insulin, C: control, C M: control with melatonin, D: diet, DM: diet with melatonin, * $p < 0.05$ (DM vs D), ** $p < 0.05$ (CM vs C), & $p < 0.05$ (D vs C), # $p < 0.01$ (DM vs D), $n = 4-6$ individual preparations/group analysed in duplicate.

6.3.3. Effect of acute melatonin and insulin treatment on cardiomyocyte signalling

To investigate which mechanism is implicated in enhancing glucose uptake, cardiomyocytes were isolated from control and diet rats fed for 20 weeks. Isolated cardiomyocytes were treated with or without melatonin for 30 min and insulin was added for an additional 30 min. Luzindole was administered for 30 min before melatonin treatment when they were administered to the same cell population.

Insulin alone or with melatonin increased the activation of PKB/Akt in control and in D groups with a more than 2.2 and 1.5 fold increase respectively (fig.6.13). In control but not in diet group, melatonin treatment significantly increased the phosphorylation of PKB/Akt (Ser-473) compared to basal levels ($p < 0.05$) (fig.6.13). Interestingly, luzindole attenuated melatonin-induced PKB/Akt phosphorylation while insulin with or without melatonin had higher PKB/Akt phosphorylation in control than in D group (fig.6.13).

AMPK and GLUT-4 expression were not affected in all groups of treatment (fig.6.14-15).

- **PKB/Akt**

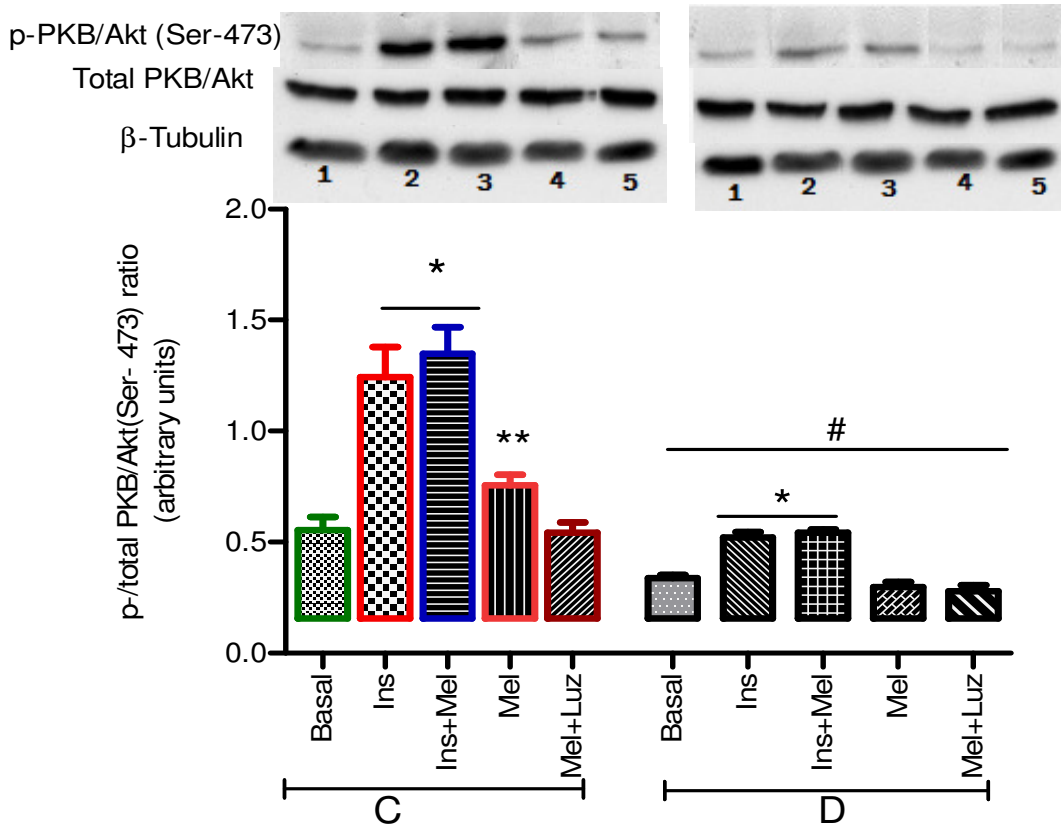


Figure 6.13 PKB/Akt phosphorylation and activation, C: control, D: diet (high calorie diet), Basal (1), Ins: insulin (2), Insulin+melatonin (3), Mel: melatonin (4), luzindole+melatonin (5), Luz: luzindole, C: * $p < 0.05$ (Ins or Ins+Mel vs basal), ** $p < 0.05$ (Mel vs basal or luzindole), D: * $p < 0.05$ (Ins or Ins+Mel vs basal), # $p < 0.05$ (D vs C), $n = 3$ individual preparations/group. Blot presented is representative. C and D performed on the same blot.

• GLUT-4

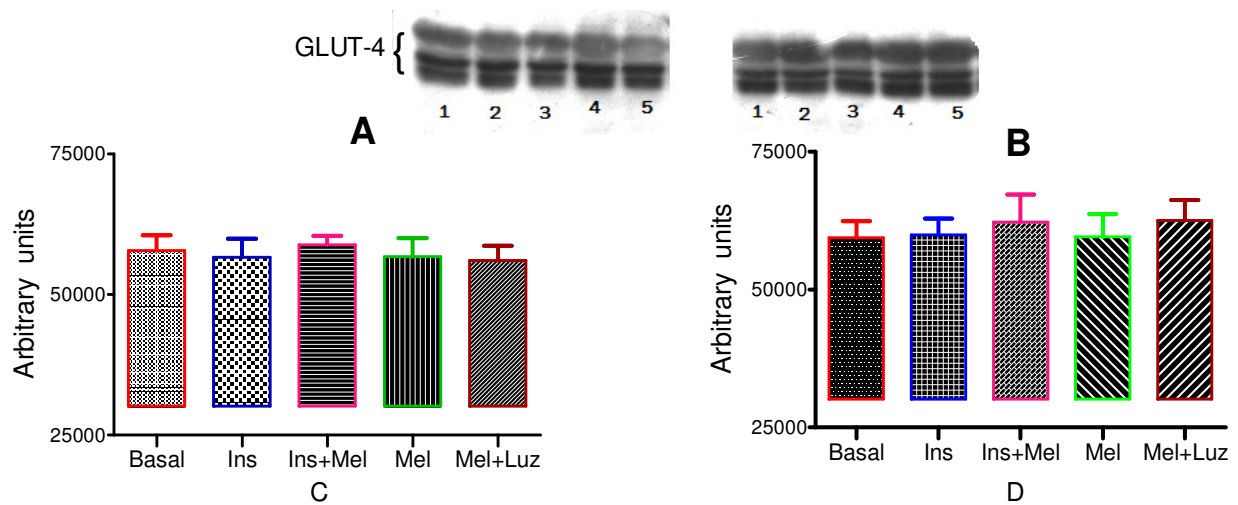


Figure 6.14 GLUT-4 expression, C: control (A), D: high calorie diet (B), basal (1), Ins: insulin (2), insulin+melatonin (3), Luz: Luzindole, Mel: melatonin (4), Luzindole + melatonin (5), n=3 individual preparations/group. Blot presented is representative. C and D performed on the same blot.

• AMPK

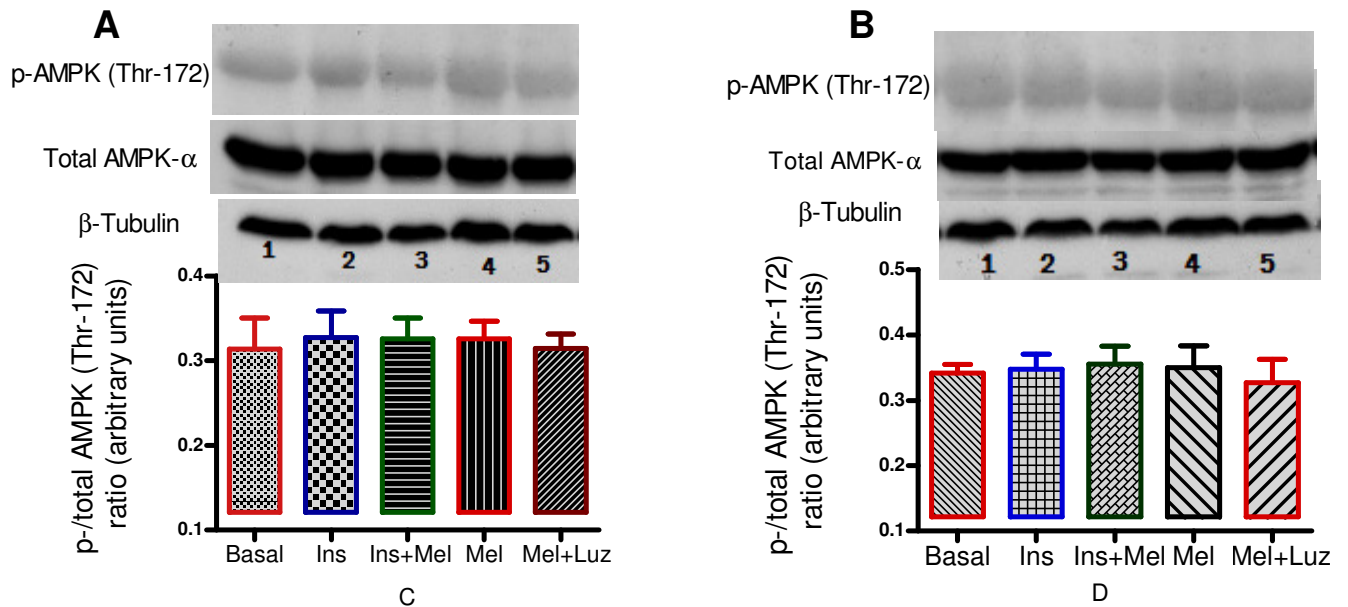


Figure 6.15 AMPK expression and phosphorylation, C: control (A), D: high calorie diet (B), basal (1), Ins: insulin (2), insulin +melatonin (3), Mel: melatonin (4), luzindole + melatonin (5), Luz: luzindole, n=3 individual preparations/group. Blot presented is representative. C and D performed on the same blot.

6.4. DISCUSSION

As mentioned in the introduction to this chapter, glucose homeostasis involves various and complex mechanisms associated with glucose availability and utilization (Norris and Rich, 2012). The first step of glucose metabolism is glucose transport across the cell membrane. In chapter 4 we focused on melatonin's effects on insulin resistance focussing on fasting blood glucose and serum insulin levels as well as glucose tolerance. It is well established that in the non-fasting condition, insulin plays a crucial role in the regulation of blood glucose levels by inhibiting endogenous glucose production and stimulating glucose uptake into peripheral tissues, including the heart, skeletal muscle and adipose tissue (Nolan et al., 2011). To gain more information regarding myocardial glucose metabolism in obesity, we studied the effects of melatonin treatment on glucose uptake by cardiomyocytes isolated from hearts from normal and insulin resistant rats.

The main findings are as follows: (i) acute melatonin treatment alone had no effect on glucose uptake by cardiomyocytes isolated from hearts from young, old or insulin resistant rats; (ii) acute melatonin treatment enhanced, in a concentration-dependent manner, the insulin-stimulated glucose uptake by cardiomyocytes isolated from the hearts of normal young rats; (iii) acute melatonin treatment did not affect insulin-stimulated glucose uptake by cardiomyocytes isolated from old control or insulin resistant animals; (iv) acute melatonin administration to cardiomyocytes isolated from hearts from control but not insulin resistant rats was associated with increased basal PKB/Akt phosphorylation with no effect on AMPK phosphorylation and GLUT-4 expression, (v) long-term (6 weeks) melatonin treatment increased basal glucose uptake by cardiomyocytes isolated from the hearts of obese rats and enhanced insulin-stimulated glucose uptake in both groups.

6.4.1. Effects of acute melatonin treatment on cardiomyocyte glucose uptake

The role of melatonin in glucose metabolism has recently attracted many investigators (Peschke, 2008; Agil et al., 2012b; de Oliveira et al., 2012; Faria et al., 2013; Karamitri et al., 2013; Zanuto et al., 2013). The implication of melatonin in glucose regulation in general and in

glucose uptake in particular has been supported by the observation that pinealectomized animals developed insulin resistance (Lima et al., 1998; Alonso-Vale et al., 2004). In this regard, pinealectomy was associated with a decrease in glucose uptake by adipose tissue (Lima et al., 1998; Alonso-Vale et al., 2004). Accordingly, administration of melatonin reversed pinealectomy-induced insulin resistance and improved glucose uptake of isolated adipose tissue (Lima et al., 1998; Alonso-Vale et al., 2004). On other hand, as expected, due to its anti-lipogenic activities, a decrease in glucose uptake was reported in PAZ6 adipocyte cells after 14 days incubation with melatonin (Brydon et al., 2001). Additionally, melatonin was shown to increase glucose uptake in C₂C₁₂ myotubules (Ha et al., 2006). However, controversially, in the present study, melatonin had no significant effect on glucose uptake by isolated cardiomyocytes from hearts from young rats (fig.6.4). Similar findings have also been reported in chick brain (Cantwell and Cassone, 2002) as well as in adipose tissue isolated from a female fruit bat (Banerjee et al., 2011). Interestingly, when insulin (1nM) was added to cells pre-treated with melatonin (100nM), it caused a further stimulation of glucose uptake (fig.6.5). This synergistic interaction between melatonin and insulin-stimulated glucose uptake is consistent with previous studies (Banerjee et al., 2011; de Oliveira et al., 2012; Wang et al., 2013) and supports the potential insulin-sensitizing effect as previously suggested (see chapter 4, section 4.4.2).

We expanded our investigation by additionally studying the effect of obesity and insulin resistance on glucose uptake, using cardiomyocytes isolated from hearts from control and diet rats fed for 16 to 19 weeks. Despite increased body weight and visceral fat mass, the diet had no effect on basal glucose uptake (table 6.2) as previously reported (Huisamen et al., 2011). In addition, as it was observed in cardiomyocytes isolated from hearts from young rats, melatonin alone was without effect. However, it enhanced the insulin stimulated glucose uptake as a trend (fig.6.6) in cardiomyocytes from control hearts, but this disappeared in the diet group (fig.6.7). This could be merely due to ageing coupled to the progression of insulin resistance as previously demonstrated in control and obese mice (Carroll et al., 2005).

In addition to above, we furthered our investigation by using cardiomyocytes isolated from hearts from control and diet rats fed for a longer period, namely 20 to 23 weeks. As it was observed in 16 to 19 weeks study, the diet and/or melatonin had no effect on basal glucose uptake (table 6.3). Additionally, insulin-stimulated glucose uptake was significantly lower in the diet group, compared with the control group (fig.6.11) which may be indicative of an impairment of insulin actions and myocardial insulin resistance. As observed in 16 to 19 weeks study, melatonin pre-treatment had no effect on insulin-stimulated glucose uptake in both groups, in contrast to what was observed in the younger animals (fig.6.11). The loss of the synergistic interaction between melatonin and insulin action is difficult to explain. It may be a result of ageing in the control group which is coupled to insulin resistance in obese (D) group as previously demonstrated in control and obese mice (Carroll et al., 2005). However, additional investigation is needed to explain this phenomenon.

To explore whether melatonin receptors are involved in glucose uptake, we used the melatonin receptor antagonist luzindole. Luzindole alone had no effect on cardiomyocyte glucose uptake (fig.6.19-10). Surprisingly, in the presence of melatonin, luzindole significantly attenuated glucose uptake (fig.6.19-10), an effect difficult to explain since melatonin had no effect on basal glucose uptake by isolated cardiomyocytes. However Luzindole has been shown to attenuate the melatonin-stimulated glucose uptake in C₂C₁₂ in myotubules (Ha et al., 2006) and this was recently confirmed in insulin resistant 3T3-L1 adipocytes (Wang et al., 2013). Although the basal levels were not shown, not only did the melatonin receptor agonist, piromelatine (Neu-P11), enhance insulin-stimulated glucose uptake as was observed with melatonin, but luzindole was also able to reverse these effects (Wang et al., 2013), supporting the involvement of the melatonin receptor in the synergistic interaction between melatonin and insulin-stimulated glucose uptake.

6.4.2. Effects of long-term melatonin treatment on cardiomyocyte glucose uptake

Long-term melatonin treatment has been shown to improve glucose homeostasis and to reduce insulin resistance (Sartori et al., 2009; Agil et al., 2012b; Wan et al., 2013; Zanuto et

al., 2013). Although the high calorie diet caused a significant increase in body weight and visceral fat mass (table 6.4), obesity had no effect on basal cardiomyocyte glucose uptake consistent with a previous study (Huisamen et al., 2011). It reduced however insulin-stimulated glucose uptake as previous reported (Huisamen et al., 2011; Flepisi et al., 2013).

As expected, melatonin treatment induced a significant weight gain inhibition in the diet group (table 6.4) with a concomitant increase in basal glucose uptake by isolated cardiomyocytes (fig.6.12). In view of the increase in basal glucose uptake, although we did not measure GLUT-1 expression, this is responsible for the basal glucose transporter in many cell types and its expression would give more insight [for review, see (Mueckler and Thorens, 2013)]. Importantly, when stimulated by insulin, cardiomyocytes isolated from both treated groups exhibited increased glucose uptake (fig.6.12). Collectively, these findings support the involvement of melatonin in glucose homeostasis at cellular levels as previously demonstrated (Sartori et al., 2009; Shieh et al., 2009; Faria et al., 2013; Wan et al., 2013). However, how melatonin affected glucose homeostasis and enhanced insulin sensitivity is complex and unclear (this has largely been discussed in chapter 4). In obesity, long-term melatonin treatment may indirectly increase insulin sensitivity through changes in the metabolic profile such as increasing adiponectin levels and reducing leptin resistance (Agil et al., 2011, Kitagawa et al., 2012). In the following section we will further discuss the potential intracellular signalling associated with the synergistic interaction between melatonin and insulin.

6.4.3. Melatonin and cardiomyocyte signalling

As discussed above, melatonin receptors may play an important role in the regulation of glucose metabolism. Melatonin receptors have been identified in cardiomyocytes (Peliciari-Garcia et al., 2011). Importantly, removal of the MT1 receptor significantly impairs the ability of mice to metabolize glucose and probably induces insulin resistance in these animals (Contreras-Alcantara et al., 2010). In view of the synergistic interaction between insulin and melatonin on glucose uptake, we aimed to explore the intracellular signalling processes that may be involved in this effect. For this particular study, we preferred to use cardiomyocytes

isolated from control and diet groups for 20 weeks as in chapters 4 and 5. To avoid the complexity of the multiple physiological factors of *in vivo* treatment, we used an *in vitro* approach to evaluate the direct effect of melatonin treatment. Isolated cardiomyocytes were treated with or without melatonin for 30 min and insulin was added for an additional 30 min, followed by evaluation of the expression and phosphorylation of PKB/Akt and AMPK as well as GLUT-4 expression. The following section will focus on their role in glucose uptake.

6.4.3.1. PKB/Akt, AMPK, GLUT-4 and glucose uptake

PKB/Akt and AMPK play important roles in insulin mediated and non-insulin mediated glucose uptake, respectively [for review, see (Montessuit and Lerch, 2013)]. As discussed in chapter 4 (section 4.4.4.4), GLUT-4 is the most prominent glucose transporter in differentiated cardiomyocytes (Montessuit and Lerch, 2013). Apart from its involvement in insulin-stimulated actions, PKB/Akt has multiple other actions, including cell survival activities where it can be activated independently of insulin actions [for review, see (Manning and Cantley, 2007)].

Our results showed that the high calorie diet reduced basal PKB/Akt phosphorylation (fig.6.13) confirming previous findings in isolated cardiomyocytes from obese animals (Huisamen et al., 2001). This could be, however, increased by melatonin alone as well as insulin with or without melatonin pre-treatment, all showing a higher increase in the control than in the diet group. The involvement of PKB/Akt phosphorylation in glucose homeostasis upon melatonin treatment has been consistently reported by several researchers using various tissues/organs (Sartori et al., 2009; Shieh et al., 2009; Faria et al., 2013; Wan et al., 2013). Our results showed that PKB/Akt phosphorylation was closely associated with glucose uptake stimulated by insulin alone or with melatonin pre-treatment (fig.6.13), confirming involvement of PKB/Akt in insulin-stimulated glucose uptake (fig.6.11).

On other hand, however, an increase in PKB/Akt phosphorylation in cells treated with melatonin alone in the absence of insulin) was also observed. This is unlikely to play a role in glucose uptake since *in vitro* melatonin treatment alone had no significant effect on basal glucose uptake (fig.6.9-10), highlighting that PKB/Akt phosphorylation does not necessarily

translate into glucose uptake. In this regard, Ha and co-workers (2006) have also shown that melatonin induced glucose uptake in C₂C₁₂ mouse skeletal muscle cells independently of PKB/Akt phosphorylation, suggesting a tissue-dependent effect. Interestingly, luzindole abolished PKB/Akt phosphorylation induced by melatonin treatment alone (fig.6.13), suggesting involvement of the melatonin receptor in melatonin-induced PKB/Akt phosphorylation. The involvement of PKB/Akt in melatonin's effects is further discussed in chapters 4 and 7 focusing on heart tissues and endothelial cells, respectively.

Supporting the observation made by Ha et al. (2006), AMPK expression and phosphorylation were not affected, suggesting that the effects of melatonin are not AMPK dependent (fig.6.15). The lack of effect of insulin on AMPK was also reported in cardiomyocytes isolated from hearts from control and obese mice (Carroll et al., 2005). The effects of melatonin on AMPK are further discussed in chapter 7 focusing on endothelial cell function.

GLUT-4 expression was not affected by either the diet or melatonin treatment (fig.6.14). This is in agreement with an *in vivo* study where melatonin did not affect basal GLUT-4 expression in skeletal muscles after 16 weeks of treatment (Mendes et al., 2013). However, although a defect in GLUT-4 expression may indicate an impaired insulin action, its translocation to the membrane is a better indicator of glucose uptake (Montessuit and Lerch, 2013). One month of calorie restriction or melatonin replacement (50µg/100g/day, i.p.) to pinealectomized rats improved insulin sensitivity and increased plasma membrane GLUT-4 protein content in adipose tissue (Zanquetta et al. 2003). However, as previously indicated, melatonin's effect may vary depending on the cell type of as well as the experimental protocol. For example, a decrease in glucose uptake and corresponding GLUT-4 gene expression was previously reported following melatonin treatment in human brown adipocyte cells lines (PAZ6) (Brydon et al., 2001).

The effect of melatonin treatment on GLUT-4 expression is further discussed in chapter 4.

6.5. CONCLUSION

Convincing evidence exists for the involvement of melatonin in glucose homeostasis (Peschke, 2008; Sartori et al., 2009; Agil et al., 2012b; Peschke et al., 2013; Zanuto et al., 2013). However, the role of melatonin in glucose metabolism is not yet clear. In the present study, we have demonstrated that acute melatonin treatment of isolated cardiomyocytes had no effect on basal glucose uptake regardless of the age of the animal or insulin sensitivity. We have also demonstrated that long-term melatonin treatment increased basal glucose uptake by cardiomyocytes isolated from the hearts of obese insulin resistant rats. Additionally, we showed that melatonin enhanced insulin-stimulated glucose uptake by cardiomyocytes when administered *in vivo* or *in vitro*. Interestingly, the increase in PKB/Akt phosphorylation induced by acute melatonin treatment *in vitro* was not associated with increased glucose uptake and further investigation into the intracellular signalling processes involved is warranted.

6.6. STUDY LIMITATION

The present investigation focused on glucose uptake by isolated cardiomyocytes under normoxic conditions. Therefore, additional investigations using isolated cardiomyocytes exposed to stimulated ischaemia or anoxia are needed to elucidate the effects of melatonin on glucose uptake/metabolism and its role in cardioprotection. In addition, since the heart is comprised of a number of different cell types in which the effects of melatonin on glucose uptake/metabolism may differ, further investigation using isolated perfused hearts (under normoxic or ischaemic conditions) is warranted to get more insight into the role of melatonin in myocardial glucose uptake.

STUDY IV

**THE EFFECTS OF ACUTE MELATONIN TREATMENT ON CARDIAC
MICROVASCULAR ENDOTHELIAL CELLS**

CHAPTER SEVEN

STUDY IV

THE EFFECTS OF ACUTE MELATONIN TREATMENT ON CARDIAC MICROVASCULAR ENDOTHELIAL CELLS

7.1. INTRODUCTION

Obesity and insulin resistance have been associated with a low grade inflammatory state (Furukawa et al., 2004; de Luca and Olefsky, 2008; Lionetti et al., 2009) which is identified by elevated circulating cytokines, including tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1 β , IL-6 and IL-8 (Picchi et al., 2006; Qatanani and Lazar, 2007; Agil et al., 2012a). TNF- α has been shown to induce vascular endothelial dysfunction in MetS and diabetes (Hotamisligil, 1999; Picchi et al., 2006). In these conditions, melatonin administration reversed the levels of inflammatory markers including TNF- α (Nishida, 2005; Agil et al., 2012a) which is known as the first pro-inflammatory cytokine to be released by an inflammatory response (Carvalho-Sousa et al., 2011). Additionally, it has recently been demonstrated that TNF- α interferes with melatonin synthesis (Carvalho-Sousa et al., 2011), supplying an explanation for the reduced levels of melatonin observed in inflammatory states (Tamura et al., 2010).

Previous studies on the effects of melatonin using endothelial cells have indicated that melatonin has beneficial effects in preventing/protecting against endothelial dysfunction (Rodella et al., 2013). Indeed, melatonin is a pleiotropic molecule able to enter all cells, including circulating platelets, and is transported everywhere in the body (Di Bella and Gualano, 2006; Hardeland et al., 2006). In the case of the vascular endothelium, it can regulate blood-tissue exchanges (Di Bella and Gualano, 2006). In this regard, melatonin has been suggested to interact with endothelial cells by regulating the release of both relaxing-factors (e.g., nitric oxide) and contracting-factors (e.g., endothelin) (Di Bella and Gualano, 2006). Importantly, compared to cells from animals with normal nocturnal levels of melatonin, endothelial cells obtained from animals with lower nocturnal melatonin levels expressed higher levels of adhesion molecules and inducible nitric oxide synthase (iNOS) (Tamura et al.,

2010). This was also associated with an increased translocation of the transcription factor nuclear factor kappa B (NF- κ B) to the nuclei (Tamura et al., 2010), indicating endothelial protection by endogenous melatonin. These melatonin-induced endothelium protective properties have been shown to be accomplished *via* diverse mechanisms including free radical scavenging activities, stimulation of antioxidant defence enzymes, normalization of lipid and blood pressure profiles and an eventual increase in nitric oxide (NO) bioavailability (Anwar et al., 2001; Grossini et al., 2011; Rodella et al., 2013).

The cardiac and coronary vascular endothelium plays an important role in cardiac function in normal and pathological conditions (Brutsaert, 2003; Strijdom and Lochner, 2009). Cardiac endothelium comprises myocardial capillary endothelial cells (cardiac microvascular endothelial cells or CMECs) and endocardial endothelial cells (Strijdom and Lochner, 2009). While the coronary endothelial cells affect the cardiomyocytes indirectly, CMECs directly influence the physiology of cardiomyocytes (Strijdom and Lochner, 2009). In addition, not only do CMECs outnumber cardiomyocytes by a ratio of 3:1 (Brutsaert, 2003), but also produce more NO than cardiomyocytes (Strijdom et al., 2006). NO, in turn, plays an important role in the regulation of the myocardial contractile performance, heart rate, metabolism and modulation of growth (Massion et al., 2003). It is also implicated in the cardioprotective response of the heart to ischaemia-reperfusion damage (Schulz et al., 2004). In the setting of IRI, endothelial cell damage leads to microvascular dysfunction [for review, see Opie (2004)]. Therefore, as CMECs represent about 90% of the total endothelial cells in the heart (Piper et al., 1990), their dysfunction may be even more detrimental in ischaemia. Surprisingly, the role of CMECs dysfunction has been overlooked or simply not explored in myocardial IRI. In this regard, few studies have been done on CMECs (Zhang et al., 2011; Qi et al., 2013). In addition, whether insulin resistance affects CMECs function has not yet been studied.

In view of this unexploited area of research, more investigations on CMECs are required. In the context of the role of melatonin in cardioprotection, considering the direct impact of CMECs on cardiomyocyte physiology (especially NO production) (Strijdom and Lochner, 2009),

investigation into the acute and long- term effects of melatonin on the CMECs NO production in normal as well as pathological conditions is warranted. Furthermore, whether the cardioprotective effects of melatonin are associated with cardiac endothelial protection remains unexplored. Thus, in this study, we investigated the effects of acute melatonin treatment on cardiac microvascular endothelial cells.

Our objectives were to determine: i) the effect of melatonin on endothelial cells focusing on NO production and cell viability, ii) the effect of melatonin on TNF- α -induced endothelial dysfunction, focusing on eNOS activation and expression and intracellular signalling as indicated by activation of ERK1/2, PKB/Akt, STAT-3, AMPK and expression of iNOS, I κ B- α and nitrotyrosine levels.

7.2. MATERIAL AND METHODS

Similar to the previous parts of our investigation, chemicals and other materials used are described in chapter 3. In this section, the cell culture technique and experimental procedure are described.

7.2.1. CARDIAC MICROVASCULAR ENDOTHELIAL CELL (CMEC) CULTURE

Adult rat CMECs were commercially purchased from VEC technologies (Rensselaer, New York, USA). Upon arrival, CMECs in 25 or 75 mL fibronectin coated tissue culture flasks were immediately cultured as previously described (Strijdom et al., 2009). A standard tissue culture incubator with an atmosphere of 21 % oxygen, 5 % carbon dioxide, 40-60 % humidity and temperature at 37 °C was used during the entire cell culture procedure. Briefly, CMEC cultures were maintained in a microvascular endothelial cell growth medium (EGM) (Clonetics EGM-2 MV; Lonza, Walkersville, MD, USA), supplemented with 10% foetal bovine serum (FBS) (Highveld Biological, RSA) and other standard endothelial cell culture supplements such as growth factors and antibiotics. Cells were grown until they were fully confluent. At this stage, passaging of primary cultures was performed with 500 BAEE units trypsin/180 μ g EDTA • 4Na per mL in Dulbecco's phosphate buffered saline (PBS) (Sigma Chemical Co, St Louis, Mo, USA). Subcultures became confluent in about 5 days. After the 4th to 7th passage, cells were

finally plated in fibronectin-coated 35mm dishes at a density of $\sim 1 \times 10^6$ cells/dish. Purity of CMEC cultures was verified by microscopic identification of typical “cobblestone” monolayer morphology (Strijdom et al., 2006; Strijdom et al., 2009b). Functional characterization was determined by measuring the uptake of fluorescently labelled Dil-ac-LDL, a marker specific for endothelial cells (Piper, 1990) by flow activated cell sorter (FACS) analysis, with demonstration of a positive staining rate throughout. No experiments were conducted on CMEC subcultures with < 80 % purity.

Prior to experimentation, the existing 10% FBS-containing EGM was removed and substituted with EGM in which the FBS was reduced to 5%.

7.2.2. DRUG ADMINISTRATION

Melatonin was first dissolved in DMSO and thereafter in growth medium to yield a final concentration of 1nM, 1 μ M or 10 μ M. These concentrations have been used in previous studies on microvascular endothelial cells (Silva et al., 2007; Tamura et al., 2009).

After determination of the effect of melatonin on NO production and cell viability, we opted to use 1nM in further experiments including the TNF- α -induced endothelial dysfunction study. This dosage is near the physiological melatonin concentration (Arendt, 2006). All tubes containing melatonin were covered by aluminium foil to protect against the light. Compared to the control (fresh growth medium), DMSO alone had no effect on cell viability and other parameters.

7.2.3. EXPERIMENTAL PROCEDURE

Figure 7.1 shows the main steps in the experimental procedure. We have divided our investigation into three parts: 1) Cell viability assessment, 2) NO production determination and 3) TNF- α -induced endothelial dysfunction.

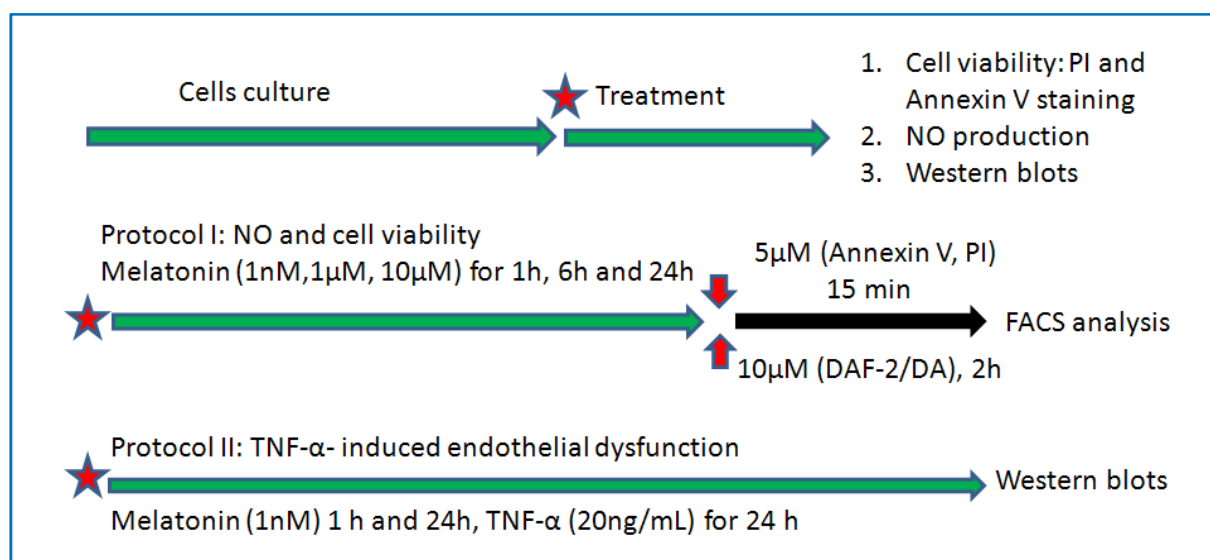


Figure 7.1 Simplified schematic representation of the experimental procedure. PI: propidium iodide, DAF-2/DA: 4, 5-diaminofluorescein-2/diacetate, FACS: flow activated cell sorter, NO: nitric oxide

7.2.3.1. Cell viability

Cell viability was assessed as previously described (Strijdom et al., 2009b). To investigate the effect of melatonin on endothelial cell viability, 5µM of propidium iodide (PI) (Sigma Chemical Co, St Louis, Mo, USA) and 5µM annexin V (conjugated with Alexa Fluor® 647) (Calbiochem San Diego, CA, USA) were administered to melatonin-pre-treated cells (at 1nM, 1µM and 10 µM for 1h, 6h and 24h incubation). After the treatment period, cells were trypsinised and washed sequentially in staining and binding buffer (BioLegend, San Diego, CA, USA) before administration of PI or Annexin V and incubation at room temperature in the dark for 15 min. This was followed by FACS analysis.

PI and Annexin V were used to determine cell viability. PI uptake identified the non-viable (necrotic) cell population. Due to loss of membrane integrity, the cell membrane becomes permeable and the PI probe enters into the cell and subsequently stains the nucleus. Annexin V staining was used as a marker for apoptotic cells. It binds to phosphatidylserine protein which is an intracellular membrane protein that translocates to the external surface of the plasma membrane in apoptotic events.

7.2.3.2. NO production determination

NO production was measured directly using the NO-specific 4, 5-diaminofluorescein-2/diacetate (DAF-2/DA) fluorescent probe as previously described by Strijdom et al. (2006). Cells were incubated with varying concentrations of melatonin (1nM, 1µM and 10 µM) and different times of incubation: 1h, 6h and 24 h. At the end of the incubation, cells were washed with PBS and incubated with 10 µM DAF-2/DA at 37°C for 2 h. Thereafter DAF-2/DA was washed out, cells trypsinised and resuspended in probe-free PBS for FACS analysis.

7.2.3.3. Flow cytometry

FACS analysis of CMECs treated with PI and Annexin V or DAF-2/DA was performed as previously described (Strijdom et al., 2009b). A FACS analyser (Becton-Dickinson FACSCalibur, Franklin Lakes, NJ) was used to quantify fluorescence in the FL1-H channel at the single-cell level, and data was analysed using Cellquest® version 3.3 (Becton, Dickinson and Co, San Jose, CA, USA) software. Briefly, selected cell populations (5,000–15,000 cells) were gated from initial acquisition populations containing 30,000–50,000 events. Selection was done by gating control samples according to their forward scatter (cell length) and side scatter (cell granularity) signals, thereby excluding non-cellular particles and debris. In order to standardize selection of the analysed cell populations between different samples, gating coordinates of control samples were used for all subsequent samples in a particular FACS session. Caution was taken to ensure that selected samples contain equal cell numbers for comparison purposes.

Fluorescence signals were recorded on a frequency histogram by logarithmic amplifiers. A probe-free absolute control sample (to determine autofluorescence) and probe-containing control samples were included in all experiments. Fluorescence data were expressed as mean fluorescence intensity (% of control, control adjusted to 100%).

7.2.3.4. TNF- α -induced endothelial dysfunction

To investigate the effect of melatonin on TNF- α -induced endothelial dysfunction, four different groups of treatments were studied: (i) untreated cells (or control), (ii) cells treated with

melatonin (1nM) for 1h or for 24 h, (iii) cells treated with TNF- α (20ng/mL) for 24h and (iv) cells pre-treated with melatonin (1nM) for 1h and thereafter treated with melatonin combined with TNF- α (20ng/mL) (Sigma Chemical Co, St Louis, Mo, USA) for 24h. All samples were incubated in fresh growth medium. After incubation (1h or 24h), samples were prepared for western blotting.

7.2.3.4.1. Western blot analysis

After trypsinization, at least four plates of cells (submitted to the same treatment protocol) were pooled to make one lysate. The proteins studied are summarized in table 7.1. Protein expression and activation were assessed using western blot analysis as indicated in chapter 3. Lysates were made using a lysis buffer containing (in mM): Tris-HCl (pH 7.5) 20, EGTA 1, EDTA 1, NaCl 150, β -glycerophosphate 1, sodium orthovanadate 1, tetra-sodium diphosphate 2.5, NaF 50, PMSF 1, 0.1% Sodium dodecylsulfate (SDS), aprotinin 10 μ g/mL; leupeptin 10 μ g/mL and 1% Triton-X100. After sonication, the protein content of each lysate was determined using the Bradford technique (Bradford, 1976).

For each cell lysate, equal amounts of protein were loaded on 7.5 and 10% SDS-polyacrylamide gels and transferred onto PVDF membranes (see table 7.1) and proteins separated as described in chapter 3.

Table 7.1 Protocol for detection of proteins

Protein	MW	Quantity	Gel	Antibody dilution	
				Primary	Secondary
PKB/Akt	60kDa	50µg	12%	1:1000 TBST	1:4000 TBST(5% milk)
ERK 42/44	42/44kDa	30µg	12%	1:1000 TBST	1:4000 TBST
STAT-3	79/86 kDa	50µg	10%	1:1000TBST	1:4000 TBST
iNOS	130 kDa	60µg	7%	1:2000TBST	1:4000TBST(5%Milk)
eNOS	140kDa	60µg	7%	1:1000 TBST	1:4000TBST(2.5%Milk)
IκB-α	39kDa	50µg	12%	1:1000 TBS	1:4000TBST
p38 MAPK	43kDa	30µg	12%	1:1000 TBST	1:4000 TBST
AMPK	62kDa	60 µg	10%	1:1000 TBST	1:4000 TBST(5% milk)
Nitrotyrosine	60kDa	50µg	10%	1:1000 TBST	1:5000TBST
β-tubulin	55kDa	-----*	10-12%	1:1000 TBST	1:4000 TBST

MW: Molecular weight, *β-tubulin was used to check the equal loading (performed after stripping the membrane).

7.2.4. DATA ANALYSIS

All statistical analyses were performed as indicated in chapter 3. For cell viability and NO production experiments, fluorescence data were expressed as percentage of control, control adjusted to 100%.

7.3. RESULTS

7.3.1. CELL VIABILITY

To test the effect of melatonin administration on endothelial cell viability, melatonin (1nM, 1 μ M or 10 μ M) was administered to CMECs for 1h, 6h and 24 h. After 1h, melatonin treatment caused a significant reduction in necrosis (PI) (1nM: 49.3 \pm 21%; 1 μ M: 24.0 \pm 1.1%; 10 μ M: 21.8 \pm 2.6%) and apoptosis (Annexin V) (1nM: 48.3 \pm 16.8%; 1 μ M: 30.1 \pm 2.8%; 10 μ M: 23.3 \pm 3%) compared to untreated cells (p <0.05) (fig.7.2).

However, after 6h, melatonin caused a dose-dependent increase in percentage of necrotic cells, becoming significant at 10 μ M (177.2 \pm 10.4%, p <0.05) (fig.7.3B). As similar observation was also made after 24h treatment, becoming significant at 1 μ M and 10 μ M (1 μ M: 125.0 \pm 9.2%; 10 μ M: 129.2 \pm 8.8%) (fig.7.4B). Melatonin, however, had no effect on the number of apoptotic cells, regardless of the concentration after 6h and 24h (fig.7.3-4).

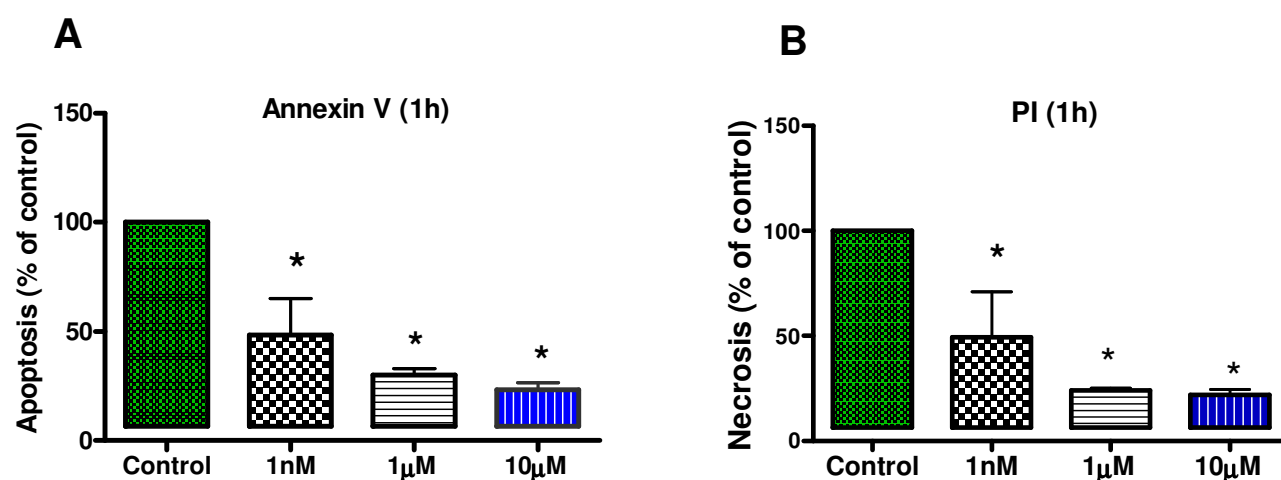


Figure 7.2 Effect of melatonin on cell viability after 1h treatment, expressed as percentage of control, control=100%, A: AnnexinV, B: propidium iodide (PI), * p <0.05 vs control, n=6-8/group.

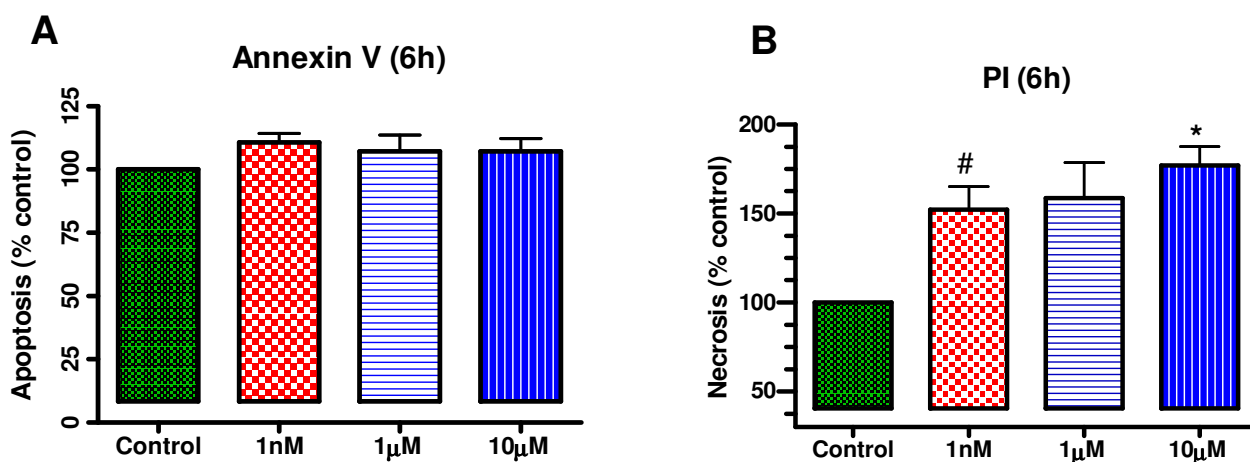


Figure 7.3 Effect of melatonin on cell viability after 6h of treatment, expressed as percentage of control, control=100%, A: AnnexinV, B: propidium iodide (PI), * $p < 0.05$ vs control, # $p = 0.07$ vs control, $n = 6-8$ /group.

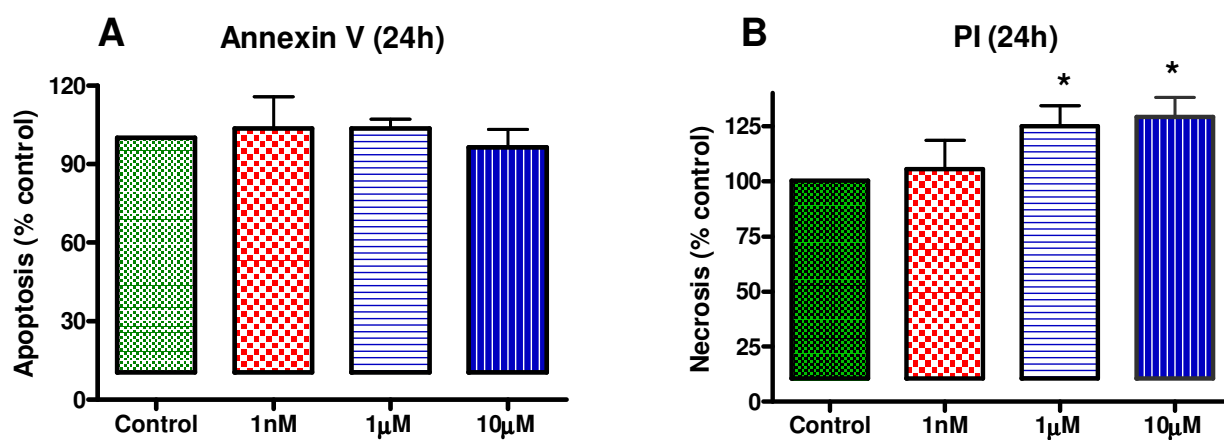


Figure 7.4 Effect of melatonin on cell viability after 24h of treatment, expressed as percentage of control, control=100%, A: AnnexinV, B: propidium iodide (PI), * $p < 0.05$ vs control, $n = 6-8$ /group.

7.3.2. NO PRODUCTION

To test the effect of melatonin administration on NO production, melatonin at the dosages of 1nM, 1 μ M and 10 μ M was administered to CMECs for 1h, 6h and 24h. After 1h, melatonin (1nM) treatment caused a significant reduction in NO levels ($91.9\pm 2.7\%$ vs control, $p < 0.05$). After 6h, melatonin at all concentrations attenuated the levels of NO (1nM: $85.3\pm 5.1\%$; 1 μ M: $69.2\pm 6.6\%$; 10 μ M: $81.4\pm 5.6\%$) compared to control ($p < 0.05$) (fig.7.6). When the treatment was extended to 24h, melatonin at 10 μ M reduced significantly the level of NO ($93.4\pm 2.1\%$) compared to control while other dosages did not affect the level of NO (fig.7.7).

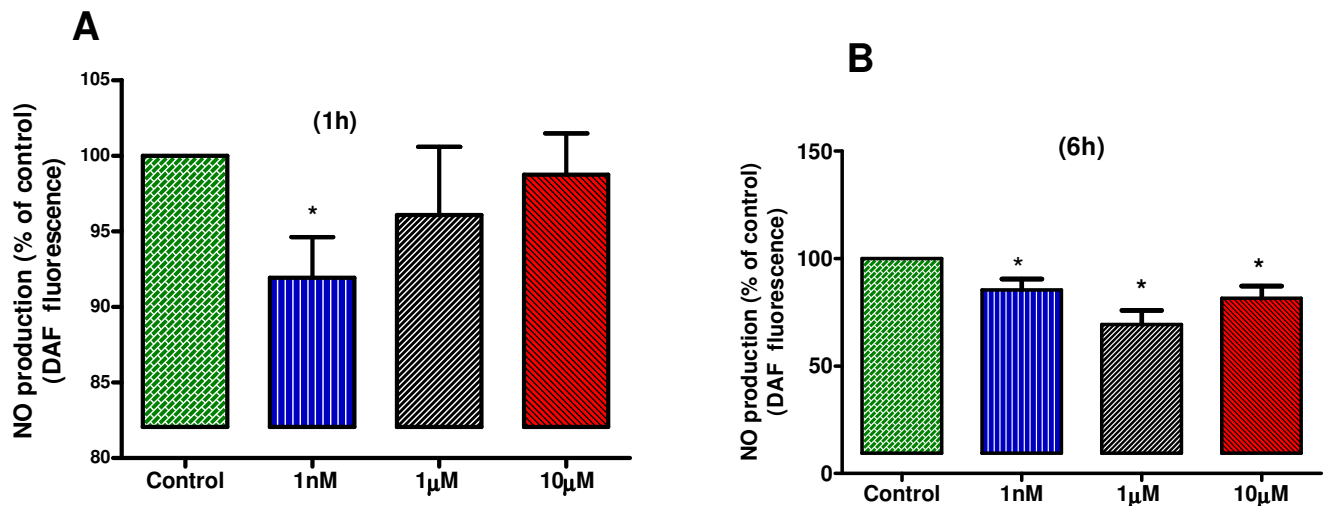


Figure 7.5 Effect of melatonin on NO production (mean DAF fluorescence) after 1h (A) and 6h (B) treatment, expressed as percentage of control, control=100%, * $p < 0.05$ vs control, $n = 4-6$ /group.

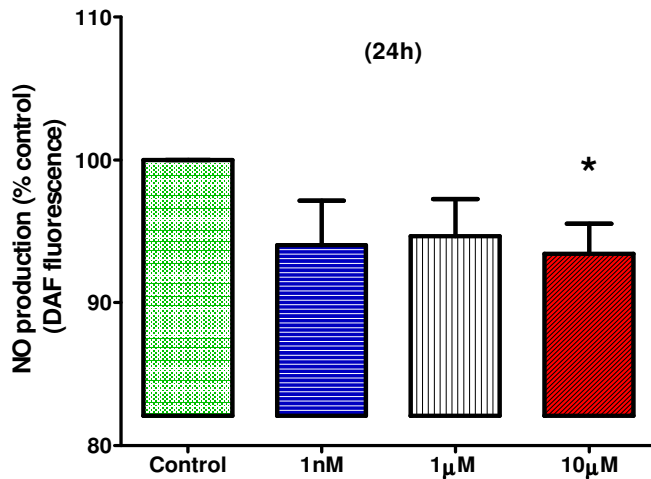


Figure 7.6 Effect of melatonin on NO production (mean DAF fluorescence) after 24h treatment, expressed as percentage of control, control=100%, *p<0.05 vs control, n=4-6/group.

7.3.3. TNF- α -INDUCED ENDOTHELIAL DYSFUNCTION

7.3.3.1. NO production

To test the effect of TNF- α on endothelial cell dysfunction, TNF- α was administered at the dosages of 0.5, 5 and 20ng/mL to CMECs for 24h. The following data are from Amanda Genis (Division of Medical Physiology, SU) who performed the experiment. TNF- α administered at the dosages of 0.5ng/mL and 20ng/mL significantly reduced NO production compared to the control (fig.7.7A). However, it had no effect on NO production when administered at 5ng/mL.

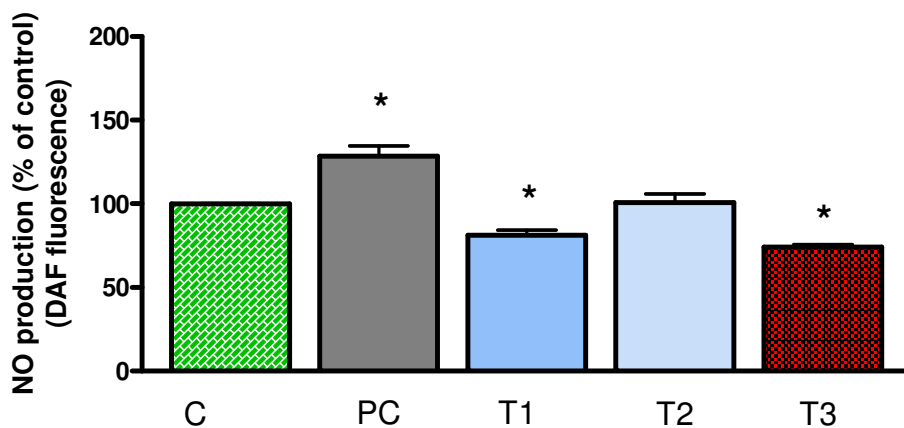


Figure 7.7A Effect of TNF- α on CMECs NO production, 24h incubation, fluorescence values expressed as percentage of the control (control =100%), C: control, PC: positive control, T1:

TNF- α (0.5ng/mL), T2: TNF- α (5ng/mL), T3: TNF- α (20ng/mL), * $p < 0.05$ vs Control, $n = 4$ /group, experiment performed 3 times.

7.3.3.2. PKB/Akt-eNOS pathway

In view of the above results on NO production, we further studied the effect of TNF- α on the PKB/Akt-eNOS pathway. Cells were incubated with TNF- α (0.5, 5 and 20ng/mL) for 24 h after which lysates were prepared for western blot analysis. Results are presented in figure 7.7B.

When TNF- α was administered at 5ng/mL, it had no effect on PKB/Akt expression and phosphorylation. This was associated with reduction in eNOS phosphorylation while its expression was increased, causing a decrease in activation. A similar trend in eNOS expression and phosphorylation was also observed when TNF- α was administered at the low dosage of 0.5ng/mL. Here, PKB/Akt expression and phosphorylation both were reduced but the phospho-/total ratio remained unchanged.

When the TNF- α dosage was increased to 20ng/mL, PKB/Akt expression and phosphorylation were also reduced but to such extent that the phospho-/total ratio was significantly lower than the control. Importantly, this was associated with a marked increase in eNOS expression and significant reduction in phosphorylation leading to a phospho-/total ratio of 18.5% of the control levels (fig.7.7B).

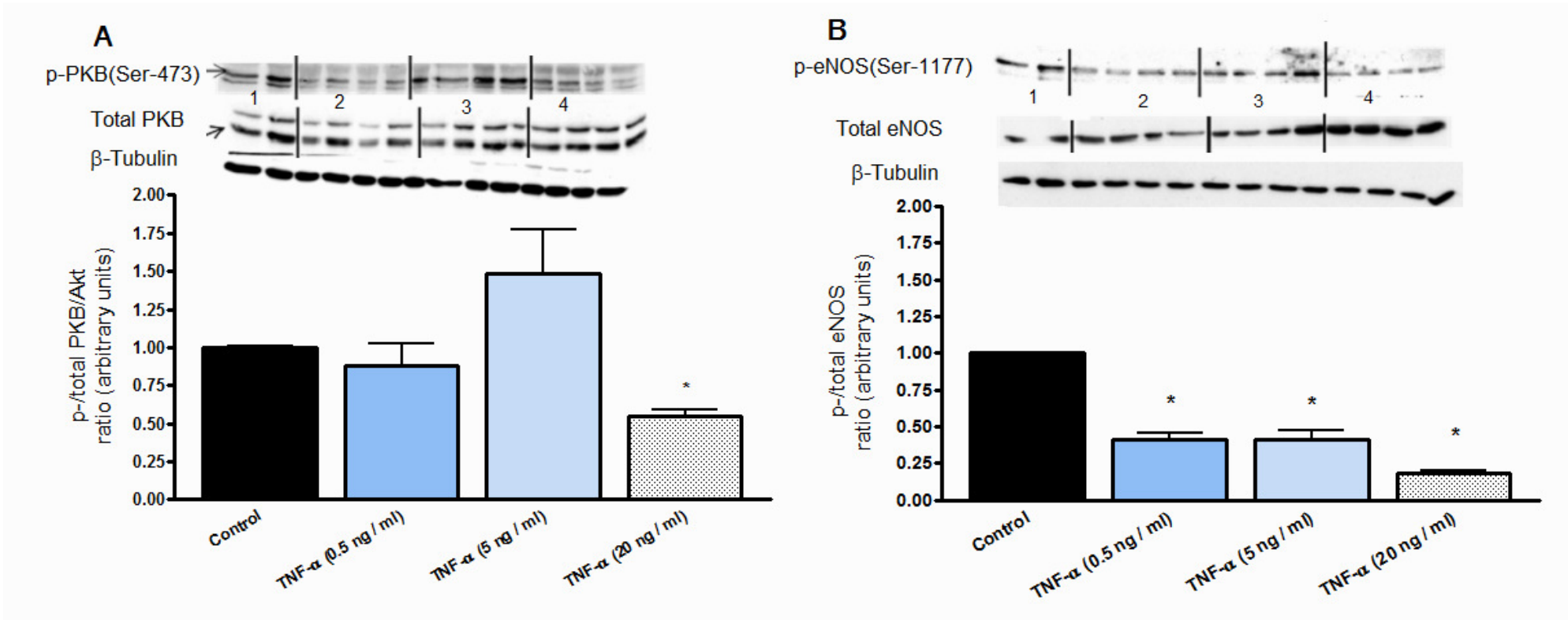


Figure 7.7B Effects of TNF- α on CMECs , 24h incubation, expression and phosphorylation of PKB /Akt (A) and eNOS (B), Representative graph, values expressed as phospho-/total ratios normalized to the control values, 1: control, 2: TNF- α (0.5ng/mL)(n=4), 3: TNF- α (5ng/mL), 4: TNF- α (20ng/mL), *p<0.05 vs control, n=4/group, blots presented are representative, experiment was performed twice (Amanda Genis).

7.3.4. EFFECT OF MELATONIN ON TNF- α -INDUCED ENDOTHELIAL DYSFUNCTION

To test the effect of melatonin administration on endothelial cell dysfunction, melatonin at the dose of 1nM was administered to CMECs for 1h before the administration of TNF- α (20ng/mL). This melatonin concentration was chosen because the other dosages had increased the levels of necrotic cells after 24h treatment (fig.7.4). The TNF- α dosage was chosen due to its pronounced effects on PKB/Akt and eNOS activation as well as NO production compared to other dosages (fig.7.7A; B). After 24h incubation, lysates were prepared for western blot analysis. Results are presented in figures 7.8-16 and summarized in the table 7.4. β -Tubulin was used to check equal loading of proteins. Its representative blot is only added on the graph where proteins are downregulated (e.g., PKB/Akt, eNOS) or if only the total protein expression was evaluated (e.g., iNOS, I κ B- α).

For all figures, C: control, M1: melatonin treatment for 1h, M: melatonin treatment for 24h, T: TNF- α treatment for 24h, TM: co-administration of melatonin with TNF- α for 24h following 1h melatonin pre-treatment.

- **PKB/Akt**

Figure 7.8 represents the effect of melatonin and TNF- α treatment on PKB/Akt phosphorylation in CMECs. Compared to the control, melatonin administration for 1h and 24h increased the phosphorylation of PKB/Akt significantly (1.5 and 1.2 fold increased phosphorylation, respectively), the phosphorylation of PKB/Akt being higher after 1h than after 24h treatment. When compared to TNF- α treatment alone, the effect of melatonin administration on PKB/Akt phosphorylation after 1h and 24 h was 1.9 and 1.5 folds higher than that of TNF- α , respectively.

TNF- α reduced significantly the phosphorylation of PKB/Akt (Ser-473) compared to control or other treatments with melatonin alone or combined with TNF- α ($p < 0.05$).

When TNF- α was co-administered with melatonin for 24h, PKB/Akt phosphorylation was significantly reduced compared to 1h and 24h melatonin and similar to the control but significantly higher than that of TNF- α alone for 24h.

Cells pre-treated with melatonin for 1h followed by 24h co-administration of TNF- α and melatonin had a significant reduction in expression of total PKB/Akt and β -tubulin when compared to the control and other treatments ($p < 0.05$). β -Tubulin blot was performed to check the equal loading on phosho-PKB/Akt membrane.

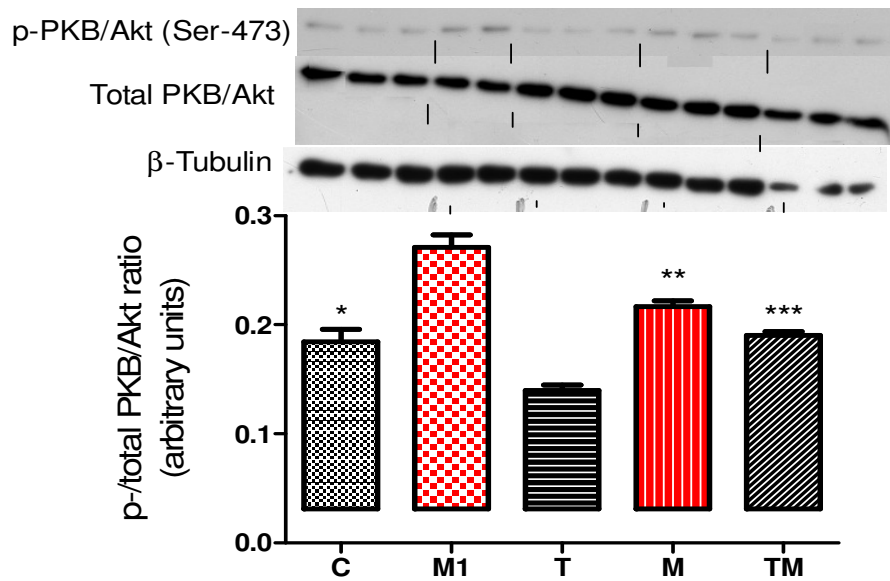


Figure 7.8 Effect of melatonin and TNF- α on the expression and phosphorylation of PKB/Akt, * $p < 0.05$ (C vs M1, T and M), ** $p < 0.05$ (M vs M1, T and TM), *** $p < 0.05$ (TM vs T), $n = 3$ /group (M1=2).

- **eNOS**

Compared to control, 1h melatonin treatment reduced eNOS phosphorylation ($p < 0.05$). However, when the treatment was extended to 24h, this reduction disappeared and the eNOS phosphorylation was similar to the control (fig.7.9).

TNF- α alone for 24h reduced eNOS phosphorylation compared to the control. However, when combined with melatonin this reduction disappeared. β -Tubulin blot was performed to check the equal loading on phosho-eNOS membrane.

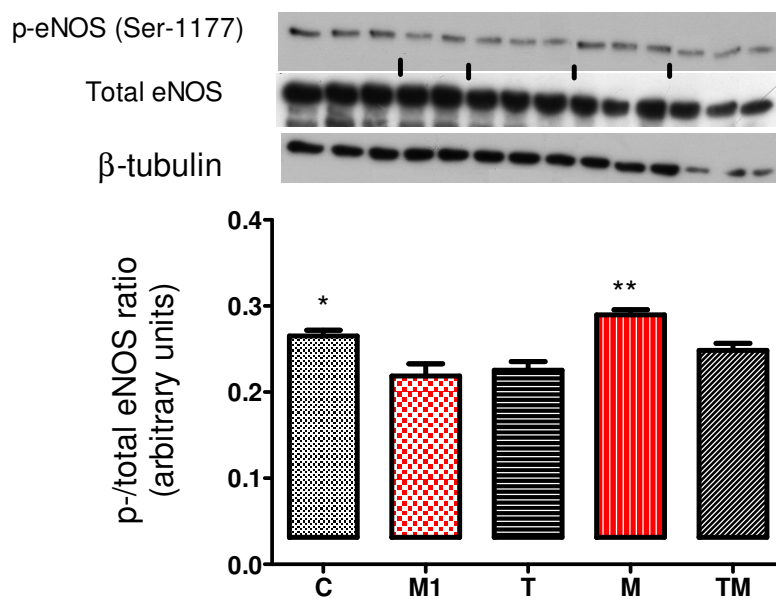


Figure 7.9 Effect of melatonin and TNF- α on eNOS expression and phosphorylation,

Figure 7.9, * $p < 0.05$ (C vs M1, T), ** $p < 0.05$ (M vs M1, T, TM), $n = 3/\text{group}$ (M1=2).

- **iNOS expression**

Figure 7.10 represents the effects of melatonin and TNF- α treatment. Melatonin administered for 1 h increased iNOS expression, while 24 h treatment was without effect. Compared to the control and 24h melatonin treatment, TNF- α treatment induced increased iNOS expression ($p < 0.05$).

Pre-treatment with melatonin for 1h followed by 24h TNF- α plus melatonin treatment caused a significant reduction in the expression of iNOS compared to control and TNF- α ($p < 0.05$). However, there was no difference between the effect of melatonin administered alone for 1h and that of TNF- α treatment after 24h ($p > 0.05$).

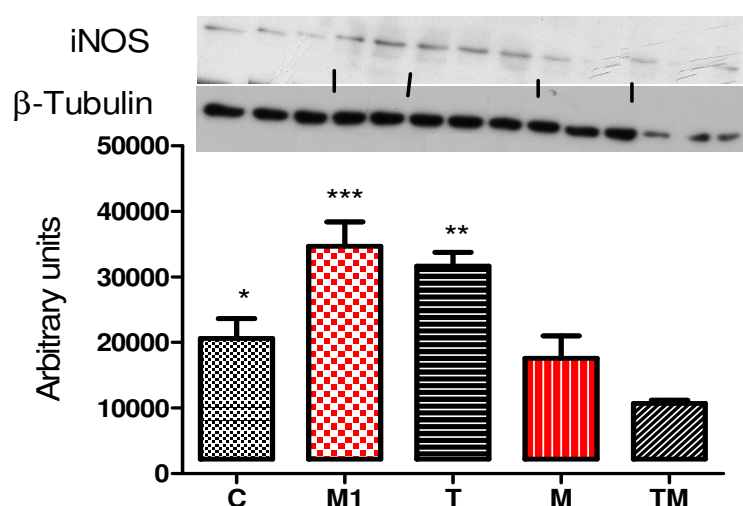


Figure 7.10 Effect of melatonin and TNF- α on iNOS expression, * $p < 0.05$ (C vs M1, T and TM), ** $p < 0.05$ (T vs M, and TM), *** $p < 0.01$ (M1 vs M, TM), $n = 3/\text{group}$ (M1=2).

- **Nitrotyrosine levels**

After 1h, melatonin reduced the levels of nitrotyrosine compared to control ($p < 0.05$). When the treatment was extended to 24h, nitrotyrosine levels were similar to that of the control cells.

When TNF- α was co-administered with melatonin in 1h melatonin pre-treated cells, after 24 h, nitrotyrosine levels were significantly reduced compared to 1h and 24 h melatonin as well as 24h TNF- α treatments.

Similar to eNOS and PKB/Akt (fig.7.9 and 10), nitrotyrosine levels and β -tubulin expression were reduced when melatonin was co-administered with TNF- α for 24 h.

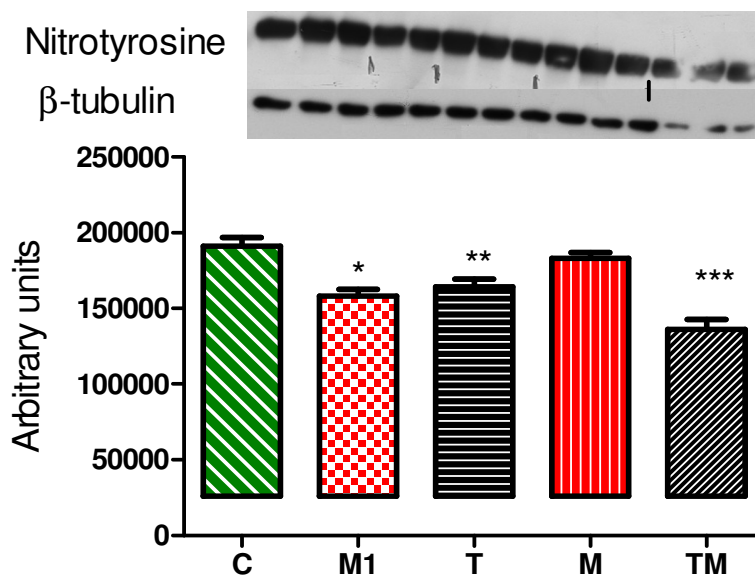


Figure 7.11 Effect of melatonin and TNF- α on nitrotyrosine levels, * $p < 0.05$ (M1 vs C, M and TM), ** $p < 0.05$ (T vs C, M, TM), *** $p < 0.01$ (TM vs C, M, $n = 3/\text{group}$ (M1=2)).

- **I κ B- α expression**

Melatonin treatment for 1h did not affect the expression of I κ B- α compared to control. When the treatment was prolonged to 24h, the expression remained unchanged. TNF- α treatment for 24h reduced significantly I κ B- α expression compared to control, 1h and 24h melatonin treatments.

When TNF- α was co-administered with melatonin in 1h melatonin pre-treated cells, after 24h, I κ B- α expression was significantly reduced compared to control, 1h and 24h melatonin but not 24h TNF- α treatments.

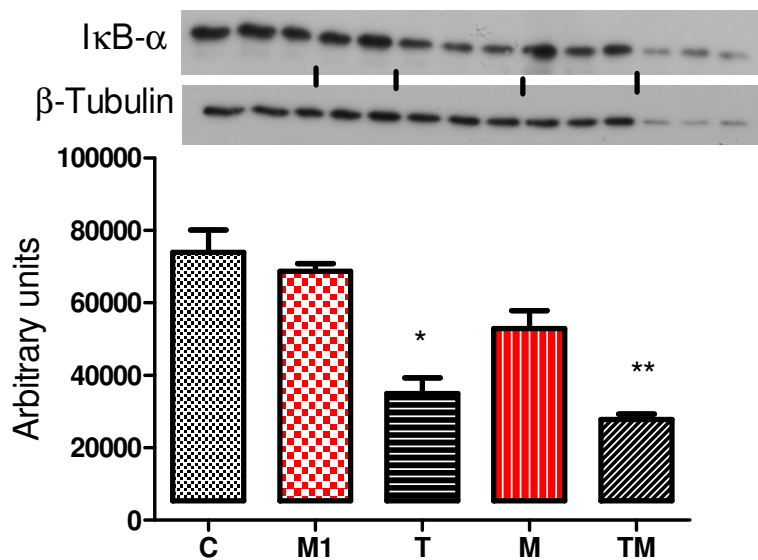


Figure 7.12 Effect of melatonin and TNF- α on expression of I κ B- α , *p<0.05 (T vs C, M1 and M), **p<0.05 (TM vs C, M1 and M), (M vs C, p>0.05), n= 3/group (M1=2)

- **ERK 42/44**

Figure 7.13 represents the effects of melatonin and TNF- α treatment on ERK44/42. Melatonin alone or combined with TNF- α did not affect the expression and phosphorylation of ERK44/42 compared to the control. TNF- α treatment for 24h reduced only the p/total ratio of ERK42 ($p < 0.05$) compared to control.

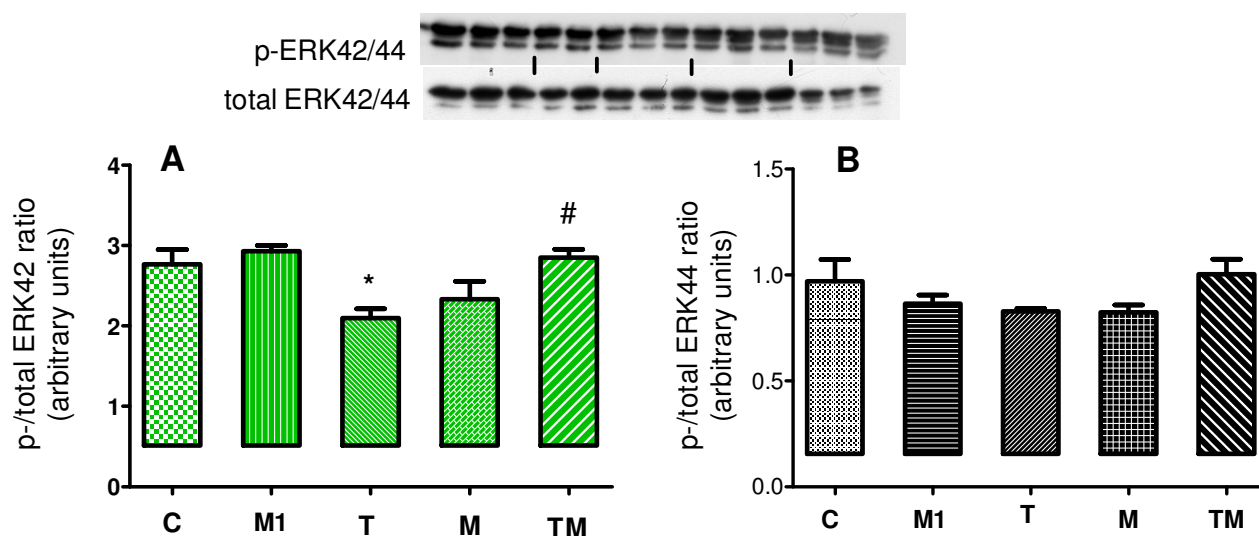


Figure 7.13 Effect of melatonin and TNF- α on expression and phosphorylation of ERK42 (A) and ERK 44 (B). A: * $p < 0.05$ (T vs C, M1), # $p < 0.05$ (TM vs T), $n = 3$ /group (M1=2).

- **AMPK**

Melatonin administered for 1h caused a significant increase in the phosphorylation of AMPK (Thr-172). After 24h treatment, this effect disappeared (fig.7.14).

Compared to the control, TNF- α treatment also induced an increase in phosphorylation of AMPK (Thr-172) ($p < 0.05$). Pre-treatment with melatonin followed by co-administration of melatonin with TNF- α for 24 h reduced significantly the phosphorylation of AMPK (Thr-172) compared to control, 1h melatonin and TNF- α treatments ($p < 0.05$). β -Tubulin blot was performed to check the equal loading on phospho-AMPK (Thr-172) membrane.

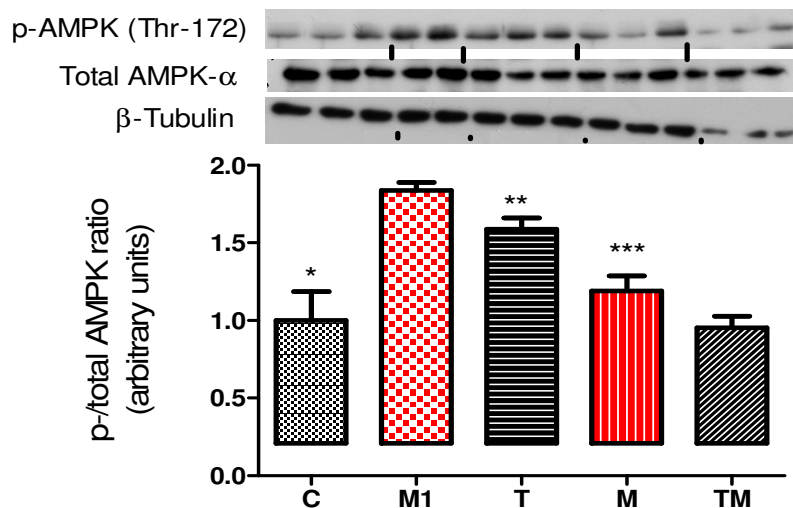


Figure 7.14 Effect of melatonin and TNF- α on expression and phosphorylation of AMPK (Thr-172), * $p < 0.05$ (C vs M1 and T), ** $p < 0.05$ (T vs M and TM), *** $p < 0.05$ (M vs M1), $n = 3$ /group (M1=2).

- **p38 MAPK**

Figure 7.15 represents the effect of melatonin and TNF- α treatment on p38MAPK. After 1h, melatonin increased the phosphorylation of p38MAPK compared to control. When the treatment was extended to 24h, the phosphorylation of p38 MAPK remained high.

TNF- α treatment for 24h caused a significant reduction in the phosphorylation of p38MAPK compared to control, 1h and 24 h melatonin treatments.

When TNF- α was co-administered with melatonin to 1h melatonin pre-treated cells, after 24 h, p38MAPK phosphorylation was significantly increased compared to control and 24h TNF- α but not 1h and 24 h melatonin treatments.

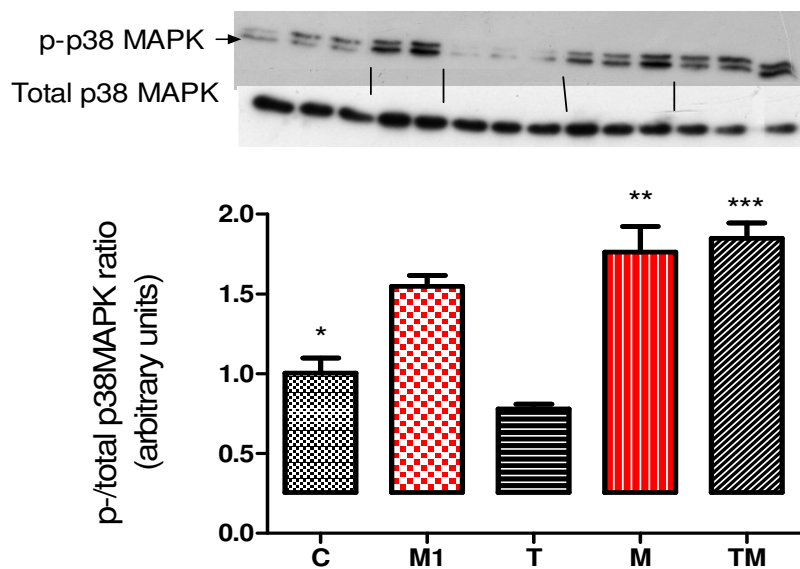


Figure 7.15 Effect of melatonin and TNF- α on expression and phosphorylation of p38MAPK, * $p < 0.05$ (C vs M1, T, M and TM), ** $p < 0.05$ (M vs T), *** $p < 0.05$ (TM vs M1 and T), $n = 3$ /group (M1=2)

- **STAT-3**

Figure 7.16 represents the effect of melatonin and TNF- α treatment on STAT-3. In this study Cell lysates without fractionation were used. Melatonin treatment after 1h and 24h did not affect the phosphorylation of STAT-3 (Ser-727) compared to control. Although 24h TNF- α treatment also had no significant effect on STAT-3 phosphorylation compared to control, its associated phosphorylation was higher than that seen after 24h melatonin treatment.

When TNF- α was co-administered with melatonin in 1h melatonin pre-treated cells, after 24 h, STAT-3 phosphorylation was significantly reduced compared to control, 1h and 24h melatonin as well as 24h TNF- α treatments.

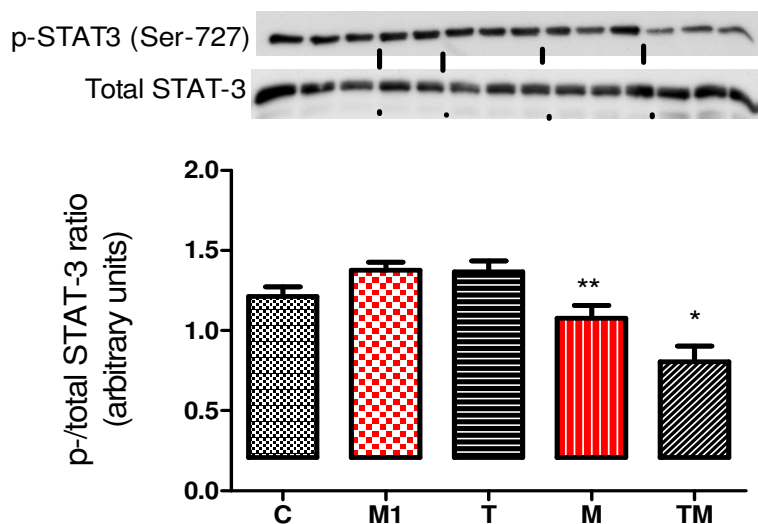


Figure 7.16 Effect of melatonin and TNF- α on expression and phosphorylation of STAT-3,

* $p < 0.05$ (TM vs C, M, T and M1), ** $p < 0.05$ (M vs T), $n = 3/\text{group}$ (M1=2).

Table 7.4 Summary of western blots results

A vs Control	Phosphorylation/activation							Expression		
	eNOS	PKB/Akt	ERK42	ERK44	p38MAPK	AMPK	STAT-3 (Ser-727)	iNOs	IκB-α	Nitrotyrosine
M1 (1h)	↓	↑	↔	↔	↑	↑	↔	↑	↔	↓
M (24h)	↔	↑	↔	↔	↑	↔	↔	↔	↔	↔
T (24)	↓	↓	↓	↔	↓	↑	↔	↑	↓	↓
T+M (24h)	↔	↔	↑	↔	↑	↔	↓	↓	↓	↓

B vs TNF-α	Phosphorylation/activation							Expression		
	eNOS	PKB/Akt	ERK42	ERK44	p38MAPK	AMPK	STAT-3 (Ser-727)	iNOs	IκB-α	Nitrotyrosine
M1 (1h)	↔	↑	↔	↔	↑	↑	↔	↔	↑	↔
M (24h)	↑	↑	↔	↔	↑	↓	↓	↓	↑	↑
T+M (24h)	↔	↑	↑	↔	↑	↓	↓	↓	↔	↓

A: compared to control, B: compared to TNF-α treatment, ↑: significant increase, ↓: significant decrease, ↔: no significant difference, M1 (1h): melatonin treatment for 1h, M (24h): melatonin treatment for 24 h, T (24): TNF-α treatment for 24h, T+M (24h): Co-administration of melatonin and TNF-α for 24 h following 1h melatonin pre-treatment; melatonin (1nM), TNF-α (20ng/mL)

Table 7.5 Summary of results of cell viability and NO production

A: Cell viability		Melatonin (dosages)		
vs. control		1nM	1 μ M	10 μ M
1h	Apoptosis (Annexin V)	↓	↓	↓
	Necrosis (Propidium iodide)	↓	↓	↓
6h	Apoptosis (Annexin V)	↔	↔	↔
	Necrosis (Propidium iodide)	↔	↔	↑
24h	Apoptosis (Annexin V)	↔	↔	↔
	Necrosis (Propidium iodide)	↔	↑	↑
B: Nitric oxide production		Melatonin (dosages)		
vs. control		1nM	1 μ M	10 μ M
1h	DAF	↓	↔	↔
6h	DAF	↓	↓	↓
24h	DAF	↔	↔	↓

A: Cell viability vs control; B: Nitric oxide (NO) production vs control, ↑: significant increase, ↓: significant decrease, ↔: no significant difference.

7.4. DISCUSSION

In the present study we investigated the effects of melatonin treatment on CMECs evaluating cell viability, NO production and TNF- α -induced endothelial dysfunction as well as intracellular signalling associated with the treatment of melatonin and TNF- α administered separately or in combination. Findings are summarized in tables 7.4-5.

7.4.1. EFFECT OF MELATONIN ON CELL VIABILITY

Cell viability was evaluated by Annexin V and PI staining as markers for apoptotic and necrotic cells, respectively. Apoptosis and necrosis are two different processes of cell death that can coexist in the same tissue [for review, see (Fadeel and Orrenius, 2005)]. Necrosis refers to the pathological or accidental mode of cell death involving irreversible swelling of the cytoplasm and distortion of organelles including mitochondria followed by rupture of the cell membrane and inflammation that damage the cells and its surrounding tissues (Fadeel and

Orrenius, 2005). Apoptosis is a sequential process where the dying cell undergoes nuclear and cytoplasmic condensation with blebbing of the plasma membrane leading to formation of apoptotic bodies which are recognized and removed *via* phagocytosis by macrophages without damaging the surrounding tissues (Fadeel and Orrenius, 2005). After 1h, melatonin (1nM, 1 μ M and 10Mm) increased the cell viability by reducing the percentage of necrotic and apoptotic cells compared to the control (fig.7.2). This is in agreement with previous studies where melatonin has been shown to increase cell viability of a resting human liver cell line (HepG2) (0.1–10 μ M/24h) (Osseni et al., 2000), sperm cells (2mM/30min) (Du Plessis et al., 2010) or bovine oviduct epithelial cells (bOECs) (1-1mM/24h) (Kim et al., 2011) and to inhibit NO-induced apoptosis in PGT- β immortalized pineal cells (0.1mM/10h) (Yoo et al., 2002). In addition, melatonin increased cell viability and protected Chinese hamster ovary (CHO) cells (300 μ M-3mM/30min-24h) (Celik and Naziroglu, 2012) and human umbilical vein endothelial cells (HUVECs) (125-500 μ M/2-8h) (Duan et al., 2013) against H₂O₂-induced injury. The hormone also protected hepatocytes against necrosis and apoptosis induced by IRI (Kim and Lee, 2008). This was also demonstrated in the hearts of senescence-accelerated mice (SAMP8) treated with melatonin (Forman et al., 2010).

When melatonin treatment was prolonged to 6h and 24h, apoptosis was no longer reduced. (fig.7.3-4). However, surprisingly, the percentage of necrotic cells was increased after 6 h (10 μ M) and 24h (1,10 μ M) (fig.7.3-4), which may reflect a dose- and time-dependent effect of melatonin as previously described in human liver cell line (HepG2) (Osseni et al., 2000). Consistent with the present study, a low concentration (0.1–10 μ M) administered for a short period increased cell viability and a high concentration (1–10,000 μ M) for a long period decreased cell viability of human liver cell line HepG2 (Osseni et al., 2000).

7.4.2. MELATONIN AND NO PRODUCTION

Nitric oxide (NO) is an important protective molecule in the cardiovascular system (Heusch et al., 2008; Strijdom et al., 2009a). It is primarily produced by the NO synthases (NOS) namely neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS

(eNOS or NOS-3) present in the heart (Balligand and Cannon, 1997). Viewed as an endothelial cell survival factor (Dimmeler and Zeiher, 1999), a reduction in NO bioavailability is considered as a marker for endothelial dysfunction (Muniyappa and Sowers, 2013). However, besides its important beneficial activities, in pathophysiological conditions excessive NO production may contribute to oxidative stress and an associated inflammatory state with eventual tissue damage (Pacher et al., 2007).

To investigate how melatonin affects endothelial function in addition to improved cell viability, we evaluated NO production of resting CMECs (non-stimulated). Similar to cell viability, melatonin treatment affected NO production in a dose-and time-dependent manner. After 1h, melatonin (1nM) reduced NO production while other concentrations (1 μ M and 10 μ M) were without effect on NO production (fig.7.5). However, when the treatment is extended to 6h, melatonin at all concentrations (1nM, 1 μ M and 10 μ M) decreased NO production (fig.7.6). After 24h, only cells treated with 10 μ M melatonin had reduced NO production compared to control (fig.7.7). As far as we know, there are no other reports on the effect of melatonin on CMECs. In contrast to our study, microvascular endothelial cells from cremaster muscle treated with 0.1nM and 1nM melatonin, had no effect on the basal NO production [probably due to the short period of incubation (1min)](Tamura et al., 2006). However, similar to the present findings, melatonin (1nM) reduced bradykinin-induced NO production in microvascular endothelial cells (Tamura et al., 2006; Silva et al., 2007). As was also observed in our study, the reduction in NO production by melatonin was dose-and time-dependent and was linked to its anti-oxidative activities (Tamura et al., 2009). Indeed, an excessive amount of NO (free radical) is known to cause cytotoxic changes and melatonin protects the cells through its free radical scavenging activities (Aydogan et al., 2006; Korkmaz et al., 2009). Interestingly, when the percentage of necrotic cells (PI levels) in CMECs reached its highest peak after 6h (compared to 1h and 24) (fig.7.2-4), the reduction in NO production appeared to be the highest (fig.7.5-7). Thus, the observed reduction in NO production after 6h melatonin treatment may be a causal factor in the reduction in cell viability seen at this time-point. However this remains to be established.

7.4.3. MELATONIN AND INTRACELLULAR SIGNALLING

To investigate the signalling associated with the effect of melatonin on cell viability and NO production, cells were treated with melatonin (1nM) and incubated for 1h and 24h. The survival pathways including PKB/Akt, eNOS and iNOS (involving NO production), MAPK including p38MAPK, ERK42/44 as well as other proteins implicated in cellular stress events such as nitrotyrosine, I κ B- α , STAT-3 and AMPK were evaluated using western blots.

7.4.3.1. NO production pathways: PKB/Akt-eNOS and iNOS

PKB/Akt is a serine/threonine kinase that has been shown to play a crucial role in cellular activities including, amongst others, cell survival, growth, proliferation, metabolism and angiogenesis (Manning and Cantley, 2007; Sussman et al., 2011). It constitutes the central node of cell signalling downstream of growth factors, cytokines, and other cellular stimuli (Manning and Cantley, 2007). After 1h, melatonin treatment at 1nM reduced the percentage apoptotic and necrotic cells in resting CMECs (fig.7.2) and this reduction was associated with an increase in PKB/Akt phosphorylation (fig.7.8), as a survival factor (Manning and Cantley, 2007). These findings are consistent with previous studies where melatonin treatment increased PKB/Akt phosphorylation in the rat hypothalamus (Anhe et al., 2004), hepatic HepG2 cells (Shieh et al., 2009), Müller cells (Jiang et al., 2012) and human neuronal cell line SK-N-MC (Choi et al., 2008).

Interestingly, melatonin inhibited NO-induced apoptosis by increasing PKB/Akt phosphorylation and Bcl-2 expression and reducing Bax expression (Choi et al., 2008; Kim et al., 2011). Since excess NO production can induce apoptosis (Choi et al., 2008; Kim et al., 2011), the anti-apoptotic effect observed in the present study may also be related to the reduction in basal NO production (fig.7.4) and eNOS phosphorylation (fig.7.10). However, the respective contributions of a reduction in eNOS phosphorylation (fig.7.9) and increased iNOS expression (fig.7.10) to the reduction in NO production induced by 1h melatonin treatment, is difficult to interpret. On the other hand, the reduction in NO production is also difficult to interpret in view of the activation of PKB/Akt and AMPK which are upstream of eNOS. However, it appears in

this context that melatonin reduced NO availability *via* its direct free radical scavenging activities (which are receptor independent) (Aydogan et al., 2006). Elevated iNOS expression is associated with an inflammatory state where eNOS expression, which is a protective enzyme, is reduced (Rodella et al., 2010; Kireev et al., 2012). In contrast to our finding, melatonin treatment in nicotine-treated rats has been shown to increase eNOS expression with a concomitant reduction in iNOS expression (Rodella et al., 2010).

When the treatment was extended to 24h, PKB/Akt phosphorylation remained significantly increased while eNOS phosphorylation and iNOS expression returned to control levels (fig.7.8-9), indicating that signalling events are also time-dependent and that PKB/Akt phosphorylation, independent of eNOS phosphorylation, was involved in the effects of melatonin on CMECs.

7.4.3.2. Melatonin and cellular stress: oxidative stress and inflammation

To investigate the effect of melatonin on basal cellular stress we evaluated the expression or phosphorylation of I κ B- α , p38MAPK, ERK42/44, STAT-3, AMPK and nitrotyrosine levels after 1h and 24h melatonin treatment. A nitrotyrosine level is an index of formation of peroxynitrite (ONOO⁻) and hence considered as a marker of oxidative stress (Pacher et al., 2007). After 1h, melatonin treatment reduced basal nitrotyrosine levels (fig.7.11), supporting melatonin's antioxidant activities (Korkmaz et al., 2009).

In addition, p38MAPK phosphorylation was increased (fig.7.15). This is the first study to evaluate the effect of melatonin treatment on p38 MAPK in CMECs. The role of p38MAPK in cell viability is controversial (Rose et al., 2010). Our laboratory has previously found that melatonin-induced cardioprotection was associated with a reduction in post-ischemic phosphorylation of p38MAPK (Genade et al., 2008; Nduhirabandi et al., 2011), indicating the pro-apoptotic effects of p38MAPK (Li et al., 2005; Luchetti et al., 2009). On other hand, p38MAPK has also been suggested to be a pro-survival kinase in LPS-induced activation of BV-2 cells (Svensson et al., 2011) and in cardioprotection (Engelbrecht et al., 2006). Furthermore, a survival role for p38MAPK has been recently reported associated with PKB/Akt, AMPK and ERK42/44 phosphorylation in neutrophils (Rossi and Lord, 2013). On the

other hand, melatonin treatment had no effect on the phosphorylation of PKB/Akt and p38MAPK in C₂C₁₂ myotubes (Ha et al., 2006) as well as AMPK in HepG2 cells (Shieh et al., 2009).

In our model of CMECs, activation of p38MAPK was found to be associated with increased cell viability (tables 7.4-5). However, the effects of p38MAPK on cell survival remain controversial and may depend on the cell type and the isoform activated.

The role of activation of AMPK in endothelial cells is complex. Viewed as a metabolic-sensing kinase, AMPK is also known as a stress-activated protein kinase (Fisslthaler and Fleming, 2009), being activated by cellular stresses associated with reduced cellular ATP levels in endothelial cells (Nagata et al., 2003; Fisslthaler and Fleming, 2009). Apart from its well-known anti-apoptotic effects and activation of eNOS (Liu et al., 2010), it exhibits its anti-inflammatory actions by inhibiting NF- κ B signalling and thus prevents the appearance of a pro-inflammatory phenotype [for review, see (Fisslthaler and Fleming, 2009; Salminen et al., 2011)]. However, whether AMPK phosphorylation plays an important role in the anti-apoptotic effect seen in our study needs further investigation.

ERK42/44 phosphorylation, I κ B- α expression and STAT-3 phosphorylation after 1h melatonin treatment were similar to the control and thus probably not involved in the improvement in cell viability observed at this time point. Increased ERK42/44 phosphorylation was recently reported to be involved in the anti-apoptotic effects of melatonin treatment in UVB-stressed U937 cells (Luchetti et al., 2009). STAT-3 is an important transcription factor which is the signalling target of pro-inflammatory cytokines and is activated by hypoxia/reoxygenation in human umbilical vein endothelial cells (HUVEC) (Mattagajasingh et al., 2012). Although its inhibition has been associated with PKB/Akt phosphorylation and protection of bovine aortic endothelial cells (bAEC) and human umbilical vein endothelial cells (HUVEC) (Neria et al., 2007), it was found to be unaffected by melatonin in CMECs and thus probably not important in the results obtained in the present study.

7.4.4. MELATONIN AND TNF- α -INDUCED ENDOTHELIAL DYSFUNCTION

Several studies have shown that vascular endothelial dysfunction results from reduced NO bioavailability, increased oxidative stress, elevated expression of pro-inflammatory and pro-thrombotic factors which lead to abnormal vasoreactivity (Muniyappa and Sowers, 2013). TNF- α has been shown to induce endothelial dysfunction in MetS and diabetes (Hotamisligil, 1999; Picchi et al., 2006).

To investigate the effect of TNF- α on endothelial function, cells were incubated with TNF- α for 24h. Interestingly, TNF- α treatment caused increased iNOS expression and AMPK phosphorylation (fig.7.10, 14) associated with a reduction in PKB/Akt and eNOS phosphorylation as well as I κ B- α expression, supporting its pro-inflammatory actions coupled to the reduced NO availability [for reviews, see (Fisslthaler and Fleming, 2009; Salminen et al., 2011)].

In agreement with the present study, a reduction in PKB/Akt and eNOS activation was also previously reported in bovine aortic endothelial cells treated with TNF- α (5ng/mL/6h) (Li et al., 2007). Interestingly, this was additionally associated with increased AMPK phosphorylation (Li et al., 2007). However, in contrast to our study, TNF- α (5ng/mL) induced increases in p38MAPK and ERK42/44 phosphorylation (Li et al., 2007). Apart from difference in TNF- α dosage, this controversial response may be linked to endothelial cell heterogeneity as recently demonstrated (Woth et al., 2013).

I κ B- α is the regulatory protein of the nuclear transcription factor NF- κ B with which it is associated in the cytoplasm of resting endothelial cells (Beg and Baldwin, 1993). TNF- α induces phosphorylation of I κ B- α leading to its degradation and subsequent translocation of NF- κ B to the nucleus, where by its transcriptional activities initiates a pro-inflammatory response such as an increase in iNOS expression (Anderson et al., 2004). Consistent with a previous study in bAECs (Anderson et al., 2004), I κ B- α expression was reduced following TNF- α treatment (fig.7.12). Interestingly, it was associated with increased iNOS expression

(fig.7.10) which may result from increased pro-inflammatory transcription following degradation of the complex NF- κ B and I κ B- α (Beg and Baldwin, 1993; Cruz et al., 2001).

To evaluate the effect of melatonin treatment on TNF- α -induced endothelial dysfunction, cells were first pre-treated with melatonin for 1h before co-administration of TNF- α and melatonin for 24h. The overall results (table 7.4A) show that melatonin treatment was able to prevent the TNF- α -induced reduction in eNOS and PKB/Akt phosphorylation (fig.7.8-9) and reversed the TNF- α -induced AMPK phosphorylation (returning it to the control levels) and iNOS expression (reduced compared to control) (fig.7.10;14), indicating potential beneficial effects of melatonin in this regard (Korkmaz et al., 2009).

The reduction in the expression of eNOS, PKB/Akt, nitrotyrosine as revealed by β -tubulin expression (fig.7.9-12) in cells where melatonin was co-administered with TNF- α for 24h is surprising. Whether co-administration of TNF- α and melatonin affects the morphological changes on β -actin and other cytoskeleton proteins as did TNF- α treatment (Kohno et al., 1993), requires further attention. The observed increased degradation of I κ B- α leading to activation of NF- κ B and its nuclear translocation may be implicated (Beg and Baldwin, 1993; Cruz et al., 2001).

Compared to the effect of TNF- α (table 7.4B), co-administration of TNF- α and melatonin was able to retain/preserve the effects observed after 1h melatonin pre-treatment (increased PKB/Akt and p38 MAPK phosphorylation); it also maintained the effect of 24h melatonin treatment compared to TNF- α (reduced phosphorylation of AMPK and STAT-3 and iNOS expression). The failure to reduce I κ B- α could be linked to the low concentration used 1nM (Sasaki et al., 2002). However, except for I κ B- α expression, the TNF- α signalling effects were lost or even reversed by melatonin treatment (table 7.4B).

7.5. CONCLUSION

We investigated the effect of acute melatonin treatment on CMECs with or without TNF- α -induced endothelial dysfunction. Our main findings showed that melatonin treatment increased cell viability of CMECs and reduced NO production in a dose-and time-dependent manner.

These effects were associated with changes in intracellular signalling which also reflected a time-dependence aspect. While TNF- α treatment affected the intracellular signalling of CMECs, co-administration with melatonin was able to reverse the signalling due to TNF- α treatment. Except for I κ B- α expression, the effects of TNF- α treatment on CMECs including PKB/Akt, eNOS, p38MAPK, AMPK phosphorylation were lost or even reversed by melatonin co-treatment. These observations could be of paramount importance in the beneficial effects of melatonin on the ischaemic/reperfused heart, taking into account the significant role of the endothelial cell in this regard.

In view of these preliminary findings, further investigation on the effect of melatonin on CMECs (in normoxic, hypoxic or anoxic condition) using specific inhibitors is needed. In this regard, the role of PKB/Akt and p38MAPK as well as the anti-apoptotic activities of melatonin need further exploration in TNF- α -induced endothelial dysfunction by determining cell viability and NO production. In addition, whether melatonin affects CMECs function *via* receptor-mediated activities also needs to be investigated.

CHAPTER VIII

CONCLUDING REMARKS

8.1. CONCLUSIONS

The present dissertation is a continuation of our previous studies on the role of melatonin in cardioprotection. Melatonin has been shown to have strong cardioprotective actions in lean animals but its effects in obesity and insulin resistance are not well established. Although our previous studies were designed and performed with particular emphasis on myocardial ischaemia-reperfusion injury, the present study additionally focussed on myocardial glucose homeostasis, mitochondrial function and microvascular endothelial function in normal or/and insulin resistant states.

We considered cardioprotection as a multifactorial process involving all the cell types in the heart in general, and cardiomyocytes and cardiac endothelial cells in particular. Thus, the present investigation comprised the effects of melatonin on the heart at baseline level or when exposed to IRI in insulin resistant rats and their age-matched controls.

The present dissertation demonstrated the effects of relatively short-term *in vivo* treatment with melatonin on the heart by evaluating its *ex vivo* basal function and intracellular signalling. Additionally, the effect of melatonin was studied in the context of glucose uptake by cardiomyocytes isolated from the hearts of healthy young and old as well as insulin resistant rats. Furthermore, the effects of melatonin on mitochondria (isolated from hearts from insulin resistant rats and their age-matched controls), which are the primary cellular sites for energy supply to the heart, were evaluated focussing on their oxidative phosphorylation capacity, respiratory function and response to exposure to anoxia. Since the cardiac endothelium may play an important role in exacerbating myocardial damage in ischaemia, the effects of melatonin on resting endothelial cells were investigated. To mimic the events in obesity and insulin resistance where circulating TNF- α levels are elevated, the effects of melatonin on TNF- α -induced endothelial dysfunction were also evaluated.

Apart from the above-mentioned baseline investigations, we studied the effects of *in vivo* melatonin treatment on the heart exposed to regional or global ischaemia, followed by reperfusion and determination of infarct size and myocardial function. In a number of hearts, mitochondria were isolated after exposure to ischaemia and their respiratory function evaluated. Additionally, effects of melatonin on myocardial signalling during reperfusion were determined. The main findings of the present investigation are presented in the figure 8.1

Using obese, insulin-resistant rats as an experimental model, the present dissertation showed for the first time that melatonin:

When administered *in vivo* for 3 or 6 weeks (in drinking water)

- protected the heart against myocardial ischaemia-reperfusion damage independent of changes in body weight, visceral fat, blood lipid and glucose levels while baseline activation of STAT-3 and the RISK pathway during reperfusion were involved in this cardioprotection
- regulated respiratory function of mitochondria isolated from non-perfused or perfused hearts exposed to ischaemic injury
- improved cardiac glucose homeostasis by increasing basal glucose uptake and enhancing insulin actions

When administered *in vitro* (acute treatment):

- had no effect on cardiomyocyte glucose uptake *per se*, but enhanced insulin-stimulated glucose uptake in normal, but not insulin resistant cardiomyocytes
- increased cell viability and reduced NO production in cardiac microvascular endothelial cells and was able to reverse the signalling induced by TNF- α treatment by increasing PKB/Akt activation independent of eNOS activation

The present dissertation also confirmed previous studies done on obese and non-insulin resistant by showing that short-term melatonin treatment

- was also cardioprotective in normal and insulin resistant rats

- reduced insulin resistance independent of body weight gain
- reduced body weight gain independent of visceral fat mass, blood lipid and glucose levels

Finally, this dissertation demonstrated that melatonin is a pleiotropic hormone, having multiple effects depending, amongst others, on the duration and dosage of the treatment as well as the type of experiment (*in vivo* or *ex vivo* setting). Unfortunately the broad scope of the studies undertaken, hampered in depth investigation of several of the interesting observations made and it is obvious that there are still many unanswered questions which need to be addressed.

8.2. PERSPECTIVES FOR THE FUTURE

In contrast to other cardioprotective strategies, the present investigation confirms the powerful cardioprotective potential of melatonin, even when administered at a late stage in the development of obesity and insulin resistance. Its marked effects on weight (not visceral fat) loss are intriguing and needs further investigation. Clinical trials are certainly warranted in this regard.

The present investigation also draws the attention to the potential beneficial effects of melatonin on mitochondrial function in insulin resistance and on cardiac microvascular endothelial cell function. Further studies are needed to shed more light on the interesting preliminary findings obtained.

Indications are that the hormone has also a profound effect on glucose homeostasis in obesity and insulin resistance. Again the need for clinical studies is evident: most, if not all, the current knowledge on this topic is derived from rodent studies, which are, in contrast to humans, active and non-fasting during the night.

Finally, melatonin is a hormone with fascinating actions. Based on current knowledge, daily intake of melatonin may have many beneficial actions in the human. Melatonin is a cheap drug and already sold over the counter. However, clinical trials are required to confirm its therapeutic use in humans.

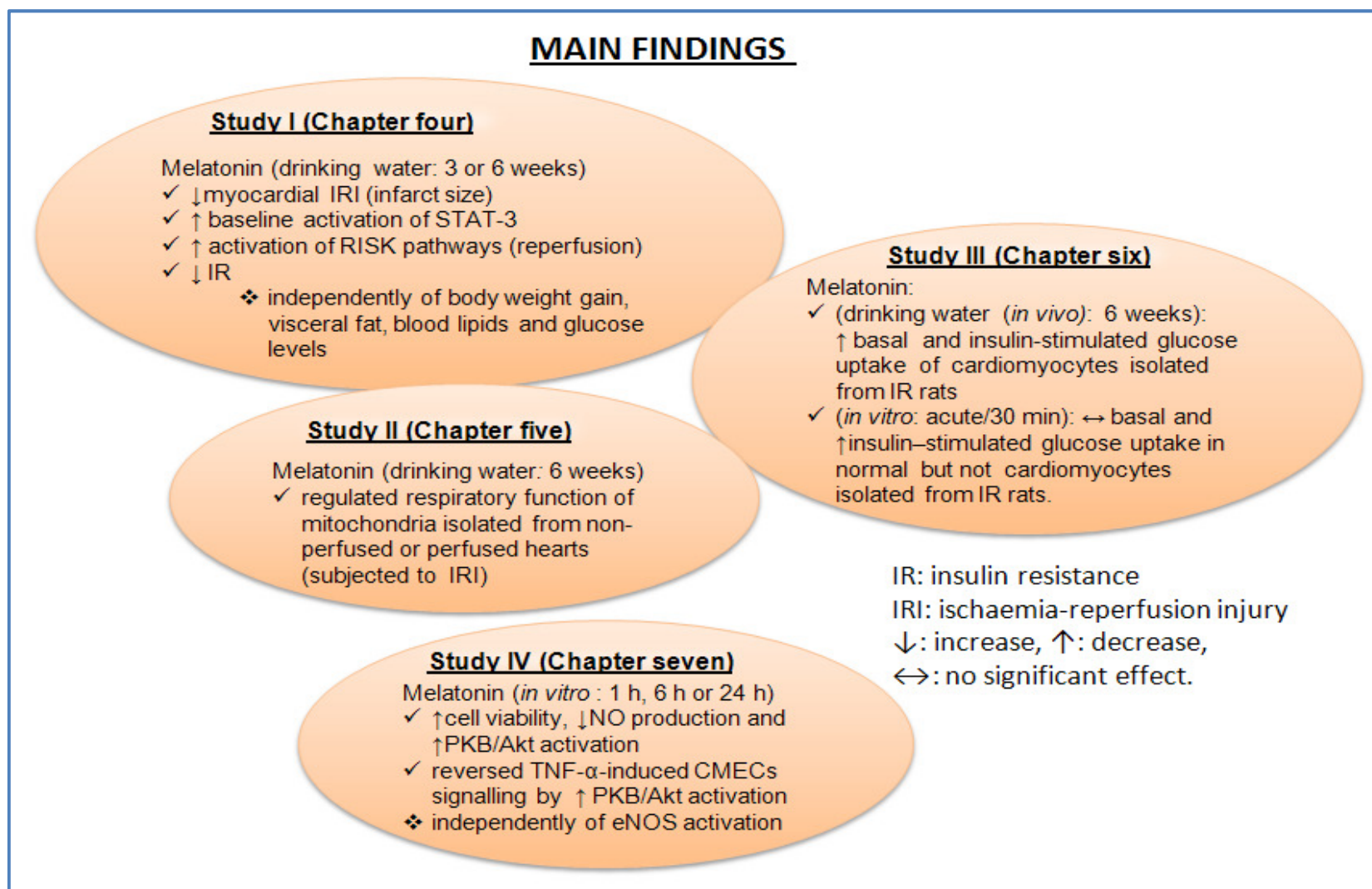


Figure 8.1 Diagram showing the main findings of the investigation. Studies I, II and III used normal and insulin resistant rats; study IV used cultured cardiac microvascular endothelial cells (CMECs).

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