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Characterisation of the relationship between Heme-oxygenase-1 and adiponectin

Mengliu Yang

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Abstract

The prevalence of obesity is growing at an alarming rate worldwide, and current therapies have limited benefits. Therefore, it is essential to increase our understanding of the underlying mechanisms so that new strategies can be developed to reduce the incidence of obesity related diseases. Adiponectin is an adipocyte-derived hormone that regulates glucose and lipid metabolism via direct and indirect mechanisms and has anti-inflammatory, anti-diabetic, anti-atherogenic and cardioprotective properties. Hypoadiponectinemia is implicated in the aetiology of obesity-related diseases making strategies to increase circulating adiponectin levels therapeutically attractive.

Emerging evidence, predominantly from preclinical studies, suggests induction of heme-oxygenase-1 (HO-1) increases adiponectin production concomitant with decreased inflammatory tone prompting the proposal of a "HO-1 – adiponectin axis." However, the underlying mechanisms of increased adiponectin production via HO-1 induction are poorly defined; indeed a direct relationship has not been demonstrated. Thus, the overarching aim of this thesis is to investigate the effects of HO-1 induction on adiponectin production as well as adipocyte and adipose tissue remodelling and metabolic parameters using cellular and mouse models.

Initial studies were designed to characterize the direct effect of HO-1 induction on adiponectin production. We found that treatment with the widely used HO-1 inducer cobalt protoporphyrin (CoPP) or hemin for 24-48 h increased HO-1 expression and activity without affecting adiponectin expression and secretion, in human mature adipocyte under a variety of experimental scenarios. Treatment of adipocytes with TNFα reduced adiponectin production and induced pro-inflammatory cytokines production. HO-1 induction failed to reverse these effects. These results do not support a direct HO-1 – adiponectin axis.

However, literature suggests that chronic induction of HO-1 via CoPP administration throughout differentiation, promotes adiponectin secretion, albeit in the context of reduced adipogenesis. While our previous findings argued against the existence of a direct "HO-1 – adiponectin axis," they did not address the potential effects of chronic induction of HO-1 on adiponectin production. Thus, we extended our study to characterise the effects of chronic HO-1 induction throughout differentiation of human preadipocytes. In this study we demonstrate that chronic induction of HO-1 with CoPP or hemin throughout differentiation results in dose-dependent inhibition of adipogenesis and adiponectin production as well as induction of additional NRF2 target genes. Co-treatment with SnMP (HO-1 activity inhibitor) and HO-1 siRNA did not prevent these effects. These findings suggest that the chronic treatment with CoPP or hemin inhibits adipogenesis and adiponectin production potentially by a HO-1-independent mechanism that may be downstream of NRF2.

However, our results contradict the majority of reports in the literature. One possibility is that the literature that supports "HO-1 – adiponectin axis" used mesenchymal stem cells, thus we propose the effect of CoPP is different in different cell types. In fact, recent reports show caloric restriction increases adiponectin production from bone marrow adipose tissue but not white adipose tissue (WAT) supports our proposal.

The above findings indicate that induction of HO-1 failed to promote adiponectin production *in vitro*, specifically in WAT. Many studies in pre-clinical models have also shown that CoPP administration in obese mice reduces body weight gain, improves insulin sensitivity, reduces adipocyte size and attenuates liver steatosis. However, it should be noted that reduction in body weight gain which, in-itself, is predicted to decrease adipose tissue as well as systemic inflammation and to increase adiponectin. Thus, we again extended our study to characterise the effects of administration of CoPP in lean and obese mice. We found that CoPP administration significantly increased inflammatory tone in white adipose tissue irrespective of obesity. In obese mice, circulating adiponectin levels were elevated by CoPP administration whereas the adiponectin gene expression in WAT was significantly reduced. Additional effects specific to obese mice included reduced food intake, weight gain, adipocyte size and liver steatosis as well as improved insulin responsiveness. Co-administration with SnMP in obese mice ameliorated CoPP effects on adipose tissue inflammation and blunted CoPP effects on circulating adiponectin, but not other parameters. This supports the hypothesis that the induction of HO-1 may, at least partly, increase circulating adiponectin levels. We propose the increase adiponectin production from bone marrow adipose tissue (BMAT) may contribute to this, which is consistent with our *in vitro* findings. Further studies are needed to investigate if BMAT plays a central role in the effects of HO-1 induction on adiponectin production.

In summary, the work presented in this thesis establishes CoPP administration *in vitro* does not have benefit on adipocytes, evidence against a direct "HO-1 – adiponectin axis" in WAT. CoPP administration *in vivo* promotes divergent effects on adipose tissue, adiponectin and insulin responsiveness. However, the majority of these effects appear to be independent of HO-1 activity. These factors suggest there is no positive effect of HO-1 induction on adiponectin production in WAT, and emphasize the potential role of BMAT in increased adiponectin production via CoPP administration.

Declaration by author

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Publications during candidature

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Contributor	Statement of contribution
Author Mengliu Yang (Candidate)	Designed experiments (40%) Wrote the paper (50%)
Author Masaki Kimura	Designed experiments (6%)
Author Choaping Ng	Designed experiments (1%)
Author Jingjing He	Designed experiments (1%)
Author Sahar Keshvari	Designed experiments (1%)
Author Felicity Rose	Designed experiments (1%)
Author Johanna Barclay	Designed experiments (20%) Wrote and edit the paper (25%)
Author Jon Whitehead	Designed experiments (30%) Edited the paper (25%)

Contributions by others to the thesis

The publications author contributions are detailed above.

Statement of parts of the thesis submitted to qualify for the award of another degree

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List of abbreviations

AA	Ascorbic Acid
AdipoR1	Adiponectin Receptor 1
AdipoR2	Adiponectin Receptor 2
AKT	Protein Kinase B
AMPK	AMP-Activated Protein Kinase
APPL1	Adaptor Protein, Phosphotyrosine Interacting, PH Domain and Leucine Zipper 1
ARE	Antioxidant response element
ATMs	Adipose Tissue Macrophages
AUC	Area Under the Curve
BACH1	BTB and CNC Homology 1
BAMBI	BMP-and Activin-Membrane-Bound Inhibitor
BAT	Brown Adipose Tissue
BCL-2	B-cell Lymphoma-2
BMAT	Bone Marrow Adipose Tissue
BMD	Bone Mineral Density
BMI	Body Mass Index
C/EBPs	CCAAT/enhancer Binding Protein
CHD	Coronary Heart Disease
CHOP	C/EBP Homologous Protein
CK2β	The Regulatory Subunit of Protein Kinase CK2
CO	Carbon Monoxide
CoPP	Cobalt protoporphyrin
COX-2	Cyclooxygenase-2
CREB	cAMP Response Element-Binding Protein
CTGF	Connective Tissue Growth Factor
CVD	Cardiac Vascular Disease
CYP450	Cytochrome 450
DEX	Dexamethasone
DMSO	Dimethylsulfoxide
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ERp46	Endoplasmic Reticulum Protein 46
FFAs	Free Fatty Acids

FoxO1	Forkhead Box Protein O1
GCLM	Glutamate-Cysteine Ligase Modified Subunit
GLUT1	Glucose Transporter 1
GLUT4	Glucose Transporter 4
GRP78	Glucose-Regulated Protein 78
GTT	Glucose Tolerance Test
H&E staning	Hematoxylin and eosin staning
HeRE	Heme-responsive Elements
HFD	High Fat Diet
HMW	High Molecular Weight
HO-1	Heme oxygenase-1
HSC	Hepatic Stellate Cells
IL-10	Interleukin-10
IL-6	Interleukin-6
ITT	Insulin Tolerance Test
JNK	c-Jun N-terminal Kinase
LMW	Low Molecular Weight
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MHO	Metabolically Healthy Obese
MIX	Methylisobutylxanthine
MS	Multiple Sclerosis
MSCs	Mesenchymal Stem Cells
MUO	Metabolically Unhealthy Obese
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NQO1	NAD(P)H-Quinone Oxidoreductase 1
NRF2	NF-E2 Related Factor 2
PAI-1	Plasminogen Activator Inhibitor-1
phPAs	Primary Human Preadipocytes
PI3k	Phosphatidylinositol 3-kinase

PPARα	Peroxisome Proliferator-Activated Receptor-Alpha
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PTMs	Post Translational Modifications
PUFA	Polyunsaturated Fatty Acid
RACK1	Protein Kinase C1
RBP4	Retinol Binding Protein 4
SCD	Standard Chow Diet
SGBS	Simpson Golabi Behmel Syndrome
sGC	Soluble Guanylyl Cylase
SnMP	Tin-mesoporphyrin
SREBP-1c	Sterol Regulatory Element Binding Protein-1c
sXBP-1	Spliced X-box Binding Protein 1
TBP	Tata Box Protein
TG	Triglyceride
TGF-β1	Transforming Growth Factor-β1
TLR-4	Toll-like Receptor-4
TNFα	Tumor Necrosis Factor Alpha
TZD	Thiazolidinediones
WAT	White Adipose Tissue
WHO	World Health Organization

Chapter 1:

General Introduction

1.1 Obesity and adipose tissue

1.1.1 Obesity

Obesity is defined by the World Health Organization (WHO) as the accumulation of abnormal or excessive body fat, resulting in adverse health outcomes[1]. It has been formally recognized as a disease by a variety of governments and non-government organizations. The prevalence of overweight and obese individuals across populations is measured by BMI (BMI = kg/m^2). A BMI greater than 25 is considered overweight while greater than 30 is obese[1]. According to the WHO, obesity is growing at an alarming rate, and the global obese population has nearly doubled since 1980[1]. It was reported that more than a quarter of Australian adults are currently obese and another 40% are overweight, and this has increased nearly four-fold over the past thirty years [2]. Obesity is associated with several chronic diseases such as non-alcoholic fatty liver disease (NAFLD) [3], CVD [4], type 2 diabetes [5], cancer [6] and bone metabolic diseases [7]. As more health care and ancillary services are needed, and the lost productivity due to obesity-related diseases increases, there is a significant increase in health and socio-economic costs. Adipose tissue, which plays an important role in obesity, was initially considered a simple fat store organ, but is now recognized as a major endocrine organ [8]. Adipose tissue has now moved center stage in obesity research, there having been a revolution in our understanding of the biological role ofadipose tissue over the past decade. However, we still have limited understanding of the underlying mechanisms involved in adipose tissue regulation. Thus, it is important to study adipose tissue biology to identifying effective therapeutic strategies.

1.1.2 Adipose Tissue

There are two types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is composed mostly of adipocytes. It also contains a variety of cell types including preadipocytes, vascular endothelial cells, fibroblasts and immune cells which make up the stromal vascular fraction. It has two main functions; the storage of excess energy and the secretion of multiple hormones and cytokines, termed adipokines [9]. BAT were mainly observed in the supraclavicular, suprarenal, paravertebral and neck tissues in adults [10, 11]. Mature BAT express high levels of uncoupling protein 1 (UCP1), a mitochondrial protein that plays an important role in heat production by uncoupling the activity of the respiratory chain from ATP synthesis [11]. The activation of BAT contributes to adaptation thermogenesis in response to cold stimulation [12]. It

has been reported that WAT can convert to BAT under cold stimulation to produce heat, and these adipocytes have been named “brite” or “beige” adipocytes [10].

Excess energy is stored in WAT primarily in the form of triglycerides (TG). These TGs are mobilized for utilization by other tissues when they require energy [13]. As a consequence of chronic positive energy balance, adipose tissue expands. This occurs either by hypertrophy or by hyperplasia [14]. Hypertrophy involves increased accretion of lipids within existing adipocytes. Hyperplasia is related to the recruitment of stem cells to become preadipocytes, and then differentiate to adipocytes for further TG storage. As adipocytes do not proliferate, new preadipocytes must be recruited [15, 16]. Preadipocytes undergo cell differentiation to become adipocytes, a process termed adipogenesis, which is regulated by several transcription factors such as CCAAT/enhancer binding protein (C/EBPs) [17]and peroxisome proliferator-activated receptor gamma (PPARγ) [18]. PPARγ has been identified as a dynamic and specific regulator during the differentiation of an adipocyte precursor cell into a fully developed adipocyte [19].

As mentioned previously, WAT was thought to be a passive depot for energy storage for decades, but now has been recognized as a major endocrine organ. It secretes a variety of bioactive molecules which have profound influence on metabolism and metabolic organs. We call these bioactive molecules adipokines, or adipocytokines [13]. Leptin and adiponectin are the two adipokines that have been most widely studied and, importantly, are produced predominantly by adipocytes. They are currently considered to play a crucial role in metabolism and inflammatory processes. However, while WAT has been recognized as an endocrine organ, much less is known about the adipokines released by brown and beige fat and their physiological functions. Therefore, we mainly investigate WAT in this thesis.
Leptin is one of the major adipose-derived hormones. It plays an important role in regulating energy intake and expenditure, including appetite and hunger, thereby regulating behaviour and metabolism. Leptin alters the expression of orexigenic and anorexigenic neuropeptides to regulate energy homeostasis by acting primarily in the hypothalamus [20]. Thus, mouse models lacking the gene encoding leptin and/or its receptor display a severe obese phenotype and are characterized by an increase in food intake and a decrease in energy expenditure [21, 22]. This phenotype can be reversed by increasing leptin signalling. In humans, serum leptin concentrations positively correlate with percentage of body fat [23]. Leptin deficiency results in obesity and insulin resistance [24], whereas recombinant leptin treatment reduces obesity and improves metabolism [25, 26]. Therefore, increasing leptin levels may reduce inflammation-related diseases such as CVD, hypertension and metabolic syndrome [27]. While high levels of leptin are found in overweight and obese individuals,

the anorexigenic signal to the brain does not function. This phenomenon is called leptin resistance. Due to the existence of leptin resistance, leptin may not be an effective therapeutic treatment.
Adiponectin is another major adipose-derived hormone which plays a key role in the regulation of glucose and lipid metabolism. Adiponectin is the most abundant adipokine in the circulation with many protective properties, including insulin-sensitization, anti-atherogenisis, anti-inflammation, anti-oxidation and cardioprotection [28]. Decreased serum adiponectin has been observed in obesity and its associated diseases [29]. Adiponectin-knockout (KO) mice exhibit diet-induced obesity and insulin resistance [30] which is reversed by adiponectin administration [31]. Therefore, pharmacological strategies to increase plasma adiponectin levels should be useful in the treatment of obesity and its related diseases. The details of adiponectin will be discussed later in this review.

In addition to leptin and adiponectin, many other adipokines have been identified such as apelin, chemerin, interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), retinol binding protein 4 (RBP4), tumor necrosis factor-alpha (TNFα) and visfatin [32-34].

1.1.3 Adipose Tissue Dysfunction

A large body of evidence indicates that adipose tissue dysfunction contributes to the development of obesity related diseases including NAFLD, CVD, type 2 diabetes, cancer and bone metabolic diseases [35, 36]. Most patients with obesity and its associate diseases have impaired adipose tissue function caused by adipocyte hypertrophy, inflammation and hypoxia. It is well-established that obesity is associated with low-grade chronic inflammation [37], as a result of altered adipocyte and macrophage secretory profile leading to an over production of pro-inflammatory adipokines and a decrease in anti-inflammatory adipokines [38]. Thus, “fit” adipocytes are necessary to maintain a healthy metabolic status.

As described previously, adipocyte hypertrophy occurs when excessive TG accumulates within the cell. For decades it was believed that the number of adipocytes is set during childhood and maintained through adulthood [39], which indicated that adipose tissue expansion is due to increases in the size of adipocytes rather than increases in the number of adipocytes. However, recent studies show that adipocytes do not have an unlimited capacity for hypertrophy [14, 40]. In addition, Spalding et al. demonstrated that up to 10% of adipocytes in humans are renewed every year by analysis of ^{14}C (derived from nuclear bomb tests) integrated in adipocyte DNA [39]. Collectively these studies suggest that weight gain initially leads to adipocyte hypertrophy, and

when surplus energy storage results in maximum adipocyte hypertrophy, continued excess nutrition triggers hyperplasia [40, 41]. The differences in adipose tissue cellularity are closely related to obesity and its associated diseases. It has been reported that people with larger subcutaneous abdominal adipocytes are more likely to experience hyperinsulinemia and be glucose intolerant compared with those with similar adipose tissue mass but smaller adipocytes [41-44]. Salans and colleagues showed that larger adipocytes displayed a diminished response to insulin, but insulin sensitivity was restored after weight loss and a reduction in fat cell size [43]. These data indicate that people who become obese mainly through hypertrophy are more susceptible to metabolic complications than those who become obese primarily through hyperplasia [42-44]. Therefore, inhibiting fat cell enlargement and increasing adipocyte number may provide a novel therapeutic strategy to improve obesity and associated diseases (Figure 1.1).

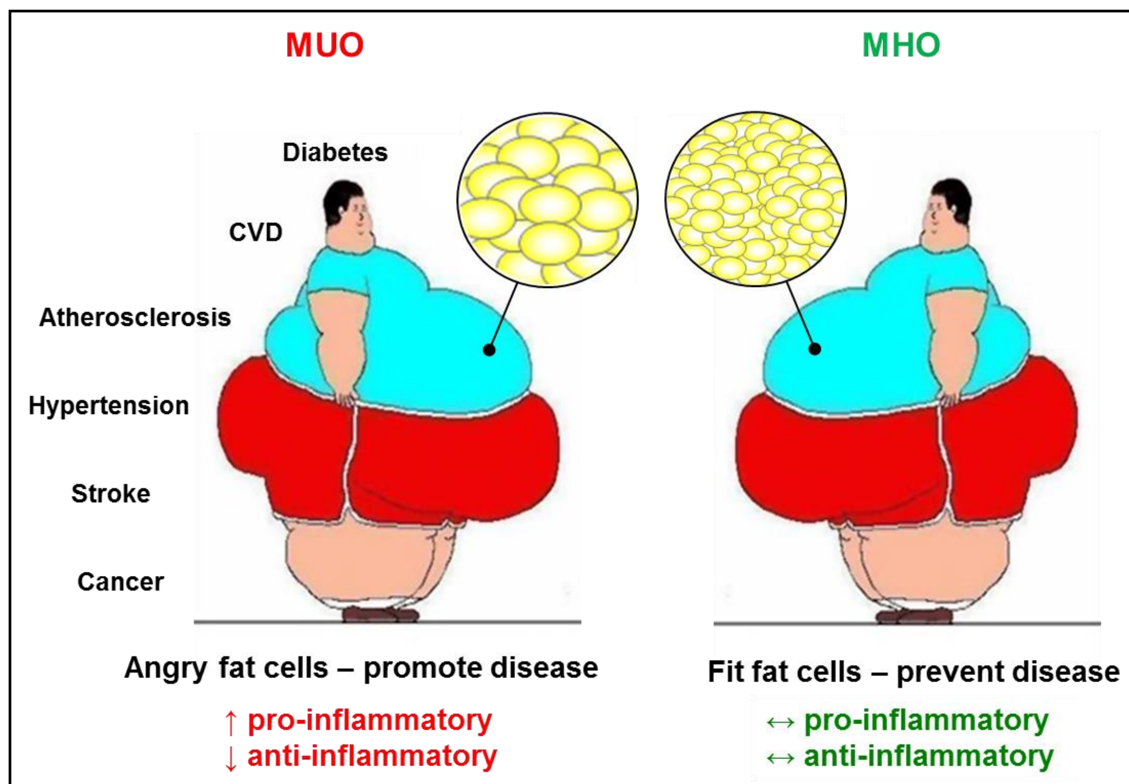

Figure 1.1 Schema showing adipocyte size from metabolically unhealthy obese (MUO) and metabolically healthy obese (MHO). Adipose tissue expands primarily through an increase in fat cell size in MUO individuals. The hypertrophic adipocyte increases pro-inflammatory factors and decreases anti-inflammatory adipokines. This promotes the development of metabolic diseases including diabetes, CVD, atherosclerosis, hypertension, stroke and cancers. Adipose tissue expands primarily through an increase in fat cell number in MHO individuals. Those newly generated small adipocytes are able to secrete balanced pro- and anti-inflammatory adipokines, and thus, they can protect MHO individuals from metabolic diseases.

Obesity and inflammation are highly interconnected with respect to the development of metabolic disease [45]. Moderate increases in circulating inflammatory cytokines and immune cell infiltration into adipose tissue has been reported in obesity and its related diseases [46]. Adipose tissue macrophages (ATMs), which are now recognized as important mediators of adipose tissue dysfunction, contribute to obesity and insulin resistance [47]. There are two types of infiltrating macrophages: M1-macrophages which are increased during obesity and secrete pro-inflammatory factors such as TNFα and IL-6; and M2-macrophages which secrete anti-inflammatory factors such as IL-10 [48]. Lumeng et al. showed that diet-induced obesity can shift the activation state of ATMs from M2 to M1 [48]. Hypertrophic adipocyte-derived free fatty acids (FFAs) can also lead to an increase in TNFα production by binding to macrophage toll-like receptor-4 (TLR-4) and activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [49, 50]. In turn, TNFα released from macrophages activates human adipocytes to further increase the levels of pro-inflammatory factors [51]. Since adiponectin acts as a potential mediator in the inhibition of the activity of NF-κB mediated by TLR [52], it is possible that adiponectin levels play a vital role in this loop.

Hypoxia occurs in adipose tissue from obese mice, and this result from adipose tissue hypoperfusion caused by the expansion of fat cells to a point exceeding the diffusion limit of oxygen [53]. It has been demonstrated that hypoxia, which induces endoplasmic reticulum (ER) stress, is partly responsible for the dysregulation of adipokine production. Meanwhile, ER stress inhibits the activity of the adiponectin promoter and PPARγ, which leads to increased adiponectin mRNA degradation. Thus, a decline in adiponectin is observed in obesity [54].

It has been well-established that weight loss and physical activity increase the production of anti-inflammatory cytokines (adiponectin and IL-10) and decrease the production of pro-inflammatory cytokines (TNFα, leptin and IL-6) in obesity [55]. However, since lifestyle interventions have been largely unsuccessful, it is very important to find other clinical strategies to reduce obesity and or related diseases. Thus, knowledge that underpins adipose tissue dysfunction may provide novel therapeutic targets to reduce obesity related diseases.

1.2 Adiponectin

1.2.1 Overview

Adiponectin is an adipocyte-derived hormone which was first identified through the analysis of a cDNA library from murine, 3T3-L1 adipocytes [56]. Given that the structure of adiponectin is similar to complement factor C1q, adiponectin was originally named Acrp30 (adipocyte complement-related protein of 30 kDa). Three independent groups identified it and called it AdipoQ, apM1and GBP28 respectively [57].

Adiponectin achieves high concentrations in the human circulation, around 5-30 ug/mL. Circulating adiponectin predominantly exists in three forms: trimers, hexamers, and HMW multimers (12-, 18-mers and larger), the latter being the most metabolically active (Fig 1.2) [58]. Importantly, the level of adiponectin is decreased in obese subjects, unlike other adipose tissue-derived proteins which are increased with obesity due to increased adipocyte mass [59]. Previous clinical studies have shown that plasma adiponectin levels negatively correlate with insulin resistant [60] and body weight [61]**.** In mouse models, adiponectin shows protective properties such as improvement of the ability of insulin to suppress hepatic glucose production as a result of decreased tissue TG, and suppression of the elevation of plasma FFAs by increased β-oxidation [9, 62, 63]. Thus, adiponectin is associated with the majority of obesity-related diseases, including NAFLD, CVD, type 2 diabetes, cancer and bone metabolic diseases.

1.2.2 Adiponectin Receptors and Signaling Pathways

Adiponectin secretes from adipose tissue and causes pleiotropic effects via two 7 transmembrane domain receptors, AdipoR1 and AdipoR2 [64, 65]. Adiponectin receptors express in liver, heart, muscle, osteoblasts, adipose tissue, pancreas and the brain [66-72]. Evidence suggest that AdipoR1 and AdipoR2 serve to transduce adiponectin signals; the increase of adiponectin receptors leads to the increase of adiponectin binding , signalling and action [73]. Studies have demonstrated that the expression of adiponectin receptors appears to be regulated by many factors. It has been shown that the expression of AdipoR1 is reduced in the adipose tissue of obese subjects [74]; feeding and insulin reduces the expression of both receptors [75, 76]; and fibrates increase the expression of both receptors in adipose tissue [77], but not skeletal muscle [78].

It has been reported that many intracellular kinases were mediated via adiponectin receptors including activation of AMP kinase (AMPK), peroxisome-proliferator activated receptor alpha

(PPARα) and P38-MAPK kinase in liver, skeletal muscle and endothelial cells [79]. Of these, AMPK acts as a major downstream component of adiponectin signaling and it is involved in adiponectin-mediated pleiotropic effects [80]. Studies show that the phosphotyrosine domain of a leucine zipper motif (APPL1) is binding to both adiponectin receptors and downstream signaling molecules [81, 82]. APPL1 is required for adiponectin-mediated activation of AMPK and p38-MAPK pathways. APPL1 is also required for adiponectin-regulated, AMPK-dependent phosphorylation and modulation of eNOS in humanendothelial cells [83, 84]. Additionally, the anti-inflammatory and cytoprotective effects of adiponectin are, at least partly, mediated via APPL1-dependent AMPK activation of the phosphatidylinositol 3-kinase (PI3K)-v-akt (Akt) signaling pathway [85].

In addition to APPL1, other adiponectin receptor interacting proteins have been identified, including the receptor for activated protein kinase C1 (RACK1) [86], the regulatory subunit of protein kinase CK2 (CK2β) [87], endoplasmic reticulum protein 46 (ERp46) [88] and lymphotoxin-β [89]. Of these, binding of lymphotoxin-β to AdipoR1 appears to be involved in adiponectin-mediated suppression of NF-κB signaling in endothelial cells [89]. Erp46 has been reported to interact specifically with AdipoR1 via non-conserved N-terminal amino acids, and it is involved in regulating adiponectin-stimulated AMPK and p38 MAPK phosphorylation [88]. However, the roles of these proteins in adiponectin signaling have not been fully defined.

1.2.3 Adiponectin Production

1.2.3.1 Molecular Structure and Multimerisation of Adiponectin

Human adiponectin is a member of the complement factor C1q family. It is a 224 amino acid protein which is organised into four domains: an amino-terminal signal peptide at the N-terminus; followed by a variable region which exhibits no homology to any other known protein; a collagenous domain which contributes to the formation of a collagen-triple helix; and a globular domain at C-terminus which shares a significant homology with C1q [90, 91]. The structure of the globular region has been determined, and shows similarity to TNFα [92].

Adiponectin exists in the circulation predominantly in three forms: Trimers and hexamers, termed as low molecular weight (LMW) multimers, and larger complexes (12-, 18-mers and larger) described as HMW multimers. Adiponectin produced in bacteria was unable to reduce hyperglycemia and restore insulin sensitivity in mice [9]. This is most likely due to the inability of bacteria to perform hydroxylation of proline residues within the collagenous domain as well as other critical post-translational modifications (PTMs), indicating that PTMs play an essential role in the multimerization and insulin-sensitizing actions of adiponectin [93]. Cys36 (Cys39 in mouse) is

a conserved cysteine residue in the amino-terminal variable region of adiponectin, which is responsible for the formation of a disulfide bond that stabilises HMW adiponectin complexes [94, 95]. Mutation of Cys39 blocks the assembly and secretion of multimers larger than trimers [94]. It has been reported that Cys39 is a key site for succination, which disrupts the multimerisation of adiponectin and may contribute to reduced circulating adiponectin in diabetics [96]. In addition to the formation of disulphide bonds, adiponectin also undergoes hydroxylation and glycosylation [97]. Four conserved lysine residues located in the collagenous domain of adiponectin are involved in this process [98, 99]. Mutation of these resides significantly attenuates multimerisation and the ability of adiponectin to suppress glucose production in hepatocytes [98, 100]. Numerous studies have demonstrated that HMW adiponectin multimers play a predominant role in the bioactive functions of adiponectin [93]. Type 2 diabetes and related diseases characteristically show a reduction in adiponectin glycosylation and a decrease in HMW multimers [101]. Previous clinical studies have shown that plasma HMW adiponectin levels negatively correlate with insulin resistance, ascribed to the effects of both chronic hyperglycemia and hyperinsulinemia [60], and body weight [61]. Pajvani et al. defined S_A as a new functional adiponectin index calculated as HMW/(HMW + LMW). They found that changes in S_A correlated more closely with improvements in hepatic insulin sensitivity than changes in total adiponectin levels [102], although it is noteworthy that this alone does not demonstrate causality. These data strongly suggest that PTMs of adiponectin are critically involved in regulating the formation of HMW multimers, which largely contribute to the insulin-sensitizing activity of adiponectin. Understanding the underlying mechanism of regulating adiponectin multimerisation and secretion could give rise to an effective therapeutic approach for the treatment of metabolic diseases.

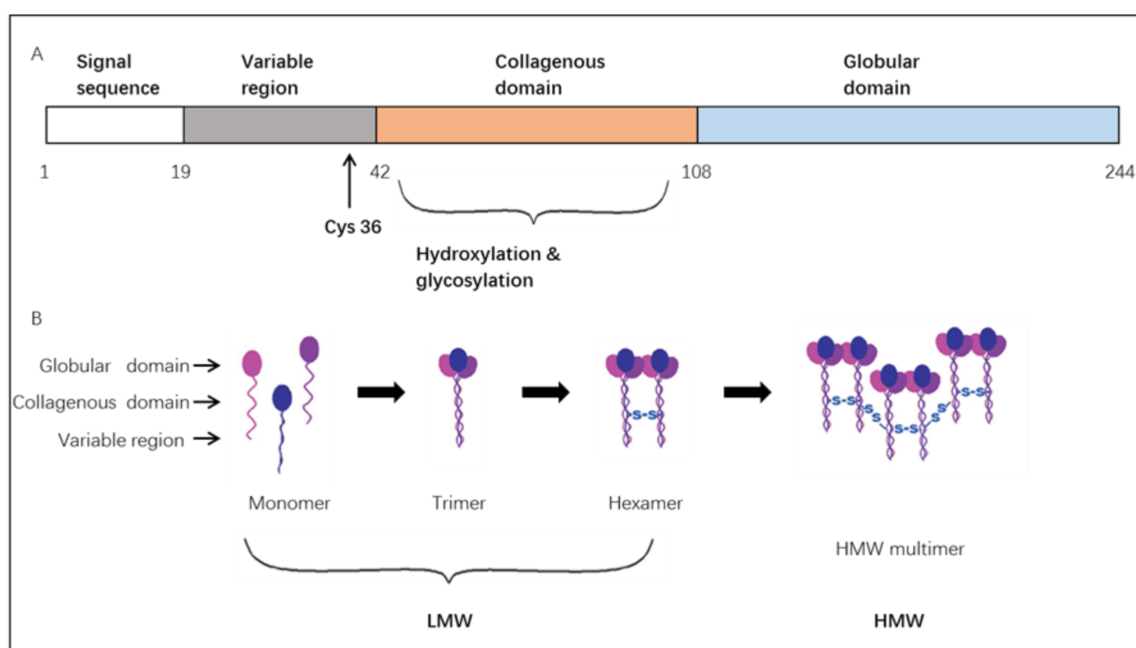

Figure 1.2 Adiponectin structure. **(A)** The four structural domains of adiponectin are shown in block form. Cys36 in the variable region of adiponectin is responsible for the formation of a disulfide bond and succination. Four conserved lysine residues located in the collagenous domain are responsible for hydroxylation and glycosylation. **(B)** Adiponectin is synthesised from a single subunit which goes through multimerisation to form LMW (trimers and hexamers) and HMW (12–18-mers) multimers before secretion. Trimers form a ball and stick-like structure. Hexamers consist of two trimers arranged in a parallel head-to-head manner and connected by disulphide bond. HMW multimers appear to consist mainly of 18-mers, it is synthesized from trimers and hexamers and also connected by disulphide bonds.

1.2.3.2 Regulation of Adiponectin Expression and Secretion

There are many factors that regulate adiponectin secretion at the transcription level and/or posttranslational level, including PPARγ, C/EBP, sterol regulatory element binding protein-1c (SREBP-1c), forkhead box protein O1 (FoxO1), cAMP response element-binding protein (CREB), nuclear factor of activated T-cells (NFAT), TNFα and interleukins.

PPARγ plays a key role in regulating adiponectin expression and secretion. It has been demonstrated that PPARγ receptors are of great importance in the regulation of adipocyte differentiation, insulin action and lipid metabolism [103]. PPARγ is expressed in several key target tissues of insulin action such as adipose, liver and skeletal muscle [104, 105]. In both the human and rodent adiponectin promoter there are putative PPARγ obligatory binding sites, and mutation of these sites lead to a reduction in adiponectin expression [103]. Furthermore, thiazolidinediones (TZDs), PPAR agonists, markedly stimulate the expression of adiponectin [106]. Oxidative stress has been report to contribute to NAFLD. It has been demonstrated that the expression and secretion of TNFα are elevated in obese subjects, which is negatively correlated with adiponectin expression and secretion [107]. Adiponectin expression in 3T3-L1 cells treated with TNFα is decreased by up to 85% [108]. TNFα exerts its strong transcriptional inhibition of adiponectin by suppressing PPARγ activity [109]. The mechanism of TNFα-regulated adiponectin suppression may be via c-Jun N-terminal kinase (JNK), which phosphorylates PPARγ and reduces its DNA-binding activity [110]. TNF-α also suppresses SREBP-1c, and C/EBP, both of which are considered to be transcriptional inducers of adiponectin [111, 112].

In addition to PPARγ and TNFα, there are many other adipogenic transcriptional factors, including C/EBP, SREBPs, FoxO1, CREB, and NFAT that regulate adiponectin secretion at the transcription and/or posttranslational level [113-118].

1.2.3.3 Strategies to Increase Adiponectin

1.2.3.3.1 Thiazolidinediones (TZDs) and Adiponectin

TZDs, novel insulin-sensitizing agents, seem to offer a useful treatment for the metabolic abnormalities encountered in obesity and associated diseases by reducing plasma glucose and insulin levels [119]. Recent studies report that TZDs elevate plasma adiponectin levels in both humans and rodents [120]. They also increase adiponectin expression and secretion in a dose-dependent manner in 3T3-L1 cells [121]. TNFα reduces adiponectin expression and secretion from adipocytes but TZDs reverse the inhibitory effect of TNFα on adiponectin [121, 122]. Conversely, studies have also shown that adiponectin inhibits the TNFα signaling pathway in endothelial cells and reduces TNFα production in macrophages [123-125]. Therefore, TZDs might induce adiponectin production via direct effects on the adiponectin promoter in combination with antagonism of the negative effects of TNFα on adiponectin production. In addition, TZDs show a close relationship with PPARγ, as described previously. Several studies have demonstrated that TZDs regulate the expression of many genes involved in glucose and lipid metabolism through binding with PPARγ [126, 127]. It has also been reported that treatment of subjects with type 2 diabetes with TZD for only 21 days is sufficient to increase the ratio of HMW adiponectin/total levels, which may contribute to the hepatic insulin-sensitizing effects of TZDs [128]. Taken together, these observations suggest that TZDs may act by inducing adiponectin production, especially the secretion of HMW adiponectin, and reducing the effect of TNF-α on the adiponectin promoter.

1.2.3.3.2 Omega3 Fatty Acids (PUFA) and Adiponectin

Omega-3 (n-3) long chain polyunsaturated fatty acids (PUFAs), namely DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid), are mainly found in fish oil. These compounds are reported to exert numerous beneficial effects, including improvements in obesity and metabolic disease, partially due to the metabolic actions of n-3 PUFAs in adipose tissue [58]. Fernández-Real et al. demonstrated that there is a strong correlation between dietary fatty acid intake and plasma adiponectin in healthy humans [128]. A small clinical trial has shown that increased $n-3$ PUFA consumption combined with reduced $n-6$ PUFA consumption has favourable outcomes, including

improvement in anti-inflammatory lipid profiles, increased plasma adiponectin and decreased pro-inflammatory TNFα [129]. Studies in cells and animals have also demonstrated that *n*−3 PUFA may increase adiponectin [130]. Dietary supplementation in rats with EPA+DHA from fish oil increases plasma concentrations of adiponectin, decreases inflammation and prevents left ventricular remodelling and dysfunction without any effect on adiposity [130]. Fish oil feeding in mice significantly induces adiponectin expression in epididymal adipose tissue within 24 h in a dose-dependent manner, in a PPARγ-dependent and PPARα-independent manner [131]. Itoh et al. demonstrated that administration of EPA in genetically obese mice and diet-induced obese mice increased serum adiponectin concentrations [132]. EPA could further reverse the macrophage-induced decrease of adiponectin from co-cultured adipocytes, indicating that EPA-induced adiponectin secretion may, at least in part, be due to the dowregulation of TNFα [132]. All these studies provide important insight into the therapeutic potential of *n*−3 PUFA. Therefore, PPARγ is a key transcription factor linking dietary intake of FAs and pharmacological agents to adiponectin expression and production.

1.2.3.3.3 Vitamin C and Adiponectin

Vitamin C, also known as ascorbic acid (AA) or simply ascorbate, is an essential nutrient for humans. It is unable to be synthesized by humans due to a mutation in the gene encoding the final enzyme for vitamin C biosynthesis, so it must be obtained through the diet. Vitamin C is well known for its antioxidant property. Accumulation of free radicals results in oxidative stress [133], which has a negative effect on CVD, hypertension, chronic inflammatory diseases, diabetes and wound healing [134-136]. Vitamin C can act as an effective scavenger of free radicals and other reactive oxygen species [137, 138].

Epidemiological studies show that one-third of people in the United States experience vitamin C deficiency or depletion [139]. A concentration below 11 μM is considered severe vitamin C deficiency while between 11 and 28 μM is marginal vitamin C deficiency. Epidemiological studies indicate that vitamin C levels are decreased in states such as obesity and type 2 diabetes [140-143]. Perhaps surprisingly then, supplementation studies have generally failed to show beneficial effects of vitamin C supplementation on cardiometabolic parameters [144, 145]. However, as discussed by Michels and Frei [146], the failure of such trials to demonstrate any positive effects may reflect limitations in trial design, most notably the lack of recruitment of a vitamin C deficient cohort that may be expected to benefit from vitamin C supplementation [146].

Hence, a number of studies have been performed to address the relationship between vitamin C and metabolic parameters. It has been reported that vitamin C plays an important role in the formation of HMW adiponectin multimers. It has been described previously that the hydroxylation and

subsequent glycosylation of four highly conserved lysine residues in adiponectin plays a key role in the formation of HMW adiponectin multimers [94, 98]. Adiponectin is a collagen-like protein that shares homology with collagen X, VIII and complement protein C1q [147]. Vitamin C is required [148] and consumed in the hydroxylation reaction catalysed by lysyl-hydroxylase enzymes (LH1, LH2 and LH3) in the biosynthesis of collagen and collagen-like protein complement component C1q [149, 150], which suggests that vitamin C plays a key role in ECM remodelling. Our research found that treatment of simpson golabi behmel syndrome (SGBS) cells, a human primary pre-adipocyte cell line, with vitamin C increased the secretion of HMW adiponectin, but not total adiponectin. Co-treatment with TZDs and vitamin C further increased the secretion of HMW adiponectin, but not total adiponectin, compared with treatment with TZDs or vitamin C alone. This phenomenon was also observed in the absence of TNFα [151]. However, whether vitamin C supplementation in deficient individuals can promote HMW adiponectin should be further studied.

1.2.4Adiponectin Function

1.2.4.1 Adiponectin in Non-alcoholic Fatty Liver Diseases (NAFLD)

NAFLD includes a broad range of liver damage ranging from simple nonalcoholic steatohepatitis (NASH) to fibrosis and cirrhosis complications. It has been recognized as the hepatic manifestation of metabolic syndrome, and insulin resistance is closely associated with the pathogenesis of NAFLD [152]. Several studies have shown that adiponectin plays an important role in NASH. Hypoadiponectinemia is recognized as an early biomarker of NASH and NAFLD and relates to the severity of liver damage [3, 153, 154]. Studies show that adiponectin has anti-steatotic, anti-inflammatory, anti-fibrotic, and anti-tumor properties in NAFLD. The anti-steatotic property is via the activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-alpha (PPAR-α) signalling. AMPK phosphorylation inhibits energy-consuming processes and promotes ATP-producing catabolic pathways [155]. PPAR-α regulates hepatic lipogenic gene expression and manipulates fatty acid oxidation [156]. Oxidative stress has been reported to contribute to NAFLD, and TNFα induces inflammation and apoptosis in the liver under oxidative stress [157]. The anti-inflammatory properties of adiponectin are achieved through the induction of anti-inflammatory cytokines and the inhibition of NF-κB activity, thereby suppressing the expression of TNFα [158, 159]. Additionally, it has been demonstrated that adiponectin attenuates liver fibrosis [160], which plays a critical role in the pathophysiology of NAFLD. The transformation of hepatic stellate cells (HSC) into myofibroblasts plays a key role in the initiation of the fibrotic process during liver damage. Adiponectin can effectively improve this process by upregulating the expression and production of BMP-and activin-membrane-bound inhibitor (BAMBI). BAMBI

upregulation leads to the suppression of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) synthesis [160-162]. In addition, Kamada and colleagues have shown that hepatic tumor formation and oxidative stress markers are enhanced in mice lacking adiponectin [163]. These results indicate that adiponectin can protect hepatocytes from hepatic steatosis, fibrosis, and hepatic tumor formation, and thus, adiponectin might be considered a possible treatment for NAFLD.

1.2.4.2 Adiponectin in Cardiovascular Diseases (CVD)

CVD is related to obesity and other metabolic disorders and is a leading cause of death globally. It is well established that adiponectin plays a critical role in coronary heart disease (CHD) [164], hypertension [165], and myocardial infarction [166]. Epidemiological studies suggest that low levels of plasma adiponectin, especially HMW multimers, may be an independent risk factor for CVD [4, 167]. By contrast, a 10-year follow up epidemiology study by Frystyk and colleagues showed that higher levels of plasma adiponectin are related to a decreased risk of CHD, which is independent of other risk factors such as waist circumference, BMI etc. [4]. Several animal studies have showed the cardioprotective effects of adiponectin in mice. The elevation of plasma adiponectin by recombinant adiponectin administration significantly attenuates atherosclerosis as well as inhibits the expression of vascular cell adhesion molecule-1 and TNFα in apoE knockout mice [168]. Adiponectin null mice exhibit a severe cardiac hypertrophy after pressure overload, diminished AMPK signalling, and impaired glucose metabolism [169]. Adiponectin deficient mice also exhibit increases in myocardial infarct size, apoptosis and expression of TNFα in an ischemia/reperfusion myocardium injury model. These morbidities are reversed after adiponectin administration [170]. Using cultured cardiomyocytes, Walsh et al. demonstrated that adiponectin activates the cyclooxygenase 2 (COX-2) pathway, regulating COX-2-derived PGI_2, and conferring vasoprotective properties [170]. Mechanistically, the protective actions of adiponectin are partly mediated by regulating the levels of nitric oxide (NO) produced from endothelial nitric oxide synthase (eNOS) through AMPK-dependent pathways [171, 172]. Furthermore, adiponectin has been shown to inhibit TNFα-induced endothelial cell inflammation [124] and suppress the proliferation and migration of aortic smooth muscle cells via the inhibition of growth factor–stimulated extracellular signal-regulated kinase (ERK) signalling [173]. Taken together, these data indicate that adiponectin acts as a multifunctional protector in vascular remodelling, with anti-atherogenic and anti-inflammatory properties.

1.2.4.3 Adiponectin in Type 2 Diabetes

Type 2 diabetes mellitus has been defined as a metabolic disorder characterised by hyperglycemia with insulin resistance and a relative lack of insulin. Adiponectin is known to have insulin-sensitising properties in type 2 diabetes, multiple sclerosis (MS), hypertension, and dyslipidaemia [174]. Epidemiological studies have found that there is a close relationship between higher adiponectin levels and lower incidence of diabetes [5, 175]. A variety of clinical studies show lower adiponectin levels in obese humans compared to lean subjects. Hotta et al. analysed the concentration of plasma adiponectin in age- and BMI–matched non-diabetic and type 2 diabetic subjects and found decreased plasma adiponectin in diabetes [61]. They also found a severe decrease in plasma adiponectin concentration in type 2 diabetic subjects with coronary artery disease, which may indicate that adiponectin plays an important role in atherosclerotic vascular damage in diabetes [61]. In the study by Weyer et al. plasma adiponectin positively correlates with several insulin sensitive parameters. Furthermore, fasting plasma insulin concentration and insulin sensitivity are the predominant determinates of adiponectin concentrations, which indicates that plasma adiponectin concentration is closely related to insulin resistance and hyperinsulinemia [60]. The most compelling evidence to identify the role of adiponectin in insulin resistance is found in the adiponectin-deficient mice when fed a HFD. These mice exhibit higher levels of TNFα and a reduction in insulin signalling mediated by muscle insulin-receptor substrate 1 (IRS-1)-associated phosphatidylinositol 3 kinase (PI3-kinase), which leads to severe diet-induced insulin resistance [31]. The hyperglycemia and hyperinsulinemia which occurs in adiponectin-deficient mice can be normalised by adiponectin administration. However, despite growing evidence to support the relationship between adiponectin and type 2 diabetes, the underlying mechanisms are still largely unknown. A recent study by Waki et al. implicated impaired adiponectin multimerisation in the development of type 2 diabetes. They found that in hepatocytes, HMW adiponectin is capable of activating AMPK, which has been reported to mediate the effects of adiponectin in glucose and lipid metabolism [94]. All of the studies on the role of adiponectin in type 2 diabetes and insulin resistance suggest that these metabolic disorders are closely associated with decreased levels of adiponectin, and the administration of adiponectin may become a potential therapeutic strategy. Further studies to understand the underlying mechanisms are necessary.

1.2.4.4 Adiponectin in Cancer

Recent studies have reported that obesity plays an important role in the development of many malignancies [6]. A large body of evidence shows that plasma adiponectin levels are negatively correlated with the risk of malignancies which have been linked to obesity and insulin resistance, such as breast cancer [176], endometrial cancer [177, 178], leukaemia [179], colon cancer [178],

gastric cancer [180] and prostate cancer [181]. Adiponectin exerts its anti-carcinogenic properties in different ways. In obesity, hypoadiponectinemia leads to insulin resistance and hyperinsulinemia and results in reduced insulin-like growth factor binding protein 1 (IGFBP1) and IGFBP2. Consequently bioavailable IGF1 is increased, contributing to carcinogenesis by promoting proliferation and inhibiting apoptosis [182]. Yokota et al. found that adiponectin plays a key role in regulating hematopoiesis and the immune system, inhibiting B-cell lymphoma 2 (Bcl-2) mediated growth and promoting apoptosis in myelomonocyte (leukaemia) cell lines [183]. Adiponectin also regulates several signalling pathways including AMPK, PPARγ, and mitogen-activated protein kinase (MAPK) in liver and skeletal muscles [184], thereby potentially interfering with carcinogenesis through growth inhibition or the promotion of cell death [185]. In summary, studies show that adiponectin plays an important inhibitory role in the development of cancer.

1.2.4.5 Adiponectin in Metabolic Bone Diseases

Bone is a dense connective tissue that supports and protects the body and organs, produces red and white blood cells, and serves as an endocrine organ for the regulation of mineral homeostasis. Clinical epidemiological studies show a discrepancy in the relationship between plasma adiponectin concentration and bone mineral density (BMD). Following adjustment for age, gender and other factors known to influence circulating adiponectin levels (smoking, race) the majority of studies [7, 186-189], but not all [190], shown an inverse relationship between adiponectin and BMD.

In *in vitro* studies, adiponectin has been reported to promote the differentiation of mesenchymal stem cells (MSCs) to preosteoblasts and inhibit differentiation to adipocytes. COX-2 seems to play a central role in this process [191]. Lee and colleagues suggested that adiponectin might activate the AdipoR1-p38/MAPK/c-Jun cascade, leading to COX-2-induced osteoblast differentiation [191]. Meanwhile, Yokota et al. found that adiponectin could suppress the differentiation of MSCs to adipocytes by the COX-2 dependent manner [192]. Oshima et al. has reported that adiponectin may increase bone mass by inhibiting osteoclastogenesis and activating osteoblastogenesis [193]. However, although several studies have suggested that adiponectin plays a role in bone marrow and mesenchymal progenitor cell development, the precise link between plasma adiponectin and metabolic bone disease still needs to be further studied.

1.3 Heme oxygenase

1.3.1 Overview

HO, a ubiquitously expressed inducible cellular stress protein, serves as a rate-limiting enzyme catalysing the oxidative degradation of heme to the bioactive molecules carbon monoxide (CO), biliverdin, and ferrous iron. Biliverdin is subsequently converted into bilirubin [194]. The ferrous iron is rapidly sequestered by ferritin and either pumped out of the cell by an ATPase pump or recycled for heme synthesis [195]. Two main isoforms of HO have been identified, the inducible isoform HO-1 and the constitutively expressed isoform HO-2 [195]. HO-1 can be induced by several stimuli, such as its substrate heme, heavy metals, inflammation, stress, hypoxia and other oxidants [196]. The anti-apoptotic and anti-inflammatory properties of HO-1 are largely due to these degradation products [197]. CO is reported to have several beneficial functions including relaxation of blood vessels [198], suppression of apoptosis [199], stimulation of blood vessel formation [200], and inhibition of pro-inflammatory cytokines [201]. Bilirubin is a potential antioxidant, and also inhibits NADP(H) oxidase, subsequently inhibiting superoxide anion production [202, 203]. Some, but not all [204] evidence suggest that induction of HO-1 has beneficial effects in several disease models including metabolic disorders such as insulin resistance, type 2 diabetes and obesity [195].Thus, the subsequent studies tried to identify effective approaches to induce HO-1 in human [205-207]. CoPP [208-210] and hemin [208, 211, 212] were used to induce HO-1 in cell culture and pre-clinical models in most studies. Unlike CoPP, hemin is the derivative of heme which is a HO-1 substrate analog that can serve as both an inducer and a substrate for HO-1 [213, 214]. The underlying mechanism for the induction of HO-1 typically involves reciprocal regulation of Bach1 and Nrf2 protein stability [213]. Both are basic leucine zipper transcription factors and the former represses HO-1 expression whilst the latter promotes its expression [213]. Interestingly, bivalent metals other than iron, including Cu, Zn, Sn and Co, are able to bind to the porphyrin ring within heme and the HO-specific binding region does not discriminate between these different metalloporphyrins [219]. As such, these alternate metalloporphyrins bind to and inhibit HO-1 enzyme activity because they are not degraded [220]. Hence, metalloporphyrins can induce HO-1 expression and compromise enzyme activity. CoPP is widely used to induce HO-1 activity as it is a strong inducer of HO-1 expression and weak inhibitor of HO-1 activity. In contrast, SnMP is used as a HO-1 inhibitor because it is a weak inducer of HO-1 expression but a strong inhibitor of HO-1 catalytic activity [219].

1.3.3 HO-1 and Obesity

Accumulating evidence has led to the suggestion that induction of HO-1 via CoPP [209, 210, 215] or Hemin [211, 212, 216] has a range of beneficial effects on obesity. For example, chronic HO-1 induction in obese mice prevents body weight gain, increases the number and decreases the size of adipocytes, increases circulating adiponectin levels and decreases circulating pro-inflammatory cytokine levels [210, 217]. Studies show that CoPP administration to rats either subcutaneously or via intra-cerebroventricular injection led to an initial decrease in food intake of between 60-80%, compared with vehicle-treated animals [218-220], and subsequently results showed a 20 to 25% reduction of body weight compared with vehicle [218-220], but this reduction in body weight was eliminated when co-treated with HO inhibitor [217]. CoPP administration and HO-1 induction via other compounds have also been demonstrated to induce weight loss. Treatment with hemin has been demonstrated to lower body weight in Zucker diabetic fatty rats [221]. Consistent with reduction in food intake and lower body weight, insulin sensitivity and glucose tolerance in obese mice were also improved [217, 222]. Systemic induction of HO-1 has been reported to lower hyperglycemia and hyperinsulinemia in several models of obesity in both rats and mice [217, 221, 223]. A recent study also showed that MC4R-deficient obese mice treated with long-term (4-23 weeks of age) CoPP administration, exhibited increased oxygen consumption, and CO_2 and heat production [224]. Increased adiponectin has also been reported and has been proposed to underpin these beneficial effects [209, 225-228]. Studies have also shown that the above benefits can be reversed by SnMP treatment [210, 217]. It has also been reported that the beneficial effects of HO-1 induction are correlated with the increase of phosphorylated AMP-activated protein-kinase (AMPK), Akt, insulin receptors and the increase of the glucose transporter-4 in both adipocytes and skeletal muscle [223, 224]. Collectively, these results indicate that improvements in basal metabolism combined with decreased food intake may underlie the sustained weight loss observed in response to HO-1 induction *in vivo*, and adiponectin seems to be a key element in these beneficial effects of HO-1 induction. However, the underlying mechanism of how the HO-1 inducer affects food intake and basal metabolism requires further investigation.

1.3.4 HO-1 and adipocytes

Adipose tissue plays an important role in obesity. As individuals become obese, their adipocytes enlarge, and adipose tissue undergoes molecular and cellular changes which affect systemic metabolism. Pro-inflammatory factors are also increased with the increase of adipose tissue in obesity [229]. Recently, emerging studies have reported that chronic induction of HO-1 can significantly alter the phenotype of adipocytes [210, 224, 230]. Induction of HO-1 by CoPP or

hemin administration in ob/ob mice or Zucker rats reduced adiposity and improved insulin sensitivity in diabetic animals [217, 231]. It was first reported by L'Abbate et al that HO-1 induction results in increased serum adiponectin in diabetic rats [232]. Subsequent studies showed that chronic HO-1 induction appears to remodel adipocytes, resulting in hyperplasia instead of hypertrophy [210]. Abraham and colleagues demonstrated that HO-1 induction in adipocytes not only leads to an increase in adiponectin, but also a decrease in inflammatory cytokines such as TNFα, IL-6, and IL1-β [210, 217, 233]. HO-1 may relieve inflammation by reducing NF-κB, which plays an important role in the development of insulin resistance. Moreover, the increased phosphorylation of AMPK and PPARγ [234], and decreased levels of monocyte chemoattractant protein-1 (MCP-1) [235], may also contribute to the reduced inflammation following HO-1 induction. As decreased serum adiponectin has been observed in subjects with obesity and its associated diseases [29]. Adiponectin-deficient mice exhibit diet-induced obesity and insulin resistance [30] which can be reversed by adiponectin administration [31]. Therefore, it is thought that the increased levels of adiponectin are the mechanism by which induction of HO-1 results in improvement of the health status in obese subjects [217, 225, 227, 236-242].

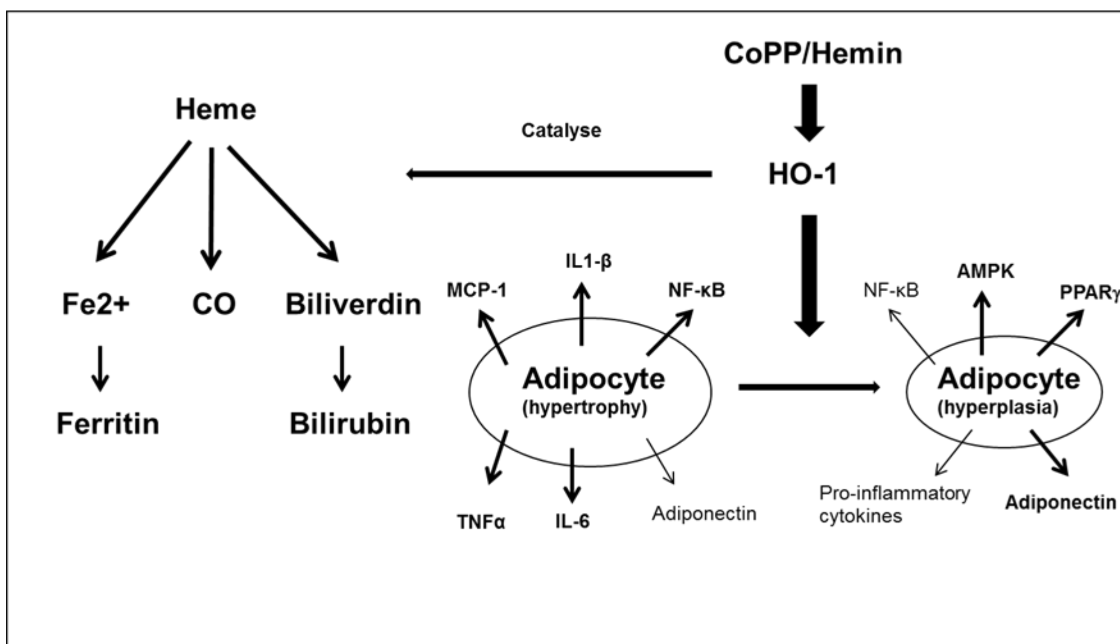

Figure 1.3 The relationship between HO-1, the adipocyte and adiponectin. HO-1 is the rate limiting enzyme that catalyses the degradation of heme into three byproducts: iron, CO and biliverdin. Iron is subsequently bound to iron-binding protein ferritin and biliverdin is rapidly converted to bilirubin. Adipocytes from obese individuals are usually expanded via hypertrophy. These adipocytes usually produce a large amount of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL1-β, and a small amount of the beneficial adipokine, adiponectin. HO-1 induction remodels adipocytes, resulting in hyperplasia instead of hypertrophy. These adipocytes increase the

production of AMPK, PPARγ, and adiponectin, at the same time decreasing the production of inflammatory markers and cytokines [209, 210, 215, 243].

1.3.5 Genetic manipulation of HO-1

Genetic studies on the increase of HO-1 activity in adipocytes have been performed by two independent research groups by using the aP2 promoter to manipulate HO-1 expression in adipocytes via lentiviral or transgenic approaches. In the paper published by Cao et al, researchers used lentiviral (aP2-HO-1) construct via intracardiac injection, which successfully increased HO-1 expression in adipose tissue and attenuated high fat diet (HFD) induced body weight increase, decreased adiposity and also improved insulin sensitivity and adiponectin production [209]. Another paper published by Huang et al, used a classic transgenic approach to increase HO-1 in adipose tissue [244]. In contrast to the previous report, this failed to attenuate HFD-induced obesity, insulin resistance or the increase in adiponectin. The explanation for these contrasting findings needs further investigation, but it is worth know that the aP2 promoter is switched on in the early stage of differentiation process, which means the increased HO-1 expression would also be seen in immature adipocytes, and it would also drive gene expression in cells other than adipocytes such as cardiomyocytes and macrophages [245]. In the papers published by Huang et al, transgenic overexpression of HO-1 via the P2 promoter resulted in an increase in HO-1 in peritoneal macrophages which is sufficient to increase M2 macrophage polarisation but not enough to protect against obesity decreased adiponectin and other metabolic dysfunctions [244].Collectively, these studies indicated there may be an association between reduced body weight and improved adiponectin levels. Moreover, recent literature published by Jais et al. provided the most challenging findings in this area [204]. Jais et al. found that HO-1 acts as a driver rather than a brake in obesity associated inflammation. In this study, they found no evidence of a major role for HO-1 in adipocyte, muscle or pancreatic β-cells [204]. However, their results indicate that inhibition of HO-1, rather than induction, may represent effective therapeutic effects in both myeloid and hepatic cells [204]. Due to the discrepancy of these studies, further studies are necessary to determine the underlying mechanisms between HO-1, insulin sensitivity, adiposity, adiponectin and inflammation.

1.3.6 The side effects of metalloporphyrins

As discussed previously, metalloporphyrins can bind to porphyrin rings to compete for heme and inhibit enzyme activity. An exception is CoPP, which is a strong HO-1 inducer, and its HO-1 synthesis effect outweighs its inhibitory effect [246]. Therefore, the major concern of the use of metalloporphyrins is the potential cytotoxicity of heme after blockade of its metabolism. It has been reported that metalloporphyrins interact with many heme-containing enzyme systems, including soluble guanylyl cylase (sGC), nitric oxide synthase (NOS), and cytochrome P450 (CYP450) [247]. They have also been found to affect steroidogenesis, matopoiesis, and the iron status of the body [247]. However, the most prominent potential side effect is the photosensitivity of majority metalloporphyrins. The photosensitinzing property of metalloporphyrins causes the formation of triplet excited states which subsequently causes the formation of singlet oxygen [248]. The excess singlet oxygen reacts with many biological substrates, including amino acids, cholesterol and fatty acids) [248, 249]. It has been reported that the photophysical parameters and singlet oxygen-sensitizing ability of SnMP is strong, and therefore it is expected to have phototoxic effects [250]. It has also been reported that mortality was observed in rats when treated with SnMP and exposure to cool white fluorescent light simultaneously [251]. The underlying mechanisms are not known. Another major concern in the use of metalloporphyrins is that they reduce the CO and free iron status of cells; therefore, they may affect hemoproteins and other enzymes. It has been reported that SnMP decreases CYP450 activity and therefore affects CYP450-related enzymes of adrenal synthesis and drug metabolism in animal models [247]. However, the underlying mechanisms are not clear.

1.4 Hypothesis and aims

Adiponectin is an adipose-derived hormone with anti-diabetic, anti-inflammatory and cardioprotective properties. Obesity and associated cardiometabolic diseases are characterized by reduced adiponectin levels. Clinical and pre-clinical studies demonstrate the therapeutic value of strategies to reverse such “hypoadiponectinemia”.

HO-1 is a ubiquitously expressed inducible cellular stress protein, which serves as a rate-limiting enzyme catalyzing the oxidative degradation of heme. Recently, HO-1 has been reported to have a protective effect under conditions of diet-induced obesity with direct effects on adipose tissue. Pharmacological induction of HO-1, by CoPP or Hemin, *in vivo* leads to a reduction in body weight and improved insulin sensitivity, increased serum adiponectin and decreased pro-inflammatory

cytokines. Subsequent *in vitro* studies also showed an increase in adiponectin and a decrease in pro-inflammatory cytokines following HO-1 induction during differentiation of MSCs. These observations have promoted the concept of a HO-1 – adiponectin axis. However, whether such an axis represents a direct effect of HO-1 on adiponectin or an indirect effect remains unknown, as do the molecular details of the putative HO-1 – adiponectin axis.

Thus, the aim of this project is to characterize details of the relationship between HO-1, adiponectin and metabolic homeostasis in human preadipocytes and in mouse models.

Hypothesis 1: Induction of HO-1 increases adiponectin production in healthy and TNFα treated human adipocytes.

Aim 1 - In control and TNFα-treated human SGBS adipocytes, characterise the effects of acute (24-48 h) CoPP-induced HO-1 induction on:

(i) Adiponectin expression (mRNA) and secretion (total and HMW)
(ii) IL-6 expression and secretion
(iii) Pro-inflammatory cytokine expression
(iv) Markers of ER stress

Aim 2 - Characterise the effects of chronic CoPP treatment on differentiation of human SGBS preadipocytes including determination of:

(i) Adiponectin expression (mRNA) and secretion (total and HMW)
(ii) Morphological changes and lipid accumulation during differentiation
(iii) Temporal mRNA expression profiles of adipogenic markers
(iv) Basal and insulin-stimulated glucose uptake in 'mature' day 14 adipocytes

Hypothesis 2: Induction of HO-1 *in vivo* alleviates obesity-induced hypoadiponectinemia, reduces circulating pro-inflammatory cytokines and improves metabolic function in mice and these effects would reverse by SnMP treatment.

Aim 3 - Examine the effect of CoPP administration to standard chow diet (SCD) and high fat diet fed (HFD) C57BL/6 mice:

(i) The changes in food intake and body weight
(ii) Circulating adiponectin profiles (total and HMW)
(iii) Circulating pro-inflammatory cytokine levels (TNFα, IL-6, MCP-1), glucose and insulin levels
(iv) Adipose tissue architecture, gene expression and macrophage infiltration
(v) The changes in steatosis and gene expression in liver
(vi) Insulin and glucose tolerance (via intra-peritoneal insulin tolerance and glucose tolerance tests)

Aim 4 - Examine the effect of CoPP and CoPP+SnMP administration to HFD C57BL/6 mice:

(i) The changes in food intake and body weight

(ii) Circulating adiponectin profiles (total and HMW)

(iii) Circulating pro-inflammatory cytokine levels (TNFα, IL-6, MCP-1), glucose and insulin levels

(iv) Adipose tissue architecture, gene expression and macrophage infiltration

(v) The changes in steatosis and gene expression in liver

(vi) Insulin and glucose tolerance

Chapter 2:

Induction of Heme-oxygenase-1 (HO-1) does not enhance adiponectin production in human adipocytes: Evidence against a direct HO-1 – adiponectin axis

2.1 Introduction to this publication

This Chapter was published as an original article in *Molecular and Cellular Endocrinology* journal. In this study, we demonstrate that in mature human adipocytes:

- Inducers of HO-1 acutely (24-48 h) increase HO-1 mRNA, protein and activity
- Acute induction of HO-1 does not enhance or rescue adiponectin production in healthy or TNFα-treated cells
- Acute induction of HO-1 does not ameliorate TNFα-stimulated expression and secretion of pro-inflammatory adipocytokines

Collectively, these findings argue against a direct HO-1 – adiponectin axis and also suggest mature adipocytes are unlikely to be acutely involved in mediating the systemic effects of HO-1 induction.

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Induction of Heme-oxygenase-1 (HO-1) does not enhance adiponectin production in human adipocytes: Evidence against a direct HO-1 – adiponectin axis

Mengliu Yang[1], Masaki Kimura[1, 2], Choaping Ng[1], Jingjing He[1], Sahar Keshvari[1], Felicity J. Rose[1], Johanna L. Barclay[1], Jonathan P. Whitehead[1,3]

[1]Mater Research Institute-UQ, Translational Research Institute, Brisbane, QLD, Australia

[2]Current address: Faculty of Pharmacy, Keio University, Tokyo, Japan

[3]Correspondence to: Jon Whitehead, Mater Research Institute-UQ, Translational Research Institute, 37 Kent Street, Brisbane, QLD 4101, Australia.

Tel 61 07 3343 7633.

Email jon.whitehead@mater.uq.edu.au

Keywords

Adiponectin; HO-1; Inflammation; Therapeutic

2.2 Abstract

Adiponectin is a salutary adipokine and hypoadiponectinemia is implicated in the aetiology of obesity-related inflammation and cardiometabolic disease making therapeutic strategies to increase adiponectin attractive. Emerging evidence, predominantly from preclinical studies, suggests induction of heme-oxygenase-1 (HO-1) increases adiponectin production and reduces inflammatory tone. Here, we aimed to test whether induction of HO-1 enhanced adiponectin production from mature adipocytes. Treatment of human adipocytes with cobalt protoporphyrin (CoPP) or hemin for 24-48 h increased HO-1 expression and activity without affecting adiponectin expression and secretion. Treatment of adipocytes with TNFα reduced adiponectin secretion and increased expression and secretion of additional pro-inflammatory cytokines, IL-6 and MCP-1, as well as expression of *sXBP-1*, a marker of ER stress. HO-1 induction failed to reverse these effects. These results demonstrate that induction of HO-1 does not directly enhance adiponectin production or ameliorate the pro-inflammatory effects of TNFα and argue against a direct HO-1 – adiponectin axis.

2.3 Introduction

Adiponectin is an adipocyte-derived hormone that regulates glucose and lipid metabolism via direct and indirect mechanisms and has beneficial anti-inflammatory, anti-diabetic, anti-atherogenic and cardioprotective properties [252-255]. Paradoxically, and in contrast to most other adipocyte-derived hormones or "adipokines", circulating adiponectin levels are reduced in obesity [256]. Although the precise mechanisms for this reduction are unclear metabolic, oxidative and or inflammatory stress are all implicated. The structural complexity of adiponectin appears to be an additional factor that makes it particularly sensitive to such cellular stresses [252]. Briefly, adiponectin is synthesised as a monomer that undergoes multimerisation to form higher order species via a coordinated process that involves a number of post-translational modifications. Efficient multimerisation to trimer, hexamer and high molecular weight (HMW) multimers is a prerequisite for efficient secretion. Moreover, functional studies suggest HMW adiponectin is the most metabolically active form [257, 258]. Evidence suggests that hypoadiponectinemia contributes to the aetiology of obesity-related cardiometabolic diseases and that this typically reflects a selective decrease in the circulating levels of HMW adiponectin [252]. Consistent with this, numerous pre-clinical and clinical studies demonstrate that reversal of hypoadiponectinemia improves a range of cardiometabolic parameters thereby establishing the adiponectin system as an attractive therapeutic target [259, 260].

Heme oxygenase-1 (HO-1), which is sometimes called heat shock protein 32 (Hsp32), is an inducible protein that serves as a rate-limiting enzyme catalysing the oxidative degradation of heme to carbon monoxide (CO), iron and biliverdin, which is subsequently converted to bilirubin [261]. Each of the products of HO-1 activity modulates various aspects of cellular function and homeostasis [261] prompting some to propose HO-1 as a dual purpose "sensor/effector" that both senses and responds to oxidative, inflammatory and metabolic stress [207]. Consistent with this most, but not all [204], investigators promote the induction of HO-1 as an attractive therapeutic strategy to ameliorate the pathophysiology of a range of human diseases including metabolic disorders such as insulin resistance, type 2 diabetes and obesity [195, 262]. As such, considerable efforts are being made to identify efficacious approaches to induce HO-1 in man [206, 207, 263].

Accumulating evidence has led to the suggestion that HO-1 may mediate at least some of its beneficial effects by increasing circulating adiponectin levels through what has been termed the "HO-1 – adiponectin axis" [217, 225, 227, 230, 239, 241, 264-268]. For example, chronic administration of obese mice with the HO-1 inducer cobalt protoporphyrin (CoPP) was reported to increase HO-1 protein, prevent weight gain and decrease fat content (in the absence of any change in food intake), reduce circulating inflammatory cytokines (including TNFα and IL-6) and increase circulating adiponectin levels [217]. Consistent with these changes insulin sensitivity and glucose

tolerance were improved in this pre-clinical model thereby providing further evidence of the potential therapeutic benefits of HO-1 induction. Complementary *ex vivo* studies on isolated bone marrow-derived mesenchymal stem cells demonstrated reduced adipogenesis and increased adiponectin production upon chronic CoPP treatment in support of a regulatory HO-1 – adiponectin axis [217].

In spite of the findings detailed above, which typically describe the effects of chronic induction of HO-1 on adiponectin levels *in vivo* or *in vitro*, to the best of our knowledge no studies have examined whether induction of HO-1 has a direct effect on adiponectin production. Thus, the purpose of the present work was to test the hypothesis that acute induction of HO-1 in mature adipocytes would increase the production of adiponectin, particularly the more metabolically active HMW multimers. Surprisingly, we found no evidence to support a direct effect of HO-1 on adiponectin production (total or HMW), or improvements in markers of cellular stress, in human adipocytes in a variety of experimental scenarios. These results argue against a direct HO-1 – adiponectin axis.

2.4 Materials and Methods

2.4.1 Reagents and antibodies

General reagents were obtained from Sigma-Aldrich (Victoria, Australia) and cell culture reagents were obtained from Invitrogen (Victoria, Australia) unless otherwise stated.

2.4.2 SGBS cell culture, differentiation and treatment

Human SGBS preadipocytes, a gift from Martin Wabitsch (University of Ulm, Ulm, Germany) [269], were maintained and differentiated in the absence of serum as described [270]. Fully differentiated cells (day 14) were treated with increasing concentrations of CoPP (0, 50, 100, and 150 nM) or Hemin (0, 1, 5 μM), or vehicle, in the presence or absence of TNFα (50 ng/mL). Cells and conditioned media were harvested after 24 h or 48 h (following a media change at 24 h for the latter). For experiments performed in the presence of serum, cells were differentiated and maintained in media containing 10% FBS and treated with increasing concentrations of CoPP (0, 5,

10 μM) in the presence or absence of TNFα (50 ng/mL) on day 14. Cells and conditioned media were harvested after 24 h.

2.4.3 Isolation, culture, differentiation and treatment of Primary Human Preadipocytes

Primary human preadipocytes (phPAs) were isolated from subcutaneous adipose tissue from two subjects (both female, age 25 & 39 years, BMI 24.1 & 23.3, metabolically healthy - no insulin resistance, diabetes or cardiovascular diseases) and cultured independently as described previously [270]. The procedure was approved by the Research Ethics Committees of the University of Queensland, the Princess Alexandra Hospital, and the Mater Adults Hospital. Both patients had given their written informed consent. Treatments were performed in fully differentiated cells (day 21) as described above (section 2.2).

2.4.4 Measurement of gene expression by qRT-PCR.

Gene expression was measured by qRT-PCR and standardized against the expression of cyclophilin essentially as previously described [270]. Briefly, total RNA was extracted using Trizol or RNA Mini Kit (Ambion Life Technologies, Victoria, Australia) according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using a cDNA synthesis kit (Bioline, NSW, Australia) and RT-PCR was performed using the SYBR Hi-ROX kit (Bioline) on a 7900HT Fast Real-time PCR system (Ambion Life Technologies).

Primer sequences were as follows (all human, listed as Forward / Reverse): *Cyclophilin* - CGCGTCTCCTTTGAGCTGTT / TCTCCAGTGCTCAGAGCACG; *HO-1* - CCAGGCAGAGAATGCTGAGTTC / AAGACTGGGCTCTCCTTGTTGC; *ADIPOQ* - AGAAAGGAGATCCAGGTCTTATTGGT / AACGTAAGTCTCCAATCCCACACT; *IL-6* - AGACAGCCACTCACCTCTTCAG / TTCTGCCAGTGCCTCTTTGCTG; *TNFα* – CTCTTCTGCCTGCTGCACTTTG / ATGGGCTACAGGCTTGTCACTC; *MCP-1* – AGAATCACCAGCAGCAAGTGTCC / TCCTGAACCCACTTCTGCTTGG; *sXBP-1* – CTGCCAGAGATCGAAAGAAGGC / CTCCTGGTTCTCAACTACAAGGC; *BIP* – CTGTCCAGGCTGGTGTGCTCT / CTTGGTAGGCACCACTGTGTTC; *CHOP* – GGTATGAGGACCTGCAAGAGGT / CTTGTGACCTCTGCTGGTTCTG.

2.4.5 Determination of HO-1, Adiponectin and IL-6 protein

Cellular HO-1 was quantitated in whole cell lysates (in 0.05-0.25 μg protein) using a HO-1 ELISA (Life Sciences, Florida, USA). Secreted total and HMW adiponectin was quantitated using an Adiponectin ELISA Kit (ALPCO, New Hampshire, USA and R&D system, Minnesota, USA). Absolute secreted total and HMW adiponectin levels are presented for each set of experiments in the Figure Legend as mean ± SEM (range: lowest - highest) ng/ml. Data presented in graphs are normalised to values from the control cells (no treatments) which were arbitrarily set at 1. Secreted IL-6 was measured in conditioned media (at a 1 in 2 dilution) using an IL-6 ELISA (R&D system, Minnesota, USA).

2.4.6 SDS-PAGE/Western blot of ferritin

SDS-PAGE and Western blotting of cleared whole cell lysates (centrifuged at 2,000 x *g* for 10 min) was performed using standard approaches [271]. Ferritin antibody was from MP Biomedicals, (Aurora, Ohio, USA - Catalogue # 65077 - used at 1:2,000) and was followed by an Alexa-800 conjugated secondary anti-rabbit antibody (Molecular Probes, VIC, Australia – used at 1:20,000) and scanned using the LI-COR Odyseey Infrared Imaging System.

2.4.7 Statistical analysis

Data are presented as mean ± SEM. One-way ANOVA followed by Tukeys was used to test for a significant effect of CoPP or hemin in cells incubated without TNFα or independently in cells treated with TNFα. When there was no effect of CoPP or hemin a Student t-test (unpaired) was used to test for a significant effect of TNFα. Differences were considered statistically significant at $p<0.05$. Statistical analyses were performed using GraphPad Prism 5.0.

2.5 Results

2.5.1 Acute CoPP treatment induces HO-1 in a dose-dependent manner in SGBS adipocytes

To investigate whether induction of HO-1 has a direct effect on adiponectin expression or secretion we first performed a series of dose response studies in mature (day 14) human SGBS adipocytes to identify optimal CoPP concentrations. We established that treatment with CoPP for 24-48 h at concentrations from 50-150 nM was sufficient to promote robust (10-50 fold) induction of *HO-1* mRNA and protein in control cells as well as cells treated with the pro-inflammatory cytokine TNFα (Fig 2.1A-D), which compromises adiponectin expression and secretion [151]. Treatment of SGBS adipocytes with higher concentrations of CoPP (from 600 nM to 10 μM) failed to promote any further significant increase in HO-1 levels, but induced signs of toxicity at concentrations greater than 2 μM (data not shown). Thus, subsequent experiments were performed using CoPP in the 50-150 nM range.

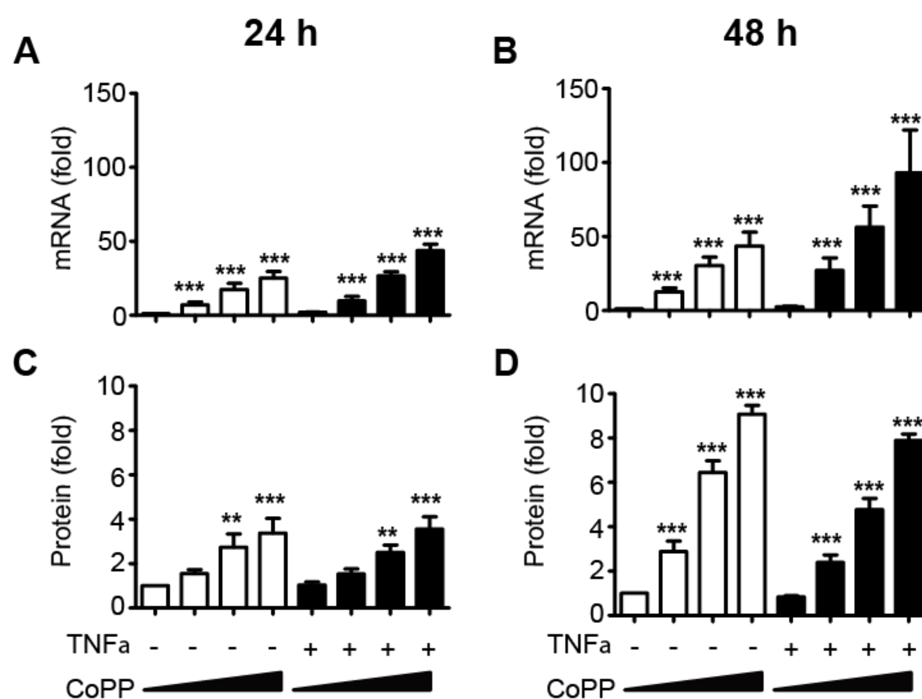

Fig 2.1 Acute CoPP treatment (24–48 h) induces HO-1 in a dose-dependent manner in SGBS adipocytes. Fully differentiated SGBS adipocytes were incubated with increasing concentrations of CoPP (50, 100 and 150 nM) in the absence or presence of TNFα (50 ng/mL) for either 24 or 48 h. *HO-1* mRNA after treatment for (**A**) 24 or (**B**) 48 h. HO-1 protein after treatment for (**C**) 24 or (**D**) 48 h. Values are presented as mean ± SEM of 4 independent experiments and expressed as fold-increase over untreated control. ** = $p < 0.01$, *** = $p < 0.001$ significant difference compared to control.

2.5.2 Acute CoPP treatment has no effect on adiponectin production in SGBS adipocytes

Having established that treatment with CoPP for 24-48 h was sufficient to induce HO-1, in the absence or presence of TNFα, we then examined the effects of CoPP treatment on adiponectin expression and secretion. There was no significant effect of CoPP treatment on adiponectin mRNA levels after 24 or 48 h treatment in either the control or the TNFα treated cells (Fig 2.2A & B). TNFα treatment significantly reduced adiponectin mRNA levels to around 50% of those in control cells. Measurement of secreted total and HMW adiponectin into the conditioned media was performed by ELISA. There was no significant effect of CoPP treatment on total or HMW adiponectin secretion after 24 or 48 h treatment in either the control or TNFα treated cells (Fig 2.2C-F). TNFα treatment significantly reduced total and HMW adiponectin secretion by around 40% and 80% at 24 and 48 h respectively. These results indicate that CoPP has no effect on adiponectin mRNA expression or protein secretion in healthy SGBS adipocytes or in TNFα-treated adipocytes where adiponectin expression and secretion is significantly compromised.

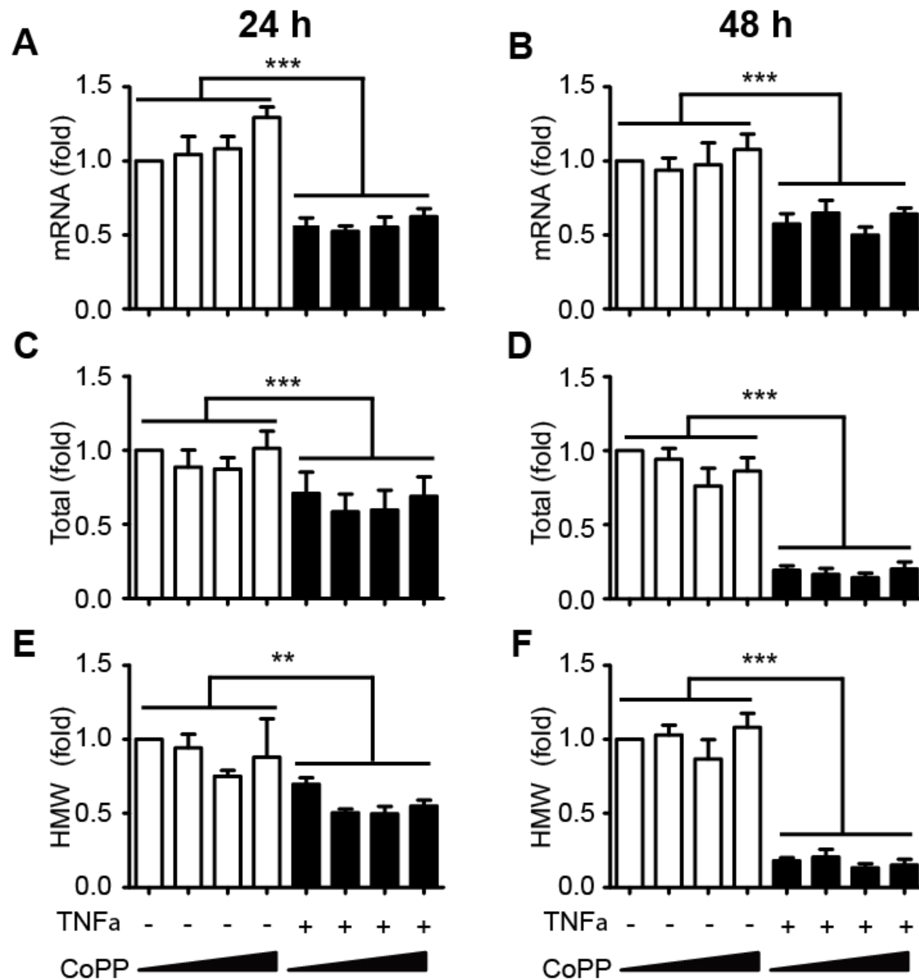

Fig 2.2 CoPP has no effect on adiponectin production in SGBS adipocytes. Fully differentiated SGBS adipocytes were incubated with increasing concentrations of CoPP (50, 100 and 150 nM) in the absence or the presence of TNFα (50 ng/mL) for either 24 h or 48 h. *Adiponectin* mRNA after treatment for 24 h (**A**) and 48 h (**B**). Total adiponectin secretion after treatment for 24 h (**C**) and 48 h (**D**). HMW adiponectin secretion after treatment for 24 h (**E**) and 48 h (**F**). Values are presented as mean ± SEM of 4 independent experiments and expressed as fold-increase over untreated control. ** = $p < 0.01$, *** = $p < 0.001$ significant difference compared to non-TNFα treated cells. Absolute secreted total/HMW adiponectin concentrations for control cells were: 24 h – total 173.5 ± 37.6 (99.1–263.5)/HMW 69.4 ± 23.7 (27.5–137.2): 48 h – total 122.3 ± 43.8 (33.5–218.0)/HMW 55.9 ± 20.8 (12.3–106.5) ng/ml.

2.5.3 Acute CoPP treatment has no effect on pro-inflammatory cytokine production in SGBS adipocytes

Induction of HO-1 has been shown to reduce the circulating levels of pro-inflammatory cytokines [238, 272]. To investigate whether HO-1 induction mediated such beneficial effects in SGBS adipocytes we examined the effects of CoPP treatment on TNFα-induced *IL-6*, *TNF*α and *MCP-1* expression and IL-6 secretion. TNFα treatment for 24-48 h resulted in a significant increase in *IL-6* mRNA and secretion (Fig 2.3A-D) as well as TNFα and *MCP-1* expression (Fig 2.3E-H). However, treatment with CoPP failed to ameliorate the pro-inflammatory effects of TNFα. These results indicate that CoPP has no effect on pro-inflammatory cytokine production in healthy SGBS adipocytes or in TNFα-treated adipocytes where pro-inflammatory cytokine production is significantly increased.

2.5.4 Acute CoPP treatment has no effect on ER stress

We next went on to investigate the effects of HO-1 induction and TNFα-treatment on markers of ER stress. TNFα treatment resulted in a significant, two-fold increase in *sXBP-1* expression and this was unaffected by HO-1 induction (Fig 2.4A & B). Treatment with CoPP or TNFα had no significant effect on *GRP78* (*BiP*) or *CHOP* expression (Fig 2.4C-F).

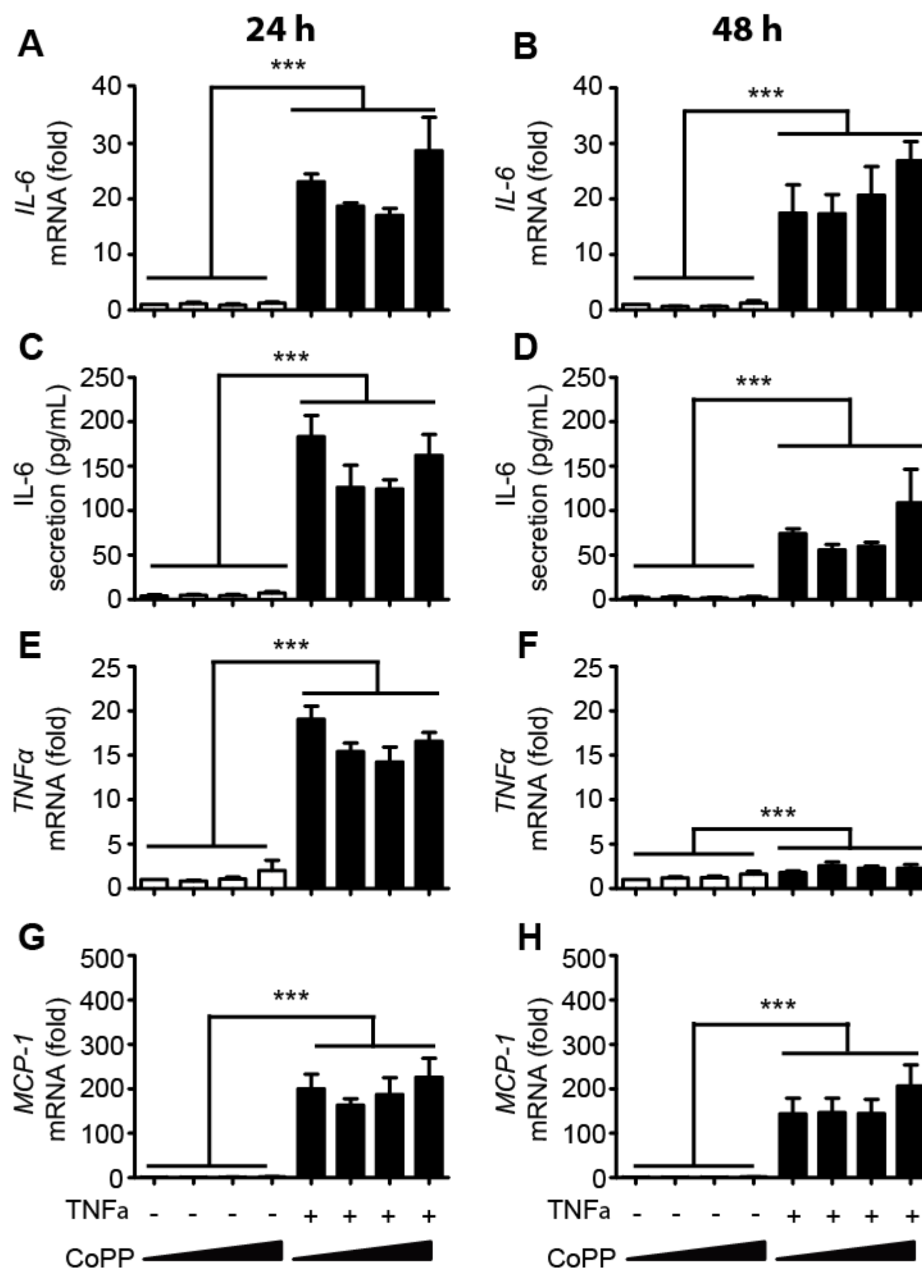

Fig 2.3 CoPP does not ameliorate TNFα-stimulated pro-inflammatory cytokine production in SGBS adipocytes. Fully differentiated SGBS adipocytes were incubated with increasing concentrations of CoPP (50, 100 and 150 nM) in the absence or presence of TNFα (50 ng/mL) for either 24 or 48 h. *IL-6* mRNA after (**A**) 24 h and (**B**) 48 h. IL-6 secretion after (**C**) 24 and (**D**) 48 h. *TNFα* mRNA after (**E**) 24 h and (**F**) 48 h. *MCP-1* mRNA after (**G**) 24 h and (**H**) 48 h. Data are presented as mean ± SEM of 4 independent experiments and expressed as fold-increase over untreated control. *** = $p < 0.001$ significant difference compared to non-TNFα treated cells.

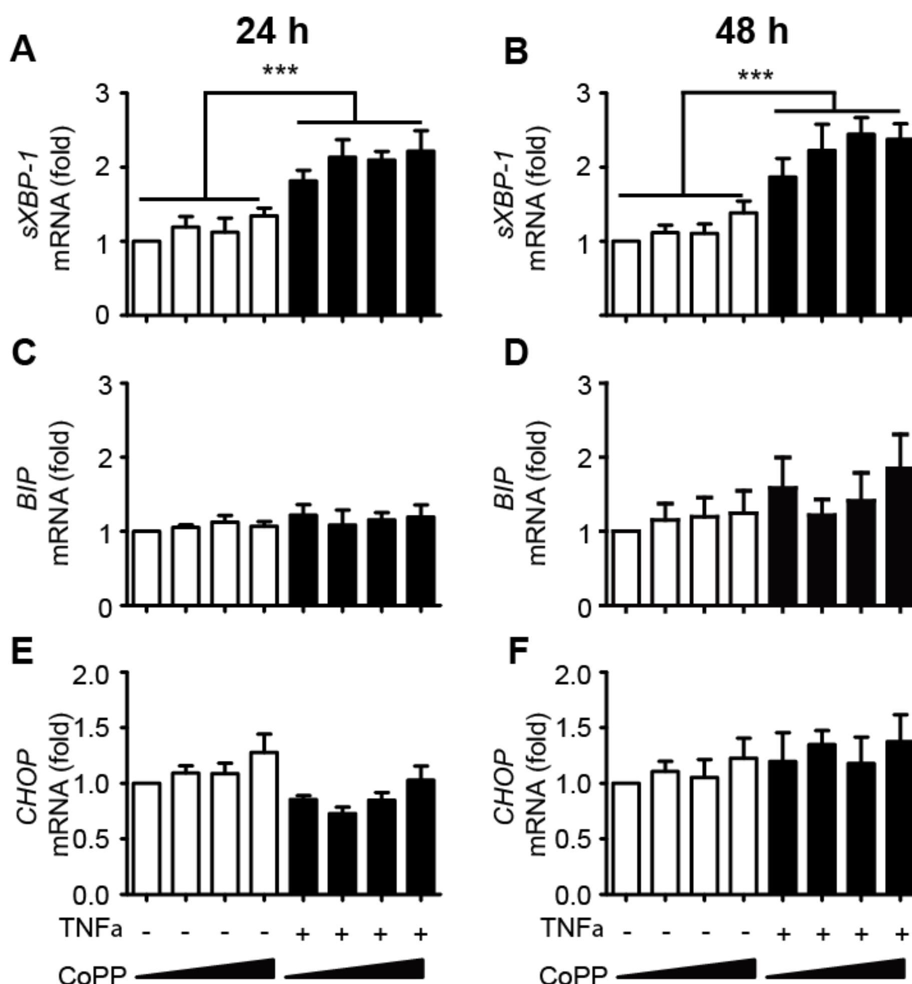

Fig 2.4 CoPP has no effect on markers of ER stress in SGBS adipocytes. Fully differentiated SGBS adipocytes were incubated with increasing concentrations of CoPP (50, 100 and 150 nM) in the absence or presence of TNFα (50 ng/mL) for either 24 or 48 h. *sXBP-1* mRNA after 24 h (**A**) and 48 h (**B**). *BIP* mRNA after 24 h (**C**) and 48 h (**D**). *CHOP* mRNA after 24 h (**E**) and 48 h (**F**). Values are presented as mean ± SEM of 4 independent experiments and expressed as fold-increase over untreated control. *** = $p < 0.001$ significant difference compared to non-TNFα treated cells.

2.5.5 Addition of exogenous substrate does not promote a response to HO-1 induction in SGBS adipocytes

The above studies were performed in SGBS adipocytes differentiated and maintained in defined medium without serum in accordance with our standard experimental approaches [270, 273, 274]. Thus, it remained plausible that the lack of any discernible effect of HO-1 induction on adiponectin or pro-inflammatory cytokine production may reflect limited substrate availability [213]. To

investigate whether this was the case we employed complementary approaches. First, we performed similar experiments in cells treated with hemin, which serves as both inducer and substrate of HO-1 [213, 275]. Treatment with hemin at concentrations from 1-5 μM for 48 h was necessary and sufficient to promote a 5-10 fold induction in cellular HO-1 protein (Fig 2.5A). However, such induction of HO-1 had no effect on adiponectin or IL-6 secretion in control or TNFα treated cells (Fig 2.5B & C). Second, we determined the effects of HO-1 induction with hemin or CoPP on ferritin protein levels, which serves as an indirect marker of HO-1 activity [213]. As expected, induction of HO-1 with either hemin or CoPP markedly induced the levels of ferritin protein (Fig 2.5D & E). Third, we performed experiments in cells that were differentiated and subsequently treated with CoPP in the presence of 10% fetal bovine serum. Treatment with CoPP induced HO-1 (Fig 2.6A), although higher concentrations (5-10 μM) were required to induce HO-1 to the levels observed following treatment in the absence of serum, probably reflecting sequestration of CoPP via binding to serum factors. TNFα treatment significantly reduced adiponectin production and increased IL-6 production, albeit to a lesser extent than was observed in the absence of serum. There was no significant effect of CoPP on adiponectin or IL-6 production in either the control or TNFα-treated cells (Fig 2.6B-E). Collectively, these data indicate that induction of HO-1, even in the presence of exogenous substrates, does not affect adiponectin production nor reduce the pro-inflammatory effects of TNFα in mature SGBS adipocytes.

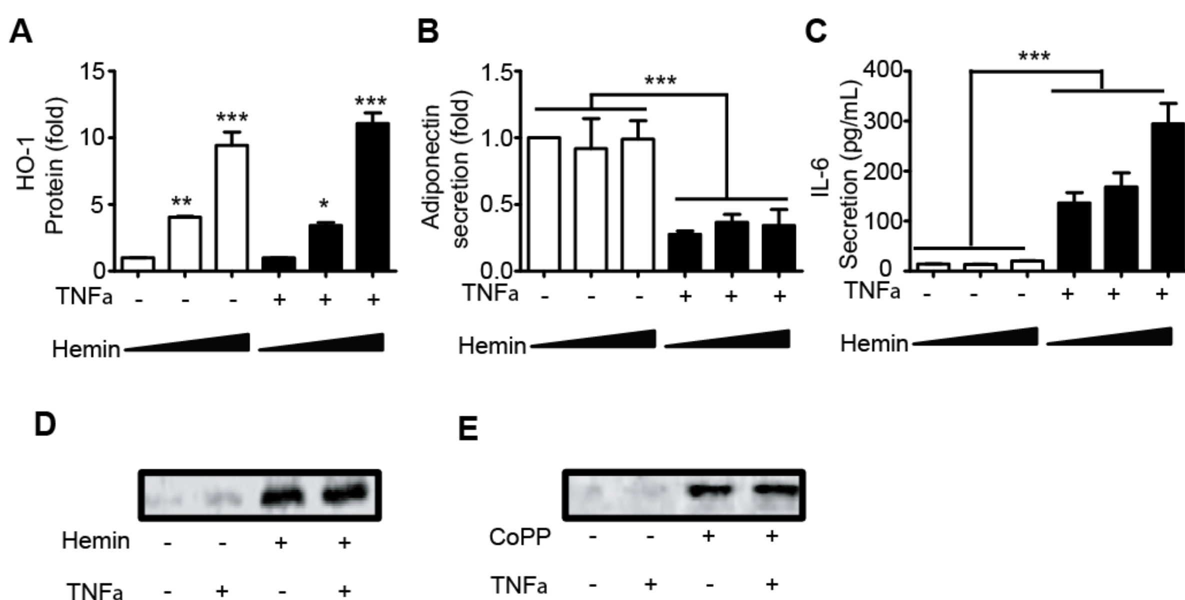

Fig 2.5 Induction of HO-1 with hemin has no effect on adiponectin or pro-inflammatory cytokine production in SGBS adipocytes. Fully differentiated SGBS adipocytes were incubated

with increasing concentrations of hemin (1 and 5 μM) in the absence or presence of TNFα (50 ng/mL) for 48 h. (**A**) Cellular HO-1 protein. (**B**) Secreted total adiponectin. (**C**) Secreted IL-6. Fully differentiated SGBS adipocytes were incubated with hemin (5 μM) or CoPP (150 nM) in the absence or presence of TNFα (50 ng/mL) for 48 h. (**D**) and (**E**) Cellular ferritin levels. Data are presented as mean ± SEM of 4 independent experiments and expressed as fold-increase over untreated control. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ significant difference compared to control or non-TNFα treated cells. Absolute secreted total adiponectin for control cells was: 577.3 ± 56 (417.9–663.6) ng/ml.

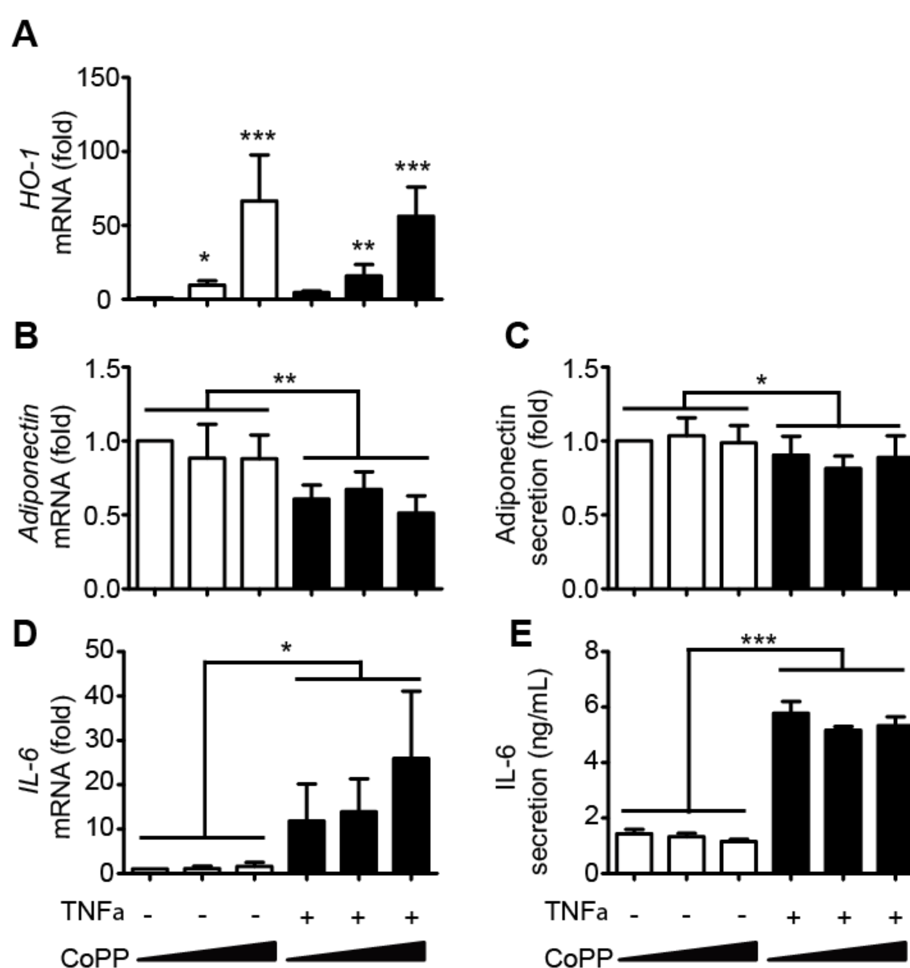

Fig 2.6 Induction of HO-1 with CoPP has no effect on adiponectin or pro-inflammatory cytokine production in SGBS adipocytes in serum. Mature SGBS adipocytes were differentiated and maintained in the presence of serum and then incubated with increasing concentrations of CoPP (5 and 10 μM) in the absence or the presence of TNFα (50 ng/mL) for 24 h (**A**) *HO-1* mRNA. (**B**) *Adiponectin* mRNA and (**C**) Adiponectin (total) secretion. (**D**) *IL-6* mRNA and (**E**) secretion. Data are presented as mean ± SEM of 4 independent experiments and expressed as fold-increase over untreated control. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ significant difference compared to

control or non-TNFα treated cells. Absolute secreted total adiponectin for control cells was: 340.8 ± 42.6 (280.6–401.1) ng/ml.

2.5.6 Acute CoPP treatment induces HO-1 in primary human adipocytes but does not affect adiponectin and IL-6 production

Next, to extend our observations from the SGBS adipocytes we performed similar experiments in primary human adipocytes treated in the absence and presence of serum. Treatment with CoPP for 24 h promoted a dose-dependent increase in *HO-1* mRNA and this was comparable in the absence or presence of TNFα (Fig 2.7A & B). Treatment with TNFα significantly reduced adiponectin production and increased IL-6 production (Fig 2.7C-J). Induction of HO-1 with CoPP had no impact on adiponectin or IL-6 production in control or TNFα treated cells in either the absence or presence of serum (Fig 2.7C-J). These results are consistent with those from the SGBS adipocytes and argue against the existence of a direct HO-1 – adiponectin axis and any major anti-inflammatory role for HO-1 in the context of mature human adipocytes.

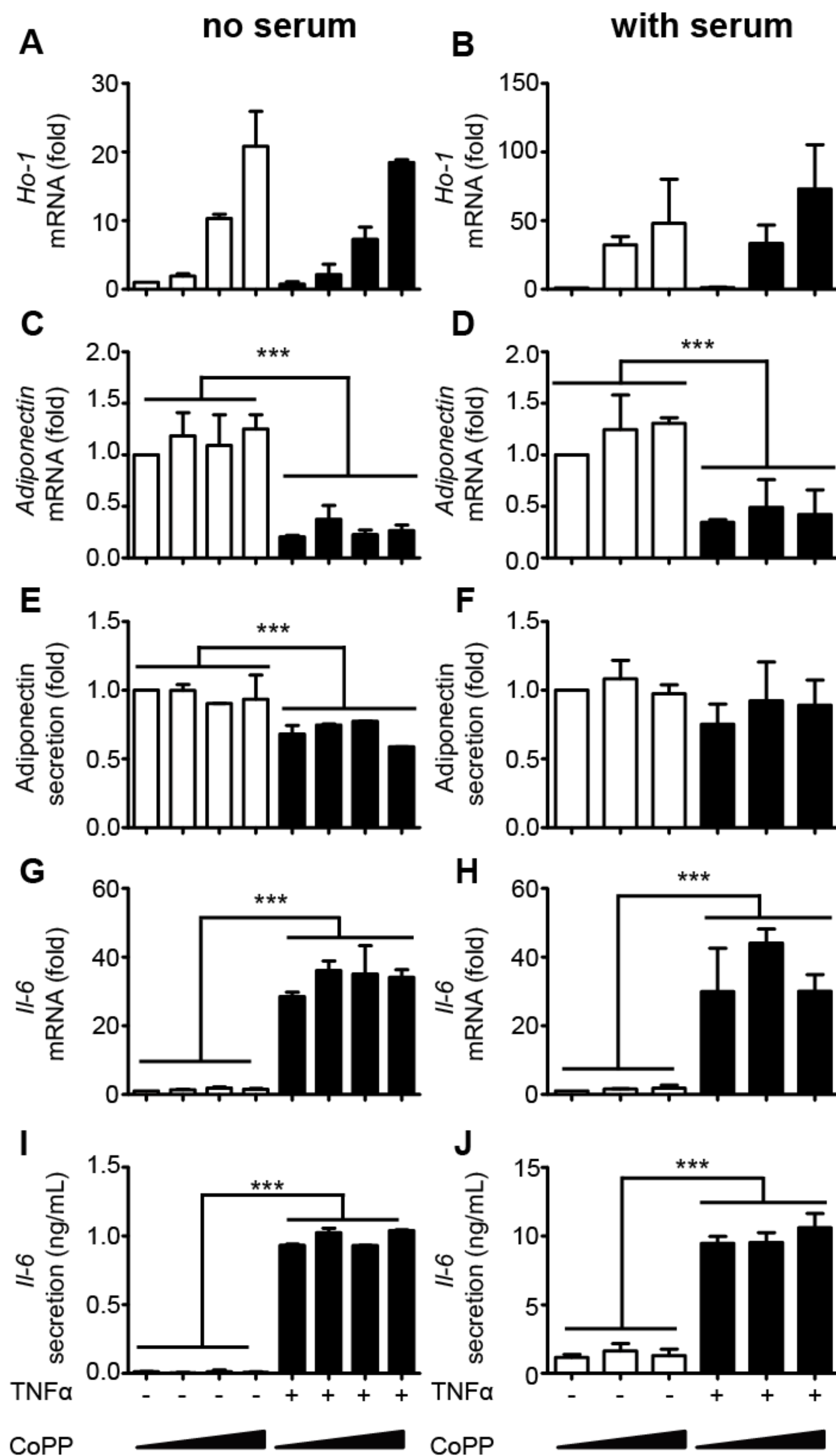

Fig 2.7 Induction of HO-1 with CoPP has no effect on adiponectin or pro-inflammatory cytokine production in primary human adipocytes. Primary human adipocytes were differentiated in the absence or presence of serum and then incubated in the same with increasing concentrations of CoPP (50, 100 and 150 nM in the absence of serum; 5 and 10 μM in the presence

of serum) in the absence or the presence of TNFα (50 ng/mL) for 24 h. *HO-1* mRNA in the (**A**) absence (**B**) and presence of serum. *Adiponectin* mRNA in the (**C**) absence and (**D**) presence of serum. Secreted total adiponectin in the (**E**) absence and (**F**) presence of serum. *IL-6* mRNA in the (**G**) absence and (**H**) presence of serum. Secreted IL-6 in the (**I**) absence and (**J**) presence of serum. Data are presented as mean ± SEM of 2 independent experiments and expressed as fold-increase over untreated control. *** = $p < 0.001$ significant difference compared to control or non-TNFα treated cells. Absolute secreted total adiponectin for control cells was: minus serum – 1143.4 ± 16.7/plus serum – 514.4 ± 14.5 ng/ml.

2.6 Discussion

In the current investigation we aimed to determine whether induction of HO-1 has a direct effect on adiponectin production from mature adipocytes, and thereby contribute to the purported HO-1 – adiponectin axis. We could find no evidence to support a direct beneficial effect of HO-1 induction on adiponectin expression or secretion in healthy or TNFα-treated SGBS or primary human adipocytes. Indeed, despite robust induction of HO-1 mRNA, protein and activity, we observed no beneficial effects of HO-1 induction on these parameters or on TNFα-induced ER stress or pro-inflammatory adipocytokine production.

Numerous reports have described an association between pharmacological induction of HO-1 and increased circulating levels of the beneficial adipokine adiponectin, in *in vivo* studies in rodents [217, 225, 227, 236-242], and *in vitro* studies, showing increased adiponectin secretion from adipocytes [238, 242]. These observations stimulated the proposal of a HO-1 – adiponectin axis [217, 225, 227, 230, 239, 241, 264-268] which could underpin, at least in part, the favourable effects of HO-1 induction reported in most pre-clinical models of obesity and related cardiometabolic disorders. Thus, we reasoned information affording a greater understanding of the cellular and molecular framework of the HO-1 – adiponectin axis would help to identify, validate and progress development of efficacious therapeutic approaches.

To this end, we performed a series of experiments on mature SGBS and primary human adipocytes differentiated *in vitro*. Treatments were limited to mature adipocytes in order to reduce the potential for confounding effects on the differentiation process, as has been reported by others [238, 242, 276, 277], and incubation periods were limited to 24-48 h to reduce the likelihood of secondary effects. As expected, treatment with two widely used inducers of HO-1, CoPP and

Hemin, resulted in increased HO-1 expression at both the mRNA and protein level. A concomitant increase in HO-1 activity was demonstrated by increased ferritin protein, the levels of which are elevated in response to the increase in cellular iron levels [213]. Perhaps surprisingly then, we found no evidence of any effect on adiponectin expression, multimerisation or secretion. This was the case in healthy control adipocytes as well as adipocytes co-treated with TNFα, a pro-inflammatory cytokine implicated in the aetiology of obesity-related cardiometabolic dysfunction, known to promote insulin resistance and compromise adiponectin production from adipocytes [151]. Furthermore, there was no indication of any impact of HO-1 induction on other markers of cellular stress stimulated by TNFα, most notably the induction of other pro-inflammatory cytokines including IL-6 and MCP-1 as well as induction of TNFα itself.

The above findings indicate that acute induction of HO-1 has no direct effect on adiponectin, or adipocytokine, production at the level of the mature adipocyte and instead support a model where the association between HO-1 induction and increased circulating adiponectin levels most likely represents a chronic or indirect effect. One possibility is that chronic induction of HO-1 may increase adiponectin via altering differentiation of preadipocytes. Consistent with this, *in vitro* studies showed that chronic induction of HO-1, via CoPP administration throughout differentiation, promoted increased adiponectin secretion, albeit in the context of reduced adipogenesis [238, 242]. Whilst it seems somewhat paradoxical that inhibition of adipogenesis would result in increased adiponectin secretion, given the dose-dependent effects of chronic CoPP treatment [238] and the finding that adiponectin secretion is highest in 'immature' adipocytes [278] it remains possible that there is an 'optimal window' for adiponectin secretion that was somewhat serendipitously established in these investigations [238, 242]. *In vivo* findings showing altered adipose tissue architecture consistent with reduced adipocyte hypertrophy and increased adipocyte number appear to support such a model, however increased adipogenesis is intrinsically required in such a situation [278]. Further investigations are required to establish whether this is the case or whether the decrease in adipocyte hypertrophy simply reflects a decrease in body weight (see below).

Another possibility is that induction of HO-1 may mediate adiponectin production from adipocytes indirectly via its effects on other cell types. For example, a recent report demonstrates that acute (24 h) induction of HO-1 via hemin increases adiponectin expression from 3T3-L1 adipocytes co-cultured with Raw264.7 macrophages [211]. The inflammatory tone of the co-cultured 3T3-L1 adipocytes and Raw264.7 macrophages was reduced upon hemin treatment with cellular markers suggesting increased levels of M2 macrophage polarisation. Complementary *in vivo* investigations showed hemin administration reduced adipose tissue inflammation in mice fed a HFD for 2 weeks concomitant with reduced markers of M1 macrophage polarisation [211]. A caveat to this and

most other *in vivo* studies is that induction of HO-1 is typically associated with a reduction in body weight and/or body weight gain [209, 211, 217, 237, 241, 265, 279, 280] which would, in-itself, be predicted to decrease adipose tissue as well as systemic inflammation and to increase adiponectin.

Genetic attempts to increase the activity of HO-1 specifically in adipocytes have also been performed. Two independent studies used the aP2 promoter to drive expression of HO-1 in adipocytes via lentiviral or transgenic approaches. In the first, intracardial injection of a lentiviral (aP2-HO-1) construct resulted in increased expression of HO-1 in adipose tissue and this was sufficient to attenuate high fat diet (HFD) induced changes in body weight, associated metabolic sequelae and improved adiponectin [209]. In the second, a classic transgenic approach was used to increase HO-1 in adipose tissue but, in contrast to the above report, this failed to ameliorate HFD-induced obesity, insulin resistance or the decrease in adiponectin [281]. The explanation for such contrasting findings remains obscure but may, at least in part, be explained by the different methodologies employed to increase HO-1. It is noteworthy that in both instances expression of HO-1 in cells other than mature adipocytes is to be expected as the aP2 promoter is switched on early in the differentiation process, meaning immature adipocytes will also express increased HO-1, and it also drives gene expression in a range of non-adipocyte cells including cardiomyocytes and macrophages [245]. Consistent with the latter, transgenic overexpression of HO-1 by aP2 resulted in increased HO-1 in peritoneal macrophages, and, although this was sufficient to affect the expression of some M2 markers in the adipose tissue of transgenic mice it was not sufficient to protect against obesity, decreased adiponectin and metabolic dysfunction [281]. Taken together these studies further highlight the association between reduced body weight and improved adiponectin levels.

Whilst the overwhelming weight of evidence argues in favour of a beneficial effect of HO-1 induction in the context of cardiometabolic disease there remain a number of elements that demand further exploration. For example, it is unclear what mechanism(s) underpin any observed changes in body weight. Reduced food intake [218, 220] and elevated metabolism, heat production and activity [279] have been proposed although, at least in some instances, these changes appear to occur in the presence of HO-1 inhibition [280, 282], arguing against a central role for HO-1 activity in this context. However, perhaps the most thought-provoking and challenging work in this area comes from the recent, elegant and comprehensive report from Pospisilik, Esterbauer and colleagues [204] which continues to put HO-1 centre-stage of obesity and insulin resistance but as a driver rather than a brake of obesity-associated inflammation. In keeping with findings from the current study, they found no evidence of a major role for HO-1 in the adipocyte or muscle or pancreatic β-cells [204]. However, they presented compelling data to indicate a predominant role

for HO-1 in both myeloid and hepatic cells that lead them to propose inhibitors, rather than inducers, of HO-1 may represent effective therapeutic agents [204].

In summary, in the current report we demonstrate that induction of HO-1 in human adipocytes has no direct effect on adiponectin production. In addition, induction of HO-1 did not ameliorate the effects of TNFα on adiponectin or pro-inflammatory adipocytokine production. These findings argue against a direct HO-1 – adiponectin axis and also suggest mature adipocytes are unlikely to be acutely involved in mediating the systemic effects of HO-1 induction.

2.7 Acknowledgements

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Chapter 3:

Chronic treatment of human preadipocytes with CoPP or hemin compromises adipogenesis and adiponectin production in a heme-oxygenase-1 (HO-1) independent manner

3.1 Introduction to this publication

This Chapter was submitted to *Scientific report* journal. In this study, we demonstrate that in human preadipocytes:

- Induction of HO-1 using two commonly-used agents, CoPP or hemin, results in:
  - Dose-dependent inhibition of adipogenesis and adiponectin production as determined
    - Morphologically
    - Genetically
    - Functionally
  - Dose-dependent induction of Nrf2 target genes, *NQO1* and *GCLM*
- Co-treatment with an inhibitor of HO-1 activity, SnMP, or siRNA-mediated knockdown of HO-1 fails to ameliorate these effects.

Collectively, these results demonstrate that chronic treatment of human preadipocytes during differentiation impairs adipogenesis and adiponectin production by a mechanism that is independent of HO-1. These results do not support the existence of a direct HO-1 – adiponectin axis.

<u>**Yang, M.,**</u> Kimura, M., Ng, C., He, J., Barclay, J. L., Whitehead, J. P., Chronic treatment of human preadipocytes with CoPP or hemin compromises adipogenesis and adiponectin production in a heme-oxygenase-1 (HO-1) independent manner. *Scientific report,* submitted

Chronic treatment of human preadipocytes with CoPP or hemin compromises adipogenesis and adiponectin production in a heme-oxygenase-1 (HO-1) independent manner

Mengliu Yang[1,2], Masaki Kimura[1,3], Choaping Ng[1], Jingjing He[1], Johanna L. Barclay[1], Jonathan P. Whitehead[1*]

[1]Mater Research Institute-UQ, Translational Research Institute, Brisbane, QLD, Australia
[2]Current address, Department of Endocrinology, the Second Affiliated Hospital, Chongqing Medical University, 400010 Chongqing, China
[3]Current address, Department of Pharmacotherapeutics, Faculty of Pharmacy, Keio University, Tokyo, Japan

[*]Correspondence to: Jon Whitehead, Mater Research Institute-UQ, Translational Research Institute, 37 Kent Street, Brisbane, QLD 4101, Australia
Tel 61 07 3343 7633
Email jon.whitehead@mater.uq.edu.au

3.2 Abstract

Adiponectin is an adipocyte derived hormone (adipokine) with a range of beneficial effects. Reduced adiponectin is implicated in the aetiology of obesity-related diseases making strategies to increase adiponectin production attractive. Induction of the cellular stress-protein heme-oxygenase-1 (HO-1), using agents such as cobalt protoporphyrin (CoPP) or hemin, has been reported to increase circulating adiponectin levels and has prompted the proposal of a 'HO-1 – adiponectin axis'. We previously reported that acute (24-48 h) induction of HO-1 did not enhance adiponectin production from mature human adipocytes. Here, we have extended these studies by characterising the effects of chronic HO-1 induction throughout differentiation of human preadipocytes. Induction of HO-1 with CoPP or hemin resulted in dose-dependent inhibition of differentiation (adipogenesis) and adiponectin production as well as induction of additional target genes of the Nuclear factor erythroid-derived 2-like 2 (NRF2) transcription factor (*NQO1* and *GCLM*). Co-treatment with a HO-1 inhibitor (SnMP) or HO-1 siRNA did not prevent these effects. Collectively, these results suggest chronic treatment with CoPP or hemin inhibits adipogenesis and adiponectin production by a HO-1-independent mechanism that may be downstream of NRF2. Furthermore, these results do not support the existence of a direct HO-1 – adiponectin axis.

Keywords: Adipocyte; Differentiation; Obesity; Therapeutic

3.3 Introduction

Adiponectin is an important adipocyte derived hormone or 'adipokine' that exhibits a range of beneficial properties that include anti-inflammatory, cardioprotective and insulin-sensitizing effects to reduce cardiometabolic disease [252, 283]. In contrast to most other adipokines circulating levels of adiponectin are reduced in obesity, type 2 diabetes and associated conditions [252, 283]. In addition, adiponectin knockout mice or humans with polymorphisms that decrease adiponectin production typically develop cardiometabolic dysfunction and or type 2 diabetes [252, 283]. For these reasons, therapeutic strategies to increase the circulating levels of adiponectin are considered attractive.

Heme oxygenase-1 (HO-1) is a stress-inducible protein that catalyses the oxidative degradation of heme to produce carbon monoxide (CO), iron and biliverdin/bilirubin [261, 284]. These catalytic products modulate diverse features of cellular function and homeostasis in response to oxidative, inflammatory and metabolic stress [207, 261, 284]. In accordance with this, induction of HO-1 is typically promoted as an attractive therapeutic approach to reduce the incidence and severity of a variety of human diseases that include obesity and type 2 diabetes [195, 262, 284]. As such, strategies to induce HO-1 in humans are under investigation [206, 207, 263].

Evidence, primarily from preclinical *in vivo* studies, supports the notion that induction of HO-1 may give rise to beneficial effects via an increase in circulating adiponectin levels leading to the proposal of a "HO-1 – adiponectin axis" [217, 225-227, 230, 236, 239, 241, 242, 265, 267, 268, 285-287]. In the absence of any evidence to support a direct effect of HO-1 on adiponectin production from adipocytes we recently performed a study to determine whether acute (24-48 h) induction of HO-1, using two commonly used agents - cobalt protoporphyrin (CoPP) or hemin, increased adiponectin production from mature human adipocytes. Surprisingly, despite robust induction of HO-1 we saw no evidence of enhanced adiponectin production (expression or secretion) from healthy adipocytes or adipocytes where adiponectin production was reduced by co-treatment with TNFα [288]. While these findings argued against the existence of a direct HO-1 – adiponectin axis they did not address the potential effects of chronic induction of HO-1 on adiponectin production. Indeed, reports suggested that chronic induction of HO-1 throughout the process of differentiation promoted increased adiponectin secretion, albeit in the face of reduced differentiation [238, 242]. Although this seemed paradoxical we previously reported that adiponectin secretion was highest in immature adipocytes differentiated *in vitro* [278], hence we reasoned that chronic HO-1 induction may arrest differentiation at a stage where adiponectin production was higher than in the mature adipocyte. Thus, in the current study we performed a

comprehensive series of investigations to determine whether chronic treatment with CoPP or hemin, across appropriate dose-response curves, could increase adiponectin production from human preadipocytes differentiated *in vitro*. We found that chronic treatment with both agents promoted dose-dependent inhibition of differentiation and also reduced the expression and secretion of adiponectin.

In an attempt to define the underlying processes we explored potential mechanisms. CoPP and hemin induce HO-1 expression, at least in part, by increasing the stability and activity of the transcription factor Nuclear factor erythroid-derived 2-like 2 (NRF2) [275]. NRF2 is a critical regulator of the antioxidant defence pathway that appears to play a complex role in the regulation of adipogenesis and adipose tissue biology [289]. The expression of two NRF2 target genes, *NQO1* and *GCLM*, confirmed dose-dependent activation of NRF2 upon CoPP or hemin treatment. Combined with investigations using a HO-1 inhibitor or HO-1 siRNA we provide evidence which demonstrates that the inhibitory effects of CoPP or hemin are independent of HO-1 but appear to be linked to some other downstream effector(s), possibly one or more NRF2 target genes.

3.4 Methods

3.4.1 Reagents and antibodies

General reagents were obtained from Sigma-Aldrich (Victoria, Australia), unless otherwise stated. Cell culture reagents were obtained from Invitrogen (Victoria, Australia).

3.4.2 Isolation, culture, differentiation and treatment of primary human preadipocytes

Primary human preadipocytes (phPAs) were isolated from subcutaneous adipose tissue from 4 subjects (all female, age 30.5 ± 6.2 years, BMI 23.45 ± 0.46, metabolically healthy - no insulin resistance, diabetes or cardiovascular disease) and cultured independently as described previously [270]. The procedure was approved by the Research Ethics Committees of the University of Queensland, the Princess Alexandra Hospital, and Mater Adults Hospital. All patients had given their written informed consent. Cells were differentiated in the continued presence of vehicle

(DMSO) or CoPP (0.1 - 0.25 μM) for 21 days. Cells and conditioned media were harvested on day 21.

3.4.3 Cell culture, differentiation, and treatment of SGBS PAs

SGBS PAs [269] were routinely maintained and differentiated as described [270]. In brief, cells were cultured in DMEM/F12 containing 10%FCS, penicillin, 33 μM biotin, and 17 μM pantothenic acid until confluent. To differentiate, the cells were then cultured in serum-free DMEM/F12 supplemented with penicillin, 33 μM biotin, 17 μM pantothenic acid, 0.01 mg/mL transferring, 0.1 μM corisol, 200 pM triiodotyronine, 20 nM human insulin, 0.25 μM dexamethasone (DEX), 500 μM methylisobutylxanthine (MIX), 2 μM rosiglitazone, 90 g/mL heparin and 1 ng/mL FGF-1 for 3 days. On day 3, medium was changed to differentiation medium without rosiglitazone and from day 7 to the completion of the differentiation, cells were cultured in the differentiation medium without rosiglitazone, MIX and DEX. SGBS PAs were differentiated in the presence of vehicle (DMSO) or CoPP (0.1 - 0.25 μM) or Hemin (0.5 - 5 μM) for 14 days. For experiments performed in the presence of serum, cells were differentiated and maintained in media containing 10% foetal bovine serum (FBS) in the continued presence of vehicle (DMSO) or CoPP (1 - 5 μM) for 14 days [288]. For experiments involving co-treatment with the HO-1 inhibitor SnMP (Frontier scientific services, New Jersey, USA), cells were differentiated in the continued presence of vehicle (DMSO), CoPP (0.25 μM) or Hemin (3 μM), with or without SnMP (5 μM) for 14 days.

For experiments involving siRNA mediated HO-1 knockdown SGBS-PAs were transfected at 80% confluence with scrambled (Scr) control siRNA (AATTCTCCGAACGTGTCACGT) or HO-1 siRNA (CAGGCAGAGGGTGATAGAAGA) at 50 nM (Qiagen, Victoria, Australia) using NanoJuice (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. 72 h post-transfection cells were induced to differentiate in the continued presence of vehicle (DMSO), CoPP (0.25 μM) or Hemin (3 μM) for 14 days. For all experiments cells and conditioned media were harvested on day 14.

3.4.4 Measurement of gene expression by qRT-PCR

Gene expression was measured by qRT-PCR and standardized against the expression of cyclophilin as previously described [270]. Briefly, total RNA extraction was performed using Trizol or RNA Mini Kit (Ambion Life Technologies, Victoria, Australia) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg total RNA using a cDNA

synthesis kit (Bioline, NSW, Australia) and RT-PCR was performed using the SYBR Hi-ROX kit (Bioline) on a 7900HT Fast Real-time PCR system (Ambion Life Technologies). Primer sequences were as follows (all human, listed as Forward / Reverse): *Cyclophilin* - CGCGTCTCCTTTGAGCTGTT / TCTCCAGTGCTCAGAGCACG; *HO-1* - CCAGGCAGAGAATGCTGAGTTC / AAGACTGGGCTCTCCTTGTTGC; *ADIPOQ* - AGAAAGGAGATCCAGGTCTTATTGGT / AACGTAAGTCTCCAATCCCACACT; *NQO1* - CCTGCCATTCTGAAAGGCTGGT / GTGGTGATGGAAAGCACTGCCT; *GCLM* - TCTTGCCTCCTGCTGTGTGATG / (R) TTGGAAACTTGCTTCAGAAAGCAG; *PPARγ* - GAAACTTCAAGAGTACCAAAGTGCAA / AGGCTTATTGTAGAGCTGAGTCTTCTC; *ADIPOR1* - CATCGTCTGTGTCCTGGGCATT / CTTGACAAAGCCCTCAGCGATAG; *ADIPOR2* - GCCTCTACATCACAGGAGCTGC / CCTGGAGGTTTGAGACACCATG; *GLUT4* - CTCTCTGGCATCAATGCTGT / ACCGAGACCAAGGTGAAGAC; *GLUT1* - TTGCAGGCTTCTCCAACTGGAC / CAGAACCAGGAGCACAGTGAAG; *IL-6* - AGACAGCCACTCACCTCTTCAG / TTCTGCCAGTGCCTCTTTGCTG

3.4.5 Determination of HO-1 and adiponectin protein

Cellular HO-1 was quantitated in whole cell lysates (in 0.05-0.25 μg protein) using an HO-1 ELISA (Life Sciences, Florida, USA). Secreted total adiponectin present in conditioned media was quantitated using a total adiponectin ELISA kit (R&D system, Minnesota, USA).

3.4.6 Measurement of 2-Deoxyglucose uptake

Glucose uptake was measured in SGBS cells on day 14 of differentiation as described [270]. In brief, cells were washed three times and incubated in warm KRH (136 mM NaCl, 4.7 mM KCl,10 mM HEPES, 1.25 mM MgSO4,1.25 mM CaCl2, pH 7.4) buffer at 37°C for 2 h. The cells were then stimulated with 100 nM insulin for 20 min at 37°C. The cells for negative control (background) were treated with cytochalasin B for 1 min prior to [3H]2-deoxyglucose (Amersham, Little Chalfont, UK) administration. After that, a mixture of 50 μM 2-deoxyglucose containing 1 μCi/mL [3H]deoxyglucose was added for 20 min at 37°C. The reaction was stopped by removal of media and addition of ice-cold PBS. The cells were harvested in 1% Triton X-100 (in PBS) and solubilized in Optiphase "supermix" liquid scintillant (Perkin Elmer, Wellesley, MA) and counted in a Microbeta Jet 1450 LSC beta counter (Perkin Elmer).

3.4.7 Statistical analysis

Data are presented as means ± SEM. One-way ANOVA was used to compare between treatment groups with Tukey's *post hoc* test. Two-way ANOVA with Bonferroni's *post hoc* test was used to compare between gene expression levels at the same CoPP concentration. Paired student t-test was used to compare the significant difference between SnMP or siRNA treated with control (Figs 2.5-7). Differences were considered statistically significant at $p<0.05$. Statistical analyses were performed in GraphPad Prism 5.0.

3.5 Results

3.5.1 Chronic treatment of primary phPAs with CoPP inhibits adipogenesis and adiponectin production

We first investigated the effects of chronic HO-1 induction on differentiation and adiponectin production from phPAs using the widely used inducer of HO-1, CoPP. Cells were differentiated for 21 days in the presence of increasing concentrations of CoPP ranging from 0.1 – 0.5 µM (determined in preliminary investigations – data not shown). The effects of CoPP were apparent morphologically, with increasing CoPP concentrations reducing lipid accumulation (Fig 3.1a). Analysis of *HO-1* expression confirmed marked induction in response to CoPP, with *HO-1* levels increased 15-30 fold (Fig 3.1b). Interestingly, the induction of two other NRF2-target genes [289], *NQO1* and *GCLM*, was more modest (Fig 3.1b). We measured mRNA expression of a number of adipogenic/adipocyte markers to further define the effect of CoPP on differentiation. Consistent with the reduction in lipid accumulation, the expression of early (*PPARγ)*, intermediate (*ADIPOR2)*, and late (*GLUT4)* markers of adipogenesis were all decreased in a dose-dependent manner, with the latter showing the greatest sensitivity to CoPP (Fig 3.1c). The expression of *ADIPOR1,* which is unchanged during differentiation (data not shown), was unaffected by CoPP treatment (Fig 3.1c) demonstrating the specificity of the effect on the differentiation process. Consistent with the effects of CoPP on adipogenesis, key indices of adiponectin production, namely expression and secretion, were also decreased by CoPP in a dose-dependent manner (Fig 3.1d). Interestingly, adiponectin secretion exhibited greater sensitivity to CoPP than adiponectin expression (Fig 3.1d). To investigate the effect of CoPP on cellular stress and inflammation we evaluated the expression levels of *GLUT1* and *IL-6* because upregulation of *GLUT1* occurs in response to cellular stress [290] and *IL-6* expression has been reported to be decreased upon chronic treatment with CoPP [291]. At the highest concentration of CoPP, expression of both *GLUT1* and *IL-6* was significantly increased (Fig 3.1e). Collectively, these findings indicate that treatment of phPAs with CoPP results in dose-dependent inhibition of differentiation and adiponectin production and, at high concentrations, induces cellular stress.

Fig 3.1

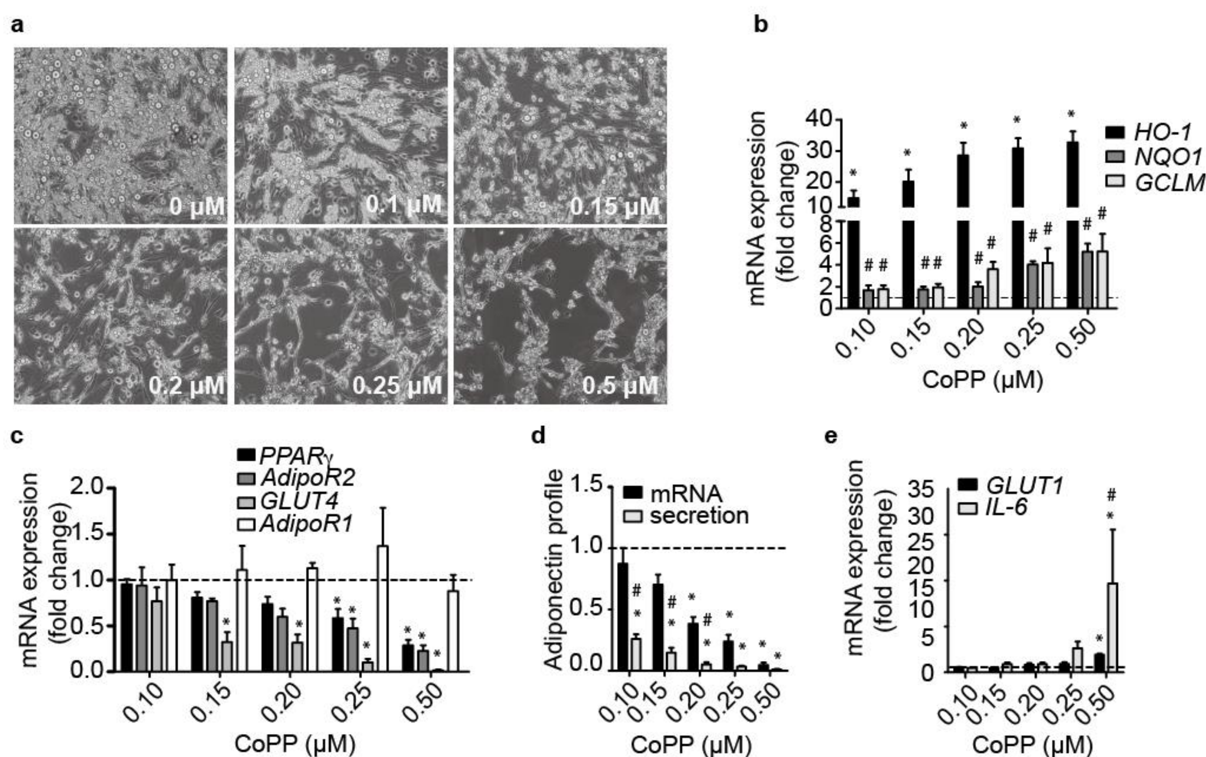

Fig 3.1 - Chronic treatment of phPAs with CoPP decreases adipogenesis and adiponectin production. phPAs were differentiated for 21 days in the presence of increasing concentrations of CoPP (0.1-0.5 μM). **(a)** Images showing cell morphology on day 21. **(b)** *HO-1*, *NQO1* and *GCLM* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test: #p<0.05 vs *HO-1* expression at the same CoPP concentration; two-way ANOVA with Bonferroni's *post hoc* test). **(c)** Adipocyte markers (*PPARγ*, *ADIPOR2*, *GLUT4*) and *ADIPOR1* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test). **(d)** Adiponectin expression and secretion determined by qRT-PCR and ELISA (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test, #p<0.05 vs adiponectin expression levels at the same CoPP concentration; two-way ANOVA with Bonferroni's *post hoc* test). **(e)** *GLUT1* and *IL-6* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one way-ANOVA with Tukey's *post hoc* test; #p<0.05 vs *GLUT*1 expression levels at the same CoPP concentration after two-way ANOVA with Bonferroni's *post hoc* test).

3.5.2 Chronic treatment of Simpson Golabi Behmel Syndrome preadipocytes (SGBS PAs) with CoPP inhibits adipogenesis and adiponectin production

To extend our observations from phPAs, we performed similar studies in the widely used human SGBS-PA cell strain [270, 278, 292, 293]. Chronic CoPP treatment throughout differentiation

reduced lipid accumulation in a dose-dependent manner (Fig 3.2a). As expected, CoPP induced the expression of *HO-1* as well as *NQO1* and *GCLM* (Fig 3.2b). Consistent with our observations in the phPAs induction of *HO-1* was greater than that observed for *NQO1* and *GCLM* and, interestingly, only induction of the latter two appeared dose-dependent under these experimental conditions. In keeping with the dose-dependent effects of CoPP on lipid accumulation and *NQO1* and *GCLM* expression, and in contrast to the effects on *HO-1* induction, we also observed dose-dependent inhibitory effects of CoPP on expression of markers of adipogenesis and adiponectin production (Fig 3.2c & d) and increased expression of markers of cellular stress and inflammation *GLUT1* and *IL-6* (Fig 3.2e). The latter probably contributes to the relatively modest induction of *HO-1* (≈10-fold) in cells treated at the highest concentration of CoPP (Fig 3.2b). Next, we measured basal and insulin-stimulated glucose uptake to explore the functional impact of these effects, particularly the changes in *GLUT1* and *GLUT4* which encode the glucose transporters responsible for basal and insulin-stimulated glucose transport in these cells. Consistent with the changes in gene expression we observed a dose-dependent increase in basal glucose uptake and a concomitant reduction in the ability of insulin to stimulate glucose uptake (Fig 3.2f). These observations confirm and extend our findings from the phPAs.

Fig 3.2

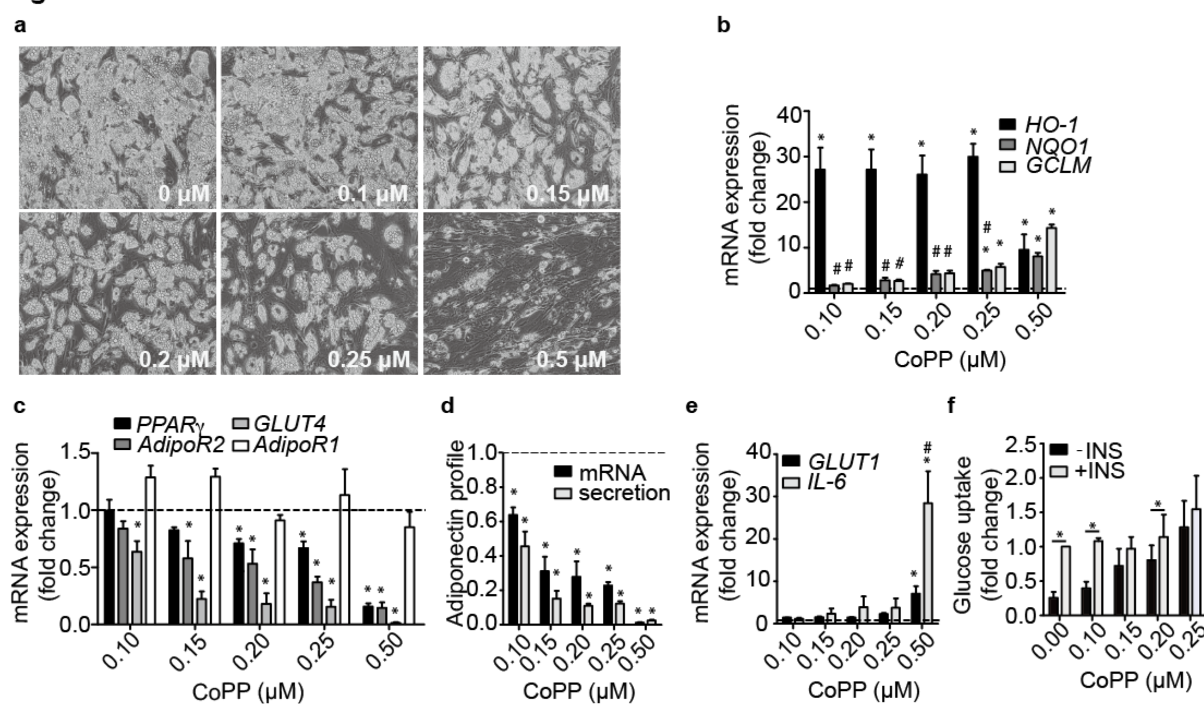

Fig 3.2 - Chronic treatment of SGBS PAs with CoPP decreases adipogenesis and adiponectin production. SGBS PAs were differentiated for 14 days in the presence of increasing concentrations of CoPP (0.1-0.5 μM). (**a**) Images showing cell morphology on day 14. (**b**) *HO-1*, *NQO1* and *GCLM* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one-way ANOVA with

Tukey's *post hoc* test: #p<0.05 vs *HO-1* expression at the same CoPP concentration; two-way ANOVA with Bonferroni's *post hoc* test). **(c)** Adipocyte markers (*PPARγ*, *ADIPOR2*, *GLUT4*) and *ADIPOR1* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test). **(d)** Adiponectin expression and secretion determined by qRT-PCR and ELISA (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test). **(e)** *GLUT1* and *IL-6* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one way-ANOVA with Tukey's *post hoc* test; #p<0.05 vs *GLUT*1 expression levels at the same CoPP concentration after two-way ANOVA with Bonferroni's *post hoc* test). **(f)** basal and insulin-stimulated glucose uptake (n=4; *p<0.05 vs non-insulin stimulated glucose uptake after two-way ANOVA with Bonferroni's *post hoc* test).

3.5.3 The addition of exogenous HO-1 substrate does not modulate the negative effects on adipogenesis or adiponectin production

In accordance with our standard experimental approaches both the phPAs and SGBS PAs were differentiated in the absence of serum [270, 278, 293] raising the possibility that the inhibitory effects of CoPP may reflect, at least in part, a shortage of substrate for HO-1 as it has been suggested that substrate may be limiting under such conditions [213]. To investigate this we employed two complementary approaches. First, we examined the effects of hemin, which serves as both HO-1 inducer and substrate [213, 275], on differentiation of SGBS PAs. Preliminary investigations identified a dose-response regime where chronic hemin treatment reduced lipid accumulation (Fig 3.3a), akin to that observed for CoPP (see Fig 3.2a). Analysis of gene expression profiles revealed that hemin dose-dependently induced *HO-1* and, again, this induction was greater than that observed for *NQO1* and *GCLM* (Fig 3.3b), although the magnitude of these effects was 3-4 fold less than those observed for CoPP. Chronic hemin treatment also dose-dependently reduced expression of adipogenic markers (Fig 3.3c) and adiponectin production (Fig 3.3d), with secretion again showing greater sensitivity than expression, and significantly increased expression of *IL-6* (Fig 3.3e).

Our second approach was to characterize the effects of CoPP on SGBS PAs in the presence of 10% foetal bovine serum, which contains substrate for HO-1 [213]. The inclusion of serum reduced the apparent sensitivity to CoPP, which most likely reflects the binding and sequestering of CoPP to serum factors, such that experiments were performed using CoPP at 1-5 μM. There was a clear

decrease in lipid accumulation in cells treated with 2.5 and 5 μM CoPP but not in cells treated with 1 μM CoPP (Fig 3.4a). Induction of *HO-1* was ≈15-fold in cells treated with 1 μM CoPP peaking at 30-35-fold in cells treated at the higher concentrations (Fig 3.4b). Induction of *NQO1* and *GCLM* was dose-dependent (ranging from 2-8-fold) but, as before, was less than that observed for HO-1 (Fig 3.4b). Interestingly, significant reductions in expression of adipogenic markers were only observed in cells treated with 5 μM CoPP, with no effects observed in cells treated at 1 μM and only non-significant trends (10-20%) in cells treated with 2.5 μM (Fig 3.4c). In contrast, adiponectin expression and secretion were significantly reduced in cells treated with 2.5 μM CoPP, by around 50 and 70% respectively (Fig 3.4d). Interestingly, *GLUT1* and *IL-6* exhibited counter-regulation with *GLUT1* significantly increased and *IL-6* significantly decreased in response to treatment with 5 μM CoPP (Fig 3.4e).

Taken together, the above results demonstrate that chronic treatment of human preadipocytes with either CoPP or hemin interferes with both differentiation and adiponectin production. However, inconsistencies between the dose-response of *HO-1* induction and inhibition of adipogenesis and adiponectin production were apparent and prompted us to investigate the putative role of HO-1 activity and HO-1 protein in these effects in more detail.

Fig 3.3

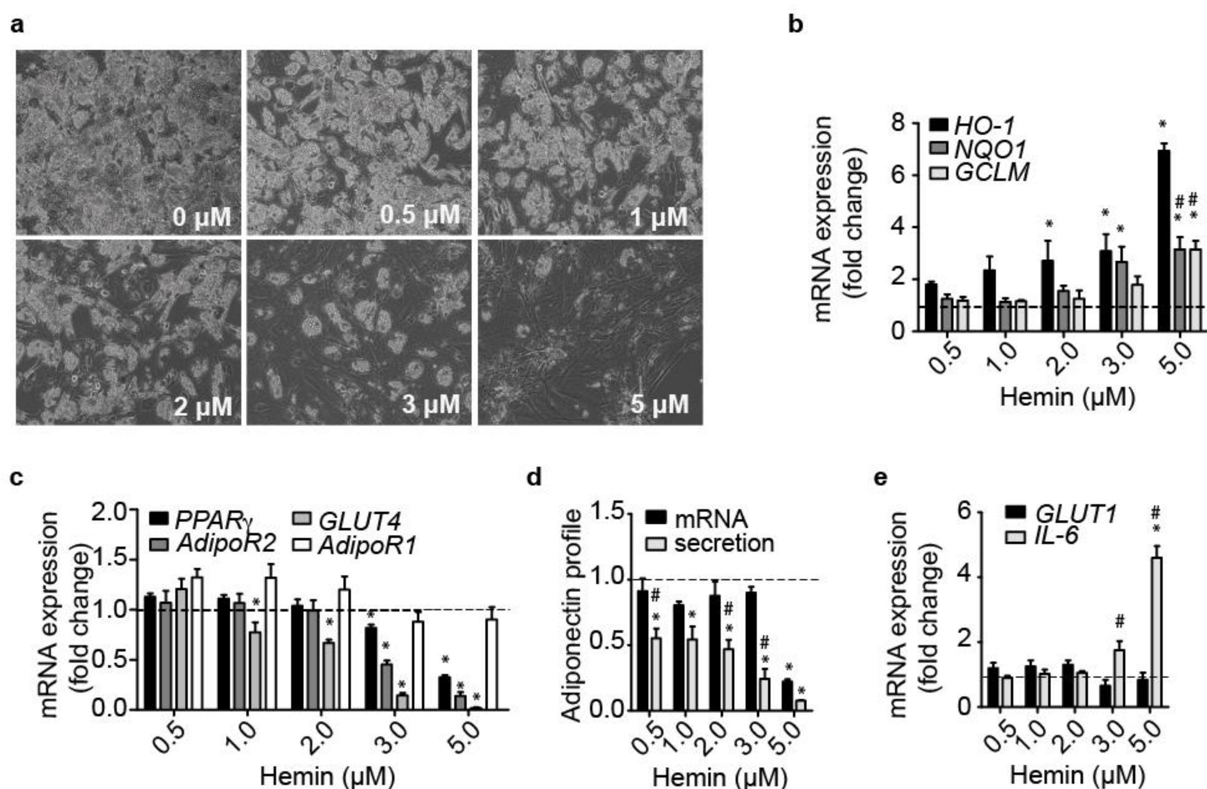

Fig 3.3 - Chronic treatment of SGBS PAs with hemin decreases adipogenesis and adiponectin production. SGBS PAs were differentiated for 14 days in the presence of increasing concentrations

of hemin (0.5-5 μM). **(a)** Images showing cell morphology on day 14. **(b)** *HO-1*, *NQO1* and *GCLM* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test: #p<0.05 vs *HO-1* expression at the same CoPP concentration; two-way ANOVA with Bonferroni's *post hoc* test). **(c)** Adipocyte markers (*PPARγ*, *ADIPOR2*, *GLUT4*) and *ADIPOR1* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test). **(d)** Adiponectin expression and secretion determined by qRT-PCR and ELISA (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test). **(e)** *GLUT1* and *IL-6* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one way-ANOVA with Tukey's *post hoc* test; #p<0.05 vs *GLUT*1 expression levels at the same CoPP concentration after two-way ANOVA with Bonferroni's *post hoc* test).

Fig 3.4

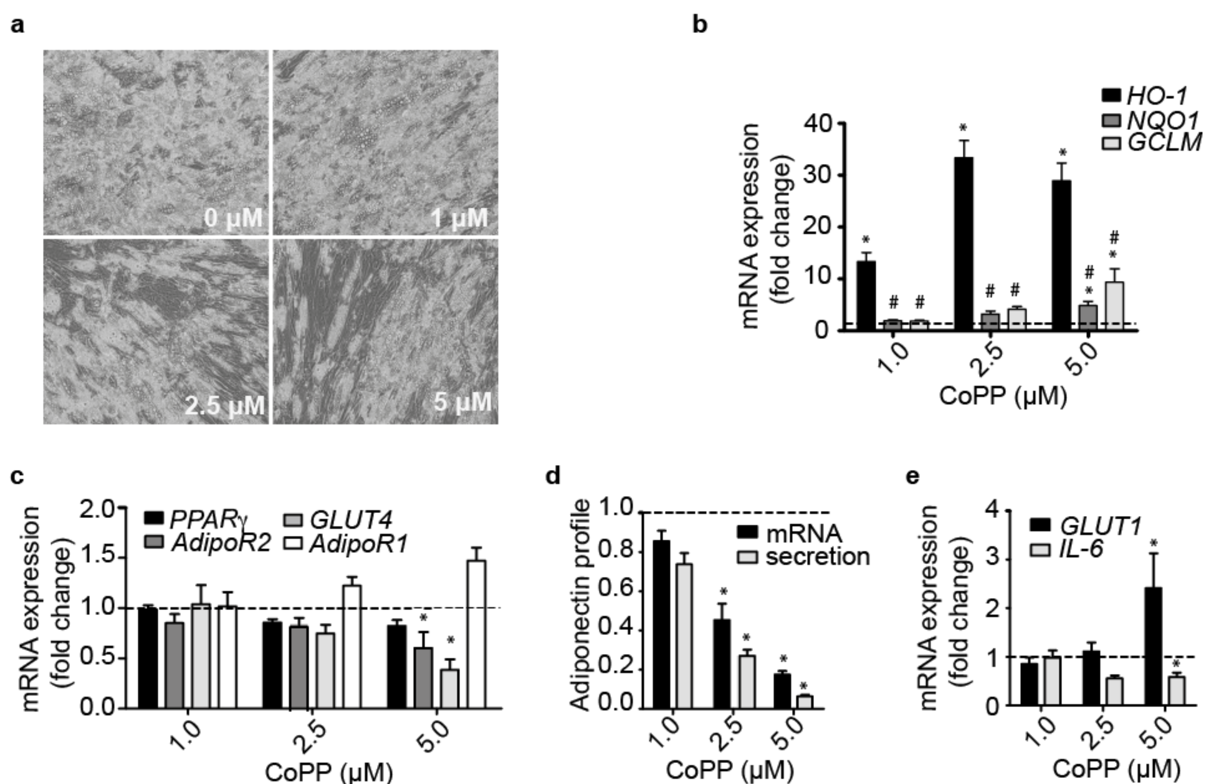

Fig 3.4 - Chronic treatment with CoPP decreases adipogenesis and adiponectin production from SGBS PAs in serum. SGBS PAs were differentiated for 14 days in serum containing medium in the presence of increasing concentrations of CoPP (1-5 μM). **(a)** Images showing cell morphology on day 14. **(b)** *HO-1*, *NQO1* and *GCLM* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test: #p<0.05 vs *HO-1* expression at

the same CoPP concentration; two-way ANOVA with Bonferroni's *post hoc* test). **(c)** Adipocyte markers (*PPARγ*, *ADIPOR2*, *GLUT4*) and *ADIPOR1* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test). **(d)** Adiponectin expression and secretion determined by qRT-PCR (n=4) and ELISA (n=3; *p<0.05 vs control; one-way ANOVA with Tukey's *post hoc* test). **(e)** *GLUT1* and *IL-6* expression determined by qRT-PCR (n=4; *p<0.05 vs control; one way-ANOVA with Tukey's *post hoc* test).

3.5.4 Co-treatment with a HO-1 inhibitor (SnMP) does not prevent the inhibitory effects of CoPP or hemin on adipogenesis or adiponectin production

To investigate whether the inhibitory effects of CoPP and hemin were associated with increased HO-1 activity we performed a series of experiments in the presence or absence of SnMP, a widely used inhibitor of HO-1 activity [243, 294]. Treatment of cells with SnMP (5 μM) had no obvious effect on lipid accumulation in control cells or in cells treated with CoPP (0.25 μM) or hemin (3 μM) (Fig 3.5a). Treatment with SnMP tended to increase the levels of HO-1 mRNA and protein under all conditions, reaching significance for *HO-1* in control and hemin treated cells (Fig 3.5b & c). This effect is consistent with other reports [214, 243] and demonstrates the efficacy of the SnMP treatment. Similar results were observed for *NQO1* and *GCLM* (Fig 3.5d & e). Consistent with these observations, whilst we typically observed no significant impact of SnMP treatment on the expression of adipogenic markers across the various conditions, co-treatment with SnMP modestly, but consistently exacerbated the effects of CoPP or hemin (reducing *PPARγ*, *ADIPOR2* and *GLUT4* expression by a further 20-30%) reaching significance for co-treatment with CoPP on *ADIPOR2* expression (Fig 3.5f-h). Similar effects were apparent when the effects of SnMP on adiponectin production were determined (Fig 3.5i & j), most notably for adiponectin secretion which was significantly reduced upon co-treatment with SnMP in the presence of either CoPP or hemin (Fig 3.5j). Similarly, co-treatment with SnMP exacerbated the effects of CoPP or hemin on *IL-6* expression (Fig 3.5k). Collectively, these observations do not support a model whereby the inhibitory effects of CoPP or hemin on adipogenesis and adiponectin production are mediated by HO-1 activity.

Fig 3.5

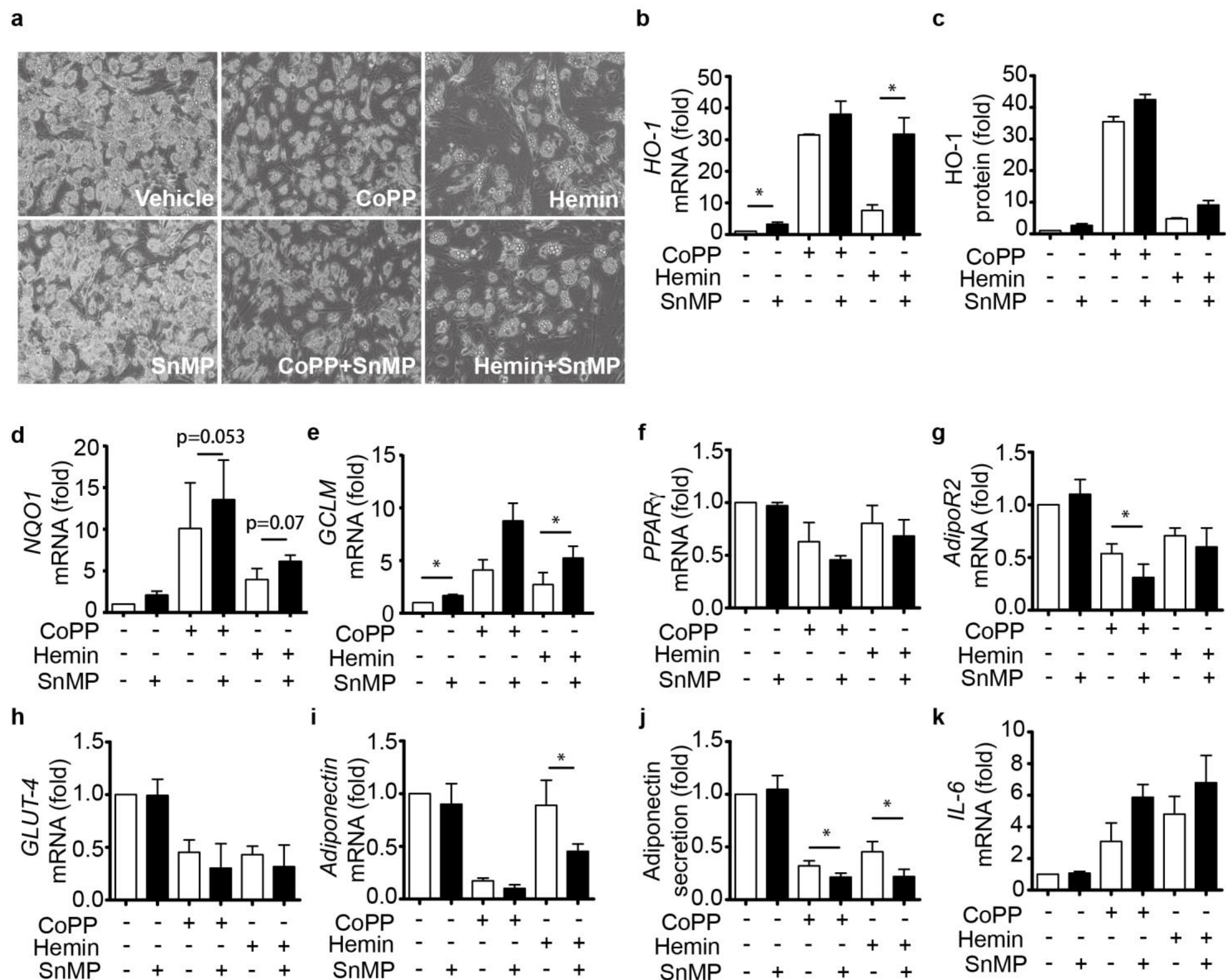

Fig 3.5 – Co-treatment with the HO-1 inhibitor SnMP does not prevent the effects of CoPP or hemin. SGBS PAs were differentiated for 14 days in the presence of CoPP (0.25 μM) or hemin (3 μM) in the presence of SnMP (5 μM) where indicated. **(a)** Images showing cell morphology on day 14. **(b)** *HO-1* expression and **(c)** HO-1 protein determined by qRT-PCR and ELISA. **(d)** *NQO1* and **(e)** *GCLM* expression determined by qRT-PCR. Expression of adipocyte markers (**f**) *PPARγ*, (**g**) *ADIPOR2* and (**h**) *GLUT4* determined by qRT-PCR. Adiponectin **(i)** expression and **(j)** secretion determined by qRT-PCR and ELISA. (**k**) *IL-6* expression determined by qRT-PR (for all graphs n=3; *p<0.05; Paired student t-test was used to determine whether treatment with SnMP had any significant effect).

3.5.5 HO-1 knockdown does not prevent the inhibitory effects of CoPP or hemin on adipogenesis or adiponectin production

Having established that increased HO-1 activity is unlikely to mediate the inhibitory effects of CoPP and hemin on adipogenesis and adiponectin production we next performed a series of experiments to investigate the effects of siRNA mediated HO-1 knockdown. Treatment with HO-1 specific siRNA had no obvious effect on lipid accumulation in control or CoPP treated cells (Fig 3.6a) despite reducing HO-1 mRNA and protein levels by 50-60% (Fig 3.6b & c). Expression of *NQO1* was significantly increased in response to HO-1 knockdown in control and CoPP cells and a similar pattern was observed for *GCLM* although this failed to reach statistical significance (Fig 3.6d & e). Collectively, these results demonstrated that the HO-1 knockdown was reasonably efficient (>50%) and was sufficient to alter expression of NRF2 target genes. In contrast, HO-1 knockdown did not reverse the effects of CoPP on the expression of adipogenic markers, adiponectin production or *IL-6* (Fig 3.6f-k).

Similar experiments were performed in cells treated with hemin and these gave comparable results. HO-1 knockdown had no obvious effect on lipid accumulation in control or hemin treated cells (Fig 3.7a), despite reducing both basal and hemin-induced HO-1 mRNA and protein by 50-60% (Fig 3.7b & c). Expression of *NQO1* and *GCLM* was generally increased upon HO-1 knockdown (Fig 3.7d & e) whilst the effects of hemin on expression of adipogenic markers, adiponectin production and *IL-6* expression were unaffected or exacerbated (Fig 3.7f-k).

These results indicate that chronic treatment of human preadipocytes with CoPP or hemin interferes with the process of adipogenesis and subsequent production of adiponectin by a mechanism that is not coupled to either HO-1 activity or protein.

Fig 3.6

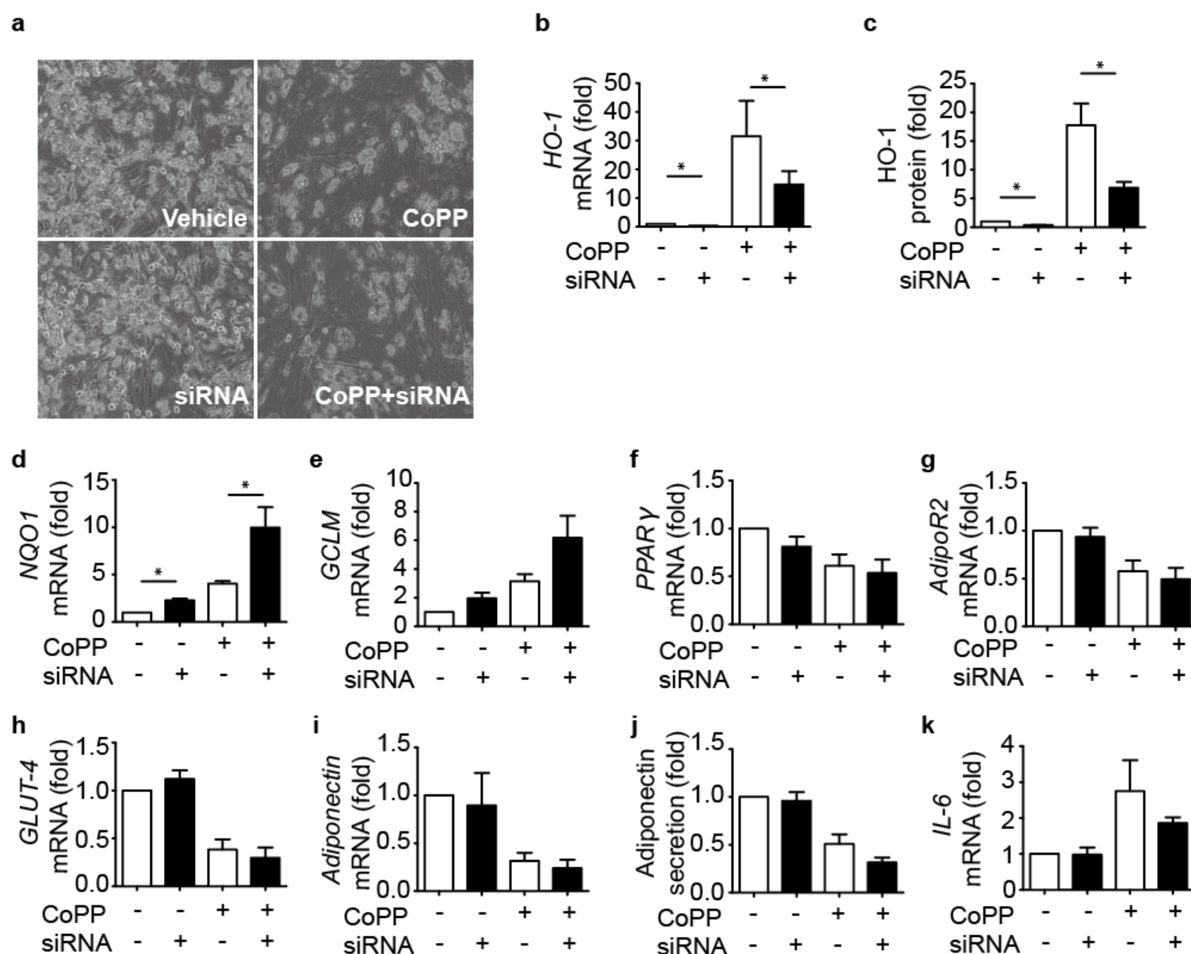

Fig 3.6 – HO-1 knockdown does not prevent the effects of CoPP. SGBS PAs were transfected with scrambled (vehicle) or HO-1 siRNA for 72 h then differentiated for 14 days in the presence of CoPP (0.25 µM). **(a)** Images showing cell morphology on day 14. **(b)** *HO-1* expression and **(c)** HO-1 protein determined by qRT-PCR and ELISA. **(d)** *NQO1* and **(e)** *GCLM* expression determined by qRT-PCR. Expression of adipocyte markers (**f**) *PPARγ*, (**g**) *ADIPOR2* and (**h**) *GLUT4* determined by qRT-PCR. Adiponectin **(i)** expression and **(j)** secretion determined by qRT-PCR and ELISA. (**k**) *IL-6* expression determined by qRT-PR (for all graphs n=3; *p<0.05; Paired student t-test was used to determine whether HO-1 knockdown had any significant effect).

Fig 3.7

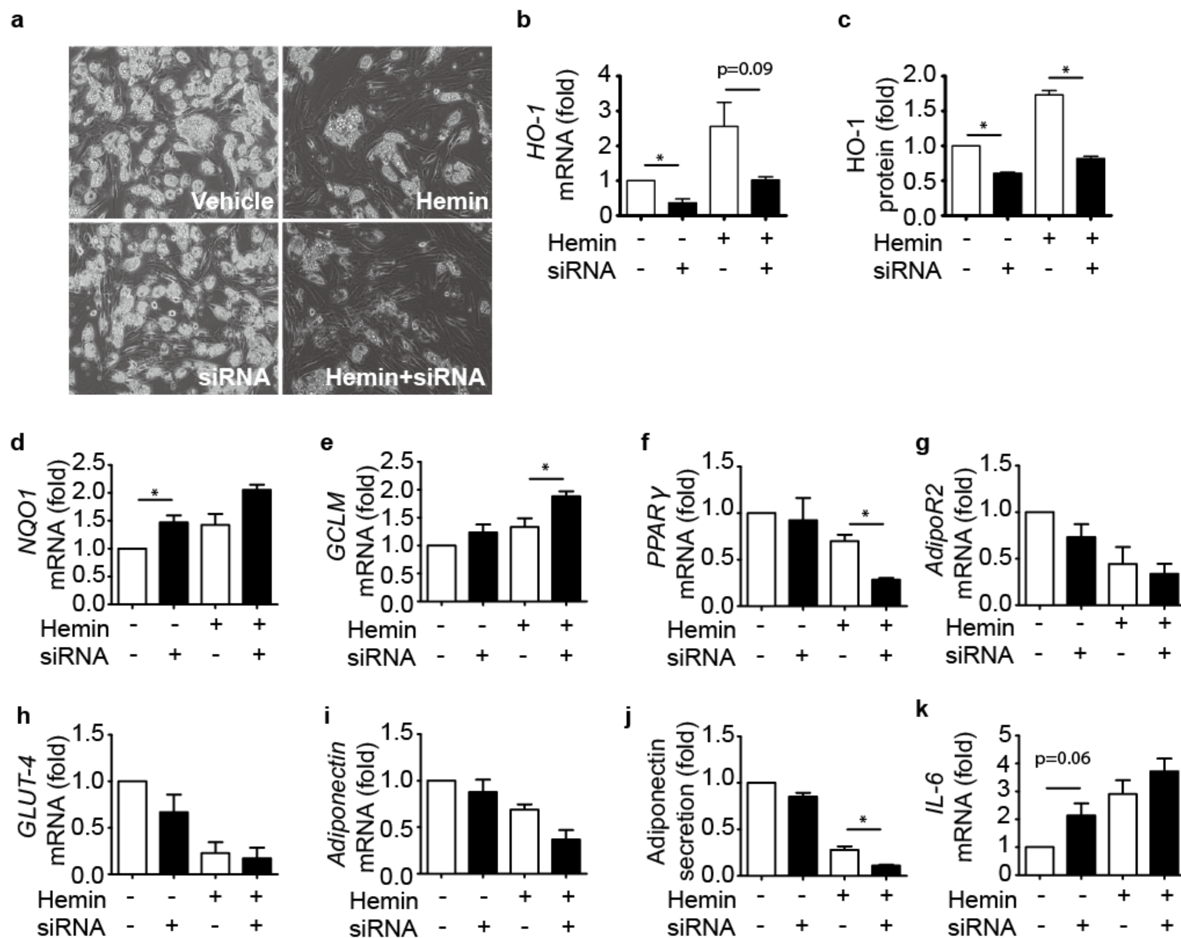

Fig 3.7 – HO-1 knockdown does not prevent the effects of hemin. SGBS PAs were transfected with scrambled (vehicle) or HO-1 siRNA for 72 h then differentiated for 14 days in the presence of hemin (3 μM). **(a)** Images showing cell morphology on day 14. **(b)** *HO-1* expression and **(c)** HO-1 protein determined by qRT-PCR and ELISA. **(d)** *NQO1* and **(e)** *GCLM* expression determined by qRT-PCR. Expression of adipocyte markers (**f**) *PPARγ*, (**g**) *ADIPOR2* and (**h**) *GLUT4* determined by qRT-PCR. Adiponectin **(i)** expression and **(j)** secretion determined by qRT-PCR and ELISA. (**k**) *IL-6* expression determined by qRT-PR (for all graphs n=3; *p<0.05; Paired student t-test was used to determine whether HO-1 knockdown had any significant effect).

3.6 Discussion

The increase in prevalence and severity of obesity related cardiometabolic diseases continues to drive efforts to identify novel and efficacious therapeutic approaches [295]. One such strategy is to identify pharmacological approaches to increase adiponectin production [283]. In the current report we have investigated the hypothesis that chronic induction of HO-1 will modulate differentiation of human preadipocytes in a manner that results in increased adiponectin production [217, 230, 239]. We demonstrate that under all conditions tested, chronic induction of HO-1 with either CoPP or hemin resulted in dose-dependent inhibition of differentiation concomitant with induction of NRF2 target genes. However, under no conditions was adiponectin production enhanced. Further investigations involving co-treatment with an inhibitor of HO-1 activity or siRNA-mediated HO-1 knockdown, combined with inconsistencies across dose responses, indicate that these effects are HO-1-independent. We propose that they are most-likely mediated by an alternate pathway, possibly involving one or more NRF2 target genes [296]. Collectively, these findings suggest that any beneficial effects following chronic treatment with CoPP or hemin *in vivo* are unlikely to be mediated by a direct positive effect of HO-1 induction on adipogenesis of preadipocytes. These findings are consistent with independent studies that found no beneficial effect when genetic approaches were used to manipulate HO-1 in adipocytes [204, 281].

An increasing number of studies have put forward the existence of a 'HO-1 – adiponectin axis'. Typically, investigations which have led to this proposal have involved chronic induction of HO-1 using pleiotropic agents such as CoPP, hemin (or derivatives) or epoxyeicosatrienoic acid (EET)-agonists *in vivo* and or *in vitro* [217, 225-227, 230, 236, 239, 241, 242, 265, 267, 268, 285-287]. Whilst a primary effect of these agents is to induce HO-1 expression and activity it is not the sole effect. Indeed, these agents induce HO-1 expression, at least in part, by modulation of broad acting transcriptional repressors and transcription factors. More specifically, they involve post-translational downregulation of BACH1 and upregulation of NRF2 [275, 291, 297, 298]. The latter is recognised as a key regulator of cellular defence against oxidative stress that is also strongly implicated in modulation of adipogenesis and adipose tissue remodelling [289, 299, 300]. Thus, when using such pleiotropic agents additional experimental strategies are required to ascribe a defined role to the induction of HO-1 in such processes.

In the current study we first performed a comprehensive series of carefully controlled *in vitro* investigations in phPAs and SGBS PAs which demonstrated that chronic treatment with CoPP or hemin resulted in dose-dependent inhibition of differentiation (as determined morphologically, genetically and functionally) and adiponectin production (expression and secretion) as well as

induction of NRF2 target genes (see Figs 3.1-3.4). Under no conditions did we observe any evidence of an increase in adiponectin production. This finding is consistent with the observed reduction in *PPARγ* expression, given the pivotal role of this transcription factor in adiponectin production [301], combined with the potential for downregulation of PPARγ activity by CDK5 [302], which is upregulated upon induction of HO-1 [303]. Nevertheless, these *in vitro* observations contrast with numerous reports based largely on studies from one group involving bone marrow derived mesenchymal stem cells (BMSCs) differentiated *in vitro* in the continued presence of CoPP [238, 242, 243, 294] or EET-agonists [230, 291, 294]. Whilst the reasons for these differences are unclear differences in experimental approaches may contribute, although such details are typically difficult to determine in the above studies. It also remains possible that adiponectin production from BMSCs may be regulated differently in response to these treatments than in preadipocytes derived from subcutaneous white adipose tissue, as were used throughout the current study. Indeed, recent findings showing caloric restriction increased adiponectin production from bone marrow adipose tissue but not white adipose tissue support such a scenario [304]. Direct comparisons between the different cell-types, under tightly controlled experimental conditions, are warranted to investigate this possibility.

Interestingly, there was no suggestion of dose-dependent induction of *HO-1* by CoPP when the SGBS PAs were differentiated in the absence of serum (see Fig 3.2), conditions where substrate is likely to be limiting [213]. This apparent disconnect, between the dose response of *HO-1* expression and all other parameters, was the first evidence that made us question the role of HO-1 *per se*. We employed two complementary approaches to address this, using SnMP to inhibit HO-1 activity directly or HO-1 siRNA to reduce HO-1 protein. These experiments were performed in cells treated with intermediate concentrations of CoPP or hemin, such that a decrease in the potency of either agent would be apparent. Perhaps surprisingly, there was no reduction of the effects of CoPP or hemin in cells co-treated with SnMP or HO-1 siRNA. Indeed, we observed a fairly consistent pattern across all readouts, some of which were significant, that indicated co-treatment with SnMP or HO-1 siRNA enhanced or exacerbated the effects of CoPP or hemin potency. Consistent with previous reports [243] treatment with SnMP typically increased HO-1 mRNA and protein. We also observed increased expression of *NQO1* and *GCLM* suggesting that NRF2 activity was increased by treatment with SnMP or HO-1 siRNA. One possible explanation for these effects is that induction of HO-1 activity represents part of a homeostatic feedback mechanism that is unbalanced by treatment with the inhibitor or siRNA, resulting in a rise in NRF2 activity, promoting increased transcription of downstream targets including *NQO1* and *GCLM*. Nonetheless, these results demonstrate that the increased levels of HO-1 activity or protein are not

responsible for the inhibitory effects of CoPP or hemin observed in the human preadipocytes. These results contrast with the findings from studies in BMSCs [230, 238, 242, 243, 291, 294]. Again, the underlying reasons for the observed differences remain obscure.

Although a large number of reports suggest the beneficial metabolic effects of administration of agents such as CoPP or hemin are mediated via increased activity of HO-1, there is an emerging body of evidence that argues against this. For example, the beneficial effects of heme arginate in diabetic (db/db) mice were unaffected by co-treatment with a HO-1 inhibitor [282]. Moreover, genetic approaches to increase HO-1 in adipocytes have typically failed to improve metabolic parameters in the context of diet-induced obesity [204, 281], including the reduction in circulating adiponectin levels [281]. The exception to this comes from a study which employed an atypical delivery approach involving intracardial injection of a lentiviral construct (aP2-HO-1) to drive adipocyte-specific overexpression of HO-1 [209]. Notably, this approach resulted in a significant decrease in weight gain with a concomitant reduction in white adipose tissue mass and inflammation [209], factors that are sufficient to increase adiponectin levels [252, 283]. It is also worth noting that these observations resemble a scenario of caloric restriction, where adiponectin production from the bone marrow is increased [304].

In summary, in the current report we have investigated the effects of chronic treatment with CoPP or hemin on the induction of HO-1 and differentiation of human preadipocytes and adiponectin production. We find that both agents inhibit differentiation and adiponectin production under all conditions tested. Inhibition of HO-1 activity or expression failed to ameliorate these effects as well as the induction of other NRF2 target genes *NQO1* and *GCLM*. These data indicate that chronic treatment with CoPP or hemin interfere with differentiation and adiponectin production from human preadipocytes in a HO-1-independent manner. We propose that induction of other NRF2 target genes, such as *NQO1* [296], may underpin the observed inhibitory effects. Finally, these findings do not support the existence of a direct HO-1 – adiponectin axis.

3.7 Acknowledgements

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3.8 Author Contributions Statement

M.Y. performed experiments, interpreted data, and drafted a manuscript. M.K. performed experiments and interpreted data. C.N. and J.H. performed experiments. J.L.B. designed the study, interpreted data, and edited the manuscript. J.P.W. designed the study, interpreted data, wrote and edited the manuscript, and is the guarantor of the article.

3.9 Competing financial interests

The authors have no competing financial interests to declare.

Chapter 4:

Divergent effects of CoPP, a HO-1 inducer, on weight gain, adipose tissue inflammation, adiponectin and insulin responsiveness in obese mice.

4.1 Introduction to this publication

This Chapter was submitted to *Scientific report* journal. In this study, we characterised the effects of administration of the widely used HO-1 inducer cobalt protoporphyrin (CoPP) in lean and obese mice:

- Treatment of obese mice with CoPP results in decreased food intake, body weight gain and adipocyte size as well as enhanced insulin sensitivity and attenuated liver steatosis.
- Obese CoPP-treated mice display increased adipose tissue inflammation and reduced adiponectin expression but circulating adiponectin levels are increased.
- Co-administration of a HO-1 inhibitor SnMP in obese mice ameliorated CoPP effects on adipose tissue inflammation and blunted CoPP effects on circulating adiponectin, while other parameters were unchanged.

Collectively, these findings indicate that CoPP administration promotes divergent effects on adipose tissue, adiponectin and insulin responsiveness. The majority of these effects appear independent of HO-1 activity.

Yang, M., Ng, C., Keshvari, S., Bulmer, A. C., Barclay, J. L., Whitehead, J. P., Divergent effects of CoPP, a HO-1 inducer, on weight gain, adipose tissue inflammation, adiponectin and insulin responsiveness in obese mice. *Scientific report*, submitted

Divergent effects of CoPP, a HO-1 inducer, on weight gain, adipose tissue inflammation, adiponectin and insulin responsiveness in obese mice.

Mengliu Yang[1,2], Choaping Ng[1], Sahar Keshvari[1], Andrew C. Bulmer[3], Johanna L. Barclay[1], Jonathan P. Whitehead[1*]

[1]Mater Research Institute-University of Queensland (MRI-UQ), Translational Research Institute, Brisbane, QLD, Australia
[2]Current address, Department of Endocrinology, the Second Affiliated Hospital, Chongqing Medical University, 400010 Chongqing, China
[3]Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Gold Coast, QLD, Australia

[*]Correspondence to: Jon Whitehead, MRI-UQ, Translational Research Institute, 37 Kent Street, Brisbane, QLD 4101, Australia
Tel 61 07 3343 7633
Email jon.whitehead@mater.uq.edu.au

4.1 Abstract

Adiponectin is a beneficial adipocyte-derived hormone with anti-inflammatory and insulin-sensitising properties. Hypoadiponectinemia contributes to obesity-related cardiometabolic diseases making strategies to increase adiponectin levels attractive. Literature suggests pharmacological approaches that promote induction of heme-oxygenase-1 (HO-1) increase adiponectin levels and reduce inflammation prompting use of the term 'HO-1 – adiponectin axis'. We previously performed *in vitro* studies using human preadipocytes/adipocytes which argue against a direct HO-1 – adiponectin axis. Here, we characterised the effects of administration of the widely used HO-1 inducer cobalt protoporphyrin (CoPP) in lean and obese mice. CoPP administration significantly increased inflammatory gene expression in white adipose tissue irrespective of obesity. In obese mice adiponectin expression was significantly reduced by CoPP yet circulating adiponectin levels (total and HMW) were elevated. Similar trends were observed in lean mice. Additional effects specific to obese mice included reduced food intake, weight gain, adipocyte size and liver steatosis and improved insulin responsiveness. Co-administration of a HO-1 inhibitor SnMP in obese mice ameliorated CoPP effects on adipose tissue inflammation and blunted CoPP effects on circulating adiponectin. Other parameters were unchanged. These findings indicate that CoPP administration promotes divergent effects on adipose tissue, adiponectin and insulin responsiveness. The majority of these effects appear independent of HO-1 activity.

Keywords: Obesity; Therapeutic; Adipokine; Inflammation

4.3 Introduction

The global prevalence of obesity-associated cardiometabolic diseases requires the development of novel therapeutic approaches [295, 305]. Adiponectin is an adipocyte-derived hormone that displays a range of beneficial cardiometabolic properties including anti-inflammatory and insulin sensitizing effects [283, 295]. Paradoxically, circulating adiponectin levels are reduced in obesity and this 'hypoadiponectinemia' contributes to the aetiology of obesity-associated diseases [283, 295]. Consistent with this, humans with genetic polymorphisms that reduce adiponectin production and mice lacking adiponectin typically develop cardiovascular dysfunction and or type 2 diabetes [252, 283]. Following on from this, the efficacy of the insulin sensitizing thialozidinediones (TZDs), which were used widely for the treatment of type 2 diabetes, correlated closely with improvements in adiponectin profiles [260]. Whilst the success of the TZDs provides proof-of-principle that strategies that target circulating adiponectin are effective this class of drug has largely been withdrawn due to adverse secondary effects, providing momentum for new therapeutic approaches in this area [301].

An emerging body of evidence suggests that pharmacological induction of the stress-induced protein heme oxygenase-1 (HO-1) may promote an increase in circulating adiponectin levels, prompting the suggestion of a "HO-1 – adiponectin axis" [217, 225-227, 230, 236, 239, 241, 242, 265, 267, 268, 285-287]. HO-1 catalyses the oxidative degradation of heme to carbon monoxide (CO), iron and biliverdin/bilirubin [261, 284], products that are known to modulate cellular function and homeostasis in response to a variety of inflammatory, oxidative and metabolic stresses [207, 261, 284]. Indeed, strategies to induce HO-1 in humans are under investigation [206, 207, 263] as this is generally considered an attractive therapeutic strategy in diseases including obesity and type 2 diabetes [195, 262, 284]. However, some lines of evidence suggest that HO-1 *per se* may not be involved [282] and may even play a negative role in the aetiology of obesity-associated inflammation and cardiometabolic complications [204].

In light of these contrasting viewpoints and the absence of any compelling evidence to support a direct effect of HO-1 on adiponectin production from adipocytes we recently performed a series of *in vitro* studies to determine the effects of acute or chronic induction of HO-1, using two commonly used agents - cobalt protoporphyrin (CoPP) or hemin, on adiponectin production from human adipocytes. In the first study, we showed that induction of HO-1 for up to 48 h did not alter adiponectin production (expression or secretion) in either healthy mature adipocytes or in unhealthy TNFα-treated adipocytes where adiponectin production was compromised [288]. In the second study, we demonstrated that chronic induction of HO-1 throughout the process of adipogenesis (where preadipocytes differentiate into mature, insulin responsive adipocytes) compromised

adipogenesis and adiponectin production (Yang et al, under review, SREP-16-40083). Whilst the latter finding contrasts with earlier reports [238, 242] mechanistic investigations, using an inhibitor of HO-1 activity or HO-1 knockdown, indicated that the inhibitory effects were independent of HO-1. One possibility is that they are mediated by downstream targets of the transcription factor Nuclear factor erythroid-derived 2-like 2 (NRF2) [275], a key regulator of the antioxidant defence pathway that plays a complex role in the regulation of adipogenesis and adipose tissue [289] that is upregulated by CoPP and hemin (Yang et al, under review, SREP-16-40083).

Collectively, our *in vitro* studies do not support the existence of a direct HO-1 – adiponectin axis. In the current report we have extended these studies by performing a series of investigations to characterise the effects of CoPP administration on white adipose tissue (WAT), adiponectin and related parameters in lean and obese mice. We find that CoPP-treatment promotes a range of divergent effects on adipose tissue and adiponectin, particularly in obese mice. Paradoxically, obese CoPP-treated mice display increased adipose tissue inflammation and reduced adiponectin expression but circulating adiponectin levels are increased. Co-treatment with a HO-1 inhibitor prevents the pro-inflammatory effect of CoPP in adipose tissue but does not restore adiponectin expression yet blunts the effect on circulating adiponectin. These complex and somewhat contradictory effects of CoPP are, perhaps surprisingly, consistent with a considered and integrated appraisal of the literature. Moreover, they support the hypothesis that the induction of HO-1 may, at least in part, increase circulating adiponectin levels.

4.4 Methods

4.4.1 Reagents and antibodies

General reagents were obtained from Sigma-Aldrich (Victoria, Australia), unless otherwise stated.

4.4.2 Animals

All experimental procedures were carried out in accordance with approved protocols by the University of Queensland Animal Ethics Unit and were conducted using the Guidelines of the National Health and Medical Research Council. Male C57BL6 mice (Queensland Biological Resource, Brisbane, QLD, Australia) were were maintained at 22.0±0.5°C under a 12-h day, 12-h

night cycle and fed standard chow diet (SCD) containing 5% of total energy from fat. At 8 weeks of age mice were maintained on SCD or switched to a high fat diet (HFD) (SF04-027, Specialty Feeds, 43% energy from fat) for 16 weeks to induce obesity. CoPP or SnMP were dissolved in 10 mmol/L Tris base and pH adjusted to pH 7.4-7.8 with 0.1 N HCl. Vehicle (Tris/HCl solution), CoPP (3 mg/kg once per week) and SnMP (20 mg/kg three times a week) were administered by intraperitoneal injection for 6 weeks such that all mice in each cohort received either 1 treatment per week (cohort 1 - Figs 4.1-4.4) or 3 treatments per week (cohort 2 - Figs 4.5-4.8). Body weight was measured every day during the first two weeks after which it was measured once a week. Energy intake was calculated by taking into account the energy value of each of the diets (2 kcal energy for SCD, and 4.71 kcal energy for HFD) and using this to calculate energy intake based on a simple equation X daily food intake (g/day) *Y (kcal/g)=Z kcal/day.

4.4.3 Glucose and Insulin Tolerance Tests (GTT and ITT)

GTT and ITT were performed in 6 h fasted mice after 5 or 6 weeks of treatment respectively (treatment strategy see below). For glucose tolerance, mice were administered glucose (2 g/kg in lean mice; 1 g/kg in obese mice) by intraperitoneal injection and blood samples were collected from the tail vein at 0, 20, 40, 60, 90 & 120 min for determination of blood glucose and serum insulin. For insulin tolerance, mice were administered human insulin (0.75 U/kg) and blood glucose was determined at 0, 20, 40, 60, 90 & 120 min. Insulin responsiveness was calculated as the change in blood glucose from 0 to 20 min.

4.4.4 Hematoxylin and eosin (H&E) staining

Adipose tissue and liver samples were fixed in 10% buffered formalin phosphate, dehydrated, embedded in paraffin, then cut into 5 μm sections for H&E staining. Sections were rehydrated in an alcohol series, stained in hematoxylin for 10-15 min, rinsed in water for 5 min, dipped in acid ethanol 10 times. Sections were then rinsed twice in water, stained in eosin for 30 sec, then dehydrated in an alcohol series and xylene. H&E staining was visualised and images captured using an inverted epifluorescence microscope (Olympus, USA). Adipocyte size (diameter) was measured using a quantitative image analysis system (Cell Sens, Olympus, USA).

4.4.5 Oil Red O staining of liver

Hepatic lipid accumulation was evaluated in frozen liver sections by Oil Red O staining. Liver samples were embedded in OCT compound (PST, Australia) and cut into 3 μm frozen pathological sections. Sections were fixed with 10% formalin for 15 min, rinsed with 60% isopropanol for 10 min, stained with Oil Red O solution for 20 min followed by Harris's hematoxylin counterstain for 2 min, and washed in tap water until blue. Sections were subsequently examined and images captured using an inverted epifluorescence microscope (Olympus, USA). Images were analysed using ImageJ.

4.4.6 RNA extraction and real-time PCR

Gene expression was measured by quantitative RT-PCR as described [288] using *Tata box protein* (*Tbp*) as the housekeeper. Primer sequences were as follows (all mouse, listed as Forward / Reverse): *Tbp* - CTCAGTTACAGGTGGCAGCA / ACCAACAATCACCAACAGCA; *Ho-1* - CACTCTGGAGATGACACCTGAG / GTGTTCCTCTGTCAGCATCACC; *Adipoq* - AGATGGCACTCCTGGAGAGAAG / ACATAAGCGGCTTCTCCAGGCT; *F480* - CGTGTTGTTGGTGGCACTGTGA / CCACATCAGTGTTCCAGGAGAC; *Cd68* - GGCGGTGGAATACAATGTGTCC / AGCAGGTCAAGGTGAACAGCTG; *Cd11b* - TACTTCGGGCAGTCTCTGAGTG / ATGGTTGCCTCCAGTCTCAGCA; *Tnfα* - GGTGCCTATGTCTCAGCCTCTT / GCCATAGAACTGATGAGAGGGAG; *Mcp-1* - GCTACAAGAGGATCACCAGCAG / GTCTGGACCCATTCCTTCTTGG; *Ferritin* - CCTCGAGTTTCAGAACGATCGC / CCTGATTCAGGTTCTTCTCCATG.

4.4.7 Determination of HO-1 protein and circulating total and HMW adiponectin protein

Tissue HO-1 was quantitated using a Mouse HO-1 ELISA (Enzo Life Sciences, NY, USA) following the manufacturer's instructions. Circulating total adiponectin was quantitated using a total adiponectin ELISA Kit (R&D systems – Cat# DY1119, Minneapolis, USA) and HMW adiponectin was quantitated using a HMW adiponectin ELISA kit (ALPCO – Cat# 47ADPMS-E01, New Hampshire, USA) according to the manufacturer's instructions. Total and HMW adiponectin levels were not comparable across the different manufacturers kits (eg SCD control mice: total adiponectin = 1.2 + 0.2 µg/ml; HMW adiponectin = 3.6 + 1.3 µg/ml) so these data are presented

as arbitrary units.

4.4.8 Statistical Analysis

Data are presented as mean ± SEM. Two-way ANOVA with Bonferroni's post hoc test was used to compare between treatment groups in both lean and obese mice (Fig 4.1-4.4). One-way ANOVA was used to compare between vehicle, CoPP and CoPP+SnMP treatment groups with Tukey's post hoc test (Fig 4.5-4.8). Unpaired student t-test was used to compare differences between vehicle and CoPP in adipocyte size and Oil Red O stain. Differences were considered statistically significant at $p<0.05$. Statistical analyses were performed in GraphPad Prism 5.0.

4.5 Results

4.5.1 CoPP administration transiently decreases food intake and prevents body weight gain in obese mice

Mice were fed either a standard chow diet (SCD) or high fat diet (HFD) for 16 weeks, to induce obesity. Body weight of lean (SCD) mice was 31.3 ± 1.11 g (n=28) and that of obese (HFD) mice was 43.6 ± 1.99 g (n=30). Mice were maintained on the same diets for a further 6 weeks and received weekly administration of CoPP (3 mg/kg once per week) (the dose of CoPP was chosen based on dosing used in similar studies [210, 217], to allow direct comparison) or vehicle. Final body weight of lean mice was 31.6 ± 1.20 g for control and 30.5 ± 1.34 for CoPP-treated mice (n=16 & 12 respectively) whilst that of obese mice was 45.2 ± 2.05 g for control and 43.5 ± 2.49 for CoPP-treated mice (n=16 & 14 respectively).

CoPP treatment had no effect on food intake in lean mice but was associated with a trend towards reduced cumulative energy intake in obese mice (Fig 4.1a & b). More detailed analysis of daily food intake in obese mice revealed significant transient reductions in daily energy intake following CoPP administration (Fig 4.1c & d). Consistent with this, transient weekly reduction in energy intake, body weight change over the 6 week intervention period was significantly reduced in the obese CoPP-treated mice compared with the obese control mice (Fig 4.1c & d). The CoPP-induced reduction in body weight is consistent with other reports [217, 237, 306].

Fig 4.1

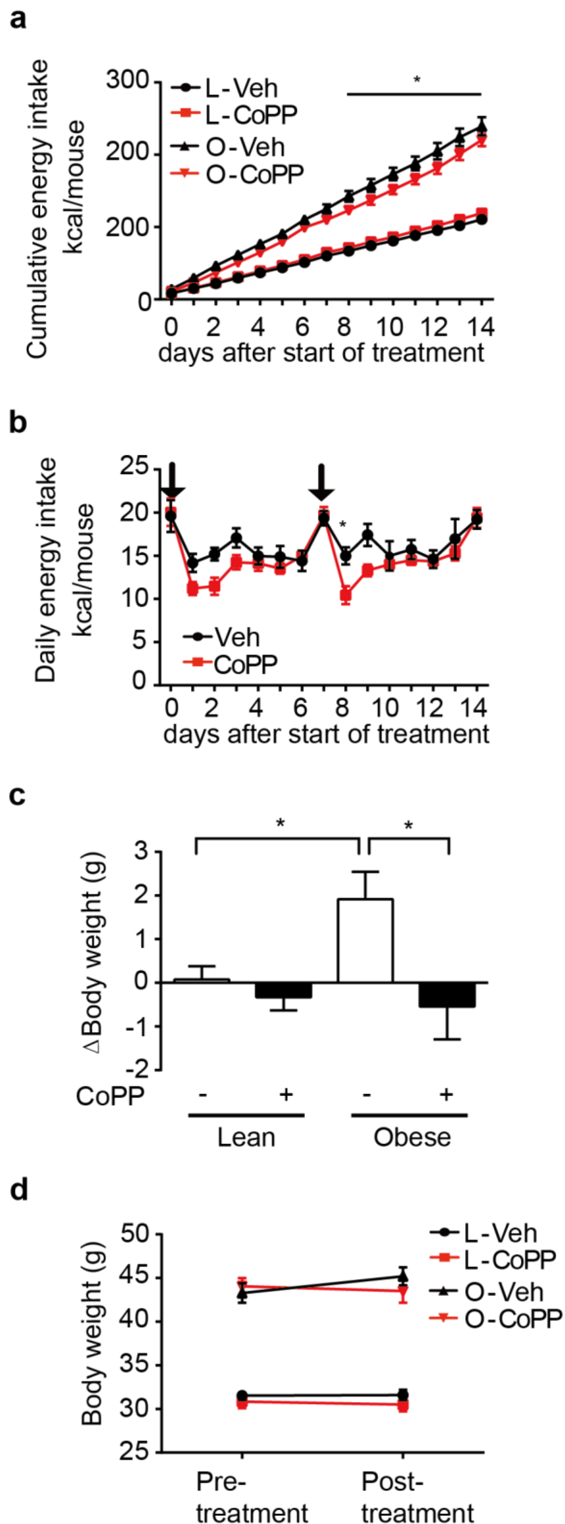

Fig 4.1 - CoPP administration decreases food intake and prevents body weight gain in obese mice. Lean (L) and obese (O) mice were maintained on chow (L) or HFD (O) and received weekly administration of vehicle (veh) or CoPP (3 mg/Kg) for 6 weeks. **(a)** Cumulative energy intake over the first 14 days of treatment in lean and obese mice (L-veh n=6; L-CoPP n=9; O-veh n=5; O-CoPP n=8). **(b)** Daily energy intake over the first 14 days of treatment in obese mice. Arrows depict

administration days. **(c)** Change in body weight after 6 weeks of treatment with or without CoPP **(d)** The absolute body weight pre and post CoPP treatment (n ⩾ 12 for all groups). *$p<0.05$; ***$p<0.001$; two-way ANOVA with a Bonferroni post hoc test.

4.5.2 CoPP administration improves insulin responsiveness and increases circulating adiponectin levels in obese mice

To investigate the potential effects of CoPP on glucose metabolism we performed glucose tolerance tests (GTT) and insulin tolerance tests (ITT) after 5 and 6 weeks of treatment respectively. CoPP administration had no effect on glucose tolerance in lean or obese mice (Fig 4.2a & b). Similarly, CoPP had no effect on insulin tolerance in lean mice (Fig 4.2c) however, CoPP significantly improved insulin tolerance in obese mice (Fig 4.2d), with insulin promoting a similar response to that observed in the lean mice as evidenced by the insulin responsiveness (Fig 4.2e). We observed an inverse correlation between change in body weight and insulin responsiveness in the obese mice (Fig 4.2f), which strongly suggests the improvement in insulin responsiveness observed in the obese CoPP-treated mice was driven by, at least in part, the reduction in weight gain.

We next examined the impact of CoPP on circulating adiponectin profiles. Perhaps surprisingly, circulating adiponectin levels were not reduced in obese mice in this study. This is consistent with other, similar diet-induced obesity studies involving mice in the host lab [307]. Notwithstanding, given adipose tissue mass increased threefold in the obese mice it seems reasonable to conclude that adiponectin production per unit of adipose tissue was reduced. Circulating levels of both total and HMW adiponectin were significantly increased in obese CoPP-treated mice as were circulating HMW adiponectin levels in lean CoPP-treated mice (Fig 4.2g & h). However, unlike the correlation between ITT and body weight change, neither total nor HMW adiponectin levels correlated with body weight change ($r = -0.173$, $p=0.418$ and $r = -0.463$, $p=0.831$ respectively). Collectively, these findings suggest that in obese mice CoPP administration improves insulin responsiveness by a mechanism associated with reduced weight gain whereas it increases circulating adiponectin levels by an independent mechanism.

Fig 4.2

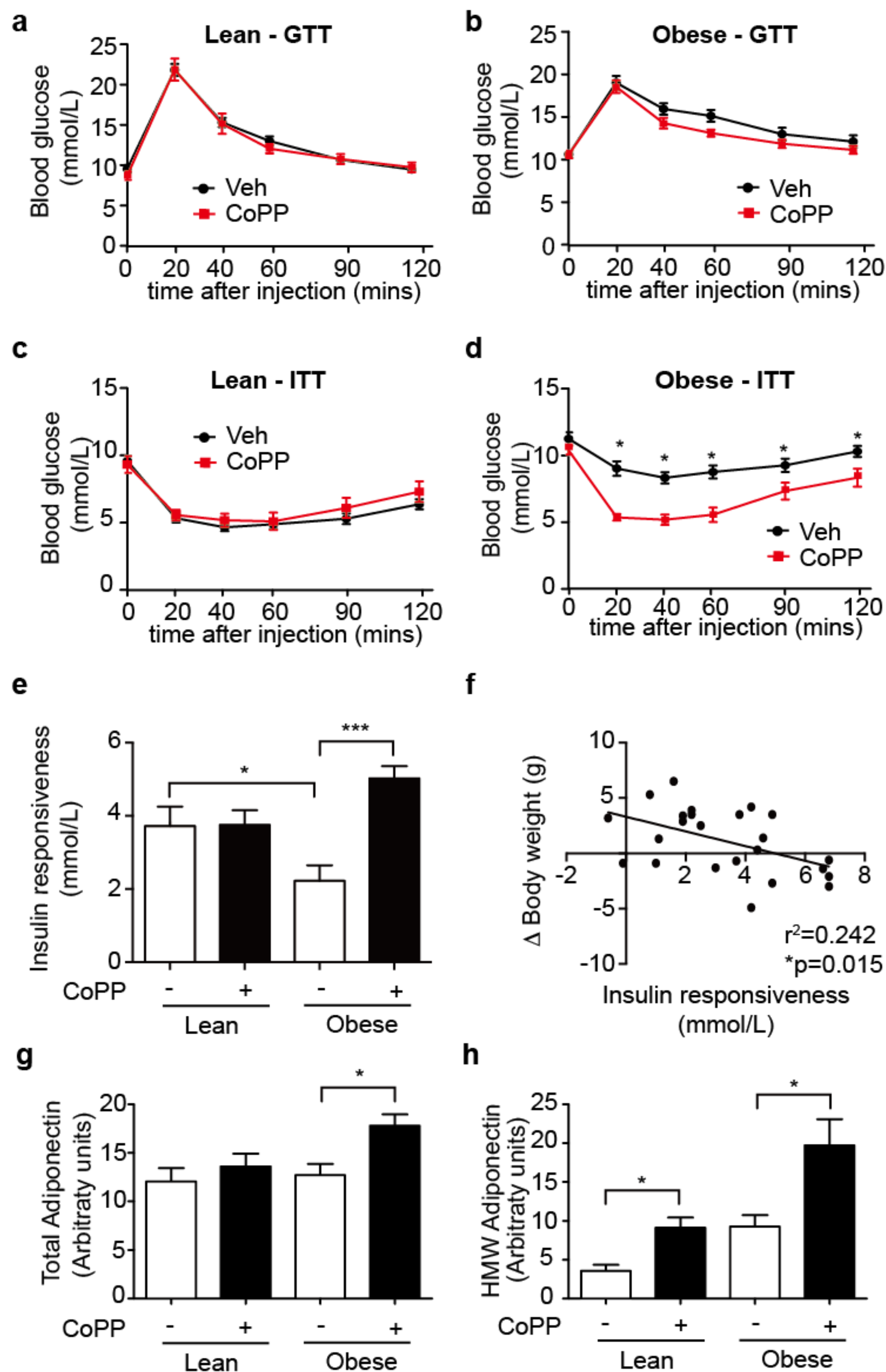

Fig 4.2 - CoPP administration improves insulin responsiveness and increases circulating adiponectin levels in obese mice. Lean and obese mice were maintained on chow or HFD respectively and received weekly administration of vehicle (veh) or CoPP (3 mg/Kg) for 6 weeks. Glucose tolerance test (GTT) in **(a)** lean and **(b)** obese mice after 5 weeks of treatment (n $\geqslant$ 11 for all groups). Insulin tolerance test (ITT) for **(c)** lean and **(d)** obese mice after 6 weeks of treatment. **(e)** Quantitation of insulin responsiveness (Δ blood glucose from 0 – 20 min post insulin). **(f)**

Correlation between Δ body weight and insulin responsiveness in obese mice. Circulating levels of **(g)** total and **(h)** HMW adiponectin after 6 weeks of treatment (n ⩾ 11 for all groups; data are presented in arbitrary units because total and HMW adiponectin were determined using ELISA kits from different manufacturers making absolute values incomparable). *p<0.05; **p<0.01; ***p<0.001; two-way ANOVA with a Bonferroni post hoc test. Total and HMW adiponectin levels were not comparable across the different manufacturers kits (eg SCD control mice: total adiponectin = 1.2 + 0.2 µg/ml; HMW adiponectin = 3.6 + 1.3 µg/ml) so these data are presented as arbitrary units.

4.5.3 CoPP administration promotes divergent effects on adipocyte size and inflammatory and adiponectin gene expression profiles in white adipose tissue

Having established that CoPP treatment increased circulating adiponectin levels in obese mice we next examined the impact of CoPP administration on adiponectin expression and inflammation in WAT. Histological analysis of epididymal adipose tissue from obese CoPP-treated mice revealed a significant reduction in adipocyte size compared to control mice (Fig 4.3a & b). It seems likely that this is also linked to the reduction in weight gain, as highlighted by correlations between adipocyte size and body weight gain as well as insulin responsiveness (Fig 4.3c & d). As expected, CoPP administration promoted robust induction of HO-1 gene and protein in epididymal adipose tissue from lean and obese mice (Fig 4.3e & f). Adiponectin gene (*Adipoq*) expression trended towards a reduction in obese verses lean mice (reduced by 40%) but this was not significant, probably due to high variability (Fig 4.3g). Perhaps surprisingly, in light of other publications [217, 237, 241, 242, 272, 279, 306] and the effects of CoPP on circulating adiponectin levels, CoPP administration reduced *Adipoq* expression by 30-40%, and this reached significance in the obese mice (Fig 4.3g). We also examined the effects of CoPP administration on markers of immune cell infiltration and inflammation, which has been widely reported to play a causative role in the aetiology of obesity induced adipose tissue dysfunction and insulin resistance [204, 308-311]. CoPP administration increased the expression of markers of immune cell infiltration (*F480*, *Cd68* and *Cd11b*) and inflammation (*Tnfα* and *Mcp-1*) in both lean and obese mice (Fig 4.3h-k). Similar results were observed following analysis of a selection of genes from subcutaneous adipose tissue from obese mice, with CoPP administration resulting in robust (15-fold) induction of *Ho-1* expression, reducing *Adipoq* expression (by 60%), and inducing *F480* (3-fold) (Supp Fig 4.9 a-c) demonstrating these effects are not specific to epididymal fat but more likely reflect CoPP-induced changes throughout WAT.

Fig 4.3

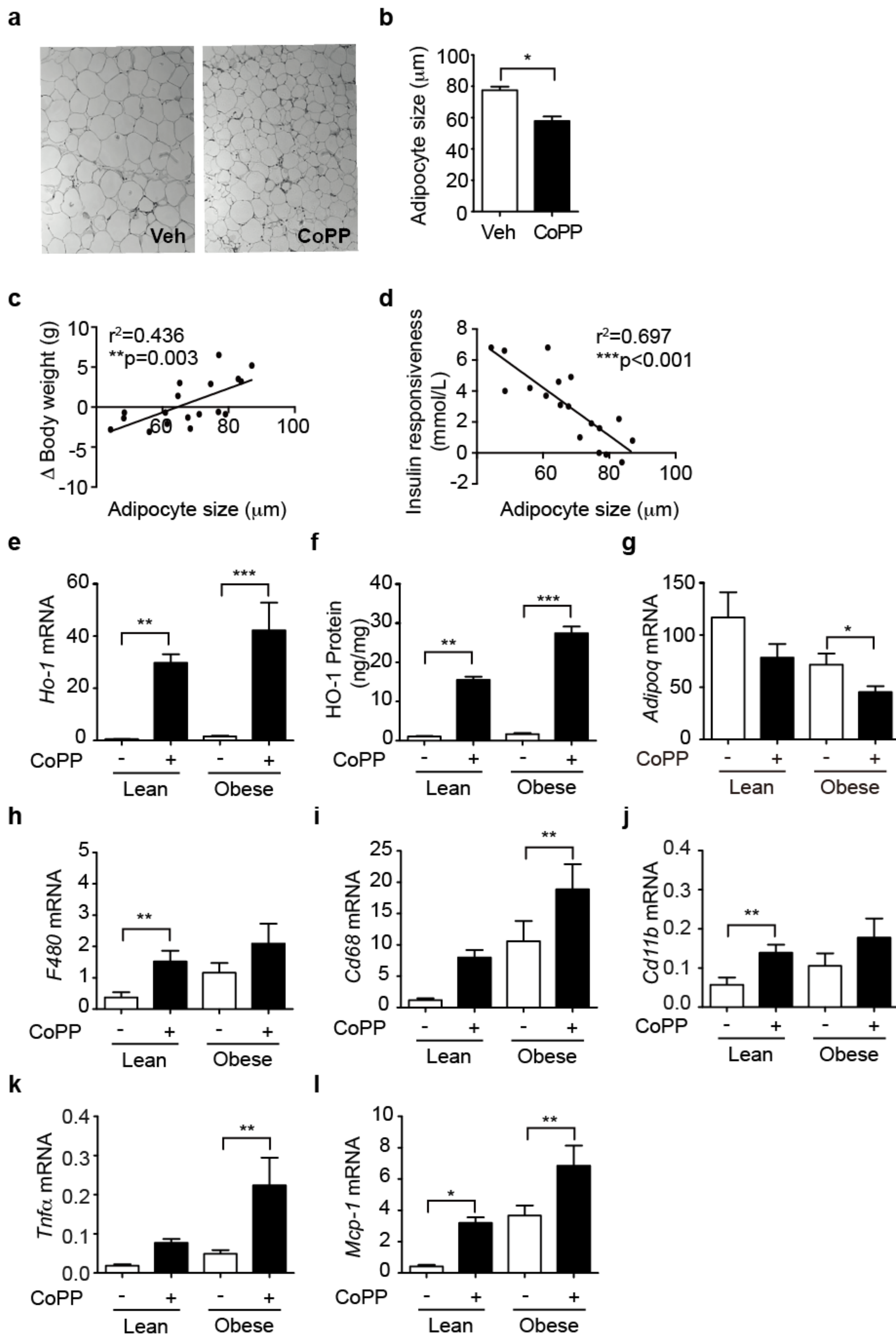

Fig 4.3 - Divergent effects of CoPP on adipocyte size and inflammatory and adiponectin gene expression profiles in epididymal adipose tissue. Lean and obese mice were maintained on chow or HFD respectively and received weekly administration of vehicle (veh) or CoPP (3 mg/Kg) for 6 weeks. **(a)** Histological and **(b)** quantitative analysis of adipocyte size in epididymal adipose tissue in obese mice. Correlations between **(c)** Δ body weight and adipocyte size and **(d)** insulin responsiveness and adipocyte size in obese mice. HO-1 **(e)** gene and **(f)** protein levels in epididymal adipose tissue from lean and obese mice. Expression of **(g)** *Adipoq*, **(h)** *F480*, **(i)** *Cd68*, **(j)** *Cd11b*, **(k)** *Tnfα* and **(l)** *Mcp-1* in epididymal adipose tissue from lean and obese mice (n≥8 for all groups). *p<0.05; **p<0.01; ***p<0.001; two-way ANOVA with a Bonferroni post hoc test.

4.5.4 CoPP administration promotes divergent effects on hepatic inflammatory gene expression and steatosis

Having established that CoPP increased the inflammatory gene signature in adipose tissue from lean and obese mice we next explored the effects of CoPP administration on similar parameters in the liver. CoPP treatment induced *Ho-1* expression at the gene and protein level in liver (Fig 4.4a & b) although this was relatively modest compared with the levels of induction seen in epididymal adipose tissue (typically 5-10 fold less). Hepatic expression levels of *F480* and *Cd68*, two macrophage cell-surface markers, were increased by obesity and by CoPP whilst hepatic expression of *Cd11b*, a neutrophil marker, was increased only by CoPP (Fig 4.4c-e). In contrast, hepatic expression of *Tnfα* and *Mcp-1* was increased only by obesity (Fig 4.4f & g). Under no conditions did CoPP promote any significant reduction in hepatic inflammatory gene expression, in either lean or obese mice. This lack of any overt beneficial effect of CoPP on hepatic gene expression profiles is even more surprising when considered in the context of obesity-associated hepatic steatosis. Histological and quantitative analysis of hepatic lipid accumulation in obese mice revealed a significant reduction in CoPP-treated mice (Fig 4.4h-j). Furthermore, the improvement in hepatic steatosis correlated with body weight gain, insulin responsiveness and adipocyte size (Fig 4.4k-m).

Fig 4.4

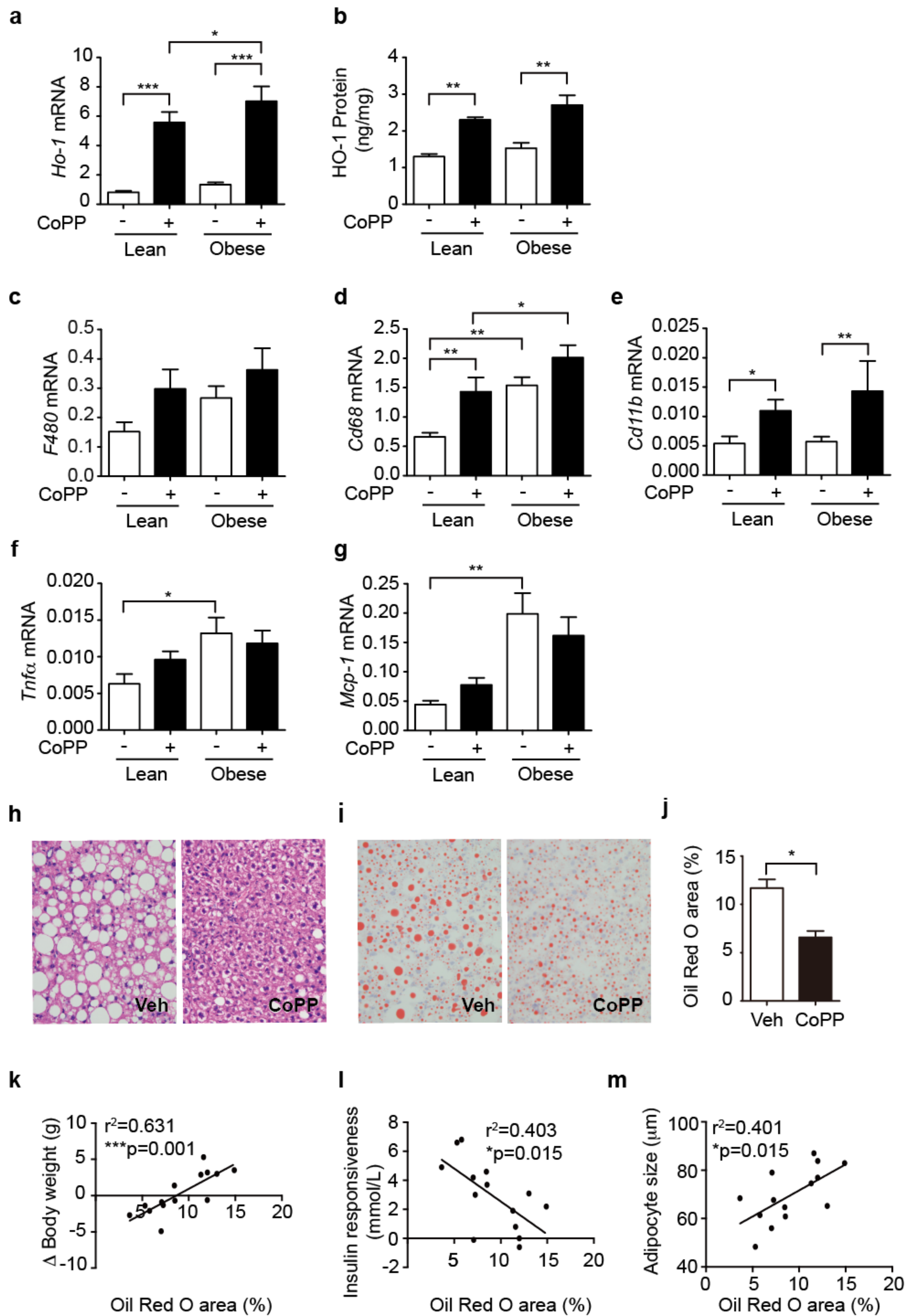

Fig 4.4 – Divergent effects of CoPP on hepatic inflammatory gene expression and steatosis. Lean and obese mice were maintained on chow or HFD respectively and received weekly administration of vehicle (veh) or CoPP (3 mg/Kg) for 6 weeks. HO-1 **(a)** gene and **(b)** protein levels in liver from lean and obese mice. Expression of **(c)** *F480*, **(d)** *Cd68*, **(e)** *Cd11b*, **(f)** *TnfR* and **(g)** *Mcp-1* in liver from lean and obese mice. **(h)** H&E and **(i)** Oil Red O staining showing hepatic lipid accumulation and **(j)** quantitation in obese mice. Correlations between **(k)** Δ body weight, **(l)** insulin responsiveness and **(m)** adipocyte size with Oil red O stain. (n ⩾ 8 for all groups). *p<0.05; **p<0.01; ***p<0.001; two-way ANOVA with a Bonferroni post hoc test.

4.5.5 Co-treatment with SnMP does not prevent the effects of CoPP on food intake and body weight in obese mice

Given the somewhat surprising effects of CoPP on a variety of parameters, particularly the increase in adipose tissue inflammation, we repeated the above experiments in a second cohort of obese mice with the inclusion of an additional treatment arm which involved co-treatment of CoPP-treated mice with the HO-1 inhibitor SnMP. Co-treatment with SnMP did not prevent the reduction in food intake by CoPP (Fig 4.5a). Consistent with this, co-treatment with SnMP did not prevent the effect of CoPP on weight change (Fig 4.5b), with weight gain in both CoPP and CoPP+SnMP mice being significantly reduced compared to that in control mice. These effects suggest that the CoPP-induced reduction in food intake and body weight gain are independent of changes in HO-1 activity.

Fig 4.5

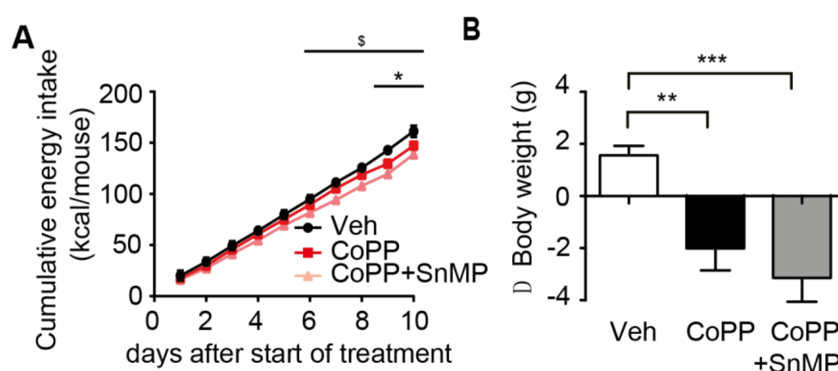

Fig 4.5 - SnMP co-treatment does not prevent the effects of CoPP on food intake and body weight in obese mice. Obese mice were maintained on a HFD and were administered CoPP (3 mg/Kg; 1 time/week) alone or in combination with SnMP (20 mg/Kg; 3 times/week) or vehicle (veh) for 6 weeks such that all mice were treated 3 times/week. **(a)** Cumulative energy intake over the

first 10 days of treatment (n ⩾ 7 for all groups). *p<0.05, vehicle vs CoPP; $^{\$}$p<0.05, vehicle vs CoPP+SnMP; one-way ANOVA with Tukey's post hoc test. **(b)** Change in body weight after 6 weeks (n ⩾ 7 for all groups). **p<0.01; ***p<0.001; one-way ANOVA with Tukey's post hoc test.

4.5.6 Co-treatment with SnMP does not prevent the effects of CoPP on insulin responsiveness but blunts the effect on circulating adiponectin levels

As before, treatment with CoPP did not affect glucose tolerance and co-treatment with SnMP was also without effect (Fig 4.6a). CoPP treatment improved insulin tolerance and this was also unaffected by co-treatment with SnMP (Fig 4.6b). The improvement in insulin responsiveness correlated inversely with the change in body weight (Fig 4.6c). These findings suggest that CoPP improves insulin responsiveness by a mechanism that is associated with the change in body weight that is independent of HO-1 activity.

We next examined the impact of co-treatment with SnMP on the effects of CoPP on circulating adiponectin profiles. In contrast to the lack of effect of co-treatment with SnMP on food intake, body weight and insulin responsiveness the effects of CoPP on circulating levels of total and HMW adiponectin were blunted by co-treatment with SnMP (Fig 4.6c & d). As before, neither total nor HMW adiponectin levels correlated with body weight change (r =-0.285, p=0.188 for both). These findings suggest that CoPP may increase circulating adiponectin levels by a mechanism that is, at least in part, dependent on HO-1 activity.

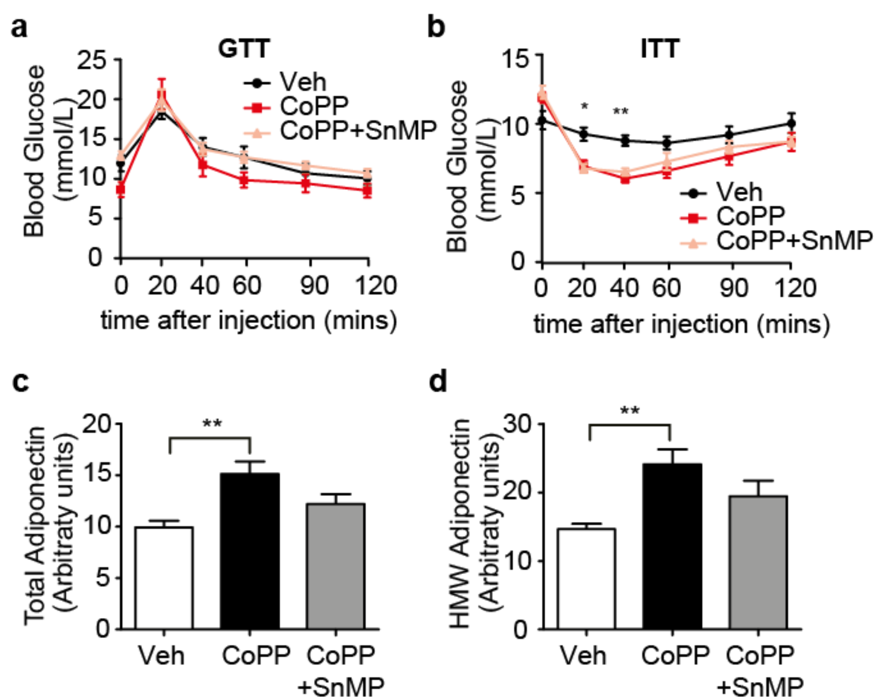

Fig 4.6 - SnMP co-treatment does not prevent the CoPP-mediated improvement in insulin responsiveness but blunts the effect on circulating adiponectin levels. Obese mice were maintained on a HFD and were administered CoPP (3 mg/Kg; 1 time/week) alone or in combination with SnMP (20 mg/Kg; 3 times/week) or vehicle (veh) for 6 weeks such that all mice were treated 3 times/week. **(a)** Glucose tolerance test (GTT) after 5 weeks and **(b)** ITT after 6 weeks of treatment (n ⩾ 7 for all groups). Circulating levels of **(c)** total and **(d)** HMW adiponectin after 6 weeks of treatment (n ⩾ 7 for all groups; data are presented in arbitrary units because total and HMW adiponectin were determined using ELISA kits from different manufacturers making absolute values incomparable). *p<0.05; **p<0.01; one-way ANOVA with Tukey's post hoc test. Total and HMW adiponectin levels were not comparable across the different manufacturers kits (eg SCD control mice: total adiponectin = 1.2 + 0.2 µg/ml; HMW adiponectin = 3.6 + 1.3 µg/ml) so these data are presented as arbitrary units.

4.5.7 Co-treatment with SnMP ameliorates the effects of CoPP on inflammatory gene expression profiles in white adipose tissue

Next we investigated whether co-treatment with SnMP altered CoPP-affected gene expression profiles in adipose tissue in obese mice. Co-treatment with SnMP typically has no effect or even exacerbates CoPP induction of HO-1 *in vitro* thus it was surprising that co-treatment with SnMP in

obese mice actually reduced induction of HO-1 at the mRNA and protein level in epididymal adipose tissue (Fig 4.7a & b). This effect of SnMP co-treatment provided the first clear evidence of an effect of SnMP co-treatment. To investigate the effect on HO-1 activity more directly we measured *Ferritin* expression, as this is induced to metabolise the iron released as a by-product of the HO-1 catalysed breakdown of heme [213]. CoPP promoted a 3-4 fold increase in *Ferritin* expression and this was reduced by around 60% with the SnMP co-treatment (Fig 4.7c), providing further evidence of the efficacy of the SnMP co-treatment.

SnMP co-treatment promoted amelioration of inflammatory gene induction in all cases, except for *Cd68* (Fig 4.7d-h). Whilst the reason for this discrepancy is unclear it is noteworthy that CoPP did not increase *Cd68* expression (which contrasts to all the other inflammatory genes in this and the previous cohort) and that *Cd68* levels in the obese mice in this cohort (which were heavier/fatter than in the first cohort) were similar to those observed in the obese CoPP-treated mice in the first cohort. Regardless, these data show that SnMP co-treatment prevented the effects of CoPP on inflammatory gene expression. *Adipoq* expression was dramatically reduced by CoPP in this cohort (by 60%) and co-treatment with SnMP appeared to partially prevent this effect (Fig 4.7i).

In an attempt to better understand the potential molecular pathways responsible for mediating these effects we performed correlation analyses of select parameters. First, we explored the association between *Ferritin* (a surrogate for local HO-1 activity) and *Tnfα* (a known repressor of adiponectin expression) [288]. *Ferritin* and *Tnfα* expression showed a positive correlation (Fig 4.7j), strongly supporting the hypothesis that increased local HO-1 activity contributed to the increase in *Tnfα* expression. As expected, subsequent analysis of *Tnfα* and *Adipoq* expression revealed an inverse correlation (Fig 4.7k). Hence, it follows that increased HO-1 activity may contribute to the reduction in *Adipoq* expression. However, correlation analysis of *Ferritin* and *Adipoq* expression does not directly support this ($r=-0.292$; $p=0.199$) and the effects of SnMP co-treatment on adiponectin expression were modest (Fig 4.7i). Taken together these findings suggest that the direct effects of local changes in HO-1 activity on *Adipoq* expression are likely to be modest and the changes in *Adipoq* expression are more likely to reflect the changes in the inflammatory milieu.

As before, analysis of a selection of genes from subcutaneous adipose tissue produced similar results (Supp Fig 4.10). Interestingly, co-administration with SnMP did not reduce CoPP-induced *Ho-1* expression (Supp Fig 4.10a) but did prevent the induction of *F480* and *Cd68* (Supp Fig 4.10b-c) whilst only partially reversing the inhibition of *Adipoq* expression (Supp Fig 4.10d). These results confirm the major effects of CoPP are common across adipose tissues whilst also demonstrating subtle differences in the magnitude of these effects and the response to co-treatment with SnMP.

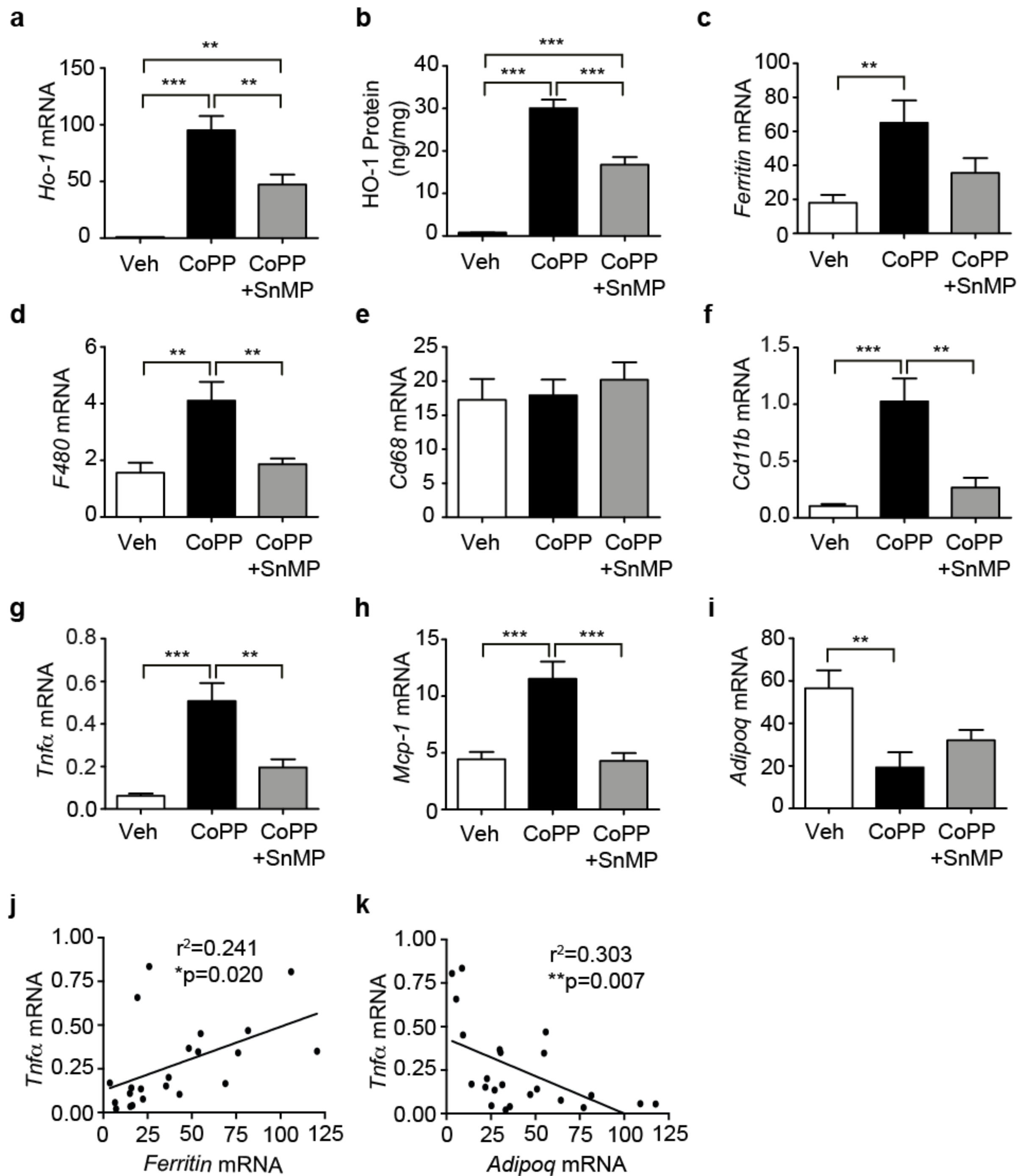

Fig 4. 7 - SnMP co-treatment ameliorates the effect of CoPP on inflammatory gene expression profiles in epididymal adipose tissue. Obese mice were maintained on a HFD and were administered CoPP (3 mg/Kg; 1 time/week) alone or in combination with SnMP (20 mg/Kg; 3 times/week) or vehicle (veh) for 6 weeks such that all mice were treated 3 times/week. HO-1 **(a)** gene and **(b)** protein levels in epididymal adipose tissue. Expression of **(c)** *Ferritin*, **(d)** *F480*, **(e)** *Cd68*, **(f)** *Cd11b*, **(g)** *Tnfα*, **(h)** *Mcp-1* and **(i)** *Adipoq* in epididymal adipose tissue. Correlations between *Tnfα* and **(j)** *Ferritin* or **(k)** *Adipoq*. ($n \geqslant 7$ for all groups). *$p<0.05$; **$p<0.01$;

***p<0.001; a – i one-way ANOVA with Tukey's post hoc test; j – k two-way ANOVA with Bonferroni's post hoc test.

4.5.8 Co-treatment with SnMP does not prevent the effects of CoPP on hepatic steatosis

Co-treatment with SnMP promoted a slight, but significant, increase in hepatic induction of *Ho-1* but this did not translate to an increase in hepatic HO-1 protein (Fig 4.8a & b). The effects of CoPP and therefore co-administration of SnMP on hepatic *Ferritin* and inflammatory gene expression profiles were relatively modest and typically failed to reach statistical significance (Fig 4.8c-g). Our previous findings revealed the most striking hepatic effect of CoPP to be the reduction in steatosis, which correlated with change in body weight and insulin responsiveness. These effects were all maintained upon co-treatment with SnMP (Fig 4.8h-k). Taken together with our findings from the first cohort, these observations suggest that CoPP may induce modest hepatic inflammation but this is not associated with the degree of hepatic steatosis.

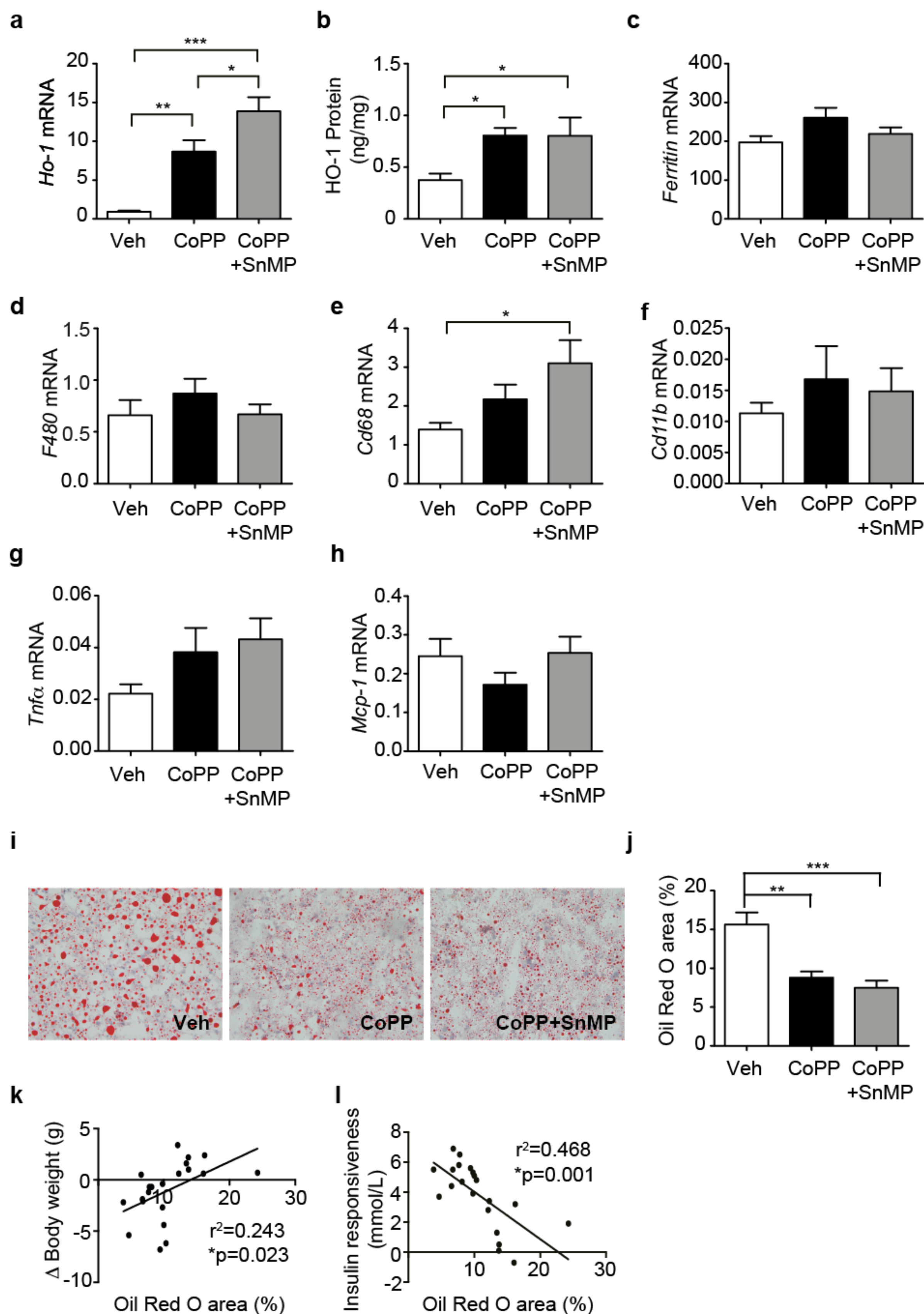

Fig 4. 8 – Co-treatment with SnMP does not prevent the effect of CoPP on hepatic steatosis. Obese mice were maintained on a HFD and were administered CoPP (3 mg/Kg; 1 time/week) alone

or in combination with SnMP (20 mg/Kg; 3 times/week) or vehicle (veh) for 6 weeks such that all mice were treated 3 times/week. HO-1 **(a)** gene and **(b)** protein levels in liver. Expression of **(c)** *Ferritin*, **(d)** *F480*, **(e)** *Cd68*, **(f)** *Cd11b*, **(g)** *Tnfα*, and **(h)** *Mcp-1*. **(i)** Oil Red O stain and **(j)** quantitation of hepatic lipid accumulation. Correlations between **(k)** Δ body weight or **(l)** insulin responsiveness and hepatic steatosis. (n ⩾ 7 for all groups). *p<0.05; **p<0.01; ***p<0.001; a – j one-way ANOVA with Tukey's post hoc test; k – l two-way ANOVA with Bonferroni's post hoc test.

4.6 Discussion

In the current report we aimed to characterise the effects of the widely-used HO-1 inducer CoPP on WAT and adiponectin in the context of a mouse model of diet-induced obesity. Our results demonstrate that, at least under the experimental conditions employed, CoPP-treatment results in diverse, often counterintuitive effects and only some of these appear to be dependent on increased HO-1 activity based on sensitivity to co-treatment with SnMP. Of particular interest, we found that CoPP administration increased the inflammatory gene signature in WAT and reduced *Adipoq* expression yet, consistent with other reports [217, 237, 241, 242, 272, 279, 306], increased circulating adiponectin levels. Co-treatment with SnMP prevented the increase in inflammation (Fig 4.7), had no significant effect on *Adipoq* expression (Fig 4.7i) yet halved the effect of CoPP on circulating adiponectin (Fig 4.6c). In addition, CoPP-treatment also prevented weight gain in obese mice and this correlated with improvements in insulin responsiveness, adipocyte size and hepatic steatosis, none of which were affected by SnMP co-treatment. Taken together, these results demonstrate that CoPP promotes divergent effects that are both HO-1-dependent and independent. Importantly, when considered alongside other reports [217, 237, 241, 242, 272, 279, 306], they support a model whereby the CoPP-induced increase in circulating adiponectin is unlikely to reflect increased production from WAT but is more likely to be due to increased adiponectin production from other sites, possibly bone marrow adipose tissue (BMAT) [239, 243, 304].

Numerous investigations in a variety of murine models of obesity have established that CoPP administration results in reduced weight gain and increased circulating adiponectin levels [217, 237, 241, 242, 272, 279, 306]. Many of these studies also provide evidence to support a role for HO-1 in these effects as they are typically prevented or blunted by co-treatment with SnMP [217, 237, 241]. Perhaps surprisingly then, the major site(s) of action and molecular details of these effects

remain poorly understood as does the precise role of HO-1. Indeed, reports involving genetic or pharmacological approaches to manipulate the 'HO-1 axis' provide results that highlight the complexity of the axis and the need for considered interpretation and subsequent integration of the findings [204, 281, 282].

In this context, given the absence of any compelling evidence to either prove or disprove a direct effect of HO-1 induction on adiponectin production from mature adipocytes we performed a series of investigations using CoPP, or hemin, that show that induction of HO-1 does not stimulate increased adiponectin production from mature human adipocytes [288]. These observations are in stark contrast to a number of studies that report that chronic induction of HO-1 during the process of adipogenesis results in increased adiponectin production [230, 238, 239, 243, 291]. In an attempt to reconcile these discordant findings we performed a comprehensive series of investigations to characterise the effects of chronic induction of HO-1 on adipogenesis and adiponectin production from human preadipocytes/adipocytes. Consistent with the literature, we found induction of HO-1 with CoPP, or hemin, compromised adipogenesis in a dose-dependent manner. However, under no conditions did we observe increased adiponectin expression or secretion (Yang et al, under review, SREP-16-40083). Moreover, studies that aimed to characterise the mechanistic processes responsible for these effects, involving SnMP or HO-1 knockdown, demonstrated these effects were independent of HO-1 activity and were more likely mediated by other targets, possibly downstream effectors of NRF2 (Yang et al, under review, SREP-16-40083).

A fundamental difference between our investigations and studies that suggest a key role for HO-1 is the cell-type. Whilst we used cells derived from subcutaneous WAT (either the SGBS cell strain or primary cells) studies that have reported a HO-1-dependent increase in adiponectin production have been performed almost exclusively in mesenchymal stem cells derived from bone marrow [230, 238, 239, 243, 291]. Thus, it remains possible that the processes governing adiponectin production are distinct in these discrete cell types. Indeed, evidence demonstrates that BMAT and, more specifically, adiponectin production from BMAT is regulated differently to that from classic WAT [304]. Carefully controlled studies investigating the effects of HO-1 induction on adiponectin production from preadipocytes/adipocytes derived from the different depots are required to investigate whether intrinsic differences between cell-types contribute to the differences described above.

Notwithstanding the observed differences in the *in vitro* systems outlined above key fundamental differences are also apparent when considering the findings from *in vivo* models. For example, in the current study we used a treatment regime used by others [217, 306] but made several contrasting observations. Like others [217, 237, 241, 242, 272, 279, 306], we found that CoPP administration

prevented weight gain in HFD-induced obese mice. However, this effect was not prevented by co-treatment with SnMP, implying HO-1-independence, whereas the effect was abolished or reduced by SnMP in studies of genetically (ob/ob mice) [217, 237] or HFD-induced [306] obese mice or rats [287]. The reasons for the differential sensitivity to SnMP is unclear. One possibility is that the mechanism by which CoPP affects weight gain, and other parameters, in the context of obesity may differ over time. Obesity is a progressive state with temporal alterations in stressors of specific cell-types and tissues, as evidenced by the dynamics of adipose tissue inflammation and development of ectopic lipid accumulation and insulin resistance [308, 312-314]. Thus, it seems reasonable to consider that the mechanisms by which pharmacological agents such as CoPP, which are known to be pleiotropic rather than specific [204], may mediate similar effects by alternate mechanisms that are context-dependent.

Given that reduced weight gain is a common effect of CoPP, or other HO-1 inducers such as heme [217, 237, 241, 242, 272, 279, 306], it is important that effects on weight are taken into account when considering related metabolic effects. Surprisingly, this seldom appears to be the case. In the current study we specifically explored whether the observed effects of CoPP correlated with its effects on weight. Perhaps reassuringly, we found that adipocyte size, liver steatosis and insulin responsiveness all correlated with change in body weight during the 6 week intervention and that none of these effects were sensitive to SnMP. Together, these findings argue that the metabolic improvements mediated by CoPP-treatment under these experimental conditions are all associated with the prevention in weight gain and are independent of HO-1 activity *per se*. The mechanism(s) by which CoPP promotes a reduction in weight gain may include a reduction in food/energy intake, as reported here and elsewhere [220], and or an increase in metabolic rates [279], possibly via increased mitochondrial biogenesis and 'browning' of white preadipocytes/adipocytes [315]. With respect to the latter, we did not observe any increase in *Ucp-1* expression, a marker of brown adipocytes, in epididymal or subcutaneous adipose tissue from CoPP-treated mice (Supp Fig 4.11a). Perhaps surprisingly, to the best of our knowledge, no study has described the effects of CoPP-treatment on WAT or adiponectin expression in this tissue. Moreover, we found that CoPP-treatment promoted an increase in the inflammatory signature in WAT and that this was independent of obesity. This effect was abolished by co-treatment with SnMP, which did not affect weight gain or insulin responsiveness, indicating that this inflammatory effect was dependent on HO-1 activity. Whilst these findings are in stark contrast to observations made in BMAT [217, 237, 241, 242, 272, 279, 306] they are supported by findings from genetic models [204, 281, 316]. Evidence indicates that conditional deletion of HO-1 from macrophages is sufficient to ameliorate diet induced insulin resistance and adipose tissue inflammation [204]. Indeed, myeloid-specific haploinsufficiency of HO-1 was sufficient to reduce infiltration of macrophages into WAT [316].

Thus, we propose that CoPP elevates HO-1 activity in myeloid-derived cells and that this subsequently results in increased macrophage infiltration and inflammation in WAT. We observed comparable, albeit more moderate, effects in liver and suggest that these are explained by a similar mechanism [204, 316]. The role of adipose tissue inflammation in the aetiology of insulin resistance is far from clear [308, 317] and our observations provide further evidence that adipose tissue inflammation does not correlate with changes in insulin sensitivity.

Adipoq expression in both epididymal and subcutaneous WAT was reduced by CoPP-treatment. This contrasts with the findings in BMAT [230, 238, 239, 243, 291] highlighting additional differences. Our correlation analyses suggest the reduction in *Adipoq* expression in WAT is associated with the increased WAT inflammation, which in turn correlated with *Ferritin*, a marker of increased HO-1 activity in WAT. However, there was no correlation between *Adipoq* expression and *Ferritin* and the effect of SnMP on *Adipoq* expression was, at best, modest. The underlying reasons for these discrepancies are currently obscure. In contrast, it is clear that the changes in *Adipoq* expression in WAT do not reflect the changes in circulating adiponectin levels, which are increased by CoPP and are blunted by co-treatment with SnMP. Whilst changes in *Adipoq* expression are not always reflected by similar changes in its secretion or the circulating levels, we are aware of no precedent where *Adipoq* expression is reduced (by 30-60%) and circulating levels are increased (by 30-50%) [252, 258]. Hence, we propose that adiponectin production from WAT is reduced following CoPP-treatment but this is not reflected in the circulating adiponectin levels due to opposite changes in adiponectin production from alternate sites. A body of evidence indicates that CoPP, or other HO-1 inducers, promotes adiponectin production from BMAT [230, 238, 239, 243, 291]. Moreover, increased adiponectin production from BMAT has been shown to underpin the increased circulating adiponectin levels reported following caloric restriction [304]. Thus, it seems reasonable to propose that the increase in circulating adiponectin levels are mediated by increased production from BMAT. Future studies are required to investigate such a model and, if correct, elaborate the molecular mechanisms responsible for the divergent effects in the different adipose tissues.

Intriguingly, in the current study the CoPP-mediated improvement in insulin responsiveness correlated with the change in weight but occurred in the absence of any enhancement in glucose tolerance. One possible explanation for these results would be if CoPP-treatment promoted a defect in insulin production. Whilst measurement of circulating insulin levels during the GTT failed to reveal such a defect (data not shown) it remains feasible that CoPP may result in increased islet inflammation, similar to the scenario described above in WAT, thereby compromising the ability of the insulin-producing β-cells to process precursor insulin efficiently, leading to secretion of immature, pro-insulin which has limited potency compared to mature insulin [321]. It may be

anticipated that the effects of CoPP on the ITT (insulin responsiveness) and GTT (glucose tolerance) would be enhanced if the tests were performed within the 24 h period post-CoPP administration, when food intake was significantly reduced although we, nor others [210, 217], have performed such investigations at this time point.

In summary, in the present study we have shown that chronic administration of CoPP in obese mice promotes a number of divergent effects and that only some of these were dependent on HO-1 activity. CoPP increased circulating adiponectin levels, by a mechanism that was at least partly dependent on HO-1 activity, despite also promoting increased inflammation and reduced adiponectin expression in WAT. Elaborating the underlying mechanisms may provide novel therapeutic strategies.

4.7 Acknowledgements

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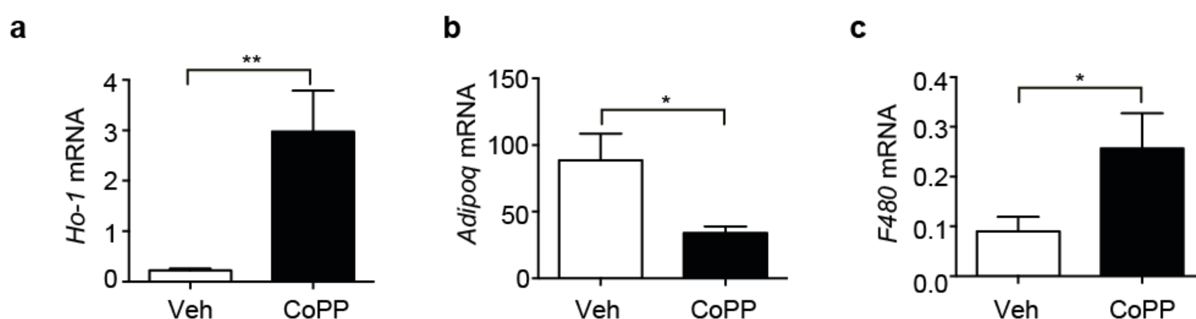
4.8 Author Contributions Statement

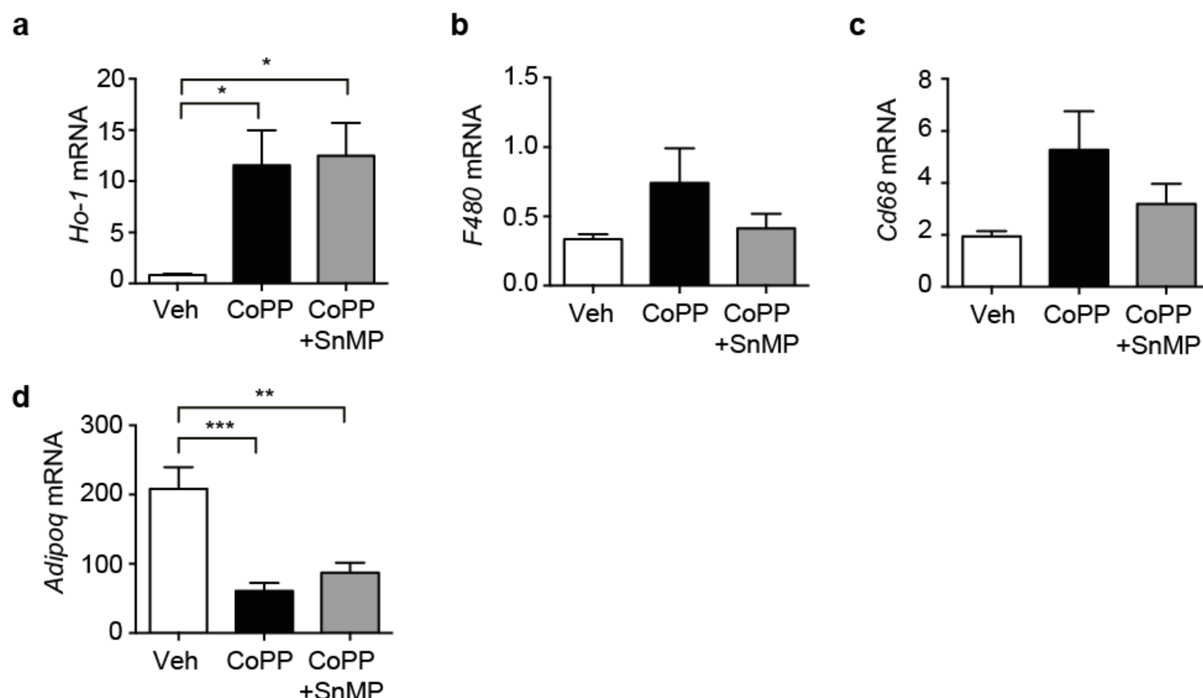
M.Y. performed experiments, interpreted data, and drafted a manuscript. C.N. performed experiments. S.K. and A.C.B. performed experiments and interpreted data. J.L.B. designed the study, interpreted data, and edited the manuscript. J.P.W. designed the study, interpreted data, wrote and edited the manuscript, and is the guarantor of the article.

Sup Fig 4.9.



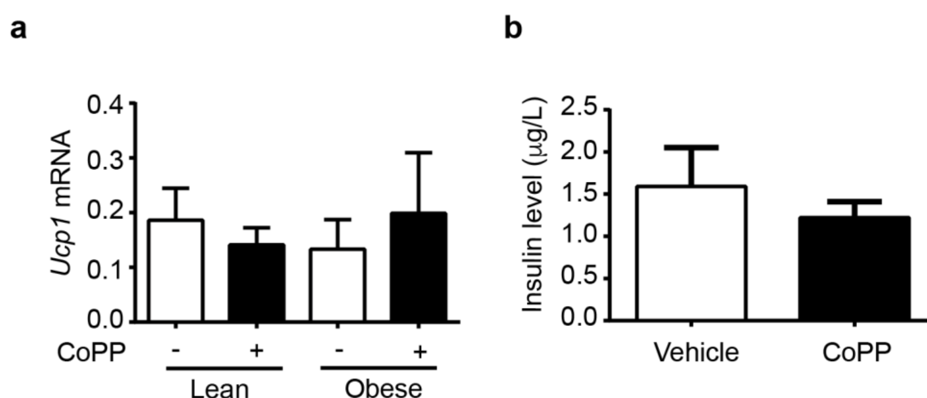
Supp Fig 4.9 – Effect of CoPP administration on gene expression profiles in subcutaneous adipose tissue. Obese mice were maintained on a HFD and treated weekly with CoPP (3 mg/Kg) for 6 weeks. Expression of **(a)** *Ho-1*, **(b)** *Adipoq* and **(c)** *F480* in subcutaneous adipose tissue after 6 weeks. n=12 for all groups; *p<0.05; **p<0.01; two-way ANOVA with Bonferroni's post hoc test.

Sup Fig 4.10



Supp Fig 4.10 - SnMP co-treatment ameliorates the effect of CoPP on inflammatory gene expression profiles in subcutaneous adipose tissue. Obese mice were maintained on a HFD and were administered CoPP (3 mg/Kg; 1 time/week) alone or in combination with SnMP (20 mg/Kg; 3 times/week) or vehicle (veh) for 6 weeks such that all mice were treated 3 times/week. Expression of **(a)** *Ho-1*, **(b)** *F480*, **(c)** *Cd68* and **(d)** *Adipoq* in subcutaneous adipose tissue. (n ⩾ 7 for all groups). *p<0.05; **p<0.01; ***p<0.001; one-way ANOVA with Tukey's post hoc test.

Supp Fig 4.11



Supp Fig 4.11 – UCP1 gene expression and insulin levels. Obese mice were maintained on a HFD and were administered CoPP (3 mg/Kg; 1 time/week). Expression of **(a)** *Ucp1* in subcutaneous fat pad, **(b)** insulin levels in CoPP-treated HFD animals.

Chapter 5:

General Discussion

5.1 Overview

The prevalence of obesity and metabolic and cardiovascular diseases has been growing at an alarming rate globally, and current therapies have brought limited benefits [322]. Thus, it is essential to develop new strategies to reduce effects of these diseases. Adiponectin is a beneficial adipokine with insulin-sensitising, anti-inflammatory and cardioprotective properties [58, 253, 254]. Hypoadiponectinemia is implicated in the aetiology of obesity-associated diseases making therapeutic strategies to increase adiponectin attractive [252]. Heme Oxygenase-1 is a rate-limiting enzyme that catalysts the degradation of heme into three by-products, carbon monoxide (CO), iron and biliverdin, which is subsequently converted to bilirubin [261]. In preclinical models, induction of HO-1 has been reported to increase circulating adiponectin levels, concomitant with a decrease in inflammatory cytokines, prompting the proposal of a "HO-1 – adiponectin axis" [217, 225, 227, 230, 239, 241, 264-268]. However, contradictory findings showed that a classic transgenic approach to increased HO-1 in adipose tissue failed to reverse the HFD-induced decrease in adiponectin [281]. Work in this area reported by Jais et al. continues to put HO-1 center-stage in obesity and insulin resistance, but as a driver rather than a brake of obesity-associated inflammation [204]. In keeping with findings from the current study, they found no evidence of a major role for HO-1 in the adipocyte or muscle or pancreatic β-cells [204]. The aim of this thesis was to determine the underlying mechanisms and the relationship between HO-1, insulin sensitivity, adiposity, adiponectin and inflammation. Key findings are outlined below:

1. Inducers of HO-1 acutely (24-48 h) increase HO-1 mRNA, protein and activity in mature adipocytes.
2. Acute induction of HO-1 does not enhance or rescue adiponectin production in healthy or TNFα-treated mature adipocytes.
3. Acute induction of HO-1 does not ameliorate TNFα-stimulated expression and secretion of pro-inflammatory adipocytokines in mature adipocytes.
4. Chronic CoPP or hemin treatment (throughout differentiation) increase HO-1 mRNA and protein, as well as other Nrf2 related gene expressions in human preadipocytes.
5. Chronic CoPP or hemin treatment decrease lipid accumulation, interferes with adipogenesis, decreases adiponectin production and increases inflammatory tone in human preadipocytes
6. Co-treatment with a HO-1 inhibitor (SnMP) or HO-1 siRNA failed to reverse the effects of chronic CoPP or hemin treatment in human preadipocytes.
7. CoPP-treatment of obese mice result in decreased food intake, body weight gain and adipocyte size as well as enhanced insulin sensitivity and attenuated liver steatosis.
8. Obese CoPP-treated mice display increased adipose tissue inflammation and reduced adiponectin expression; however, circulating adiponectin levels are increased.

9. Co-administration of a HO-1 inhibitor SnMP in obese mice ameliorated CoPP effects on adipose tissue inflammation and blunted CoPP effects on circulating adiponectin, while other parameters were unchanged.

5.2 General discussion and further directions

During the course of this research, our findings do not support a direct “HO-1 – adiponectin axis” in WAT. They also suggest that WAT is unlikely to be involved in the systemic beneficial effects of CoPP or hemin treatment.

In the earlier sections of this thesis, we aimed to establish the existence of direct “HO-1 – adiponectin axis”. We treated mature adipocytes with widely-used HO-1 inducers CoPP or hemin acutely (24-48 h). We expected an increase in adiponectin production in this experiment. However, our results revealed that HO-1 induction has no direct effect on adiponectin production in human adipocytes *in vitro*. In addition, induction of HO-1 did not ameliorate the effects of TNFα on adiponectin production or inflammatory tone in human adipocytes. These findings do not support a direct “HO-1 – adiponectin axis.” Instead, our results indicated that the association between HO-1 inductions and increased circulating adiponectin levels are most likely to be a chronic or indirect effect. One possibility is that chronic HO-1 inductionmay increase adiponectin indirectly by altering differentiation of preadipocytes. Consistent with this hypothesis, evidence in the literature shows that chronic CoPP treatment throughout the process of differentiation increases adiponectin production, albeit it reduces adipogenesis [238, 242]. It seems unlikely that inhibition of adipogenesis would result in increased adiponectin production. However, we previously reported that adiponectin secretion was highest in immature adipocytes [278]. Thus, it remains possible that chronic CoPP treatment may arrest differentiation at a stage where adiponectin production is higher than in the mature adipocytes. To find out whether chronic CoPP or hemin treatment, across appropriate dose-response curves, could increase adiponectin production at a “specific concentration,” we extended these studies to characterize the dose-dependent effects of chronic CoPP and hemin treatment throughout differentiation of human preadipocytes. To our surprise, results showed that chronic CoPP and hemin treatment inhibited differentiation in a dose-dependent manner, but under no condition was adiponectin production increased. Rather more surprising is that co-treatment with a HO-1 activity inhibitor (SnMP) or HO-1 siRNA failed to reverse these effects. These results strongly suggested that the effects of chronic CoPP and hemin administration in adipocytes are HO-1-independent.

The above findings indicat that induction of HO-1 fails to promote adiponectin production in human adipocytes *in vitro*. In contrast, the majority of preclinical studies suggest that induction of HO-1

increases adiponectin production and improves metabolic status via a "HO-1 – adiponectin axis" [217, 225, 227, 230, 239, 241, 264-268]. To test this ourselves, we performed a series of experiments in preclinical models. We found that CoPP administration to obese mice significantly decreased body weight gain, and this change is significantly correlated with changes in adipocyte size, and liver steatosis. The ITT significantly improves in obese mice following CoPP treatment, but there is no significant change in GTT, which suggests improvements in muscle insulin sensitivity but not necessarily improvements in the liver. Moreover, we found that CoPP-treatment promotes a range of divergent effects on adipose tissue and adiponectin, particularly in obese mice (Table 5.1). One possibility is that the decrease in adiponectin gene expression in adipose tissue that occurs concomitant with an increase in circulating adiponectin levels may reflect effects of CoPP on circulating adiponectin via direct effects on other tissues. Indeed, other studies showing CoPP administration increases adiponectin are mainly from studies involving BMSCs treated with CoPP [238, 242, 243, 294] or EET-agonists [230, 291, 294]. The fundamental difference between the experiments is the use of cells from different origins. Thus, it is tempting to speculate that adiponectin production from cells derived from BMSCs may be regulated differently than in human preadipocytes derived from adipose tissues. Indeed, and consistent with this, it has been reported that caloric restriction increased adiponectin production from bone marrow adipose tissue but not white adipose tissue which supports our proposal [304]. Based on these observations, we propose that CoPP does mediate the increase in circulating adiponectin levels via a mechanism that is at least partly HO-1 dependent, and may involve induction of adiponectin production from BMAT. However, further studies are required to investigate such a model.

		CoPP (1)	Correlation Δbody wt	Hemin	Correlation Δbody wt
	food intake	↓*	UD	↓*	UD
	cumulative food intake	↓*	UD	↓*	UD
	Δbody wt	↓**		↔	
	insulin sensitivity	↑***	***	↔	↔
	circulating adiponectin	↑*	↔	↔	↔
	HMW adiponectin	↑*	↔	↔	↔
	liver steatosis	↓***	**	↔	↔
	adipocyte size	↓***	**		
	fat pad & liver weight	↔	↔	↔	↔
	bilirubin	UD		↔	
	Haem	UD		↔	
epi mRNA	HO-1	↑***		↑*	
	HO-1 protein	↑***		↑***	
	intracelluar adiponectin(Epi)	↔		UD	
	NQO1 &GCLM mRNA	UD		GCLM(↑)	
	adiponectin	↓*		↔	
	pro-inflam cytokines	↑**(TNF, IL-6, CD68, MCP1);↔(CD11b)		↔	
subQ mRNA	HO-1	↑**		↔	
	adiponectin	↓*		↓***	
	pro-inflam cytokines	↑*(F480);↔(MCP1,IL-6)		↔	
liver mRNA	HO-1	↑***		UD	
	HO-1 protein	↑***		↔	
	NQO1 &GCLM mRNA	UD		↔	
	pro-inflam cytokines	↔		UD	
	ferritin	UD		↔	
HO-1 protein	brain	UD		↔	
	pancreas	UD		↑**	
	quad	UD		↔	
	heart	UD		↔	
insulin levels		UD			

Table 5.1 – Effect of CoPP or hemin in obese mice.
UD-Undetermined

Treatment of obese mice with hemin did not result in changes in body weight gain, liver steatosis or insulin responsiveness, which further support the hypothesis that the phenotype induced by CoPP administration is HO-1 activity independent (Table 5.1). Moreover, CoPP administration in obese mice significantly increased inflammatory gene expression and decreased adiponectin gene expression in WAT, which was slightly ameliorated by SnMP treatment (Table 5.2). Hemin treatments showed a similar pattern, but moderate effects on WAT, which suggested that these effects were, at least partly, HO-1 activity dependent. Thus, the *in vivo* results argue against a positive effect of HO-1 activity on adiponectin production in WAT but do confirm that CoPP increases circulating adiponectin levels. This contrasts with the *in vitro* results that argue against the "HO-1 – adiponectin axis," which may due to the fact that different cell types have different response to CoPP. The hepatic effects of both CoPP and SnMP are relatively modest when

compared to those in adipose tissue. The underlying mechanisms remain unclear but may reflect a greater impact on influx of circulating inflammatory cells in adipose tissue (via direct effects on the inflammatory cells) than liver.

		CoPP	CoPP & SnMP
	food intake	↔	↔
	cumulative food intake	↓*(from day7)	↓*from day7
	Δ body wt	↓**	↔
	insulin sensitivity	↑**	↔
	circulating adiponectin	↑**	↔
	HMW adiponectin	↑*	↔
	liver steatosis	↓**	↔
	adipocyte size		
	fat pad& liver weight	↔	↔
	bilirubin	↔	↔
	Haum	↑*	↔
epi mRNA	HO-1	↑***	↓**
	HO-1 protein	↑***	↓***
	intracelluar adiponectin(Epi)	UD	UD
	NQO1&GCLM mRNA	↑***	NQO1(↓**)
	adiponectin	↓**	↔
	pro-inflam cytokines	↑***(TNF,CD11b, MCP-1);↑**(F480);↔CD68	↓**(TNF,F480,CD11b);↓***(MCP-1);↔CD68
	Ferritin	↑**	↔
subQ mRNA	HO-1	↑*	↔
	adiponectin	↓***	↔
	pro-inflam cytokines	↔	↔
liver mRNA	HO-1	↑**	↑*
	HO-1 protein	↑***	↓*
	NQO1&GCLM mRNA	GCLM(↑**)	↔
	pro-inflam cytokines	↔	↔
	ferritin	↔	↔
HO-1 protein	brain	↔	↔
	pancreas	↑***	↓**
	quad	↑***	↓*
	heart	↔	↔
insulin levels		↔	↔

Table 5.2 – Effects of CoPP and SnMP in obese mice.

UD-Undetermined

It is well established that CoPP induces HO-1 by modulating the transcriptional repressors and transcription factors, the downregulation of BACH1 and upregulation of NRF2 [275, 291, 297, 298]. Thus, CoPP is not a direct HO-1 specific inducer. In *in vitro* experiments, we found that the *NQO1* and *GCLM* gene expressions were increased by SnMP or HO-1 siRNA treatment, which suggests that NRF2 activity was increased by SnMP or HO-1 siRNA treatment. Considering the effects of

CoPP and hemin were not reversed by SnMP or HO-1 siRNA treatment, it is possible that HO-1 is not responsible for the effects of CoPP or hemin in human preadipocytes. These findings suggest that the chronic treatment with CoPP or hemin potentially inhibits adipogenesis and adiponectin production by a HO-1-independent mechanism that may be downstream of NRF2. However, our results contradict the literatures that support “HO-1 – adiponectin axis.”

Collectively, *in vitro* studies demonstrated that CoPP treatment does not have any beneficial effect on adipocytes and does not support a direct “HO-1 – adiponectin axis” in WAT. *In vivo* studies showed that CoPP increased circulating adiponectin levels, by a mechanism that was at least partly dependent on HO-1 activity, despite also promoting increased inflammation and reduced adiponectin expression in WAT. Taken together with the current literature, these results suggest that BMAT, not WAT, plays an important role in the effect of HO-1 induction on adiponectin production.

5.3 Conclusions

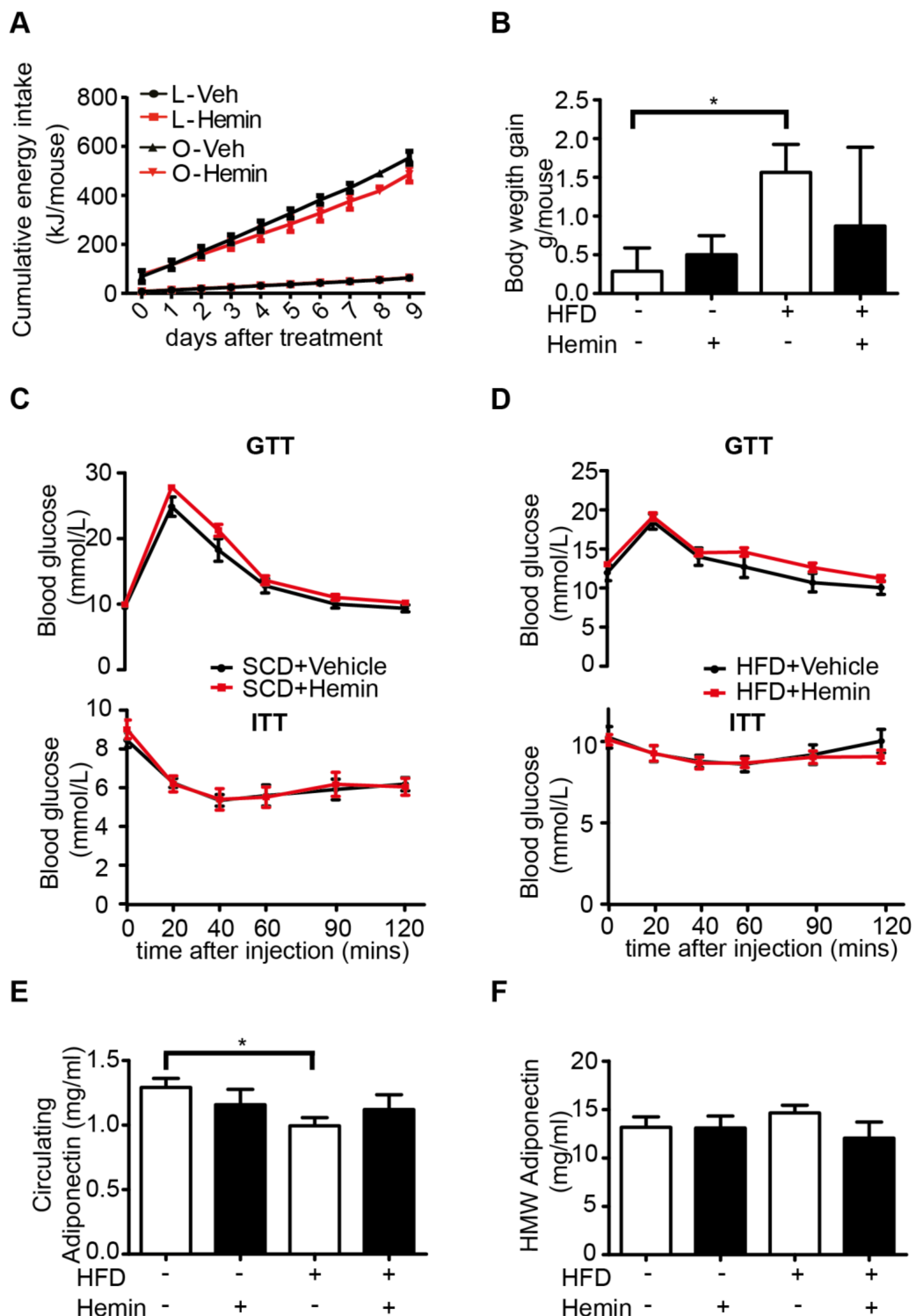
Increasing in circulating adiponectin levels is recognized as an attractive potential target for the treatment of metabolic disorders. Nevertheless, current therapeutic strategies are not suitable due to a series of side effects. Thus it is essential to develop other therapeutic strategies. Despite numerous studies describing the existence of a HO-1-adiponectin axis, there is no direct evidence showing that HO-1 directly induces adiponectin production. The first aim of this study was to investigates whether HO-1 induces adiponectin production directly *in vitro*. Secondly, we investigate whether adipocytes are involved in the systemic beneficial effects of CoPP and hemin treatment. Further, we utilized a preclinical mouse model to investigate whether the beneficial effects of HO-1 induction are, at least partly, dependent on adiponectin. In this project we demonstrated (i) HO-1 induction via CoPP and hemin treatment have no direct effect on adiponectin production in mature adipocytes, (ii) Chronic HO-1 induction via CoPP and hemin treatment compromises adipogenesis in a manner which appears incompatible with enhanced adipocyte function and reduces adiponectin production and (iii) Chronic CoPP administration in obese mice promotes a number of divergent effects, only some of which were dependent on HO-1 activity. Although further studies are required to determine the mechanism for improving metabolic status by CoPP treatment, current studies established that CoPP treatment offer several benefits in the preclinical models although the majority of them are HO-1-independent. This work provides a foundation for future studies so that CoPP may become a new therapeutic strategy for metabolic disorders such as type 2 diabetes and CVD.

5.4 Further directions

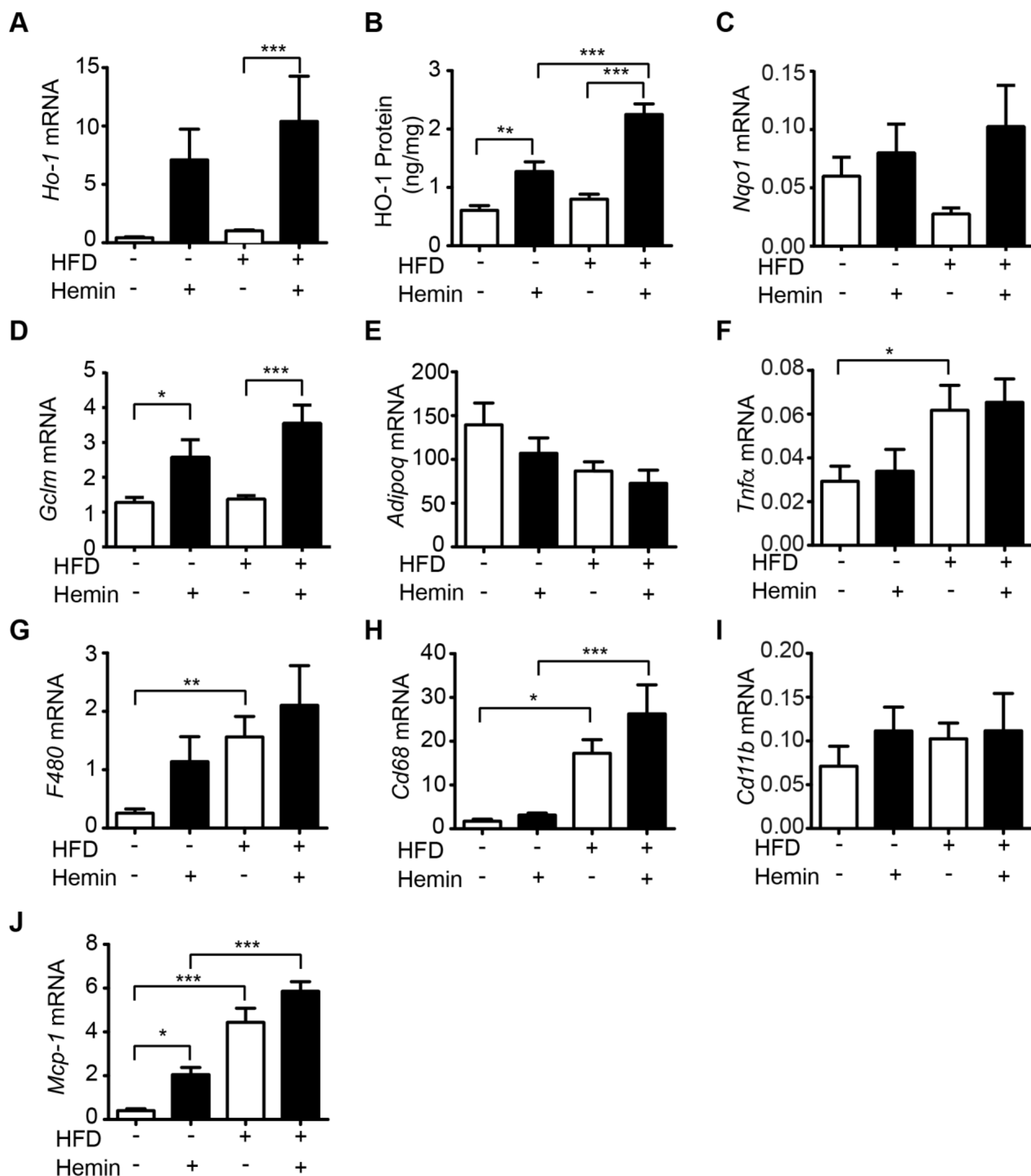
This thesis characterized the relationship between HO-1 and adiponectin, and it has disproved a main postulate in the field that shows a HO1-adiponectin axis. We performed a series of experiments to determine the effects of HO-1 agonists in mature adipocytes, differentiating adipocytes, and in an *in vivo* model. We found all three methods support the same conclusion. It is obvious that our results contradict previous research. Therefore, further studies are necessary. First of all, the reason that CoPP treatment increases adiponectin in MSCs but not human adipocytes is not clear and it is important to find out the reasons that different cell types have different response to CoPP. Secondly, in our previous reports, we found that CoPP and hemin treatments inhibit adipogenesis and decreases adipogenesis markers, but none of these effects were due to HO-1. We also found that CoPP and hemin are not specific inducers of HO-1, but they do induces other NRF2 related genes. Therefore, what role NRF2 plays in this system requires further investigation.

In *in vivo* study, we found there is a significant decrease in food intake in CoPP-treated obese mice and other parameters were correlated with body weight reduction due to food intake decrease. Whether all of these changes were due to body weight reduction can be verified by feeding a group of mice the same amount of food eaten by CoPP-treated mice and measuring those parameters. In addition, we found circulating adiponectin levels were increased but adiponectin gene expression in WAT were decreased. Therefore, it is important to discover how circulating adiponectin levels increase under CoPP treatment in future studies.

Supp Fig 5.1

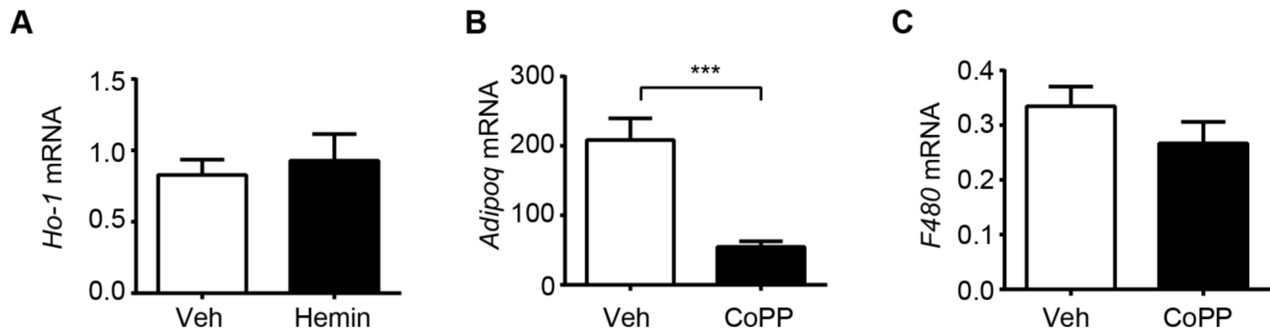


Supp Fig 5.1 – Hemin administration in mice. Lean (L) and obese (O) mice were administered hemin (20 mg/Kg; 3 time/week) or vehicle (veh) for 6 weeks such that all mice were treated 3 times/week. **(A)** Cumulative energy intake over the first 9 days of treatment in lean and obese mice (n=2). **(B)** Change in body weight after 6 weeks of treatment with or without hemin. GTT and ITT in **(C)** lean and **(D)** obese mice after 5 weeks of treatment (n = 8 for all groups). Circulating levels of **(E)** total and **(F)** HMW adiponectin after 6 weeks of treatment (n = 8 for all groups). *p<0.05; two-way ANOVA with a Bonferroni post hoc test.



Supp Fig 5.2 - Divergent effects of hemin on inflammatory and adiponectin gene expression profiles in epididymal adipose tissue. Lean (L) and obese (O) mice were administered hemin (20 mg/Kg; 3 time/week) or vehicle (veh) for 6 weeks such that all mice were treated 3 times/week. HO-1 **(A)** gene and **(B)** protein levels in epididymal adipose tissue from lean and obese mice. Expression of **(C)** Nqo1, **(D)** Gclm, **(E)** *Adipoq*, **(F)** *Tnfα,* **(G)** *F480*, **(H)** *Cd68*, **(I)** *Cd11b* and **(J)** *Mcp-1* in epididymal adipose tissue from lean and obese mice (n≥8 for all groups). *p<0.05; **p<0.01; ***p<0.001; two-way ANOVA with a Bonferroni post hoc test.

Supp Fig 5.3



Supp Fig 5.3 - The effect of hemin on inflammatory gene expression profiles in subcutaneous adipose tissue. Lean (L) and obese (O) mice were administered hemin (20 mg/Kg; 3 time/week) or vehicle (veh) for 6 weeks such that all mice were treated 3 times/week. Expression of **(A)** *Ho-1*, **(B)** *Adipoq* and **(C)** *F480* in subcutaneous adipose tissue. (n = 8 for all groups). ***p<0.001; one-way ANOVA with Tukey's post hoc test.

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