# Adipocyte Heme Oxygenase-1 Induction Attenuates Metabolic Syndrome In Both Male And Female Obese Mice

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**Supplementary Material**

**Materials and Methods**

***Animal Care and CoPP Administration***

Male and female *obese* mice (B6v-Lep *obese*/J) were purchased from Harlan (Chicago, IL) at the age of 7 weeks. Lean mice, (age-matched B6.V, lean, Harlan Chicago, IL) were used as control. Sex matched lean and ob mice were fed a normal laboratory animal diet and had free access to water. Body weight of *obese* and lean mice were 34 ± 5 g and 26 ± 3 g, respectively and glucose levels were 229 ± 21 and 154 ± 9 mg/dl, respectively, at the start of the experiments. At 8 weeks of age after *obese* mice established diabetes, CoPP (3mg/kg/once a week), or SnMP (3mg/kg/three times a week), was administered intraperitoneally for 6 weeks to 48 *obese* mice (24 males and 24 females) and 20 lean mice (10 males and 10 females). There were eight groups of animals: A) male lean, B) male *obese*, C) male *obese*- CoPP treated, D) male *obese*-CoPP+ treated, E) female lean, F) female *obese*, G) female *obese*-CoPP treated, and H) female *obese*-CoPP+SnMP treated. There was no difference in food intake in any of the treatment groups. The Animal Care and Use Committee of New York Medical College approved all experiments.

***Preparation of Tissue for Histological Analysis of Adipocyte Cell Size and Adipocyte Cell Number***

Subcutaneous and visceral aortic adipose fat tissues were isolated and fixed with 4% paraformaldehyde for 24-30 hours at room temperature and embedded in paraffin. 5-μm thick paraffin-embedded tissue sections were then deparaffinized and rehydrated in graduated alcohol in distilled water. Digital images of adipose tissue sections were capture using a light microscope (Olympus, Germany) at 20X magnification. For each group, five fields from each of five different ematoxylin-eosin stained section per animal were analyzed. The number of adipocytes within each field was determined using image analysis software as previously described [1, 2] (Image Pro Plus, Milan, Italy). Data are presented as means ± SD. Differences between experimental groups were evaluated with ANOVA with Bonferroni corrections. Statistical significance was set at $P<0.05$.

***HO-1 Immunohistochemistry***

Alternate sections of adipose tissues were deparaffinized, rehydrated and then incubated in 1% bovine serum albumin; subsequently sections were incubated with polyclonal rabbit antibody against HO-1 (diluted 1:500; Stressgene Bioreagents, Victoria, BC, Canada) for 1h at room temperature and overnight at 4°C. Then, sections were labeled using anti-mouse Alexa Fluor 546 and anti-rabbit Alexa Fluor 488 conjugated secondary antibodies(1:200, Invitrogen, UK). Finally, the samples were counter-stained with DAPI, mounted and observed with a confocal microscope (LSM 510 Zeiss, Germany) at a final magnification of 40X. The immunofluorescent control was perform by omitting the primary antibody and in presence of isotype matched IgGs.

### *Blood Pressure Measurements*

Blood pressure was measured by the tail-cuff method before and every 7 days after CoPP administration.

### *Determination of HO Activity*

HO activity was assayed as described previously [3]. Using a technique in which bilirubin, the end product of heme degradation, was extracted from chloroform, and its concentration was determined spectrophotometrically (dual UV/VIS beam spectrophotometer lambda 25; PerkinElmer Life and Analytical Sciences, Wellesley, MA) using the difference in absorbance at a wavelength from λ460 to λ530 nm with an absorption coefficient of 40 $mM^{-1}cm^{-1}$. Under these conditions, HO activity was linear with protein concentration, time-dependent, and substrate-dependent [4].

### *Cytokine, Glucose and LDL Measurements*

Adiponectin (high molecular weight, HMW), TNFα, IL-1β, and IL-6 were determined in mouse serum using an ELISA assay (Pierce Biotechnology, Woburn, MA). Glucose was measured using an automated analyzer (Lifescan Inc., Milpitas, CA). Serum LDL (low density lipoprotein) levels were measured using LDL/VLDL Quantification Kits (Biovision, Mountainview, CA). The assays were performed according to manufacturer's guidelines.

### *Glucose and Insulin Tolerance Tests*

After a 12 hour fast, mice were injected intraperitoneally with glucose (2.0g/kg body weight) and for insulin tolerance test, mice were injected with insulin (2.0 units/kg). Blood samples were taken at various time points (0-120 min), and fasting blood glucose levels were measured. Due to limitation in blood volumes for glucose determinations measurement of glucose levels (fasting) in Figures 3 and 4 were carried out on different groups of animals 2 weeks apart.

### *Western Blot Analysis of HO-1, pAMPK, pAKT, and PPARγ expression*

At sacrifice, subcutaneous and visceral fat in the abdomen (the visible mesenteric fat, fat around the liver, the kidney, the spleen and the heart were dissected, pooled for each mouse and used to isolate adipocyte cells. Specimens were stored at -140°C until assayed. To isolate mouse adipocyte cells, adipose tissues were washed with phosphate-buffered saline (PBS) and digested at 37°C for 30 min with 0.075% type II collagenase[1]. Frozen tissues were pulverized and placed in a homogenization buffer as previously described [5]. Homogenates (20-50 µg of protein) were examined by protein immunoblot. HO-1, HO-2, AMPK, pAMK, AKT, pAKT, and PPAR gamma (Cell Signaling) levels were determined as previously described [6].

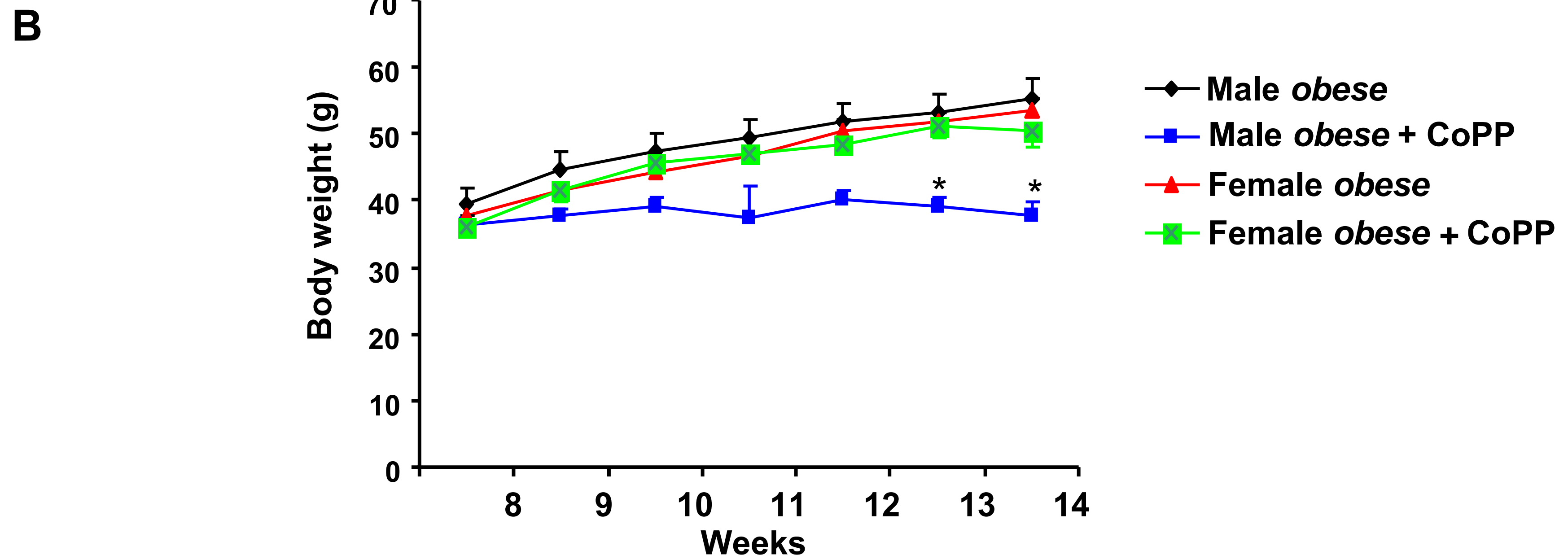
### *Statistical analyses*

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

**Figure S1**

**A** Male *obese* | Male *obese* +CoPP | Female *obese* | Female *obese* +CoPP

1cm



## Figure S1

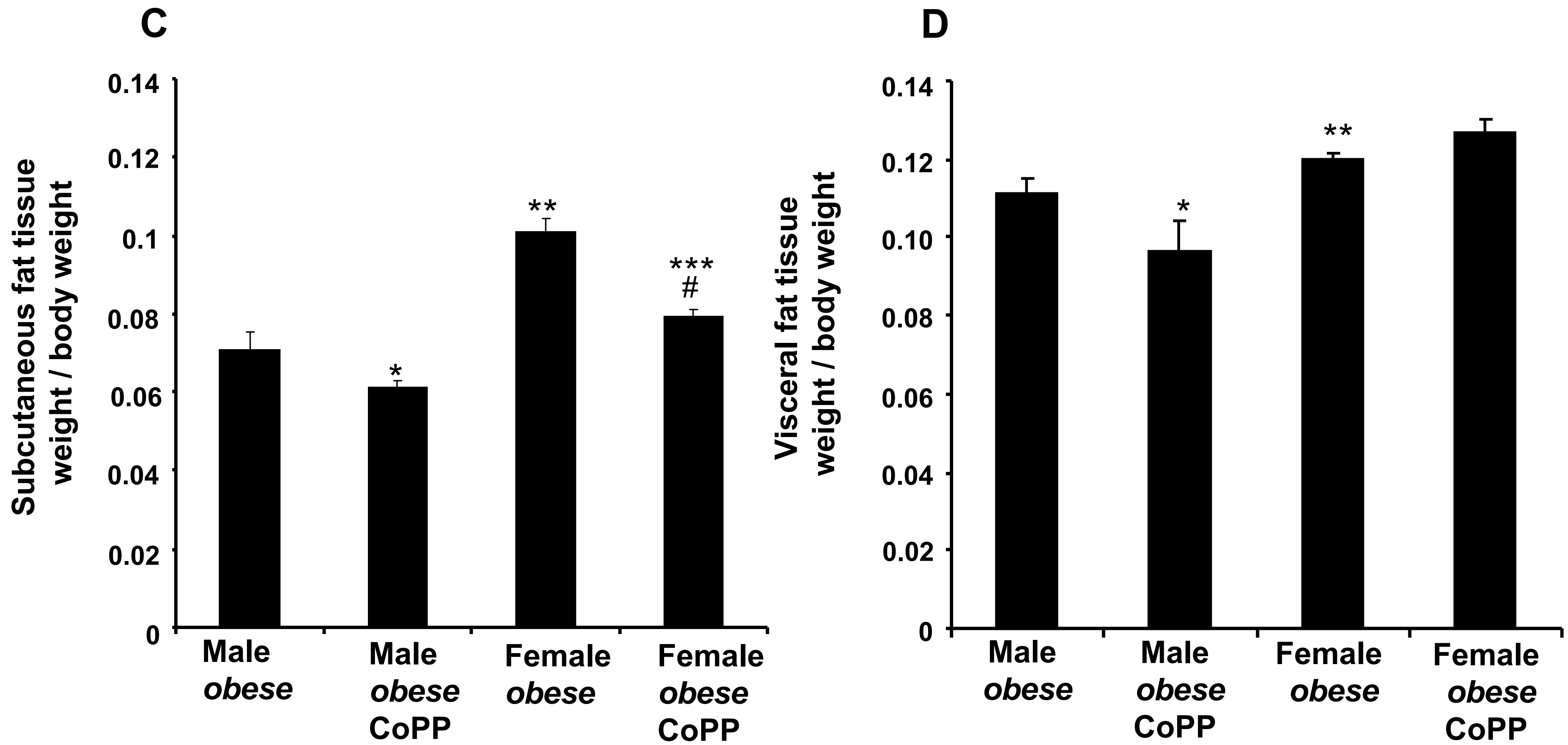
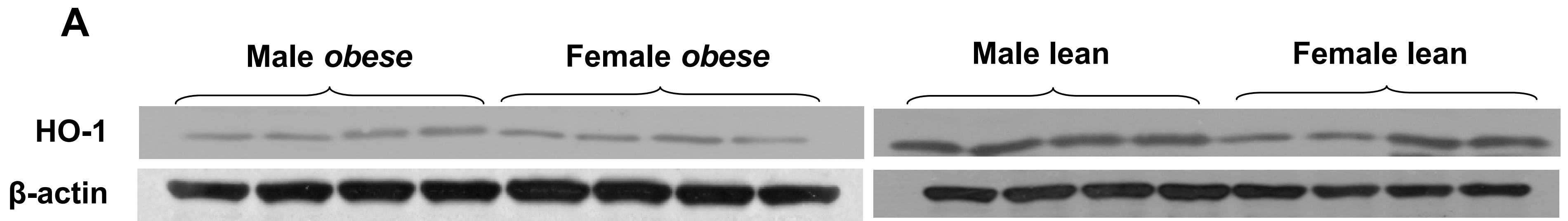
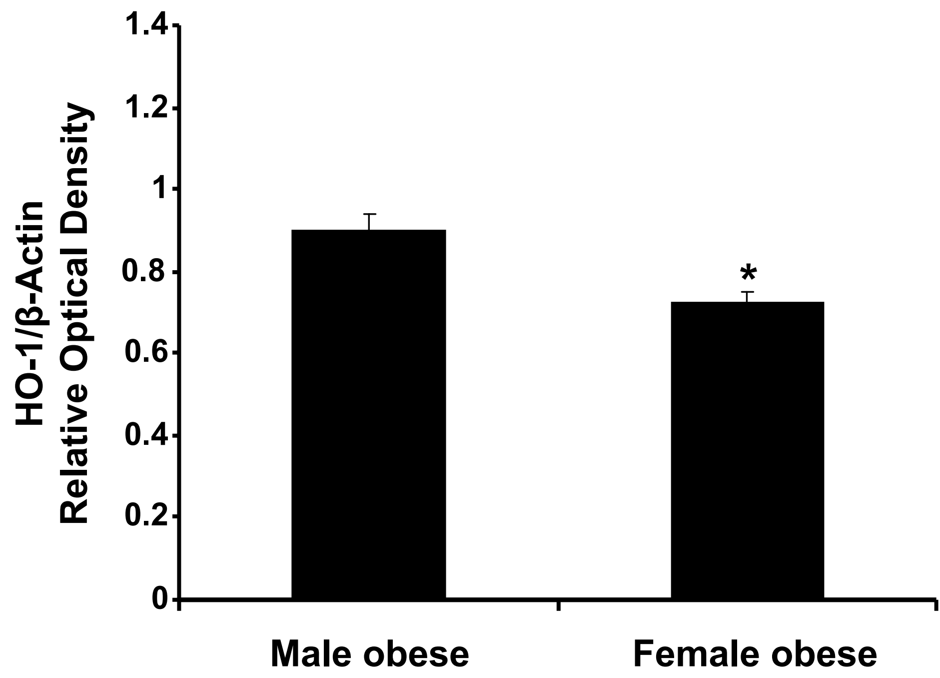


**Figure S1 A-D:** Effect of HO-1 induction on body weight, subcutaneous and visceral fat content in *obese* mice after 6 weeks of CoPP treatment (3mg/Kg/once a week) or vehicle solution **A)** *obese* male and female mice, respectively, were treated and weight determined (average of two independent experiments) n=6 for control and n=8 for treatment in each group, representative photograph for untreated male *obese*, male *obese* + CoPP, and untreated female *obese* and female *obese* + CoPP after 6 weeks of treatment **B)** Effect of CoPP treatment on body weight at the end of the treatment (6 weeks), $p<0.05$ versus male *obese*. **C)** Effect of CoPP on subcutaneous fat. Results are by 2-way ANOVA. Levels of significance: *$p<0.05$ versus male *obese*; $^{\#}p<0.05$ versus female *obese*; **$p<0.01$ versus male *obese*; and ***$p<0.01$ versus male *obese* + CoPP. **D)** Effect of CoPP on visceral fat. Level of significance: *$p< 0.05$ versus male *obese*; **$p< 0.01$ versus male *obese*.

Figure S2



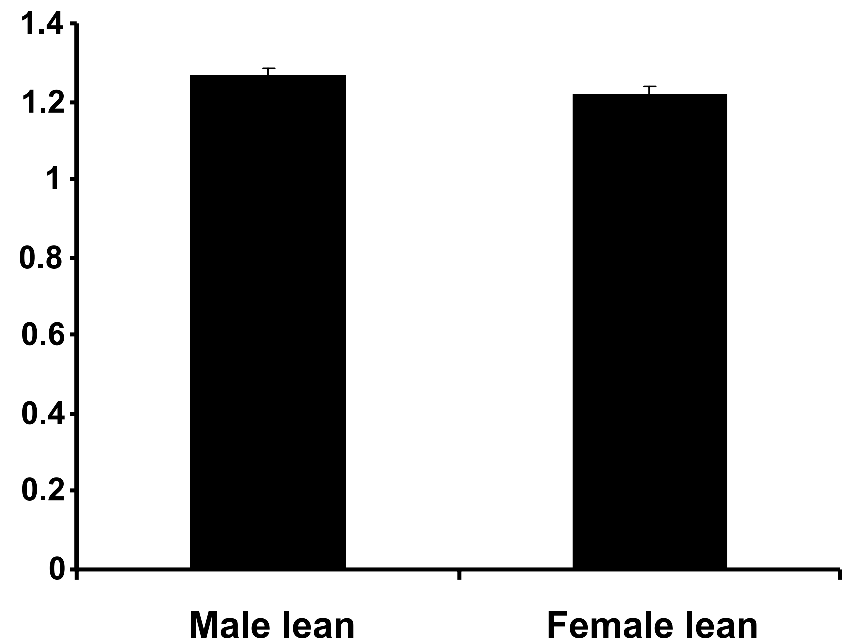
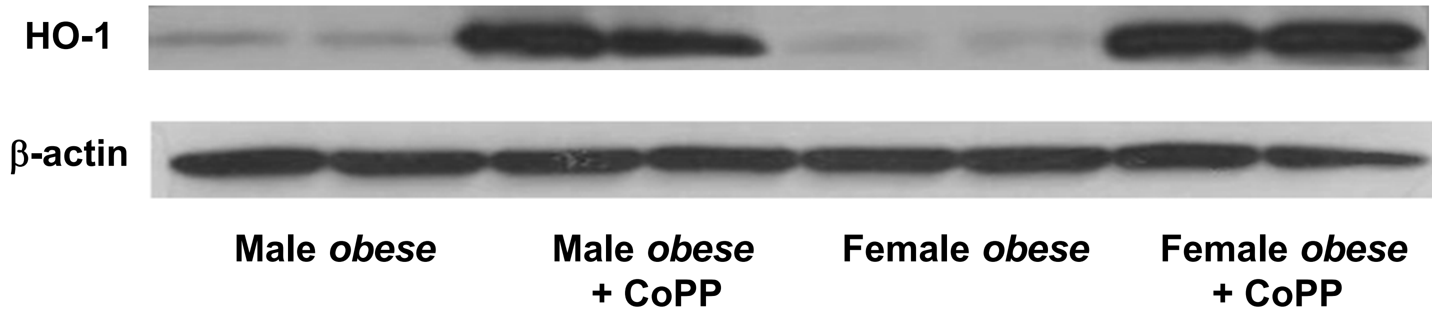



**Figure S2**

**B**



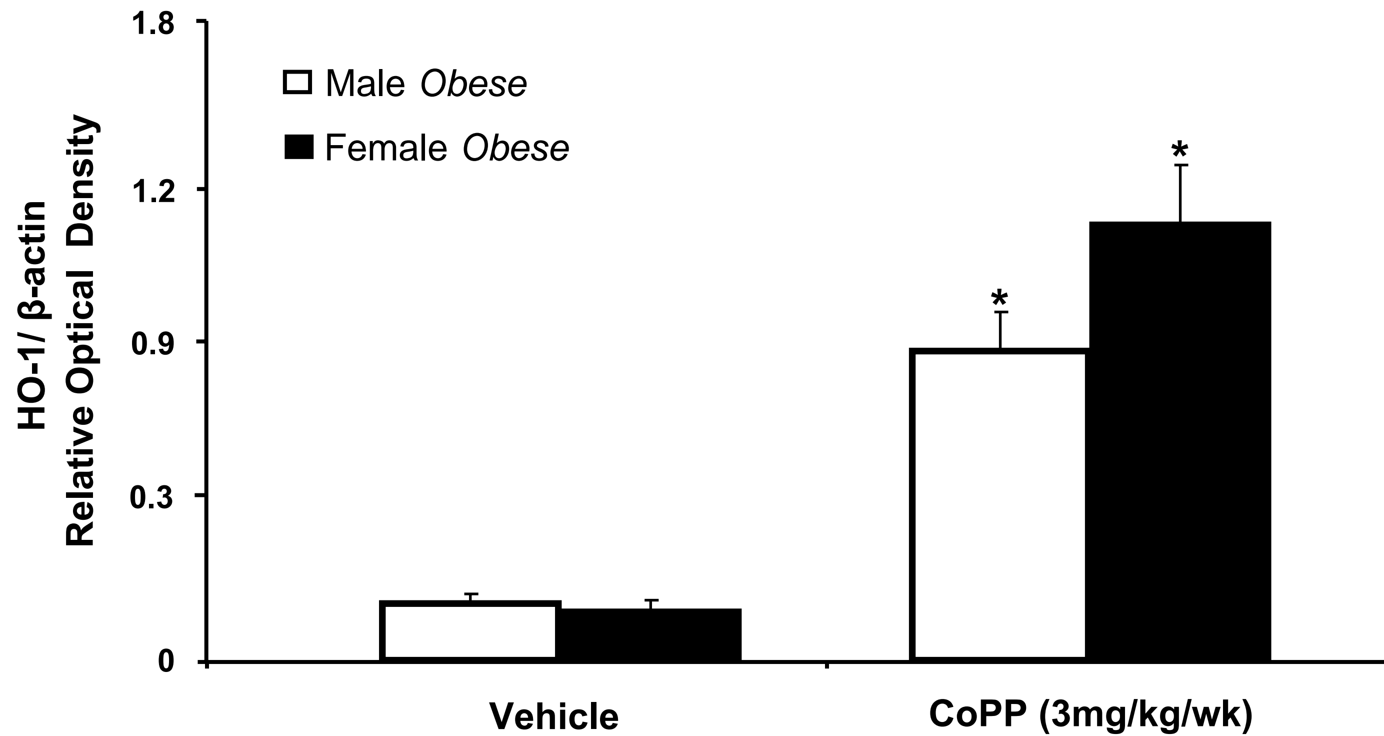



**Figure S2**: **A, B)** Expression of HO-1 in renal cells isolated from lean and *obese* female mice. Renal samples were subjected to Western blotting for the determination of HO-1 protein expression and densitometry analysis of HO-1/actin ratio. Results are expressed as means $\pm$ SE, n=4-6. Levels of significance: * $p<0.05$ versus untreated female *obese* mice, B**)** Effect of CoPP on HO-1 expression in renal cells of *obese* female mice, * $p<0.001$ versus *obese*; ** $p<0.05$ versus *obese* male; ***$p<0.01$ versus *obese*.

**References**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