Treatment of Obese Diabetic Mice with an Heme Oxygenase Inducer Reduces Visceral and Subcutaneous Adiposity, Increases Adiponectin Levels and Improves Insulin Sensitivity and Glucose Tolerance

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Running Title: Role of HO-1 induction in obesity and diabetes

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ABSTRACT

Objective: We hypothesized that the induction of heme oxygenase-1 (HO-1) and increased HO activity, which induces arterial anti-oxidative enzymes and vasoprotection in a mouse and a rat model of diabetes, would ameliorate insulin resistance, obesity and diabetes in the ob mouse model of type 2 diabetes.

Research Design and Methods: Lean and ob mice were administered (intraperitoneally) the HO-1 inducer CoPP (3 mg/kg) with and without the HO inhibitor SnMP (2 mg/100g) once a week for 6 weeks. Body weight, blood glucose and serum cytokines and adiponectin were measured. Aorta, adipose tissue, bone marrow and mesenchymal stem cells were isolated and assessed for HO expression and adipogenesis.

Results: HO activity was reduced in ob mice compared to age-matched lean mice. Administration of CoPP caused a sustained increase in HO-1 protein, prevented weight gain, decreased visceral and subcutaneous fat content (p<0.03 and p<0.01 respectively compared to vehicle animals), increased serum adiponectin and decreased plasma TNF, IL-6 and IL-1ß levels (p<0.05). HO-1 induction improved insulin sensitivity and glucose tolerance and decreased insulin levels. Upregulation of HO-1 decreased adipogenesis in bone marrow in vivo as well as in cultured mesenchymal stem cells, and increased adiponectin levels in the culture media. Inhibition of HO activity decreased adiponectin and increased secretion of TNF, IL-6 and IL-1ß levels in ob mice.

Conclusions: This study provides strong evidence for the existence of an HO-1-adiponectin regulatory axis that can be manipulated to ameliorate the deleterious effects of obesity and the metabolic syndrome associated with cardiovascular disease and diabetes.
Oxidative stress has been implicated in the pathogenesis of insulin resistance, type 2 diabetes and its cardiovascular complications (1,2). Excessive generation of reactive oxygen species (ROS) in diabetes is the underlying mechanism of endothelial injury, resulting in an accelerated rate of apoptosis and endothelial cell sloughing (3,4). In addition, reduced plasma adiponectin levels have been documented in patients with coronary artery disease and diabetes, presumably as a result of an increase in ROS (5,6). Lin et al highlighted the importance of ROS production in adipocytes and the associated insulin resistance and changes in serum levels of adiponectin (7,8), suggesting that increases in ROS are associated with an induced inflammatory response in adipocytes.

Adipose tissue plays an important role in insulin resistance through the production and secretion of a variety of proteins, including TNF-α, IL-6, leptin and adiponectin (9,10). Of these proteins, adiponectin has recently attracted much attention as it has insulin-sensitizing properties that enhance fatty acid oxidation, liver insulin action, glucose uptake and positively affects serum triglyceride levels (10-12). Adiponectin is exclusively secreted from adipose tissue, and its expression is higher in subcutaneous compared to visceral adipose tissue. It circulates in the blood at very high concentrations and is found as both low-molecular weight (LMW) oligomers and high-molecular weight (HMW) multimers (12,13). Several studies report that HMW adiponectin is more active and correlates more significantly with glucose and insulin levels when compared to LMW and even total adiponectin (13-15). Low plasma levels of HMW adiponectin have been consistently associated with obesity, insulin resistance, type 2 diabetes and coronary artery disease (16,17). Recent data have revealed that adiponectin possesses a vascular protective role by preserving endothelial cell function in diabetic and non-diabetic patients with the metabolic syndrome (18). In addition to modulating atherogenesis through its insulin-sensitizing actions, it also has a direct anti-atherogenic effect on the arterial wall. Increase in adiponectin has been shown to improve the beneficial effects of antihypertensive agents in hypertensive patients (19). The PPAR response was found to increase expression of adiponectin (20) and also to regulate the expression of HO-1 in human vascular cells (21).

The HO system provides both antioxidant and anti-apoptotic properties due to its byproducts, bilirubin/biliverdin and carbon monoxide (CO), respectively (22). HO-1 is induced by oxidant stress and plays a crucial role in protection against oxidative insult in diabetes and cardiovascular disease (22). Upregulation of HO-1 gene expression prevents vascular dysfunction and endothelial cell death through decreases in ROS levels (23). Recently, L’Abbate et al. (24) have shown that induction of HO-1 was associated with a parallel increase in the serum levels of adiponectin, which has well documented anti-inflammatory properties (24). Adiponectin has been ascribed anti-oxidative properties (25). These observations also serve to define some of the key mechanisms by which HO-1 is involved in diabetes and the metabolic syndrome.

In the present study, we report that in obese mice, there is a reduction in HO activity, an increase in TNF and IL-1 and a decrease in adiponectin levels when compared to age-matched lean animals. The increases in HO-1 and adiponectin resulted in a concomitant decrease in serum levels of TNF and IL-1. Using mesenchymal stem cells, we showed that induction of HO-1 decreased adipocyte size and TNF, IL-1 and IL-6 release in culture media, a process that is reversed by inhibition of HO activity. We extended these
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observations to an in vivo setting and demonstrated increased adipogenesis in bone marrow cells of ob mice and that upregulation of HO-1 diminished the number of fat cells. Further, an increase of adipocyte HO-1 protein resulted in a decrease of adipogenesis which is reversed by inhibition of HO activity. We demonstrated, for the first time, that an increase in HO-1-adiponectin levels prevents obese-diabetes-induced inflammatory cytokine formation both in vivo and in vitro. We propose that HO-1 mediated increase in adiponectin plays a crucial role in improvement in the metabolic profile, including the diabetic phenotype.

MATERIALS AND METHODS

Animal protocols. Male obese (ob) mice (B6v-Lep ob/J) were purchased from Harlan (Chicago, IL) at the age of 7 weeks and used at the age of 8 weeks. Age- and sex-matched lean mice (B6.V, lean, Harlan, Chicago, IL) were used as controls. Mice were fed a normal chow diet and had free access to water and food. Body weight of ob and lean mice at the beginning of the treatment were 34±5 g and 26±3 g, respectively. Glucose levels were 229±21 and 154±9 mg/dl for ob and lean mice, respectively.

Glucose monitoring was performed using an automated analyzer (Lifescan Inc., Milpitas, CA). Beginning at 9 weeks, when all ob mice have established diabetes, CoPP (3 mg/kg once a week), or CoPP (3 mg/kg once a week) plus SnMP (2 mg/100 gm BW, three times a week) were administered intraperitoneally for 6 weeks. Metalloporphyrins were dissolved in 10mM tris base and the pH adjusted to pH 7.8 with 0.1 N HCl. A tris/HCL solution free of metalloporphyrins was used to inject control animals. There were six groups of animals: A) lean, B) lean-CoPP, C) lean-CoPP-SnMP, D) ob control, E) ob-CoPP, and F) ob-CoPP-SnMP. Food intake did not change in the mice treated with various treatments. The Animal Care and Use Committee of New York Medical College approved all experiments.

Tissue preparation for Western blot of adipocyte stem cells, heart, kidney and aorta. At the time of sacrifice, subcutaneous and visceral fat in the abdomen (the visible mesenteric fat, fat around the liver, fat around the kidney and fat around the spleen) were dissected free, pooled for each mouse, weighed and used to isolate adipocyte mesenchymal stem cells. Cells were frozen until needed for protein measurements. Aorta, heart and kidney were also harvested drained of blood and flash frozen in liquid nitrogen. Specimens were maintained at -80°C until needed. Frozen aorta and kidney segments were pulverized and the supernatant was isolated and used for HO activity, HO-1 and HO-2 protein (26).

Heme Oxygenase Activity Assay and CO Generation. HO activity was assayed in homogenates of various tissues or mesenchymal stem cells as previously described (27). 300-µg protein were used for CO measurement in the presence and absence of heme and NADPH-generating system (28,29).

Cytokine and insulin measurements. Multiples assay kits were used for quantification of the proteins in mice serum and were done according to the manufacturers protocol. Plates were analyzed using a luminex 100IS analyzer (Lumines Inc. Austun, Tx). The data were saved and evaluated as Median Fluorescence Intensity (MFI) using appropriate curve-fitting software (Luminex 100IS software version 2.3). Certain measurement including Adiponectin (HMW), TNF , IL-1 and IL-6 were determined in mouse serum using an ELISA assay (Pierce Biotechnology, Inc., Woburn, MA). Insulin was measured using an ELISA Kit (Millipore, Billerica, MA).

Glucose and insulin tolerance test. After a 12 hour fast, mice were injected intraperitoneally with glucose (2.0g/kg body weight). Blood
Samples were taken at various time points (0-120 min), and blood glucose levels and serum insulin levels were measured. For insulin tolerance test, mice were injected intraperitoneally with insulin (2.0 U/kg). Blood samples were taken at various time points (0-90 min) and blood glucose levels were measured.

**Oil red O staining of bone marrow.** Bone marrow smears made from the fibia were stained with 0.5% oil red O in isopropanol (w/v) for 10 min, and lipid droplets were then evaluated using a light microscope digitalized with a charge-coupled device camera and an image analysis system (Imaging & Computers, Milan, Italy). Mean number of lipid droplets was calculated from 6 different fields.

**Measurement of O₂⁻ levels in aorta and bone marrow of obese mice.** Lean, obese and diabetic aorta or bone marrow were placed in plastic scintillation minivials, containing 5 µM lucigenin for the detection of O₂⁻, as described (30,31).

**Isolation of mice bone marrow-derived MSCs and CFU-F determination** The bone marrow cells were collected from the mice femur and tibia of ob mice. Non-adherent cells were removed on day 4, and adherent cells were further incubated in fresh IMEM including 10% FBS (Invitrogen Corporation, Carlsbad, CA) and 1% antibiotic-antimycotic solution (Invitrogen). The cultured cells with spindle-like morphology after three passages were defined as MSCs, and were induced to differentiate into adipocyte cells **in vitro.** Colony forming units-fibroblasts (CFU-F) were determined using MesenCult media according to manufacturer's instruction (Stem cell technology, Vancouver, Canada).

**Induction of adipogenesis in MSCs.** MSCs were cultured in adipogenic medium (high glucose DMEM containing 10 ug/ml insulin, 0.1 mM dexamethasone, 0.2 mM indomethacin, 10% FBS and 1% antibiotic-antimycotic solution). At 50% confluence, CoPP (2 M) and SnMP (5 M) were added, and adipogenesis was measured using the oil red O staining as described (32). Briefly, cells were fixed by ice cold 10% formalin in PBS for 10 minutes, rinsed with distilled water and stained with oil red O solution for 20 minutes. Cells were placed in absolute propylene glycol for 5 minutes, rinsed in 85% propylene glycol followed by distilled water and air-dried. Oil red O stain was extracted with isopropanol and optical absorbance was measured at 490-520 nm.

**Detection of MSC cell markers by FACS analysis.** The ISCT has provided the following minimum criteria for defining multipotent mesenchymal stromal cells. MSC are normally plastic-adherent under standard culture conditions and express CD105, CD73 and CD90. MSC must lack the expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR and be able to differentiate into osteoblasts, adipocytes and chondroblasts in vitro (33). The MSCs presented as a homogeneous fibroblastoid cell population. Expression of stem cell markers assessed with RT-PCR showed that after passage 2, these cells were negative for hematopoietic cell markers (CD34 and CD45) and positive for CD90, CD105, CD166 (ALCAM), markers of MSCs. Flow cytometric analysis of passage 4 cells confirmed that cells were negative for CD34 and CD45 and that cells were positive for CD29 (1-integrin) and CD90 (Thy-1) (34).

**STATISTICAL ANALYSES**

Statistical significance between experimental groups was determined by the Fisher method of analysis of multiple comparisons (p<0.05). For comparison between treatment groups, the null hypothesis was tested by a single-factor ANOVA for multiple groups or unpaired t test for two groups. Data are presented as mean ± SE.
RESULTS

Effect of CoPP on HO-1 expression and HO activity in the aorta. Western blot analysis showed a significant decrease (p<0.05) in the ratio of HO-1 and actin in the aorta of ob compared to lean mice (Figure 1A). CO production is the aorta of lean and obese mice was measured by GCMS and was found to be significantly (p<0.05) lower in obese mice compared to lean mice. This reflects the less active HO system in obese mice compared to lean mice. A weekly injection of CoPP for 6 weeks resulted in a significant increase in HO-1 protein levels in both lean and ob mice (Figure 1B).

CoPP administration increased aortic HO-1 protein in lean mice by 4.3-fold (p<0.01) and in ob mice by 9-fold (p<0.001) (Figure 1B). HO activity was measured in aortas isolated from these animals (Figure 1C). There was a significant (p<0.05) decrease in HO activity in ob mice compared to age-matched lean controls (0.27±0.02 and 0.40±0.03 nmol bilirubin/mg/h, respectively). CoPP administration increased bilirubin formation in the aorta of both lean and obese mice (1.9±0.35 and 1.67±0.28 nmol bilirubin/mg/h, respectively). SnMP abolished the increase in HO activity (results not shown). This pattern of HO expression and activity was also observed in kidneys and heart (data not shown).

Effect of CoPP on serum adiponectin levels. There was a significant decrease in serum adiponectin levels in ob mice compared to age-matched lean controls (Figure 2): adiponectin levels in ob mice were 2.51±0.33 g/ml compared to 3.86±0.46 g/ml (p<0.029) in lean animals. CoPP administration resulted in a marked 4-fold increase (p<0.009) in the levels of serum adiponectin in ob mice compared to untreated ob animals. Serum adiponectin increased to 10.2±1.66 g/ml following HO-1 induction compared to 2.51±0.33 g/ml in untreated ob mice. The concomitant administration of SnMP with CoPP blocked the increase in serum adiponectin.

Effect of CoPP on IL-6, IL-1 and TNF levels. Obese mice exhibited a significant increase in serum IL-6 levels (620±6 pg/ml) when compared to age-matched lean controls (89±23 pg/ml) (Figure 3). Administration of CoPP resulted in a significant decrease in serum IL-6 levels in ob mice when compared to untreated ob mice. This decrease was blocked by administration of SnMP. IL-6 serum levels in CoPP in combination with SnMP were 600±122 pg/ml compared to 257±48 pg/ml in CoPP-treated ob mice. Similar results were observed with serum TNF and IL-1 levels (Figure 3B and 3C).

Effect of CoPP on formation of O$_2^-$ in isolated aorta. The levels of O$_2^-$ in aorta obtained from ob mice treated with vehicle alone were 8.0±0.6 (x10$^4$ cpm) compared to 1.1±0.3x10$^4$ cpm (p<0.03) in vehicle treated lean mice (Figure 3D). CoPP-treated ob mice showed a significant decrease in O$_2^-$ levels in the aorta compared to vehicle treated ob mice. The levels in the CoPP treated obese mice decreased to 1.7±0.4x10$^4$cpm (p<0.039). Inhibition of HO activity by administration of SnMP to CoPP-treated mice increased superoxide production to 6.3±0.4x10$^4$ cpm.

Effect of induction of HO-1 on obese and lean mice body weight, appearance and fat content. As seen in Figure 4A, CoPP-treatment prevented weight gain in ob mice when compared to age-matched controls. The prevention of body weight gain was manifested by a reduction in visceral fat in obese mice. In contrast, control lean mice administered CoPP did not lose as much weight (30.33±0.53 vs 27.42±0.66 g for control and CoPP treated lean mice, respectively). The prevention of weight gain in obese mice following treatment with CoPP was partially reversed by co-administration of SnMP. Food intake in the treatment and control groups was comparable (Figure 4B). As seen in Figures 4C and 4D, fat and body...
appearance of obese mice confirmed the reduction in fat content and body weight loss. Visceral fat in obese mice was decreased by CoPP treatment from 6.1±0.1 gm to 3.9±0.4 gm (p<0.03). Subcutaneous fat in obese mice was decreased from 3.8±0.2 gm to 2.5±0.2 gm (p<0.01). The ability of SnMP to reverse the CoPP-induced loss of body weight as well as the increased adiponectin levels in ob mice suggests that the decrease in adiponectin and the resultant weight gain was dependent on decreased HO activity.

**Effect of changes in HO-1-adiponectin levels on glucose tolerance and insulin sensitivity.** Glucose tolerance and insulin sensitivity were determined after development of insulin resistance (Figure 5A & 5B). Plasma glucose levels at all time points in obese mice were higher than those in obese mice treated with CoPP. Blood glucose levels in obese mice were significantly elevated (p<0.05) 30 minutes after glucose administration and remained elevated. In CoPP treated obese mice, blood glucose levels were significantly elevated (p<0.05) after 30 minutes but returned to initial levels at 120 minutes. Insulin administration to CoPP-treated obese mice produced a rapid decrease in glucose but not in the vehicle treated obese mice suggesting improved insulin sensitivity in CoPP-treated obese mice.

**Effect of HO-1 expression on obesity/diabetes on bone marrow adiponectin and HO activity and response to CoPP.** As seen in Figure 6A, bone marrow cells from obese mice stained for oil Red O displayed a significant increase in adipocytes (middle panel) compared with obese mice treated with CoPP. Adipogenesis as measured by the number of oil red O lipid droplets was markedly decreased in ob mice treated with CoPP to levels similar to those seen in lean mice (Figure 6B), suggesting that HO-1 induction exhibits an inhibitory effect on adipogenesis in vivo.

As seen in Figure 6C, HO activity in the marrow from lean mice was significantly lower in ob mice compared with lean mice (0.45±0.042 vs 0.69±0.05 nmol bilirubin/mg/h, respectively, p<0.05). CoPP treatment increased HO activity in both lean and obese mice to 1.93±0.26 and 1.74±0.24 nmol bilirubin/mg/h, respectively (Figure 6C). Superoxide levels in bone marrow increased (p<0.05) in obese mice compared to lean. CoPP treatment resulted in a significant (p<0.001) decrease in superoxide levels compared to obese mice (Figure 6D).

**Effect of HO-1 expression on isolation of MSC as measured by cell surface markers.** MSCs cells were stained with CD 45, CD4, CD166, CD90 and CD105, and then measured by FACS. Confirmation of the MSC phenotype was made by the presence of positive markers, 83.6% of the cells were positive for CD 166, 76.2% of the cells for CD90 and 57.26% of cells for CD105 positive. Also, the absence of CD34, a hematopoietic stem cell marker and CD 45, a lymphocytic marker (Figure 7A), indicate that were less than 0.2% cells positive for the negative markers, CD45 and CD34, suggesting that the MSC were pure and not contaminated.

**Effect of HO-1 expression on mesenchymal stem cell clonal efficiency.** Bone marrow mononuclear cells of ob fat mice (3x10^5/well in 6 well plate) were cultured for 14 days, and stained with Gimesa. The CFU-F colony numbers increased in the presence of high levels of glucose and SnMP when compared to control. CoPP administration resulted in a decrease in CFU-F when compared to the SnMP, and high glucose groups (Figure 7B and 7C) and the levels were comparable with the control group (15±1) indicating that inhibition of HO-1 increased CFU-F, whereas the induction of HO-1 decreased CFU-F. Inhibition of HO-1 by high glucose or SnMP resulted in increased MSC clonal efficiency.
In contrast, induction of HO-1 decreased clonal efficiency. **Effect of glucose and HO-1 expression on bone marrow mesenchymal stem cell-mediated adipogenesis.** As seen in Figure 8A, glucose increases adipogenesis in MSC similar to that seen in bone marrow. Induction of HO-1 inhibited adipogenesis in MSC (p<0.002). In contrast, inhibition of HO-1 did not significantly affect adipogenesis (Figure 8A and 8B). This may be due to inhibition of HO activity by glucose (35).

Figure 8C shows representative Western blots depicting HO-1 expression in MSC before and after glucose exposure. In MSC exposed to glucose, levels of HO-1 protein were barely detectable; this is similar to control MSC cell homogenates (Figure 8C, lower panel). CoPP and SnMP increased HO-1 in a similar manner, markedly inducing the levels of HO-1 protein.

**DISCUSSION**

We report in obese diabetic mice a decrease in HO-1 protein levels and HO activity when compared to age-matched lean control mice, and that increases in HO-1 protein levels following CoPP administration resulted in increased insulin sensitivity and decreased glucose tolerance. Further, increases in HO-1 protein expression were paralleled by increases in serum adiponectin levels, decreases in visceral and abdominal fat content and decreased plasma TNF, IL-6 and IL-1ß levels. Induction of HO-1 also resulted in decreased aorta adipocyte and bone marrow superoxide production (Figures 3D and 6D). Upregulation of HO-1 by CoPP treatment caused a decrease in adipogenesis in bone marrow both in vivo and in vitro, in cultured mesenchymal stem cells and a decrease in secretion of adiponectin in the culture media. This effect was blocked by the inhibitor of HO activity, SnMP, clearly delineating the existence of an HO-1-adiponectin axis that is responsible for the beneficial changes that occur in the metabolic syndrome. This hypothesis is bolstered by the observation that the increased activity of the HO-1-adiponectin axis was associated with increased insulin sensitivity and increased glucose tolerance. An increase in adiponectin levels inhibited the diabetes-mediated increase in TNF, IL-1 and IL-6. The metabolic syndrome and obesity are characterized by increases in serum levels of inflammatory cytokines such as TNF and IL-6, which decrease insulin sensitivity (36,37). Additionally, a decrease in serum adiponectin (ACR30) is the result of an increase in ROS, thus contributing to the pathogenesis of insulin resistance (38).

We report here a prevention of both body weight gain (Figure 4A) and visceral fat content (Figure 4C) that parallel the increase in adiponectin. The latter is an indicator of improvements in the metabolic syndrome which, in turn, leads to decreases in arterial diseases, heart disease and increased insulin sensitivity (3,10,37,39-41). While increases in obesity are considered a risk factor for cardiovascular complications (37), obesity-associated improvement in the diabetic phenotype, including increases in insulin sensitivity and glucose tolerance, may occur through adipose tissue and increased adiponectin secretion (10,42). We believe that an increase in the activity of the HO-1-adiponectin axis is crucial in providing adipocyte cells with tolerance to ROS.

The effect of CoPP on body weight with normal food intake is not unexpected. Multiple low dose regimens result in a prolonged prevention in body weight gain in genetically obese rats and mice (43,44). Low dose regimes of CoPP are not associated with alterations in endocrine homeostasis or hepatic heme metabolism or food intake as confirmed (Figure 4A).

The mechanism by which HO-1 is involved in increased adiponectin levels is, we believe, related to the function of HO-1 as
a stress response/chaperone protein as well as its ability to decrease ROS by increasing glutathione and EC-SOD levels (4,22,45) and by decreasing $O_2^-$ production (23,24). PPAR agonists, are shown to induce both HO-1 (21), and the rate-limiting chaperone protein EroL (46,47). PPAR agonist which increases adiponectin may do so by increasing the levels of EroL chaperone protein (46). Since PPAR also increases HO-1 protein levels (21) and HO-1 is known as a chaperone protein, it is possible that one of the mechanisms by which HO-1 can increase adiponectin levels is through more efficient adiponectin stabilization and protection. This would confirm the report of Wang et al who showed that the chaperone protein EroL increased adiponectin (47). We also have previously shown that upregulation of HO-1 protein in diabetic rats provided both cardio- and vascular- protection against ROS (24).

The seminal finding of a decrease in HO activity with a resultant decrease in CO generation (Figure 1A) in obesity reported in this study suggests that CO may be involved as a signaling molecule in adiponectin secretion. This result is in contrast to a previous report that indicated that the metabolic syndrome increases endogenous CO production promoting hypertension and endothelial dysfunction in obese Zucker rats (48). These investigators measured HO-derived CO by Hb-CO but exhaled CO was determined by GC/MS. In the present study, CO was measured using GC/MS. The difference in results remains to be elucidated, but could be explained by the use of different animal models, the high concentration of inhibitors of HO system (48).

The effect of HO-1 induction on the bone marrow fat cell is an excellent indicator of the effect on adipogenesis. HO-1 induction decreased mesenchymal stem cell-derived adipocytes in bone marrow and may decrease circulating adipocyte stem cells in the body. As a result it decreases adipogenesis in bone marrow as well as diminishing adipogenesis in visceral and subcutaneous fat and promoted the loss of body weight even with normal food intake. This was associated with an increase in adiponectin, insulin sensitivity and improvement in glucose tolerance (Figure 5). The mechanism by which HO-1 increases insulin sensitivity may be related to HO-1 mediated reduction in IL-1ß, TNF and IL-6 levels. The reduction in IL-1ß, TNF and IL-6 (Figure 3) levels in vivo after HO-1 expression by CoPP treatment could also contribute to a reduction in oxidative stress and thus contribute to the increase in adiponectin levels insulin sensitivity. TNF, IL-1 and IL-6 expression induced insulin resistance in human obesity (49,50). Because insulin acts centrally to decrease body weight, it has been suggested that the obesity state observed in ob mice is, in part, due to insulin resistance (37). The results we present here demonstrate that CoPP treatment decreased IL-1ß, improved insulin sensitivity and glucose tolerance (Figure 5). The HO-1-mediated increase in adiponectin may play an important role in modulating glucose tolerance and insulin sensitivity in these mice. Our results support recent reports of the beneficial effect of increased levels of adiponectin in the metabolic syndrome and obesity (10,22,37). The present study is of considerable interest from a clinical and basic science perspective, clearly defining the existence of an HO-1-adiponectin regulatory axis that can be manipulated to ameliorate the deleterious effects of increased insulin resistant, obesity and the metabolic syndrome in critical areas of cell damage associated with cardiovascular disease and diabetes.

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FIGURE LEGENDS

**Figure 1.** Effect of CoPP on HO-1 protein levels in aortas of lean and obese mice. **A)** Western blot and densitometry analysis of HO-1 protein in untreated lean and obese mice. Results are the mean ± SE of the band density normalized to actin, n=4, *p<0.05 vs lean. CO generation (pmol/mg/h) in aortas isolated from lean and obese mice, n=4, *p<0.05 vs lean. **B)** Western blot and densitometry analysis of HO-1 and HO-2 proteins in lean and obese mice treated with CoPP. Results are the mean ± SE of the band density normalized to actin; n=4; *p<0.01 CoPP-treated lean vs untreated lean; †p<0.01 CoPP-treated vs untreated ob mice; #p<0.01 obese CoPP-treated vs. lean CoPP-treated. **C)** HO activity in aorta of lean and obese mice treated with CoPP, n=4, #p<0.01 CoPP-treated vs vehicle-treated control.

**Figure 2.** Effect of CoPP on serum adiponectin levels in lean and ob mice. CoPP and SnMP were administered weekly for 6 weeks and serum samples were obtained immediately prior to sacrifice. The results are mean ± SE; n=8-12; **p<0.05 vs lean; *p<0.009 vs vehicle-treated ob mice; †p<0.02 vs vehicle-treated ob mice; †p<0.02 vs vehicle-treated ob mice; #p<0.05 vs CoPP-treated ob mice.

**Figure 3.** Effect of CoPP and SnMP on serum cytokines in lean and ob mice. Results are mean ± SE, n=8-12. **A)** IL-6; *p<0.001 vs lean; #p<0.001 vs vehicle-treated ob mice; **p<0.01 vs CoPP-treated ob mice. **B)** TNF ; *p<0.001 vs lean; #p<0.001 vs vehicle-treated ob mice; **p<0.001 vs CoPP-treated ob mice. **C)** IL-1 ; *p<0.05 vs lean; †p<0.05 vs vehicle-treated ob mice; **p<0.001 vs CoPP-treated ob mice. **D)** Superoxide generation *p<0.001 vs lean; #p<0.001 vs CoPP-treated ob mice.

**Figure 4.** Effect of HO-1 induction on body weight and visceral fat content in lean and obese mice. **A)** Effect of CoPP and SnMP administration on body weight of obese mice. Results are mean ± SE, n=6-10; *p<0.05 and **p<0.01 vs corresponding vehicle-treated mice. **B)** food intake in obese mice during the first two weeks of treatment. **C)** The weight of subcutaneous and visceral fat after CoPP treatment, *p>0.05 vs vehicle treated obese animal. **D)** Representative photographs showing mouse from each group after 6 weeks of treatment.

**Figure 5.** Effect of HO-1 expression on glucose tolerance and insulin sensitivity. Intraperitoneal glucose tolerance (IPGTT) **A)** and Intraperitoneal insulin sensitivity (IPITT) **B)** tests were performed as described in the methods. The results are expressed mean ± SEM, n=3.

**Figure 6. Oil red O staining on bone marrow** Evaluation of lipid content measured as percentage of Oil Red O staining. **A)** Representative photographs of lipid droplets in bone marrow of lean and ob mice treated with CoPP. **B)** Quantitative analysis of bone marrow lipid content. Results are mean ± SE, n=3, *p<0.05 vs vehicle-treated lean mice, #p<0.05 vs vehicle-treated ob mice. **C)** HO activity. Bone marrow cells were harvested and HO activity measured as described (35). Results are mean ± SE, n=4, #p<0.05 vs vehicle-treated lean mice. * p<0.01 vs vehicle-treated ob mice. **D)** Superoxide generation in bone marrow cells, bone marrow cells homogenates was harvested and O_2^- levels were determined as described in Methods. , *p<0.05 obese vs vehicle lean; #p<0.001 vs vehicle treated obese.
Figure 7. Effect of high glucose, CoPP and SnMP on bone marrow-derived MSC CFU-F. A) Surface markers of cultured cells were detected by a FACScan after staining with the mAbs listed in the figures. MSCs were negative for CD45 and CD4, while they were positive for CD166, CD90 and CD105. B) Representative photographs of CFU-F from obese bone marrow MSCs treated with and without high glucose, CoPP (2 M) and SnMP (5 M). The CFU-F colony was detected by staining with Giemsa solution. C) Effect of HO-1 expression on CFU-F number. Colonies were microscopically counted; *p<0.01 vs vehicle control, # p<0.02 and p<0.005 vs vehicle control. The data shown are Mean ± SEM, n= 4, *p<0.03 vehicle treated lean mice vs vehicle treated obese mice; #p<0.039 vehicle treated obese mice vs CoPP treated obese mice.

Figure 8. Effect of HO-1 expression on MSC-derived adipogenesis. A) Adipogenesis was measured as the relative absorbance of oil red O at day 10 after inducing adipogenesis as described in Methods. Results are mean±SE, n=4; *p<0.01 vs control medium; #p<0.005 vs high glucose. B) Representative photographs of MSC-derived adipocytes stained with oil red O. C) Western blot and densitometry analysis of HO-1 and actin proteins in MSC control, MSC treated with glucose in combination with CoPP or SnMP. Blots are representative of 4 separate experiments and D) Mean band density normalized relative to actin to HO-1 (*p < 0.001 vs MSC exposed to glucose).

NOTE: The figures for this article can be found using the link entitled “Figures”. (Available at http://dx.doi.org/10.2337/db07-1764.)