

Heme Oxygenase-Mediated Increases in Adiponectin Decrease Fat Content and Inflammatory Cytokines Tumor Necrosis Factor- α and Interleukin-6 in Zucker Rats and Reduce Adipogenesis in Human Mesenchymal Stem Cells

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ABSTRACT

Adiponectin, an abundant adipocyte-derived plasma protein that modulates vascular function in type 2 diabetes, has been shown to provide cytoprotection to both pancreatic and vascular systems in diabetes. Therefore, we examined whether up-regulation of heme oxygenase (HO)-1 ameliorates the levels of inflammatory cytokines and influences serum adiponectin in Zucker fat (ZF) rats. ZF rats displayed a decrease in both HO activity and HO-1 and HO-2 protein levels and an increase in tumor necrosis factor (TNF)- α and interleukin (IL)-6 compared with Zucker lean (ZL) rats. Treatment of ZF animals with 2 mg/kg cobalt protoporphyrin IX (CoPP) increased protein levels of HO-1 and HO activity, but HO-2 was unaffected. The increase in HO-1 was associated with a decrease in superoxide levels ($p < 0.05$) and an increase in plasma adiponectin ($p < 0.005$), compared with untreated ZF rats. CoPP treatment de-

creased visceral and s.c. fat content, and it reduced weight gain ($p < 0.01$). In addition, the inflammatory cytokines TNF- α and IL-6 were decreased ($p < 0.04$ and $p < 0.008$, respectively). Treatment of human bone marrow-derived adipocytes cultured with CoPP resulted in an increase in HO-1 and a decrease in superoxide levels. Up-regulation of HO-1 caused adipose remodeling, smaller adipocytes, and increased adiponectin secretion in the culture medium of human bone marrow-derived adipocytes. In summary, this study demonstrates that the antiobesity effect of HO-1 induction results in an increase in adiponectin secretion, in vivo and in vitro, a decrease in TNF- α and IL-6, and a reduction in weight gain. These findings highlight the pivotal role and symbiotic relationship of HO-1 and adiponectin in the modulation of the metabolic syndrome phenotype.

Oxidative stress has been implicated in the pathogenesis and cardiovascular complications of insulin resistance in type 2 diabetes (Wellen and Hotamisligil, 2005; Namikoshi et al., 2007). Excessive generation of reactive oxygen species (ROS) is the underlying mechanism of endothelial injury, resulting in an accelerated rate of apoptosis and endothelial cell sloughing (Kruger et al., 2005; Bahia et al., 2006; Kim et al., 2007a). In addition, reduced plasma adiponectin levels have been documented in patients with coronary artery disease and diabetes, presumably as a result of an increases in

ROS (Bakkaloglu et al., 2006; Ohashi et al., 2006; Haider et al., 2007). Lin et al. (2005) highlighted the importance of ROS production in adipocytes and the associated insulin resistance and changes in serum levels of adiponectin, suggesting that the increases in ROS are associated with an induced inflammatory response in the adipocyte.

Adipose tissue plays an important role in insulin resistance through the production and secretion of a variety of proteins such as tumor necrosis factor (TNF)- α , IL-6, leptin, and adiponectin (Berg and Scherer, 2005). Of these proteins, adiponectin has recently attracted much attention because it has insulin-sensitizing properties that reduce serum triglyceride levels and enhance fatty acid oxidation, insulin activity in the liver, and hepatic glucose uptake (Berg et al., 2001; Kim et al., 2007b). Adiponectin is exclusively secreted from

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ABBREVIATIONS: ROS, reactive oxygen species; TNF, tumor necrosis factor; IL, interleukin; HMW, high molecular weight; PPAR, peroxisome proliferator-activated receptor; HO, heme oxygenase; CoPP, cobalt protoporphyrin IX; SnMP, tin mesoporphyrin; ZL, Zucker lean; ZF, Zucker fat; FBS, fetal bovine serum; MSC, mesenchymal stem cell; O₂⁻, superoxide; FACS, fluorescence-activated cell sorting; DMEM, Dulbecco's modified Eagle's medium; h, human.

adipose tissue and its expression is higher in s.c. compared with visceral adipose tissue (Fain et al., 2004). It circulates in the blood, and it is found as both low-molecular-weight oligomers and high-molecular-weight (HMW) multimers (Basu et al., 2007). HMW adiponectin is reported to be more active and to correlate more significantly with glucose and insulin levels compared with both low-molecular-weight and total adiponectin (Lara-Castro et al., 2006). Low plasma levels of HMW adiponectin have been consistently associated with obesity, insulin resistance, type 2 diabetes, and coronary artery disease (Arita et al., 1999). L'Abbate et al. (2007) recently reported that increased adiponectin levels associated with increased expression of HO-1 resulted in enhanced cardiac protection from ROS. PPAR γ -response element has been found to increase expression of adiponectin (Iwaki et al., 2003) and also to regulate the expression of HO-1 in human vascular cells (Krönke et al., 2007).

The HO system provides both antioxidant and antiapoptotic properties due to its products bilirubin/biliverdin and CO (Ollinger et al., 2007). HO-1 is induced by oxidant stress, and it plays a crucial role in protection against oxidative insult in diabetes and cardiovascular disease (Abraham and Kappas, 2008). Up-regulation of HO-1 gene expression prevents vascular dysfunction and endothelial cell death through decreases in ROS levels (Abraham et al., 2004).

In the present study, we report that HO activity and HO-1 protein expression were decreased in obese rats, and we hypothesized that induction of HO-1 might serve to counteract the negative effects of type 2 diabetes mellitus and the metabolic syndrome. Using cobalt protoporphyrin IX (CoPP), an inducer and tin mesoporphyrin (SnMP, an inhibitor) to manipulate HO activity, we report here, for the first time, that induction of HO-1 was associated with reduced fat content and prevention of weight gain as a result of reduced adipogenesis in both in vitro and in vivo models of type 2 diabetes mellitus.

Materials and Methods

Animal Protocols. Male Zucker lean (ZL) rats (Charles River Laboratories, Wilmington, MA) and Zucker obese rats (Charles River Laboratories) were maintained on a standard rat diet and tap water ad libitum. In the first protocol, we used Zucker obese but not diabetic rats and in the second protocol, we used 11-week-old Zucker fat (ZF) rats and age-matched ZL controls (six animals/group). In the second protocol, we examined the effects of HO-1 and adiponectin preconditioning on the expression of HO-1, adiponectin, and the vascular phenotype ($n = 6$ /group). Chemical structures of most of the compounds were described previously (Martasek et al., 1988).

Glucose monitoring was performed using an automated analyzer (Lifescan Inc., Milpitas, CA). Beginning at 11 weeks when all rats had established diabetes, CoPP, SnMP (Frontier Science, Logan, UT), or both were given weekly at a dose of 2 and 5 mg/kg once and three times a week, respectively, by i.p. injection for 6 weeks. Control animals were administered an equal volume of vehicle (0.1 M sodium citrate buffer, pH 7.8) i.p. Six groups of Zucker rats were used: 1) ZL, 2) ZL-CoPP, 3) ZL-CoPP + SnMP, 4) ZF, 5) ZF-CoPP, and 6) ZF-CoPP + SnMP. There was no difference in the food intake among the treatment groups. 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, s.c. and visceral fat in the abdomen (visible mesenteric fat, fat around the liver, fat around the kidney, and fat around the spleen) was 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 then they were placed in homogenization buffer [10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Tergitol (Mallinckrodt Baker, Phillipsburg, NJ), pH 7.5] and homogenized using a standard glass homogenizer and pestle. Homogenates were centrifuged at 27,000g for 10 min at 4°C . The supernatant was isolated, and protein levels were assayed by Bradford method (Bradford, 1976). The supernatant was used for measurement of HO-1 and HO-2 (Nventa Biopharmaceuticals, San Diego, CA). Protein levels were visualized by immunoblotting with antibodies against each specific mouse protein. Actin was used to ensure adequate sample loading for all Western blots. Antibodies were prepared in the following dilution: HO-1 and HO-2, 1:1000. In brief, 20 μg of lysate supernatant was separated by 12% SDS-polyacrylamide gel electrophoresis, and then it was transferred to a nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, UK) with a semidry transfer apparatus (Bio-Rad, Hercules, CA). The membranes were incubated with 10% milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 buffer at 4°C overnight. After they were washed with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20, the membranes were incubated with anti-HO-1 or anti-HO-2 for 1 h at room temperature, with constant shaking. The filters were washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (GE Healthcare). Chemiluminescence detection was performed with the Amersham enhanced chemiluminescence detection kit, according to the manufacturer's instructions.

Aortic HO activity was assayed as described previously (Abraham et al., 2003) using a technique in which bilirubin, the end product of heme degradation, was extracted with chloroform, and its concentration was determined spectrophotometrically (dual UV-visible beam spectrophotometer Lambda 25; PerkinElmer Life and Analytical Sciences, Waltham, MA) using the difference in absorbance at a wavelength from 460 to 530 nm, with an extinction coefficient of 40 $\text{mM}^{-1}\text{cm}^{-1}$.

Human Bone Marrow-Derived Adipocyte Mesenchymal Stem Cells. Frozen bone marrow mononuclear cells were purchased from Allcells (Emeryville, CA). After thawing the cells, mononuclear cells were resuspended in an α -minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen) and 1% antibiotic/antimycotic solution (Invitrogen). The cells were plated at a density of 1 to 5×10^6 cells per 100- cm^2 dish. The cultures were maintained at 37°C in a 5% CO_2 incubator, and the medium was changed after 48 h and every 3 to 4 days thereafter. When the MSCs were confluent, the cells were recovered by the addition of 0.25% trypsin/EDTA (Invitrogen). MSCs (passages 2–3) were plated in a 60- cm^2 dish at a density of 1 to 2×10^4 and cultured in α -minimal essential medium with 10% FBS for 7 days. The medium was replaced with adipogenic medium, and the cells were cultured for an additional 21 days. The adipogenic media consisted of complete culture medium supplemented with DMEM-high glucose, 10% (v/v) FBS, 10 $\mu\text{g}/\text{ml}$ insulin, 0.5 mM dexamethasone (Sigma-Aldrich, St. Louis, MO), 0.5 mM isobutyl methylxanthine (Sigma-Aldrich), and 0.1 mM indomethacin (Sigma-Aldrich).

Oil Red O Staining. For Oil Red O staining, 0.5% Oil Red O solution (Sigma-Aldrich) was used. In brief, adipocytes were fixed in 1% formaldehyde, washed in Oil Red O for 20 min, rinsed with 85% propylene glycol (Sigma-Aldrich) for 3 min, washed in distilled water, and then mounted with aqueous mounting medium (Bavendiek et al., 2005).

Measurement of Mesenchymal $\text{O}_2^{\cdot -}$ Levels and Inflammatory Cytokines and Adiponectin. Using previously described methods, control and fat mesenchymal stem cells, 0.3 mg of protein, approximately 3×10^5 cells, were placed in plastic scintillation minivials

containing 5 μM lucigenin for the detection of O_2^- and other additions in a final volume of 1 ml of air-equilibrated Krebs' solution buffered with 10 mM HEPES-NaOH, pH 7.4. Lucigenin chemiluminescence was measured in a liquid scintillation counter (LS6000IC; Beckman Coulter, Fullerton, CA) at $\sim 37^\circ\text{C}$, and data are reported as counts per minute per milligram of protein after background subtraction.

Adiponectin (HMW), TNF- α , and IL-6 were determined in rat serum using an enzyme-linked immunosorbent assay. Multiple assay kits were used for quantification of the proteins in rat serum, and assays were conducted according to the manufacturer's protocol (Pierce, Woburn, MA). Plates were analyzed using a Luminex 100IS analyzer (Luminex Inc., Austin, TX). The data were evaluated as the median fluorescence intensity using appropriate curve-fitting software. A five-parameter logistic method with weighing was used. All measurements were performed in triplicate. Cytokine assays were done according to instructions provided by BD Gentest (Woburn, MA).

Detection of MSC Cell Markers by FACS Analysis. Human MSCs are defined by an array of positive and negative markers. MSCs are normally plastic-adherent under standard culture conditions, expressing CD105, CD73, and CD90. MSCs must lack expression of CD45, CD34, CD14, or CD11b, CD79 or CD19, and human leukocyte antigen DR-1. In addition, MSCs must be able to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro (Keating, 2006).

Human MSC phenotype was confirmed by flow cytometry (Elite ESP 2358; Beckman Coulter) using several markers known to be found on MSCs. The negative markers used were anti-CD34 and anti-CD45 (BD Biosciences Pharmingen, San Diego, CA), also known to be expressed as hematopoietic stem cell marker and common lymphocyte antigen. CD90, CD105, and CD166 were used as positive

markers for MSCs. The data were analyzed using WinMDI 2.8 software (<http://facs.scripps.edu/software.html>).

Culture Conditions of Adipocytes and Effect of HO-1 Inducers and Inhibitors. Adipogenic differentiation of hMSCs was induced by incubation in an adipogenesis induction medium (25 mM DMEM-high glucose supplemented with 10 $\mu\text{g}/\text{ml}$ insulin, 1 μM dexamethasone, 0.2 mM indomethacin, 10% FBS, and 1% antibiotic-antimycotic solution). Medium was changed every 3 to 4 days (Novikoff et al., 1980; Tondreau et al., 2005). In addition, treatment with 5 μM SnMP and glucose was administered every 2 days. CoPP (2 μM) treatment and media changes were applied every 4 days. The conditioned media was harvested after 6 days of culture, and the levels of adiponectin were determined.

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 analysis of variance for multiple groups or unpaired t test for two groups. Data are presented as mean \pm S.E.

Results

Levels of HO-1 Protein in Aorta and Adipocytes of ZL and ZF Animals. The levels of HO-1 and HO-2 protein were measured in 17-week-old animals. In the aorta, levels of HO-1 protein in ZF animals were significantly decreased compared with age-matched ZL animals ($p < 0.04$). The ratio of HO-1/actin declined from 0.57 ± 0.11 in lean animals to

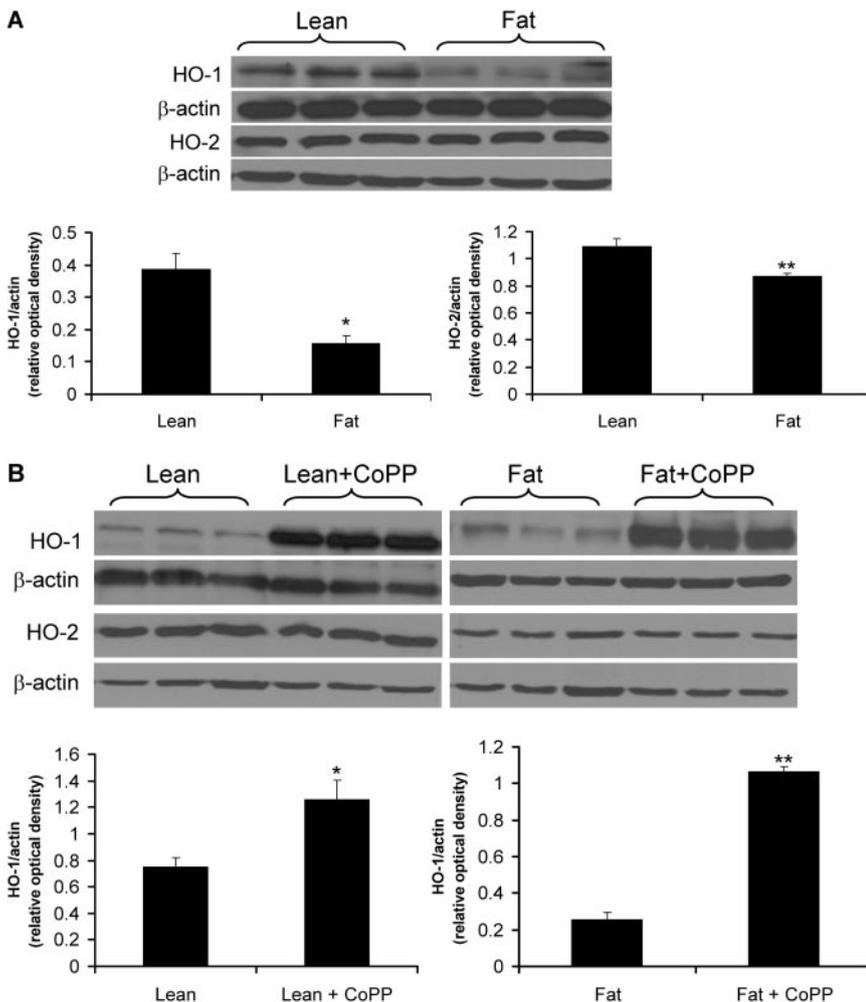


Fig. 1. A, HO-1 and HO-2 expression in ZF and ZL rats. Aortic samples were subjected to Western blotting for the determination of HO-1 and HO-2 protein and densitometry analyses of HO-1 and HO-2/actin ratio. *, $p = 0.04$, fat versus lean; **, $p = 0.02$, fat versus lean. B, effect of CoPP on HO-1 and HO-2 expression in the aorta of lean and fat rats. CoPP was administered once a week for 6 weeks. Aortic samples were subjected to Western blotting for the determination of HO-1 and HO-2 protein expression and densitometry analysis of HO-1/actin ratio. Levels of significance: *, $p < 0.01$, lean versus CoPP-treated lean; *, $p < 0.005$, fat versus CoPP-treated fat.

0.28 ± 0.02 in ZF animals (Fig. 1A). The levels of HO-2 protein were also lower ($p < 0.02$) in ZF animals compared with age-matched ZL animals (Fig. 1A), indicating a decrease in both the inducible and constitutive forms of HO in this animal model of obesity.

Moreover, in a separate set of experiments, CoPP administration, once a week for 6 weeks resulted in a significant increase in HO-1 protein levels in both sets of animals. HO-1 protein was increased 4.3-fold in ZF CoPP-treated animals ($p < 0.005$) compared with ZF animals and 1.3-fold in CoPP-treated ZL animals ($p < 0.05$) compared with ZL animals (Fig. 1B). The levels of HO-2 were unchanged after CoPP administration. Similar results were seen in heart and kidney (data not shown).

HO activity was measured in the aorta of ZF and ZL rats after CoPP and CoPP + SnMP treatment. CoPP administration, in ZF rats, increased HO activity 4- to 5-fold compared with untreated ZF rats (Fig. 2). As seen in Fig. 2, HO activity was increased in ZF rats after CoPP treatment (2.3 ± 0.1 nmol bilirubin formed/mg protein/h; $p < 0.001$) compared with control (0.34 ± 0.04 nmol bilirubin formed/mg protein/h). A similar effect on HO activity was seen in ZL animals. Treatment with the HO inhibitor SnMP in combination with CoPP inhibited HO activity in both ZF and ZL animals compared with ZF and ZL animals treated with CoPP alone (Fig. 2). It should be noted that HO activity was lower on ZF animals compared with ZL animals ($p < 0.05$). This parallels the decrease in HO-1 and HO-2 protein levels described above (Fig. 1, A and B). Similar results were seen in heart and kidney (data not shown).

Effect of CoPP on Serum Adiponectin Levels. To determine the effect of HO induction on adiponectin, we measured the adiponectin level in ZF and ZL rats before and after treatment with CoPP for 6 weeks. As seen in Fig. 3A, adiponectin was notably lower in 18-week-old ZF animals compared with ZL animals ($p < 0.05$). After the treatment with CoPP, serum adiponectin increased in ZF rats to 6.7 ± 0.5 µg/ml compared with 2.5 ± 0.3 µg/ml in control ZF rats ($p < 0.005$). Moreover, the increase in adiponectin in ZF rats treated with CoPP attained a level significantly ($p < 0.01$) above that in ZL controls (Fig. 3A). When CoPP and SnMP were coadministered to ZF animals, adiponectin levels did not increase, and they remained at the levels of ZF controls (Fig. 3A).

Effect of CoPP on IL-6 and TNF-α. To investigate the connection between HO induction and classic risk factors of

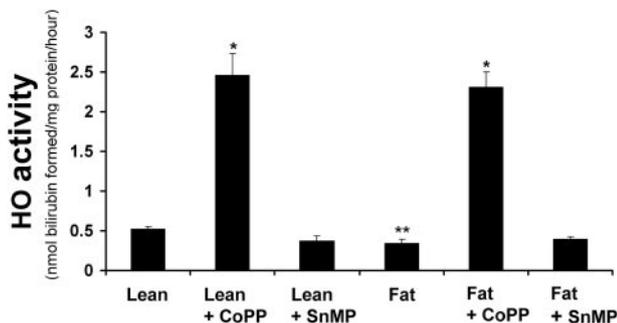


Fig. 2. Effect of CoPP and SnMP on HO activity in the aorta of lean and fat rats. HO activity was determined in samples prepared from aorta (see *Materials and Methods*). Levels of significance: *, $p < 0.001$, lean versus CoPP-treated lean and fat versus CoPP-treated fat; **, $p < 0.05$, lean versus fat.

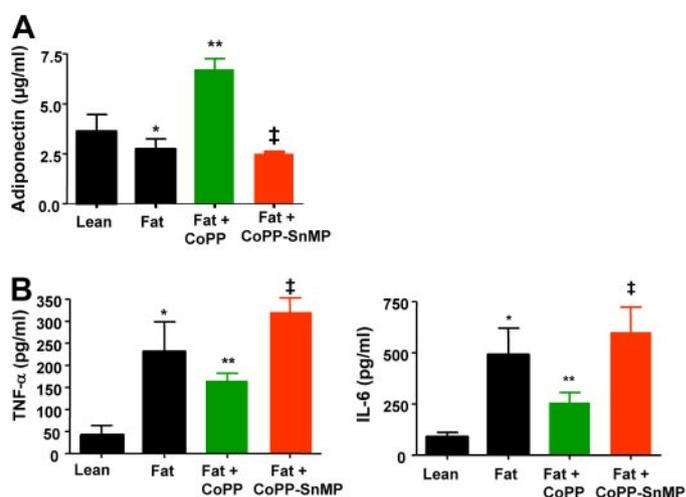


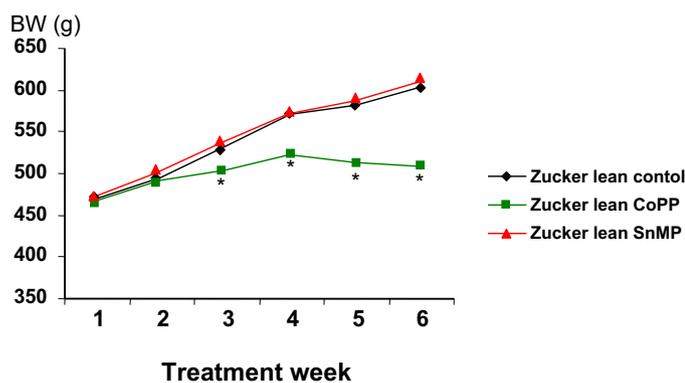
Fig. 3. A and B, effect of CoPP on serum adiponectin levels in lean and fat rats. CoPP and SnMP were administered weekly for 6 weeks, and serum samples were obtained immediately before sacrifice. The results are expressed as picograms per milliliter of serum. Levels of significance: *, $p < 0.05$, lean versus fat; **, $p < 0.005$, fat versus CoPP-treated fat; ‡, $p < 0.001$, CoPP-treated fat versus SnMP-treated fat. The results are expressed as picograms per milliliter of serum for IL-6 and TNF-α. B, effect on serum IL-6 levels in lean and fat rats. Levels of significance for IL-6: *, $p < 0.03$, lean versus fat; **, $p < 0.008$ lean versus fat + CoPP; ‡, $p < 0.04$, fat versus fat + CoPP + SnMP. Effect on serum TNF-α levels in lean and fat rats. Levels of significance for TNF-α: *, $p < 0.002$, lean versus fat; **, $p < 0.0001$, lean versus fat + CoPP; ‡, $p < 0.04$, fat versus fat + CoPP; $p < 0.014$, fat versus fat + CoPP + SnMP.

type 2 diabetes, we assessed IL-6 and TNF-α levels in ZF and ZL animals in response to treatment with CoPP for 6 weeks. Cytokines TNF-α and IL-6 were significantly ($p < 0.002$ and $p < 0.03$, respectively) increased in ZF animals compared with ZL animals (Figs. 3B). After treatment with CoPP, the levels of both proinflammatory cytokines (IL-6 and TNF-α) were significantly decreased in CoPP-treated ZF rats compared with control ZF rats ($p < 0.008$ and $p < 0.04$, respectively). These effects were blocked by coadministration of SnMP with CoPP (Fig. 3B).

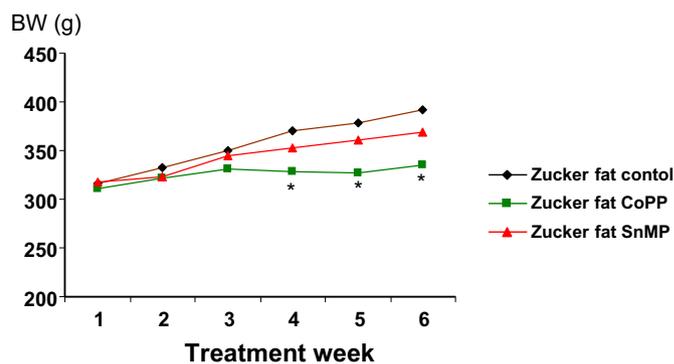
Effect of CoPP on Body Weight and Fat Content. In examining the effects of HO-1 on body weight, we used age and weight-matched ZL and age and weight matched ZF animals. Both the vehicle-treated and the CoPP-treated rats gained weight. In the ZF controls, weight was increased to a final value of 637 ± 22.6 g. The rate of body weight gain in ZF rats treated with CoPP was less, and a difference ($p < 0.05$) was seen after as little as 2 weeks. Subsequently, the weight curves continued to diverge, and they were different at every subsequent time point (Fig. 4A). The coadministration of SnMP with CoPP blocked the effect of CoPP on weight gain. Similar results were also found in the ZL group. After treatment, ZL rats gained weight, whereas CoPP-treated Zucker lean rats maintained the same approximate body weight. Weight gain was significantly ($p < 0.05$) less in the CoPP-treated rats even after 2 weeks, and the weight curves continued to diverge throughout the next 6 weeks (Fig. 4B). There was no difference in food intake during the 6-week treatment period between ZF and ZL rats treated with either vehicle or CoPP (data not shown).

As seen in Fig. 4, C and D, fat and body appearance of obese rat 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 to 3.9 ± 0.4 g ($p < 0.001$). Subcu-

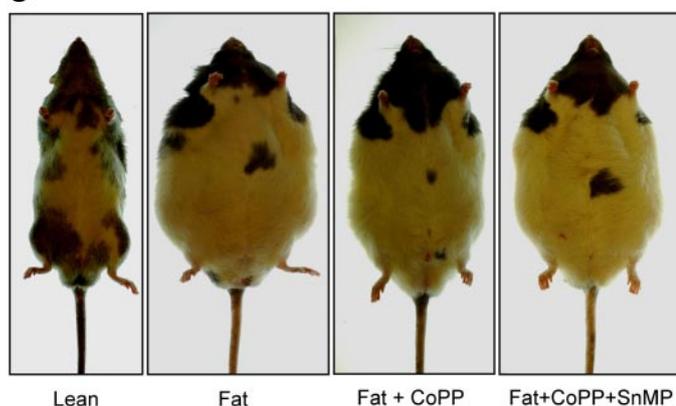
A Body weight of Zucker fat rat



B Body weight of Zucker lean rat



C



D

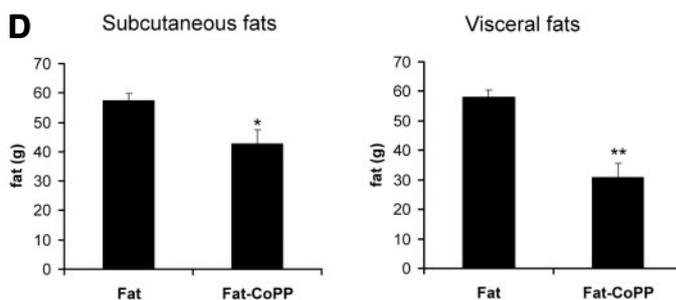


Fig. 4. A to D, effect of up-regulation of body weight and visceral fat content in lean and obese rats after 6 weeks of CoPP treatment (2 mg/kg/week) or vehicle solution. Obese (A) and lean (B) rats were treated, and weight was determined (average of two independent experiments). $n = 6$ for control and $n = 10$ for treatment in each group; *, $p < 0.05$, CoPP-treated versus control. C, representative photograph for untreated obese rats, obese-CoPP, and obese-CoPP + SnMP after 6 weeks of treatment. D, weight of s.c. and visceral fat after 6 weeks of CoPP treatment (6 weeks). Level of significance of Zucker fat rat treated with CoPP. *, $p < 0.05$ fat + CoPP versus fat. **, $p < 0.001$ fat + CoPP versus fat.

taneous fat in obese mice was decreased from 3.8 ± 0.2 to 2.5 ± 0.2 g ($p < 0.05$). SnMP reversed the CoPP-induced loss of body weight as well as the increased adiponectin levels, suggesting that the decrease in adiponectin and the resultant weight gain was dependent on decreased HO activity.

Determination of Human Mesenchymal Stem Phenotype and HO-1 Expression in Human MSCs. hMSCs were examined for various positive and negative markers by flow cytometry to establish the identity of mesenchymal stem cells. Confirmation of hMSC phenotype was made by the presence of positive markers CD 90, CD105, and CD166. The absence of CD34, a hematopoietic stem cell marker, and CD 45, a lymphocytic marker, confirmed that MSCs were not contaminated (Fig. 5). Our population of hMSCs was found to be 99.7% positive for CD90, 87.5% positive for CD105, and 96.9% positive for CD166. There was less than 0.2% positivity for both negative markers, CD45 and CD34 (Fig. 5).

To determine HO-1 expression in hMSCs during adipogenesis, we measured HO-1 protein expression through Western blot and densitometry analysis. CoPP and SnMP treatment, increased HO-1 expression by more than 50- and 60-fold, respectively, as shown by Western blot and densitometry analysis (Fig. 6). Densitometry analysis showed that CoPP and SnMP increased the ratio of HO-1/

actin, demonstrating the potent effect of CoPP and SnMP on HO-1 gene expression.

Effect of CoPP on Mesenchymal Stem Cell O_2^- Levels. Measurements of superoxide levels in mesenchymal stem cells demonstrated increased levels of O_2^- in these cells isolated from ZF animals compared with cells isolated from ZL animals ($p = 0.04$, $n = 4$) (Fig. 7). CoPP-treated ZF rats showed a significant decrease in O_2^- levels compared with untreated ZF rats, from 5.67 ± 3.1 to 3.47 ± 0.7 ($p = 0.03$). These results show the effect of CoPP treatment resulting in decreased levels of O_2^- .

Effect of HO-1 Expression on Adipogenesis in Human Bone Marrow-Derived Mesenchymal Stem Cells. Lipid O staining and Image-Pro analysis (Media Cybernetics, Inc., Bethesda, MD) revealed that hMSCs treated with CoPP (301.8 ± 16.8 cells) showed a decrease in adipogenesis, resulting in a significantly decreased number of adipocytes compared with hMSCs treated with glucose and CoPP (374 ± 10.2) ($p < 0.004$) (Fig. 8, A and B). HO-1 induction by CoPP led to a decrease in adipocyte number (271 ± 3.8) similar to the number of adipocytes seen with control (301.8 ± 16), with the difference between being statistically insignificant. The number of adipocytes formed after treatment with CoPP and glucose was also measured by flow cytometry staining with

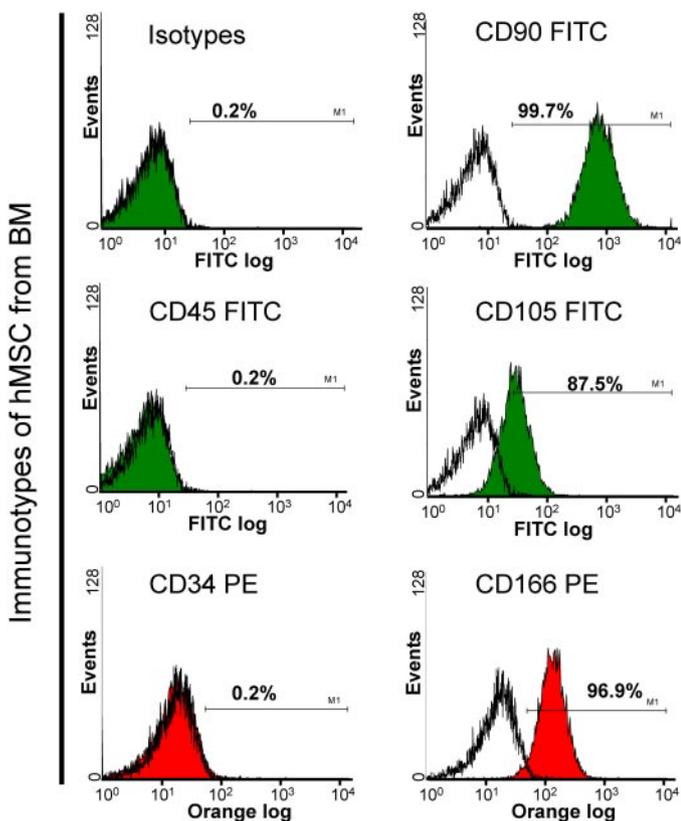


Fig. 5. Surface expression of CD90, CD105, and CD166 on hMSCs were monitored by FACS analysis. Percentage of cells expressing positive markers CD90 (Thy-1), CD105 (endoglin), and CD166 (activated leukocyte cell adhesion molecule) were 99.7, 87.5, and 96.9%, respectively. CD45 and CD34 were shown to be expressed in less than 0.2% of cells, the same as unstained isotypes. CD45 (common lymphocytes antigen) and CD34 (hematopoietic stem cell marker) were used as negative markers. FITC, fluorescein isothiocyanate; BM, bone marrow; PE, R-phycoerythrin.

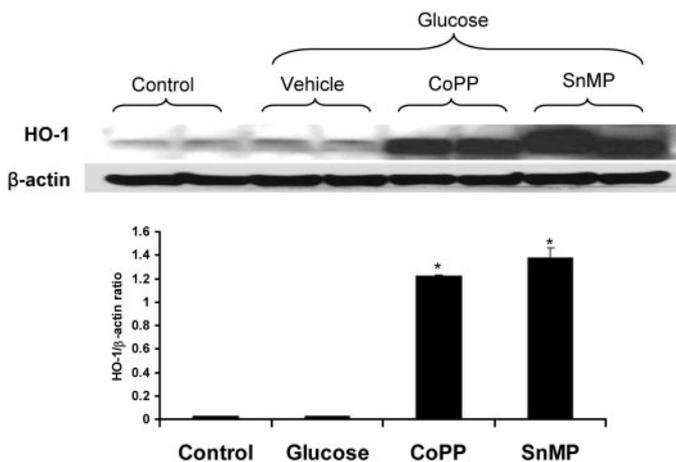


Fig. 6. Western blot analysis of HO-1 in hMSCs during adipogenesis. Quantitative densitometry analysis of protein levels was determined, and data are expressed as a ratio to β -actin (mean \pm S.E.; $n = 3$). Representative Western blots for HO-1 and actin along with densitometry analysis for HO-1/actin. HO-1 expression was increased in CoPP and SnMP. We measured HO-1 expression from hMSCs during adipogenesis. Control, adipogenesis media (2 mM DMEM-high glucose); CoPP, 5 μ M; glucose, additional 10 mM glucose. *, $p < 0.001$ versus control.

BODIPY after 2 weeks of treatment (Fig. 9A). There was decreased adipogenesis in CoPP-treated cells compared with hMSCs treated with glucose. We also observed a significant

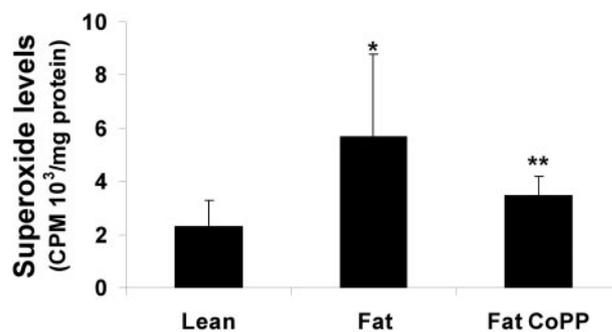


Fig. 7. Effect of CoPP on superoxide levels in mesenchymal stem cells from ZL and ZF (mean \pm S.E.; $n = 4$). *, $p < 0.04$ versus ZL control; **, $p = 0.03$ versus ZF untreated versus ZF untreated.

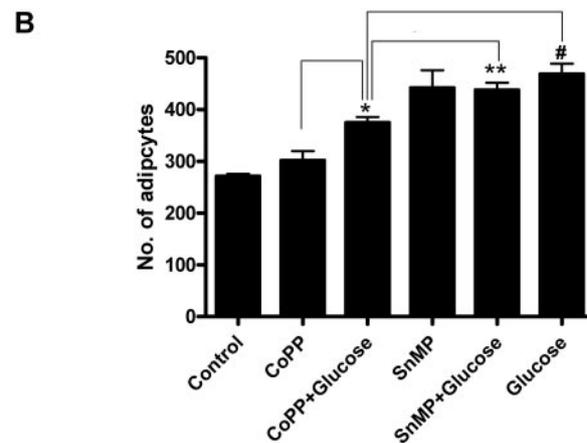
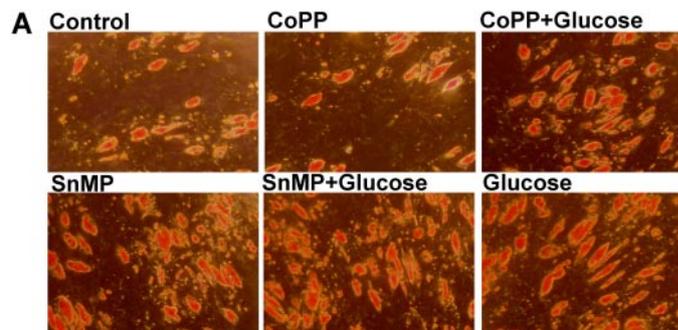


Fig. 8. A, effect of glucose and HO-1 expression on adipogenesis. We measured adipocytes with Oil Red O (staining is specific for lipid droplets) staining after adipogenesis from hMSCs. Control, adipogenesis media (25 mM DMEM-high glucose); CoPP, 2 μ M; CoPP represents 2 μ M CoPP treated group in adipogenic media for 2 weeks; CoPP + glucose, 2 μ M CoPP + additional 10 mM glucose. B, effect of HO-1 expression on the number of adipocytes in the presence of glucose and CoPP. Calculated cell numbers by Image-Pro software. *, $p < 0.004$ CoPP versus CoPP + glucose; **, $p < 0.01$ CoPP + glucose versus SnMP + glucose; #, $p < 0.005$ CoPP + glucose versus glucose.

shift to left indicating decreased adipogenesis in CoPP-treated glucose treated cells compared with cells treated with glucose alone ($p < 0.05$) (Fig. 9A). To confirm the role of HO-1 on adiponectin secretion, we studied the effect of CoPP. CoPP administration resulted in a significant increase ($p < 0.04$) in adiponectin secretion compared with both control cells and cells treated with glucose (Fig. 9B).

Discussion

This study suggests a novel role for HO-1 in ameliorating obesity-mediated inflammation and for the existence of an HO-

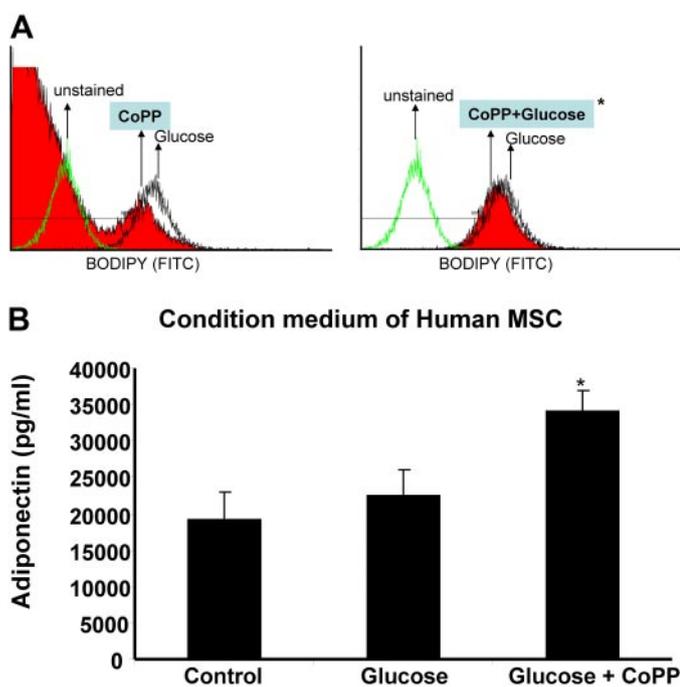


Fig. 9. A, FACS analysis of BODIPY of mesenchymal stem cells during adipogenesis in presence and absence of HO-1 inducers. Adipocytes measured with BODIPY (staining specific to lipid droplets) by FACS after adipogenesis from hMSCs. CoPP represents 2 μ M CoPP-treated group in adipogenic media for 2 weeks, CoPP + glucose, 2 μ M CoPP + additional 10 mM glucose for 2 weeks. HO-1 expression (A) by CoPP causes shift of mesenchymal stem cells to the left, and glucose increases lipid droplets causing shift to the right; *, $p < 0.05$. Effect of CoPP induced HO-1 expression (B) causes a decrease in number of adipocytes and shift to the left; $p < 0.005$. B, effect of CoPP on adiponectin secretion from adipocytes. The conditions used are described in the legend to Fig. 8. $p < 0.04$, glucose + CoPP versus glucose.

1-adiponectin axis that is central to the reversal of the metabolic derangement that occurs in obesity. Four observations in the Zucker obese rat model substantiate this conclusion. First, obese rat aorta display lower HO activity, and lower HO-1 and HO-2 protein levels, compared with aorta isolated from ZL animals (Figs. 1A and 2). This is associated with a decrease in serum adiponectin levels (Fig. 3). Obese rats treated with CoPP, an inducer of HO-1, demonstrated robust increases in HO activity, HO-1 protein, and adiponectin levels but no change in HO-2 protein levels. Second, the increase in adiponectin levels seen in obese rats was associated with a reduction in visceral and s.c. fat content and in weight gain. This effect was blocked by SnMP, an inhibitor of HO activity, clearly delineating the inter-relationship of HO-1 with adiponectin in the modulation of the metabolic syndrome. Third, the HO-1-adiponectin-mediated increase seen in obese rats was associated with a decrease in the serum levels of the inflammatory cytokines TNF- α and IL-6. Fourth, up-regulation of HO-1 by CoPP treatment caused a decrease in droplet formation and adipogenesis in human bone marrow-derived adipocyte stem cells but increased secretion of adiponectin in the culture media. This important observation is bolstered by the demonstration that the increased HO activity resulted in a reciprocal decrease in secretion of TNF- α and IL-6. These decreases in cytokine levels when considered with increases in adiponectin signify that a favorable outcome can be achieved by increased levels of HO-1 in obesity.

The effect of CoPP on body weight, achieved with normal food intake, is not unexpected. Multiple low-dose regimens

result in a prolonged decrease in the rate of body weight gain in genetically obese rats and mice (Galbraith and Kappas, 1990, 1991). Low-dose regimes are associated with no reduction in food intake. The action of CoPP is thought to be a central nervous system action (Galbraith et al., 1992; Li et al., 2006). The effect of CoPP on body weight (Fig. 4A) in obese rats confirms these studies, and it extends them by demonstrating the potential role of HO-1-mediated increase in adiponectin in a reduction in weight gain. The HO-1 mediated increase in serum adiponectin is manifest by an improvement in the metabolic syndrome, which, in turn, may lead to improvements in arterial and heart disease (Bahia et al., 2006; Kim et al., 2007b). Increases in obesity are considered a risk factor for cardiovascular complications (Lazar, 2005). Metabolic syndrome and obesity are characterized by an increase in serum levels of inflammatory cytokines such as TNF- α and IL-6, with a resultant decrease in insulin sensitivity (Muse et al., 2007). Increases in adiponectin levels and the associated reduction in body weight and both s.c. and visceral fat content (Fig. 4D) are important antagonists of the metabolic syndrome, resulting in a decrease in arterial and heart disease (Bahia et al., 2006; Fontana et al., 2007). The beneficial effects of adiponectin in a variety of cardiovascular diseases have been reviewed by Hopkins et al. (2007).

The mechanism by which HO-1 is involved in increased adiponectin levels may be related to the function of HO-1 as a stress response/chaperone protein as well as its ability to increase antioxidants levels via an increase in glutathione and extracellular superoxide dismutase levels (Abraham et al., 2008). Galinier et al. (2006) reported that an increase in antioxidants resulted in the promoted accumulation of triglycerides in the adipocyte (Galiner et al., 2006). The latter suggest that obesity is associated with an increase in intracellular antioxidants even under the stressful conditions of obesity (Galiner et al., 2006). This result is not contradictory to the present findings, because the levels of the antioxidant may not be sufficient; furthermore, the levels of antioxidants measured may be dependent on the stage of obesity. The mechanism by which HO-1 acts as an anti-obesity agent is not only due to its antioxidant properties of heme removal but also the effect of HO-1-derived product activity. Up-regulation of HO-1 increases the levels of numerous signaling molecules, including phospho-AKT and endothelial NO synthase, increases mitochondrial transport, restores mitochondrial cytochrome oxidase, inhibits caspase activity, and decreases the number of CD11c dendritic cells (Di Noia et al., 2006; Kruger et al., 2006; Li et al., 2007). Up-regulation of HO-1 caused adipose tissue remodeling, resulting in smaller adipocytes and inhibition of adipogenesis. In addition, increased levels of HO-1 protein were mirrored by increased levels of adiponectin.

PPAR γ agonists are known to induce both HO-1 (Krönke et al., 2007) and the rate-limiting chaperone protein EroL (Wang et al., 2007). PPAR γ agonist, which increases adiponectin, may do so by increasing the levels of EroL chaperone protein. Because PPAR γ also increases HO-1 protein levels (Krönke et al., 2007) 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 add substance to the report that the chaperone protein EroL increased adiponectin levels (Wang et al., 2007).

The seminal finding described in this report is the crucial role of HO-1-adiponectin axis in ameliorating obesity-mediated increases in plasma levels of inflammatory cytokines. This is associated with increased secretion of adiponectin in vivo and in vitro. However, the critical effect of antioxidants and HO-1 expression may be related to adipocyte compensation mechanism to increase adiponectin levels (Figs. 3 and 9B). In cultured mesenchymal stem cells, CoPP increases adiponectin during the early stages of adipocyte stem cell growth. However, CoPP inhibits adipogenesis and decreases adiponectin and the number of adipocytes due to the inhibition of adipogenesis. The mechanism remains to be clarified. However, these results support recent reports that describe the beneficial effect of increases in adiponectin in metabolic syndrome and obesity (Kim et al., 2007b; Li et al., 2008). The present data are 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 obesity and metabolic syndrome and prevent critical areas of cell damage associated with cardiovascular disease.

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