Hyperglycaemic-Induced Regulation of SIRT3 and Downstream Antioxidant Profile

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DECLARATION

This study represents the original work by the author and has not been submitted in any form to another University. The use of work by others has been duly acknowledged in the text.

The research described in this study was carried out in the Discipline of Medical Biochemistry, Faculty of Health Sciences, University of KwaZulu-Natal, under the supervision of Prof. A.A. Chuturgoon and Dr. D. Moodley.

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Gounden,S, Phulukdaree,A, Moodley, D, Chuturgoon, A.A. College of Health Science Research Symposium 2011, UKZN. *[Poster presentation]*

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LIST OF ABBREVIATIONS

µM	Micromolar
mM	Millimolar
MTT	Methyl thiazol tetrazolium
ºC	Degrees Celsius
µL	Microlitre
nm	Nanometer
•OH	Hydroxyl radical
8-oxoG	7, 8-dihydro-8- oxoguanine
AGE	Advanced glycation end product
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
AO	Antioxidant
APAF-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BER	Base excision repair
CASPASES	Cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases
cDNA	Complementary DNA
CI	Confidence Interval
COX2	Cyclooxygenase
CREB	cAMP response element-binding protein
Ct	Cycle threshold

DAG	Diacylglycerol
DMI	Type 1 diabetes mellitus
DMII	Type 2 diabetes mellitus
ERR-α	Estrogen-related receptor-alpha
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide
FOXO	Forkhead box O
GLUT2	Glucose transporter 2
GPx1	Glutathione peroxidase 1
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HEK 293	Human embryonic kidney 293 cells
HEPG2	Human hepatocellular carcinoma cell line
HO-1	Heme oxygenase 1
hr	Hour
HRP	Horseradish peroxidase
HSE	Heat shock element
HSF	Heat shock factors
HSP	Heat shock protein
HSP27	Heat shock protein 27
HSP70	Heat shock protein 70
HSR	Heat shock response

IDF	International Diabetes Federation
IDH2	Isocitrate dehydrogenase 2
KD	Knockdown
LON	Lon protease
mg	Milligram
min	Minutes
ml	Millilitre
MnSOD/SOD2	Manganese superoxide dismutase
MPP	Mitochondrial processing peptidase
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting sequence
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAM	Nicotinamide
NF-κB	Nuclear factor kappa B
NO•	Nitric oxide
NRF2	Nuclear factor erythroid 2 [NF-E2]-related factor 2
O_2•	Superoxide radical
OCT1	Organic cation transporter 1
OGG1	8-Oxoguanine glycosylase
ONOO	Peroxynitrite radical
OS	Oxidative stress
OXPHOS	Oxidative phosphorylation

PGC-1α	Peroxisome proliferator-activated receptor gamma co-activator 1 alpha
PKC	Protein kinase C
qPCR	Quantitative polymerase chain reaction
RBD	Relative band density
RLU	Relative light units
ROS	Reactive oxygen species
s	Second
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SIRT	Sirtuin
TCA	Tricarboxylic acid cycle
TNFα	Tumor necrosis factor alpha

ABSTRACT

Hyperglycaemia increases reactive oxygen species (ROS) production and mitochondrial dysfunction which are involved in metabolic disorders. Sirtuin 3 (SIRT3) is a primary mitochondrial deacetylase that regulates mitochondrial function and antioxidant (AO) defence.

We investigated the role of SIRT3 in AO defence under hyperglycaemic conditions in HepG2 cells in the presence and absence of metformin and curcumin. We also examined cell protective mechanisms that counterbalance apoptotic stress under these oxidative conditions.

HepG2 cells were cultured with 5mM (control), 19.9mM mannitol (OC), 10mM glucose, 30mM glucose (hyperglycaemic), 10mM nicotinamide (NAM) at 24hr and 72hr time points in the absence or presence of curcumin (5μM and 10μM) or metformin (3mM).

Increased expressions of SIRT3, peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), mitochondrial AO enzymes glutathione peroxidase 1 (GPx1), superoxide dismutase 2 (SOD2), uncoupling protein 2 (UCP2) and mtDNA repair enzyme 7, 8-dihydro-8-oxoguanine (OGG1) were observed under hyperglycaemic conditions. The same trend was observed for all parameters following metformin and curcumin treatment. In addition, curcumin also increased expressions of nuclear factor-kappa B (NF-κB), lon protease (Lon) and heat shock protein 70 (Hsp70). These were optimally expressed in the 10μM curcumin-treated groups. We also showed that under hyperglycaemic conditions, apoptosis was initiated but may not have been fully executed due to the induction of stress proteins (heat shock protein 27, nuclear factor erythroid-derived 2-like 2) and AO defence that counterbalance apoptotic stress.

The results suggest that SIRT3 modulates AO defence and confers resistance to oxidative stress (OS)-induced damage under hyperglycaemic conditions in HepG2 cells. Our data also suggests that metformin and curcumin may work synergistically with SIRT3, or through SIRT3-mediated mechanisms, to improve AO defence. Our model shows that hyperglycaemia may induce apoptosis; however, apoptotic stress may be counterbalanced by cell survival mechanisms that include stress response proteins and the downstream activation of AO defence.

Mitochondria are susceptible to OS, which is involved in metabolic disorders. SIRT3 may, therefore, be therapeutically targeted as a potential cyto-protective factor. Modulation of SIRT3 function, by chemical or natural therapeutics, may also improve disease outcomes.

INTRODUCTION

Type 2 diabetes mellitus (DMII) is a global health issue characterised by hyperglycaemia and insulin resistance. It is associated with an increased risk of multiple micro- and macro-vascular complications.

DMII has reached epidemic proportions and is predicted to increase by 55% by 2035 (*1*). Three-and-a-half million South Africans (about 6% of the population) suffer from DMII (*1*).

Evidence has shown that increased production of reactive oxygen species (ROS) may play a role in the progression of DMII and associated complications (*2-4*).

Excessive ROS, due to diminished antioxidant (AO) defence, can damage critical macromolecules (*5*, *6*). The electron transport chain (ETC) is the primary site for ROS generation. The energy from the ETC shuttles protons across the membranes and this creates a proton gradient that drives ATP synthesis (*7*). Unpaired electrons leak from the respiratory complexes and interact with molecular oxygen producing superoxide anions (*8*).

Under hyperglycaemic conditions, the tricarboxylic acid (TCA) cycle produces more electron transfer donors which enter the ETC (*9*). This causes partial inhibition of the ETC at complex III, increasing the levels of superoxide radicals (*9*).

Mitochondrial DNA (mtDNA) is vulnerable to oxidative damage as it is (a) closely located to the ETC, (b) lacks protective histones and (c) has limited DNA repair systems (*10*). Mitochondrial DNA damage compromises mitochondrial function and AO activity (*11*) which has been observed in metabolic disorders (*12*). The increased production of superoxide radicals further compromise the ETC resulting in persistent mtDNA damage and genomic instability (*9*).

Mitochondrial dysfunction and oxidative stress (OS), induced by hyperglycaemia, are key factors that contribute to the progression of DMII and associated complications (*13*).

In order to improve DMII, mitochondrial function needs to be maintained and OS must be reduced. Sirtuins (SIRTs), a group of class III NAD^+-dependent histone deacetylases, contribute to the network of stress response proteins. Studies have shown that SIRTs play an essential role in mitochondrial protection against OS (*14-16*). Sirtuins are involved in various biological functions such as DNA repair (*17*), apoptosis (*18*) and regulation of metabolic enzymes (*19*).

SIRT3 is located in mitochondria and regulates enzymes involved in the TCA cycle, fatty acid metabolism and glycogen metabolism (*20*).

SIRT3 regulates cell defence and survival under oxidative conditions (*14, 15*); it decreases mitochondrial ROS production and protects against mtDNA damage (*16, 21*).

SIRT3 over-expression in murine adipocyte cell lines (*22*) and mice cardiomyocytes (*15*) resulted in reduced levels of ROS. Similar results were observed in human oral keratinocytes (HOK) where SIRT3 transcript and protein levels were increased following OS induction (*23*). These findings are consistent with similar studies in primary neurons (*24*) and adipocytes of mice (*22*) as well as HEK 293 cells (*14*).

SIRT3 reduces ROS levels by modulating AO enzymes (manganese superoxide dismutase (SOD2) and isocitrate dehydrogenase 2 (IDH2)), thereby protecting against oxidative damage.

The SOD2 lysine residues are deacetylated by SIRT3 which increases SOD2 activity and reduces ROS levels (*14*). SIRT3 and SOD2 levels were down-regulated following severe OS in human mesenchymal stromal/stem cells (hMSCs) whilst over-expression of SIRT3 improved hMSCs resistance under oxidative conditions (*25*). SIRT3 also plays a role in modulating SOD2 activity in skeletal muscle of obese pregnant women with gestational diabetes mellitus (*26*).

SIRT3 was also found to activate IDH2 in mice and HEK 293 cells (*27-30*). It has been stated that SIRT3 is dependent on IDH2 to protect cells from OS (*30*). IDH2 is the enzyme involved in the conversion of isocitrate to α-ketoglutarate in mitochondria, generating NADPH from $NADP^+$. NADPH is required for the regeneration of reduced glutathione (GSH); therefore, increased IDH2 activity enhances GSH AO defence.

SIRT3 slowed the progression of age related hearing loss (AHL) by promoting the GSH-mediated mitochondrial AO defence in mice (*31*). SIRT3 also reduced oxidative DNA damage by enhancing GSH AO defence system in mice (*28*).

SIRT3 can also regulate AO defence indirectly through the transcriptional co-activator peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), which interacts with transcription factors involved in AO defence (*32*). SIRT3, which is a target gene of PGC-1α, also mediates the role of PGC-1α in the AO defence system (*33*).

In addition to AO defence, SIRT3 is involved in improving genomic stability. Cheng *et al.* (2013) showed, in human glioblastoma cell lines (LN229), that SIRT3 deacetylates and stabilizes the 8-oxoguanine DNA glycosylase (OGG1) protein, thereby maintaining its ability to repair mtDNA (*16*).

SIRT3 may therefore be seen as a pro-survival factor that plays an essential role in improving metabolic disorders by suppressing OS. This, however, has not been fully investigated in the human system.

Metformin inhibits mitochondrial ROS production by blocking the reverse electron flow through complex I of the ETC (*34*).

In vitro data showed that metformin could also induce AO enzymes, such as SOD2, thereby suppressing OS and improving DMII outcomes (*35, 36*). Metformin was also shown to significantly decrease serum levels of 7, 8-dihydro-8- oxoguanine (8-oxoG); a marker of oxidative damage in women with polycystic ovary syndrome (*37*).

Metformin may regulate both mitochondrial function and AO activity through SIRT3-mediated mechanisms; however, the molecular mechanisms underlying this process are unclear.

Natural compounds, such as curcumin, have become promising agents to reduce the risk of DMII. Curcumin is a bi-functional AO as it scavenges ROS species and indirectly up-regulates cyto-protective and AO enzymes (*38, 39*).

Curcumin administration has been shown to prevent DMII complications (*40*), prevent the decrease in the AO capacity and prevent an increase in oxidative damage in the retina of diabetic rats (*41*). Treatment with curcumin reduced renal dysfunction and OS in diabetic animals (*42*). It also improved hepatic and renal function markers in DMII rats (*43*).

The mechanisms by which curcumin inhibits OS are still being investigated. It is unknown whether the AO effect of curcumin is due to its chelating property or through regulation of SIRT3.

Hyperglycaemia-induced mitochondrial dysfunction causes apoptosis. Under oxidative conditions, mitochondria become permeabilised and release cytochrome *c*, which together with caspase-9 and apoptotic protease activating factor 1 (Apaf-1), form a complex known as the apoptosome which recruits and activates pro-caspase 9 (*44*). Apoptosis has been observed in the pancreatic beta cells of diabetic patients (*45*). Hyperglycaemia was shown to be the direct cause of apoptosis in diabetic myocardium and cultured cardiac myoblast cells (*46*).

Although it has been established that hyperglycaemia causes cell death, there is still a network of cell protective mechanisms, induced in response to OS, which may counteract apoptosis and ensure cell survival. These mechanisms have yet to be elucidated in HepG2 cells.

Rationale:

SIRT3 plays a key role in OS-associated disorders as it modulates AO defence and improves mitochondrial function and genome integrity.

SIRT3 has been investigated in several metabolic disorders *in vivo* and *in vitro.* However, with regard to hyperglycaemic-induced OS, limited studies have interrogated the role of SIRT3 and stress response in the human liver, the primary organ involved in glucose homeostasis.

Given that mitochondria are vulnerable to OS, SIRT3 may be therapeutically targeted as a potential cyto-protective factor to improve mitochondrial function and disease outcomes. It is also important to determine whether modulation of SIRT3, by chemical or natural therapeutics, may improve OS-associated disorders.

Hypothesis:

$H_{1:}$ Hyperglycaemic stimulation increases SIRT3 expression and stress response under hyperglycaemic conditions in the human hepatoma cell line.

CHAPTER 1 –LITERATURE REVIEW

1.1 Global prevalence of diabetes

There are 387 million people that have diabetes aged between 40 and 59 years. The International Diabetes Federation (IDF) predicts the number of people with diabetes to increase to 592 million in less than 25 years (*1*) (Fig 1.1).

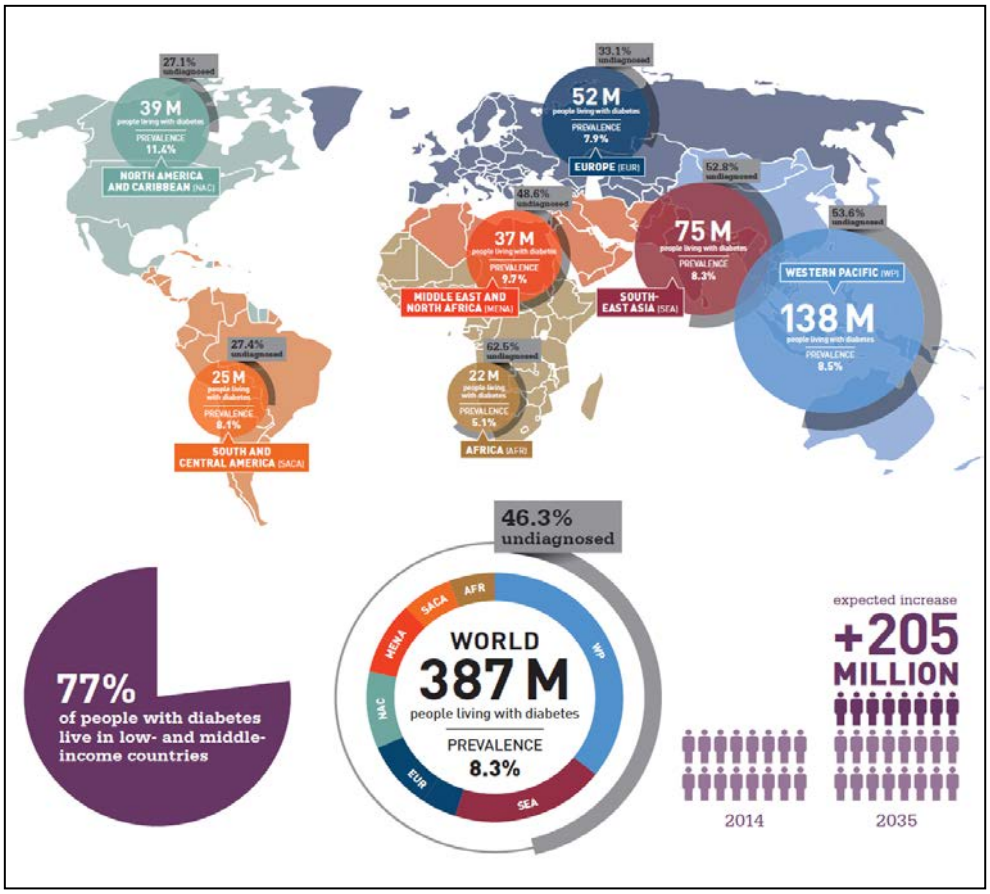

Figure 1.1: Global prevalence of diabetes mellitus (*1*)

Type 2 diabetes mellitus (DMII) has reached epidemic proportions and is predicted to increase by 55% by 2035 (*1*). Interestingly, 80% of people with diabetes live in low- and middle-income countries where economic development shifts lifestyle resulting in high rates of obesity and diabetes (*1*).

Three-and-a-half million South Africans (about 6% of the population) suffer from DMII (*1*). Five million South Africans have pre-diabetes which is a condition where insulin resistance causes blood glucose levels to be higher than normal, but not high enough to be DMII (*1*). The highest prevalence of diabetes

is among the Indian population in South Africa (11-13%) as this group has a strong genetic predisposition for diabetes. This is followed by 8-10% in the coloured community, 5-8% among blacks and 4% among whites (*1*).

1.1.1 *Pathophysiology*

Diabetes is a metabolic disorder characterised by hyperglycaemia and insulin resistance or insulin deficiency (due to pancreatic beta cell dysfunction) (*1*). This prevents glucose from entering cells and being converted to energy for proper functioning of tissues (*1*). This results in increased levels of glucose circulating in the blood (hyperglycaemia) damaging tissues over time leading to complications such as heart disease, kidney failure, and other micro-vasculature complications (*1*).

1.1.2 *Type 1 diabetes mellitus (DMI)*

Type 1 diabetes mellitus (also known as insulin-dependent diabetes) is characterized by insulin deficiency due to autoimmune pancreatic beta cell destruction (*1*).
People living with DMI can lead healthy lives through daily insulin administration, a healthy diet and regular physical exercise. The incidence of DMI increases each year and this may be due to early events in the womb, diet early in life or viral infections (*1*).

1.1.3 *Type 2 diabetes mellitus (DMII)*

Type 2 diabetes mellitus is the most prevalent form of diabetes. Obesity, poor diet, sedentarinism, familial incidence and ethnicity are risk factors for DMII (*1*). DMII is characterized by insulin resistance and increased production of glucose by the liver (*1*).

1.1.4 *Diabetic complications*

Type 2 diabetes mellitus associated complications include micro-vascular complications (retinopathy, nephropathy and neuropathy) and macro-vascular damage (cardiovascular, peripheral vascular and cerebro-vascular disease) (*1*).

1.2 Oxidative stress and DMII

Increased reactive oxygen species (ROS) generation plays a role in the progression of DMII and associated complications (*2-4*).
ROS are highly reactive species that are produced from oxygen metabolism. These include superoxide radical ($O_2^{\bullet}$), the hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), as well as nitric oxide (NO•).

Basal levels of ROS are required for signalling, cellular differentiation, apoptosis and defence mechanisms. Excessive ROS levels, due to diminished antioxidant (AO) defence, can react with macromolecules and cause DNA damage (*5*, *6*). Oxidative stress (OS) occurs when ROS exceeds the AO capacity.

Hyperglycaemia activates several biochemical pathways that increase ROS production which may be the link between DMII and associated complications (*13*).

Three key metabolic pathways contribute to hyperglycaemic-induced cell damage (*13*) (Fig 1.2):

(1) Increased polyol pathway flux; (2) activation of protein kinase C (PKC) and (3) increased advanced glycation end product (AGE) formation.

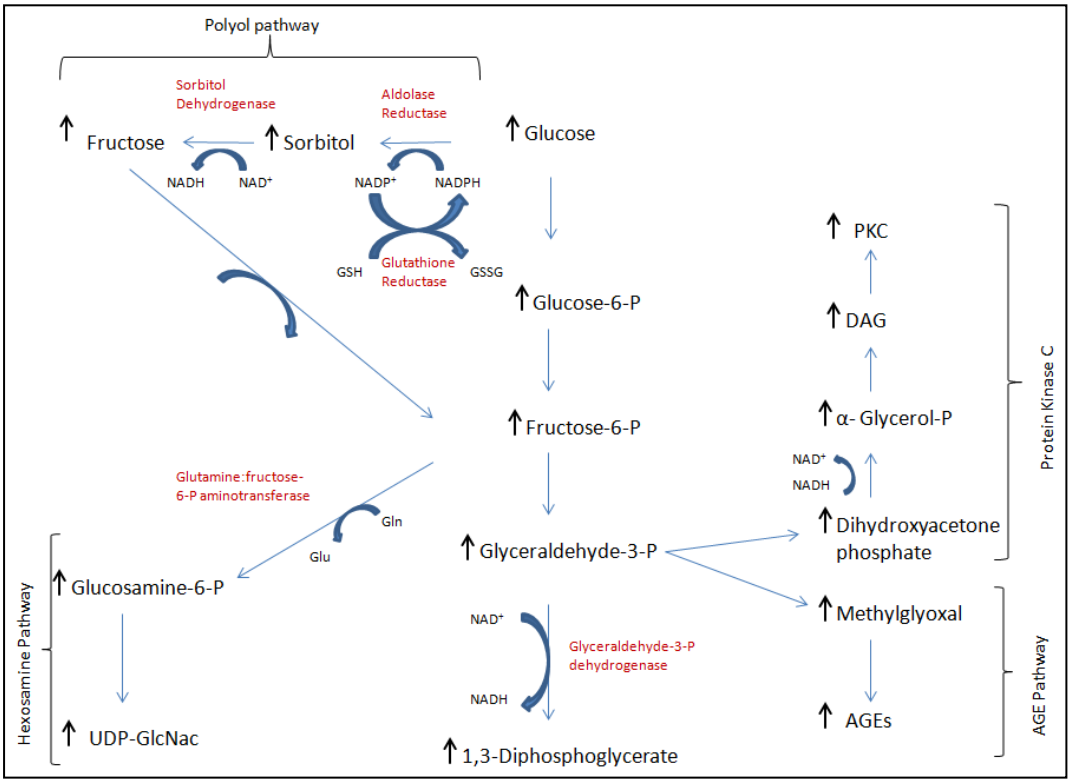

Figure 1.2: Mechanism of hyperglycaemic-induced damage (*9*)

When intracellular glucose increases, it is converted to sorbitol by aldolase reductase resulting in a decrease in NADPH and glutathione, which make cells vulnerable to OS. Sorbitol is converted to fructose by sorbitol dehydrogenase and increases the ratio of NADH/ NAD^+ (*2*). This results in *de novo* synthesis of diacylglycerol (DAG) which activates protein kinase C (PKC). Protein kinase C results in the production of proteins involved in DMII complications (*2*). Hyperglycaemia-induced ROS also increases methylglyoxal levels which form AGEs (*2*).

1.3 Mitochondrial ROS production

Mitochondrial dysfunction is a key factor in metabolic disorders such as DMII. Mitochondria generate ATP by oxidative phosphorylation (OXPHOS) (Fig 1.3) and produce electron transfer donors (NADH and $FADH_2$) through the TCA cycle.

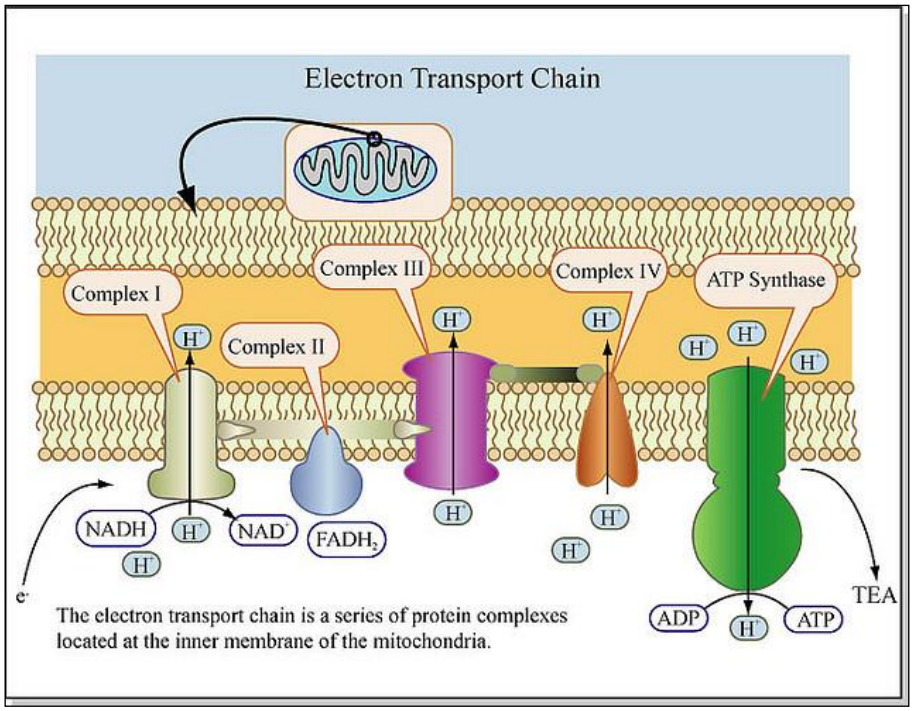

Figure 1.3: Diagram of oxidative phosphorylation (thebiochemsynapse.wordpress.com)

Oxidative phosphorylation begins with entry of electrons into the electron transport chain (ETC) which is made up of 5 multi-subunit components (I-V) (*47*) (Fig 1.3). These complexes are embedded in the inner mitochondrial membrane and transport electrons from NADH and $FADH_2$ (*47*). NADH donates electrons to complex I (NADH dehydrogenase) which transfers electrons to ubiquinone (*47*). $FADH_2$ donates electrons to complex II (succinate dehydrogenase) which also transfers the electrons to ubiquinone (*47*). Reduced ubiquinone transfers electrons to complex III (ubiquinol:cytochrome c oxidoreductase). While this occurs, a proton gradient is generated in the inter-membrane space that drives ATP synthesis by

complex V (*47*). Molecular oxygen (O_2), which is a terminal electron acceptor, is reduced at complex IV to produce water (H_2O) (*47*).

Unpaired electrons leak from the complexes and interact with molecular oxygen producing superoxide anions which mainly occurs at complex I (NADH-dehydrogenase) and at complex III (cytochrome c reductase) (*8*).

1.3.1 *Mitochondria under hyperglycaemic conditions*

Under hyperglycaemic conditions, the TCA cycle generates more electron transfer donors which enter the ETC.
The proton gradient causes partial inhibition at complex III resulting exacerbated production of superoxide radicals (*9*). Excessive ROS generated from mitochondria damage macromolecules (proteins, DNA and unsaturated lipids in membrane components) and causes mitochondrial dysfunction.

Hyperglycaemia-induced mitochondrial dysfunction and OS are factors that contribute to the progression of DMII and associated complications (*13*).

1.3.2 *Mitochondrial dysfunction*

Mitochondrial DNA (mtDNA) is vulnerable to oxidative damage as it is (a) closely located to the ETC, (b) lacks protective histones and (c) has limited DNA repair systems (*10*). Mitochondrial DNA damage compromises mitochondrial transcription, OXPHOS protein synthesis and AO activity (*11*) which has been observed in metabolic disorders (*12*). The increased production of superoxide radicals further compromise the ETC components resulting in persistent mtDNA damage and genomic instability (*8*).

1.4 Oxidative stress and mitochondrial DNA damage

Oxidative stress can induce DNA base damage such as 7, 8-dihydro-8-oxoguanine (8-oxoG) (*48*). Following OS, a hydroxyl group is added to the 8th position of guanine resulting in the formation of 8-oxoG, one of the major forms of ROS-induced lesions (*48*) (Fig 1.4).

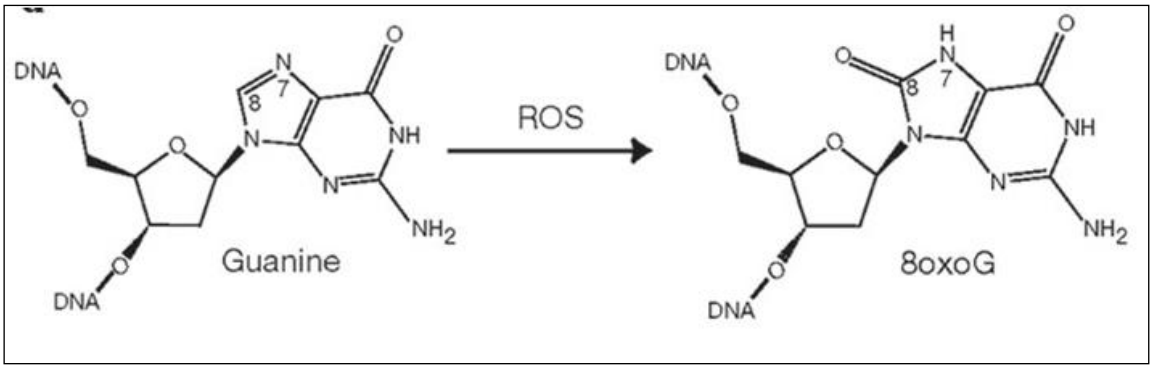

Figure 1.4: Structure of 7, 8-dihydro-8- oxoguanine (*49*)

The formation of 8-oxoG increases the frequency of spontaneous G.C- -T.A transversion mutations due to lack of base pairing specificity (*50*).

Mutations in mtDNA are involved in mitochondrial diseases and are associated with cancer (*51*) and aging (*52*). These mtDNA mutations result in mitochondrial dysfunction characterised by abnormal expression of mtDNA encoded proteins, impaired OXPHOS and TCA cycle (*53*).

8-oxoG is seen as a biomarker of oxidative DNA damage in DMII. Elevated concentrations of 8-oxoG were found in urine and in leukocyte DNA (*54*). These were associated with diabetic nephropathy and retinopathy (*54*).

1.4.1 *Repair Mechanisms: OGG1*

The base excision repair (BER) pathway repairs oxidized bases in mtDNA and preserves genomic stability, which is required for long-term cell survival (*55*).
The BER pathway recognises and removes 8-oxoG, which is base-paired with cytosine, by DNA glycosylase OGG1 (*55*) (Fig 1.5).

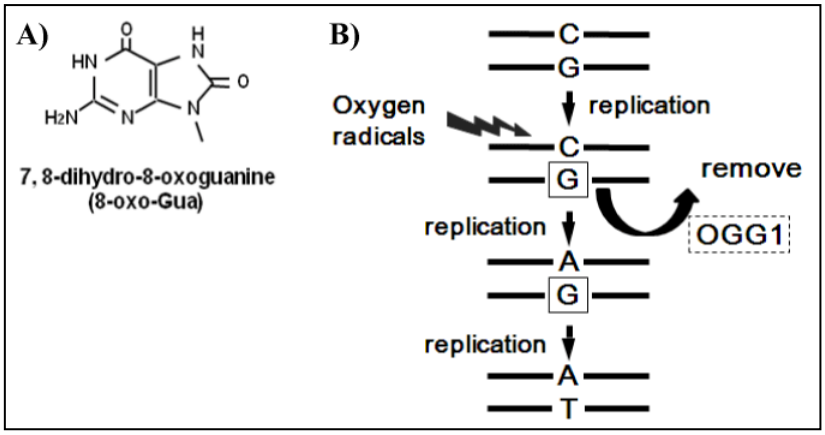

Figure 1.5: Diagram of OGG1 (8-oxoguanine DNA glycosylase) (*56*)

Mitochondria are the primary sites of OGG1 DNA repair activity (*57*). In OGG1-deficient mice, genes involved in the TCA cycle were significantly reduced. Deficiency of OGG1 was also correlated with increased susceptibility to obesity and the metabolic syndrome (*58*).

Studies have demonstrated that over-expression of mitochondria-targeted OGG1 prevented mtDNA damage and intrinsic apoptosis in vascular endothelial cells and asbestos-exposed cells (*59, 60*).
OGG1 over-expression resulted in mtDNA repair and reduced ROS production following palmitate-induced mitochondrial dysfunction in primary cultures of skeletal muscle (*61*).
Hyperlgycaemic-induced mitochondrial dysfunction is known to cause apoptosis which results in the progression of DMII and associated complications.

1.5 Caspases

Caspases (cysteine-aspartic proteases) are a family of cysteine proteases that cleave target proteins at sites next to aspartic acid residues (*44*).
Caspases are classified as initiators (caspase-8 or caspase-9) or executioners (caspase 3/7). They are produced as inactive pro-caspases and require cleavage to be activated. The initiator caspases are activated first in the death pathway and, thereafter, they activate the executioner caspases (*44*).

1.5.1 *Intrinsic pathway*

The intrinsic pathway is activated in response to mitochondrial damage (*44*). Upon OS, mitochondria become permeabilised and release cytochrome *c*. Cytochrome *c*, together with caspase-9 and the apoptotic protease activating factor 1 (Apaf-1), form the apoptosome which recruits and activates pro-caspase 9. Caspase-9 cleaves and activates executioner caspases (caspase-3 and caspase-7) (*44*) (Fig. 1.6).

1.5.2. *Extrinsic pathway*

This pathway is initiated by ligands binding to death receptors that are located at the cell surface (*44*). This leads to caspase-8 activation, which, thereafter, cleaves and activates executioner caspases (caspase-3 and caspase-7) (Fig. 1.6).

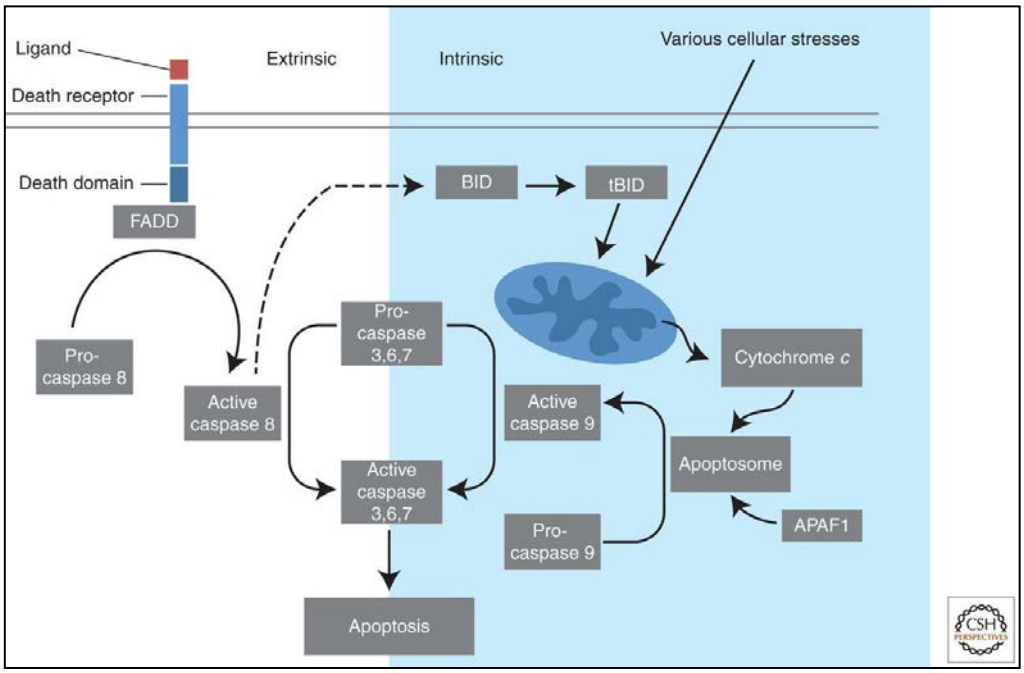

Figure 1.6: Extrinsic and intrinsic pathways of apoptosis (*62*)

Hyperglycaemia-induced ROS may trigger the apoptotic process (*63*). Apoptosis has been observed in multiple organs, *in vivo* and *in vitro*, as a result of DMII (*64*, *65*). Increased apoptosis was observed in the pancreatic beta cells of diabetic patients (*45*).

Apoptosis occurred in diabetic myocardium and this was a direct result of high glucose levels. Mitochondrial cytochrome *c* release and increased caspase-3 activation were associated with hyperglycaemia-induced myocardial apoptosis (*46*).

1.6 Lon protease

Oxidative damage can create carbonyl groups and cross-links which may impair enzymes and structural proteins and cause cellular toxicity if not removed. Protein misfolding and aggregation are associated with diseases and aging (*66*, *67*).

Lon is an ATP-dependent protease located in the mitochondrial matrix (*68*). Lon may be regulated by transcription factors associated with mitochondrial biogenesis and OS (*69*). Binding sites have been identified on the LON gene, PRSS15, for transcription factors such as nuclear factor erythroid 2 [NF-E2]-related factor 2 (Nrf2) and nuclear factor-kappa B (NF-κB) (*69*).

Under oxidative conditions, Lon is transcribed in the nucleus and translated as a precursor polypeptide that carries an amino-terminal mitochondrial targeting sequence (MTS) (*70*). This allows the Lon precursor to translocate across the mitochondrial membranes and into the matrix. In the matrix, MTS is cleaved off resulting in a processed protein (*70*). Lon degrades oxidized and damaged proteins, assists in the assembly and folding of mitochondrial proteins and is involved in mtDNA maintenance and replication (*70*).

In rhabdomyosarcoma cells, exposure to heat shock, serum starvation and OS resulted in increased Lon protein levels (*71*). Induction of Lon prevented the accumulation of carbonylated proteins resulting in improved cell survival and mitochondrial function (*71*). Silencing of Lon led to increased levels of carbonylated proteins, mitochondrial dysfunction and decreased cell survival (*71*).

In cultured human cells, Lon knockdown resulted in impaired mitochondrial function, damaged mitochondrial morphology, apoptosis and necrosis (*72*).

The activity of the Lon protease was shown to be lower in the streptozotocin (STZ) animals. STZ is an antibiotic that can cause pancreatic β-cell destruction and is used to experimentally induce DMI (*73*). Muscle from diabetic rats showed reduced mitochondrial protein quality and increased levels of modified proteins which led to mitochondrial dysfunction (*73*).

Lon is an important regulator of mitochondrial function as it responds to changes in the mitochondrial environment.

1.7 Heat shock proteins

Heat shock proteins (Hsps) are a highly conserved family of stress response proteins (*74*). Their expression is increased under OS through the heat shock response (HSR) (*74*) (Fig. 1.7). Heat shock proteins can function as molecular chaperones as they are involved in protein folding and prevent protein aggregation (*74*).

The inducible Hsp component is regulated by heat shock factors (HSFs), where heat shock factor-1 (HSF-1) is the major regulator (*75*). Under normal conditions, HSF-1 is inhibited and inactive as it is associated

with Hsps. Following OS, the Hsps dissociate from HSF-1 and bind to misfolded proteins. The HSF-1 monomers form active trimers, allowing for DNA binding. The trimers undergo stress-induced serine phosphorylation and translocate to the nucleus. In the nucleus, HSF-1 binds to the heat shock element (HSE), which results in heat shock responsive gene transcription (*75*) (Fig. 1.7).

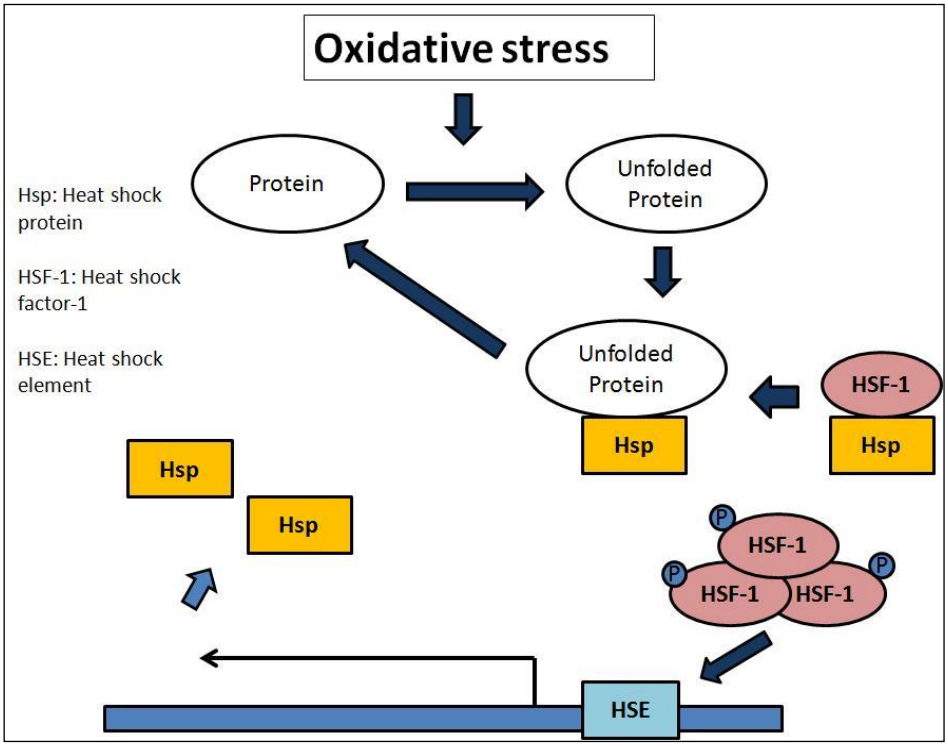

Figure 1.7: Heat shock response (Author's own work)

1.7.1 *Heat shock protein 70 (Hsp70)*

Heat shock protein 70 is the most closely linked to cyto-protection in mammalian cells (*74*). They form part of the cell protective network, together with Lon, to recognise and remove damaged proteins (*76*). In addition, Hsp70 expression improves AO defence and inhibits stress-induced apoptosis (*77*). Hsp70 induction assists in the recovery from diseases such as inflammatory diseases; diabetes and neurodegenerative damage (*78-80*).

Both Hsp70 and Lon are involved in protein quality control that maintains mitochondrial integrity (*76*).

1.7.2 *Heat shock protein 27 (Hsp27)*

During OS, Hsp27 increases the levels of intracellular glutathione as it can maintain glutathione in its reduced form under oxidative conditions (*81*).

Hsp27 also interacts with cytochrome *c* and prevents the correct formation of the apoptosome complex

and activation of caspase-3 (*82*).

1.8 Antioxidant defence in mitochondria

Increased production of ROS plays a role in mitochondrial dysfunction by compromising the ETC and causing persistent damage to mtDNA. Reducing ROS by increasing AO defence is an effective method to improve mitochondrial-associated metabolic disorders.

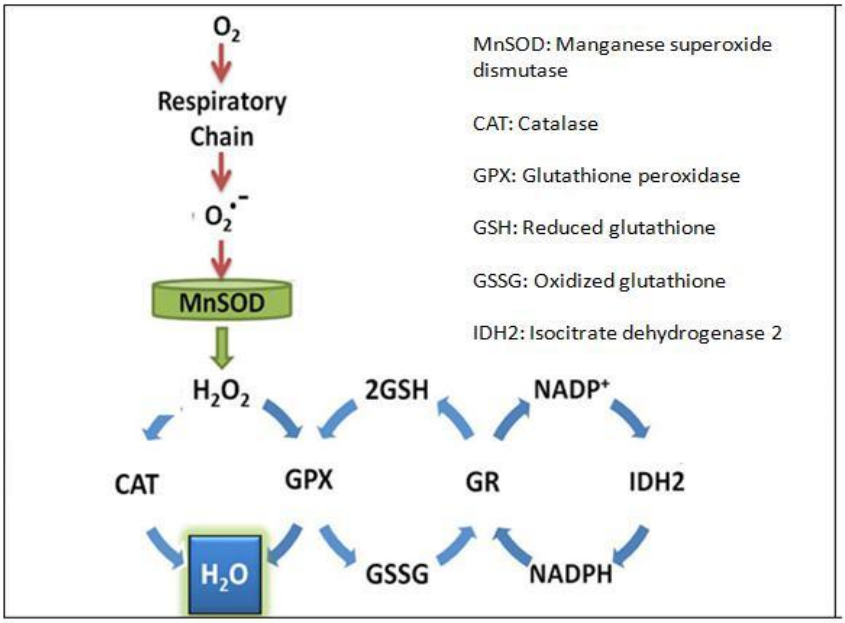

Figure 1.8: Mitochondrial ROS generation and AO defence system (*83*)

1.8.1 *Manganese superoxide dismutase (SOD2)*

Manganese superoxide dismutase is located in the mitochondrial matrix and converts O_2 ⋅ to hydrogen peroxide (H_2O_2) (*83*) (Fig. 1.8). There are three major families of superoxide dismutase: Cu/Zn (which binds both copper and zinc) found in cytoplasm and nucleus, Fe and Mn types (which bind iron or manganese) found in mitochondria, and the Ni type, which binds nickel.

1.8.2 *Glutathione*

Glutathione is also located in the mitochondrial matrix and cytoplasm. It scavenges free radicals and is a co-substrate for glutathione peroxidase (GPx) activity. Glutathione exists in two forms – reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) (*83*).

GSH reduces H_2O_2 to H_2O and thereafter is oxidized to GSSG by glutathione peroxidase 1 (GPx1). GSSG is converted to GSH by the glutathione reductase (GR) enzyme. NADPH protects cells against ROS by

providing electrons to GSSG in order to regenerate GSH (*83*) (Fig. 1.8).

Patients with DMII were found to have decreased GSH synthesis; however, following GSH supplementation, GSH synthesis was restored and hyperglycaemic-induced OS was reduced (*84*).

1.8.3 *Glutathione peroxidase 1*

Glutathione peroxidase 1 (GPx1) is found in the cytoplasm, mitochondria and nucleus. It metabolises H_2O_2 to H_2O by using GSH as a hydrogen donor (*83*).

1.9 Improving mitochondrial function and DMII

To improve DMII, mitochondrial function and mitochondrial genome integrity need to be maintained and OS must be reduced.
Sirtuins (SIRTs) are part of the network of cellular stress response proteins that improve these factors. Studies revealed that SIRTs have an essential role in mitochondrial protection against OS (*14-16*).

1.9.1 *Mammalian sirtuins*

Sirtuins or Silent information regulator proteins (Sir) are NAD^+-dependent class III histone/protein deacetylases that remove acetyl groups from the ε-amino group of lysine residues (*85*). The acetyl group is transferred from the target protein to the ADP-ribose component of NAD^+ resulting in the formation of O-acetyl-ADP-ribose, nicotinamide (NAM) and the deacetylated protein (*85*) (Fig. 1.9).
Sirtuins are involved in (*86*), DNA repair (*17*), apoptosis (*18*) and the control of metabolic enzymes (*19*).

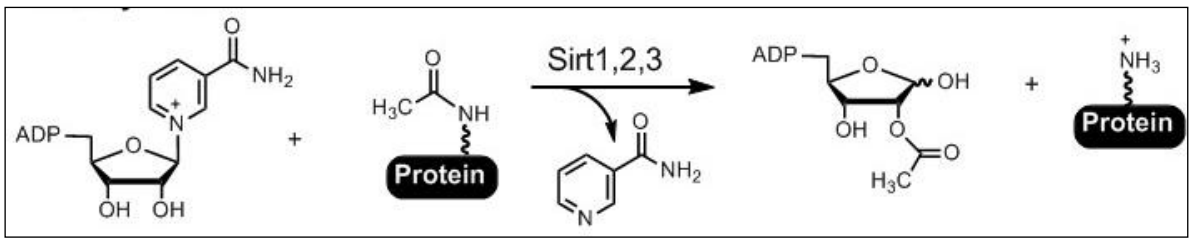

Figure 1.9: Mechanism of protein deacetylation by sirtuins (lin.chem.cornell.edu)

1.9.2 *Classification of sirtuins*

Seven SIRTs found in mammalian cells have different cellular locations and functions (*87*). They share a conserved catalytic core domain and have variable amino- and carboxyl- terminal extensions that

contribute to their differential cellular locations (*87*).

SIRT1 is located in the nucleus and regulates transcription factors such as p53 and NF-kB. SIRT2 is located in the microtubules and deacetylates α-tubulin. SIRT 6 regulates telomeric chromatin and SIRT7 is localised in the nucleoli. SIRT3, 4 and 5 are localised in the mitochondria, although SIRT3 can also be found in the nucleus (*87*).

1.10 Mitochondrial sirtuins

Amongst the mitochondrial SIRTs, SIRT3 is the main deacetylase. In SIRT3 knockout mice, high levels of mitochondrial protein acetylation were observed suggesting that SIRT3 is the primary mitochondrial deacetylase (*88*).

Lysine acetylation is involved in mitochondrial function and metabolism; this shows that SIRT3 plays a key role in modulating these pathways (*89*). SIRT3 has been shown to bind, deacetylate and activate metabolic and respiratory enzymes involved in mitochondrial processes (*20*) (Fig 1.10).

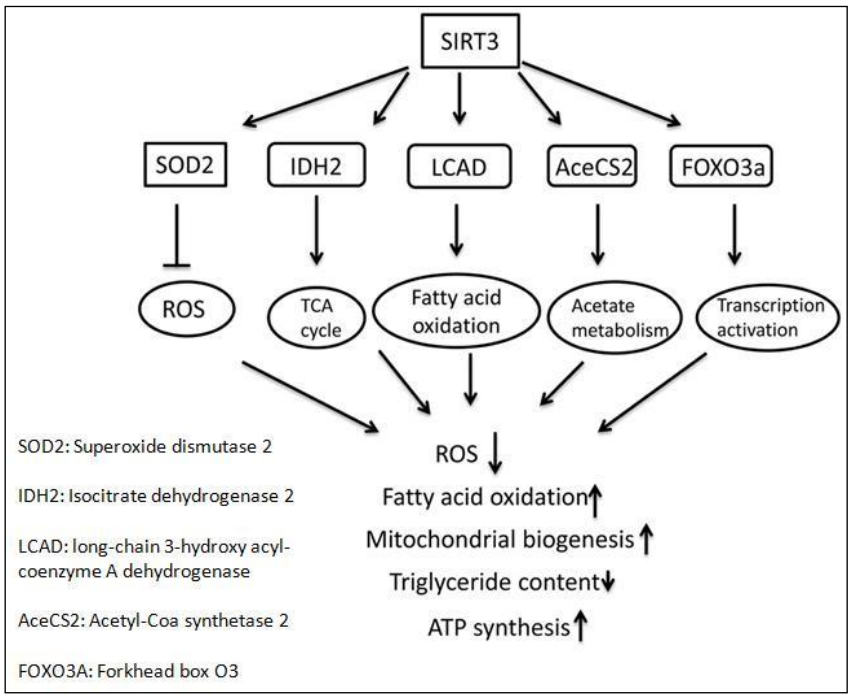

Figure 1.10: Schematic overview of molecular targets of SIRT3 and its role in mitochondrial metabolism (*90*).

1.10.1 *SIRT3 activity and localisation*

SIRT3 is nuclear-encoded and synthesised on ribosomes as larger precursors. It contains a pre-sequence called the MTS at the N-terminus which allows it to translocate into mitochondria (*91*).

Under OS conditions, full length (FL) SIRT3 is transcribed and transported into mitochondria where the MTS is cleaved off by a matrix-located metalloproteinase, called mitochondrial processing peptidase (MPP), resulting in activated SIRT3 (*92*) (Fig 1.11).

Over-expressed SIRT3 is localised in mitochondria and smaller fractions are localised to the nucleus, but both exhibit deacetylase activity (*92*). Full-length SIRT3 accumulates in the nucleus and deacetylates histones (*88*).

In mitochondria SIRT3 regulates energy metabolism and is highly expressed in metabolically active tissues such as the liver, muscle, kidney, heart, and brain.

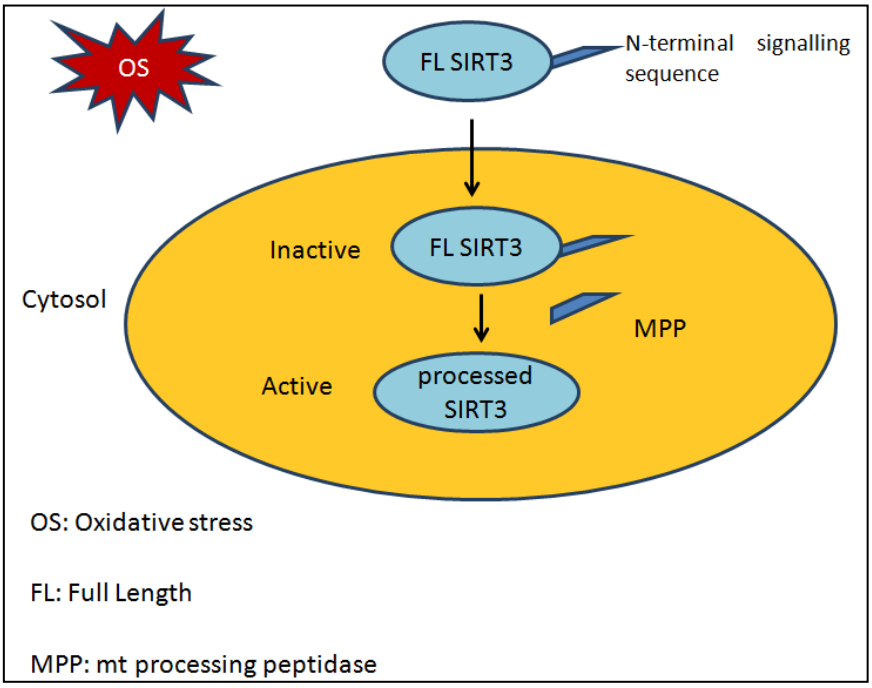

Figure 1.11: Mechanism of SIRT3 activation in mitochondria (Author's own work)

1.10.2 *SIRT3 and ETC*

Decreased SIRT3 leads to 74% and 60% reduction in complex III and IV activities respectively, suggesting that SIRT3 is necessary for efficiency of the ETC (*93*). In SIRT3 knockout mice, reduced global ATP was observed (*93*). Taken together, SIRT3 plays a role in regulating the ETC as well as ROS generation.

1.10.3 *SIRT3 in mitochondrial oxidative stress*

SIRT3 regulates cell defence and survival in response to stress (*14, 15*). It decreases mitochondrial ROS production and protects against mtDNA damage (*16, 21*).

Altered mitochondrial function and increased levels of OS are a result of decreased SIRT3 expression in SIRT3 knockdown (KD) mouse myoblast (C_2C_{12}) cells (*94*). SIRT3 KD in human luteinized granulosa cells also showed elevated ROS generation (*95*).

SIRT3 over-expression in murine adipocyte cell lines (*22*) and mouse cardiomyocytes (*15*) resulted in reduced levels of ROS. Similar results were observed in human oral keratinocytes (HOK) (*23*) where SIRT3 transcript and protein levels were increased following OS. A study by Shulyakova *et al.* (2014) showed significantly decreased levels of ROS following SIRT3 over-expression in PC12 and HEK 293 cells (*96*). These findings are consistent with the studies in primary neurons (*24*), adipocytes of mice (*22*) and HEK 293 cells (*14*).

These studies suggest that SIRT3 acts as a cyto-protective factor by suppressing OS.

1.10.3.1. *SIRT3 and AO defence*

SIRT3 reduces ROS levels by modulating AO enzymes (SOD2 and IDH2), thereby protecting against oxidative damage (Fig 1.12).

The SOD2 lysine residues are deacetylated by SIRT3, which increases SOD2 activity and reduces ROS levels (*14*). It is considered the first line of defence against OS. SIRT3 deficiency in mouse embryonic fibroblasts and liver tissue resulted in reduced SOD2 activity and increased ROS production (*27*).

SIRT3 and SOD2 levels were down-regulated following severe OS in human mesenchymal stromal/stem cells (hMSCs) whilst over-expression of SIRT3 improved hMSCs resistance to OS (*25*). SIRT3 regulates SOD2 activity in skeletal muscle of obese pregnant women with gestational diabetes mellitus (*26*).

In SIRT3-deficient cardiomyocytes, elevated ROS levels were observed following stress-induced cardiac hypertrophy (*15*). However, in cardiomyocytes over-expressing SIRT3, SOD2 and catalase levels were up-regulated (*15*). Furthermore, HEK 293 cells over-expressing SIRT3 displayed elevated GSH levels, which protected cells from H_2O_2-induced insult (*28*).

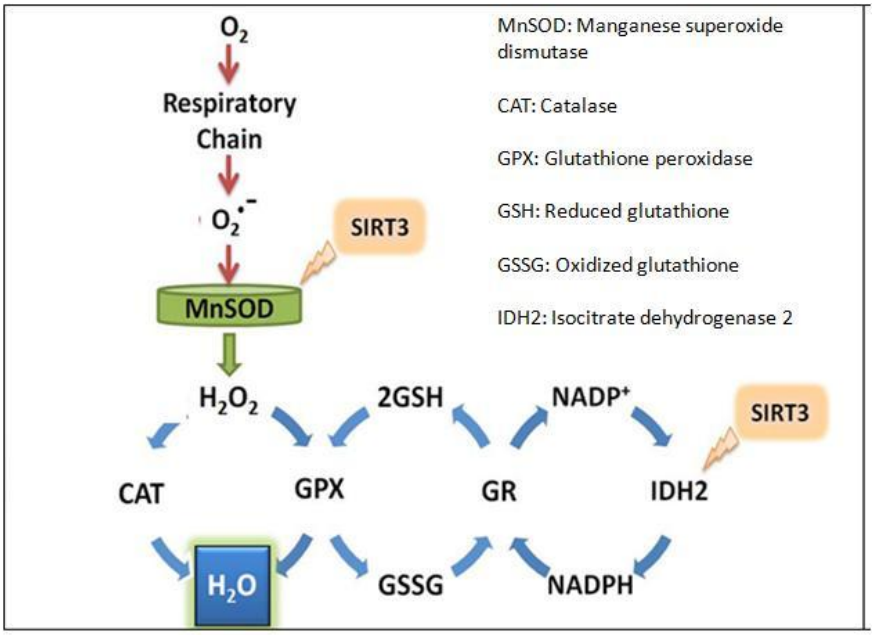

Figure 1.12: SIRT3 promotes antioxidant defence (*83*)

SIRT3 was also found to deacetylate IDH2, resulting in increased activity of IDH2 in both mice and HEK 293 cells (*27-30*). It has been stated that SIRT3 is dependent on IDH2 to protect cells from OS (*30*).

IDH2 is the enzyme required for the conversion of isocitrate to α-ketoglutarate in mitochondria, generating NADPH from $NADP^+$. NADPH is required for the regeneration of GSH; therefore, increased IDH2 activity enhances the activity of GSH in AO defence (*83*).

Han and Someya (2013) showed that SIRT3 slowed the progression of age related hearing loss (AHL) by promoting the GSH AO defence in mice (*31*). An earlier study showed that SIRT3 reduced oxidative DNA damage by enhancing GSH AO defence in mice as well (*28*). They also found that over-expressing SIRT3 and/or IDH2 increases NADPH levels and protects cells from OS-induced cell death (*28*).
SIRT3-depleted HepG2 cells showed impaired ETC functioning and reduced mitochondrial membrane potential (*97*). Increasing SIRT3 levels ameliorated these effects.

A synergistic relationship has been established between mitochondrial SIRT3 and AO defence to improve mitochondrial function and cell survival in response to stress (*94*).

1.10.4 *SIRT3 and transcription factors*

1.10.4.1 *PGC-1α*

Peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) is a transcriptional co-activator that is activated under oxidative conditions and interacts with transcription factors that are involved in mitochondrial biogenesis and AO defence (*32*).

cAMP response element-binding protein (CREB) is a nuclear transcription factor that is involved in mitochondrial biogenesis as it controls the expression of PGC-1α (*98*). Phosphorylation of CREB at serine-133 is essential for its activation.

In primary endothelial cells, PGC-1α expression resulted in up-regulated mitochondrial numbers, mitochondrial activity and AO enzymes (*99*). It has been shown that PGC-1α is specific to the mitochondria (*99*) and that it regulates *Sirt3* gene expression (*33*).

A sequence in the *Sirt3* promoter is recognized by the orphan nuclear receptor, estrogen-related receptor-alpha (ERRα). PGC-1α mediates the binding of ERRα to the *Sirt3* promoter which promotes *Sirt3* gene transcription (*33*). SIRT3, which is as a target gene of PGC-1α, also mediates the effects of PGC-1α on AO defence (*33*) (Fig 1.13).

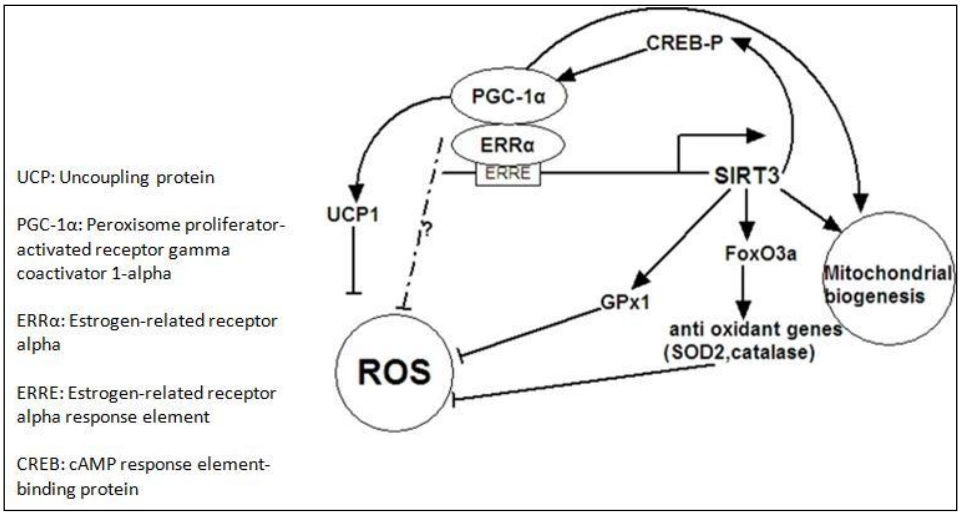

Figure 1.13: Positive feedback cycle between SIRT3 and PGC-1α (*33*)

The positive feedback cycle that exists between SIRT3 and PGC-1α further reduces ROS levels and increases AO defence under oxidative conditions. A study using HEK 293 and HepG2 cells showed that SIRT3 expression is required to induce AO enzymes by PGC-1α (*33*). The expressions of SOD2 and GPx1 were reduced in SIRT3 KD cell lines, resulting in decreased ROS protection by PGC-1α (*33*).

In pre-diabetic rats, the levels of PGC-1α, SIRT3 and AO capacity were decreased whilst lipid peroxidation was increased (*100*). Pre-diabetes repressed the PGC-1α/ SIRT3 axis, reduced respiratory capacity and increased OS, thereby compromising mitochondrial function (*100*).

1.10.4.2 *Nuclear factor-kappa B*

Nuclear factor-kappa B is a transcription factor that regulates immune response and inflammation.
The NF- κB family consists of five proteins: p105 (p50 when processed), p100 (p52 when processed), and the Rel subfamily consisting of RelA (p65), RelB, and c-Rel (*101*). These proteins form homo- or heterodimers that have distinct functions (*101*). Only p65, c-Rel, and RelB possess C-terminal transactivation domains that confer the ability to initiate transcription (*101*).

NF- κB is located in the cytoplasm as an inactive NF-κB/IκB complex. IκB kinases phosphorylate IκBs and target them to ubiquitination and proteasomal degradation. This enables NF- κB to translocate to the nucleus and transcribe genes responsible for inflammation and survival (*102*, *103*).
Activation of NF- κB occurs in response to bacterial products, viral infection, OS and therapeutics.
NF- κB mediates destruction and protection signals; however, the main role of NF- κB is considered to be protective (*104*).

Oxidative stress induced by reperfusion insult in cultured neurons was associated with NF- κB inducing a protection signal rather than death signals (*105*). Other studies have also shown that activation of NF- κB led to neuro-protection, whereas inhibition led to apoptosis (*106*, *107*).

It was also found, in an embryonal rat heart-derived cell line, that NF- κB is a target of SIRT3 (*108*). Activation of NF- κB by SIRT3 increased the expression of the downstream genes of NF- κB (*SOD2, Bcl-2 and Bax*) making cells resistant to OS (*108*). This demonstrated that SIRT3 protected cardiomyocytes from OS-induced apoptosis by activating the NF- κB pathway.

1.10.5 *SIRT3 and genomic integrity*

SIRT3 deficiency may also result in genomic instability. Cheng *et al.* (2013) demonstrated in human glioblastoma cell lines (LN229) the ability of SIRT3 to deacetylate OGG1 (*16*). Loss of SIRT3 resulted in increased OGG1 acetylation, degradation and loss of OGG1 activity. SIRT3 stabilized the OGG1 protein and sustained its capacity to repair mtDNA (*16*).

Interestingly, it has also been shown that SIRT3 deacetylates and activates Lon in mitochondria (*109*) (Fig. 1.14). Lon not only degrades oxidized and damaged proteins, but also participates in mtDNA maintenance and replication.

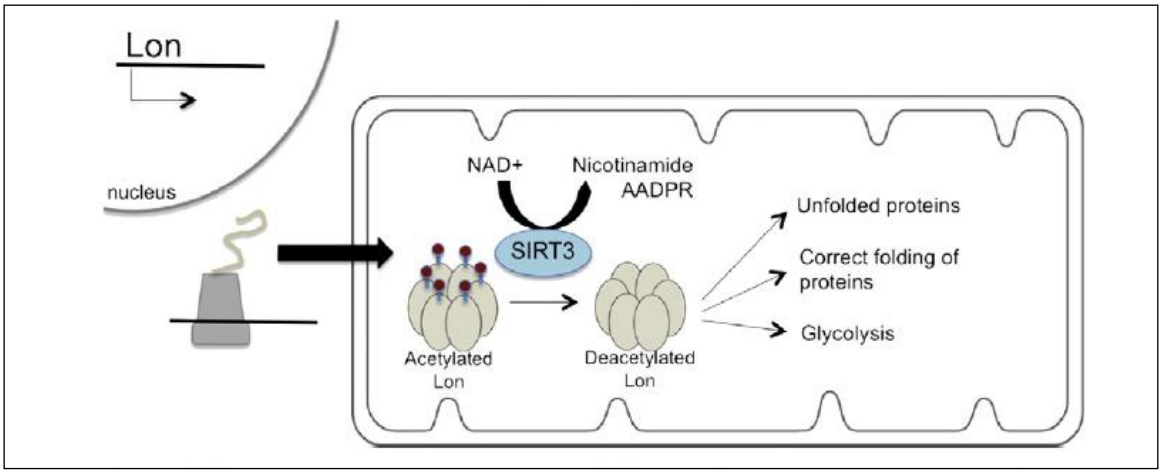

Figure 1.14: Lon is regulated by SIRT3 deacetylation (*109*)

1.11 Metformin and DMII

Metformin (1, 1-dimethylbiguanide) is a biguanide class drug used for the treatment of DMII. It lowers blood glucose concentrations in DMII without causing hypoglycaemia.

1.11.1 *Site of action*

The liver is an important target organ in DMII as it is exposed to high concentrations of metformin through the portal circulation following oral ingestion. The hepatocytes express high levels of organic cation transporter 1 (OCT1), which facilitates uptake of metformin (*110*). Once inside the cytosol, mitochondria are the primary target of metformin (*34*).

1.11.2 *Mechanism of action*

Metformin lowers blood glucose levels by reducing gluconeogenesis in the liver, which is an ATP-dependent process (*111*). Therefore, metformin elicits its activity by decreasing ATP synthesis in the ETC.

The proposed mechanism for metformin action is the inhibition of mitochondrial respiration at complex I of the ETC (*112*). This, however, does not affect any other steps of OXPHOS (*112*) (Fig 1.15).

This inhibition results in decreased NADH oxidation, decreased proton gradient, reduced ATP synthesis and, as a result, elevated concentrations of ADP (*112*). This elevates the concentration of AMP which activates adenosine monophosphate-activated protein kinase (AMPK), an enzyme involved in glucose uptake. A new mechanism has been observed where AMP deaminase may be inhibited by metformin (*113*) (Fig 1.16).

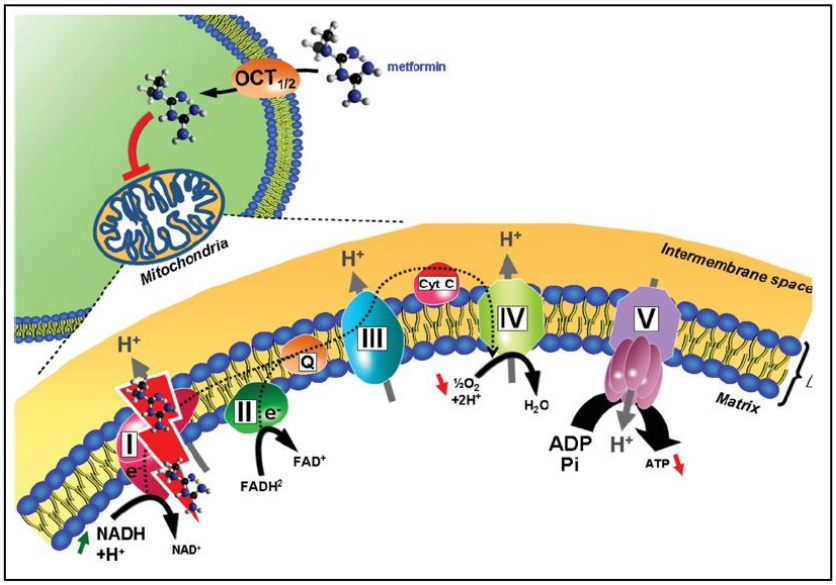

Figure 1.15: Metformin targets the mitochondrial respiratory chain complex I (*112*)

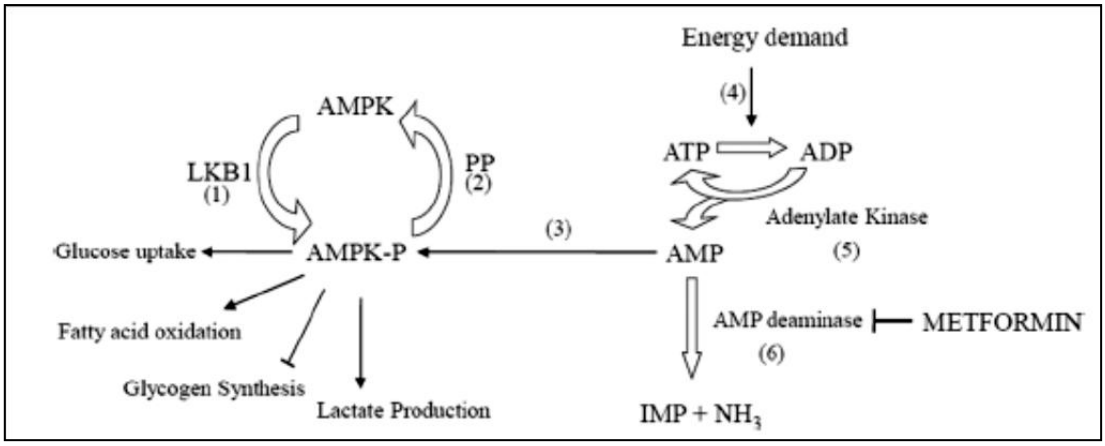

Figure 1.16: Proposed mechanism of action of metformin AMP deaminase inhibition (*113*)

1.11.3 *Metformin and ROS*

Metformin inhibits mitochondrial ROS production by blocking the reverse electron flow through complex I of the ETC (*34*).

The regulatory role of metformin was investigated in inflammatory and AO pathways in global cerebral ischemia (*114*). It was found that pre-treatment with metformin inhibited inflammatory factors (TNFα, NF- κB and COX2) and increased the Nrf2 AO pathway (*114*). Metformin also modulated GSH activity (*114*). *In vitro* data have shown that metformin could induce AO enzymes, such as SOD2, and suppress OS (*35*).

Metformin was also shown to significantly decrease serum levels of 8-oxoG, a marker of oxidative stress in DNA, in women with polycystic ovary syndrome (*37*).

Chronic metformin treatment resulted in healthier mice with longer lifespan; therefore the length of exposure to metformin may improve health and lifespan in humans (*115*). Metformin reduced OS by increasing AO enzyme activity (*36*). These findings also show that the dosage of metformin should be higher in order to observe additional beneficial results (*36*).

To date, the exact molecular mechanisms of metformin action are still poorly understood especially regarding modulation of AO defence.

1.11.4 *Metformin and SIRT3*

SIRT3 regulates mitochondrial metabolism and it may also be involved in the pharmacological effects of metformin. The molecular mechanisms underlying this process are unclear.

A study has shown that over-expression of SIRT3 in HepG2 cells reduced the inhibition of ATP synthesis by metformin (*116*). A separate study evaluated the effects of SIRT3 on OS in skeletal muscle and they found that metformin increased the expression of SIRT3 and SOD2 (*117*). Knockdown of SIRT3 significantly reversed the metformin-induced increase in SOD2 (*117*).

These studies suggest that metformin could induce AO defence through SIRT3 up-regulation. Metformin, however, may also down-regulate SIRT3 to inhibit complex I of the ETC as part of its mechanism. Therefore, SIRT3 may be differentially regulated by metformin.

1.12 Curcumin in DMII

Natural compounds, such as curcumin, have become promising agents to reduce the risk of OS-induced diseases.

Turmeric rhizome includes three analogue components: curcumin, demethoxy curcumin and *bis* demethoxy curcumin which collectively are called curcuminoids. Of the three curcuminoids, curcumin is the most abundant and diffuses freely into cells (*118*).

Curcumin is an important therapeutic agent in traditional medicine and possesses multiple biological properties including AO (*119*), antibacterial (*120*) and anti-inflammatory (*121*). Furthermore, curcumin is a therapeutic against neurodegenerative (*122*), cardiovascular (*123*), hepatic (*124*), and renal diseases (*125*).

1.12.1 *Structure*

Curcumin is a β-diketone molecule (1, 7-bis (4-hydroxy-3-methoxyphenol)-1, 6- heptadiene-3, 5-dione) that contains two ferulic acid residues linked by a methylene bridge and has two hydrophobic phenyl regions (*126*) (Fig. 1.17).

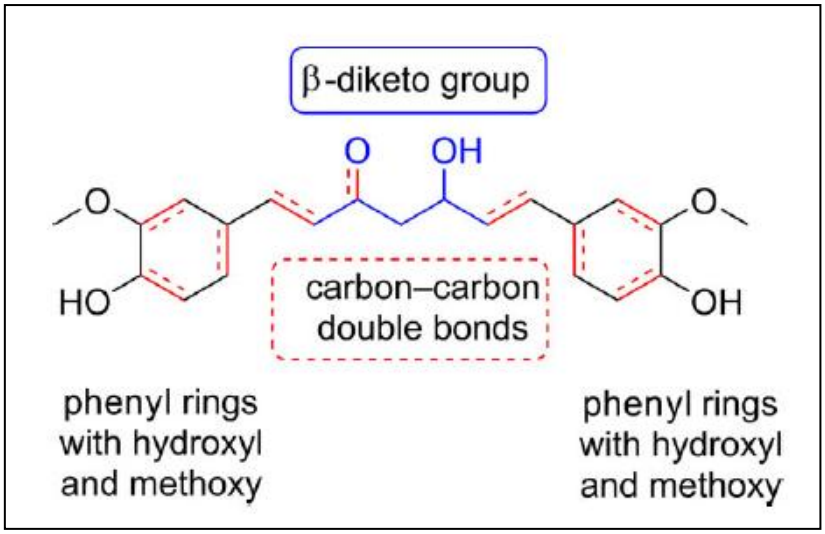

Figure 1.17: Functional groups of curcumin (*126*).

1.12.2 *Mechanism of action*

Curcumin is a bi-functional AO as it scavenges ROS and RNS species and also indirectly up-regulates cyto-protective and AO gene transcription (*126*) (Fig 1.18).

(a) ROS scavenger**:**

Curcumin is an effective AO that scavenges O_2 •, •OH, H_2O_2 and (NO•) (*38*).
The scavenging activity of curcumin is associated with (a) the β-diketone group, (b) carbon–carbon double bonds, (c) phenyl rings and (d) keto and enol form in aqueous solutions.

(b) Indirect AO properties of curcumin:

Curcumin induces the expression of cyto-protective proteins such as SOD, GPx1, heme oxygenase-1 (HO-1), glutathione-S-transferase (GST) and g-glutamylcysteine ligase (gGCL), which is involved in biosynthesis of GSH (*39*). The transcription factor Nrf2 is the major regulator of these AO enzymes (*39*) (Fig 1.18).

Curcumin reduced arsenic-induced hepatic injuries and OS in experimental mice through the Nrf2 pathway (*127*). Curcumin also protected human neuroblastoma cells against acrolein toxicity (*128*).

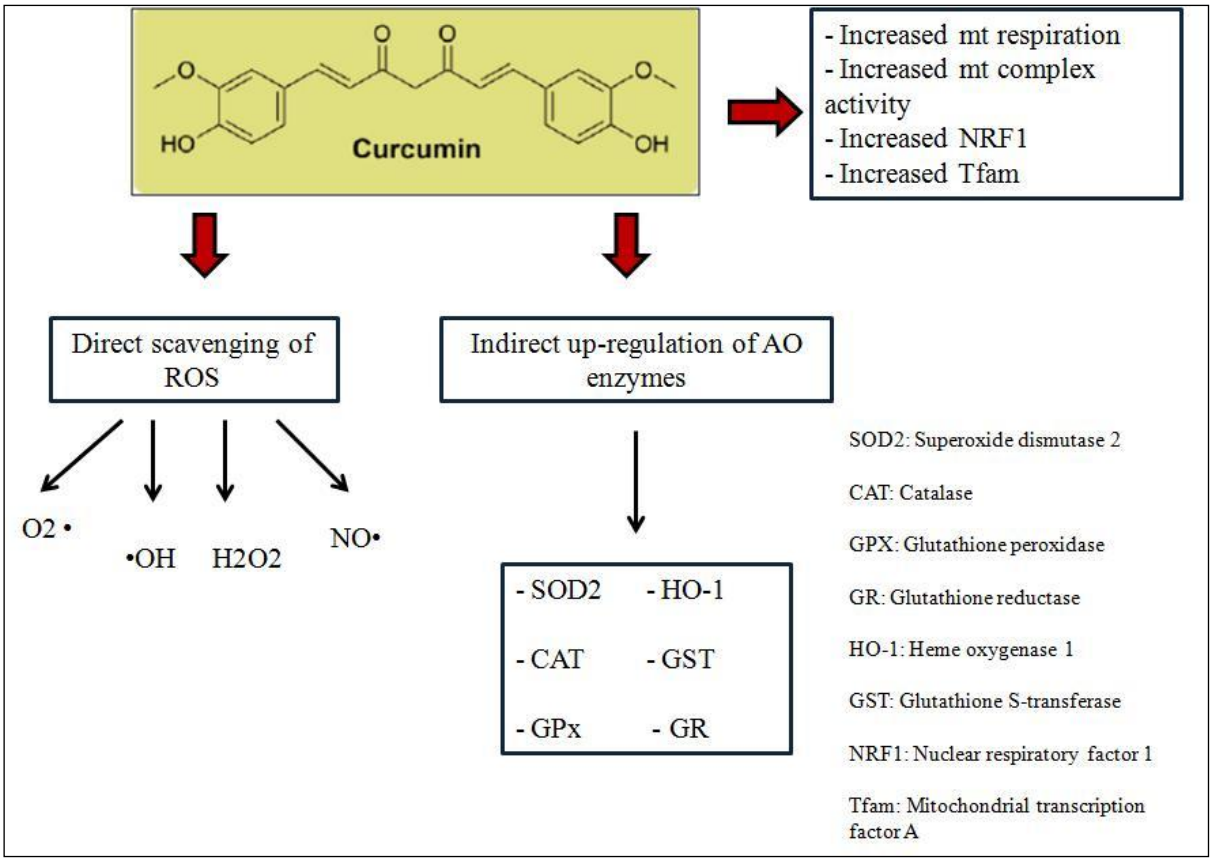

Figure 1.18: The bi-functional AO properties of curcumin (*126*)

1.12.3 *Mitochondrial dysfunction and curcumin*

Curcumin pre-treatment prevented OS and mitochondrial dysfunction in potassium dichromate- induced nephrotoxicity by inducing Nrf2 (*129*). Curcumin increased aconitase activity, a TCA cycle enzyme that functions as a marker of stress in mitochondria (*126*).

Oxidative phosphorylation in rat heart-mitochondria was also increased following treatment with curcumin (*123, 130*). Curcumin increases the expression of genes involved in mitochondrial biogenesis, such as PGC-1α, nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (Tfam) (*131*).

1.12.4 *Curcumin and diabetes*

Curcumin improves metabolic disorders and glycaemic control in DMII mouse models (*132*).
Curcumin decreases blood glucose levels and increases plasma insulin levels in STZ-induced diabetic rats by suppressing OS (*133, 134*).

In mouse embryos, curcumin reduced hyperglycaemic-induced neural tube defects by suppressing OS and caspase activation (*135*). Curcumin administration in pancreatic tissue of STZ rats reduced blood glucose levels and reduced OS markers by increasing Nrf2 and HO-1 as well as glucose transporter 2 (GLUT 2) (*136*).

Curcumin administration has also been shown to prevent the AGE-induced complications of DMII (*40*), prevent the decrease in the AO capacity and prevent an increase in 8-oxoG in the retina of diabetic rats (*41*).

Treatment with curcumin for two weeks reduced renal dysfunction and OS in diabetic animals (*42*). It was also found to improve hepatic function markers and protein levels in experimental DMII rats (*43*).
In cultured monocytes exposed to high levels of glucose, curcumin supplementation decreased proinflammatory cytokines and glycosylated haemoglobin (*137*).

The mechanisms by which curcumin inhibits OS have yet to be fully investigated. Understanding the molecular mechanisms could lead to the development of natural therapeutics in OS-associated diseases.

Aims:

(i) To determine SIRT3 and downstream antioxidant expression under hyperglycaemic conditions.

(ii) To determine the effect of metformin treatment on SIRT3 and antioxidant expression under hyperglycaemic conditions.

(iii) To determine the effect of curcumin treatment on SIRT3 expression, antioxidant defence, lon protease and heat shock protein 70 under hyperglycaemic conditions.

(iv) To determine the effect of cell survival genes on apoptotic stress under hyperglycaemic conditions.

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CHAPTER 2

Increased SIRT3 Expression and Antioxidant Defence under Hyperglycaemic Conditions in HepG2 Cells

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2.1 Abstract

Background: Hyperglycaemia exacerbates the production of mitochondrial reactive oxygen species and this contributes to a variety of pathological conditions (*1*). SIRT3 has been shown to play a role in decreasing oxidative stress, and improving disease outcomes, by regulating antioxidant defence (*2*). Our understanding of molecular events during oxidative stress under chronic hyperglycaemia in the liver is limited. We postulated that SIRT3 may play a role in antioxidant defence under hyperglycaemic conditions in HepG2 cells.

Methods: Cell viability was determined in HepG2 and HEK 293 cells cultured in the presence of 5mM glucose (control), 19.9mM mannitol (OC), 10mM glucose, 30mM glucose (hyperglycaemic) and NAM (10mM) at 24hr and 72hr time points. SIRT3, PGC-1α, p-CREB protein expressions were measured by western blot. Mitochondrial antioxidant enzymes GPx1, SOD2, UCP2 and mtDNA repair enzyme OGG1 were evaluated by qPCR.

Results: Increased cell viability and protein expressions of SIRT3, p-CREB and PGC-1α were observed under hyperglycaemic conditions at 24hr. These were further elevated at the 72hr time point. We also observed higher fold changes of *SIRT3, GPx1, SOD2, UCP2* and *OGG1* in the treated groups. Treatment with NAM decreased protein and gene expressions of SIRT3, p-CREB, PGC-1α, GPx1, SOD2, UCP2 and OGG1 at both time points in the hyperglycaemic groups.

Conclusions: Our data may allude to the relationship that has been established between SIRT3 and PGC-1α with regard to increased antioxidant defence during oxidative stress. This suggests that SIRT3 may play a role in increasing antioxidant defence and conferring resistance to oxidative stress-induced damage under hyperglycaemic conditions in the human hepatoma cell line.

2.2 Introduction

Reactive oxygen species (ROS) are a natural by-product of cellular respiration. An imbalance between production of ROS and the cell's ability to detoxify ROS disturbs the cellular reducing environment resulting in oxidative stress (OS). This can lead to accumulated damage of various components of the cell including DNA, proteins and lipids resulting in pathological conditions such as cancer, diabetes and cardiac disease.

Mitochondria are the primary source of ROS which are generated through the electron transport chain (ETC), during oxidative phosphorylation (OXPHOS). The low levels of ROS generated from the ETC are necessary for signalling pathways. However, under hyperglycaemic conditions more electron transfer donors are generated from the tricarboxylic acid (TCA) cycle resulting in a flux of electrons entering the ETC. This causes partial inhibition of the ETC at complex III leading to accumulation of electrons, resulting in increased production of superoxide radicals (*1*).

Mitochondrial DNA (mtDNA) is prone to oxidative damage as it is (a) closely located to the ETC, (b) lacks protective histones and (c) has limited DNA repair systems (*3*). Mitochondrial DNA damage compromises mitochondrial transcription, OXPHOS protein synthesis and antioxidant (AO) activity (*4*) resulting in a highly oxidative environment which has been observed in metabolic disorders and disease conditions (*5*). This may be improved by reducing OS.

Cells have developed a complex network of cellular stress response mechanisms viz. sirtuins (SIRTs). Sirtuins are a highly conserved family of NAD^+-dependent histone deacetylases localised throughout the cell. They target a variety of substrates to co-ordinate cellular metabolism, genomic integrity and stress resistance (*6*).

Sirtuin 3 (SIRT3) functions as a primary mitochondrial stress-responsive protein deacetylase. SIRT3 deacetylates several metabolic and respiratory enzymes that regulate mitochondrial processes such as the TCA cycle and OXPHOS (*7*). It can also reduce ROS levels by directly modulating key AO enzymes, such as superoxide dismutase 2 (SOD2), thereby acting as a shield against oxidative damage (*2*).

SIRT3 also regulates AO defence through peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), a transcriptional co-activator that regulates respiration, mitochondrial biogenesis, antioxidant activation (glutathione peroxidase 1 (GPx1) and SOD2) and ROS attenuating genes such as uncoupling protein 2 (UCP2).

Oxidative stress increases the expression of PGC-1α which induces SIRT3 expression through activation of estrogen-related receptor alpha (ERR-α). In turn, SIRT3 stimulates PGC-1α expression through phosphorylated c-AMP response element binding protein (p-CREB) thereby forming a positive feedback cycle and increasing AO defence (*8*). Although no direct regulation of PGC-1α by SIRT3 has been reported, a study showed that SIRT3 expression is required for the induction of AO enzymes by PGC-1α (*9*).

Studies have shown the ability of SIRT3 to protect cells against oxidative damage in different cell lines. SIRT3 has been shown to play an essential role in mediating cell survival in cardiac myocytes (*10*). Kong *et al*. (2010) showed that over-expression of SIRT3 in mouse muscle cells decreased ROS levels, whereas knockdown of SIRT3 increased basal ROS levels (*9*). Over-expression of SIRT3 also reduced oxidative damage and increased glutathione AO defence in mice (*11*).

Oxidative stress can induce many types of DNA base damage including 7, 8-dihydro-8-oxoguanine (8-oxoG) (*12*). This results in a lack of base pairing specificity and an increase in the frequency of spontaneous G.C- -T.A transversion mutations. Mutations in mtDNA are an underlying factor in many mitochondrial diseases.

The mammalian DNA glycosylase, OGG1, recognizes and removes 8-oxoG that is base-paired with cytosine. Mitochondria have been shown to be the primary site of OGG1 DNA repair activity (*13*). Several groups have demonstrated that over-expression of mitochondria-targeted OGG1 prevents mtDNA damage (*14, 15*). Interestingly, it has been shown that SIRT3 directly deacetylates and activates OGG1 enabling DNA repair and genomic stability (*16*) under oxidative conditions.

Oxidative stress is the leading cause of various pathological conditions; however, our understanding of molecular events during OS under long term hyperglycaemia in the liver is limited. This is important as the liver is the primary organ involved in glucose homeostasis. This is the first study to our knowledge that investigated the role of SIRT3 in stress response under long term hyperglycaemic conditions in a human hepatoma cell line.

2.3 Materials and Methods

Cell Culture and Treatments

HepG2 cells were cultured to confluency in 25cm^3 flasks (5% CO_2, 37°C) in complete culture media (CCM, Eagles minimum essential medium, supplemented with 10% foetal calf serum, 1% L-Glutamine and 1% penstrepfungizone). We also used the human embryonic kidney (HEK 293) cell line as a positive control. HEK 293 cells were cultured to confluency in 25cm^3 flasks (5% CO_2, 37°C) in complete culture media (Dulbecco's Modified Eagle's medium, supplemented with 10% foetal calf serum, 1% L-Glutamine and 1% penstrepfungizone).

The HepG2 and HEK 293 cells (2.5 x 10^4 cells/ well) were cultured in the presence of control (5.5mM glucose), osmotic control (OC) (19.9mM mannitol) and hyperglycaemic (10mM glucose (intermediate dose) , 30mM (hyperglycaemic dose) glucose) conditions for 24hr and 72hr in 30mm^3 cell culture plates. Cells were treated with the SIRT3 inhibitor nicotinamide (NAM) at a concentration of 10mM.

Cell Viability Assay

Following treatment, the cells (3 replicates) were incubated with methyl thiazol tetrazolium (MTT) salt solution (5mg/mL in 0.1M phosphate buffered saline (PBS)) and CCM (4h, 37ºC). Following incubation, the supernatants were aspirated and dimethyl sulphoxide was added (100 µL/well) and incubated at 37ºC for a further 1h. Optical density of the formazan product was measured by a microplate reader (Bio-tek µQuant) at 570 nm with a reference wavelength of 690nm.

Quantitative Polymerase Chain Reaction (q-PCR)

Total RNA was extracted from cultured cells using the Triazol reagent (Ambion). cDNA was synthesised by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad).
Quantitative PCR was performed on the CFX96 Real-Time System (Bio-Rad) by using iQ SYBR Green supermix (BioRad) with primer sequences as listed in Table 2.1. The PCR was initiated with the following thermocycler profile: an initial denaturation for 8min at 95°C followed by 39 cycles of 95°C denaturation for 15s, annealing for 1min and extension of 72°C for 30s. A final extension at 60°C was performed for 31s. Each measurement was done in triplicate and normalized against 18S rRNA Ct values. The qPCR data is represented as a relative fold change, calculated using the method described by Livak and Schmittgen (*17*).

Table 2.1: Primer sequences and optimised annealing temperature

Gene	Sense (5'-3')	Antisense (5'-3')	Annealing temp (°C)
18S rRNA	ACACGGACAGGATTGACAGA	CAAATCGCTCCACCAACTAA	58°C
SIRT3	GCATTCCAGACTTCAGATCGC	GTGGCAGAGGCAAAGGTTCC	50°C
CREB	GATGGACAGCAGATCTTAGTGCC	TGCTGTGCAAATCTGGTATGTT	65°C
PGC-1α	CCAAACCAACAACTTTATCTCTTCC	CACACTTAAGGTGCGTTCAATAGTC	65°C
GPx1	GACTACACCCAGATGAACGAGC	CCCACCAGGAACTTCTCAAAG	60°C
SOD2	GAGATGTTACAGCCCAGATAGC	AATCCCCAGCAGTGGAATAAGG	58°C
UCP2	GACCTATGACCTCATCAAG	ATAGGTGACGAACATCACCACG	50°C
OGG1	GCATCGTACTCTAGCCTCCAC	AGGACTTTGCTCCCTCCAC	60°C

Western blot analysis

Sample proteins were isolated using a Cytobuster™ (Novagen) supplemented with protease and phosphatase inhibitors (Roche). Proteins were quantified using the bicinchoninic acid (BCA) assay (Sigma) and standardised to 1mg/ml. Protein extracts were prepared in Laemlii buffer (dH_2O, 0.5M Tris-Cl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% resolving gel) and electrotransferred to nitrocellulose membrane. Membranes were blocked with 5% BSA in Tris-Buffered Saline and Tween 20 (TTBS) [137mM NaCl, 2.7mM KCl, 24mM Tris, 0.5% Tween 20]. Membranes were probed using the following antibodies in 5% bovine serum albumin (BSA in TTBS): rabbit anti- SIRT3 (Abcam, ab86671, 1:1000); goat anti-PGC-1α (Abcam, ab72230, 1:1000) and goat anti phospho-CREB (Cell Signalling Technology, cat. No 9191, 1:1000). All membranes were incubated in primary antibodies overnight at 4°C. A horseradish peroxidase (HRP)-conjugated goat polyclonal antibody specific for β-actin was used for the loading control and the normalisation process (1:5000 in 5% BSA in TTBS). Membranes were developed using LumiGlo® Chemiluminescent Substrate System (KPL) and images were captured on the Alliance 2.7 documentation system (UViTech). The density of the bands was quantified using UViBand Advanced Image Analysis Software (UViTech). The experiment was performed in triplicate and repeated thrice. Results are presented as relative band intensity and normalised against β-actin.

Statistical analysis

Each experiment was performed in triplicate and repeated thrice. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one way ANOVA followed by Bonferroni test for multiple group comparison. Differences with $p<0.05$ were considered statistically significant.

2.4 Results

Mitochondrial output increases under hyperglycaemic conditions

In order to determine the effect of hyperglycaemia on mitochondrial output, cell viability was measured in both HepG2 and HEK 293 cells.

In the HepG2 cells, cell viability was significantly higher in the hyperglycaemic treatments at both 24hr (p=0.0140, 95% CI: control 0.1898- 0.8632 O.D; 10mM 0.6564- 0.8596 O.D, p=0.0119, 95% CI: control 0.1898- 0.8632 O.D; 30mM 0.6793- 0.8762 O.D **Fig 2.1A**) and 72hr time points compared to their respective controls (p=0.0036, 95% CI: control 0.1898- 0.8632 O.D; 10mM 0.3869-1.067 O.D, p=0.0106, 95% CI: control 0.1898- 0.8632 O.D; 30mM 0.6420- 0.9850 O.D **Fig 2.1A**).

In the HEK 293 cells, the hyperglycaemic treatments were relatively higher at both 24hr and 72hr time points than their respective controls; however these did not reach statistical significance (**Fig 2.1B**). NAM-treated cells resulted in decreased cell viability in the hyperglycaemic treatments in both the HepG2 and HEK 293 cells at both time points (**Fig 2.1A and Fig 2.1B**).

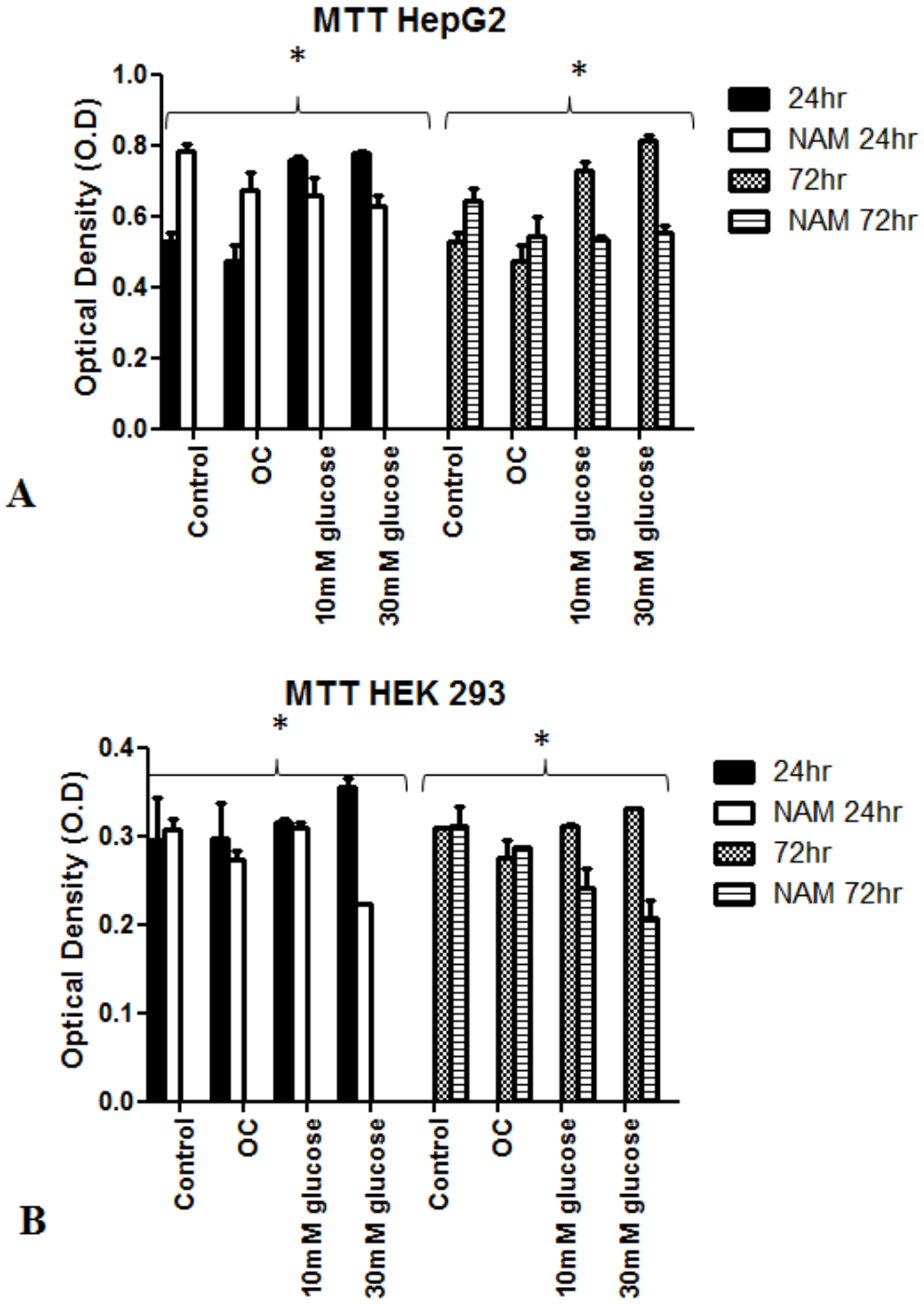

Figure 2.1: The effect of hyperglycaemia on cell viability. (A) HepG2 cells (normal and NAM-treated) and (B) HEK 293 cells (normal and NAM-treated) were subjected to a colorimetric assay that measured cell viability. Values are expressed as mean ± SEM. *$p<0.05$ vs. Control.

Increased SIRT3 expression under hyperglycaemic conditions

To determine whether hyperglycaemic conditions had an effect on SIRT3 expression, western blot and qPCR were used to measure protein and gene expression of SIRT3, respectively.

In HepG2 cells, higher SIRT3 protein expression was observed in the hyperglycaemic treatments relative to the control at the 24hr time point; however these were not statistically significant **Fig 2.2A**.

At the 72hr time point, higher protein expression was seen in both hyperglycaemic treatments relative to the control with 30mM reaching statistical significance (p= 0.0007; 95% CI: control 0.7559- 3.290 RBD; 30mM 4.732- 7.197 RBD **Fig 2.2A**). Quantitative PCR data also showed higher levels of SIRT3 in the 24hr and 72hr 10mM (p=0.0002) and 30mM (p=0.0030) groups relative to the control (5.3-fold and 6.4-fold respectively) **Fig 2.2B**.

In the HEK 293 cells, higher SIRT3 protein expression was seen in the hyperglycaemic groups at both 24hr (p=0.0003; 95% CI: control 0.3952-1.263 RBD; 30mM 1.899- 2.425 **Fig 2.3A**) and 72hr time points (p=0.0098; 95% CI: control 0.7908- 1.369 RBD; 10mM 1.218- 3.921; p=0.0194; 95% CI: control 0.7908- 1.369 RBD; 30mM 0.8772- 4.578 **Fig 2.3A).** SIRT3 transcript fold changes were also higher in the 72hr 10mM (p=0.0002) and 30mM (p<0.0001) groups relative to the control (5.1-fold and 9.1-fold respectively) **Fig 2.3B**.

NAM-treated cells resulted in lower SIRT3 protein expression in the 10mM and 30mM groups at 24hr and 72hr time points in both HepG2 and HEK 293 cells **Fig 2.2A and Fig 2.3A**. Quantitative PCR also showed decreased gene expression in the hyperglycaemic groups over 24hr and 72hr in both cell lines **Fig 2.2B and Fig 2.3B**.

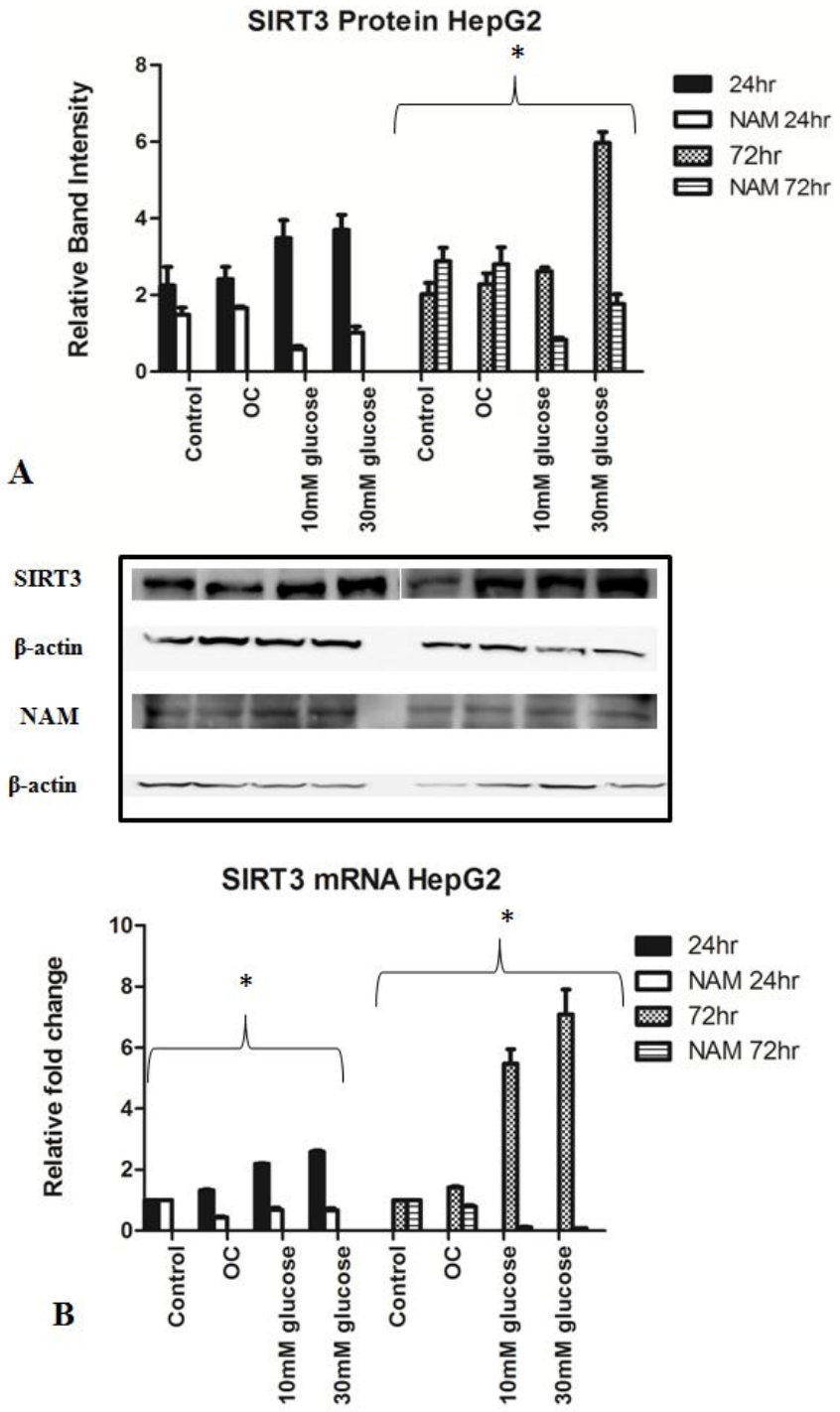

Figure 2.2: The effect of hyperglycaemia on SIRT3 expression in normal and NAM-treated HepG2 cells. (A) SIRT3 protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of SIRT3 band intensity normalised to β-actin. (B) Gene expression for SIRT3 was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *$p<0.05$ vs. Control.

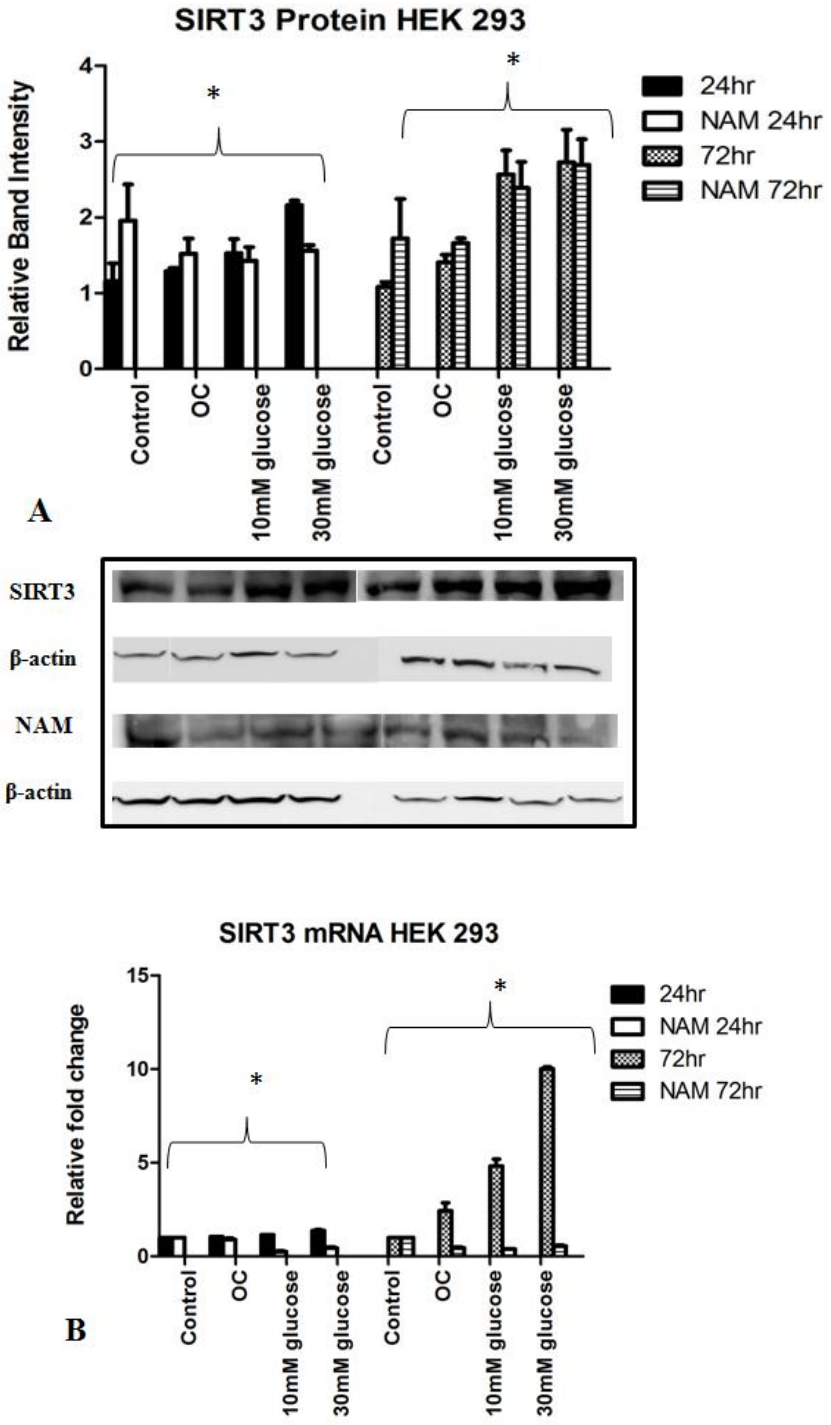

Figure 2.3: The effect of hyperglycaemia on SIRT3 expression in normal and NAM-treated HEK 293. (A) SIRT3 protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of SIRT3 band intensity normalised to β-actin. (B) Gene expression for SIRT3 was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *$p<0.05$ vs. Control.

p-CREB expression increases under hyperglycaemic conditions

To investigate the effect of hyperglycaemia in HepG2 cells on p-CREB expression, western blot was used to determine protein expression and mRNA expression was determined by qPCR.

The hyperglycaemic treatments induced significantly higher levels of p-CREB protein expression at both 24hr and 72hr time points compared to the controls (24hr: $p<0.0001$; 95% CI: control 0.8551 - 0.9128 RBD; 30mM 0.9128 1.207 RBD; 72hr $p= 0.0195$; 95% CI: control 0.4997 - 0.7535 RBD; 10mM 0.8187 - 1.388 RBD; $p= 0.0002$; 95% CI: control 0.4997 - 0.7535 RBD; 30mM 1.823 - 2.526 RBD respectively, **Fig 2.4A**).

Quantitative PCR data showed increased CREB mRNA expression in the 24hr and 72hr 10mM (1.2-fold and 1.5-fold respectively) and 30mM groups (5-fold; $p<0.0001$ and 8.4-fold; $p<0.0001$ respectively) compared to the respective controls **Fig 2.4B**.
Following NAM treatment over 24hr and 72hr, both CREB protein and mRNA expressions significantly decreased in the hyperglycaemic groups ($p<0.0001$) **Fig 2.4A and Fig 2.4B**.

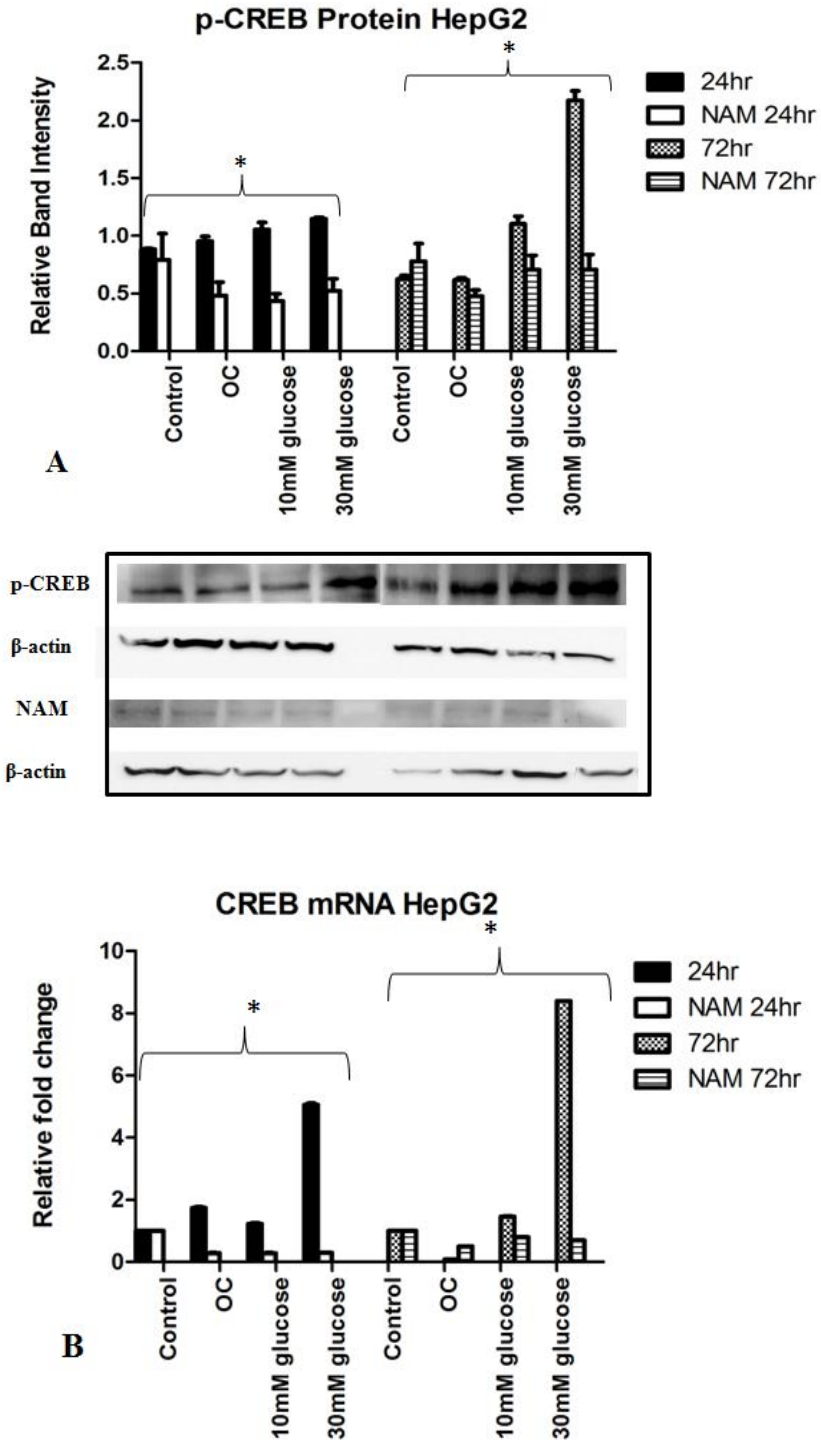

Figure 2.4: The effect of hyperglycaemia on p-CREB expression in normal and NAM-treated HepG2 cells. (A) p-CREB protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of p-CREB band intensity normalised to β-actin. Data expressed as mean±SEM. (B) Gene expression for CREB was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *$p<0.05$ vs. Control.

PGC-1α expression increases under hyperglycaemic conditions

To investigate the effect of hyperglycaemia in HepG2 cells on PGC-1α expression, western blot and qPCR were used to determine protein and gene expression respectively.

Higher levels of PGC-1α protein expression were observed at both 24hr and 72hr time points compared to the respective controls (72hr p= 0.0035; 95% CI: control 1.503 - 3.405 RBD; 10mM 3.501 - 5.255 RBD; p=0.0012; 95% CI: control 1.503 - 3.405 RBD; 30mM 2.915- 8.027 RBD respectively, **Fig 2.5A**).

PGC-1α mRNA levels were also significantly higher in the hyperglycaemic treatments at the 24hr and 72hr time points compared to the control ($p<0.0001$) **Fig 2.5B**. However, both protein and gene expressions were significantly decreased in the hyperglycaemic groups following NAM treatment ($p<0.0001$) **Fig 2.5A and Fig 2.5B.**

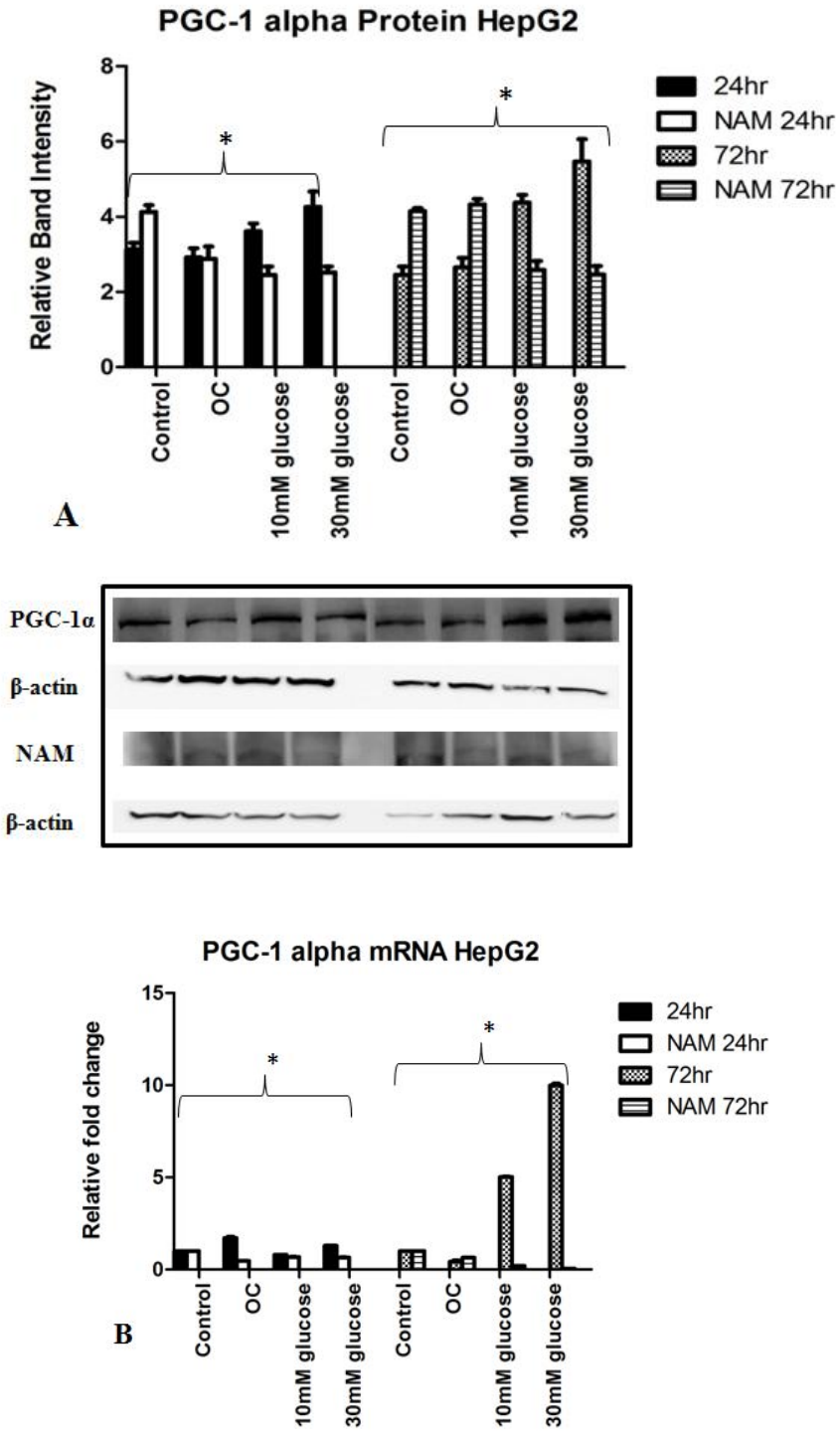

Figure 2.5: The effect of hyperglycaemia on PGC-1α expression in normal and NAM-treated HepG2 cells. (A) PGC-1α protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of PGC-1α band intensity normalised to β-actin. Data expressed as mean±SEM. (B) Gene expression for PGC-1α was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *$p<0.05$ vs. Control.

Antioxidant defence increases under hyperglycaemic conditions

In order to investigate the effect of hyperglycaemia on antioxidant activity in HepG2 cells, gene expressions for *GPx1*, *SOD2* and *UCP2* were determined by qPCR.

Increased expressions of *GPx1*, *SOD2* and *UCP2* were observed in both hyperglycaemic treatments at the 24hr and 72 time point.

GPx1 was significantly higher in the 30mM treatment at 72hr (3.4-fold; $p<0.0001$); NAM treatment reduced gene expression in the hyperglycaemic treatments over both time points **Fig 2.6A**. At 72hr, gene expression was significantly decreased in the 10mM and 30mM groups ($p=0.0011$ and $p=0.0070$ respectively) **Fig 2.6A**.

Hyperglycaemia induced significantly higher *SOD2* expression over 24hr and 72hr time points ($p<0.0001$). At 72hr, *SOD2* gene expression in both 10mM and 30mM treatments was 5.6-fold and 6.4-fold higher relative to the control, respectively **Fig 2.6B**. NAM treatment was found to reduce gene expression in both hyperglycaemic groups. *SOD2* expression was 3.3-fold lower in the 30mM treatment at both time points relative to the control **Fig 2.6B**.

Significantly higher *UCP2* expressions were observed in both hyperglycaemic groups over 24hr and 72hr ($p<0.0001$) **Fig 2.6C**. Gene expressions in the hyperglycaemic groups at both time points were significantly reduced following NAM treatment ($p<0.0001$) **Fig 2.6C.**

As a marker of mtDNA repair following oxidative damage, *OGGI* expression was determined in both hyperglycaemic treatments by qPCR. Significantly higher gene expressions were observed in the hyperglycaemic groups at 24hr and 72hr time points relative to the respective controls ($p<0.0001$) **Fig 2.6D**; however following NAM treatment, *OGGI* expressions were significantly reduced in the 30mM groups of both time points (<0.0001) **Fig 2.6D**.

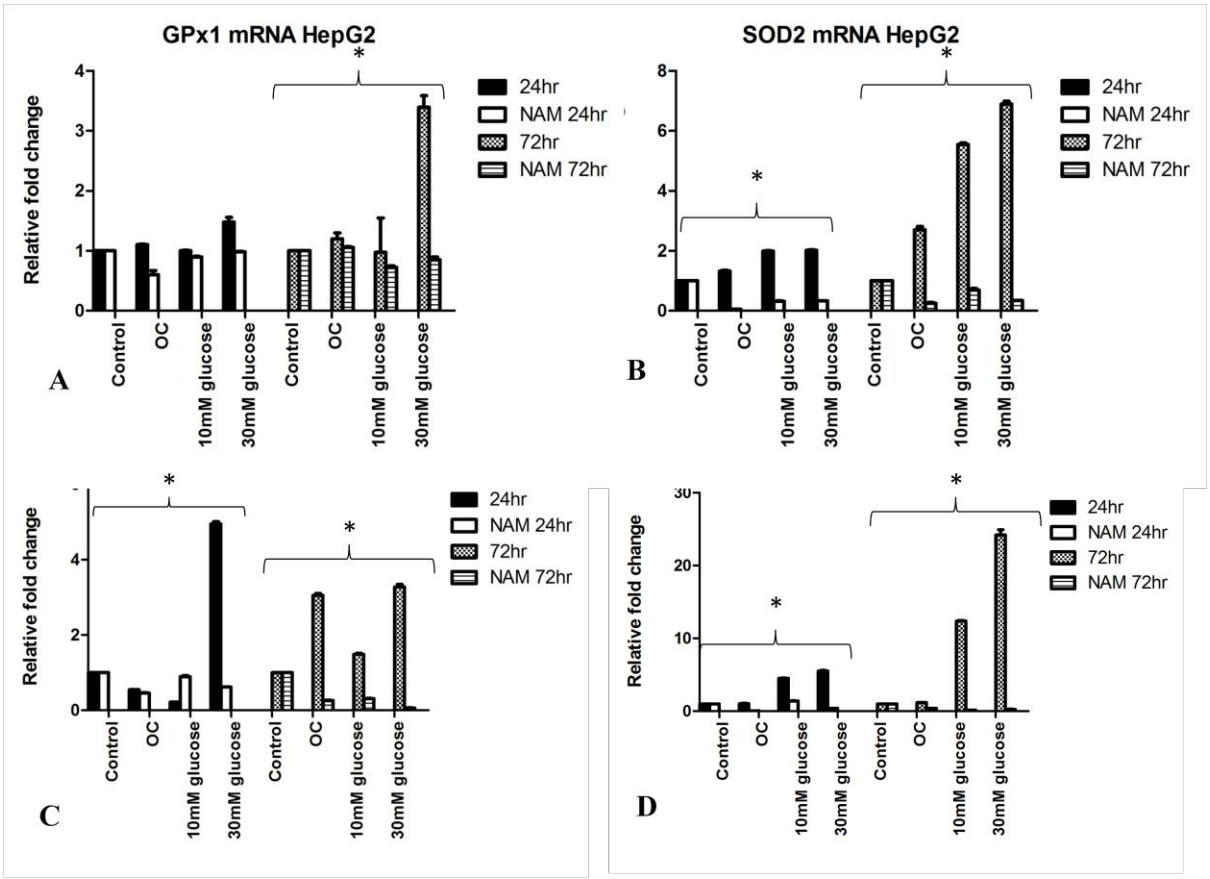

Figure 2.6: The effect of hyperglycaemia on antioxidant mRNA expression in normal and NAM-treated HepG2 cells. Gene expressions for (A) GPx1, (B) SOD2, (C) UCP2 and (D) OGG1 were assessed with quantitative PCR using specific primers. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *$p<0.05$ vs. Control.

2.5 Discussion

Oxidative stress is the leading cause of various pathological conditions. However, our understanding of the mechanisms that allow cells to protect themselves during OS under hyperglycaemic conditions in the liver is limited. This is important as the liver is the primary organ involved in glucose homeostasis. The liver plays a unique role in controlling carbohydrate metabolism by maintaining glucose concentrations in the normal range over long or short periods of time. Increases in glucose concentrations are regulated by suppression of gluconeogenic pathways, hepatic glucose uptake and an increase in glycogen storage and glucose metabolism.

To compare the effect of hyperglycaemia on SIRT3 expression and downstream AO defence in HepG2 cells, a non-carcinoma HEK 293 cell line was used. We found that the response under oxidative conditions, with regard to SIRT3 and downstream AO defence, were similar between both cell lines (see Supplementary Data).

Mitochondria are the primary sources of ROS as superoxide radicals are a natural by-product of the ETC. ROS that are physiologically produced in the mitochondria participate in critical signalling pathways. Exposure to excess ROS, which may arise as a consequence of an imbalance between production and detoxification pathways, leads to accumulated oxidative damage of critical macromolecules, such as DNA, RNA, proteins and lipid. This may play a role in physiological and pathophysiological conditions such as diabetes.

Type 2 diabetes mellitus is characterised by hyperglycaemia, OS and insulin resistance (IR) which is caused by abnormalities within the insulin signalling pathway. There is a strong correlation between IR and OS (induced by hyperglycaemia). Studies have shown that treatment of insulin-responsive cell lines with H_2O_2 caused a significant decrease in insulin sensitivity (*18-20*). This may be due to activation of mitogen-activated protein kinases (MAPKs), such as c-jun N terminal kinase (JNK), which in turn cause inhibition of insulin signalling. Oxidative stress could also lead to IR by promoting the expression of several pro-inflammatory cytokines which causes phosphorylation of insulin receptor substrate 1 (IRS-1) at inhibitory sites and promotes a significant decline in insulin sensitivity (*21*). Insulin resistance was prevented by administration of AO compounds.

Cells sustained under hyperglycaemic conditions have more electron donors produced by the TCA cycle and results in a large flux of substrates entering the ETC, leading to electron leakage and increased production of superoxide radicals (*1*).

mtDNA is susceptible to ROS-induced damage due to its close proximity to the ETC, its lack of protective histones and limited DNA repair activity (*3*). Studies have shown that exacerbated ROS production alters OXPHOS resulting in decreased mitochondrial function, decreased capacity of cells to maintain ATP levels (*22*) and a marked reduction in AO activity (*23*). These factors need to be improved in order to ameliorate disease conditions.

We observed increased cell viability under hyperglycaemic stimulation, which represents an oxidative environment. This observation was interpreted as a marker of increased mitochondrial output. This is interesting as studies have shown decreased cell viability under oxidative conditions; this is improved by activation of the endogenous AO system (*24, 25*). Inhibition of SIRT3 resulted in decreased cell viability under hyperglycaemic conditions which suggests, in our model, that SIRT3 is integral to mitochondrial function.

Cells have developed a network of regulated stress-response mechanisms. Genes encoding components involved in managing OS play a key role in this network. Sirtuins could be considered among those genes as they are sensors of redox state.

SIRT3 resides primarily in the mitochondria and has been shown to bind and deacetylate several metabolic and respiratory enzymes that regulate important mitochondrial functions.

Many studies have shown that SIRT3 regulates cell defence and survival in response to stress, such as high glucose (*16, 26-28*). Our study showed increased expressions of SIRT3 in the hyperglycaemic group. Interestingly, the levels of expression were much higher under longer hyperglycaemic stimulation.

SIRT3 expression is required for the induction of AO genes by PGC-1α (*9*). PGC-1α is a transcriptional co-activator that is activated in response to OS and plays an important role in regulating mitochondrial function and AO defence enzymes such as SOD2 (detoxifies superoxide radicals that are formed as a by-product of OXPHOS), GPx1 (enzyme that detoxifies H_2O_2 to H_2O) and UCP2, a proton carrier in the inner mitochondrial membrane that attenuates ROS-mediated damage (*1, 29, 30*). Suppression of PGC-1α expression in endothelial cells resulted in a strong reduction in the levels of the mitochondrial detoxification proteins (*31*).

In an oxidative environment, PGC-1α induces SIRT3 expression through activation of ERR- α and in turn, SIRT3 stimulates PGC-1α expression through p-CREB thereby forming a positive feedback cycle and increasing AO defence (*32, 33*).

We observed increased protein and gene expressions of PGC-1α, p-CREB and mitochondrial AO enzymes SOD2, GPx1, and UCP2 under hyperglycaemic conditions. Further elevations in expressions were observed under longer hyperglycaemic stimulation. Interestingly, chemical inhibition of SIRT3 resulted in the reduction of protein and gene expressions of PGC-1α, p-CREB and mitochondrial AO enzymes under hyperglycaemic conditions. This may suggest that SIRT3 is a central component that modulates AO response under oxidative conditions.

In addition to regulating AO defence by PGC-1α, SIRT3 also regulates detoxification of ROS through deacetylation and activation of SOD2 (*2, 11*). Over-expression of SIRT3 was shown to reduce ROS in an adipocyte cell line while increased ROS production was observed in SIRT3 deficient mice (*28, 34*).

Oxidative stress induces DNA base damage such as 8-oxoG which results in an increase in the frequency of spontaneous G.C- -T.A transversion mutations (*12*). Mutations in mtDNA are an underlying factor in many mitochondrial diseases as it results in abnormal expression of mtDNA encoded proteins and defective OXPHOS. OGG1 recognizes and removes 8-oxoG that is base-paired with cytosine. SIRT3 directly deacetylates and activates OGG1 enabling DNA repair and genomic stability under oxidative conditions (*16*).

As a marker of mtDNA repair under oxidative conditions, we have shown increased *OGG1* expression under hyperglycaemic conditions with levels being much higher under longer hyperglycaemic stimulation. Inhibition of SIRT3, however, resulted in a decrease in *OGG1* expression in the hyperglycaemic groups. This may illustrate the role of SIRT3 in genomic stability under oxidative conditions.

Our data has shown increased expressions of SIRT3, PGC-1α, mitochondrial AO and repair enzymes under hyperglycaemic conditions. We found a further increase in these expressions under longer hyperglycaemic stimulation, which represents oxidative conditions. Interestingly, we also found reduced protein and gene expressions following SIRT3 inhibition. This suggests that SIRT3 is a central component in modulating AO defence and conferring resistance to OS-induced damage under hyperglycaemic conditions in the human hepatoma cell line.

Resveratrol (a known SIRT3 activator) was found to attenuate oxidative injury in endothelial cells mediated through activation of SIRT3 signalling pathways (*35*). SIRT3 was also shown to protect against acute kidney injury by reducing OS and mitochondrial damage (*36*). Viniferin is a natural product that decreased ROS levels and prevented loss of mitochondrial membrane potential in cells expressing Huntingtin protein. SIRT3 was shown to mediate the neuro-protection of viniferin (*37*).

Therefore, regulating SIRT3 activity through chemical or natural therapeutics may be beneficial in improving several mitochondrial-associated diseases.

This study could be improved by using primary hepatocytes or an *in vivo* hyperglycaemic mouse model to establish a holistic response. Longer hyperglycaemic stimulations and SIRT knockout models may provide greater insight to SIRT3 modulation of AO defence under chronic OS.

Future work may include interrogating SIRT3 in more defined pathways that are altered in metabolic disorders, such as mitochondrial biogenesis or DNA repair. We may also look at chemical and natural therapeutics and their effects on SIRT3 expression and activity under oxidative conditions.

2.6 Conclusion

Oxidative stress-induced damage has been implicated in several metabolic disorders and diseases and SIRT3 may play a role in reducing OS by regulating AO defence and repair. Therefore, SIRT3 may be used as a therapeutic target to treat diseases associated with OS.

2.7 Acknowledgements

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Author Disclosure Statement

No competing financial interests exist.

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SUPPLEMENTARY DATA

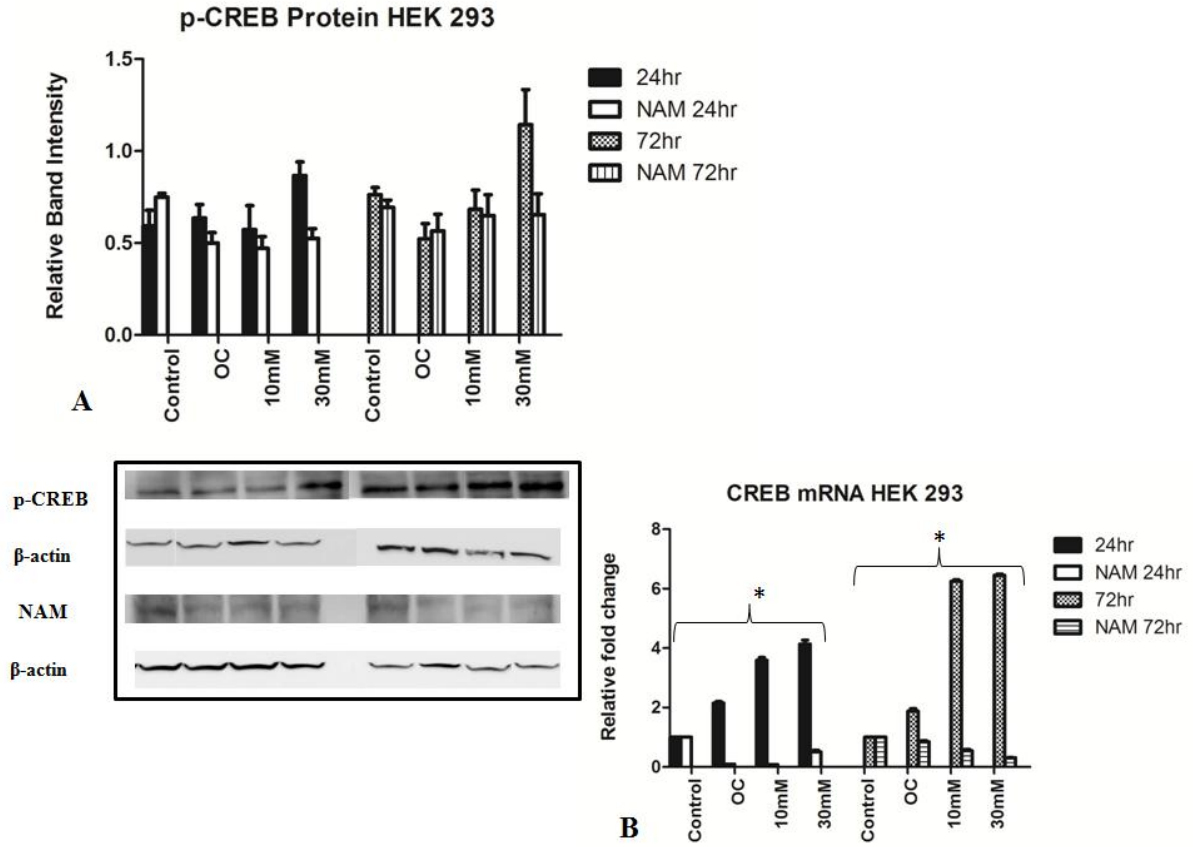

Figure s2.7: The effect of hyperglycaemia on p-CREB expression in normal and NAM-treated HEK 293 cells. (A) p-CREB protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of p-CREB band intensity normalised to β-actin. Data expressed as mean±SEM. (B) Gene expression for CREB was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *$p<0.05$ vs. Control.

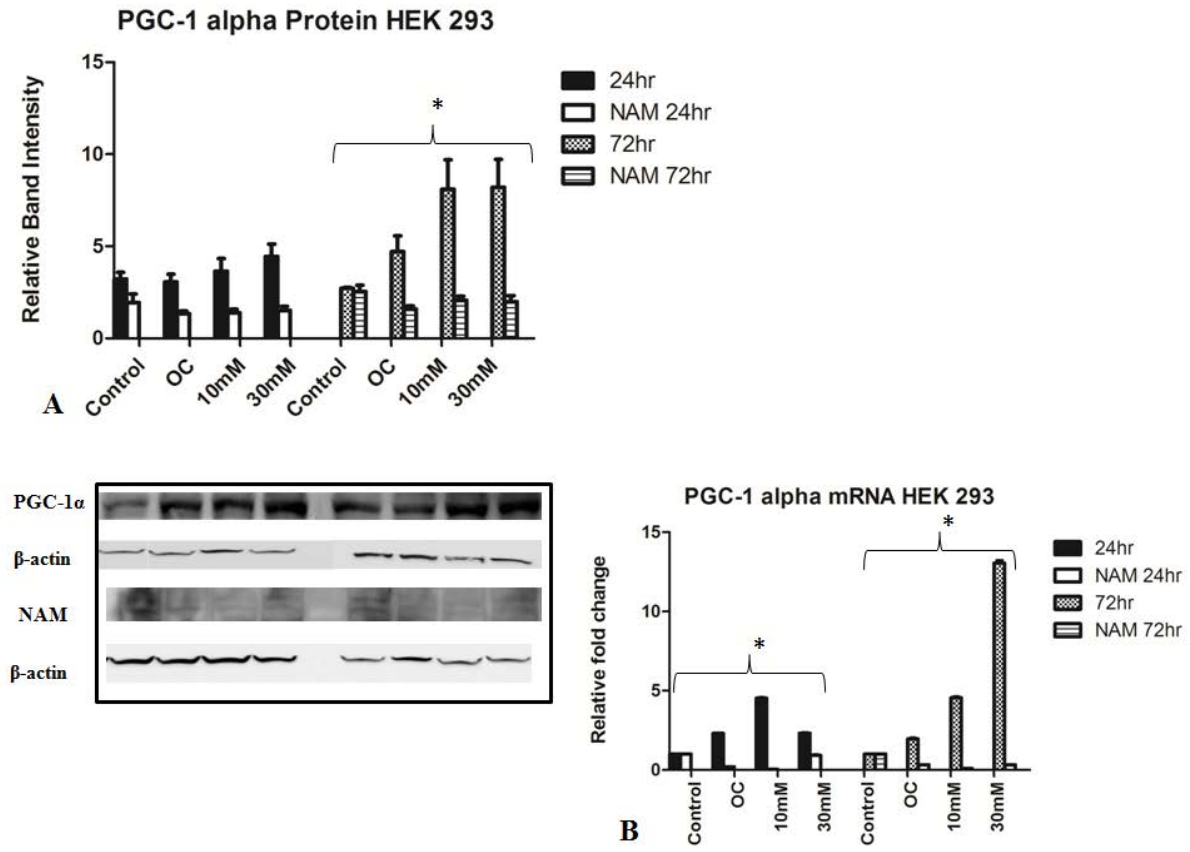

Figure s2.8: The effect of hyperglycaemia on PGC-1α expression in normal and NAM-treated HEK 293 cells. (a) PGC-1α protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of PGC-1α band intensity normalised to β-actin. Data expressed as mean±SEM. (b) Gene expression for PGC-1α was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *$p<0.05$ vs. Control.

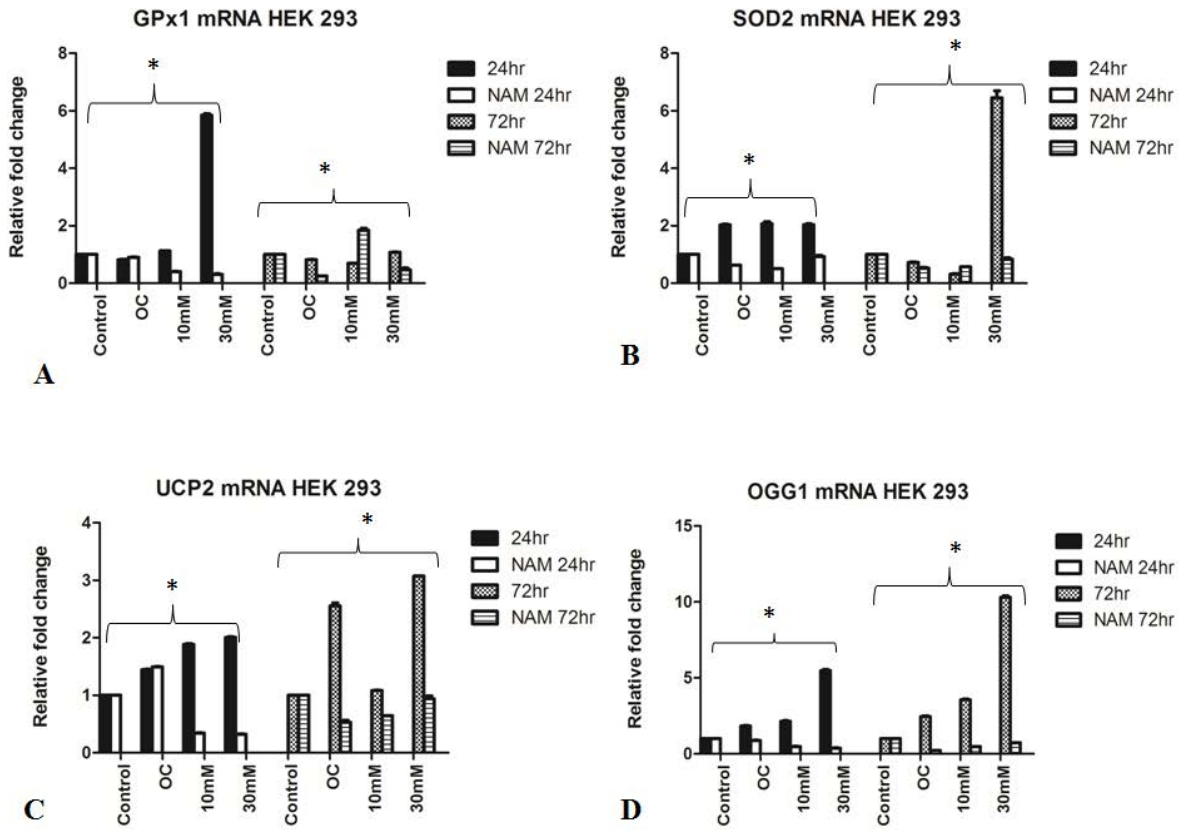

Figure s2.9: The effect of hyperglycaemia on antioxidant mRNA expression in normal and NAM-treated HEK 293 cells. Gene expressions for (A) GPx1, (B) SOD2, (C) UCP2 and (D) OGG1 were assessed with quantitative PCR using specific primers. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *$p<0.05$ vs. Control.

CHAPTER 3

Metformin Increases SIRT3 and Antioxidant Expression under Hyperglycaemic Conditions in HepG2 Cells

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3.1 Abstract

Background: Oxidative stress is involved in metabolic disorders. SIRT3 decreases oxidative stress by regulating antioxidant (AO) defence. Metformin is an anti-diabetic agent that possesses AO properties;. We postulated that metformin may increase AO defence under hyperglycaemic conditions in HepG2 cells through SIRT3-mediated mechanisms.

Methods: Cell viability was determined in HepG2 cells cultured in the presence of 5.5 mM glucose (control), 19.9mM mannitol (OC), 10mM glucose, 30mM glucose, NAM (10mM) and metformin (3mM) over 24hr. SIRT3 and p-CREB protein expressions were measured by western blot. Gene expressions of *SIRT3*, *CREB*, *GPx1*, *SOD2*, *UCP2* and *OGG1* were evaluated by qPCR.

Results: Increased cell viability and protein expressions of SIRT3 and p-CREB were observed in the metformin-treated groups. These were further elevated in hyperglycaemic groups treated with metformin. We also observed higher fold changes of *SIRT3*, *CREB*, *PGC-1α*, *GPx1*, *SOD2*, *UCP2* and *OGG1* in the hyperglycaemic groups treated with metformin. Treatment with NAM resulted in decreased protein expressions of SIRT3 and p-CREB and decreased gene expressions of *SIRT3, CREB, PGC-1α, GPx1, SOD2, UCP2* and *OGG1*.

Conclusions: Metformin increases SIRT3 and AO expression. Our data suggests that metformin may work synergistically with SIRT3 to increase AO defence.

3.2 Introduction

Type 2 diabetes (DMII) is characterized by insulin resistance, hyperglycaemia and oxidative stress (OS). Oxidative stress is a result of increased production/ decreased scavenging of reactive oxygen species (ROS) which plays a key role in DMII and associated complications (*1-4*).

ROS generation occurs primarily through the electron transport chain (ETC). Basal ROS levels are required for signalling, however, under hyperglycaemic conditions more electron transfer donors are generated from the tricarboxylic acid (TCA) cycle resulting in large amounts of reducing equivalents entering the ETC (*5*). This causes partial inhibition of the ETC at complex III and increased production of superoxide radicals (*5*).

Mitochondrial DNA (mtDNA) is vulnerable to oxidative damage as it is (a) closely located to the ETC, (b) lacks protective histones and (c) has limited DNA repair systems (*6*). Mitochondrial DNA damage compromises oxidative phosphorylation (OXPHOS) and antioxidant (AO) activity (*7*) which have been observed in metabolic disorders (*8*). These need to be improved in order to ameliorate metabolic disorders.

Metformin (1, 1-dimethylbiguanide) is an anti-hyperglycaemic agent used for the treatment of DMII. The proposed mechanism for metformin action is inhibition of complex I of the ETC (*9*). This would decrease ATP production, elevate the concentration of ADP and, in turn, increase the concentration of AMP (*9*). This results in active AMP-activated protein kinase (AMPK), which increases glucose uptake in peripheral tissues (*9*).

Metformin also inhibits mitochondrial ROS production by blocking the reverse electron flow through complex I of the ETC (*10*). Metformin modulated the intracellular production of ROS in bovine aortic endothelial cells (BAECs) stimulated with high concentrations of glucose (*11*).

In vitro data have shown that metformin could induce AO enzymes, such as superoxide dismutase 2 (SOD2) and glutathione (GSH) (*12-14*). Metformin was also shown to significantly decrease serum levels of 7, 8-dihydro-8-oxoguanine (8-oxoG), a marker of OS in DNA, in women with polycystic ovary syndrome (*15*).

These studies allude to the AO properties of metformin; however the AO effects of metformin have not been well characterised.

SIRT3 is as a primary mitochondrial stress-responsive protein deacetylase that regulates

mitochondrial function and AO defence (*16*). It reduces ROS levels by regulating AO enzymes, such as SOD2, and therefore protects against oxidative damage (*17*).

SIRT3 modulates AO defence through peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), a transcriptional co-activator that regulates respiration, mitochondrial biogenesis and AO defence (glutathione peroxidase 1 (GPx1) and SOD2).

Mutations in mtDNA are involved in many mitochondrial diseases. SIRT3 activates the mammalian DNA glycosylase OGG1 enabling DNA repair and genomic stability under oxidative conditions (*18*). A recent study has shown that SIRT3 may be involved in the AO effects of metformin (*19*); however, this has yet to be fully interrogated.

Molecular events that occur during OS under hyperglycaemic conditions in the liver are limited. The liver is the primary organ involved in glucose homeostasis and is also one of the major sites targeted during metformin therapy in DMII. This is the first study to investigate the effect of metformin on SIRT3 expression and downstream AO defence under normo- and hyperglycaemic conditions in the human hepatoma cell line.

3.3 Materials and Methods

Cell Culture and Treatments

HepG2 cells were cultured to confluency in 25cm^3 flasks (5% CO_2, 37°C) in complete culture media (CCM, Eagles minimum essential medium, supplemented with 10% foetal calf serum, 1% L-Glutamine and 1% penstrepfungizone). The cells (2.5 x 10 ^{4} cells/ well) were cultured in the presence of the hyperglycaemic control (5.5mM glucose), osmotic control (OC) (19.9mM mannitol) and hyperglycaemic (10mM, 30mM glucose) conditions in the absence (untreated/ normal) or presence of 3mM metformin (MET) for 24hr in 30mm^3 cell culture plates. Cells were treated with the SIRT3 inhibitor nicotinamide (NAM) at a concentration of 10mM.

Cell Viability Assay

Following treatment, the cells (3 replicates) were incubated with methyl thiazol tetrazolium (MTT) salt solution (5mg/mL in 0.1M phosphate buffered saline (PBS)) and CCM (4h, 37ºC). Following incubation, the supernatants were aspirated and dimethyl sulphoxide was added (100 µL/well) and incubated at 37ºC for a further 1h. Optical density of the formazan product was measured by a microplate reader (Bio-tek µQuant) at 570 nm with a reference wavelength of 690nm.

Quantitative Polymerase Chain Reaction (q-PCR)

Total RNA was extracted from cultured cells using the Triazol reagent (Ambion). cDNA was synthesised by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad).
Quantitative PCR was performed on the CFX96 Real-Time System (Bio-Rad) by using iQ SYBR Green supermix (BioRad) with primer sequences as listed in Table 3.1. The PCR was initiated with the following thermocycler profile: an initial denaturation for 8min at 95°C followed by 39 cycles of 95°C denaturation for 15s, annealing for 1min and extension of 72°C for 30s. A final extension at 60°C was performed for 31s. Each measurement was done in triplicate and normalized against 18S rRNA Ct values. The qPCR data is represented as a relative fold change, calculated using the method described by Livak and Schmittgen (*20.*)

Table 3.1: Primer sequences and optimised annealing temperature

Gene	Sense (5'-3')	Antisense (5'-3')	Annealing temp (°C)
18S rRNA	ACACGGACAGGATTGACAGA	CAAATCGCTCCACCAACTAA	58°C
SIRT3	GCATTCCAGACTTCAGATCGC	GTGGCAGAGGCAAAGGTTCC	50°C
CREB	GATGGACAGCAGATCTTAGTGCC	TGCTGTGCAAATCTGGTATGTT	65°C
PGC-1α	CCAAACCAACAACTTTATCTCTTCC	CACACTTAAGGTGCGTTCAATAGTC	65°C
GPx1	GACTACACCCAGATGAACGAGC	CCCACCAGGAACTTCTCAAAG	60°C
SOD2	GAGATGTTACAGCCCAGATAGC	AATCCCCAGCAGTGGAATAAGG	58°C
UCP2	GACCTATGACCTCATCAAG	ATAGGTGACGAACATCACCACG	50°C
OGG1	GCATCGTACTCTAGCCTCCAC	AGGACTTTGCTCCCTCCAC	60°C

Western blot analysis

Sample proteins were isolated using a Cytobuster™ (Novagen) supplemented with protease and phosphatase inhibitors (Roche). Proteins were quantified using the bicinchoninic acid (BCA) assay (Sigma) and standardised to 1mg/ml. Protein extracts were prepared in Laemlii buffer (dH_2O, 0.5M Tris-Cl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% resolving gel) and electrotransferred to nitrocellulose membrane. Membranes were blocked with 5% BSA in Tris-Buffered Saline and Tween 20 (TTBS) [137mM NaCl, 2.7mM KCl, 24mM Tris, 0.5% Tween 20]. Membranes were probed using the following antibodies in 5% bovine serum albumin (BSA in TTBS): rabbit anti- SIRT3 (Abcam, ab86671, 1:1000) and goat anti phospho-CREB (Cell Signalling Technology, cat. No 9191, 1:1000). All membranes were incubated in primary antibodies overnight at 4°C. A horseradish peroxidase (HRP)-conjugated goat polyclonal antibody specific for β-actin was used for the loading control and the normalisation process (1:5000 in 5% BSA in TTBS). Membranes were developed using LumiGlo® Chemiluminescent Substrate System (KPL) and images were captured on the Alliance 2.7 documentation system (UViTech). The density of the bands was quantified using UViBand Advanced Image Analysis Software (UViTech). The experiment was performed in triplicate and repeated thrice. Results are presented as relative band intensity and normalised against β-actin.

Statistical analysis

Each experiment was performed in triplicate and repeated thrice. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one way ANOVA followed by Bonferroni test for multiple group comparison. Differences with $p<0.05$ were considered statistically significant.

3.4 Results

Metformin treatment increases mitochondrial output under normo- and hyperglycaemic conditions

In order to determine the effect of hyperglycaemia and metformin treatment on mitochondrial output, cell viability was measured in HepG2 cells. Compared to the untreated control, cell viability was significantly higher in the metformin-treated control (p= 0.0057, 95% CI: control 0.4417 - 0.5937; control+ metformin 0.7297 - 0.9923 **Fig 3.1**).

Both hyperglycaemic groups treated with metformin also showed significantly higher cell viability compared to the untreated control (p= 0.0002, 95% CI: control 0.4417 - 0.5937; 10mM glucose+ metformin 0.9650 - 1.068; p= 0.0017, 95% CI: control 0.4417 - 0.5937; 30mM glucose+ metformin 0.7603- 0.9037 **Fig 3.1**) however, only the 10mM glucose+ metformin group was significantly higher than the treated control (p= 0.0220).

NAM treatment resulted in a significant decrease in cell viability in the metformin-treated hyperglycaemic groups (10mM+ metformin: p<0.0001 and 30mM+ metformin: p= 0.0010 (**Fig 3.1**)).

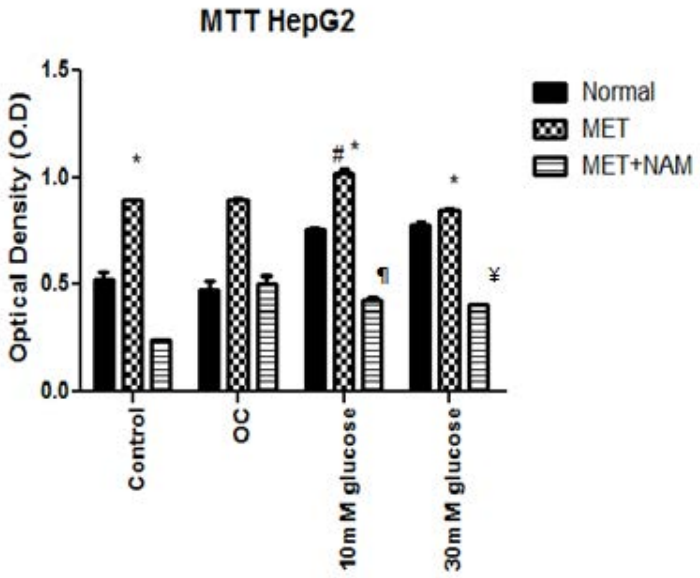

Figure 3.1: The effect of hyperglycaemia on cell viability in HepG2 cells (untreated, metformin-treated and NAM-treated). Values are expressed as mean ± SEM. *p<0.05 vs. untreated control, #p<0.05 vs. metformin -treated control, ¶ p<0.05 vs. metformin -treated 10mM glucose, ¥ p<0.05 vs. metformin -treated 30mM glucose.

Metformin treatment increases SIRT3 expression under normo- and hyperglycaemic conditions

To determine whether hyperglycaemia and metformin had an effect on SIRT3 expression, western blot and qPCR were used to measure protein and gene expression respectively.

Significantly higher SIRT3 protein expression was observed in the metformin-treated control compared to the untreated control (p= 0.0402; 95% CI: control 0.1317- 4.364 RBD; control+ metformin 4.683- 6.399 RBD **Fig 3.2A**).

Higher SIRT3 protein expression was also observed in the hyperglycaemic groups treated with metformin relative to the untreated control (p= 0.0356; 95% CI: control 0.1317- 4.364 RBD; 10mM glucose + metformin 5.595- 16.41 RBD; p= 0.0194; 95% CI: control 0.1317- 4.364 RBD; 30mM glucose + metformin 6.649- 12.65 RBD **Fig 3.2A**).

A similar pattern was observed in the metformin-treated hyperglycaemic groups relative to the treated control (p= 0.0357; 95% CI: control+ metformin 4.683- 6.399 RBD; 10mM glucose + metformin 5.595- 16.41 RBD; p= 0.0173; 95% CI: control+ metformin 4.683- 6.399 RBD; 30mM glucose + metformin 6.649- 12.65 RBD **Fig 3.2A**).

Upon comparison to the untreated control, qPCR data also showed higher transcript levels of *SIRT3* in the treated control (p= 0.0312; 1.7-fold) and treated hyperglycaemic groups (10mM+ metformin: p= 0.0038; 2.5-fold and 30mM+ metformin: p=0.0015; 2.4-fold) **Fig 3.2B**. Both treated hyperglycaemic groups showed significantly higher *SIRT3* expression compared to the treated control (10mM glucose+ metformin: p=0.0026; 30mM glucose+ metformin: p= 0.0264).

In the NAM groups, significantly lower SIRT3 protein expression was observed in the 10mM + metformin and 30mM glucose + metformin groups (p=0.0145 and p= 0.0051 respectively **Fig 3.2A**). Quantitative PCR also showed a significant decrease in gene expression in both treated hyperglycaemic groups (10mM glucose+ metformin: p=0.0008; 30mM glucose+ metformin: p= 0.0007 **Fig 3.2B**).

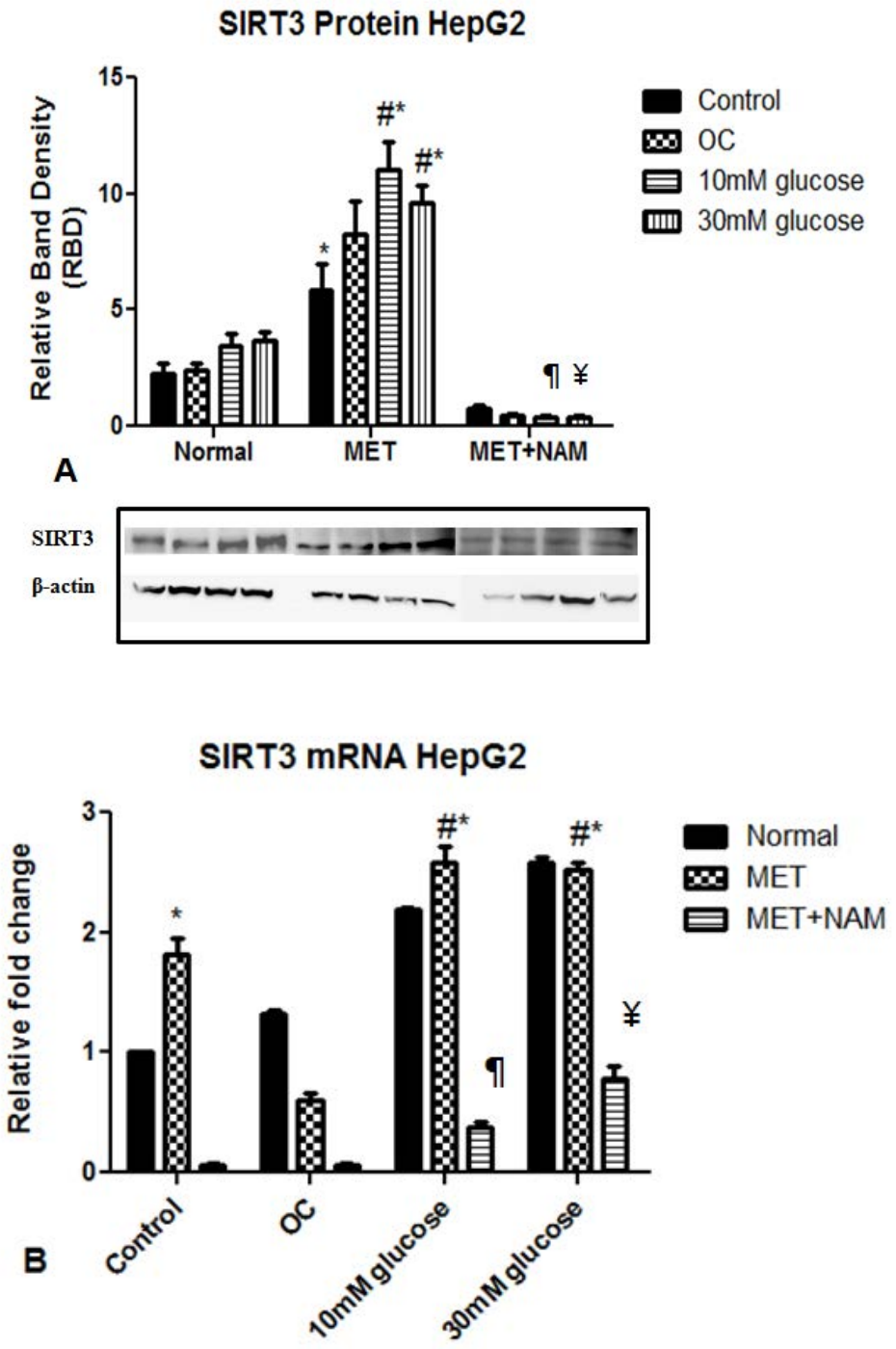

Figure 3.2: The effect of hyperglycaemia on SIRT3 expression in untreated, metformin -treated and NAM-treated HepG2 cells. (A) SIRT3 protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of SIRT3 band intensity normalised to β-actin. (B) Gene expression for SIRT3 was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *p<0.05 vs. untreated control, #p<0.05 vs. metformin-treated control, ¶ p<0.05 vs. metformin -treated 10mM glucose, ¥ p<0.05 vs. metformin -treated 30mM glucose.

Metformin treatment increases p-CREB expression under normo- and hyperglycaemic conditions

To investigate the effect of hyperglycaemia and metformin on p-CREB expression, western blot and qPCR were used to determine protein and mRNA expression respectively.

Compared to the untreated control, significantly higher p-CREB protein expression was observed in the metformin -treated control (p= 0.0002; 95% CI: control 0.8551 - 0.9128 RBD; control + metformin 6.936 - 7.754 RBD **Fig 3.3A**).

The hyperglycaemic groups treated with metformin also showed significantly higher p-CREB protein expression relative to the untreated control (p= 0.0255; 95% CI: control 0.8551 - 0.9128 RBD; 10mM glucose + metformin 2.359 - 9.098 RBD; p= 0.0458; 95% CI: control 0.8551 - 0.9128 RBD; 30mM glucose + metformin 1.135 - 10.32 RBD **Fig 3.3A)** but were lower when compared to the metformin-treated control.

NAM significantly decreased p-CREB protein expression in both treated hyperglycaemic groups (10mM glucose + metformin p= 0.0226 and 30mM glucose + metformin p= 0.0385 respectively **Fig 3.3A**).

Quantitative PCR data showed a significant 2.4-fold increase in CREB mRNA expression in the metformin -treated control compared to the untreated control (p= 0.0005) **Fig 3.3B**. Both metformin-treated hyperglycaemic groups also showed higher *CREB* levels compared to the untreated control (10mM glucose + metformin p= 0.0012 and 30mM glucose + metformin p= 0.0035 respectively).

Following NAM treatment, CREB mRNA expressions were significantly decreased in the hyperglycaemic groups treated with metformin (10mM glucose + metformin p= 0.0003 and 30mM glucose + metformin p= 0.0052) **Fig 3.3B**.

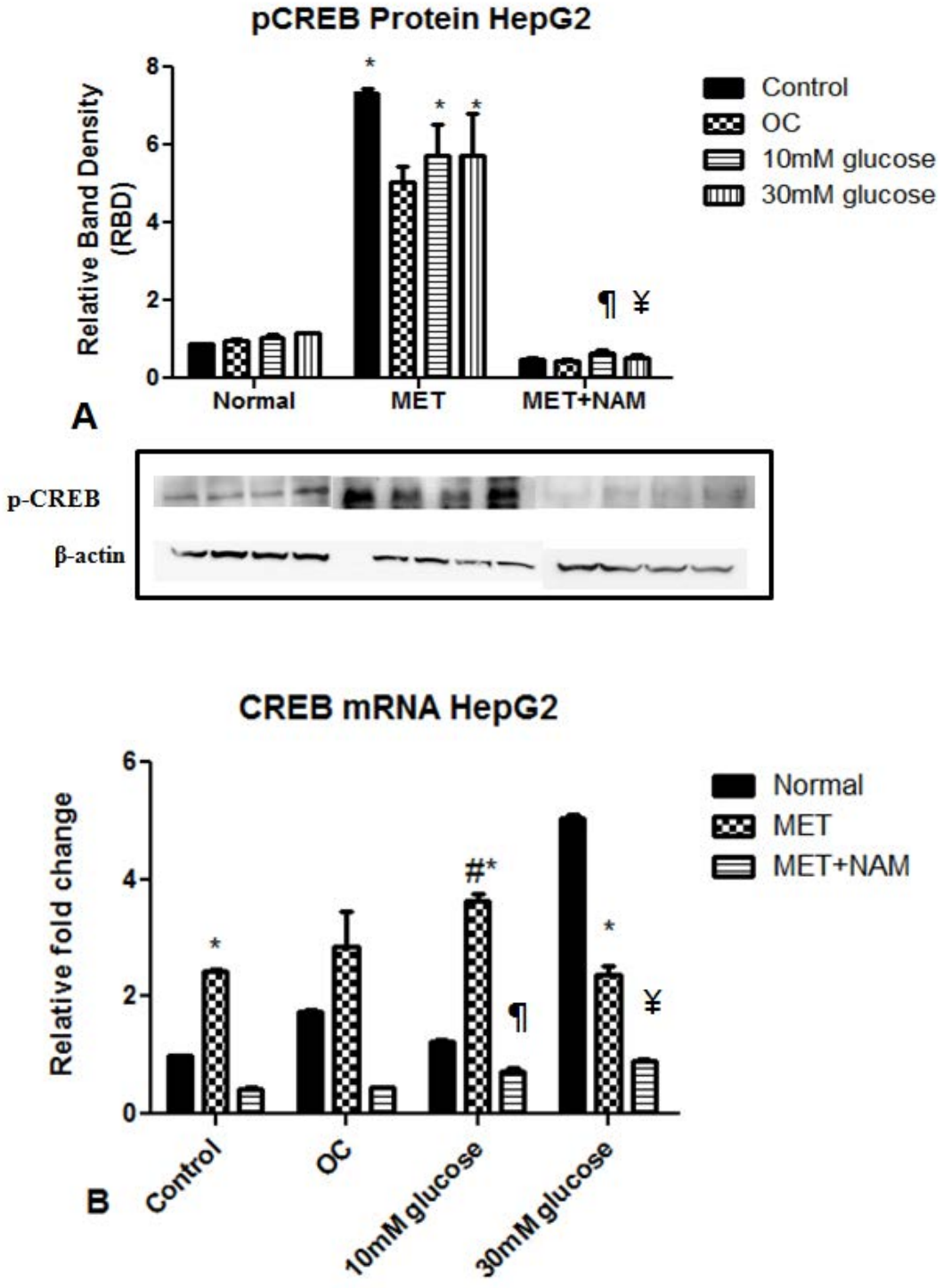

Figure 3.3: The effect of hyperglycaemia on p-CREB expression in untreated, metformin -treated and NAM-treated HepG2 cells. (A) p-CREB protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of p-CREB band intensity normalised to β-actin. Data expressed as mean±SEM. (B) Gene expression for CREB was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *p<0.05 vs. untreated control, #p<0.05 vs. metformin -treated control, ¶ p<0.05 vs. metformin -treated 10mM glucose, ¥ p<0.05 vs. metformin -treated 30mM glucose.

PGC-1α expression increases under normo and hyperglycaemic conditions following metformin treatment

To investigate the effect of hyperglycaemia and metformin on PGC-1α mRNA levels, qPCR was used to determine gene expression.

Higher *PGC-1α* levels were observed in the metformin-treated control relative to the untreated control but this did not reach statistical significance. The hyperglycaemic groups treated with metformin also showed higher *PGC-1α* levels when compared to the untreated control (10mM glucose + metformin p= 0.0161; 4.5-fold and 30mM glucose + metformin p= 0.0439; 1.9-fold **Fig 3.4**), however only the 10mM glucose + metformin group showed higher levels relative to the treated control (p= 0.0343; 2.1-fold).

PGC-1α gene expressions were significantly decreased in hyperglycaemic groups treated with metformin following NAM treatment (10mM glucose + metformin p= 0.0025 and 30mM glucose + metformin p= 0.0263 respectively **Fig 3.4).**

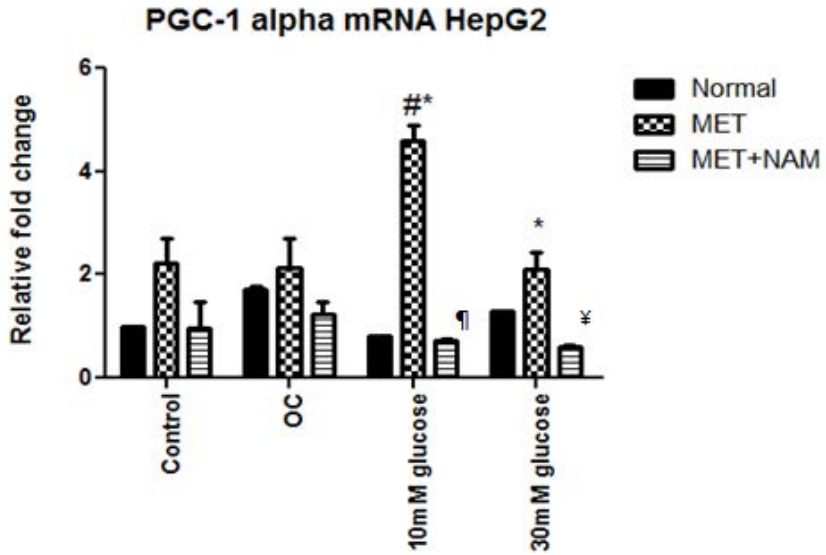

Figure 3.4: The effect of hyperglycaemia on PGC-1α expression in untreated, metformin -treated and NAM-treated HepG2 cells. Gene expression for PGC-1α was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *p<0.05 vs. untreated control, #p<0.05 vs. metformin -treated control, ¶ p<0.05 vs. metformin -treated 10mM glucose, ¥ p<0.05 vs. metformin -treated 30mM glucose.

Antioxidant defence increases under normo- and hyperglycaemic conditions following metformin treatment

In order to investigate the effect of hyperglycaemia and metformin on antioxidant activity in HepG2 cells, gene expressions for *GPx1*, *SOD2* and *UCP2* were determined by qPCR.

Increased expressions of *GPx1*, *SOD2* and *UCP2* were observed in hyperglycaemic groups treated with metformin relative to the untreated and metformin-treated controls.

Transcript levels of *GPx1* was significantly higher in the metformin-treated control compared to the untreated control (p= 0.0002; 2-fold). The hyperglycaemic groups treated with metformin also showed significantly higher levels of *GPx1* relative to the untreated control (10mM glucose + metformin p= 0.0053; 4.4-fold and 30mM glucose + metformin p= 0.0069; 1.9-fold respectively). The 10mM glucose+ metformin group showed higher levels when compared to the treated control (p= 0.0096; 4.3-fold).

NAM treatment significantly reduced gene expression in both hyperglycaemic groups treated with metformin (10mM glucose + metformin p= 0.0043 and 30mM glucose + metformin p= 0.0039 **Fig 3.5A)**.

The treated control showed a 1.2-fold increase in *SOD2* transcript levels relative to the untreated control but no statistical difference was reached. The hyperglycaemic groups treated with metformin, however, were significantly higher than both the untreated control (10mM glucose + metformin p= 0.0456; 2.8-fold and 30mM glucose + metformin p= 0.0107; 4.3-fold **Fig 3.5B**) and the metformin-treated control (10mM glucose + metformin p= 0.0468 and 30mM glucose + metformin p= 0.0170 **Fig 3.5B**).

SOD2 expression was significantly decreased following NAM treatment in both treated hyperglycaemic groups (10mM glucose + metformin p= 0.0352 and 30mM glucose + metformin p= 0.0105 **Fig 3.5B**).

The *UCP2* expression in the untreated control was significantly higher than the metformin-treated control (p= 0.0062) however, the metformin-treated hyperglycaemic groups showed significantly higher transcript levels relative to both the untreated control (10mM glucose + metformin p= 0.0050; 3.2-fold and 30mM glucose + metformin p= 0.0318; 1.5-fold **Fig 3.5C**) and the metformin -treated control (10mM glucose + metformin p= 0.0015; 9-fold and 30mM glucose + metformin p= 0.0029; 4-fold **Fig 3.5C**).

Gene expressions in the metformin-treated hyperglycaemic groups were significantly reduced following NAM treatment (10mM glucose + metformin p= 0.0079 and 30mM glucose + metformin p= 0.0220)**.**

As a marker of mtDNA repair following oxidative damage, *OGGI* expression was determined by qPCR.

Significantly higher *OGGI* expressions were observed in the metformin-treated control and metformin-treated hyperglycaemic groups relative to the untreated control (control+ metformin p= 0.0007; 3.1-fold, 10mM glucose + metformin p= 0.0046; 4.1-fold and 30mM glucose + metformin p= 0.0020; 1.5-fold respectively **Fig 3.5D**). The treated hyperglycaemic groups showed significantly higher mRNA levels than the treated control (10mM glucose + metformin p= 0.0101; 2-fold and 30mM glucose + metformin p= 0.0136; 1.3-fold respectively **Fig 3.5D**) but were also significantly decreased following NAM treatment (10mM glucose + metformin p= 0.0019 and 30mM glucose + metformin p= 0.0092 respectively).

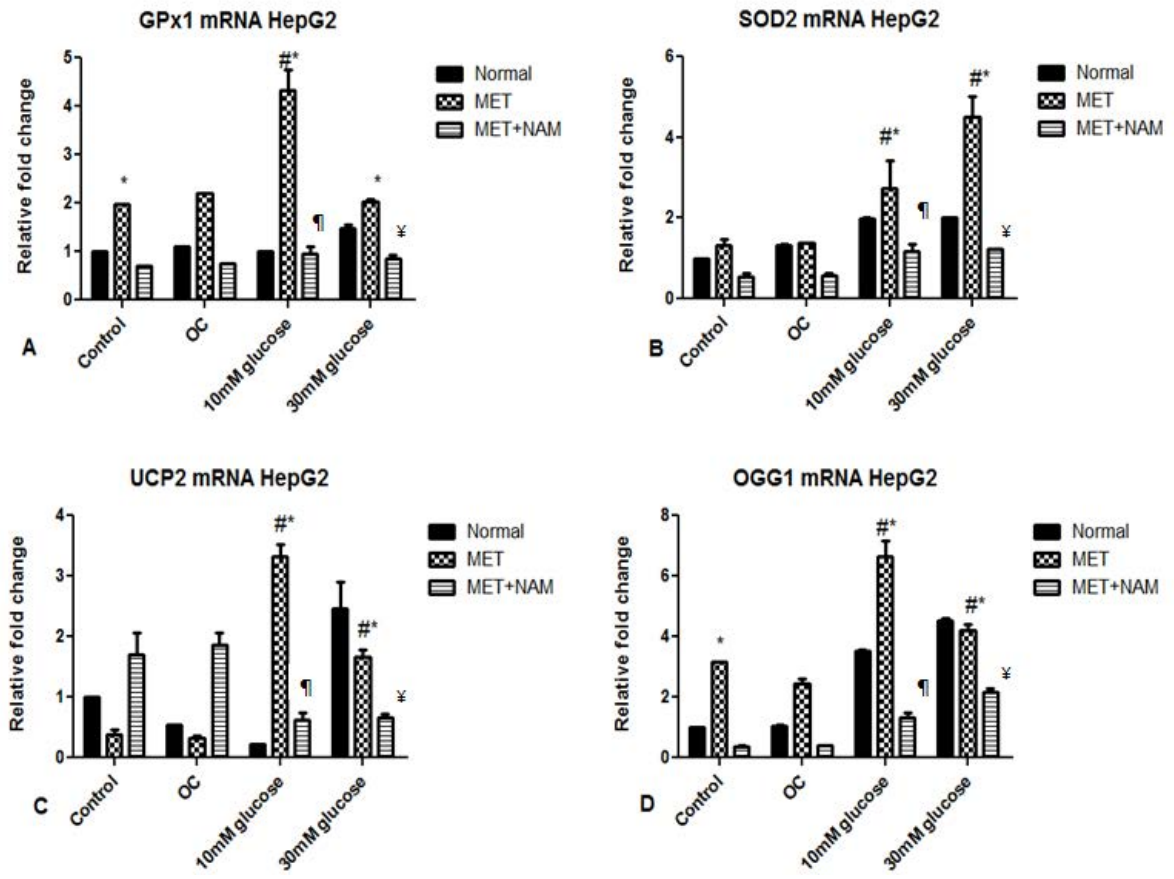

Figure 3.5: The effect of hyperglycaemia on antioxidant mRNA expression in untreated, metformin -treated and NAM-treated HepG2 cells. Gene expressions for (A) GPx1, (B) SOD2, (C) UCP2 and (D) OGG1 were assessed with quantitative PCR using specific primers. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *p<0.05 vs. untreated control, #p<0.05 vs. metformin -treated control, ¶ p<0.05 vs. metformin -treated 10mM glucose, ¥ p<0.05 vs. metformin -treated 30mM glucose.

3.5 Discussion

Type 2 diabetes (DMII) is characterised by hyperglycaemia and OS, which is a result of increased production/ decreased scavenging of ROS (*1-4*). The mechanisms that protect cells against OS under hyperglycaemic conditions in the liver are limited. This is important as the liver is the primary organ involved in glucose homeostasis.

The ETC in mitochondria is the primary source of ROS generation. Exposure to excess ROS, leads to oxidative damage of macromolecules which play a role in metabolic disorders such as DMII. Under hyperglycaemic conditions, more electron transfer donors are produced by the tricarboxylic acid (TCA) cycle and enter the ETC, leading to electron leakage and increased production of superoxide radicals.(*5*)

Mitochondrial DNA is vulnerable to ROS-induced damage as it is located closely to the ETC (*6*). Excessive ROS production alters OXPHOS resulting in decreased mitochondrial function, decreased ATP levels (*21*) and reduced AO activity.(*22*) These factors need to be improved in order to ameliorate DMII.

Metformin is an oral anti-diabetic agent that is used for DMII treatment. It inhibits mitochondrial ROS production by blocking the reverse electron flow through complex I of the ETC (*10*). A study showed that metformin treatment restored the AO status, enzymatic activity and inflammatory parameters that were altered in DMII (*23*). To study the effect of metformin in HepG2 cell culture, we chose 3mM which is more related to the range of intrahepatic metformin concentrations than those observed in plasma. The liver receives the majority of its blood through the portal vein, which may contain concentrations of metformin substantially higher than those present in the general circulation. *In vitro* studies have used metformin concentrations between 2mM and 10mM.

SIRT3 is part of a network of regulated stress-response mechanisms. It is located in mitochondria and deacetylates enzymes that regulate mitochondrial function. Studies have shown that SIRT3 regulates cell defence and survival in response to stress (*18, 24-26*).

We had previously observed increased expressions of SIRT3 and AO defence under long and short term hyperglycaemic stimulation. Further, we had established that SIRT3 is the central component that modulates AO defence and confers resistance to OS-induced damage under hyperglycaemic conditions in the HepG2 cell line (*27*).

Since SIRT3 has been shown to directly regulate mitochondrial function and enhance AO defence,

metformin could regulate AO activity through SIRT3 mechanisms.

We observed increased cell viability following metformin treatment under normo- and hyperglycaemic conditions. This observation was interpreted as a marker of increased mitochondrial output. This is interesting as metformin has been shown to directly inhibit complex I (NADH dehydrogenase). This inhibition of complex 1, however, does not affect any other steps of OXPHOS (*9*). Inhibition of complex1 is also mild (about 10%) and studies have favoured AMP deaminase inhibition as an alternative mechanism of metformin action (*28, 29*).

Inhibition of SIRT3 by NAM resulted in decreased cell viability in the metformin-treated groups which suggests, in our model, that SIRT3 may be integral in improving mitochondrial output following metformin treatment.

We showed increased SIRT3 expression in the metformin-treated groups. Interestingly, the levels of expression were much higher in the metformin-treated hyperglycaemic groups.

SIRT3 induces AO defence through PGC-1α (*30*). PGC-1α is a transcriptional co-activator that is activated under oxidative conditions and regulates mitochondrial function and AO defence such as SOD2 (detoxifies superoxide radicals that are formed as a by-product of OXPHOS), GPx1 (enzyme that detoxifies H_2O_2 to H_2O) and UCP2, a proton carrier in the inner mitochondrial membrane that attenuates ROS-mediated damage (*5, 31, 32*).

Following OS, PGC-1α induces SIRT3 expression through activation of estrogen-related receptor alpha (ERR- α) and in turn, SIRT3 stimulates PGC-1α expression through p-CREB thereby forming a positive feedback cycle and increasing AO defence (*33, 34*).

We observed increased protein and gene expressions of PGC-1α, p-CREB and mitochondrial AO enzymes SOD2, GPx1, and UCP2 following metformin treatment. Higher expressions were observed in the metformin-treated hyperglycaemic groups. Interestingly, inhibition of SIRT3 resulted in reduced protein and gene expressions of PGC-1α, p-CREB and mitochondrial AO enzymes in the metformin-treated groups. This suggests that SIRT3 may be involved in modulating AO response under oxidative conditions following metformin treatment.

A study showed that metformin reduced hepatic expression of SIRT3 in order to regulate energy metabolism (*35*). A more recent study, however, evaluated the effects of SIRT3 on the regulation of OS in skeletal muscle *in vitro* (*19*). They found that metformin increased the expression of SIRT3

and SOD2. Knockdown of SIRT3 significantly reversed the metformin-induced increase in SOD2. SIRT3 may, therefore, be differentially regulated by metformin.

Oxidative stress induces DNA base damage such as 8-oxoG which results in increased frequency of spontaneous G.C- -T.A transversion mutations leading to mitochondrial diseases **(*36*)**. The DNA glycosylase OGG1 removes 8-oxoG that is base-paired with cytosine. SIRT3 has been shown to activate OGG1 thereby maintaining genomic stability under oxidative conditions (*18*).

As a marker of mtDNA repair under oxidative conditions, we have shown increased *OGG1* expression following metformin treatment with levels being much higher in the metformin-treated hyperglycaemic groups. Inhibition of SIRT3, however, resulted in a decrease in *OGG1* expression in the metformin-treated hyperglycaemic groups. This suggests that SIRT3 may be involved in genomic stability under oxidative conditions following metformin treatment. Metformin has been shown to significantly decrease serum levels of 8-oxoG in women with polycystic ovary syndrome (*15*).

Our data has shown increased expressions of SIRT3, PGC-1α, mitochondrial AO and repair enzymes in metformin-treated groups. We found a further increase in these expressions in the metformin-treated hyperglycaemic groups, where hyperglycaemia represents oxidative conditions. Interestingly, we also found reduced protein and gene expressions following SIRT3 inhibition in the metformin-treated groups.

This suggests that metformin may work synergistically with SIRT3, or through SIRT3-mediated mechanisms, to increase AO defence.

This study could be improved by using primary hepatocytes or an *in vivo* hyperglycaemic mouse model to establish a holistic response. Longer hyperglycaemic stimulations may provide greater insight to SIRT3/metformin modulation of AO defence under oxidative conditions. A range of metformin concentrations over a longer time period may also provide an optimal response in an *in vivo* model.

Future work may include combination treatments with metformin and polyphenols known to up-regulate SIRT3, such as resveratrol. We could also interrogate the role of metformin in genomic stability (DNA methylation and regulation of histone deacetylases) under oxidative conditions.

3.6 Conclusion

Oxidative stress-induced damage has been implicated in several metabolic disorders and diseases. Metformin increases AO defence under oxidative conditions and this may be through SIRT3-mediated mechanisms.

3.7 Acknowledgements

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Declaration of Conflicting Interests

The Author(s) declare(s) that there is no conflict of interest

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CHAPTER 4

Curcumin Up-regulates Antioxidant Defence, Lon Protease and Heat Shock Protein 70 under Hyperglycaemic Conditions in Human Hepatoma Cells

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4.1 Abstract

Objective: Reactive oxygen species, produced by mitochondria, are involved in metabolic disorders. Sirtuin 3 (SIRT3) decreases oxidative stress by regulating mitochondrial antioxidant (AO) defence. Curcumin is a polyphenol known for its protective functions; however the mechanisms underlying its effects on ROS- associated diseases have yet to be fully elucidated. We postulated that curcumin increases AO defence, repair and protein quality control under hyperglycaemic conditions in HepG2 cells through SIRT3-mediated mechanisms.

Methods: Cell viability (MTT assay) was determined in HepG2 cells cultured with 5mM glucose (control), 19.9mM mannitol (OC), vehicle control (0.1% DMSO), 10mM glucose and 30mM glucose in the absence or presence of curcumin (5μM and 10μM) for 24hr. SIRT3, nuclear factor-kappa B (NF-κB), heat shock protein 70 (Hsp70) and Lon protein expressions were determined using western blot analysis. The gene expression of *SIRT3*, *peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α*), *cAMP response element-binding protein (CREB*), mitochondrial antioxidant enzymes *glutathione peroxidase 1* (*GPx1*), *superoxide dismutase 2 (SOD2), uncoupling protein 2 (UCP2*) and mtDNA repair enzyme *OGG1* were measured by qPCR.

Results: Significantly higher cell viability and SIRT3 protein expression were observed in the curcumin-treated groups. Transcript levels of *SIRT3, PGC-1α, CREB, GPx1, SOD2, UCP2* and *OGG1* were also significantly increased in the curcumin-treated hyperglycaemic groups relative to the untreated control. Protein expressions of NF-κB, Lon and Hsp70 were significantly elevated in the 10 μM curcumin-treated hyperglycaemic groups.

Conclusions: Since curcumin and SIRT3 both improve mitochondrial function and AO defence, SIRT3 may be involved in the protective effects of curcumin

4.2 Introduction

Type 2 diabetes mellitus (DMII) and associated complications are linked to oxidative stress (OS) and mitochondrial dysfunction. Oxidative stress impairs mitochondrial function leading to reduced cellular energetic efficiency and apoptosis.

Mitochondrial proteins are vulnerable to oxidative damage as they are closely situated to the electron transport chain (ETC). Oxidative damage can form adducts of proteins with carbohydrates and lipids and may create carbonyl groups and intra-molecular cross-links. These may impair or inactivate enzymes and structural proteins which, if not removed, can aggregate and cause significant cellular toxicity. Protein misfolding and aggregation are associated with diseases (*1, 2*).

Research has placed more focus on evaluating dietary antioxidant (AO) supplementation that may improve DMII conditions. For example, purified anthocyanin supplementation was shown to reduce OS and attenuate insulin resistance and DMII in animal models (*3*).

Curcumin is a phytochemical compound extracted from the rhizome of *Curcuma longa* and has been shown to possess multiple biological properties including AO (*4*), antibacterial (*5*) and anti-inflammatory properties (*6*). It is a bi-functional AO that (a) reacts directly with ROS and (b) indirectly induces up-regulation of cyto-protective and AO proteins through nuclear translocation of nuclear factor erythroid 2-related factor (Nrf2) (*7*).

Mitochondria are targeted for protection by curcumin under oxidative conditions (*8*). Curcumin treatment prevented OS and mitochondrial dysfunction in potassium dichromate- induced nephrotoxicity (*9*). It also decreased malondialehyde and protein carbonyl levels and prevented the decrease in the activity of hepatic AO enzymes viz. superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPx1)(*10*).

Curcumin preserves mitochondrial parameters such as mitochondrial membrane potential, activity of complexes I–IV of the ETC, and ATP production following toxicity (*8*). It also indirectly increases the expression of mitochondrial biogenesis genes and attenuates OS by Nrf2.

Sirtuin 3 (SIRT3) is a mitochondrial stress-responsive protein deacetylase. It regulates mitochondrial processes such as the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) (*11*). It reduces ROS levels by modulating AO enzymes, such as SOD2, thereby protecting against oxidative damage (*12*).

SIRT3 also regulates AO enzymes through transcriptional co-activator peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α). SIRT3 activates the mammalian DNA glycosylase OGG1 enabling mtDNA repair and genomic stability under oxidative conditions (*13*).

The transcription factor nuclear factor-kappa B (NF-κB) is also deacetylated and activated by SIRT3. Activation of NF- κB increases the expression of SOD2 thus making cells resistant to OS-mediated cell damage (*14*).

SIRT3 deacetylates and activates Lon, a major mitochondrial protease, which degrades oxidized and damaged proteins and is involved in mtDNA maintenance and replication (*15*). Heat shock protein 70 (Hsp70) forms part of this protein quality control network as it has the ability to prevent protein aggregation and other protein modifications (*16*).

Since curcumin and SIRT3 both improve mitochondrial function and AO defence, SIRT3 may be involved in the protective effects of curcumin, although this has yet to be fully elucidated.

Molecular events that occur under hyperglycaemic conditions in the liver are not fully known. This is important to interrogate as the liver is the primary organ involved in glucose homeostasis. This is the first study to investigate the effect of curcumin on SIRT3 expression, downstream AO defence and protein quality control under hyperglycaemic conditions in a human hepatoma cell line (HepG2).

4.3 Materials and Methods

Cell Culture and Treatments

HepG2 cells were cultured to confluency in 25cm^3 flasks (5% CO_2, 37°C) in complete culture media (CCM, Eagles minimum essential medium, supplemented with 10% foetal calf serum, 1% L-Glutamine and 1% penstrepfungizone).

The HepG2 cells (2.5 x 10 ^{4} cells/ well) were cultured in the presence of control (5.5mM glucose), osmotic control (19.9mM mannitol), vehicle control (0.1% DMSO) and hyperglycaemic (10mM (intermediate), 30mM glucose) conditions in the absence or presence of curcumin (5 μM and 10 μM) for 24hr in 30mm^3 cell culture plates.

Cell Viability Assay

Following treatment, 3 replicates were incubated with methyl thiazol tetrazolium (MTT) salt solution (5mg/mL in 0.1M phosphate buffered saline (PBS)) and complete culture media (4h, 37ºC). Following incubation, the supernatants were aspirated and dimethyl sulphoxide was added (100 μL/well) and incubated at 37ºC for a further 1h. Optical density of the formazan product was measured by a microplate reader (Bio-tek μQuant) at 570 nm with a reference wavelength of 690nm.

Quantitative Polymerase Chain Reaction (q-PCR)

Total RNA was extracted from cultured cells using the Triazol reagent (Ambion). cDNA was synthesised by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad).

Quantitative PCR was performed on the CFX96 Real-Time System (Bio-Rad) by using iQ SYBR Green supermix (BioRad) with primer sequences as listed in Table 4.1. The PCR was initiated with the following thermocycler profile: an initial denaturation for 8min at 95°C followed by 39 cycles of 95°C denaturation for 15s, annealing for 1min and extension of 72°C for 30s. A final extension at 60°C was performed for 31s. Each measurement was done in triplicate and normalized against 18S rRNA Ct values. The qPCR data is represented as a relative fold change, calculated using the method described by Livak and Schmittgen.(*17*)

Table 4.1: Primer sequences and optimised annealing temperature

Gene	Sense (5'-3')	Antisense (5'-3')	Annealing temp (°C)
18S rRNA	ACACGGACAGGATTGACAGA	CAAATCGCTCCACCAACTAA	**58°C**
SIRT3	GCATTCCAGACTTCAGATCGC	GTGGCAGAGGCAAAGGTTCC	**50°C**
CREB	GATGGACAGCAGATCTTAGTGCC	TGCTGTGCAAATCTGGTATGTT	**65°C**
PGC-1α	CCAAACCAACAACTTTATCTCTTCC	CACACTTAAGGTGCGTTCAATAGTC	**65°C**
GPx1	GACTACACCCAGATGAACGAGC	CCCACCAGGAACTTCTCAAAG	**60°C**
SOD2	GAGATGTTACAGCCCAGATAGC	AATCCCCAGCAGTGGAATAAGG	**58°C**
UCP2	GACCTATGACCTCATCAAG	ATAGGTGACGAACATCACCACG	**50°C**
OGG1	GCATCGTACTCTAGCCTCCAC	AGGACTTTGCTCCCTCCAC	**60°C**

Western blot analysis

Sample proteins were isolated using a Cytobuster™ (Novagen) supplemented with protease and phosphatase inhibitors (Roche). Proteins were quantified using the bicinchoninic acid (BCA) assay (Sigma) and standardised to 1mg/ml. Protein extracts were prepared in Laemlii buffer (dH_2O, 0.5M Tris-Cl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% resolving gel) and electrotransferred to nitrocellulose membrane. Membranes were blocked with 5% BSA in Tris-Buffered Saline and Tween 20 (TTBS) [137mM NaCl, 2.7mM KCl, 24mM Tris, 0.5% Tween 20]. Membranes were probed using the following antibodies in 5% bovine serum albumin (BSA in TTBS): rabbit anti- SIRT3 (Abcam, ab86671, 1:1000), rabbit anti-LONP1 (Sigma- HPA002192, 1:1000), rabbit anti-NF-kB p65 (CST- #3033, 1:1000) and mouse anti-HSP70 (CST- #4872, 1:1000). All membranes were incubated in primary antibodies overnight at 4°C. A horseradish peroxidase (HRP)-conjugated goat polyclonal antibody specific for β-actin was used for the loading control and the normalisation process (1:5000 in 5% BSA in TTBS). Membranes were developed using LumiGlo® Chemiluminescent Substrate System (KPL) and images were captured on the Alliance 2.7 documentation system (UViTech). The density of the bands was quantified using UViBand Advanced Image Analysis Software (UViTech). The experiment was performed in triplicate and repeated thrice. Results are presented as relative band intensity (RBD) and normalised against β-actin.

Statistical analysis

Each experiment was performed in triplicate and repeated thrice. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one way ANOVA followed by Bonferroni test for multiple group comparison. Differences with $p<0.05$ were considered statistically significant.

4.4 Results

Curcumin treatment increases mitochondrial output under normo- and hyperglycaemic conditions

In order to determine the effect of curcumin on mitochondrial output, cell viability was measured in HepG2 cells.

Significantly higher cell viability was observed in the 10μM curcumin-treated hyperglycaemic groups relative to the untreated control (10mM: 0.7703±0.0095 O.D, p-value=0.0107 and 30mM 0.7207± 0.0006 O.D, p-value=0.0069. **Table 4.2**, **Fig. 4.1A**).

Table 4.2: Cell viability, protein and gene expression results in HepG2 cells following 10μM curcumin treatment in the hyperglycaemic groups. * p<0.05 vs. untreated control, #p<0.05 vs. curcumin-treated control, N.S (not significant)

	Average ± SEM				*** p-value**		**# p-value**	
	Untreated control	**Treated control**	**10mM + 10μM curcumin**	**30mM + 10μM curcumin**	**10mM + 10μM curcumin**	**30mM + 10μM curcumin**	**10mM + 10μM curcumin**	**30mM + 10μM curcumin**
Cell Viability (O.D)	0.5177 ±0.01767	0.8223 ±0.01198	0.7703 ±0.0095	0.7207± 0.0006	0.0107	0.0069	0.0989 N.S	0.0125
SIRT3 Protein (RBD)	2.581 ±0.1805	4.555 ±0.3311	6.771 ±0.5016	5.991 ±0.4698	0.0218	0.0293	0.0125	0.0229
NF-κB Protein (RBD)	0.7184 ±0.06260	1.565 ±0.1158	2.002 ±0.1556	1.700 ±0.0698	0.0052	0.0002	0.0468	0.2509
LonP Protein (RBD)	2.124 ±0.08364	5.051 ±0.1750	7.462 ±0.2422	6.231 ±0.09705	0.0021	P<0.0001	0.0199	0.0053
Hsp70 Protein (RBD)	1.521 ±0.3309	2.098 ±0.3841	2.987 ±0.2805	3.589 ±0.6159	0.0581 N.S	0.0220	0.1156 N.S	0.0233
SIRT3 mRNA (Fold Change)	1.000 ±0.0000	2.339 ±0.4480	3.174 ±0.2435	0.7374 ±0.0686	0.0123	0.0621 N.S	0.0551 N.S	0.0519 N.S

CREB mRNA (Fold Change)	1.000 ±0.0000	4.772 ±0.5102	5.890 ±0.4388	3.920 ±0.2053	0.0080	0.0049	0.3345 N.S	0.1963 N.S
PGC-1α mRNA (Fold Change)	1.000 ±0.0000	4.200 ±0.2999	5.114 ±0.3553	1.953 ±0.3994	0.0074	0.1396 N.S	0.0077	0.0056
GPx1 mRNA (Fold Change)	1.000 ±0.0000	4.472 ±0.08289	3.120 ±0.2114	3.204 ±0.1073	0.0098	0.0024	0.0441	0.0162
SOD2 mRNA (Fold Change)	1.000 ±0.0000	4.580 ±0.2072	6.114 ±0.2227	10.04 ±0.0827	0.0019	P<0.0001	0.0008	0.0026
UCP2 mRNA (Fold Change)	1.000 ±0.0000	3.904 ±0.4294	8.141 ±0.6427	3.174 ±0.1787	0.0080	0.0067	0.0033	0.3466 N.S
OGG1 mRNA (Fold Change)	1.000 ±0.0000	6.130 ±0.4487	1.115 ±0.1722	11.02 ±0.4409	0.5721 N.S	0.0019	0.0031	0.0001

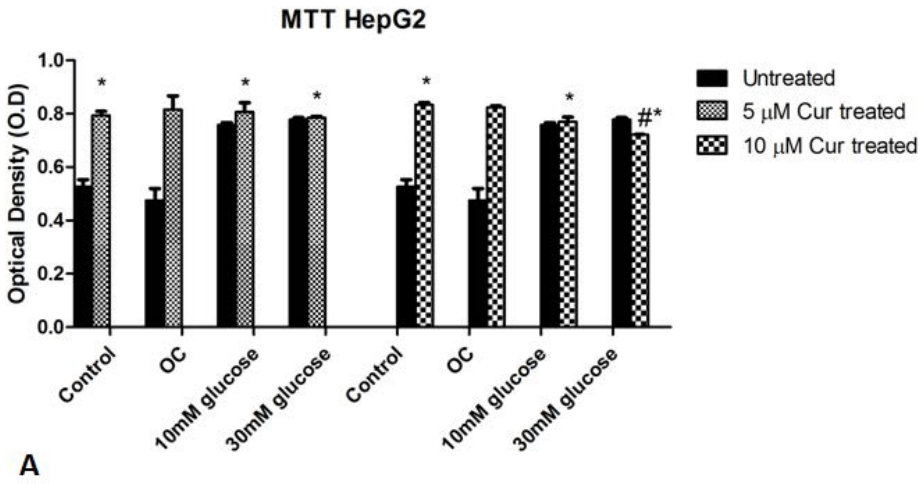


Figure 4.1: The effect of curcumin on cell viability was determined in HepG2 cells (untreated and curcumin-treated). Values are expressed as mean ± SEM. *p<0.05 vs. untreated control, #p<0.05 vs. curcumin-treated control.

Curcumin increases SIRT3 expression under normo- and hyperglycaemic conditions

To determine whether 10μM curcumin treatment had an effect on SIRT3 expression, western blot and qPCR were used to measure protein expression and mRNA levels of SIRT3 respectively in HepG2 cells.

In the curcumin treated hyperglycaemic groups, significantly higher SIRT3 protein expression was observed (10mM: 6.771±0.5016 RBD, p= 0.0218; 30mM: 5.991±0.4698 RBD, p= 0.0293, **Table 4.2, Fig. 4.2A**) as well as increased *SIRT3* levels (10mM: 3.1-fold, p= 0.0123, **Table 4.2, Fig. 4.2B**) relative to the untreated control. The treated hyperglycaemic groups also showed statistically higher SIRT3 protein expression when compared to the treated control (10mM: p=0.0125; 30mM: p=0.0229, **Table 4.2, Fig. 4.2A**).

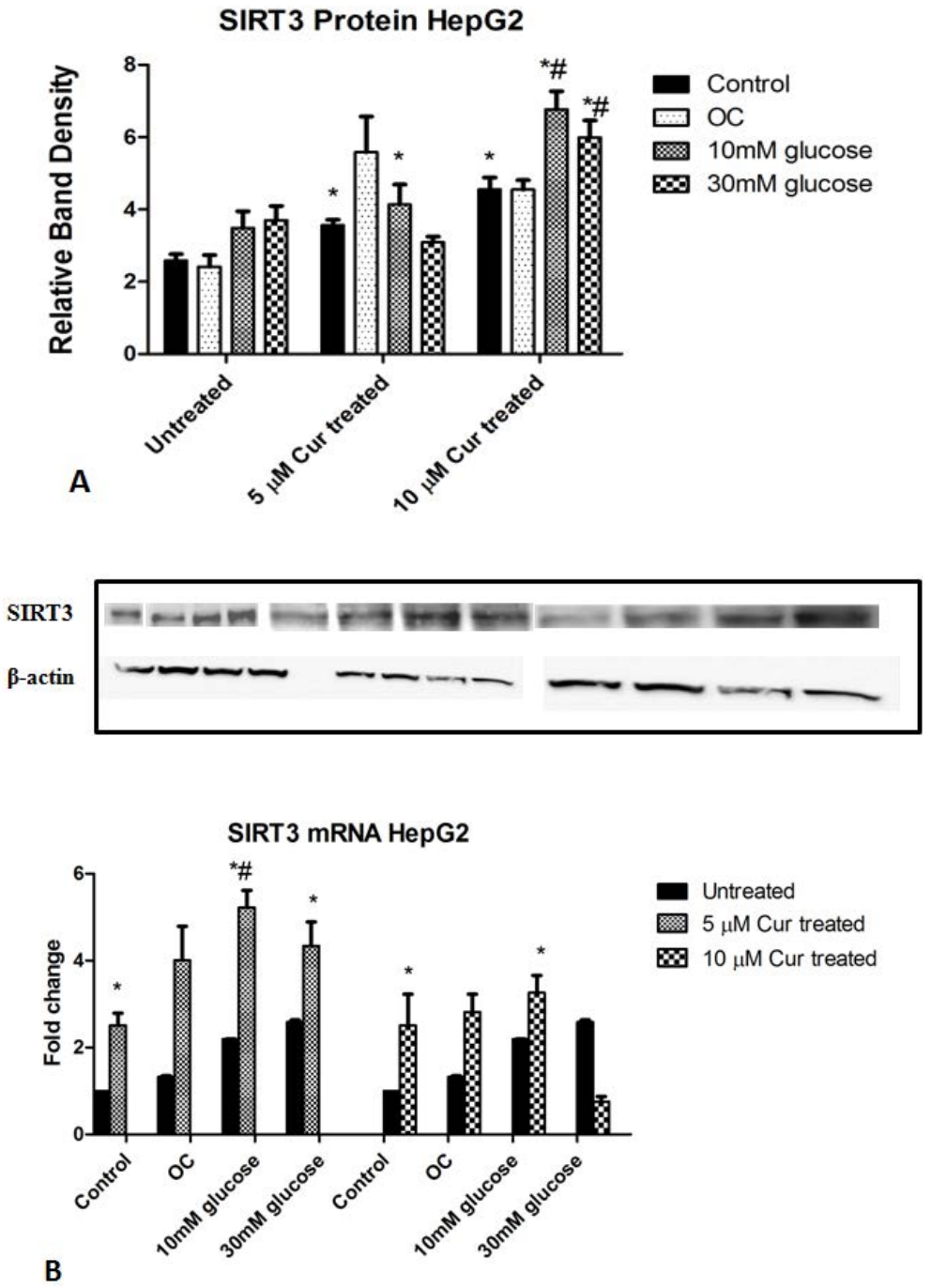


Figure 4.2: The effect of curcumin on SIRT3 expression in untreated and curcumin-treated HepG2 cells. (A) SIRT3 protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of SIRT3 band intensity normalised to β-actin. (B) Gene expression for SIRT3 was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *p<0.05 vs. untreated control, #p<0.05 vs. curcumin-treated control.

Curcumin increases CREB expression under normo- and hyperglycaemic conditions

To investigate the effect of hyperglycaemia and 10μM curcumin treatment on CREB expression, qPCR was used to measure transcript levels of *CREB* in HepG2 cells.

Significantly higher *CREB* levels were observed in the curcumin treated hyperglycaemic groups, relative to the untreated control (10mM: 5.9-fold, p= 0.0080; 30mM: 3.9-fold, p= 0.0049 **Table 4.2**, **Fig. 4.3A**). The intermediate hyperglycaemic group showed relatively higher *CREB* expression when compared to the treated control but this did not reach statistical significance.

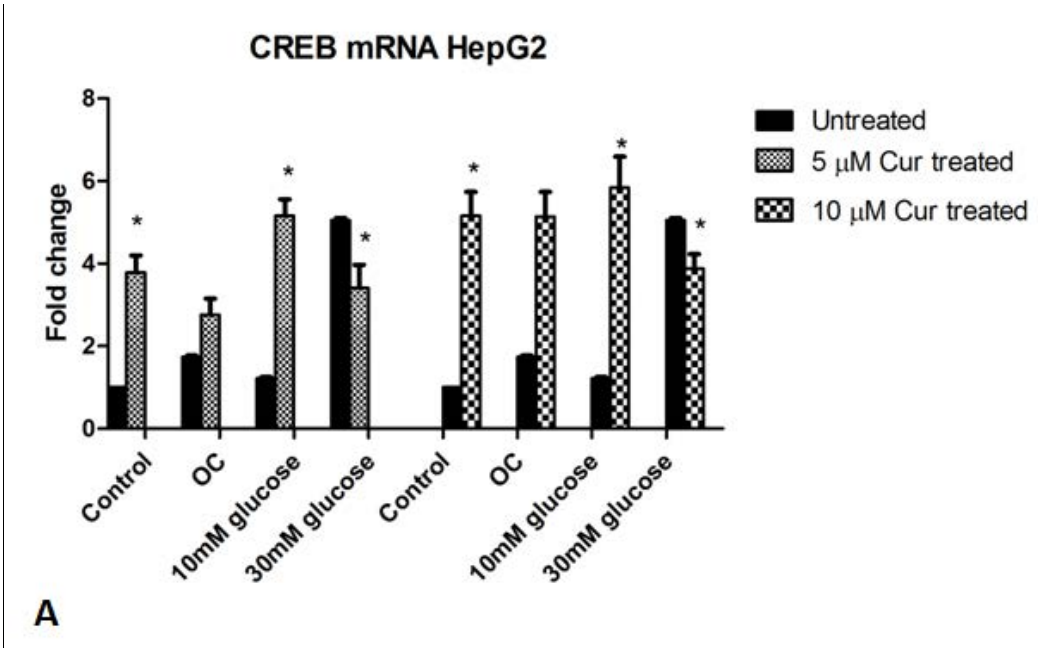


Figure 4.3: The effect of curcumin on CREB expression in untreated and curcumin-treated HepG2 cells. (a) Gene expression for CREB was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *p<0.05 vs. untreated control.

Curcumin increases PGC-1α expression under normo- and hyperglycaemic conditions

The effect of 10μM curcumin treatment on *PGC-1α* gene expression was investigated in HepG2 cells using qPCR.

Higher *PGC-1α* levels were observed in both curcumin treated hyperglycaemic groups relative to the untreated control with only the intermediate hyperglycaemic group reaching statistical significance (5.1-fold, p= 0.0074 **Table 4.2**, **Fig. 4.4A**). When compared to the treated control, the intermediate hyperglycaemic group also showed significantly higher *PGC-1α* expression (1.2-fold, p=0.0077 **Table 4.2**, **Fig. 4.4A**).

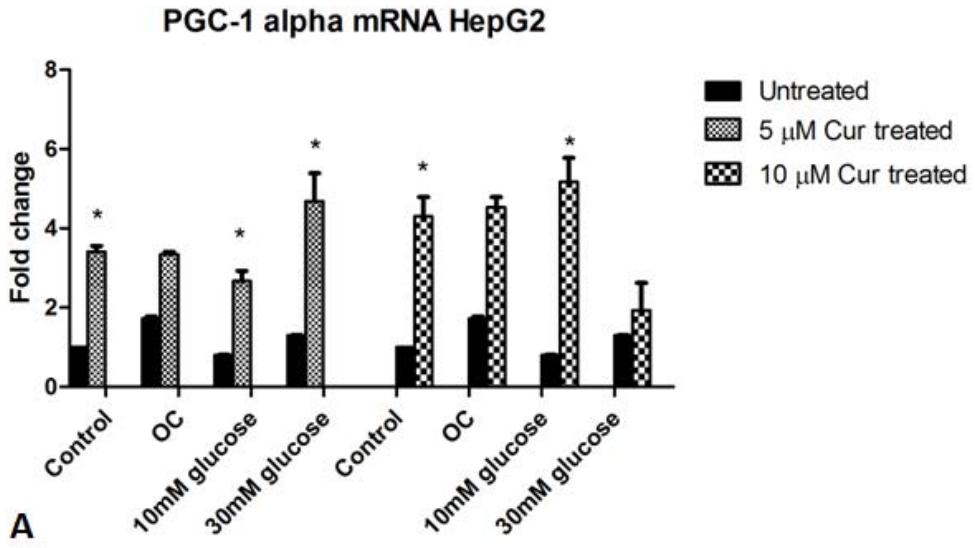


Figure 4.4: The effect of curcumin on PGC-1α expression in untreated and curcumin-treated HepG2 cells. Gene expression for PGC-1α was assessed. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *p<0.05 vs. untreated control.

Curcumin increases antioxidant defence under normo- and hyperglycaemic conditions

In order to determine the effect of 10μM curcumin treatment on antioxidant activity in HepG2 cells, gene expressions for *GPx1*, *SOD2* and *UCP2* were determined by qPCR.

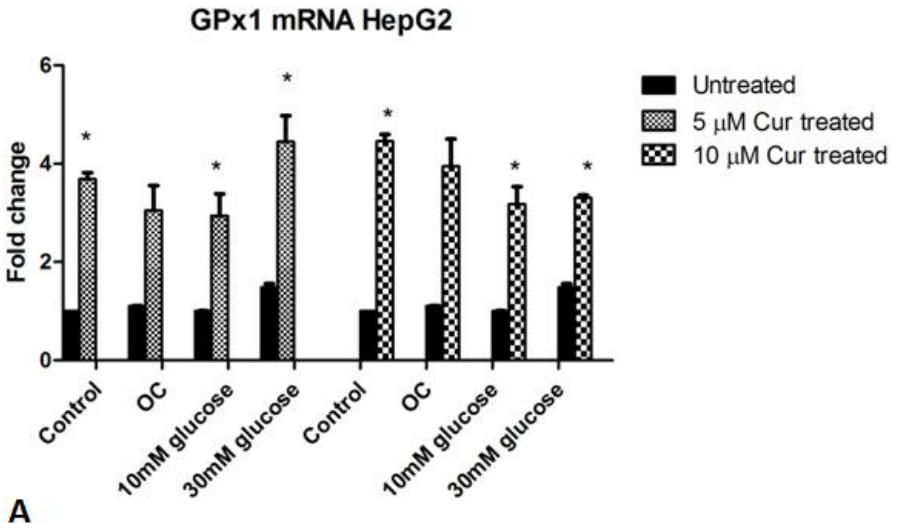
Increased expressions of *GPx1*, *SOD2* and *UCP2* were observed in the curcumin treated hyperglycaemic groups.

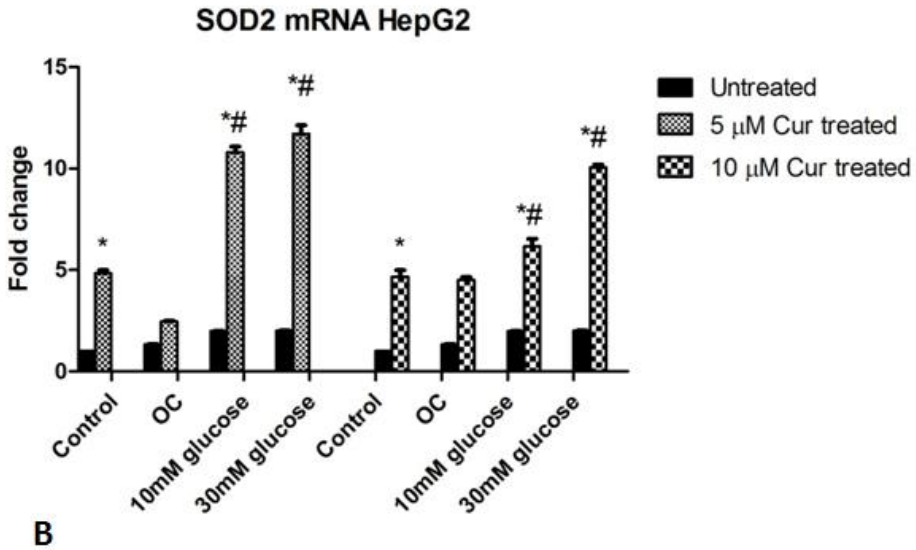
GPx1 levels were higher in the treated hyperglycaemic groups relative to the untreated control (10mM: 3.1-fold, p=0.0098; 30mM: 3.2-fold, p=0.0024 **Table 4.2**, **Fig. 4.5A**). A similar pattern was observed with *SOD2* and *UCP2* mRNA expression.

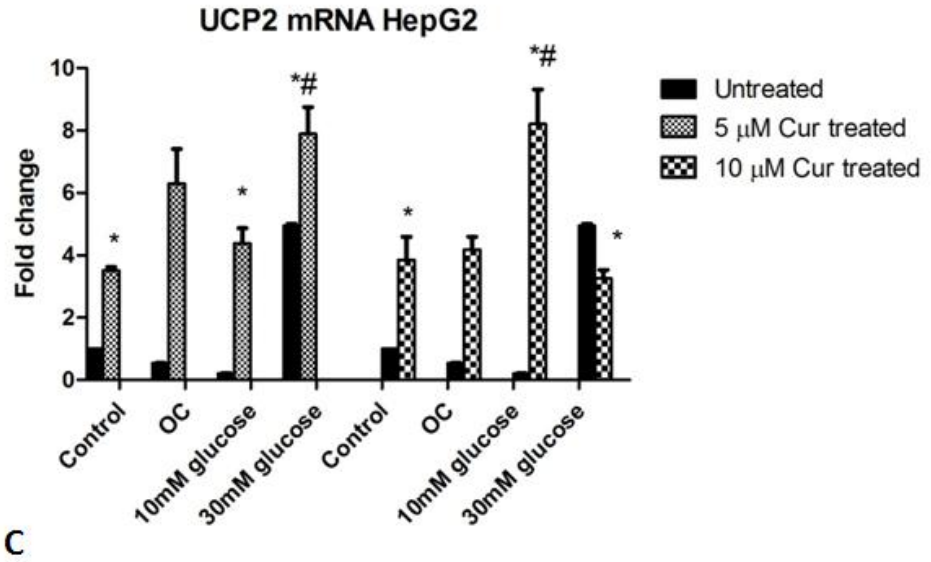
SOD2 levels were significantly higher in the treated hyperglycaemic groups relative to both the untreated and treated controls (Untreated 10mM: 6.1-fold, p=0.0019; 30mM: 10-fold, p<0.0001; Treated 10mM: 1.4-fold, p=0.0008; 30mM: 2.2-fold, p=0.0026 respectively **Table 4.2**, **Fig. 4.5B**).
There was also a significant 8.1-fold and 2-fold increase in *UCP2* mRNA levels in the intermediate group relative to the untreated and treated controls respectively (p=0.0080 and p=0.0033 respectively **Table 4.2**, **Fig. 4.5C)**.

As a marker of mtDNA repair following oxidative damage, *OGGI* expression was determined.

Higher *OGGI* levels were observed in both treated hyperglycaemic groups when compared to the untreated control, however, only the curcumin-treated 30mM glucose group was statistically different (11-fold, p=0.0019 **Table 4.2**, **Fig. 4.5D**). The *OGGI* level in this group was also significantly higher than the treated control (p=0.0001, 1.8-fold).







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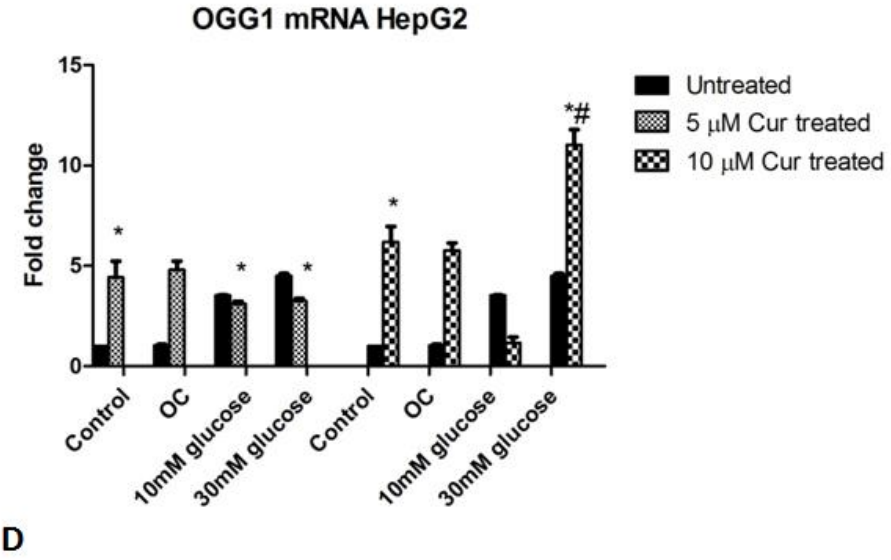

D

Figure 4.5: The effect of curcumin on antioxidant mRNA expression in untreated and curcumin-treated HepG2 cells. Gene expressions for (A) GPx1, (B) SOD2, (C) UCP2 and (D) OGG1 were assessed with quantitative PCR using specific primers. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *$p<0.05$ vs. untreated control, #$p<0.05$ vs. curcumin-treated control.

Curcumin increases NF- κB expression under normo- and hyperglycaemic conditions

We investigated the effect of 10μM curcumin on NF-κB protein expression in HepG2 cells by western blot.

The NF-κB protein expressions in both treated hyperglycaemic groups were significantly increased relative to the untreated control (10mM: 2-fold, p=0.0052; 30mM: 1.7-fold, p=0.0002,) and treated control (10mM: 1.3-fold, p=0.0468; 30mM: 1.1-fold, p=0.2509) **Table 4.2**, **Fig. 4.6A**).

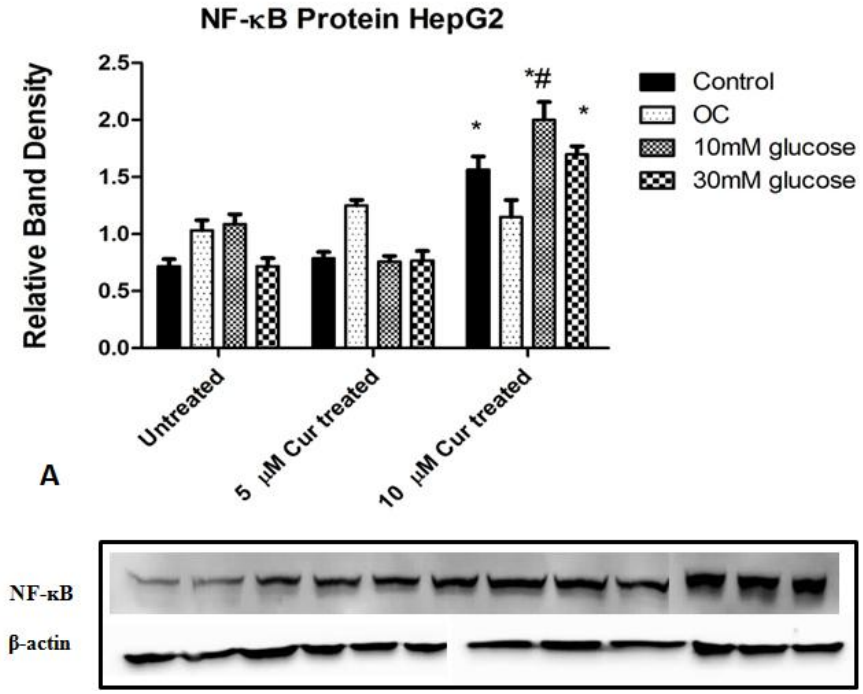

Figure 4.6: The effect of curcumin on NF- κB expression in untreated and curcumin-treated HepG2 cells. NF- κB protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of NF- κB band intensity normalised to β-actin. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *p<0.05 vs. untreated control, #p<0.05 vs. curcumin-treated control.

Curcumin increases Lon protease and Hsp70 expression under normo- and hyperglycaemic conditions

In order to determine the effect of 10μM curcumin on protein quality control, both Lon and Hsp70 protein expressions were measured.

Lon protein expression in the treated hyperglycaemic groups was relatively higher than the untreated control (10mM: 7.5-fold, p=0.0021; 30mM: 6.2-fold, p<0.0001) and treated control (10mM: 1.5-fold, p=0.0199; 30mM: 1.24-fold, p=0.0053 **Table 4.2**, **Fig. 4.7A**). These differences were statistically significant

The treated hyperglycaemic groups also showed higher protein expression of Hsp70 relative to the untreated and treated controls; but only the curcumin-treated 30mM glucose group reached significance in both untreated and treated controls (p=0.0220 and p=0.0233 respectively **Table 4.2**, **Fig. 4.8A**).

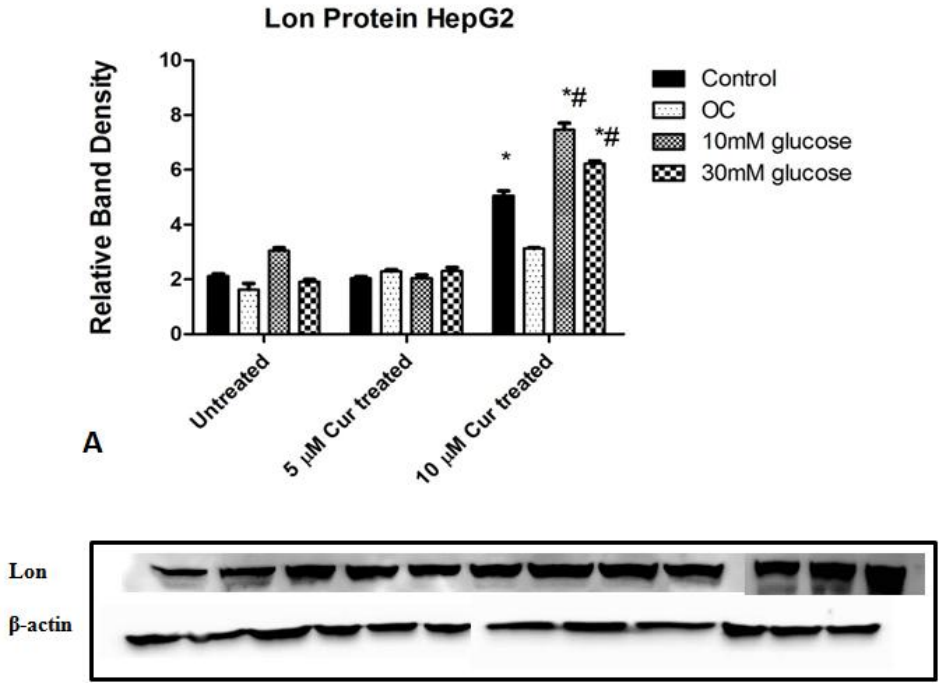

Figure 4.7: The effect of curcumin on Lon protease expression in untreated and curcumin-treated HepG2 cells. Lon protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of Lon band intensity normalised to β-actin. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *p<0.05 vs. untreated control, #p<0.05 vs. curcumin-treated control.

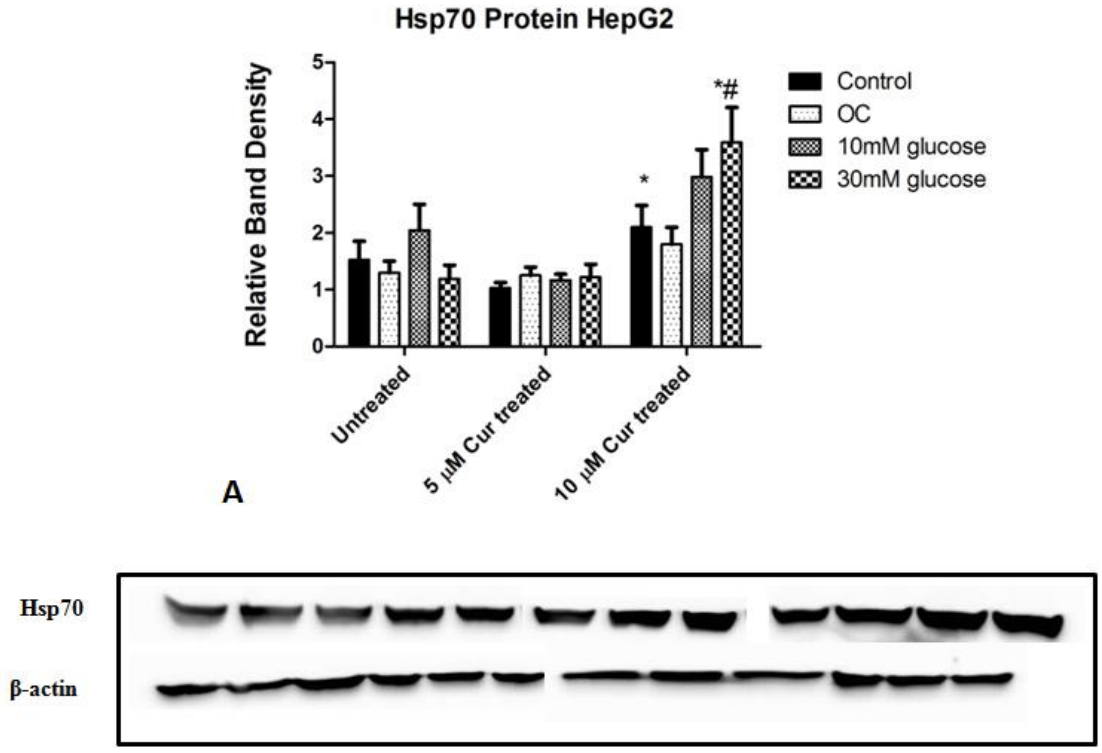

Figure 4.8: The effect of curcumin on Hsp70 expression in untreated and curcumin-treated HepG2 cells. Hsp70 protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of Hsp70 band intensity normalised to β-actin. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *$p<0.05$ vs. untreated control, #$p<0.05$ vs. curcumin-treated control.

4.5 Discussion

Mitochondria are the primary source of ROS as superoxide radicals are formed from the ETC. Basal levels of ROS are involved in signalling, whereas excessive ROS leads to oxidative damage of macromolecules, which play a role in metabolic disorders such as DMII.

Mitochondrial DNA is vulnerable to ROS-induced damage as it is located closely to the ETC, lacks protective histones and has limited DNA repair activity (*18*). Excessive ROS impairs OXPHOS leading to decreased mitochondrial function, decreased ATP levels (*19*) and a marked reduction in AO activity (*20*). These factors need to be improved in order to ameliorate disease conditions.
Oxidative stress may be reduced through the use of exogenous compounds with AO properties that may prevent mitochondrial dysfunction.

Curcumin is a phytochemical compound extracted from the rhizome of *Curcuma longa*. It is a bi-functional AO as it reacts directly with ROS and RNS and also indirectly induces up-regulation of cyto-protective and AO proteins such as SOD2, GPx1 and GSH (*7*). The presence of phenolic and β-diketone functional groups in the structure of curcumin allows it to scavenge superoxide anions, hydrogen peroxide, hydroxyl radicals and nitric oxide (*7, 21, 22*).

It has been shown that curcumin also induces endogenous AO defence by modulating Nrf2 (*23*). Curcumin modifies the inhibitor of Nrf2, allowing for the translocation of Nrf2 to the nucleus where it binds to the antioxidant response element in DNA to initiate transcription of AO genes. It has been shown that curcumin significantly ameliorates experimental diabetes and restores pancreatic AO defence (*24*).

Mitochondria are targets for protection by curcumin under oxidative conditions (*8*). In a kidney model, curcumin maintained mitochondrial function by reducing ROS production and lipid peroxidation (*10*). Curcumin also increased mitochondrial biogenesis in obese mice with liver steatosis (*25*) and reduced oxidative-induced mitochondrial damage in mice neuronal cells (*26*).
Curcumin has been shown to increase the levels of SIRT3, which is part of an intrinsic network of stress-response mechanisms and regulates mitochondrial function (*27*).

We previously showed increased expressions of SIRT3 and AO defence enzymes under long and short term hyperglycaemic stimulation. We established that SIRT3 may be the central component that modulates AO defence and confers resistance to OS-induced damage under hyperglycaemic conditions in the HepG2 and HEK 293 cell lines (*28*).

Since both curcumin and SIRT3 target mitochondrial function, we postulated that SIRT3 may have an effect on the protective effects of curcumin under short term hyperglycaemic stimulation in HepG2 cells.

As a marker of increased mitochondrial output we investigated cell viability, following curcumin treatment, using the MTT assay which measures the reduction of the tetrazolium salt by succinate dehydrogenase. Succinate dehydrogenase is bound to the inner mitochondrial membrane and is involved in the TCA cycle and the ETC. The tetrazolium salt enters mitochondria and is reduced to an insoluble formazan product which is then solubilised by an organic solvent and measured spectrophotometrically. Reduction of the tetrazolium salt only occurs in metabolically active cells and the level of activity is a measure of cell viability and mitochondrial function. We observed increased cell viability in all curcumin-treated groups with higher levels seen in the curcumin-treated hyperglycaemic groups. This suggests that curcumin may maintain mitochondrial function under oxidative conditions.

SIRT3 induces AO genes by PGC-1α, a transcriptional co-activator that is activated in response to OS (*29*). It regulates mitochondrial function and AO defence enzymes such as SOD2 (detoxifies superoxide radicals that are formed as a by-product of OXPHOS), GPx1 (enzyme that detoxifies H_2O_2 to H_2O) and UCP2, a proton carrier in the inner mitochondrial membrane that attenuates ROS-mediated damage (*30-32*).

In an oxidative environment, PGC-1α induces SIRT3 expression through activation of estrogen related receptor alpha (ERR- α) and in turn, SIRT3 stimulates PGC-1α expression via phosphorylated cAMP response element-binding protein (p-CREB), thereby forming a positive feedback cycle and increasing AO defence (*33, 34*).

In this study, increased expressions of SIRT3, CREB, PGC-1α and AO enzymes were observed in the curcumin-treated hyperglycaemic groups following 5μM and 10μM curcumin administration. Although the expression levels were differentially regulated by both concentrations of curcumin under hyperglycaemic conditions, the more pronounced effect was observed in the 10μM curcumin group.

Oxidative stress induces DNA base damage such as 7,8-dihydro-8-oxoguanine (8-oxoG) (*35*) increasing the frequency of spontaneous G.C- -T.A transversion mutations which are involved in mitochondrial diseases (*36*). The DNA glycosylase, OGG1, recognizes and removes 8-oxoG that is base-paired with cytosine (*37*). SIRT3 directly deacetylates and activates OGG1 enabling DNA repair and genomic stability under oxidative conditions (*13*).

As a marker of mtDNA repair under oxidative conditions, we have shown increased *OGG1* expression following curcumin treatment in the hyperglycaemic groups with levels peaking in the 10μM curcumin group.

Interestingly, we found a distinct increase in NF- κB expression in the 10μM curcumin-treated hyperglycaemic groups. Studies have shown that curcumin inhibits NF- κB activity as it is a key transcription factor involved in the inflammatory response (*38, 39*). Curcumin, however, has also been shown to induce ROS generation in various cell lines (*40, 41*) and this may modulate the NF- κB response.

The main role of NF- κB is considered to be protective and is involved in cell protective pathways that suppress OS (*42*). A study showed that OS induced by reperfusion insult, in cultured neurons, was associated with NF- κB inducing a protective signal rather than death signals(*43*). Other studies have also shown that activation of NF- κB led to neuro-protection, whereas NF- κB inhibition led to apoptosis (*44, 45*). It has also been shown that NF- κB is deacetylated and activated by SIRT3. Activation of NF- κB by SIRT3 increased the expression of SOD2 thus making cells resistant to OS-mediated cell damage (*14*). The target genes of NF- κB may, therefore, vary depending on the cellular context.

Oxidative damage can create carbonyl groups and intra-molecular cross-links. These may impair enzymes and structural proteins which, if not removed, can aggregate and cause significant cellular toxicity. Mitochondrial proteins are vulnerable to oxidative damage.

Lon is the major mitochondrial protease that may be regulated by NF- κB and SIRT3 (*46, 47*). Lon degrades oxidized and damaged proteins and assists in the assembly and folding of mitochondrial proteins (*48*). A study observed that Lon increased protection against accumulated carbonylated proteins and increased cell viability during OS (*49*).

In this study, we found increased expressions of NF- κB and Lon in the 10μM curcumin-treated hyperglycaemic groups, similar to SIRT3 expression, which may suggest regulation of Lon by NF-κB and SIRT3. No differences were observed in the groups treated with 5μM curcumin when compared to the untreated group.

We also observed increased Hsp70 expression in the 10μM curcumin-treated hyperglycaemic groups. Hsp70 is closely linked to cyto-protection has the ability to prevent protein aggregation and protein modifications. It may also prevent oxidative injury by improving AO defence (*50*). Hsp70 has been shown to be part of the protein quality control and cell protective network with Lon in the removal of

damaged proteins (*16*).

Following curcumin administration, our study showed increased expressions of SIRT3, AO defence, the repair enzyme and protein quality control under hyperglycaemic conditions, which represent an oxidative environment. These were optimally expressed in the 10μM curcumin-treated hyperglycaemic groups. Further, the 10mM glucose treatment yielded higher SIRT3 and AO expressions than the 30mM glucose treatment following 10μM curcumin administration. These results suggest that selective concentrations of curcumin may improve the AO response and cell survival under intermediate hyperglycaemic conditions.

Since SIRT3 has already been shown to increase AO defence and cell survival, the protective effects of curcumin may occur through SIRT3-mediated mechanisms.

This study may be improved by using a diabetic *in vivo* model which may help us establish a more holistic response. A wider range of curcumin concentrations over a chronic time period may also help us better elucidate the effect of curcumin in a diabetic model. Inhibition of SIRT3 is required in order to determine the exact role SIRT3 in the protective effects of curcumin.

Future studies include interrogating a more defined cell survival pathway following curcumin administration. Investigating the effect of curcumin on the epigenome may enable us to determine differential gene regulation during pre-diabetes and DMII. It may also be useful to investigate the effect of chemical therapeutics co-administered with curcumin in a diabetic model.

4. 6 Conclusion

Mitochondrial dysfunction and OS play a key role in the pathogenesis of several metabolic disorders. Curcumin targets mitochondria and AO defence and, therefore, may improve disease conditions by conferring resistance to OS-induced damage through SIRT3 mediated mechanisms.

4.7 Acknowledgements

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CHAPTER 5

Cell Survival Genes Counterbalance Apoptotic Stress Under Hyperglycaemic Conditions In HepG2 Cells

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5.1 Abstract

Objective: Hyperglycaemia increases mitochondrial production of oxidants which contributes to a variety of diseases. Hyperglycaemia has been shown to induce cell death; however, the cell protective mechanisms that may counterbalance apoptotic stress have yet to be fully elucidated.

Methods: Cell viability was determined in HepG2 cells cultured with 5mM glucose (control), 19.9mM mannitol (OC) and 30mM glucose for 24hr. Caspase activity (3/7, 8 and 9) was measured whilst early stage apoptosis was determined by the Annexin V assay. Protein expressions for heat shock protein 27 (Hsp27) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) were determined using western blot analysis. Gene expressions for *superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPx1), p300* and *CREB binding protein (CBP)* were evaluated by qPCR. Reduced glutathione (GSH) concentrations were measured luminometrically.

Results: Increased cell viability was observed in the hyperglycaemic group. Hyperglycaemia induced significantly higher caspase-8 and caspase-9 activity; however caspase-3/7 activity was 35-fold lower than caspase-9. The Annexin V assay also showed significantly lower percentage of apoptotic cells in this group. The protein expressions for Hsp27 and Nrf2 were both significantly elevated in the hyperglycaemic group whilst significantly higher transcript levels were observed for *p300*, *CBP*, *SOD2* and *GPx1*. The concentrations of reduced GSH were significantly increased in the hyperglycaemic group.

Conclusions: Although hyperglycaemia induces apoptosis, the apoptotic stress may be counterbalanced by cell survival mechanisms regulated by stress response proteins and downstream activation of AO defence.

5.2 Introduction

Hyperglycaemia activates several biochemical pathways that contribute to production of reactive oxygen species (ROS) which may be the causal link between type 2 diabetes mellitus (DMII) and associated complications (*1*).

Oxidative stress (OS) occurs when ROS exceeds the antioxidant (AO) capacity. Oxidative stress affects mitochondrial function resulting in reduced cellular energetic efficiency and apoptosis. This may play a role in metabolic disorders such as DMII.

Mitochondrial proteins are at risk of oxidative damage as they are situated near the electron transport chain (ETC).

Hyperglycaemia-induced mitochondrial dysfunction has been shown to induce apoptosis. Following OS, mitochondria become permeabilised and release cytochrome *c* which, together with pro-caspase-9 and the apoptotic protease activating factor 1 (Apaf-1), form a complex known as the apoptosome, which recruits and activates pro-caspase 9 (*2*). Cytochrome *c* activates caspase-3, which is required to execute apoptosis (*3*). Caspase-8, which initiates extrinsic apoptosis, also plays a role in the intrinsic (mitochondrial) pathway. These all form part of apoptotic stress.

Hyperglycaemic-induced cell death has been observed in multiple organs (*4*, *5*). Apoptosis was observed in the pancreatic beta cells of diabetic patients (*6*). Hyperglycaemia was shown to be the direct cause of apoptosis in diabetic myocardium and cultured cardiac myoblast cells (*7*). Hyperglycaemia was also shown to induce cell death in neurons by activating caspase-3 (*8*).

Although it has been established that the hyperglycaemia may lead to cell death, there is still a network of cell protective mechanisms that may counteract apoptosis and ensure cell survival.

Heat shock proteins (Hsps) are a highly conserved family of stress response proteins and their expressions are increased in response to OS. Heat shock proteins can function as molecular chaperones and protect against apoptosis. During OS, heat shock protein 27 (Hsp27) increases the levels of intracellular glutathione as it holds glutathione in its reduced form under oxidative conditions (*9*). Heat shock protein 27 also interacts with cytochrome *c* thus preventing the formation of the apoptosome and activation of caspase-9 (*10*).

The p300 and CREB binding proteins (CBP) are transcriptional co-activators that also have histone acetyltransferase (HAT) activity which results in relaxed chromatin that is associated with increased gene transcription.

In response to OS, transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is acetylated by p300 and CBP. This enables Nrf2 to translocate to the nucleus and bind to the antioxidant response element which results in the transcription of AO genes such as superoxide dismutase 2 (SOD2), glutathione peroxidase 1(GPx1) and reduced glutathione (GSH) (*11*). It has been shown that high glucose levels activate p300 and CBP (*12*). Over-expression of Nrf2 has been shown to prevent the onset of DMII and reduces OS (*13*).

Hyperglycaemia has been shown to cause cell death, however the cell protective mechanisms that counteract this have yet to be fully elucidated in HepG2 cells.

Molecular events that occur during hyperglycaemia in the liver, the primary organ involved in glucose regulation, have yet to be fully interrogated. This study investigates the effect of short term hyperglycaemic stimulation on downstream cell protective mechanisms in HepG2 cells.

5.3 Materials and Methods

Cell Culture and Treatments

HepG2 cells were cultured to confluency in 25cm^3 flasks (5% CO_2, 37°C) in complete culture media (CCM, Eagles minimum essential medium, supplemented with 10% foetal calf serum, 1% L-Glutamine and 1% penstrepfungizone).

The HepG2 cells (2.5 x 10 ^{4} cells/ well) were cultured in the presence of the hyperglycaemic control (5.5mM glucose), osmotic control (OC) (19.9mM mannitol), and 30mM glucose conditions for 24hr in 30mm^3 cell culture plates.

Cell Viability Assay

Following treatment, 3 replicates were incubated with methyl thiazol tetrazolium (MTT) salt solution (5mg/mL in 0.1M phosphate buffered saline (PBS)) and complete culture media (4h, 37ºC). Following incubation, the supernatants were aspirated and dimethyl sulphoxide was added (100 µL/well) and incubated at 37ºC for a further 1h. Optical density of the formazan product was measured by a microplate reader (Bio-tek µQuant) at 570 nm with a reference wavelength of 690nm.

Caspase-3/7, 8, 9 Assessments

Caspase-3/-7, -8 and -9 activities were detected with Caspase-Glo® assays (Promega, Madison, USA). As per manufacturer's protocol, Caspase-Glo®-3/-7, -8 and -9 reagents were reconstituted and added to wells (in six replicates) of an opaque 96-well microtitre plate (40 µl of reagent per 100 µl of 10,000 cells/well). Samples were mixed and incubated in the dark (30 min, RT). The luminescent signal was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). Caspase-3/-7, -8 and -9 activities were expressed as relative light units (RLU).

Annexin-V-Fluos assay

The annexin-V-Fluos assay (Roche) was used to determine phosphatidylserine (PS) translocation. To each flow cytometry tube, 100 µl of staining buffer, 100 µl of annexin-V-Fluos labeling solution (annexin-V: propidium iodide (PI): staining buffer (1:1:50 vol/vol/vol)) and 100 µl of cell suspension was added, and incubated in the dark (15 min, RT). Samples were analyzed on a FACS Calibur (BD Biosciences) flow cytometer. Data were analyzed using CellQuest PRO v4.02 software (BD Biosciences). Cells were gated to exclude cellular debris using FlowJo v7.1 software (Tree Star, Inc).

Approximately 50,000 events were analyzed for apoptotic (annexin-V + ve, PI -ve), necrotic (annexin-V + ve, PI + ve) and live cells (annexin-V -ve, PI -ve). The results were expressed as percentage of the total events.

Quantitative Polymerase Chain Reaction (q-PCR)

Total RNA was extracted from cultured cells using the Triazol reagent (Ambion). cDNA was synthesised by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad).
Quantitative PCR was performed on the CFX96 Real-Time System (Bio-Rad) by using iQ SYBR Green supermix (BioRad) with primer sequences listed in Table 5.1. The PCR was initiated with the following thermocycler profile: an initial denaturation for 8min at 95°C followed by 39 cycles of 95°C denaturation for 15s, annealing for 1min and extension of 72°C for 30s. A final extension at 60°C was performed for 31s. Each measurement was done in triplicate and normalized against 18S rRNA Ct values. The qPCR data is represented as a relative fold change, calculated using the method described by Livak and Schmittgen (*14*).

Table 5.1 Primer sequences and optimised annealing temperature

Gene	**Sense (5'-3')**	**Antisense (5'-3')**	**Annealing temp (°C)**
18S rRNA	ACACGGACAGGATTGACAGA	CAAATCGCTCCACCAACTAA	58°C
GPx1	GACTACACCCAGATGAACGAGC	CCCACCAGGAACTTCTCAAAG	60°C
SOD2	GAGATGTTACAGCCCAGATAGC	AATCCCCAGCAGTGGAATAAGG	58°C
p300	GCTTTGTCTACACCTGCAA	TGCTGGTTGTTGCTCTCATC3	50°C
CBP	CACCAGCAGATGAGGACTCT	TACACCGGTGCTAGGAGGAG	50°C

Western blot analysis

Sample proteins were isolated using a Cytobuster™ (Novagen) supplemented with protease and phosphatase inhibitors (Roche). Proteins were quantified using the bicinchoninic acid (BCA) assay (Sigma) and standardised to 1mg/ml. Protein extracts were prepared in Laemlii buffer (dH_2O, 0.5M Tris-Cl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% resolving gel) and electrotransferred to nitrocellulose membrane. Membranes were blocked with 5% BSA in Tris-Buffered Saline and Tween

20 (TTBS) [137mM NaCl, 2.7mM KCl, 24mM Tris, 0.5% Tween 20]. Membranes were probed using the following antibodies in 5% bovine serum albumin (BSA in TTBS): rabbit anti- Nrf 2 (Abcam, ab31163, 1:750) and mouse anti-Hsp27 (Santa Cruz, sc-51956, 1:1000). All membranes were incubated in primary antibodies overnight at 4°C. A horseradish peroxidase (HRP)-conjugated goat polyclonal antibody specific for β-actin was used for the loading control and the normalisation process (1:5000 in 5% BSA in TTBS). Membranes were developed using LumiGlo® Chemiluminescent Substrate System (KPL) and images were captured on the Alliance 2.7 documentation system (UViTech). The density of the bands was quantified using UViBand Advanced Image Analysis Software (UViTech). The experiment was performed in triplicate and repeated thrice. Results are presented as relative band intensity (RBD) and normalised against β-actin.

Glutathione Assay

Intracellular concentrations of GSH were measured using the GSH-Glo™ Glutathione Assay (Promega, Madison, USA). Briefly, cultured cells were transferred to an opaque microtitre plate (50 µL of 1x10 ^{4}cells/well, 3 replicates). GSH standards (0 µM – 50 µM) were prepared from a 5 mM stock solution diluted in deionised water. The 2X GSH-Glo™ Reagent was prepared according to the manufacturer's instructions, added to the experimental wells (50 µL/well), and incubated at room temperature (RT), 30min. Reconstituted Luciferin Detection Reagent (50 µL) was added to each well and incubated at RT for 15 minutes. The luminescence was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). A standard curve was derived using the GSH standards (0 µM – 5 µM) and the GSH concentration in each treatment was extrapolated from the equation.

Statistical analysis

Each experiment was performed in triplicate and repeated thrice. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one way ANOVA followed by Bonferroni test for multiple group comparison. Differences with $p<0.05$ were considered statistically significant.

5.4 Results

Mitochondrial output increases under hyperglycaemic conditions

In order to determine the effect of hyperglycaemia on mitochondrial output, cell viability was measured in HepG2 cells.

Significantly higher cell viability was observed in the hyperglycaemic groups relative to the control (p=0.0382, control: 0.7297±0.0614 O.D and 30mM: 1.056±0.0061 O.D, **Fig. 5.1**).

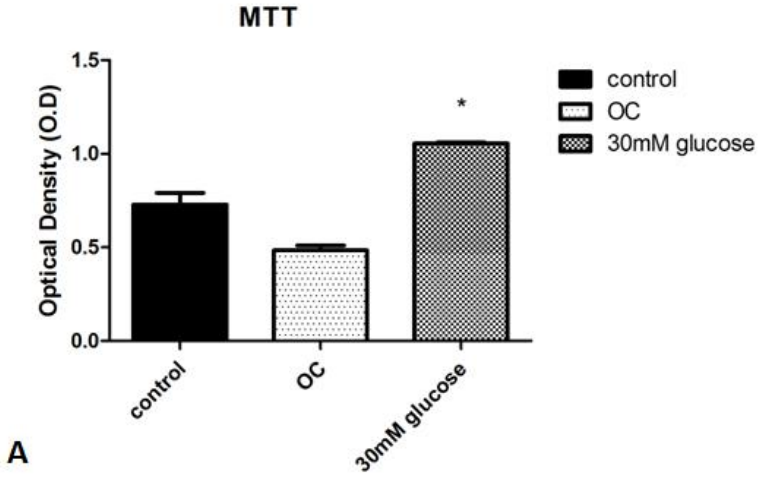

Fig. 5.1 The effect of hyperglycaemia on cell viability was determined in HepG2 cells. Values are expressed as mean ± SEM. *p<0.05 vs. control

Apoptotic markers under hyperglycaemic conditions

In order to investigate the effect of hyperglycaemia on the apoptotic cascade, caspase-8, caspase-9 and caspase 3/7 activities were measured.

Significantly higher caspase-8 and caspase-9 activities were observed in the hyperglycaemic group relative to the control (caspase-8: p= 0.0019, control: 3500000± 213800 RLU and 30mM: 5754000± 271800 RLU **Fig. 5.2a**; caspase-9: p= 0.0010, control: 7342000 ± 191700 RLU and 30mM: 9137000 ± 231700 RLU **Fig 5.2b**). Caspase-9 activity was 1.6-fold higher than caspase-8 following hyperglycaemic treatment.

Although significantly higher caspase-3/7 activity was observed in the hyperglycaemic group relative

to the control (p= 0.0030, control: 165800 ± 10510 RLU and 30mM: 257100 ± 10090 RLU **Fig. 5.2c**), the activity levels were 35-fold lower than caspase-9 activity.

The Annexin V assay was used to measure early-stage apoptosis in HepG2 cells. Interestingly, significantly lower percentage of apoptotic cells were observed in the hyperglycaemic group relative to the control (p= 0.0006, control: 9.543% and 30mM: 5.153% **Fig. 5.2d**).

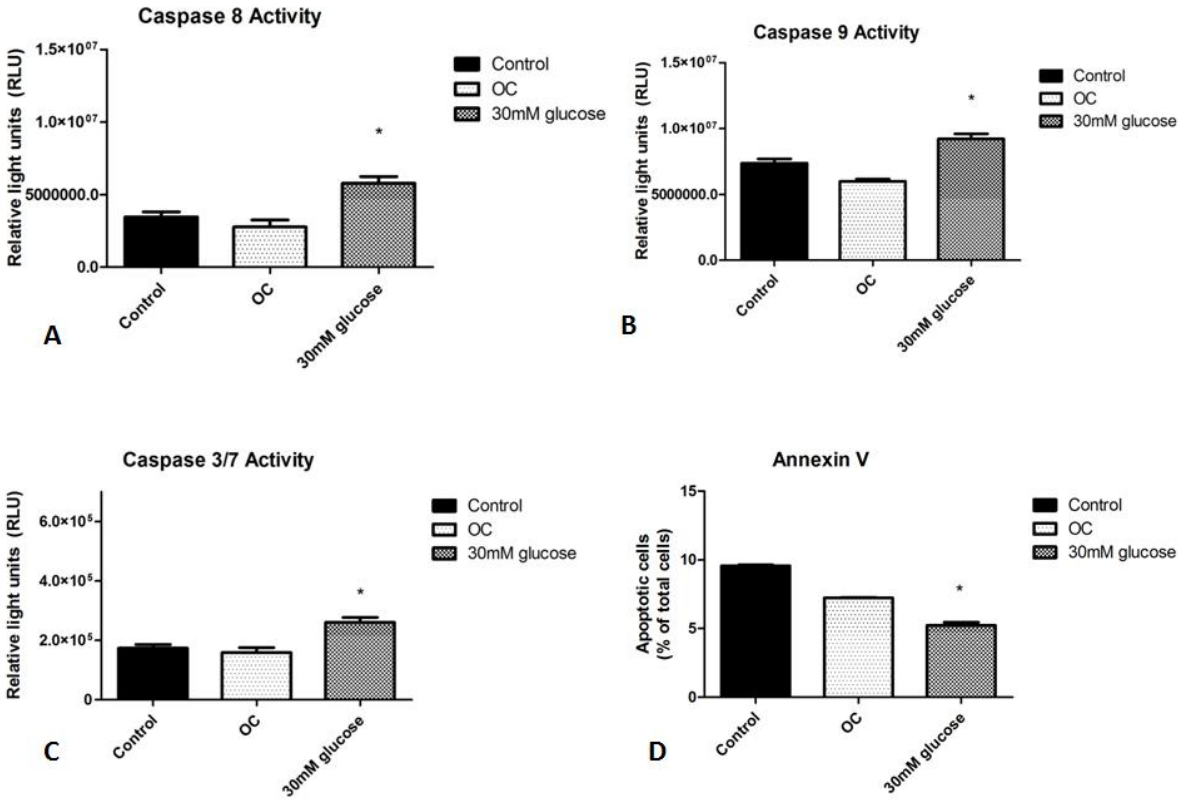

Fig. 5.2 The effect of hyperglycaemia on (a) caspase-8 activity, (b) caspase-9 activity, (c) caspase 3/7 activity and (d) percentage of apoptotic in HepG2 cells. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *p<0.05 vs. control

Increased Hsp27 expression following hyperglycaemic treatment

Hsp27 protein expression was measured by western blot analysis. The hyperglycaemic treatment yielded significantly higher protein expression upon comparison to the control (p=0.0153, control: 0.3067±0.0039 RBD and 30mM: 0.3487±0.0092 RBD **Fig. 5.3)**

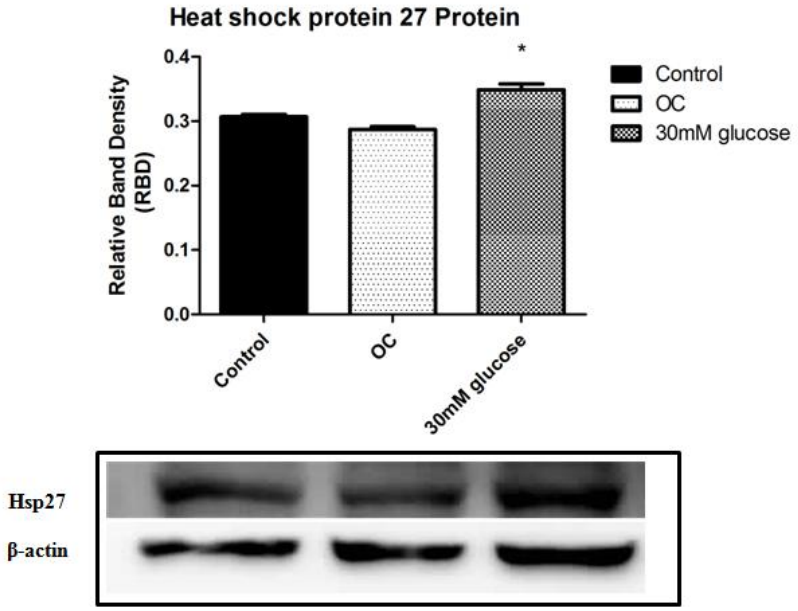

Fig. 5.3 The effect of hyperglycaemia on Hsp27 protein expression in HepG2 cells. (a) Hsp27 protein expression was determined by western blot. A representative immunoblot is shown, along with summarised data of Hsp27 band intensity normalised to β-actin. Each bar represents the mean ± SEM of 3 replicates. Data expressed as mean±SEM. *p<0.05 vs. control

Increased expression of histone acetyltransferases p300 and CBP under hyperglycaemic conditions

In order to determine the effect of hyperglycaemia on histone acetyltransferase expression, gene expressions for *p300* and *CBP* were determined by qPCR.

The expressions of both *p300* and *CBP* were higher in the hyperglycaemic group relative to the control but these did not reach statistical significance, **Fig. 5.4a and Fig. 5.4b** respectively.

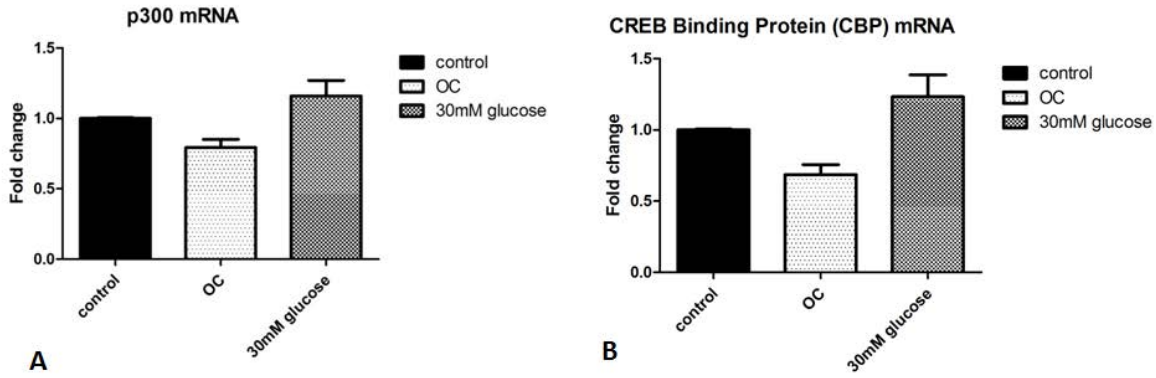

Fig. 5.4 The effect of hyperglycaemia on mRNA expressions of (a) p300 and (b) CBP in HepG2 cells. Values are expressed as fold changes relative to the control. Each bar represents the mean ± SEM of 3 replicates. *p<0.05 vs. control

Increased antioxidant expression under hyperglycaemic conditions

In order to determine the effect of hyperglycaemic treatment on the antioxidant profile, Nrf2 protein expression was measured by western blot; transcript levels of *SOD2* and *GPx1*were determined by qPCR and reduced GSH was measured luminometrically.

Slightly higher Nrf2 protein expression was observed in the hyperglycaemic group compared to the control; however, this was not statistically significant, **Fig. 5.5a**. The gene expressions for *SOD2* and *GPx1* were 2-fold (p<0.0001, **Fig. 5.5b**) and 1.5-fold (p= 0.0091, **Fig. 5.5c**) higher than the control respectively. The concentration of reduced GSH was also found to be significantly higher following hyperglycaemic treatment when compared to the control (p= 0.0019, control: 9.173± 0.2088µM and 30mM: 11.04± 0.1582µM, **Fig. 5.5d**).

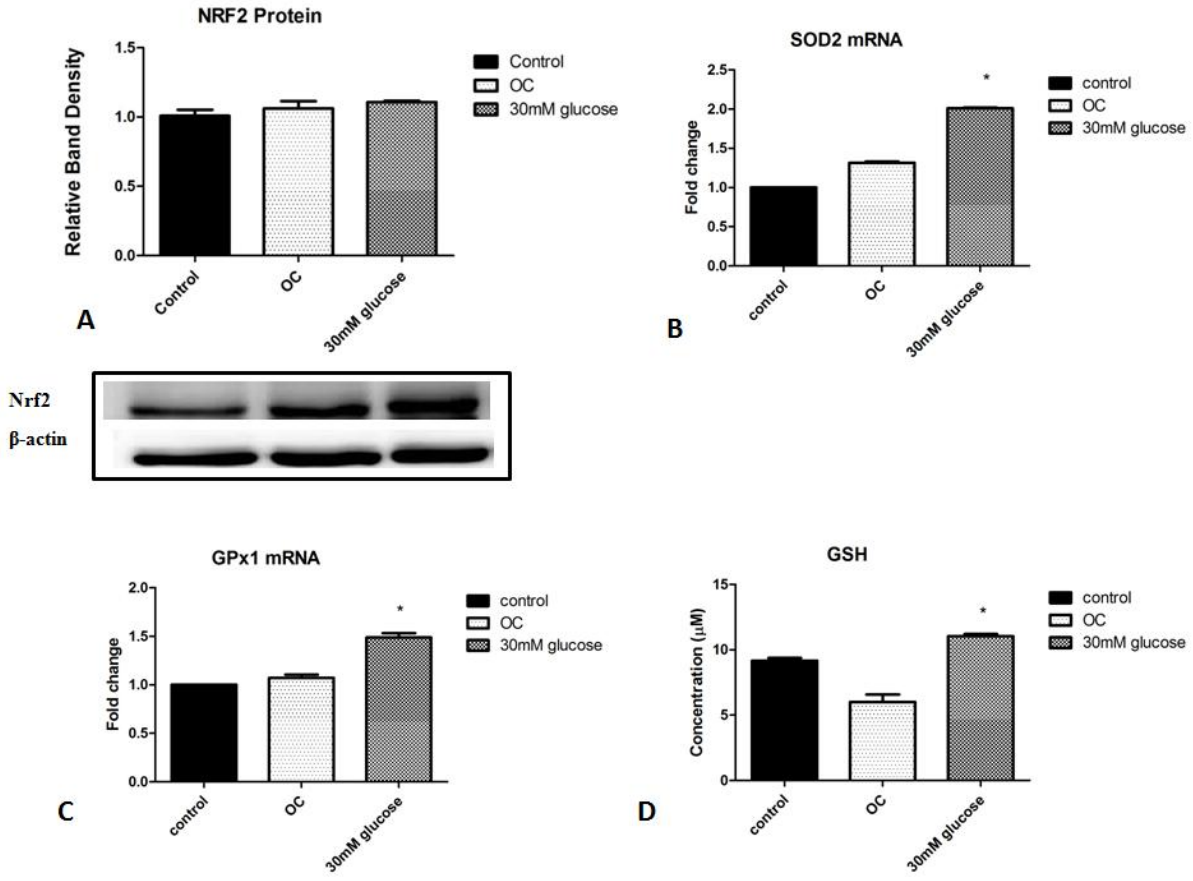

Fig. 5.5 The effect of hyperglycaemia on antioxidant profile in HepG2 cells. (**a**) Protein expression for Nrf2 was determined using western blot. A representative immunoblot is shown, along with summarised data of Nrf2 band intensity normalised to β-actin. Gene expressions for (**b**) SOD2 and (**c**) GPx1 were assessed with quantitative PCR using specific primers. Values are expressed as fold changes relative to the control. (**d**) Concentrations of GSH were determined luminometrically. Each bar represents the mean ± SEM of 3 replicates. *$p<0.05$ vs. control

5.5 Discussion

Evidence has shown that elevated ROS is involved in the progression of DMII (*15-17*). Hyperglycaemia activates several biochemical pathways that have been suggested to contribute to the formation of ROS and may be the causal link between DMII and associated complications.

Hyperglycaemia increases the production of reducing equivalents from the tricarboxylic acid (TCA) cycle. These enter the ETC resulting in electron leakage and increased production of superoxide radicals (*18*). Excessive ROS damages macromolecules and this may lead to metabolic disorders such as DMII.

Mitochondrial DNA (mtDNA) is vulnerable to oxidative damage as it is situated closely to the ETC; does not have protective histones and has limited DNA repair activity (*19*). Excessive ROS production compromises oxidative phosphorylation (OXPHOS) leading to mitochondrial dysfunction, decreased capacity of cells to maintain ATP levels (*20*), reduced AO activity(*21*) and increased apoptosis (*7*). These factors need to be improved and maintained in order to ameliorate disease conditions.

Hyperglycaemia-induced mitochondrial dysfunction has been shown to cause apoptosis. Caspases are a family of cysteine proteases that cleave target proteins at sites next to aspartic acid residues. They are known as initiators (caspase-8 and caspase-9) or executioners (caspase-3/7).

Following OS, mitochondria release cytochrome *c* which, together with caspase-9 and Apaf-1, form a complex known as the apoptosome which recruit and activate caspase-9 (*2*). Cytochrome *c* activates caspase-3 which is required to execute apoptosis (*3*). Caspase-8, which initiates extrinsic apoptosis, also plays a role in the intrinsic (mitochondrial) pathway though Bid. These all form part of apoptotic stress.

Hyperglycaemia has been shown to be a direct cause of apoptosis in diabetic myocardium and cultured cardiac myoblast cells (*7*).

In this study we investigated the effect of short term hyperglycaemic stimulation on mitochondrial output in HepG2 cells. This was done by the MTT assay which measures the reduction of the tetrazolium salt by succinate dehydrogenase. Succinate dehydrogenase is bound to the inner mitochondrial membrane and is involved in the TCA cycle and the ETC. The tetrazolium salt enters mitochondria and is reduced to an insoluble formazan product which is then solubilised by dimethyl sulphoxide and measured spectrophotometrically. Tetrazolium salt is reduced in cells that are

metabolically active and the level of activity is a measure of cell viability and mitochondrial function.

We observed increased cell viability and increased ATP levels (*22*) in the hyperglycaemic group which may suggest that mitochondrial function is maintained under oxidative conditions.
A previous study, however, had shown decreased cell viability, increased caspase-3 activity and increased apoptosis following 72hr hyperglycaemic stimulation (*23*).

Interestingly, we showed increased caspase-8 and caspase-9 activity, with caspase-9 activity being higher in the hyperglycaemic group. This may suggest that following hyperglycaemic stimulation, the intrinsic (mitochondrial) apoptotic cascade is the main pathway activated. However, caspase-3/7 activity was 35-fold lower than caspase-9. This could mean that although apoptosis is initiated, it may not be fully executed.

We found a lower percentage of apoptotic cells in the hyperglycaemic group using the annexin assay which tests the quantity of cells undergoing apoptosis and is used as an early marker of apoptosis.
The decreased number of apoptotic cells that we had observed in the hyperglycaemic group may be explained by the decreased caspase-3/7 activity.

Another reason for the decreased number of apoptotic cells could be due to the asymmetry of the plasma membrane. Phosphatidylserine (PS) is located on the inner portion of the plasma membrane (*24*). When apoptosis is initiated, PS is translocated to the extracellular membrane and can therefore be detected by Annexin V in a calcium-dependent manner (*24*).

Apoptosis requires a loss of plasma membrane asymmetry through a process called scrambling by enzymes called scramblases (*25*). A study showed that once apoptosis is initiated, caspase-3/7 cleaves and activates a scramblase called Xk-Related Protein 8 (Xkr8). This would lead to loss of asymmetry of the lipid bilayer and externalisation of PS enabling annexin to detect apoptotic cells (*26*).

The decreased caspase-3/7 activity that we observed may have resulted in reduced cleavage and activation of Xkr8, reduced externalisation of PS and, consequently, reduced annexin binding.

There are also several cell survival mechanisms that may account for the reduced caspase-3/7 activity and reduced apoptosis.
Heat shock proteins are a conserved family of stress response proteins which are highly expressed in response to OS. Heat shock proteins can function as molecular chaperones and protect against apoptosis.

Individual heat shock proteins can interact with the apoptotic pathway on different levels. HSPA1 (Hsp70) attenuates the intrinsic pathway by inhibiting BAX activation, reducing cytochrome *c* release and preventing the activation of caspase-3. HSPB5 negatively regulates apoptosis by preventing activation of caspase-3 whilst HSP90 can bind to Apaf-1 thereby preventing apoptosome formation. During OS, Hsp27 increases the levels of intracellular glutathione, which detoxifies hydrogen peroxide (H_2O_2) to water (H_2O). This is due to the ability of Hsp27 to hold glutathione in its reduced form under oxidative conditions (*9*). It also interacts with cytochrome *c* and prevents the correct formation of the apoptosome complex and subsequent activation of caspase-3/7 (*10*). Our study showed higher Hsp27 protein expression in the hyperglycaemic groups and this may account for the reduced caspase-3/7 activity and increased AO defence under hyperglycaemic conditions.

The p300 and CBP proteins are transcriptional co-activators that also have HAT activity which transfers an acetyl group to the ε-amino group of a lysine residue. This would result in a relaxed chromatin state leading to increased gene transcription.

In response to OS, transcription factor Nrf2 is acetylated by p300 and CBP. This enables Nrf2 to bind to the antioxidant response element which results in the transcription of AO genes such as SOD2 (detoxifies superoxide radicals), GPx1 (detoxifies H_2O_2 to H_2O) and GSH (*11*). We observed increased expressions of *p300*, *CBP*, Nrf2, *SOD2*, *GPx1* and increased GSH concentrations in the hyperglycaemic groups.

We had previously established that histone deacetylase, SIRT3, may be the central component that modulates AO defence, mtDNA repair and confers resistance to OS-induced damage under hyperglycaemic conditions in the HepG2 cells (*22*).

Taken together, this may suggest that in response to OS, the cell mounts a strong AO defence and induces the synthesis of key stress proteins ensuring temporary cellular survival.

Our study showed that under hyperglycaemic conditions, the apoptotic cascade may have been initiated through the intrinsic pathway. The activity of caspase-3/7 and the percentage of apoptotic cells were considerably lower suggesting that apoptosis was not fully executed. This may have been due to the induction of Hsp27 and AO defence which counter both ROS and apoptosis. In the hyperglycaemic model we show that although apoptosis may be induced as a result of oxidative damage, cell survival mechanisms counterbalance apoptotic stress.

Although previous studies have shown high apoptosis under hyperglycaemic conditions, we could not confirm these findings in our model. Our data is consistent with a study that showed no loss of

neurons in 12-month streptozotocin-induced diabetes (STZ-D) rats (*27*) and a study that observed no induction of apoptosis-related genes in acutely STZ-D rats (*28*). A previous study observed apoptotic stress in dorsal root ganglion cells but showed that apoptotic stress was counterbalanced by survival elements in sub-acute type 1 diabetic BB/ Wor rats (*29*).

This study may be improved by using a diabetic *in vivo* model which may help us establish a more holistic response. A wider range of glucose concentrations over a chronic time period may also help us better elucidate the effect of hyperglycaemia in a diabetic model.

Future studies include interrogating a more defined cell survival pathway following hyperglycaemic administration. Investigating the effect of hyperglycaemia on the epigenome may enable us to determine differential gene regulation during pre-diabetes and DMII.

5.6 Conclusion

Although hyperglycaemia may induce apoptosis, as a result of oxidative damage, the apoptotic stress may be counterbalanced by cell survival mechanisms regulated by stress response proteins (Hsp27, Nrf2 and SIRT3) and downstream AO defence.

5.7 Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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CHAPTER 6- DISCUSSION AND CONCLUSION

Hyperglycaemia increases mitochondrial ROS production which contributes to a variety of pathological conditions (*1*). Exposure to excess ROS results in oxidative damage of macromolecules and this leads to metabolic disorders such as DMII. In order to improve disease conditions, mitochondrial function and AO defence need to be maintained.

The networks of cell protective mechanisms that exist under these oxidative conditions have yet to be fully elucidated.

SIRT3 is a mitochondrial deacetylase that forms part of the cell protective network in response to OS. It deacetylates and activates enzymes that regulate mitochondrial function. SIRT3 regulates cell defence and survival in response to stress (*2-5*).

We showed increased protein expressions of SIRT3, PGC-1α and p-CREB as well as increased gene expressions of *SIRT3*, *CREB*, *PGC-1α*, *GPx1*, *SOD2*, *UCP2* and *OGG1* under hyperglycaemic conditions. Further increases in these expressions were observed under longer hyperglycaemic stimulation. Our data suggests that SIRT3 is a central component in modulating AO defence and conferring resistance to OS-induced damage under hyperglycaemic conditions in HepG2 cells.

Following treatment with the diabetic drug, metformin, we found increased protein expressions of SIRT3 and p-CREB as well as increased gene expressions of *SIRT3*, *CREB*, *PGC-1α*, *GPx1*, *SOD2*, *UCP2* and *OGG1* under hyperglycaemic conditions. We postulated that metformin may work synergistically with SIRT3, or through SIRT3-mediated mechanisms, to improve AO defence.

We also treated HepG2 cells with curcumin, which has bi-functional AO properties and is known to improve mitochondrial function. Following curcumin administration, the data showed increased SIRT3, NF-κB, Lon and Hsp70 protein expressions as well as increased transcript levels of *SIRT3, PGC-1α, CREB, GPx1, SOD2, UCP2* and *OGG1* under hyperglycaemic conditions. These genes were optimally expressed following 10μM curcumin treatment in the hyperglycaemic groups. Further, the 10mM glucose treatment yielded higher expressions of SIRT3 and stress response proteins than the 30mM glucose treatment following 10μM curcumin administration.

Since SIRT3 was shown to increase AO defence and cell survival, the protective effects of curcumin may occur through SIRT3-mediated mechanisms.

Hyperglycaemia is known to induce apoptosis. In our study, although hyperglycaemia induced significantly higher caspase-8 and caspase-9 activity, significantly lower caspase-3/7 activity was observed when compared to caspase-9. The percentage of apoptotic cells in this group was also significantly lower.

This suggests that the intrinsic apoptotic pathway was initiated but apoptosis was not fully executed due to the low activity of caspase 3/7. Another reason may be the induction of key stress proteins such as Hsp27 and AO defence that counterbalance apoptotic execution. Heat shock protein 27 functions as an AO by increasing levels of intracellular glutathione. This is due to the ability of Hsp27 to hold glutathione in its reduced form under oxidative conditions. Heat shock protein 27 also interacts with cytochrome *c*, preventing the formation of the apoptosome complex and activation of caspase-3.

Mitochondrial dysfunction and diminished AO defence are some of the key features in DMII and other metabolic disorders. SIRT3 regulates several stress proteins and AO enzymes and, therefore, plays a central role in the network that ensures temporary cell survival under oxidative conditions.

Clinical Implications:

Following metformin and curcumin administration under hyperglycaemic conditions, AO defence was improved and this was possibly mediated through SIRT3.

Resveratrol (a known SIRT3 activator) decreased oxidative injury in endothelial cells and this was mediated by SIRT3 (*6*). SIRT3 also protected against acute kidney injury by reducing OS and mitochondrial damage (*7*). Viniferin is a natural product that decreased ROS levels and prevented loss of mitochondrial membrane potential in cells expressing Huntingtin protein. SIRT3 was shown to mediate the neuro-protection of viniferin (*8*).

SIRT3 function may, therefore, be modulated by chemical or natural compounds and can be therapeutically targeted as a potential cyto-protective factor to improve OS-associated disorders.

Future work:

We have used the HepG2 cell line as an *in vitro* model to represent human hepatocytes in cell culture. This cell line can maintain its function and structure and exhibits most genotypic features of normal hepatocytes.

This study could be improved by using primary hepatocytes or an *in vivo* hyperglycaemic mouse model in order to establish a holistic response.

A larger range of glucose and insulin concentrations over chronic time periods can be used as this is an accurate representation of DMII. This may provide greater insight to SIRT3 modulation of AO defence under chronic OS.

It is also useful to interrogate SIRT3 in more defined pathways that are altered in metabolic disorders, such as mitochondrial biogenesis or mitochondrial DNA repair. These pathways are necessary for optimum mitochondrial function and cellular energetic efficiency.

It will be interesting to investigate the role of SIRT3 on the epigenome (DNA methylation and regulation of histone deacetylases) to determine differential gene regulation during pre-diabetes and DMII. This would give us better insight on tailor-made therapeutic interventions, based on patient genome, to enable optimum patient response to therapies.

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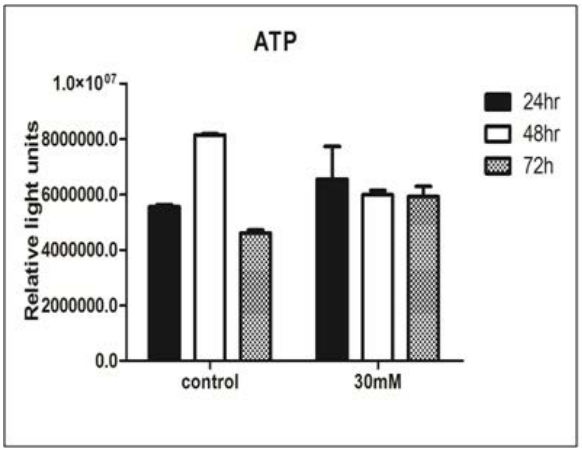
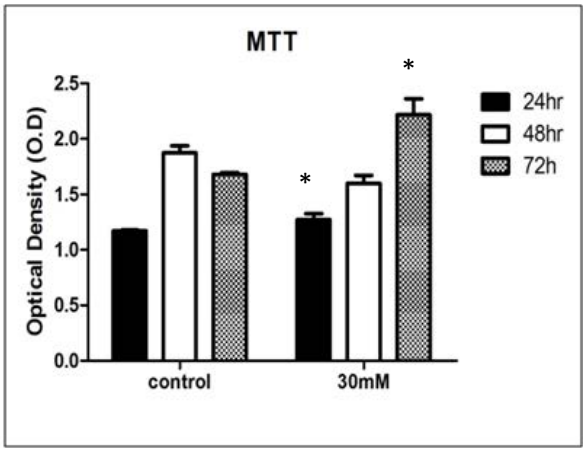
APPENDIX

APPENDIX A: ATP and MTT assays measured in HepG2 cells over 24, 48 and 72 hours





We investigated hyperglycaemia over 24, 48 and 72 hours and found that the ATP and MTT results were inconsistent in the 48hr time period. We worked with 24hr hyperglycaemic stimulation as an acute hyperglycaemic model. Longer hyperglycaemic stimulations are preferably carried out at 72 hours or longer.

APPENDIX B: Markers of mitochondrial function over 24 and 72 hours

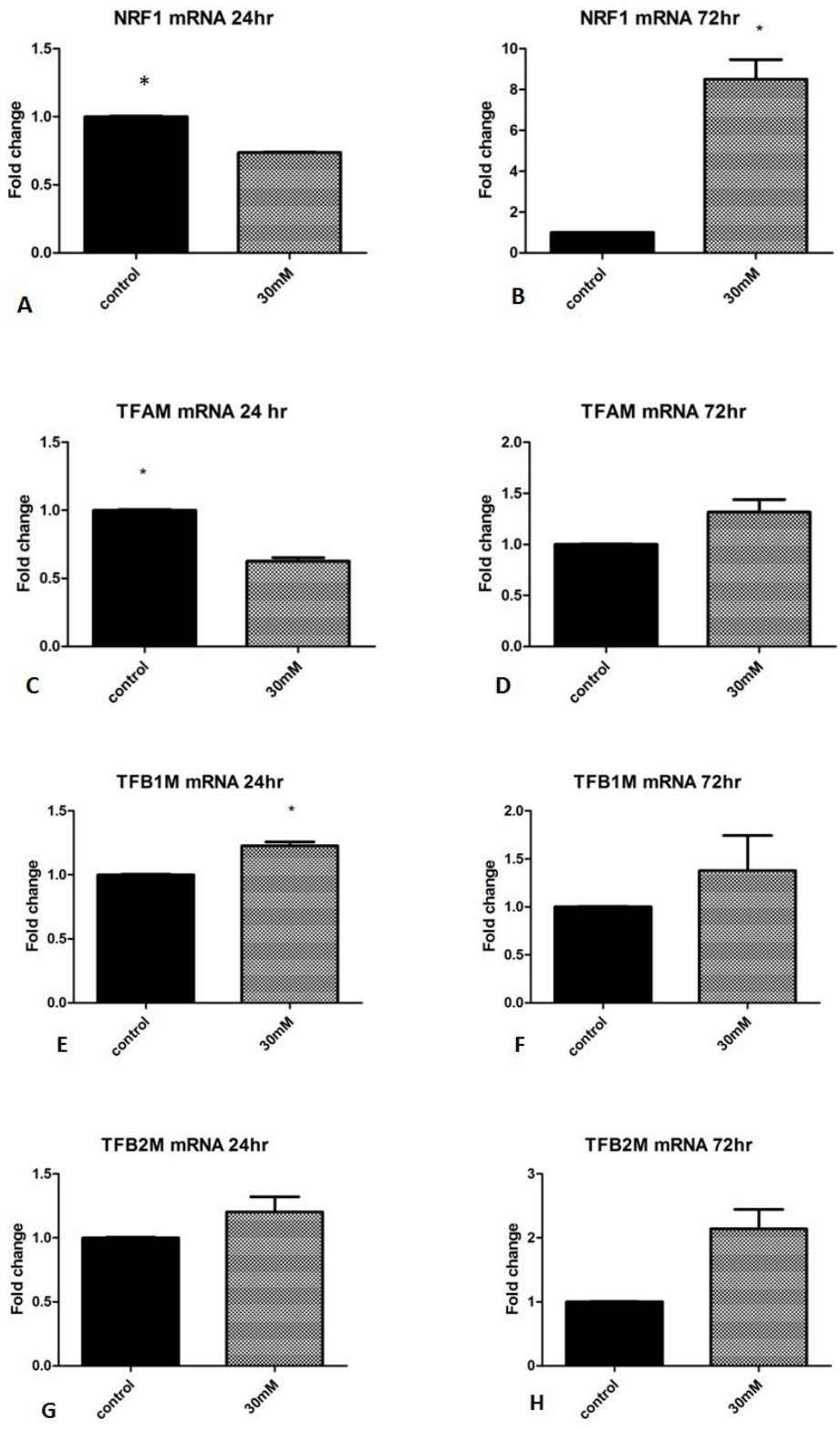


Table 1: Fold changes for mitochondrial genes

Gene	**Fold change** (control vs. 30mM)		**p-value** (control vs. 30mM)	
	24hr	**72hr**	**24hr**	**72hr**
NRF1	1.35	1.85	0.0011	0.0160
TFAM	1.61	1.31	0.0079	0.1141
TFB1M	1.22	1.38	0.0234	0.4028
TFB2M	1.20	2.14	0.2360	0.0629

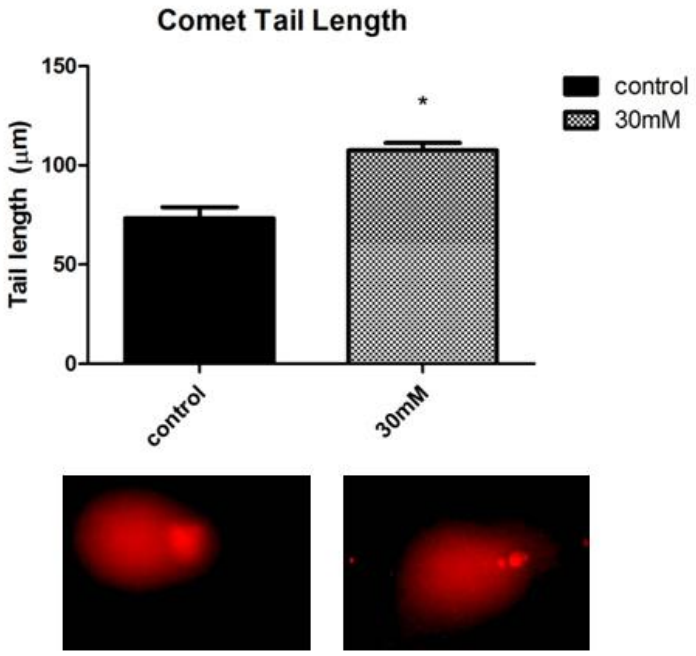
In order to investigate mitochondrial function under hyperglycaemic conditions, we determined gene expressions for nuclear respiratory factor-1 (NRF1), mitochondrial transcription factor A (TFAM), mitochondrial transcription factor B1 (TFB1M) and mitochondrial transcription factor B2 (TFB2M) over 24 and 72 hours.

NRF1 regulates nuclear-encoded mitochondrial proteins and controls the expression of ETC complexes as well as TFAM, which is involved in mitochondrial biogenesis.

The core machinery of mitochondrial gene expression are TFAM, RNA polymerase gamma, TFB1M and TFB2M. Both TFB1M and TFB2M are required to initiate biogenesis as both have recognition sites for NRF1.

Although NRF1 and TFAM were lower in the 24 hour treatment under hyperglycaemic conditions (1.35-fold and 1.61-fold respectively), all other genes were elevated at 24 and 72 hours in the 30mM group suggesting that mitochondrial function is maintained under hyperglycaemic conditions.

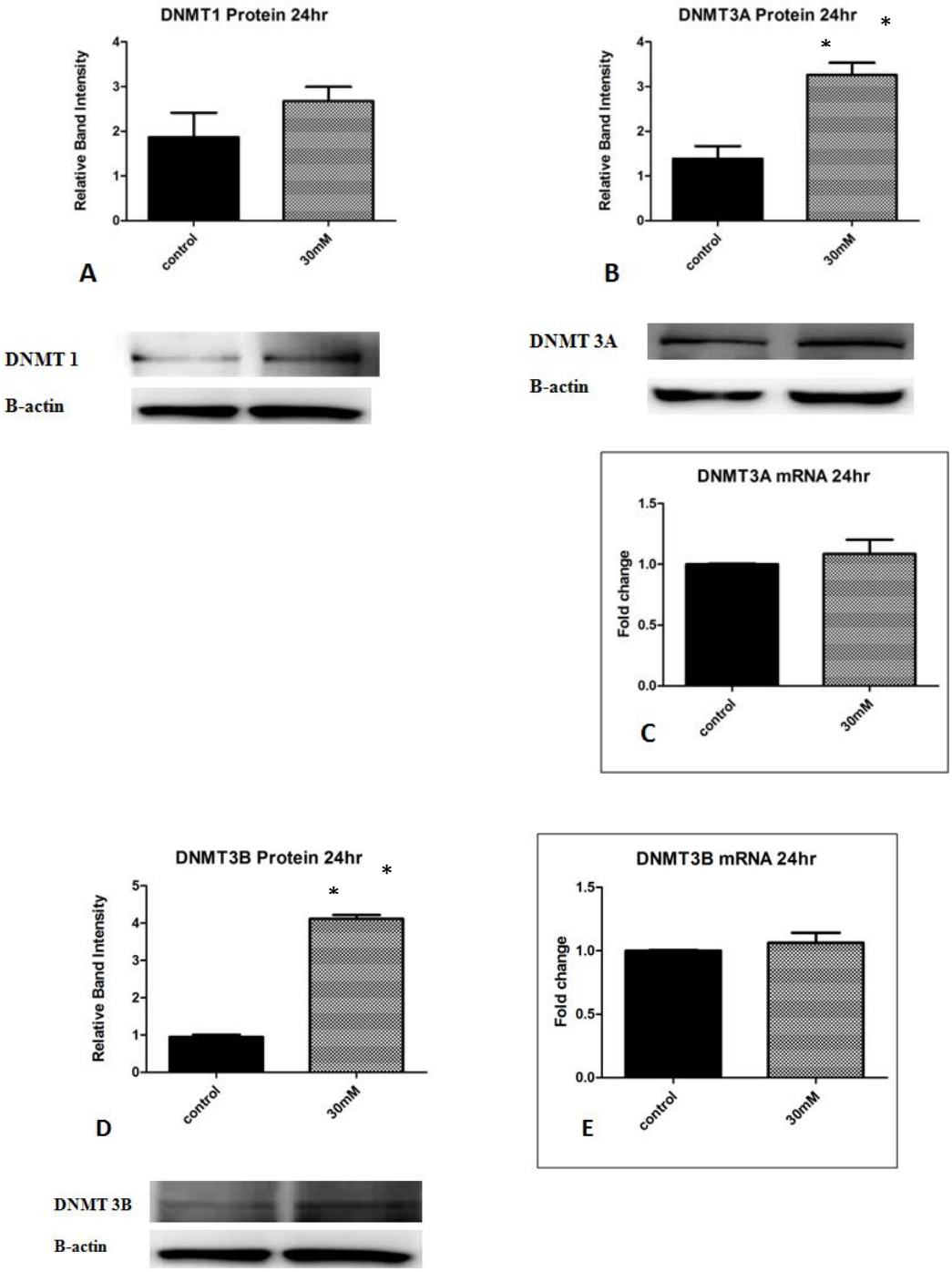
APPENDIX C: Marker of nuclear DNA damage



Nuclear DNA damage is detected by the comet assay. (Singh NP, McCoy MT, Tice RR, Schneider EL: **A simple technique for quantitation of low levels of DNA damage in individual cells.** *Exp Cell Res* 1988, **1751:**184–191). Damaged DNA migrates out the cell toward the anode and this is observed as a comet tail.

Longer tail lengths were seen in the hyperglycaemic group when compared to the control (70.10μm vs. 106.5μm respectively, p=0.0002) suggesting increased single strand breaks. This, however, may also denote DNA unwinding.

APPENDIX D: DNA methylation under hyperglycaemic conditions



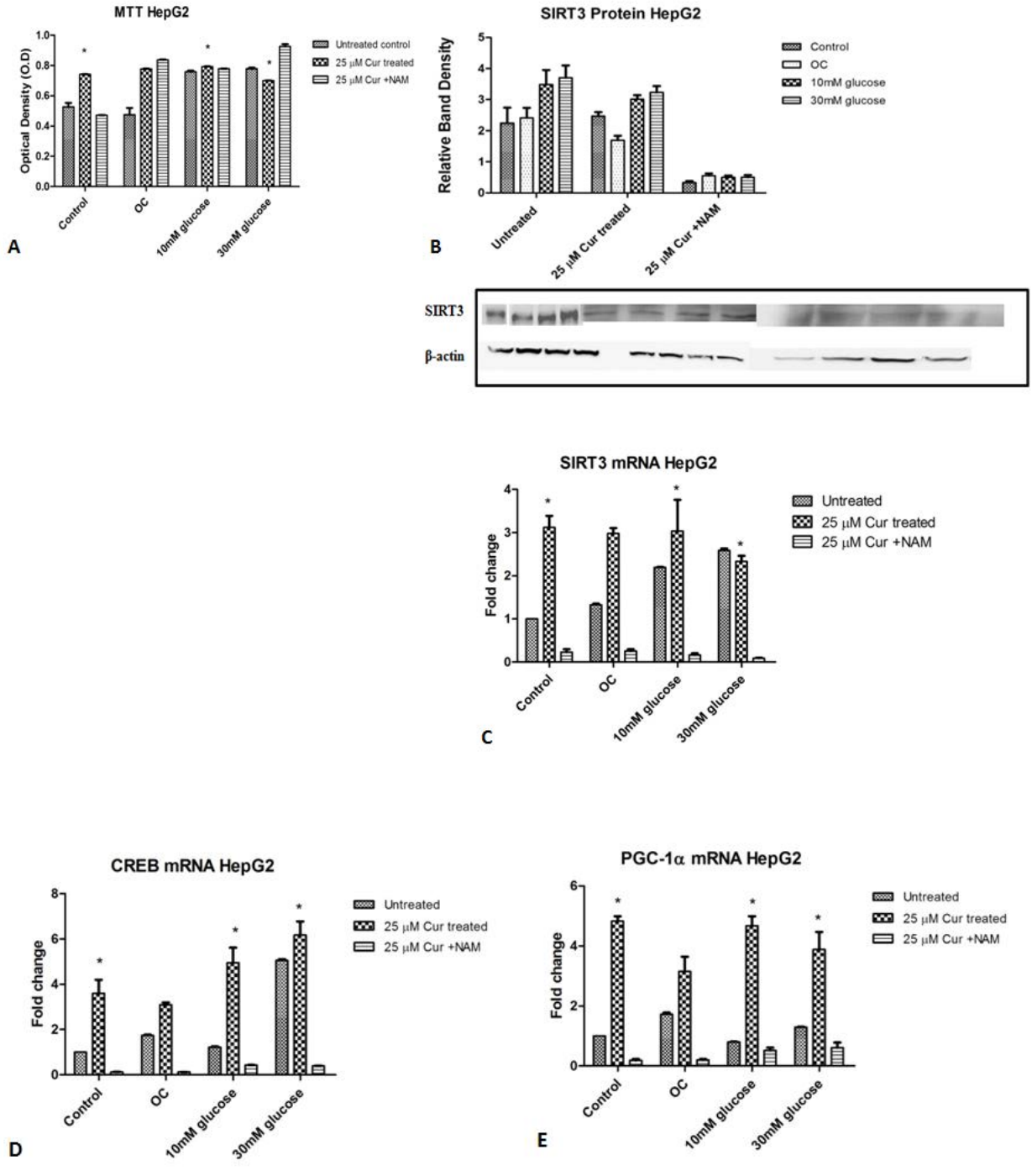
DNA methylation is a major regulator of transcriptional activity. Methylation changes the interactions between proteins and DNA by collaborating with proteins that modify nucleosomes.

This alters chromatin conformation leading to decreased transcription. DNA methyltransferases (DNMTs) transfer a methyl group from the methyl donor, *S*-adenosyl-L-methionine, to the 5-position of cytosine residues in DNA.

Higher DNMT1, 3A and 3B protein expressions were observed in the hyperglycaemic groups relative to the control (DNMT 3A: p=0.0190 and DNMT 3B: p=0.0004). Transcript levels of *DNMT 3A* and *DNMT 3B* expressions were also higher in the hyperglycaemic groups when compared to the control (1.08-fold and 1.06-fold respectively).

The elevated DNMTs in the hyperglycaemic group could result in transcriptional repression of genes involved in pathways that elevate cell death and oxidative stress.

APPENDIX E: SIRT3 and antioxidant defence under hyperglycaemic conditions following 25μM curcumin administration



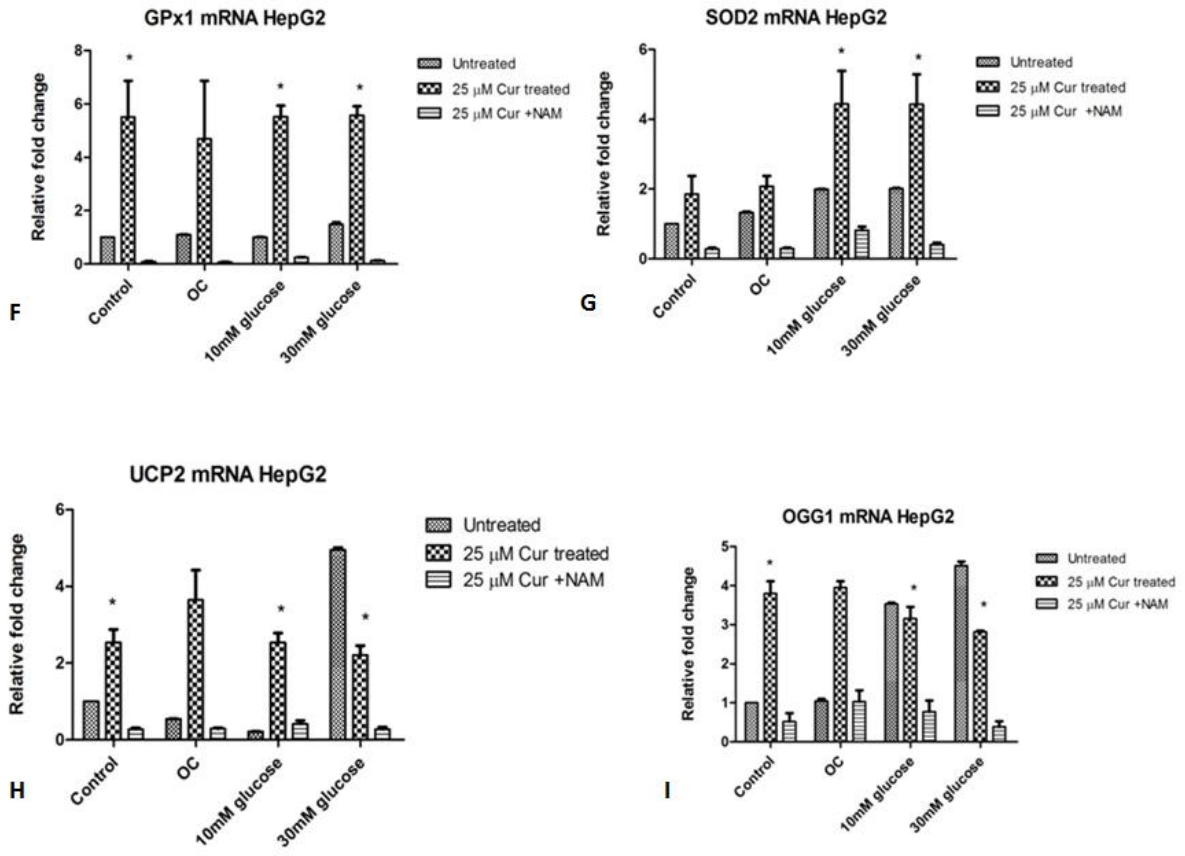


Table 2: Cell viability, protein and gene expression in HepG2 cells following 25μM curcumin treatment in the hyperglycaemic groups. * $p<0.05$ vs. untreated control

	p-value	
	10mM	**30mM**
MTT	0.0035	0.0089
SIRT3 Protein	0.3063	0.2886
SIRT 3 mRNA	0.0400	0.0118
CREB mRNA	0.0093	0.0047
PGC-1α mRNA	0.0033	0.0134
GPx1	0.0046	0.0039
SOD2	0.0248	0.0206
UCP2	0.0147	0.0183
OGG1	0.0074	0.0011

Following curcumin treatment, the hyperglycaemic groups showed relatively higher cell viability, increased protein expression of SIRT3 and increased transcript levels of *SIRT3*, *CREB*, *PGC-1α*, *GPx1*, *SOD2*, *UCP2* and *OGG1* when compared to the untreated and treated control.

These results were not included in the curcumin chapter due to more significant results yielded from the 5μM and 10μM curcumin concentrations.

APPENDIX F: The effect of metformin on SIRT3 expression and antioxidant defence in HEK 293 cells

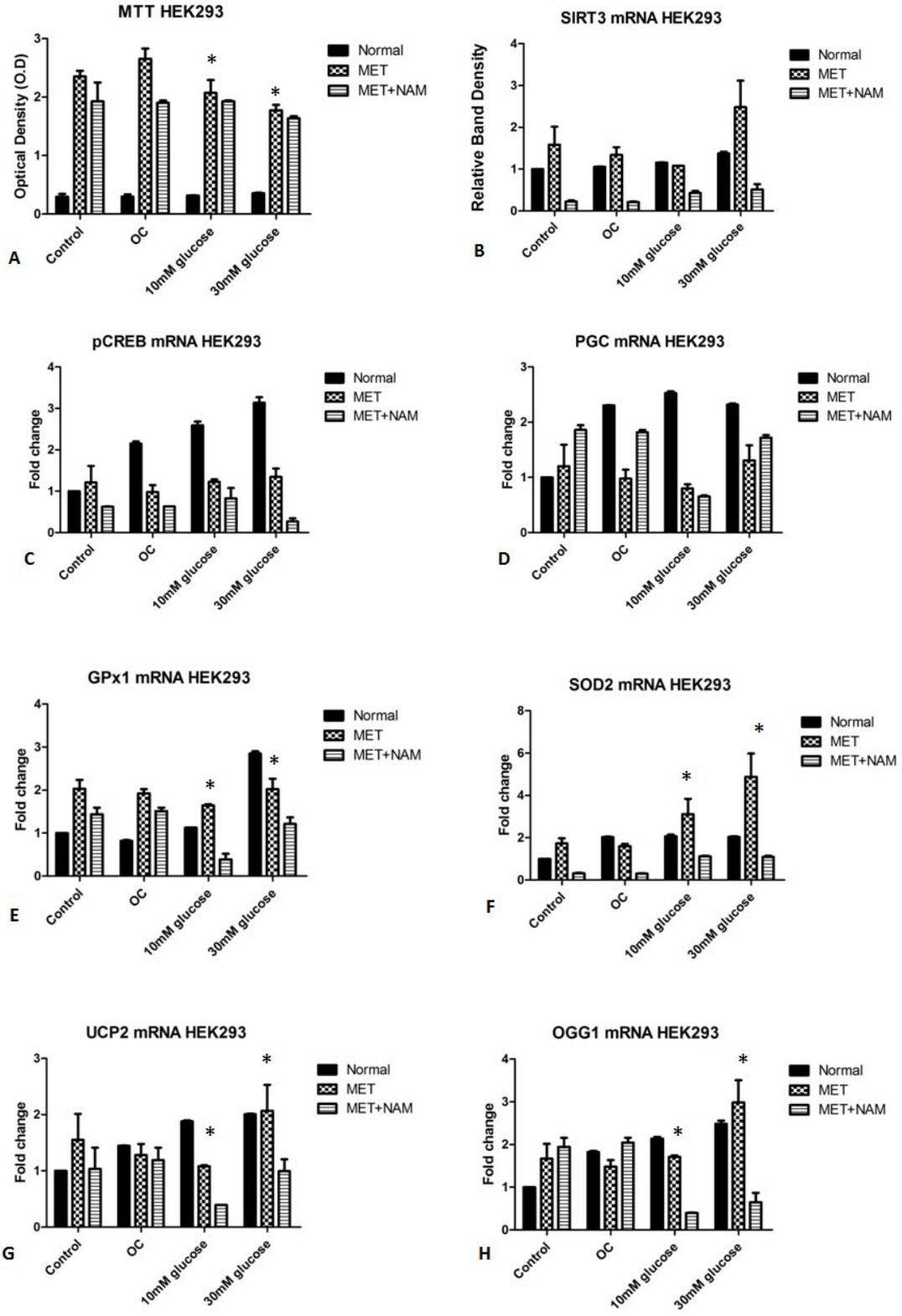


Table 2: Cell viability and gene expression in HEK 293 cells following 3mM metformin treatment in the hyperglycaemic groups. * $p<0.05$ vs. untreated control

	p-value	
	10mM	**30mM**
MTT	0.0079	0.0050
SIRT 3 mRNA	0.1835	0.0808
CREB mRNA	0.1991	0.2948
PGC-1α mRNA	0.2591	0.3882
GPx1	0.0011	0.0197
SOD2	0.0396	0.0363
UCP2	0.0669	0.0607
OGG1	0.0112	0.0226

To compare the effect of hyperglycaemia on SIRT3 expression and downstream AO defence in HepG2 cells, a non-carcinoma HEK 293 cell line was used.

Following metformin administration, higher cell viability and increased transcript levels of *SIRT3*, *CREB*, *PGC-1α*, *GPx1*, *SOD2*, *UCP2* and *OGG1* were observed in the hyperglycaemic group treated with metformin relative to the untreated control.

The response under oxidative conditions, with regard to SIRT3 and downstream AO defence, were similar between both cell lines.