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

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Abstract

Increases in visceral fat are associated with increased inflammation, dyslipidemia, insulin resistance, glucose intolerance and vascular dysfunction. We examined the effect of the potent heme oxygenase (HO)-1 inducer, cobalt protoporphyrin (CoPP), on regulation of adiposity and glucose levels in both female and male *obese* mice. Both lean and *obese* mice were administered CoPP intraperitoneally, (3mg/kg/once a week) for 6 weeks. Serum levels of adiponectin, TNFα, IL-1β and IL-6, and HO-1, PPARγ, pAKT, and pAMPK protein expression in adipocytes and vascular tissue were measured. While female *obese* mice continued to gain weight at a rate similar to controls, induction of HO-1 slowed the rate of weight gain in male *obese* mice. HO-1 induction led to lowered blood pressure levels in *obese* males and females mice similar to that of lean male and female mice. HO-1 induction also produced a significant decrease in the plasma levels of IL-6, TNF-α, IL-1β and fasting glucose of *obese* females compared to untreated female *obese* mice. HO-1 induction increased the number and decreased the size of adipocytes of *obese* animals. HO-1 induction increased adiponectin, pAKT, pAMPK, and PPARγ levels in adipocyte of *obese* animals. Induction of HO-1, in adipocytes was associated with an increase in adiponectin and a reduction in inflammatory cytokines. These findings offer the possibility of treating not only hypertension, but also other detrimental metabolic consequences of obesity including insulin resistance and dyslipidemia in *obese* populations by induction of HO-1 in adipocytes.

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Keywords

adipocyte pAMPK; female obesity; heme oxygenase inducers; inflammation

Introduction

Moderate to severe obesity is associated with increased risk for cardiovascular complications and insulin resistance in humans[1, 2] and animals[3, 4]. Cardiovascular risk is specifically associated with increased intra-abdominal fat. Women in their reproductive years have a higher BMI than males, which largely reflects increased overall subcutaneous adipose tissue or “gynoid” obesity, this is not associated with increased cardiovascular risk[5]. However, due to increases in visceral fat with aging, after the age of 60 the fat distribution in females more closely resembles that in males[6]. Increased androgen levels also often occur after the menopausal transition. These changes in visceral fat content and androgen levels correlate with both central obesity and insulin resistance and are an important determinant of cardiovascular risk[7].

Heme oxygenase (HO) catalyzes the breakdown of heme, a potentially harmful pro-oxidant, into its products biliverdin and carbon monoxide, with a concomitant release of iron (reviewed in[8]). While HO-2 is expressed constitutively, HO-1 is inducible in response to oxidative stress and its induction has been reported to normalize vascular and renal function[9–11]. Further, induction of HO-1 slows weight gain, decreases levels of TNF-α and IL-6 and increases serum levels of adiponectin in *obese* rats and *obese* diabetic mice[4, 9, 12]. The association observed between HO-1 and adiponectin has led to the proposal of the existence of a cytoprotective HO-1/adiponectin axis[4, 13].

Previously, L'Abbate et al,[14] have shown that induction of HO-1 is associated with a parallel increase in the serum levels of adiponectin, which has well-documented insulin-sensitizing, antiapoptotic, antioxidative and anti-inflammatory properties. Adiponectin is an abundant protein secreted from adipocytes. Once secreted, it mediates its actions by binding to a set of receptors, such as adipoR1 and adipoR2, and also through induction of AMPK signaling pathways[15, 16]. In addition, increases in adiponectin play a protective role against TNF mediated endothelial activation[17].

In this study, we evaluated the effect of CoPP, a potent inducer of HO-1, on visceral and subcutaneous fat distribution in both female and male *obese* mice. We show for the first time a resistance to weight reduction upon administration of CoPP in female *obese* mice but a significant decrease in inflammatory cytokines. Despite continued obesity, CoPP normalized blood pressure levels, decreased circulating cytokines, and increased insulin sensitivity in *obese* females. CoPP treatment of *obese* mice increased the number and reduced the size of adipocytes. CoPP treatment of both male and female *obese* mice reversed the reduction in adiponectin levels seen in obesity. This study suggests that in spite of continued obesity, HO-1 induction in female *obese* mice serves a protective role against obesity associated metabolic consequences via expansion of healthy smaller insulin-sensitive adipocytes.

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 *obese* mice were fed a normal laboratory animal diet and

had free access to water. At 8 weeks of age after *obese* mice established diabetes, CoPP(3 mg/Kg/once a week) or stannous mesoporphyrin (SnMP), a potent inhibitor of HO activity, (3mg/Kg/3 times a week), were administered intraperitoneally for 6 weeks to 48 *obese* mice (24 males and 24 females) and 20 lean mice (10 males and 10 females). Measurements of glucose and insulin tolerance, body weight, and fasting blood glucose (BG) were made during the course of the study. Animal tissues and serum were then collected for additional studies. For evaluation of adipocyte size analysis, digital images of adipose tissue sections were captured using a light microscope (Olympus, Germany) at 20× magnification. For each group, three fields from each of five different haematoxylin-eosin stained sections per animal were analyzed. Individual adipocyte areas (μm^2) within each field were determined using image analysis software (Image Pro Plus, Immagini e Computer, Milan, Italy). For the quantitative analysis, adipocyte areas were calculated in arbitrary fields, measuring fifty adipocytes for each section. Other methodological details are provided in the online Data Supplement (available at http://hyper.ahajournals.org). 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.

Results

Effect of induction of HO-1 on body weight, appearance, and fat content of female and male *obese* mice

Previously, we have shown CoPP treatment results in the prevention of weight gain in several male models of obesity including *obese* and *db/db* mice and *Zucker fat* rats[4, 12]. We extended our studies to examine the effect of CoPP on weight gain in female *obese* mice. CoPP-treatment prevented weight gain in male *obese* mice when compared to age-matched male controls (Figure S1). The prevention of body weight gain was accompanied by a reduction in visceral fat in male *obese* mice. However, female *obese* mice administered CoPP did not lose weight but continued to gain weight at the same rate as untreated female *obese* mice (Figure S1). This was in spite of food intake being comparable between the two groups. CoPP administration decreased subcutaneous fat content in both *obese* males and females ($p<0.05$; $p<0.05$, respectively). CoPP produced a decrease ($p<0.05$) in visceral fat in male but not in female *obese* mice when compared to untreated *obese* mice (Figure S1D).

We examined adipocyte size by haematoxilin-eosin staining in both lean, obese and CoPP treated *obese* female mice (Figure 1A, upper panel). CoPP treatment resulted in a decrease in adipocyte size ($p<0.05$) compared to untreated *obese* animals (Figure 1A, lower left panel). We then examined the number of adipocytes in lean, *obese* and CoPP-treated *obese* female mice. The number of adipocytes (mean±SE) in lean, *obese* and CoPP-treated *obese* animals was 40.83±3.50, 18.33±1.80 and 32.00±1.67 respectively indicating that CoPP treatment of *obese* mice increased the number of adipocytes to levels similar to those in lean animals (Figure 1A, lower right panel). Similar results were seen in male animals.

The induction of HO-1 was associated with a reduction in blood pressure (BP). Systolic blood pressure in *obese* female mice was 142 ± 6.5 mm Hg compared to *obese*-CoPP treated, 109 ± 8.1 mm Hg, $p<0.05$. The value in *obese* female mice treated with CoPP is similar to the blood pressure seen in lean female mice (110 ± 9.6 mm Hg). The systolic blood pressure in obese male mice was 144± 4.5 mm Hg compared to *obese*-CoPP treated, 104 ± 3.6 mm Hg, $p<0.05$.

We further examined whether CoPP affects HO-1 expression in adipocyte using immunohistochemistry and western blot analysis. Immunostaining showed increased levels of HO-1 (green staining), located on the surface of adipocytes, after CoPP treatment ($p<0.05$), compared with female *obese* mice, Figure 1B. As seen in Figure 1C, HO-1 and

HO-2 levels in adipocyte isolated from lean, untreated female *obese* mice or female *obese* mice treated with CoPP. Densitometry analysis showed that HO-1 was increased significantly in female *obese* mice treated with CoPP, compared to non-treated female *obese* mice, $p<0.05$, which is in agreement with immunohistochemistry results. This pattern of HO expression in obesity occurs in other tissues, including aortas, kidneys and hearts of male *obese* mice[4, 13].

Effect of CoPP on HO-1 expression and HO activity in female and male *obese* mice

HO-1 protein levels were increased by CoPP treatments in liver and renal tissues similar to that seen in adipocytes. Western blot analysis showed significant differences ($p<0.05$) in the ratio of HO-1 to actin in renal of male and female *obese* and lean mice (Figure S 2A). Obesity decreased HO-1 levels in both sexes when compared to age matched lean animals. In addition, HO-1 levels were significantly ($p<0.05$) lower in *obese* females compared to *obese* males (Figure S 2A). This reflects a less active HO system in both male and female *obese* animals compared to age matched lean controls. Next, we compared the effect of CoPP on male and female HO-1 gene expression in adipocytes. CoPP increased HO-1 expression in both male and female *obese* animals compared to untreated *obese* animals (Figure S 2B, $p<0.001$ and $p<0.001$, respectively). Similar results of HO-1 expression were seen in liver tissues (Result not shown).

Effect of CoPP on cytokine levels in female and male *obese* mice

CoPP administration resulted in a significant increase in the levels of plasma adiponectin in both female ($p<0.001$) and male *obese* ($p<0.001$) mice (Figure 2A). Untreated female *obese* animals exhibited a significant ($p<0.05$) increase in plasma IL-6 levels when compared to age-matched male *obese* mice (Figure 2B). CoPP decreased plasma IL-6 levels in both female and male *obese* mice ($p<0.05$, $p<0.01$, respectively) when compared to untreated *obese* mice. Similar results were observed with plasma TNF-α and IL-1β levels (Figure 2C and 2D). These results indicate that though female *obese* mice exhibited elevated serum levels of inflammatory cytokines compared to male *obese* mice, CoPP acts with equal efficacy in both female and male *obese* animals in reducing inflammation while simultaneously increasing serum adiponectin levels (Figure 2).

Effect of CoPP on blood glucose and LDL levels in female and male *obese* mice

Fasting glucose levels were determined after the development of insulin resistance. CoPP produced a decrease in glucose levels in both fasting female ($p<0.05$) and male ($p<0.001$) *obese* mice when compared to untreated *obese* control animals (Figure 3A). CoPP reduced LDL levels in both male ($p<0.01$) and female ($p<0.05$) *obese* mice when compared to untreated *obese* controls (Figure 3B). Treatment with SnMP, increased LDL levels. In separate experiments two weeks apart, glucose levels and insulin sensitivity were determined after development of insulin resistance (Fig. 4A and B). Blood glucose levels in female *obese* mice were elevated ($p<0.01$) 30 min after glucose administration and remained elevated. In CoPP-treated female *obese* mice, blood glucose levels decreased significantly 60–120 min after glucose administration ($p<0.01$). Insulin administration to CoPP-treated female *obese* mice produced a decrease in glucose but not in the vehicle-treated female *obese* mice ($p<0.01$).

Effect of Obesity on Protein Expression Levels of pAKT, pAMPK, and PPARγ levels in female and male *obese* mice

Western blot analysis of adipocytes harvested from fat tissues, showed significant differences in basal protein expression levels of pAKT and pAMPK in untreated female *obese* mice compared to untreated *obese* male mice. pAMPK levels were higher in *obese*

females compared to *obese* males (Figure 5A, $p< 0.05$). This was also the case for pAKT protein levels, where increased levels of pAKT were seen in *obese* females compared to *obese* males (Figure 5B, $p<0.05$). CoPP treatment increased pAMPK and pAKT levels in bothe *obese* females and *obese* males. In addition, CoPP administration increased PPARγ levels, in both male ($p<0.001$) and female ($p<0.05$) *obese* mice (Figures 5C).

Discussion

In the current study, we show for the first time that induction of HO-1 regulates adiposity in both male and female animals via an increase in adipocyte HO-1 protein levels. A second novel finding is that induction of HO-1 was associated not only with a decrease in adipocyte cell size but with an increase in adipocyte cell number. Further, induction of HO-1 affects visceral and subcutaneous fat distribution and metabolic function in male *obese* mice differently than in female *obese* mice. Despite continued obesity, upregulation of HO-1 induced major improvements in the metabolic profile of female *obese* mice exhibiting symptoms of Type 2 diabetes including: high plasma levels of proinflammatory cytokines, hyperglycemia, dyslipidemia, and low adiponectin levels. CoPP treatment resulted in increased serum adiponectin levels and decreased blood pressure. Adiponectin is exclusively secreted from adipose tissue, and its expression is higher in subcutaneous rather than in visceral adipose tissue. Increased adiponectin levels reduce adipocyte size and increase adipocyte number[12], resulting in smaller, more insulin sensitive adipocytes. 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[18–21]. Levels of circulating adiponectin are inversely correlated with plasma levels of oxidized LDL in patients with Type 2 diabetes and coronary artery disease, which suggests that low adiponectin levels are associated with an increased oxidative state in the arterial wall[22]. Thus, increases in adiponectin mediated by upregulation of HO-1 may account for improved insulin sensitivity and reduced levels of LDL and inflammatory cytokines (TNF-α, IL-1β, and IL-6 levels) in both male and female mice.

Females continued to gain weight in spite of the metabolic improvements. One plausible explanation for this anomaly is the direct effects of HO-1 on adiponectin mediating clonal expansion of pre-adipocytes. This supports the concept that expansion of adipogenesis leads to an increased number of adipocytes of smaller cell size; smaller adipocytes are considered to be healthy, insulin sensitive adipocyte cells that are capable of producing adiponectin[23]. This hypothesis is supported by the increase in the number of smaller adipocytes seen in CoPP-treated female *obese* animals without affecting weight gain when compared to female *obese* animals. Similar results for the presence were seen in males indicating that this effect is not sex specific.

Upregulation of HO-1 was also associated with increased levels of adipocyte pAKT, and pAMPK and PPARγ levels. Previous studies have indicated that insulin resistance and impaired PI3K/pAKT signaling can lead to the development of endothelial dysfunction[24]. In the current study, increased HO-1 expression was associated with increases in both AKT and AMPK phosphorylation; these actions may protect renal arterioles from insulin mediated endothelial damage. By this mechanism, increased levels of HO-1 limit oxidative stress and facilitate activation of an adiponectin-pAMPK-pAKT pathway and increased insulin sensitivity. Induction of adiponectin and activation of the pAMPK-AKT pathway has been shown to provide vascular protection[25, 26]. A reduction in AMPK and AKT levels may also explain why inhibition of HO activity in CoPP-treated *obese* mice increased inflammatory cytokine levels while decreasing adiponectin. The action of CoPP in increasing pAKT, pAMPK and PPARγ is associated with improved glucose tolerance and decreased insulin resistant. Insulin resistance is an independent factor for the development of

both endothelial[24] and vascular dysfunction[27, 28]. CoPP treated improved vascular function as manifest by increases in both insulin sensitivity and pAKT and pAMPK levels. Others have shown that increased phosphorylation of insulin receptors and vascular function may be a response to the increase in pAMPK and pAKT crosstalk[29–31]. Further activation of pAMPK and pAKT increase glucose transport, fatty acid oxidation and mitochondrial function[32–34]. pAKT and AMPK act as fuel sensors in the regulation of energy balance and the resultant the crosstalk of AMPK-AKT has been shown to regulate NO bioavailability and vascular function[30, 35, 36]. Furthermore, activated AMPK alone has been suggested as therapeutic target to ameliorate endothelial dysfunction[37–39].

The novel effect of CoPP on the HO-1-adiponectin-pAKT-pAMPK-module i.e., an increase in HO-1, increases in adiponectin and the subsequent increase in AKT-AMPK crosstalk and signaling pathway provide a beneficial mechanistic basis for CoPP mediated vascular protection. Thus CoPP appears capable of reprogramming adipocytes resulting in the expression of a new phenotype containing adipocytes of reduced cell size, increased number and restored insulin sensitivity. Although CoPP caused induction of HO-1 in various tissues, it is HO-1 induction in adipocytes that may be crucial for reversal of vascular dysfunction. HO-1 upregulation in adipocytes increases the release of adiponectin, with subsequent improvement in insulin sensitivity and a marked decrease in inflammatory cytokines. Therefore, targeting adipocytes with an HO-1 gene, we might be able to address obesity mediated metabolic derangements and restore vascular function.

Perspectives

We have demonstrated that HO-1 induction in adipocyte stem cells not only ameliorates obesity associated metabolic consequences including hypertension independent of body weight, but improves glucose tolerance in both male and female *obese* mice. The ability of HO-1 to cause an increase in adipocyte cell number and expansion of healthy adipocytes in *obese* mice and reduce inflammatory cytokines appears to be primarily responsible for these effects. This appears to involve HO-1, adiponectin and the pAKT/pAMPK signaling pathway acting in unison. These novel findings underscore the importance of targeting HO-1 to attenuate hypertension, insulin resistance, dyslipidemia, and subsequent cardiovascular risk within *obese* populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Angela Burgess and Ming Li contributed equally to this work

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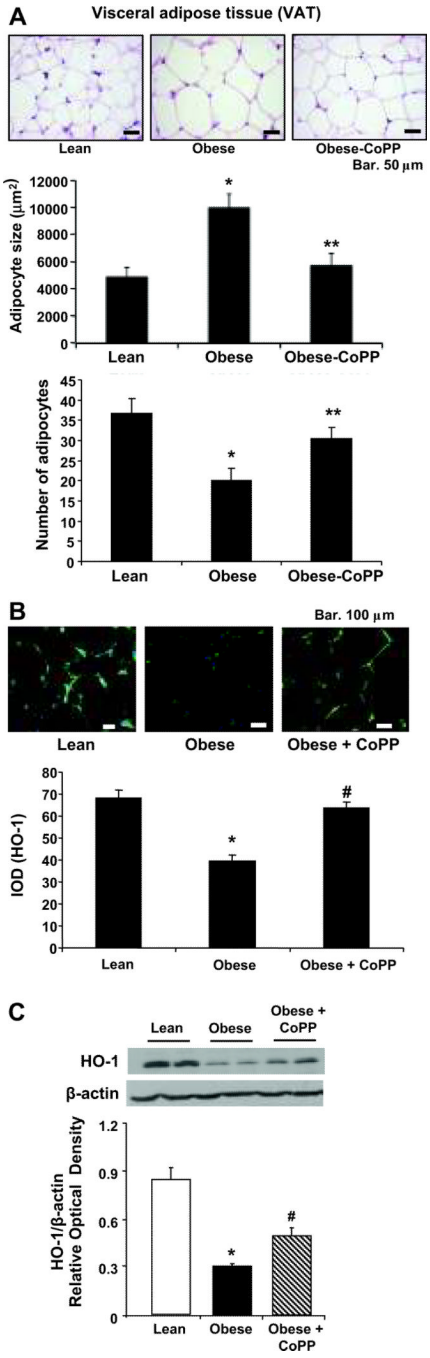

Figure 1A–C.

A) Morphological haematoxilin eosin staining of visceral (VAT) aorta surrounding fat harvested from lean, untreated *obese*, and CoPP- treated *obese*. Bar, 50 μM $p < 0.05$ compared with untreated *obese* mice, lower Panel, adipocyte size, and number of adipocytes VAT, *$p<0.05$ compared to lean or *obese* treated with CoPP, **; $p<0.05$ vs obese. n=3–4 sections per group, **B)** Representative images of immunostained adipocytes from each experimental group. n=3–4 sections per group. Female immunohistochemistry of HO-1 and IOD determination of HO-1 expression in visceral (VAT) aorta fat lean, untreated *obese*, and CoPP- treated *obese*.*$p<0.05$ (Similar results were seen in male, data not presented) and **C)** Effect of CoPP on HO-1 protein levels in adipocyte isolated from pooled visceral fat of

female lean and *obese* mice. Western blot and densitometry analysis of HO-1 protein in adipocyte isolated from fat tissues of lean and *obese* female mice treated with CoPP. Results are the mean ± SE of the band density normalized to actin; n=4; *p<0.01 vs lean; #p<0.05 vs obese using one way ANOVA.

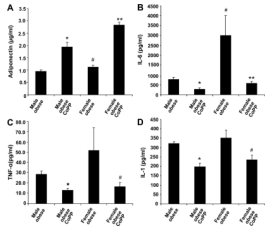

Figure 2A–D.

A) Effect of CoPP on serum adiponectin levels in male and female *obese* mice. CoPP was administered once a week for 6 weeks and serum samples were obtained immediately prior to sacrifice. Results are by 2-way ANOVA, n=6–8.Levels of significance: * $p<0.001$ versus male *obese*; # $p<0.05$ versus male *obese*; **$p<0.001$ versus female *obese*. **B)** Effect on serum IL-6 levels in male and female *obese* mice. Levels of significance for IL-6: * $p<0.01$ versus male *obese*; **$p<0.05$ versus female *obese*, # $p<0.05$ vs male obese and **C)** Effect on serum TNF-α levels in *obese* mice. Levels of significance for TNF-α: *$p<0.01$ versus male *obese*; #$p<0.05$ versus female *obese*. **D)** Effect on IL-1β serum levels in male and female *obese* mice. Levels of significance for IL-1β: *$p<0.01$ versus male *obese*; #$p<0.05$ versus female *obese*.

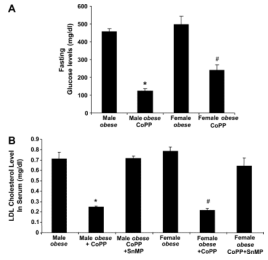

Figure 3A–B.

A) Effect of CoPP treatment on glucose levels in male and female *obese* mice, *p<0.001 versus male *obese*; #p<0.05 versus female *obese*. **B)** Effect of CoPP and SnMP treatment on LDL cholesterol levels in male and female *obese* mice. CoPP (administered once a week) and SnMP were administered three times/week for 6 weeks, and serum samples were obtained immediately before mice were sacrificed. Results are by 2-way ANOVA. n=6–8.Levels of significance: *p<0.01 versus male *obese*; #p<0.05 versus female *obese*.

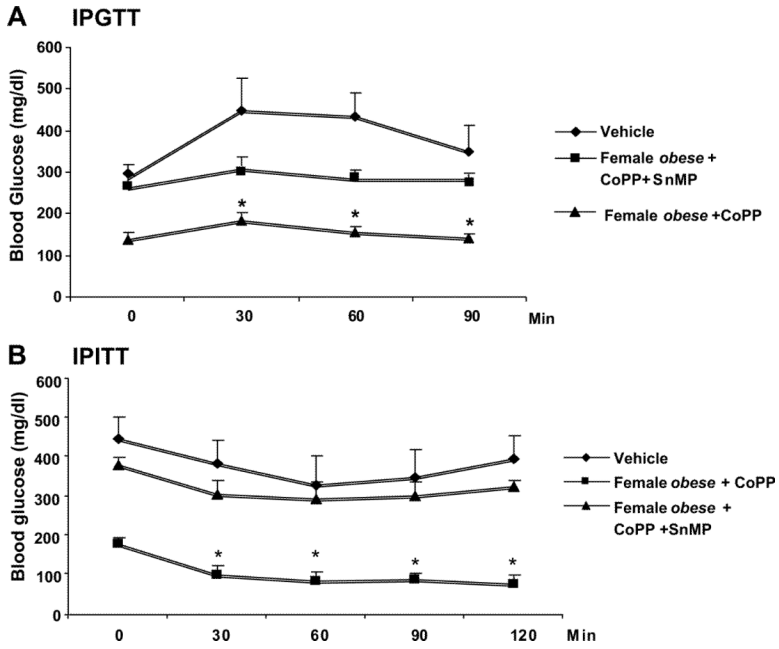

Figure 4A–B.

Effect of HO-1 expression on glucose tolerance and insulin sensitivity. Intraperitoneal glucose tolerance (IPGTT); **A)** and intraperitoneal insulin sensitivity (IPITT; **B)** The results means ± SE, n= 6–8 mice per group. Levels of significance: *p<0.01 versus female *obese*.

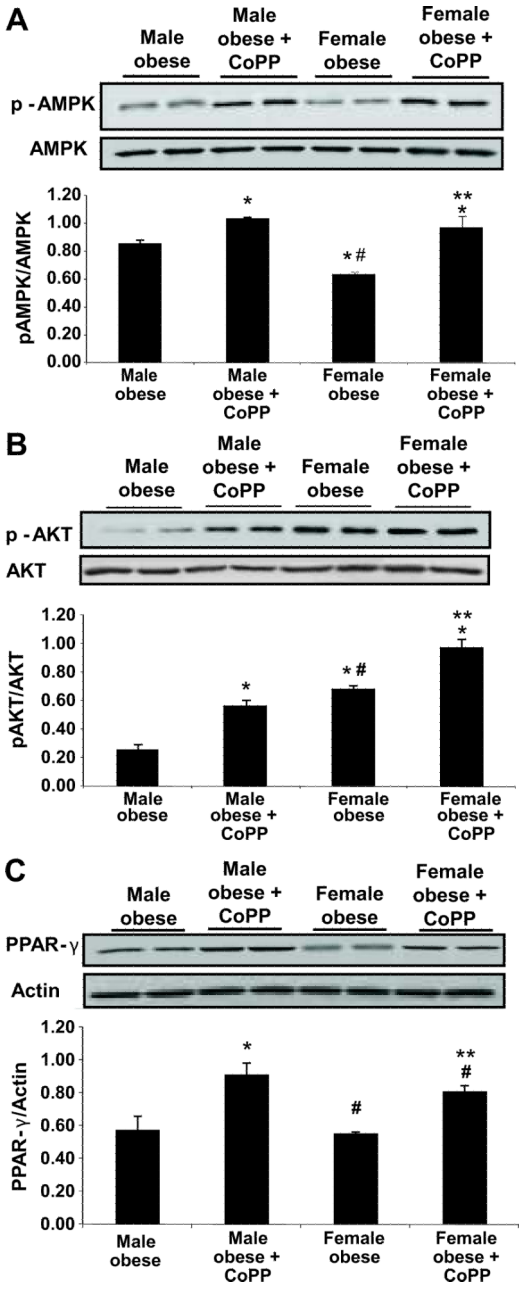

Figure 5A–C.

A) Effect of CoPP treatment on pAMPK in the adipocyte of male and female fat tissue obtained from *obese* mice. Adipocyte harvested from fat tissues samples were subjected to Western blotting for the determination of pAMPKα protein expression and densitometry analysis of pAMPKα/AMPK ratio. N=6. Levels of significance: * $p < 0.05$ versus male *obese*; # $p < 0.05$ versus *obese* male, ***$p<0.05$ vs female *obese*. **B)** Effect of CoPP treatment on pAKT in the adipocyte of male and female *obese* mice. Adipocyte samples were subjected to Western blotting for the determination of pAKT/AKT protein expression and densitometry analysis of p ratio. Levels of significance: * $p < 0.05$ versus male *obese*; *** $p < 0.05$ versus female *obese*;
# $p < 0.05$ versus *obese* male. **C)** Effect of CoPP treatment on PPARγ expression in the adipocyte of male and female *obese* mice. Adipocyte samples were subjected to Western

blotting for the determination of PPARγ protein expression and densitometry analysis of p ratio. Levels of significance: * $p < 0.001$ vs male *obese*; ** $p < 0.05$ vs male *obese*; $^{\#}p<0.05$ versus female *obese*.