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

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OBJECTIVE—We hypothesized that the induction of heme oxygenase (HO)-1 and increased HO activity, which induces arterial antioxidative 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 intraperitoneally administered the HO-1 inducer cobalt protoporphyrin (3 mg/kg CoPP) with and without the HO inhibitor stannous mesoporphyrin (2 mg/100 g SnMP) 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 (MSCs) were isolated and assessed for HO expression and adipogenesis.

RESULTS—HO activity was reduced in *ob* mice compared with 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 0.01, respectively, compared with vehicle animals), increased serum adiponectin, and decreased plasma tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β 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 and in cultured MSCs and increased adiponectin levels in the culture media. Inhibition of HO activity decreased adiponectin and increased secretion of TNF-α, IL-6, and IL-1β 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. ***Diabetes*** **57:1526–1535, 2008**

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CFU-F, colony forming units–fibroblasts; CoPP, cobalt protoporphyrin; FBS, fetal bovine serum; GCMS, gas chromatography–mass spectrometry; HO, heme oxygenase; IL, interleukin; MSC, mesenchymal stem cell; PPAR, peroxisome proliferator–activated receptor; ROS, reactive oxygen species; SnMP, stannous mesoporphyrin; TNF-α, tumor necrosis factor-α.

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. (7,8) highlighted the importance of ROS production in adipocytes and the associated insulin resistance and changes in serum levels of adiponectin, 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 tumor necrosis factor-α (TNF-α), interleukin (IL)-6, leptin, and adiponectin (9,10). Of these proteins, adiponectin has recently attracted much attention because it has insulin-sensitizing properties that enhance fatty acid oxidation, liver insulin action, and glucose uptake and positively affect serum triglyceride levels (10–12). Adiponectin is exclusively secreted from adipose tissue, and its expression is higher in subcutaneous compared with visceral adipose tissue. It circulates in the blood at very high concentrations and is found as both low–molecular weight oligomers and high–molecular weight multimers (12,13). Several studies report that high–molecular weight adiponectin is more active and correlates more significantly with glucose and insulin levels when compared with low molecular weight and even total adiponectin (13–15). Low plasma levels of high–molecular weight 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 nondiabetic 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 peroxisome proliferator–activated receptor (PPAR)γ response was found to increase expression of adiponectin (20) and also

to regulate the expression of heme oxygenase (HO)-1 in human vascular cells (21).

The HO system provides both antioxidant and anti-apoptotic properties because of 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 antioxidative properties (25,26). 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 *ob* mice, there is a reduction in HO activity, an increase in TNF and IL-1β, and a decrease in adiponectin levels when compared with age-matched lean animals. The increases in HO-1 and adiponectin resulted in a concomitant decrease in serum levels of TNF-α and IL-1β. Using mesenchymal stem cells (MSCs), we showed that induction of HO-1 decreased adipocyte size and TNF, IL-1β ,and IL-6 release in culture media, a process that is reversed by inhibition of HO activity. We extended these 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. Furthermore, 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 adioponectin plays a crucial role in improvement in the metabolic profile, including the diabetic phenotype.

RESEARCH DESIGN 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) were used as controls. Mice were fed a normal chow diet and had free access to water and food. Body weights of *ob* and lean mice at the beginning of the treatment were 34 ± 5 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, Milpitas, CA). Beginning at 9 weeks, when all *ob* mice have established diabetes, cobalt protoporphyrin (CoPP) (3 mg/kg once per week) or CoPP plus stannous mesoporphyrin (SnMP) (2 mg/100 g body wt three times per week) were administered intraperintoneally for 6 weeks. Metalloporphyrins were dissolved in 10 mmol/l Tris base, and the pH was 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: *1*) lean, *2*) lean-CoPP, *3*) lean-CoPP-SnMP, *4*) *ob* control, *5*) *ob*-CoPP, and *6*) *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. When mice were killed, 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 MSCs. 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).

HO activity assay and CO generation. HO activity was assayed in homogenates of various tissues or MSCs as previously described (27). 300 μg protein was 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 manufacturer's protocol. Plates were analyzed using a Luminex 100IS analyzer (Lumunes, Austin, TX). The data were saved and evaluated as median fluorescence intensity using appropriate curve-fitting software (Luminex 100IS software version 2.3). Certain measurements, including adiponectin (high molecular weight), TNF-α, IL-1β, and IL-6, were determined in mouse serum using an ELISA assay (Pierce Biotechnology, Woburn, MA). Insulin was measured using an ELISA kit (Millipore, Billerica, MA).

Glucose and insulin tolerance test. After a 12-h fast, mice were injected intraperitoneally with glucose (2.0 g/kg body wt). 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 units/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 tibia 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 six different fields.

Measurement of O_2^- 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 μmol/l lucigenin for the detection of O_2^-, as described previously (30,31).

Isolation of mice bone marrow–derived MSCs and colony forming units–fibroblasts determination. The bone marrow cells were collected from the mice femur and tibia of *ob* mice. Nonadherent cells were removed on day 4, and adherent cells were further incubated in fresh Iscove's modified Dulbecco's medium, including 10% fetal bovine serum (FBS; Invitrogen, 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 Dulbecco's modified Eagle's medium containing 10 μg/ml insulin, 0.1 mmol/l dexamethasone, 0.2 mmol/l indomethacin, 10% FBS, and 1% antibiotic-antimycotic solution). At 50% confluence, 2 μmol/l CoPP and 5 μmol/l SnMP were added, and adipogenesis was measured using the oil red O staining as described previously (32). Briefly, cells were fixed by ice-cold 10% formalin in PBS for 10 min, rinsed with distilled water, and stained with oil red O solution for 20 min. Cells were placed in absolute propylene glycol for 5 min, 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 fluorescence-activated cell sorter analysis. The International Society for Cellular Therapy 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. MSCs 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, and 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 means ± 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 to actin in the aorta of *ob* compared with lean mice (Fig. 1*A*). CO production in the aorta of lean and obese mice was measured by gas chromatography–mass spectrometry

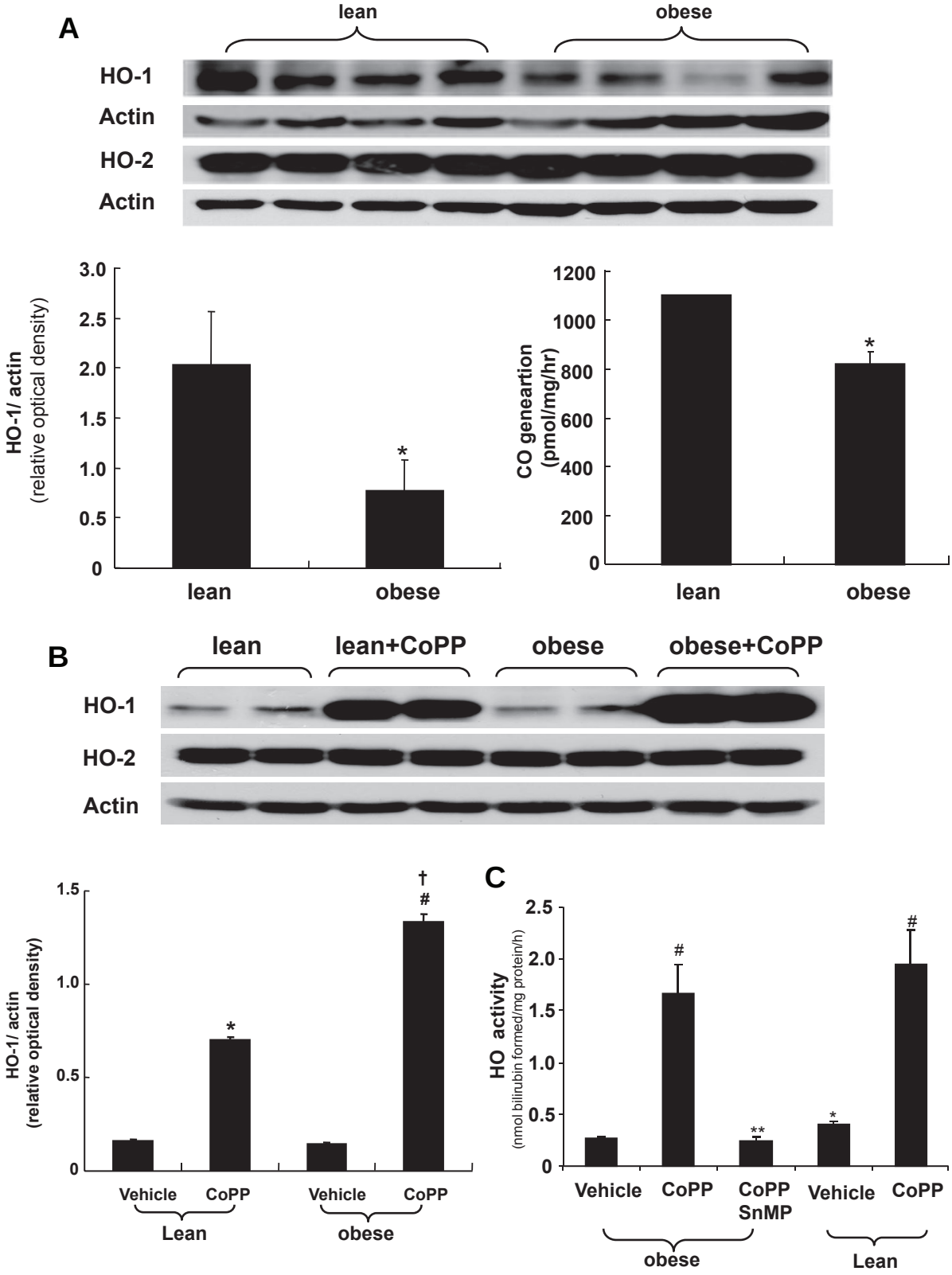

FIG. 1. Effect of CoPP on HO-1 protein levels in aortas of lean and obese mice. *A*: Western blot and densitometry analysis of HO-1 protein in untreated lean and obese mice. Results are the means ± SE of the band density normalized to actin, $n = 4$. *$P < 0.05$ vs. lean. CO generation (picomoles per milligram per hour) 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 means ± 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.

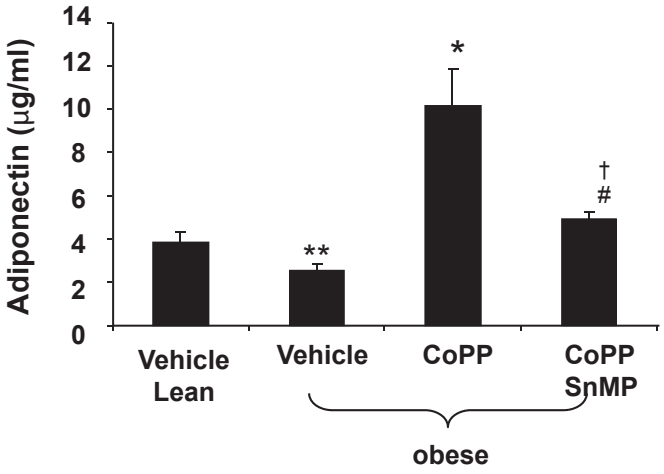

FIG. 2. Effect of CoPP on serum adiponectin levels in lean and *ob* mice. CoPP and SnMP were administered weekly for 6 weeks, and serum samples were obtained immediately before mice were killed. The results are means ± SE; $n = 8–12$. **$P < 0.05$ vs. lean; *$P < 0.009$ vs. vehicle-treated *ob* mice; †$P < 0.02$ vs. vehicle-treated *ob* mice; #$P < 0.05$ vs. CoPP-treated *ob* mice.

(GCMS) and was found to be significantly ($P < 0.05$) lower in obese mice compared with lean mice. This reflects the less active HO system in obese mice compared with 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 (Fig. 1*B*).

CoPP administration increased aortic HO-1 protein in lean mice by 4.3-fold ($P < 0.01$) and in *ob* mice by 9-fold ($P < 0.001$) (Fig. 1*B*). HO activity was measured in aortas isolated from these animals (Fig. 1*C*). There was a significant ($P < 0.05$) decrease in HO activity in *ob* mice compared with age-matched lean controls (0.27 ± 0.02 and 0.40 ± 0.03 nmol bilirubin · mg^{-1} · h^{-1}, 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^{-1} · h^{-1}, 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 with age-matched lean controls (Fig. 2); adiponectin levels in *ob* mice were 2.51 ± 0.33 μg/ml compared with 3.86 ± 0.46 μg/ml ($P < 0.029$) in lean animals. CoPP administration resulted in a marked four-fold increase ($P < 0.009$) in the levels of serum adiponectin in *ob* mice compared with untreated *ob* animals. Serum adiponectin increased to 10.2 ± 1.66 μg/ml after HO-1 induction compared with 2.51 ± 0.33 μg/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-1β, and TNF-α levels. Obese mice exhibited a significant increase in serum IL-6 levels (620 ± 6 pg/ml) when compared with age-matched lean controls (89 ± 23 pg/ml) (Fig. 3). Administration of CoPP resulted in a significant decrease in serum IL-6 levels in *ob* mice when compared with untreated *ob* mice. This

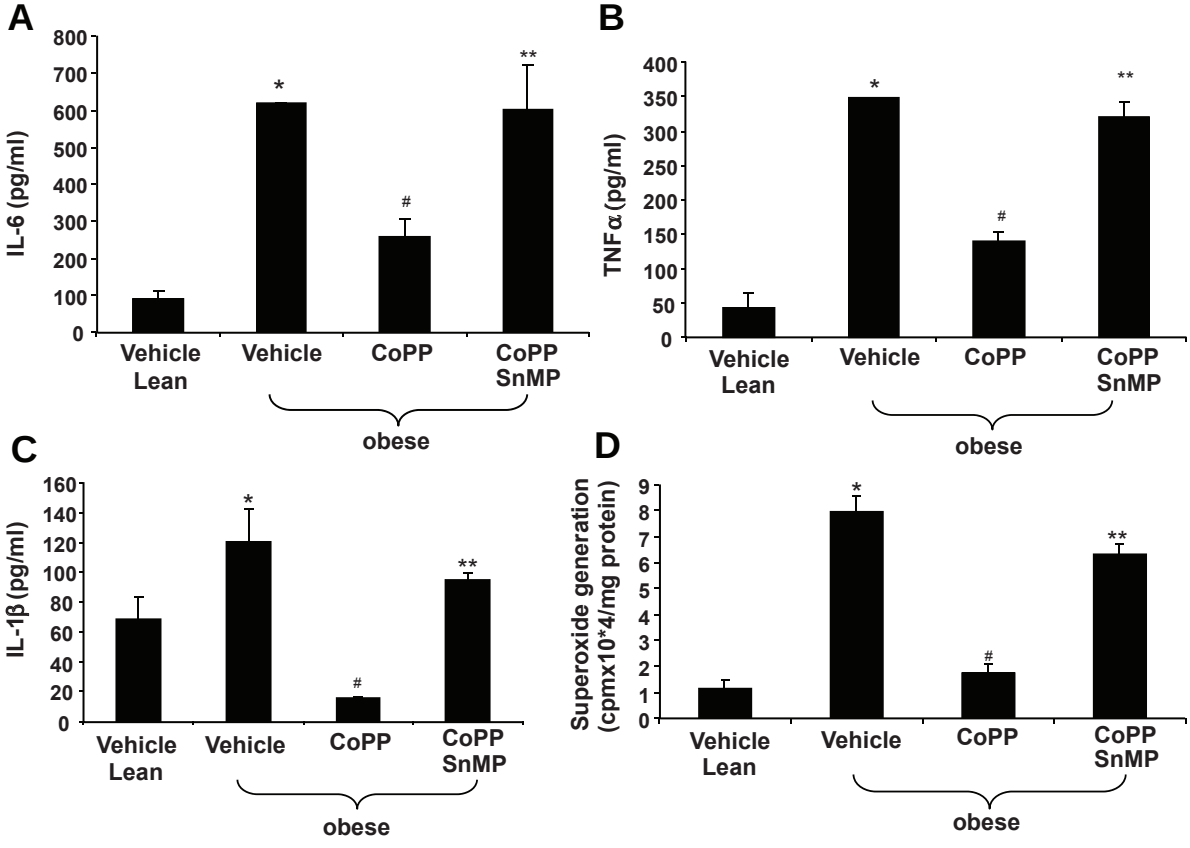

FIG. 3. Effect of CoPP and SnMP on serum cytokines in lean and *ob* mice. Results are means ± SE, $n = 8–12$. *A*: IL-6. *$P < 0.001$ vs. lean; #$P < 0.001$ vs. vehicle-treated *ob* mice; **$P < 0.01$ vs. CoPP-treated *ob* mice. *B*: TNF-α. *$P < 0.001$ vs. lean; #$P < 0.001$ vs. vehicle-treated *ob* mice; **$P < 0.001$ vs. CoPP-treated *ob* mice. *C*: IL-1β. *$P < 0.05$ 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. vehicle-treated obese mice; **$P < 0.001$ vs. CoPP-treated obese mice.

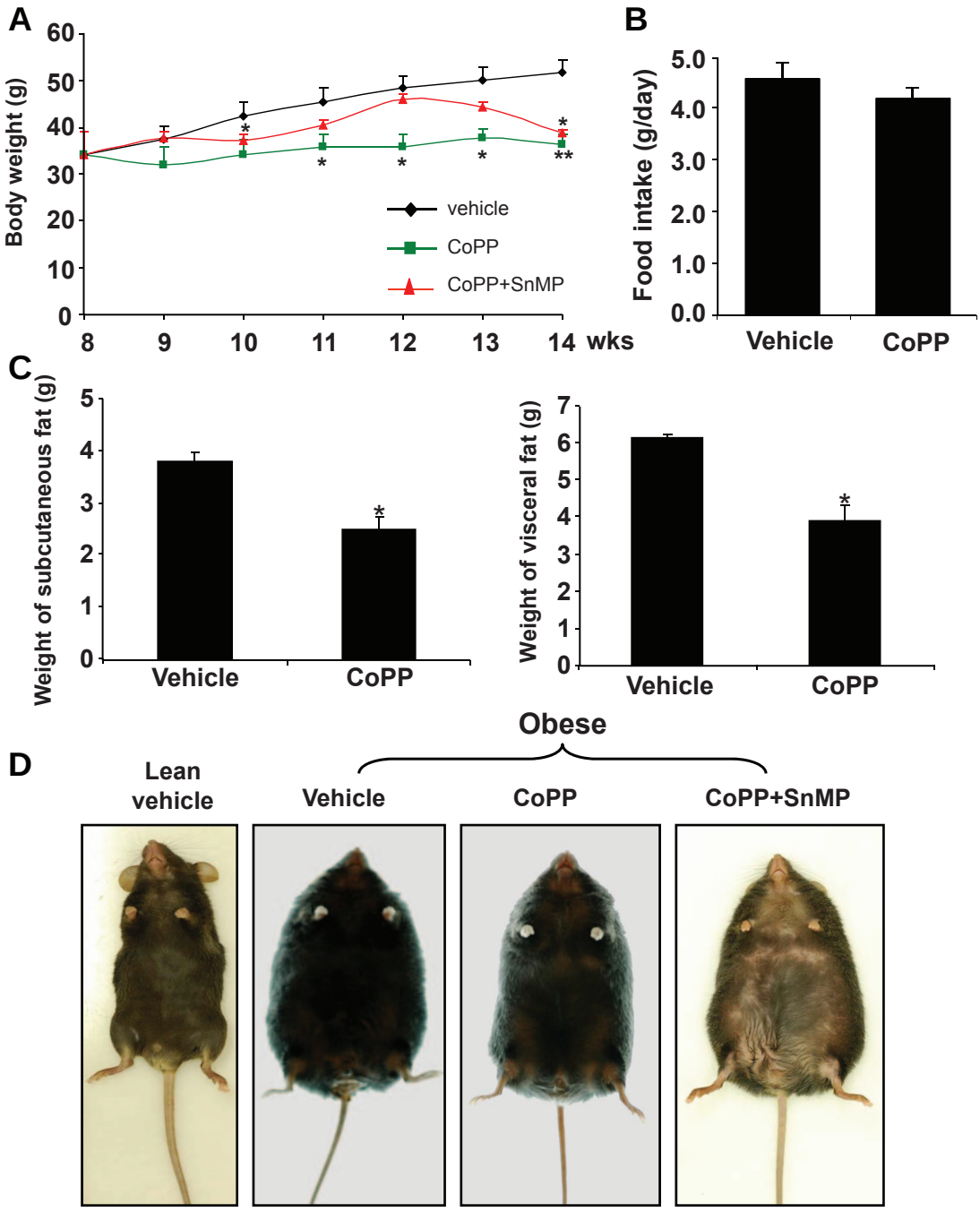

FIG. 4. Effect of HO-1 induction on body weight and visceral fat content in lean and obese mice. *A*: Effect of CoPP and SnMP administration on body weight of obese mice. Results are means ± SE, $n = 6–10$. *$P < 0.05$ and **$P < 0.01$ vs. corresponding vehicle-treated mice. *B*: Food intake in obese mice during the first 2 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.

decrease was blocked by administration of SnMP. IL-6 serum levels in CoPP in combination with SnMP were 600 ± 122 pg/ml compared with 257 ± 48 pg/ml in CoPP-treated *ob* mice. Similar results were observed with serum TNF-α and IL-1β levels (Fig. 3*B* and C).

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 ($\times 10^4$ cpm) compared with $1.1 + 0.3 \times 10^4$ cpm ($P < 0.03$) in vehicle-treated lean mice (Fig. 3*D*). CoPP-treated *ob* mice showed a significant decrease in O_2^- levels in the aorta compared with vehicle-treated *ob* mice. The levels in the CoPP-treated obese mice decreased to $1.7 \pm 0.4 \times 10^4$ cpm ($P < 0.039$). Inhibition of HO activity by administration of SnMP to CoPP-treated mice increased superoxide production to $6.3 \pm 0.4 \times 10^4$ cpm.

Effect of induction of HO-1 on body weight, appearance, and fat content of obese and lean mice. As seen in Fig. 4*A*, CoPP treatment prevented weight gain in *ob* mice when compared with 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-

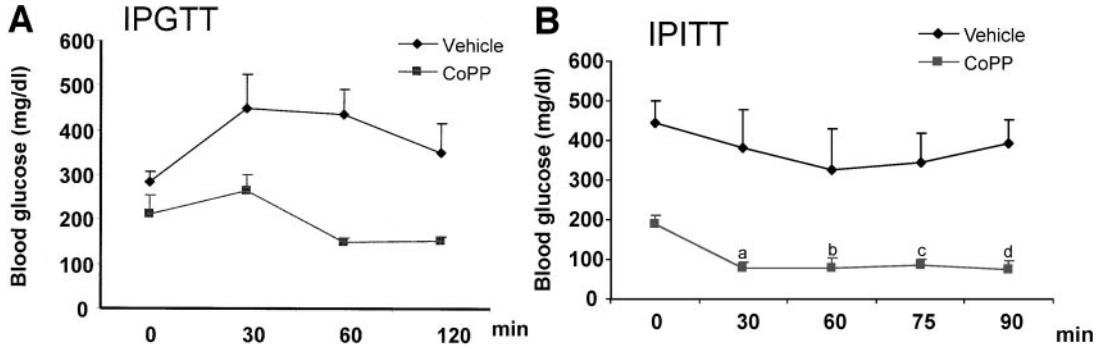

FIG. 5. Effect of HO-1 expression on glucose tolerance and insulin sensitivity. Intraperitoneal glucose tolerance (IPGTT; *A*) and intraperitoneal insulin sensitivity (IPITT; *B*) tests were performed as described in RESEARCH DESIGN AND METHODS. The results are means ± SE, $n = 3$.

treated lean mice, respectively). The prevention of weight gain in obese mice after treatment with CoPP was partially reversed by co-administration of SnMP. Food intake in the treatment and control groups was comparable (Fig. 4*B*). As seen in Fig. 4*C* and *D*, 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 to 3.9 ± 0.4 g ($P < 0.03$). Subcutaneous fat in obese mice was decreased from 3.8 ± 0.2 to 2.5 ± 0.2 g ($P < 0.01$). The ability of SnMP to reverse the CoPP-induced loss of body weight and the increased adiponectin levels in *ob* mice suggests that the decrease in adiponectin and the resultant weight gain were 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 (Fig. 5*A* and *B*). 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 min after glucose administration and remained elevated. In CoPP-treated obese mice, blood glucose levels were significantly elevated ($P < 0.05$) after 30 min but returned to initial levels at 120 min. 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 Fig. 6*A*, bone marrow cells from obese mice stained for oil red O displayed a significant increase in adipocytes (Fig. 6*A*, *middle*) compared with obese mice treated with CoPP or lean mice. 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 (Fig. 6*B*), suggesting that HO-1 induction exhibits an inhibitory effect on adipogenesis in vivo.

As seen in Fig. 6*C*, 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^{-1} · h^{-1}, 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^{-1} · h^{-1}, respectively (Fig. 6*C*). Superoxide levels in bone marrow increased ($P < 0.05$) in obese mice compared with lean. CoPP treatment resulted in a significant ($P < 0.001$) decrease in superoxide levels compared with obese mice (Fig. 6*D*).

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

Effect of HO-1 expression on MSC clonal efficiency. Bone marrow mononuclear cells of *ob* fat mice (3×10^5/well in six-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 with control. CoPP administration resulted in a decrease in CFU-F when compared with the SnMP and high glucose groups (Fig. 7*B* and *C*), 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 MSC-mediated adipogenesis. As seen in Fig. 8*A*, glucose increases adipogenesis in MSCs similar to that seen in bone marrow. Induction of HO-1 inhibited adipogenesis in MSCs ($P < 0.002$). In contrast, inhibition of HO-1 did not significantly affect adipogenesis (Fig. 8*A* and *B*). This may be due to inhibition of HO activity by glucose (35).

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

DISCUSSION

In obese diabetic mice, we report a decrease in HO-1 protein levels and HO activity when compared with age-matched lean control mice, and that increases in HO-1

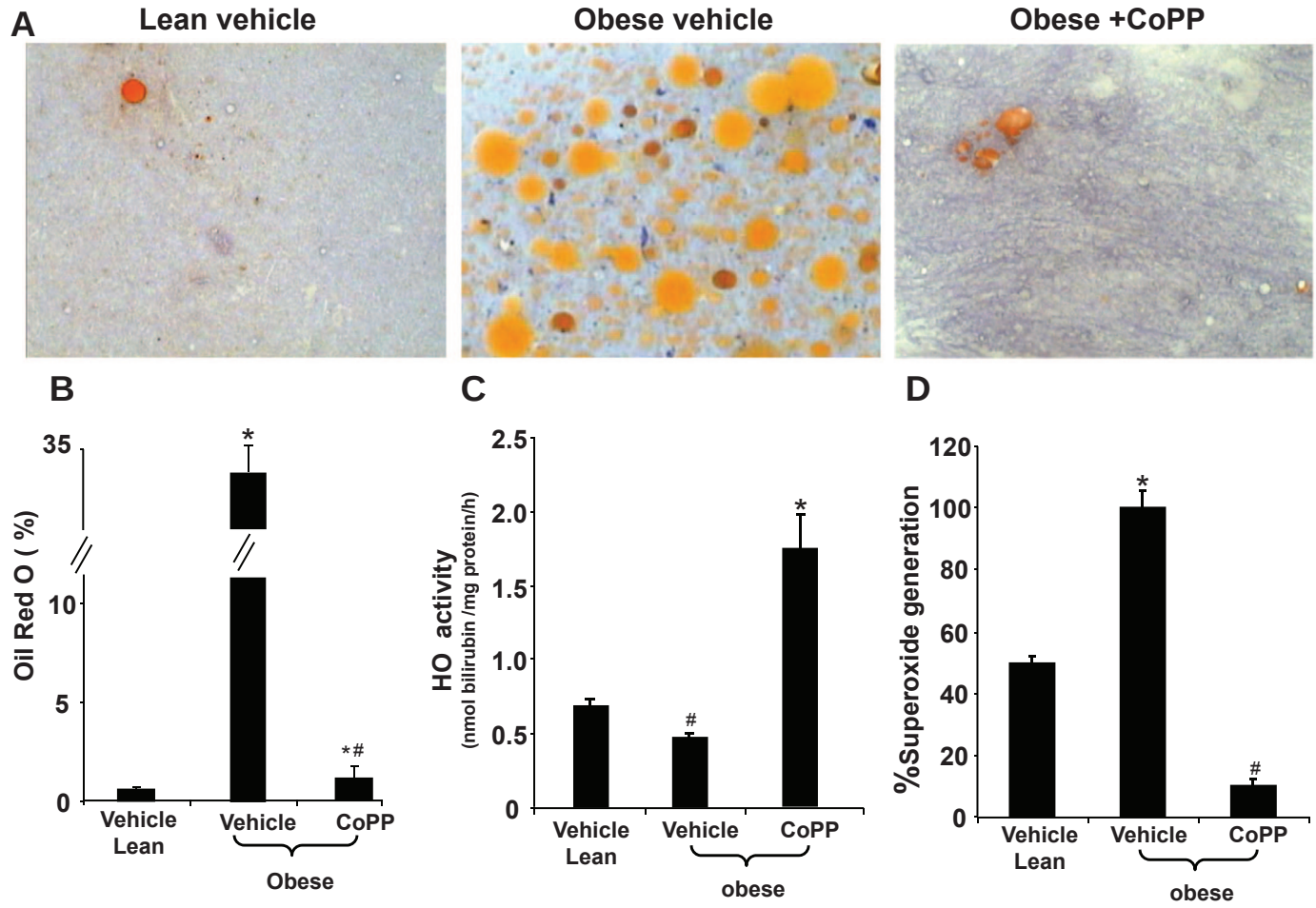

FIG. 6. Oil red O staining on bone marrow evaluation of lipid content measured as percentage of oil red O staining. *A*: Representative photographs of lipid droplets in bone marrow of lean and *ob* mice treated with CoPP. *B*: Quantitative analysis of bone marrow lipid content. Results are means ± SE, $n = 3$. *$P < 0.05$ 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 previously (35). Results are means ± 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 cell homogenates were harvested, and O_2^- levels were determined as described in RESEARCH DESIGN AND METHODS. *$P < 0.05$ obese vs. vehicle lean; #$P < 0.001$ vs. vehicle-treated obese.

protein levels after CoPP administration resulted in increased insulin sensitivity and decreased glucose tolerance. Furthermore, 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-α, IL-6, and IL-1β levels. Induction of HO-1 also resulted in decreased aorta adipocyte and bone marrow superoxide production (Figs. 3*D* and 6*D*). Upregulation of HO-1 by CoPP treatment caused a decrease in adipogenesis in bone marrow both in vivo and in vitro in cultured MSCs 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-α, IL-1β, and IL-6. The metabolic syndrome and obesity are characterized by increases in serum levels of inflammatory cytokines such as TNF-α 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 (Fig. 4*A*) and visceral fat content (Fig. 4*C*) that parallels the increase in adiponectin. The latter is an indicator of improvements in the metabolic syndrome which, in turn, leads to decreases in arterial diseases and heart disease and increased insulin sensitivity (3,10,37,39–41). Although 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 regimens of CoPP are not associated with alterations in endocrine homeostasis or hepatic heme metabolism or food intake as confirmed (Fig. 4*A*).

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 and to its ability to decrease ROS by increasing glutathione and extracellular superoxide dismutase levels (4,22,45) and by

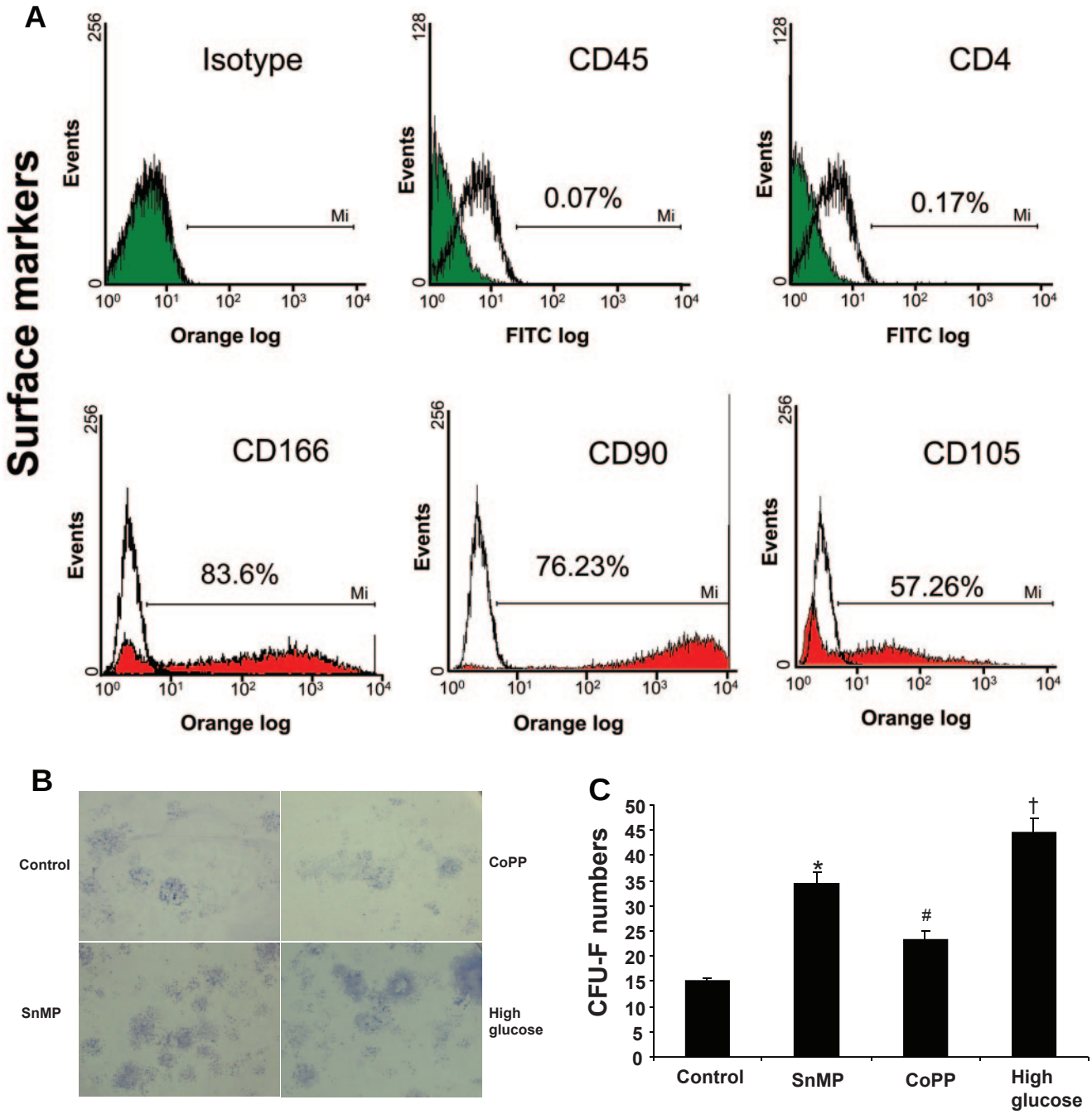

FIG. 7. Effect of high glucose, CoPP, and SnMP on bone marrow–derived MSC CFU-F. *A*: Surface markers of cultured cells were detected by a FACScan after staining with the monoclonal antibodies listed in the figures. MSCs were negative for CD45 and CD4, whereas they were positive for CD166, CD90, and CD105. *B*: Representative photographs of CFU-F from obese bone marrow MSCs treated with and without high glucose, 2 μmol/l CoPP, and 5 μmol/l SnMP. 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 are means ± SE, $n = 4$. *$P < 0.03$ vehicle-treated lean mice vs. vehicle-treated obese mice; #$P < 0.039$ vehicle-treated obese mice vs. CoPP-treated obese mice.

decreasing O_2^- production (23,24). PPARγ agonists are shown to induce both HO-1 (21) and the rate-limiting chaperone protein EroL (46,47). PPARγ agonist that increases adiponectin may do so by increasing the levels of EroL chaperone protein (46). Because PPARγ 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. (47) that showed that the chaperone protein EroL increased adiponectin. We also have previously shown that upregulation of HO-1 protein in diabetic rats provided both cardio-protection and vascular protection against ROS (24).

The seminal finding of a decrease in HO activity with a resultant decrease in CO generation (Fig. 1*A*) 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

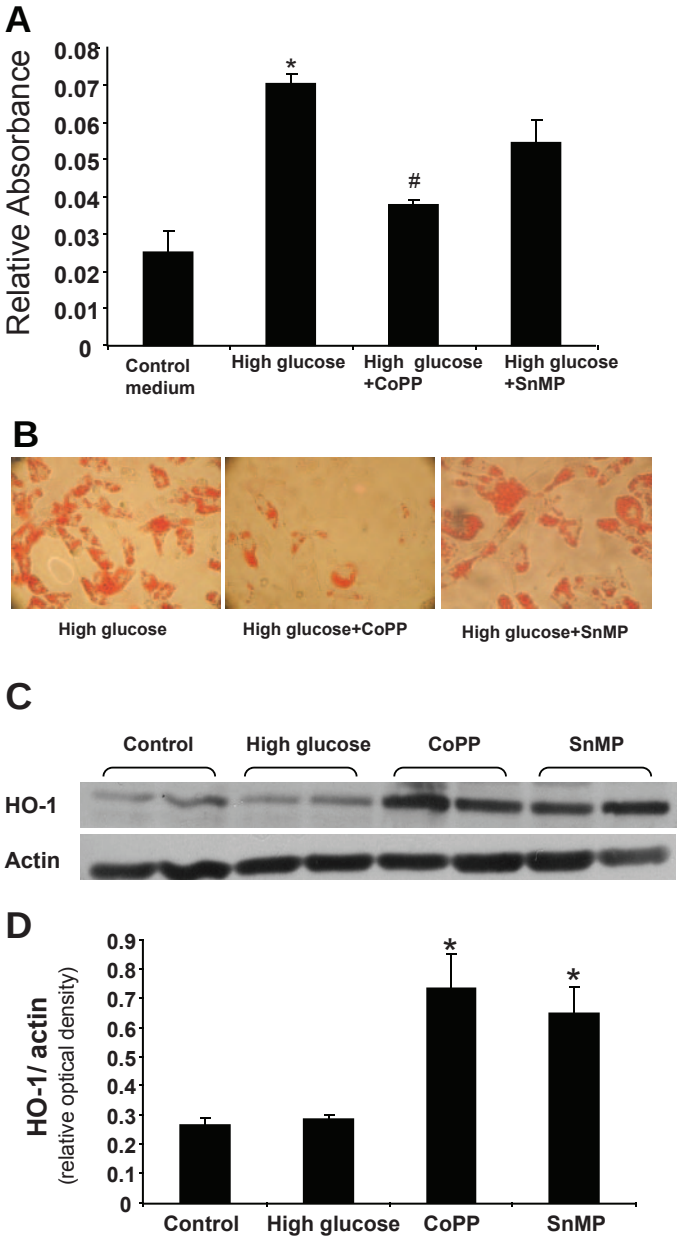

FIG. 8. Effect of HO-1 expression on MSC-derived adipogenesis. *A*: Adipogenesis was measured as the relative absorbance of oil red O at day 10 after inducing adipogenesis as described in RESEARCH DESIGN AND METHODS. Results are means ± SE, $n = 4$. *$P < 0.01$ vs. control medium; #$P < 0.005$ vs. high 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 and MSC treated with glucose in combination with CoPP or SnMP. Blots are representative of four separate experiments. *D*: Mean band density normalized relative to actin to HO-1 (*$P < 0.001$ vs. MSC exposed to glucose).

in obese Zucker rats (48). These investigators measured HO-derived CO by Hb-CO, but exhaled CO was determined by GCMS. In the present study, CO was measured using GCMS. The difference in results remains to be elucidated but could be explained by the use of different animal models and 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 MSC-derived adipocytes in bone marrow and may decrease circulating adipocyte stem cells in the body. As a result, it decreases adipogenesis in bone marrow, diminishes adipogenesis in visceral and subcutaneous fat, and promotes 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 (Fig. 5). The mechanism by which HO-1 increases insulin sensitivity may be related to HO-1–mediated reduction in IL-1β, TNF, and IL-6 levels. The reduction in IL-1β, TNF, and IL-6 (Fig. 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-1β, 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 that we present here demonstrate that CoPP treatment decreased IL-1β and improved insulin sensitivity and glucose tolerance (Fig. 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 resistance, obesity, and the metabolic syndrome in critical areas of cell damage associated with cardiovascular disease and diabetes.

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