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

Short title: 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

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.

Keywords: Adiponectin; HO-1; Inflammation; Therapeutic

1. 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 (Esmaili et al., 2014; Hickman and Whitehead, 2012; Parker-Duffen and Walsh, 2014; Tao et al., 2014). Paradoxically, and in contrast to most other adipocyte-derived hormones or "adipokines", circulating adiponectin levels are reduced in obesity (Ye and Scherer, 2013). 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 (Hickman and Whitehead, 2012). 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 (Simpson and Whitehead, 2010; Wang et al., 2008). 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 (Hickman and Whitehead, 2012). 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 (Lim et al., 2014; Pajvani et al., 2004).

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 (Wegiel et al., 2014). Each of the products of HO-1 activity modulates various

aspects of cellular function and homeostasis (Wegiel et al., 2014) prompting some to propose HO-1 as a dual purpose "sensor/effector" that both senses and responds to oxidative, inflammatory and metabolic stress (Motterlini and Foresti, 2014). Consistent with this most, but not all (Jais et al., 2014), 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 (Hosick and Stec, 2012; Son et al., 2013). As such, considerable efforts are being made to identify efficacious approaches to induce HO-1 in man (Bharucha et al., 2010; Li et al., 2007; Motterlini and Foresti, 2014).

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" (Cao et al., 2012a; Cao et al., 2011; Issan et al., 2012; Kim et al., 2010; Li et al., 2008; Ndisang and Chibbar, 2014; Ndisang et al., 2014; Ndisang and Jadhav, 2014; Salamone and Li Volti, 2010; Seow et al., 2011; Vanella et al., 2012). 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 (Li et al., 2008). 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 (Li et al., 2008).

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. Material and Methods

2.1. 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.

2.2. SGBS cell culture, differentiation and treatment

Human SGBS preadipocytes, a gift from Martin Wabitsch (University of Ulm, Ulm, Germany) (Wabitsch et al., 2001), were maintained and differentiated in the absence of serum as described (Newell et al., 2006). 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.

106 *2.3. Isolation, culture, differentiation and treatment of Primary Human Preadipocytes*

107 Primary human preadipocytes (phPAs) were isolated from subcutaneous adipose tissue from
108 two subjects (both female, age 25 & 39 years, BMI 24.1 & 23.3, metabolically healthy - no
109 insulin resistance, diabetes or cardiovascular diseases) and cultured independently as
110 described previously (Newell et al., 2006). The procedure was approved by the Research
111 Ethics Committees of the University of Queensland, the Princess Alexandra Hospital, and the
112 Mater Adults Hospital. Both patients had given their written informed consent. Treatments
113 were performed in fully differentiated cells (day 21) as described above (section 2.2).

114 *2.4. Measurement of gene expression by qRT-PCR.*

115 Gene expression was measured by qRT-PCR and standardized against the expression of
116 cyclophilin essentially as previously described (Newell et al., 2006). Briefly, total RNA was
117 extracted using Trizol or RNA Mini Kit (Ambion Life Technologies, Victoria, Australia)
118 according to the manufacturer's instructions. cDNA was synthesized from 1□ µg total RNA
119 using a cDNA synthesis kit (Bioline, NSW, Australia) and RT-PCR was performed using the
120 SYBR Hi-ROX kit (Bioline) on a 7900HT Fast Real-time PCR system (Ambion Life
121 Technologies). Primer sequences are available on request.

122 *2.5. Determination of HO-1, Adiponectin and IL-6 protein*

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

2.6. 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 (Richards et al., 2006). 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.

2.7. 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.

3. Results

3.1. 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 1A-D), which compromises adiponectin expression and secretion (Rose et al., 2010). 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.

3.2. 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 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 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.

3.3. 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 (Burgess et al., 2010; Vanella et al., 2013). 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*, *TNFα* 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 3A-D) as well as TNFα and *MCP-1* expression (Fig 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.

3.4. 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 4A & B). Treatment with CoPP or TNFα had no significant effect on *GRP78* (*BiP*) or *CHOP* expression (Fig 4C-F).

3.5. 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 (Hutley et al., 2011; Newell et al., 2006; Widberg et al., 2009). 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 (Sheftel et al., 2007). 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 (Shan et al., 2006; Sheftel et al., 2007). 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 5A & data not shown). However, such induction of HO-1 had no effect on adiponectin or IL-6 secretion in control or TNFα treated cells (Fig 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 (Sheftel et al., 2007). As expected, induction of HO-1 with either hemin or CoPP markedly induced the levels of

ferritin protein (Fig 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 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 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.

3.6. 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 7A & B). Treatment with TNFα significantly reduced adiponectin production and increased IL-6 production (Fig 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 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.

4. 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 (Cao et al., 2012a; Cao et al., 2011; Cao et al., 2012c; Hinds et al., 2014; Kim et al., 2008; L'Abbate et al., 2007; Li et al., 2008; Ndisang and Jadhav, 2014; Vanella et al., 2012; Vanella et al., 2013), and *in vitro* studies, showing increased adiponectin secretion from adipocytes (Kim et al., 2008; Vanella et al., 2013). These observations stimulated the proposal of a HO-1 – adiponectin axis (Cao et al., 2012a; Cao et al., 2011; Issan et al., 2012; Kim et al., 2010; Li et al., 2008; Ndisang and Chibbar, 2014; Ndisang et al., 2014; Ndisang and Jadhav, 2014; Salamone and Li Volti, 2010; Seow et al., 2011; Vanella et al., 2012) 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 (Kim et al., 2008; Peterson et al., 2009; Vanella et al., 2010; Vanella et al., 2013), 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 (Sheftel et al., 2007). 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 (Rose et al., 2010). 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 (Kim et al., 2008; Vanella et al., 2013). Whilst it seems somewhat paradoxical that inhibition of adipogenesis would result in increased adiponectin secretion, given the dose-dependent effects of chronic CoPP treatment (Vanella et al., 2013) and the finding that adiponectin secretion is highest in ‘immature’ adipocytes (Luo et al., 2012) it

remains possible that there is an 'optimal window' for adiponectin secretion that was somewhat serendipitously established in these investigations (Kim et al., 2008; Vanella et al., 2013). *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 (Luo et al., 2012). 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 (Tu et al., 2014). 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 (Tu et al., 2014). 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 (Cao et al., 2012a; Cao et al., 2012b; Csongradi et al., 2012; Hinds et al., 2014; Li et al., 2008; Ndisang et al., 2014; Ndisang and Tiwari, 2015; Tu et al., 2014) 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 (Cao et al., 2012b). 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 (Huang et al., 2013). 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 (Wang et al., 2010). 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 (Huang et al., 2013). 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 (Galbraith and Kappas, 1989, 1991) and elevated metabolism, heat production and activity (Csongradi et al., 2012) have been proposed although, at least in some instances, these changes appear to occur in the presence of HO-1 inhibition (Choudhary et al., 2013; Ndisang and Tiwari, 2015), 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 (Jais et al., 2014) 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 (Jais et al., 2014). 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 (Jais et al., 2014).

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.

Acknowledgements

This work was funded by a University of Queensland International Research Scholarship to MY and an Australian National Health and Medical Research Council Fellowship to JPW (511104).

5. References

Bharucha, A.E., Kulkarni, A., Choi, K.M., Camilleri, M., Lempke, M., Brunn, G.J., Gibbons, S.J., Zinsmeister, A.R., and Farrugia, G. (2010). First-in-Human Study Demonstrating Pharmacological Activation of Heme Oxygenase-1 in Humans. Clin Pharmacol Ther *87*, 187-190.

Burgess, A., Li, M., Vanella, L., Kim, D.H., Rezzani, R., Rodella, L., Sodhi, K., Canestraro, M., Martasek, P., Peterson, S.J., *et al.* (2010). Adipocyte Heme Oxygenase-1 Induction Attenuates Metabolic Syndrome in Both Male and Female Obese Mice. Hypertension *56*, 1124-1130.

Cao, J., Inoue, K., Sodhi, K., Puri, N., Peterson, S.J., Rezzani, R., and Abraham, N.G. (2012a). High-Fat Diet Exacerbates Renal Dysfunction in SHR: Reversal by Induction of HO-1–Adiponectin Axis. Obesity *20*, 945-953.

Cao, J., Peterson, S.J., Sodhi, K., Vanella, L., Barbagallo, I., Rodella, L.F., Schwartzman, M.L., Abraham, N.G., and Kappas, A. (2012b). Heme oxygenase gene targeting to adipocytes attenuates adiposity and vascular dysfunction in mice fed a high-fat diet. Hypertension *60*, 467-475.

Cao, J., Sodhi, K., Puri, N., Monu, S.R., Rezzani, R., and Abraham, N.G. (2011). High fat diet enhances cardiac abnormalities in SHR rats: Protective role of heme oxygenase-adiponectin axis. Diabetol Metab Syndr *3*, 37.

Cao, J., Vecoli, C., Neglia, D., Tavazzi, B., Lazzarino, G., Novelli, M., Masiello, P., Wang, Y.-t., Paolocci, N., Puri, N., *et al.* (2012c). Cobalt Protoporphyrin Improves Heart Function by Attenuating Cardiac Beta-oxidation and Restoring Redox Balance in an Animal Model of Experimental Diabetes. Frontiers in Physiology *3*.

Choudhary, A.K., Rennie, J., Cairns, C., Borthwick, G., Hughes, J., Morton, N.M., Kluth, D., and Conway, B.R. (2013). Administration of heme arginate ameliorates murine type 2 diabetes independently of heme oxygenase activity. PLoS One *8*, e78209.

Csongradi, E., doCarmo, J.M., Dubinion, J.H., Vera, T., and Stec, D.E. (2012). Chronic HO-1 induction with cobalt protoporphyrin (CoPP) treatment increases oxygen consumption, activity, heat production and lowers body weight in obese melanocortin-4 receptor-deficient mice. Int J Obes *36*, 244-253.

Esmaili, S., Xu, A., and George, J. (2014). The multifaceted and controversial immunometabolic actions of adiponectin. Trends Endocrinol Metab *25*, 444-451.

Galbraith, R.A., and Kappas, A. (1989). Regulation of food intake and body weight by cobalt porphyrins in animals. Proc Natl Acad Sci U S A *86*, 7653-7657.

Galbraith, R.A., and Kappas, A. (1991). Regulation of food intake and body weight in rats by the synthetic heme analogue cobalt protoporphyrin. Am J Physiol *261*, R1388-1394.

Hickman, I.J., and Whitehead, J.P. (2012). Structure, signalling and physiologic role of adiponectin - dietary and exercise-related variations. Current Medicinal Chemistry *19*, 5427-5443.

Hinds, T.D., Jr., Sodhi, K., Meadows, C., Fedorova, L., Puri, N., Kim, D.H., Peterson, S.J., Shapiro, J., Abraham, N.G., and Kappas, A. (2014). Increased HO-1 levels ameliorate fatty liver development through a reduction of heme and recruitment of FGF21. Obesity (Silver Spring) *22*, 705-712.

Hosick, P.A., and Stec, D.E. (2012). Heme oxygenase, a novel target for the treatment of hypertension and obesity? Am J Physiol Regul Integr Comp Physiol *302*, R207-214.

Huang, J.-Y., Chiang, M.-T., and Chau, L.-Y. (2013). Adipose Overexpression of Heme Oxygenase-1 Does Not Protect against High Fat Diet-Induced Insulin Resistance in Mice. PLoS One *8*, e55369.

Hutley, L.J., Newell, F.S., Kim, Y.H., Luo, X., Widberg, C.H., Shurety, W., Prins, J.B., and Whitehead, J.P. (2011). A putative role for endogenous FGF-2 in FGF-1 mediated differentiation of human preadipocytes. Mol Cell Endocrinol *339*, 165-171.

Issan, Y., Hochhauser, E., Kornowski, R., Leshem-Lev, D., Lev, E., Sharoni, R., Vanella, L., Puri, N., Laniado-Schwartzman, M., Abraham, N.G., *et al.* (2012). Endothelial Progenitor Cell Function Inversely Correlates With Long-term Glucose Control in Diabetic Patients: Association With the Attenuation of the Heme Oxygenase-Adiponectin Axis. Can J Cardiol.

Jais, A., Einwallner, E., Sharif, O., Gossens, K., Lu, T.T., Soyal, S.M., Medgyesi, D., Neureiter, D., Paier-Pourani, J., Dalgaard, K., *et al.* (2014). Heme oxygenase-1 drives metaflammation and insulin resistance in mouse and man. Cell *158*, 25-40.

Kim, D.H., Burgess, A.P., Li, M., Tsenovoy, P.L., Addabbo, F., McClung, J.A., Puri, N., and Abraham, N.G. (2008). Heme oxygenase-mediated increases in adiponectin decrease fat content and inflammatory cytokines tumor necrosis factor-alpha and interleukin-6 in Zucker rats and reduce adipogenesis in human mesenchymal stem cells. J Pharmacol Exp Ther *325*, 833-840.

Kim, D.H., Vanella, L., Inoue, K., Burgess, A., Gotlinger, K., Manthati, V.L., Koduru, S.R., Zeldin, D.C., Falck, J.R., Schwartzman, M.L., *et al.* (2010). Epoxyeicosatrienoic acid agonist regulates human mesenchymal stem cell-derived adipocytes through activation of HO-1-pAKT signaling and a decrease in PPARgamma. Stem Cells Dev *19*, 1863-1873.

L'Abbate, A., Neglia, D., Vecoli, C., Novelli, M., Ottaviano, V., Baldi, S., Barsacchi, R., Paolicchi, A., Masiello, P., Drummond, G.S., *et al.* (2007). Beneficial effect of heme oxygenase-1 expression on myocardial ischemia-reperfusion involves an increase in adiponectin in mildly diabetic rats. Am J Physiol Heart Circ Physiol *293*, H3532-3541.

Li, C., Hossieny, P., Wu, B.J., Qawasmeh, A., Beck, K., and Stocker, R. (2007). Pharmacologic induction of heme oxygenase-1. Antioxid Redox Signal *9*, 2227-2239.

Li, M., Kim, D.H., Tsenovoy, P.L., Peterson, S.J., Rezzani, R., Rodella, L.F., Aronow, W.S., Ikehara, S., and Abraham, N.G. (2008). Treatment of obese diabetic mice with a heme oxygenase inducer reduces visceral and subcutaneous adiposity, increases adiponectin levels, and improves insulin sensitivity and glucose tolerance. Diabetes *57*, 1526-1535.

Lim, S., Quon, M.J., and Koh, K.K. (2014). Modulation of adiponectin as a potential therapeutic strategy. Atherosclerosis *233*, 721-728.

Luo, X., Hutley, L.J., Webster, J.A., Kim, Y.-H., Liu, D.-F., Newell, F.S., Widberg, C.H., Bachmann, A., Turner, N., Schmitz-Peiffer, C., *et al.* (2012). Identification of BMP and Activin Membrane-Bound Inhibitor (BAMBI) as a Potent Negative Regulator of Adipogenesis and Modulator of Autocrine/Paracrine Adipogenic Factors. Diabetes *61*, 124-136.

Motterlini, R., and Foresti, R. (2014). Heme oxygenase-1 as a target for drug discovery. Antioxid Redox Signal *20*, 1810-1826.

Ndisang, J.F., and Chibbar, R. (2014). Heme Oxygenase Improves Renal Function by Potentiating Podocyte-Associated Proteins in N omega-Nitro-l-Arginine-Methyl Ester (l-NAME)-Induced Hypertension. Am J Hypertens.

Ndisang, J.F., Chibbar, R., and Lane, N. (2014). Heme oxygenase suppresses markers of heart failure and ameliorates cardiomyopathy in L-NAME-induced hypertension. Eur J Pharmacol *734*, 23-34.

Ndisang, J.F., and Jadhav, A. (2014). Hemin therapy improves kidney function in male streptozotocin-induced diabetic rats: role of the heme oxygenase/atrial natriuretic peptide/adiponectin axis. Endocrinology *155*, 215-229.

Ndisang, J.F., and Tiwari, S. (2015). Induction of heme oxygenase with hemin improves pericardial adipocyte morphology and function in obese Zucker rats by enhancing proteins of regeneration. Exp Biol Med (Maywood) *240*, 45-57.

Newell, F.S., Su, H., Tornqvist, H., Whitehead, J.P., Prins, J.B., and Hutley, L.J. (2006). Characterization of the transcriptional and functional effects of fibroblast growth factor-1 on human preadipocyte differentiation. FASEB J *20*, 2615-2617.

Pajvani, U.B., Hawkins, M., Combs, T.P., Rajala, M.W., Doebber, T., Berger, J.P., Wagner, J.A., Wu, M., Knopps, A., Xiang, A.H., *et al.* (2004). Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione -mediated improvement in insulin sensitivity. J Biol Chem *279*, 12152-12162.

Parker-Duffen, J.L., and Walsh, K. (2014). Cardiometabolic effects of adiponectin. Best Pract Res Clin Endocrinol Metab *28*, 81-91.

Peterson, S.J., Kim, D.H., Li, M., Positano, V., Vanella, L., Rodella, L.F., Piccolomini, F., Puri, N., Gastaldelli, A., Kusmic, C., *et al.* (2009). The L-4F mimetic peptide prevents insulin

resistance through increased levels of HO-1, pAMPK, and pAKT in obese mice. J Lipid Res *50*, 1293-1304.

Richards, A.A., Stephens, T., Charlton, H.K., Jones, A., Macdonald, G.A., Prins, J.B., and Whitehead, J.P. (2006). Adiponectin multimerisation is dependent on conserved lysines in the collagenous domain: Evidence for regulation of multimerisation by alterations in post-translational modifications. Mol Endocrinol *20*, 1673-1687.

Rose, F.J., Webster, J., Barry, J.B., Phillips, L.K., Richards, A.A., and Whitehead, J.P. (2010). Synergistic effects of ascorbic acid and thiazolidinedione on secretion of high molecular weight adiponectin from human adipocytes. Diabetes Obes Metab *12*, 1084-1089.

Salamone, F., and Li Volti, G. (2010). Targeting heme oxygenase/adiponectin axis for chronic hepatitis C treatment. Hepatology *52*, 801.

Seow, K.M., Lin, Y.H., Hwang, J.L., Wang, P.H., Ho, L.T., and Juan, C.C. (2011). Expression levels of haem oxygenase-1 in the omental adipose tissue and peripheral blood mononuclear cells of women with polycystic ovary syndrome. Hum Reprod *26*, 431-437.

Shan, Y., Lambrecht, R.W., Donohue, S.E., and Bonkovsky, H.L. (2006). Role of Bach1 and Nrf2 in up-regulation of the heme oxygenase-1 gene by cobalt protoporphyrin. FASEB J *20*, 2651-2653.

Sheftel, A.D., Kim, S.F., and Ponka, P. (2007). Non-heme induction of heme oxygenase-1 does not alter cellular iron metabolism. J Biol Chem *282*, 10480-10486.

Simpson, F., and Whitehead, J.P. (2010). Adiponectin-It's all about the modifications. Int J Biochem Cell Biol *42*, 785-788.

Son, Y., Lee, J.H., Chung, H.T., and Pae, H.O. (2013). Therapeutic roles of heme oxygenase-1 in metabolic diseases: curcumin and resveratrol analogues as possible inducers of heme oxygenase-1. Oxid Med Cell Longev *2013*, 639541.

Tao, C., Sifuentes, A., and Holland, W.L. (2014). Regulation of glucose and lipid homeostasis by adiponectin: effects on hepatocytes, pancreatic beta cells and adipocytes. Best Pract Res Clin Endocrinol Metab *28*, 43-58.

Tu, T.H., Joe, Y., Choi, H.S., Chung, H.T., and Yu, R. (2014). Induction of heme oxygenase-1 with hemin reduces obesity-induced adipose tissue inflammation via adipose macrophage phenotype switching. Mediators Inflamm *2014*, 290708.

Vanella, L., Kim, D.H., Asprinio, D., Peterson, S.J., Barbagallo, I., Vanella, A., Goldstein, D., Ikehara, S., Kappas, A., and Abraham, N.G. (2010). HO-1 expression increases mesenchymal stem cell-derived osteoblasts but decreases adipocyte lineage. Bone *46*, 236-243.

Vanella, L., Li, M., Kim, D., Malfa, G., Bellner, L., Kawakami, T., and Abraham, N.G. (2012). ApoA1: mimetic peptide reverses adipocyte dysfunction in vivo and in vitro via an increase in heme oxygenase (HO-1) and Wnt10b. Cell Cycle *11*, 706-714.

Vanella, L., Sodhi, K., Kim, D.H., Puri, N., Maheshwari, M., Hinds, T.D., Jr., Bellner, L., Goldstein, D., Peterson, S.J., Shapiro, J.I., *et al.* (2013). Increased heme-oxygenase 1 expression in mesenchymal stem cell-derived adipocytes decreases differentiation and lipid accumulation via upregulation of the canonical Wnt signaling cascade. Stem Cell Res Ther *4*, 28.

Wabitsch, M., Brenner, R.E., Melzner, I., Braun, M., Moller, P., Heinze, E., Debatin, K.M., and Hauner, H. (2001). Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. Int J Obes Relat Metab Disord *25*, 8-15.

Wang, Y., Lam, K.S., Yau, M.-h., and Xu, A. (2008). Post-translational modifications of adiponectin: mechanisms and functional implications. Biochemical Journal *409*, 623-633.

Wang, Z.V., Deng, Y., Wang, Q.A., Sun, K., and Scherer, P.E. (2010). Identification and characterization of a promoter cassette conferring adipocyte-specific gene expression. Endocrinology *151*, 2933-2939.

Wegiel, B., Nemeth, Z., Correa-Costa, M., Bulmer, A.C., and Otterbein, L.E. (2014). Heme oxygenase-1: a metabolic nike. Antioxid Redox Signal *20*, 1709-1722.

Widberg, C.H., Newell, F.S., Bachmann, A.W., Ramnoruth, S.N., Spelta, M.C., Whitehead, J.P., Hutley, L.J., and Prins, J.B. (2009). Fibroblast growth factor receptor 1 is a key regulator of early adipogenic events in human preadipocytes. Am J Physiol Endocrinol Metab *296*, E121-131.

Ye, R., and Scherer, P.E. (2013). Adiponectin, driver or passenger on the road to insulin sensitivity? Mol Metab *2*, 133-141.

6. Figure Legends

Fig 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.

Fig 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.

Fig 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. **=$p<0.01$, ***=$p<0.001$ significant difference compared to non-TNFα treated cells.

Fig 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.

Fig 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 (50ng/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 (50ng/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. #=p<0.05 significant difference compared to TNFα treatment. Absolute secreted total adiponectin for control cells was: 577.3 ± 56 (417.9 – 663.6) ng/ml.

Fig 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.

Fig 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

579 independent experiments and expressed as fold-increase over untreated control. ***=p<0.001
580 significant difference compared to control or non-TNFα treated cells. Absolute secreted total
581 adiponectin for control cells was: minus serum - 1143.4 ± 16.7 / plus serum - 514.4 ± 14.5
582 ng/ml

583

Highlights

We report 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

Figure 1.

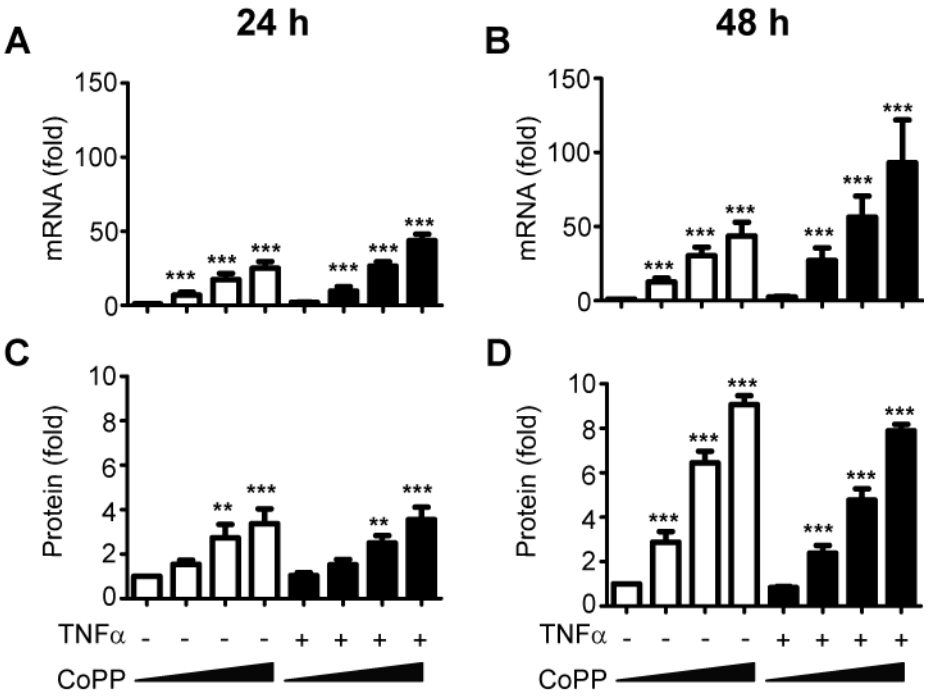

A
24 h
B
48 h
mRNA (fold)
150
100
50
0
***
C
D
Protein (fold)
10
8
6
4
2
0
**
TNFα - - - - + + + +
CoPP

Figure 2.

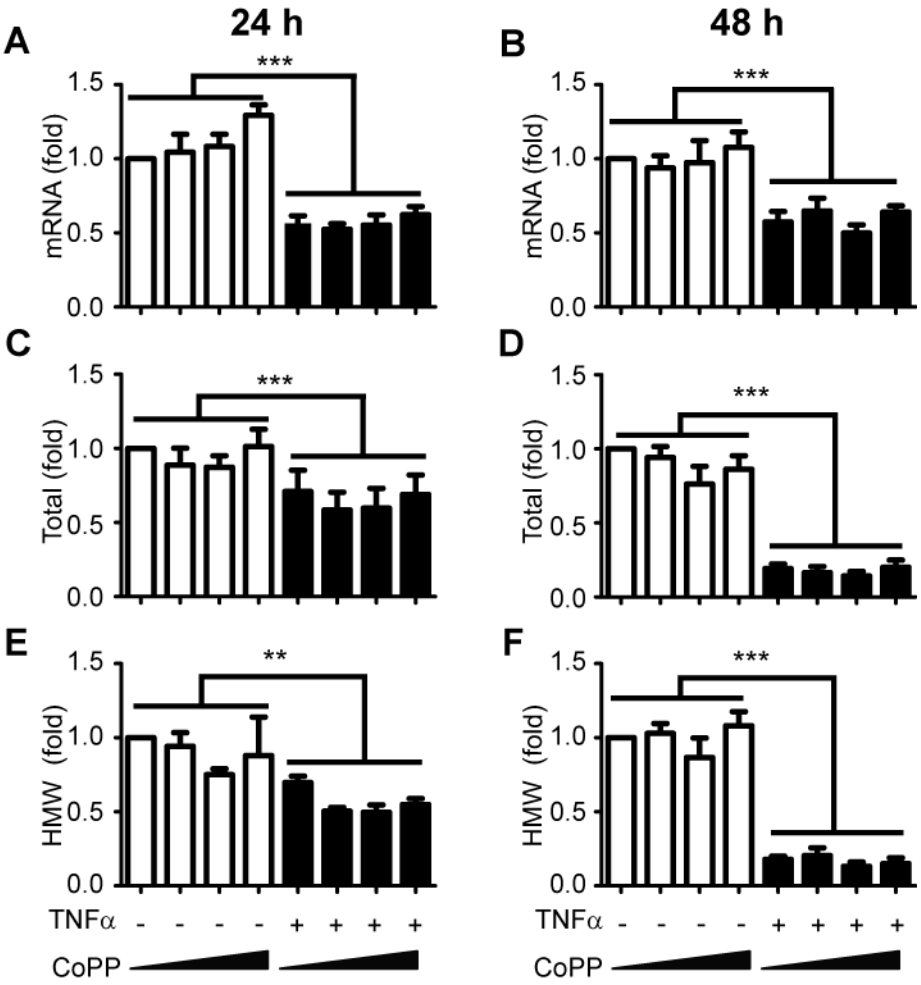

A
24 h
***
mRNA (fold)
1.5
1.0
0.5
0.0
B
48 h
***
mRNA (fold)
C
***
Total (fold)
D
***
Total (fold)
E
**
HMW (fold)
F
***
HMW (fold)
TNFα - - - - + + + +
CoPP

Figure 3.

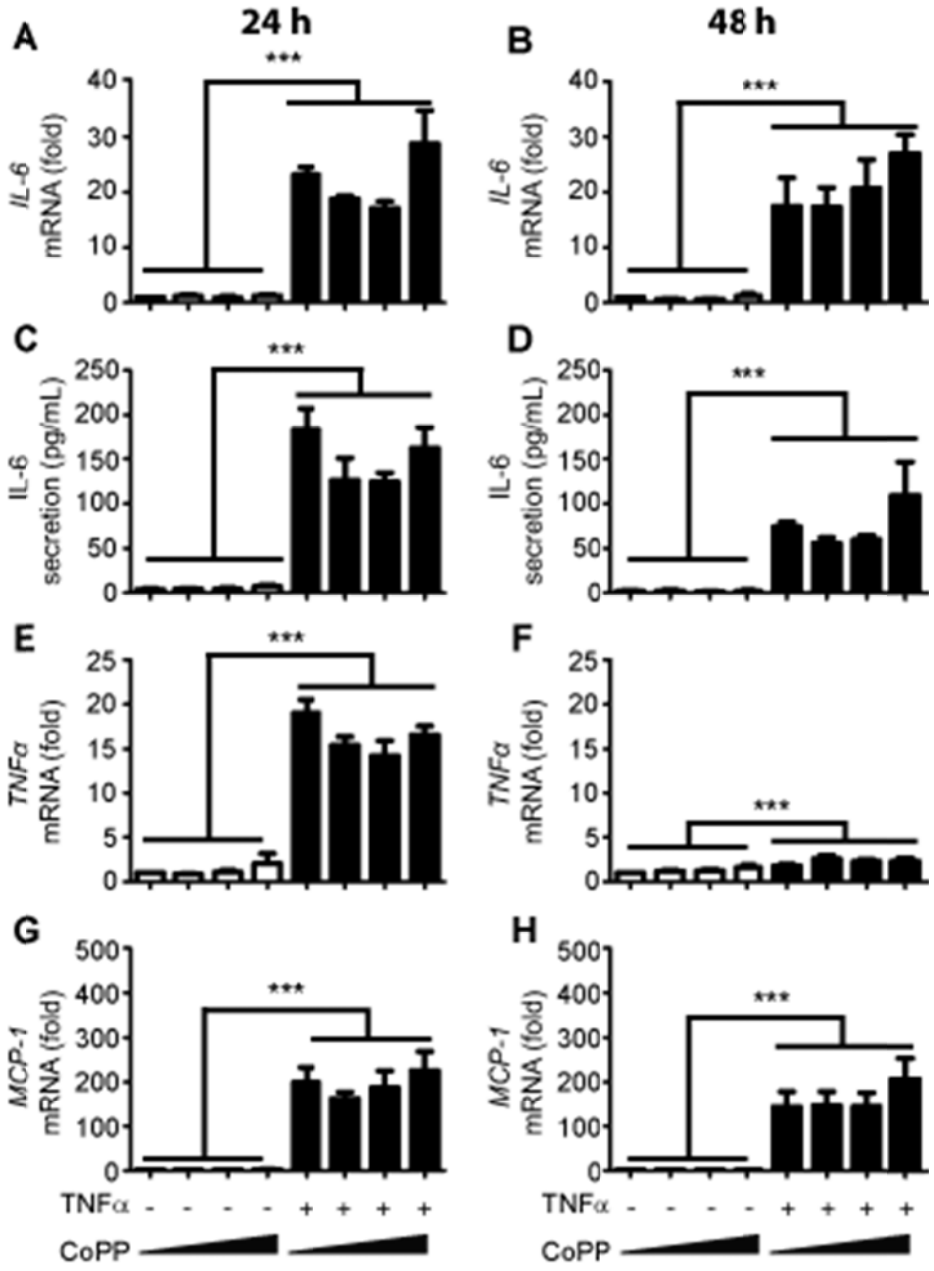

24 h
48 h
A
B
C
D
E
F
G
H
IL-6 mRNA (fold)
IL-6 secretion (pg/mL)
TNFα mRNA (fold)
MCP-1 mRNA (fold)
***
TNFα - - - - + + + +
CoPP

Figure 4.

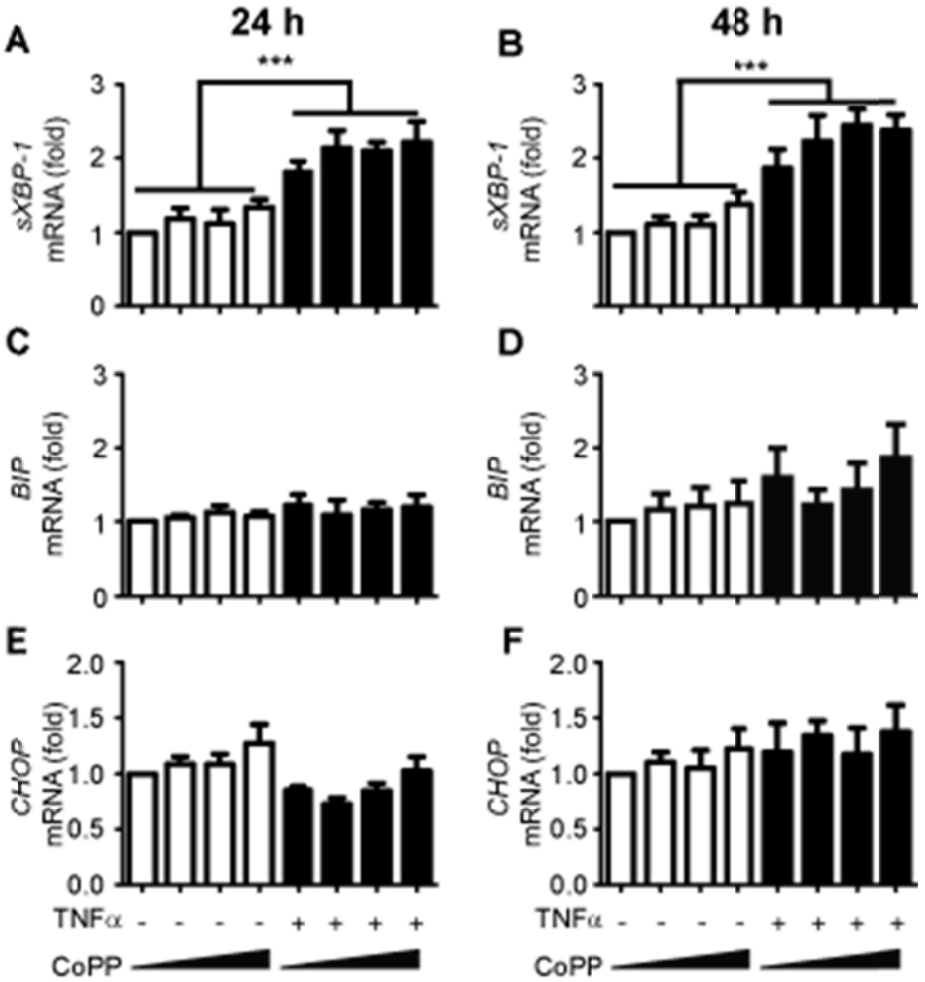

24 h
48 h
A
B
C
D
E
F
***
***
sXBP-1 mRNA (fold)
BIP mRNA (fold)
CHOP mRNA (fold)
TNFα - - - - + + + +
CoPP

Figure 5.

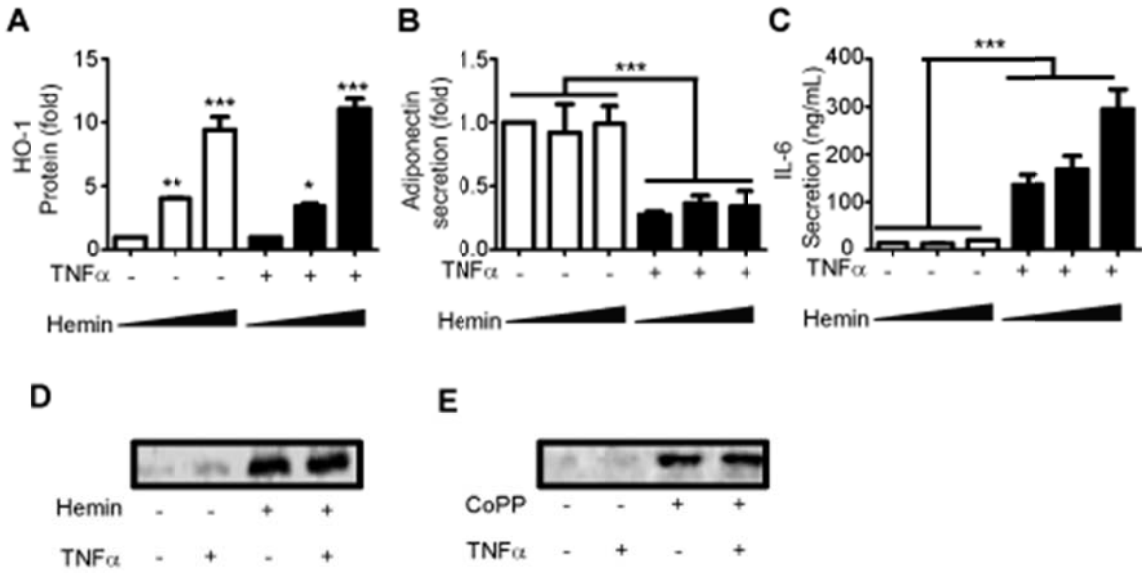

A
HO-1 Protein (fold)
15
10
5
0
**
***
*
***
TNFα - - - + + +
Hemin
B
Adiponectin secretion (fold)
1.5
1.0
0.5
0.0
***
TNFα - - - + + +
Hemin
C
IL-6 Secretion (ng/mL)
400
300
200
100
0
***
TNFα - - - + + +
Hemin
D
Hemin - - + +
TNFα - + - +
E
CoPP - - + +
TNFα - + - +

Figure 6.

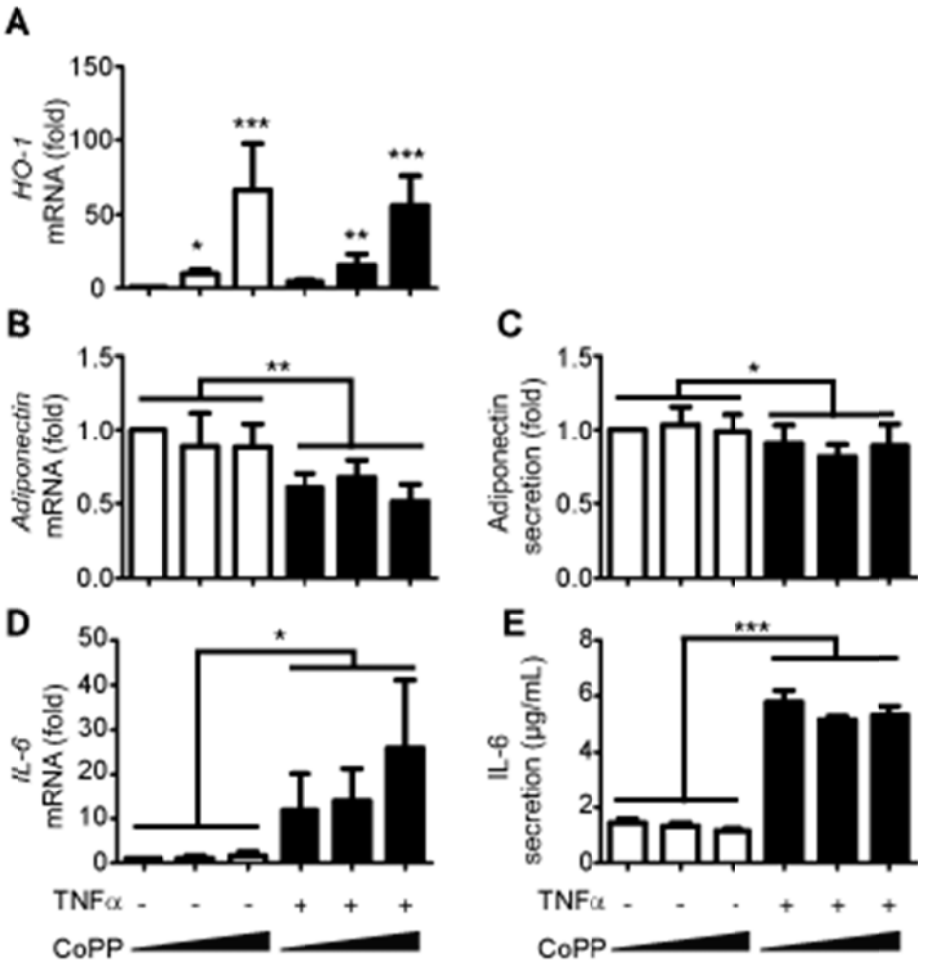
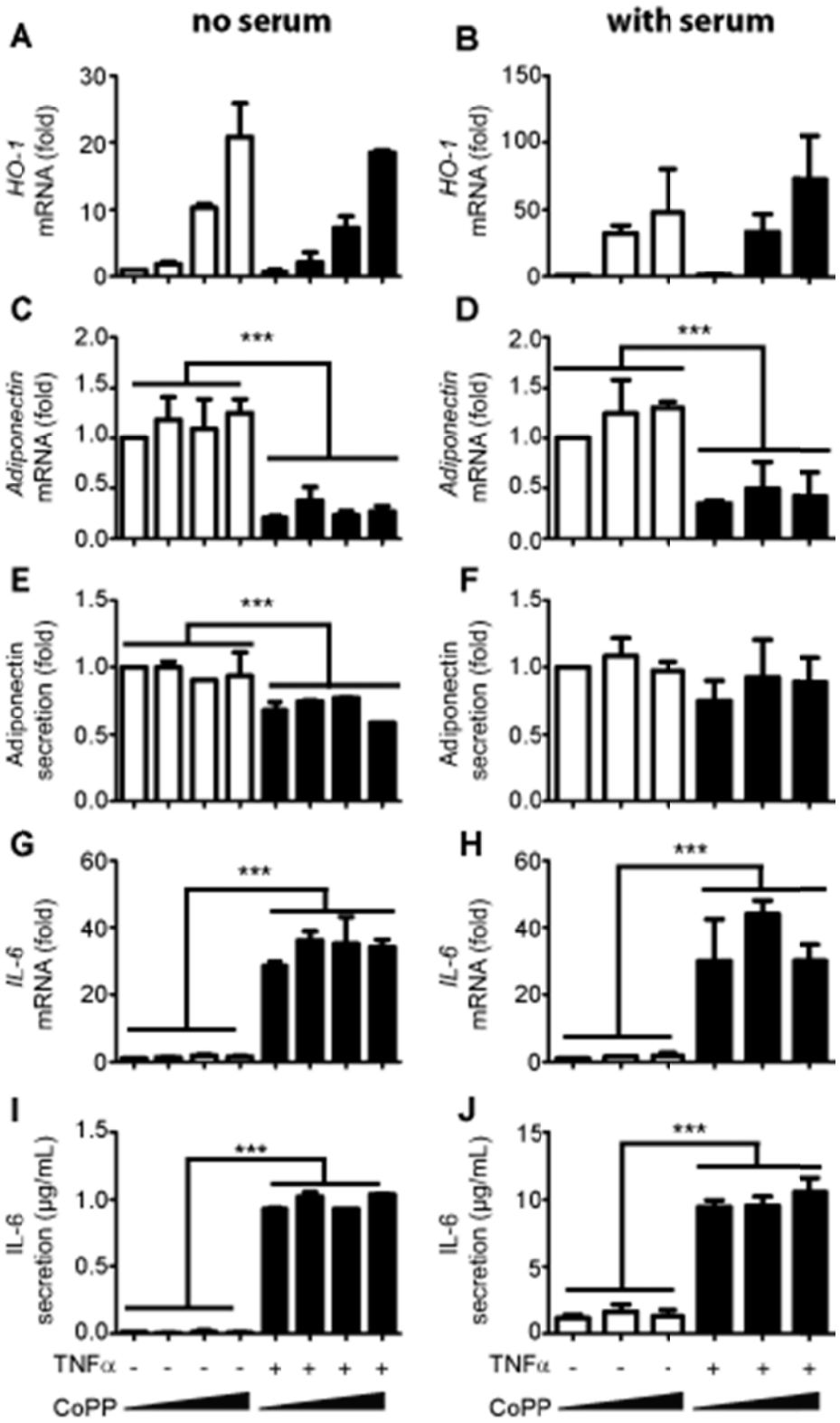

A
HO-1 mRNA (fold)
150
100
50
0
*
***
**
***
B
Adiponectin mRNA (fold)
1.5
1.0
0.5
0.0
**
C
Adiponectin secretion (fold)
1.5
1.0
0.5
0.0
*
D
IL-6 mRNA (fold)
50
40
30
20
10
0
*
E
IL-6 secretion (µg/mL)
8
6
4
2
0
***
TNFα - - - + + +
CoPP

Figure 7.


no serum
with serum
A
B
C
D
E
F
G
H
I
J
HO-1 mRNA (fold)
Adiponectin mRNA (fold)
Adiponectin secretion (fold)
IL-6 mRNA (fold)
IL-6 secretion (µg/mL)
***
TNFα - - - - + + + +
CoPP