

Heme Oxygenase Gene Targeting to Adipocytes Attenuates Adiposity and Vascular Dysfunction in Mice Fed a High-Fat Diet

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Abstract—We examined the hypothesis that adipocyte dysfunction in mice fed a high-fat (HF) diet can be prevented by lentiviral-mediated and adipocyte specific-targeting delivery of the human heme oxygenase-1 (aP2-HO-1). A bolus intracardial injection of aP2-HO-1 resulted in expression of human HO-1 for up to 9.5 months. Transduction of aP2-HO-1 increased human HO-1 expression in fat tissues without affecting murine HO-1. In mice fed a HF diet, aP2-HO-1 transduction attenuated the increases in body weight, blood glucose, blood pressure, and inflammatory cytokines, as well as the content of both visceral and subcutaneous fat. Transduction of aP2-HO-1 increased the numbers of adipocytes of small cell size ($P<0.05$), insulin sensitivity ($P<0.05$), adiponectin levels, as well as vascular relaxation to acetylcholine compared with HF mice administered the aP2-green fluorescent protein. Adipocytes of mice fed a HF diet expressed high levels of peroxisome proliferator activator receptor, aP2, C/EBP, and Wnt5b proteins and displayed marked increases in Peg1/Mesoderm specific transcript ($P<0.03$). Transduction of aP2-HO-1 lowered the elevated levels of these proteins and increased Sonic hedgehog, Wnt10b, and β -catenin ($P<0.05$). Inhibition of HO activity by administration of tin mesoporphyrin to HF-fed mice transduced with the aP2-HO-1 reversed the decrease in Peg1/Mesoderm-specific transcript, TNF α , and MCP-1 levels. Collectively, this novel study demonstrates that adipocyte-specific overexpression of HO-1 attenuates HF-mediated adiposity and vascular dysfunction; increases insulin sensitivity; and improves adipocyte function by increasing adiponectin, Shh, and WNT10b, and by decreasing inflammatory cytokines. These effects are reversed by the HO activity inhibitor, stannous mesoporphyrin. (*Hypertension*. 2012;60:467-475.) • [Online Data Supplement](#)

Key Words: HO-1 ■ adiposity ■ Wnt 10b ■ Peg1/Mest ■ lentivirus ■ SnMP

Obesity is a metabolic disorder and a risk factor commonly associated with endothelial dysfunction and the development of vascular diseases, including hypertension and other cardiovascular complications. Excessive visceral and subcutaneous fat is predictive of vascular disease and associated complications,^{1,2} including vascular dysfunction, insulin resistance, and decreased levels of adiponectin.^{3,4} Obesity increases oxidative stress and simultaneously decreases expression and activity of key cytoprotective systems, including heme oxygenase (HO) and adiponectin, while increasing inflammatory cytokines and insulin resistance.^{3,5-8} These consequences of obesity-mediated adipocyte dysfunction may be far-reaching, as changes in adipocyte-derived paracrine factors, including adipokines and cytokines, may impact

the function of other organs, and, in particular, the vasculature. Obesity is associated with vascular dysfunction, which is a prelude to vascular disease and hypertension.^{5,9,10}

Fat-tissue-derived adipocytes express several regulatory proteins, such as Wnts and β -catenin, as well as Sonic hedgehog (Shh), which potentially works upstream of these known differentiation factors to induce osteogenesis in mesenchymal stem cells (MSCs).¹¹ Hedgehog signaling exerts its pleiotropic effects through regulation of the cell cycle,¹² direction of cell differentiation,¹³ and alteration of cell survival.¹⁴ Increased Shh signaling promotes osteogenesis in various bone-forming cells in vitro.^{12,15,16} Conversely, Shh signaling represses adipogenic differentiation in preadipocytes.^{17,18} Wnts, β -catenin, and Shh are essential to regu-

Received March 5, 2012; first decision March 21, 2012; revision accepted June 2, 2012.

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The online-only Data Supplement is available with this article at <http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.112.193805/-DC1>.

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Hypertension is available at <http://hyper.ahajournals.org>

DOI: 10.1161/HYPERTENSIONAHA.112.193805

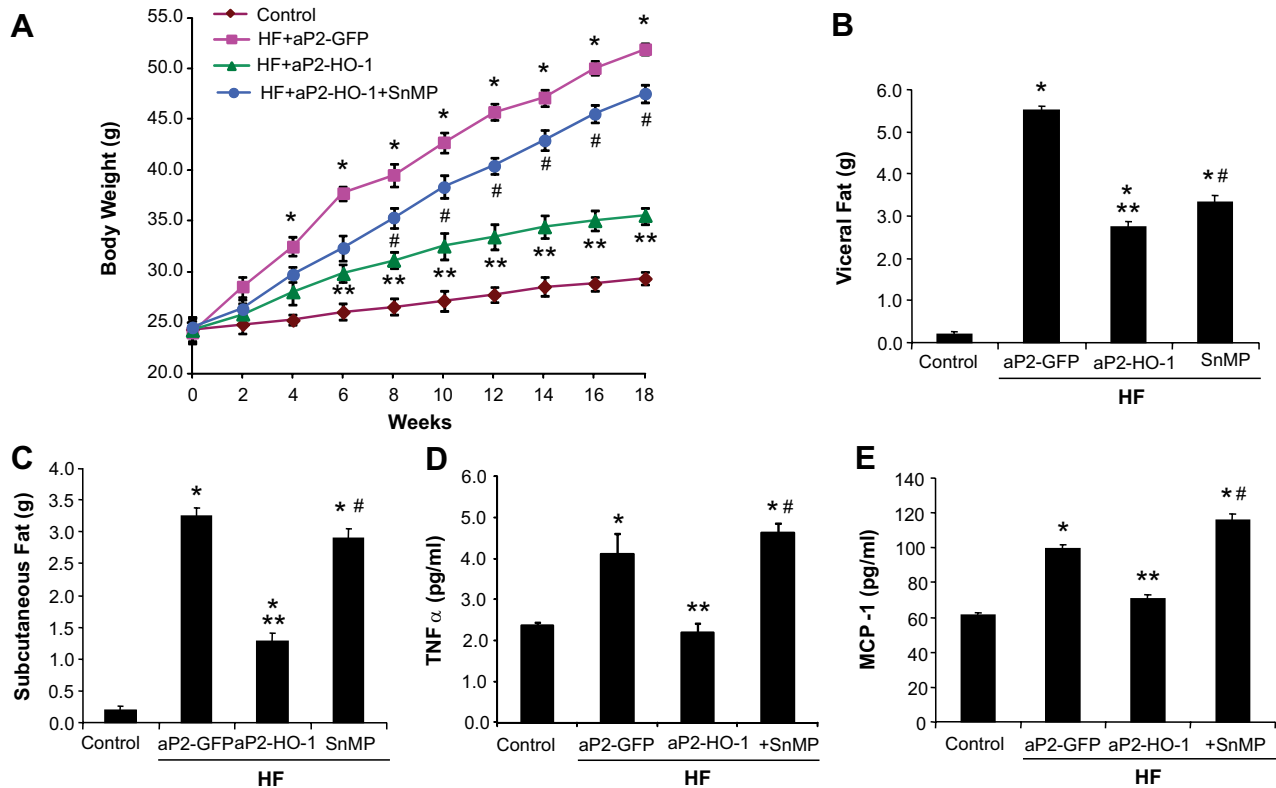


Figure 1. A, body weight; B, visceral fat and C, subcutaneous fat; D, serum TNF α ; and E, serum MCP-1 in mice fed a normal diet (control) and mice fed a HF diet and transduced with the aP2-GFP (aP2-GFP) or aP2-HO-1 untreated (aP2-HO-1) and treated with SnMP (+SnMP; * P <0.05 versus control; ** P <0.05 versus HF+aP2-GFP; # P <0.05 versus HF+aP2-HO-1).

late the conversion of preadipocytes to adipocytes.^{19,20} Wnt10b expression is increased in preadipocytes and blocks adipocyte differentiation.^{20,21} Increases in Wnt/ β -catenin inhibit adipogenic transcription factors and peroxisome proliferator activator receptor (PPAR γ), and represses adipogenesis.^{19,22–24} Conversely, paternally expressed gene 1 (Peg1)/Mesoderm specific transcript (Mest),²⁵ when upregulated, results in adipocyte enlargement during adipose tissue expansion²⁶ that is associated with increased release of IL-1 and enhanced insulin resistance.^{3,5,27,28}

Given that HO-1 gene therapy brings about extended and uninterrupted vascular protection, the present study examined the effect of HO-1 overexpression at the onset of adipocyte insult following high-fat (HF) intake in mice. Induction of HO-1 gene expression is associated with increased preadipocytes, a reduction in the number of enlarged adipocytes, increased adiponectin levels, and small adipocytes,^{3,28,29} which are regarded as healthy insulin-sensitive adipocytes, ie, expansion of adipocytes.^{27,30} In contrast, suppression of HO-1 expression results in increased insulin resistance and adiposity in rodents.^{3,31} Although obesity is associated with oxidative stress and increased reactive oxygen species levels, obesity decreases HO-1 expression.^{3,27,32,33} HO-1 and HO-2 are expressed in adipocytes, and the induction of HO-1 decreases adipocyte-mediated O₂^{•−} formation.^{3,27,32} The goal of this study was to determine the effect of a stable adipocyte-specific HO-1 gene expression as an interdependent module in preserving adipocyte function in a model of diabetes/obesity, ie, mice fed a HF diet. Herein, we demonstrate that targeted overexpression of HO-1 in adipocytes lasts for about 9 months and is sufficient to reduce

adiposity. We also identify new molecular targets of HO-1 that may be involved in reprogramming of adipocyte cells to a new phenotype as evidenced by increases in adiponectin, which is derived solely from adipocytes, and Wnt10b. The latter and its protein signaling is probably the molecular switch that governs adipogenesis. In summary, this is the first study to demonstrate that HO-1 gene targeting adipocytes decreases glucose levels and increases insulin sensitivity. These actions are reversed by the HO activity inhibitor, stannous mesoporphyrin (SnMP).

Materials and Methods

A detailed description of experimental protocols, methods, and materials is included in the Online Supplement.

Results

Adipocyte-Specific Transduction

The lentiviral construct aP2-HO-1, in which the expression of the human HO-1 is driven by the adipocyte-specific promoter aP2, is described in Supplemental Figure S1A. Cell-specific transduction was determined using cultured human microvascular endothelial cells, vascular smooth muscle cells, and adipocytes isolated from mice. After transduction with either aP2-HO-1 or aP2-green fluorescent protein (GFP), the expression of human HO-1 or GFP was only detected in adipocytes; no signals for these proteins were detected in human microvascular endothelial cells and vascular smooth muscle cells (Supplemental Figure S1B). The expression of HO-1 persisted for 3 days, the duration of the culture protocol. Transduction of aP2-HO-1 in adipocytes was asso-

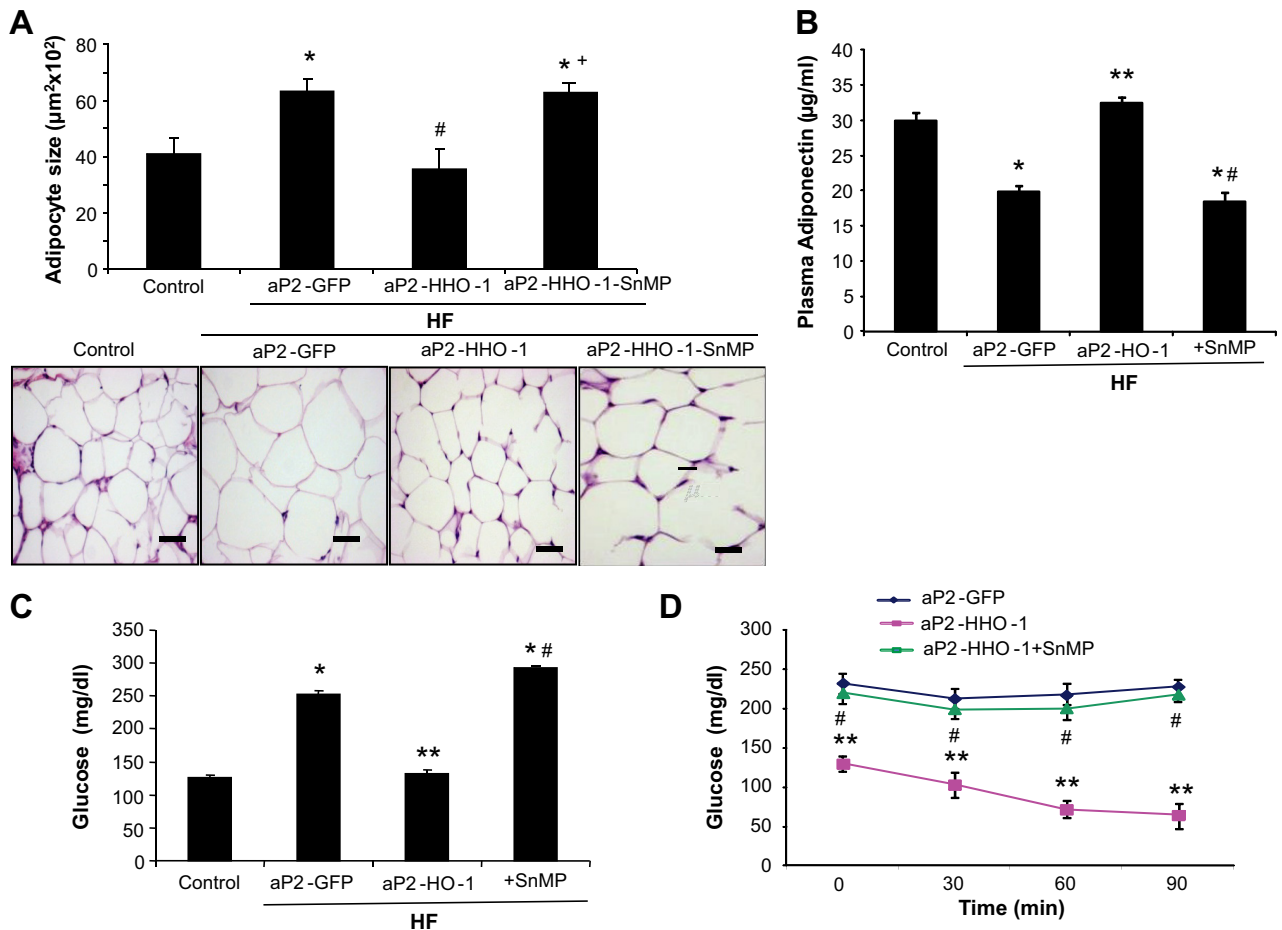


Figure 2. **A**, Hematoxylin-eosin staining of visceral fat and quantitative analysis of adipocyte size in visceral fat surrounding the aorta in mice fed a normal diet (control) and mice fed a HF diet and transduced with the aP2-GFP (aP2-GFP) or aP2-HO-1 (aP2-HO-1). Results are mean \pm SE; * P < 0.05 versus control; # P < 0.05 versus HF+aP2-GFP, + P < 0.05 versus aP2-HO-1. **B**, Plasma adiponectin levels **C**, blood glucose levels and **D**, intraperitoneal insulin tolerance test in mice fed a normal diet (control) and mice fed a HF diet and transduced with the aP2-GFP (aP2-GFP) or aP2-HO-1 untreated (aP2-HO-1) and treated with SnMP (+SnMP). Results are mean \pm SE; n = 4; * P < 0.05 versus control; ** P < 0.05 versus aP2-GFP; # P < 0.05 versus aP2-HO-1.

ciated with a 3-fold increase in HO activity when compared with adipocyte transfected with aP2-GFP (Supplemental Figure S1C). Immunohistochemistry of fat tissue adipocytes isolated from aP2-GFP- and aP2-HO-1-treated rats fed a HF diet showed expression of GFP and human HO-1, respectively (Supplemental Figure S2A through S2D); this demonstrates the cell-specific targeting of the viral vectors. The levels of the endogenous HO-1 were decreased by HF diet (Supplemental Figure S2F), a phenomenon that has been previously shown and has been attributed to prolonged oxidative stress that suppresses HO-1 expression. Interestingly, overexpression of HO-1 by aP2-HO-1 prevented the decrease in endogenous HO-1, suggesting that such an overexpression provides antioxidative and cytoprotective properties that maintain the levels and/or prevent the suppression of the endogenous HO-1 (Supplemental Figure S2E and S2F). SnMP has been shown to activate transcriptionally the HO-1 gene while inhibiting HO activity.³⁴ Given that transduced human HO-1 is under the control of a different promoter, this effect of SnMP is not displayed for the human HO-1 protein.

Effect of Adipocyte-Specific Human HO-1 Expression on Body Weight, Fat Content, and Inflammatory Cytokines

As seen in Figure 1A, body weight of aP2-GFP-HF mice increased by 61% over a period of 18 weeks when compared with mice on a normal diet (from 29.4 ± 2.5 g to 52.0 ± 1.0 g). The mice transduced with aP2-HO-1 on 18 weeks of a HF diet showed a reduction in body weight compared with aP2-GFP-HF mice (P < 0.05). The reduction in weight gain in aP2-HO-1-HF mice was reversed by coadministration of SnMP, increasing the body weight to levels similar to those of aP2-GFP-HF mice. There were no significant changes in food intake among the groups.

The reduction in weight gain by aP2-HO-1 was also manifested by significant decreases in visceral and subcutaneous fat content in obese mice (Figure 1B and 1C, respectively). Mice receiving aP2-GFP on a HF diet showed marked increase in visceral and subcutaneous fat content (5.5 ± 0.15 g and 3.30 ± 0.20 g, respectively) compared with mice fed a normal diet (0.29 ± 0.19 g and 0.23 ± 0.25 g, respectively; P < 0.05). Moreover, the reduction in visceral and subcutane-

ous fat in aP2-HO-1-HF mice, which amounted to 55% and 60%, respectively, compared with aP2-GFP-HF mice, was attenuated and reversed by administration of the HO inhibitor SnMP (Figure 1B and 1C).

To study the effect of HF diet on systemic inflammation, measurement of inflammatory cytokines, including TNF α (Figure 1D) and MCP-1 (Figure 1E), was performed. Transduction with aP2-HO-1 in mice fed a HF diet resulted in lowered ($P<0.05$) levels of these cytokines when compared with those in aP2-GFP-HF mice. Administration of SnMP reversed the positive effect of aP2-HO-1 transduction, suggesting that increased adipocyte HO-1 expression and activity has an anti-inflammatory effect on mice fed a HF diet.

Effect of Adipocyte HO-1 Gene Targeting on Adipocyte Size, Plasma Adiponectin, and Insulin Sensitivity

Histological examination of visceral fat revealed that adipocyte cell size significantly decreased in tissues obtained from aP2-HO-1-HF mice (Figure 2A). Adipocyte cell size in the visceral fat of mice fed a normal diet was significantly lower compared with that in aP2-GFP-HF mice (Figure 2A). In addition, aP2-HO-1-HF mice showed a significant reduction in adipocyte cell size, an indicator of healthy adipocytes, compared with mice receiving aP2-GFP and on a HF diet. Moreover, aP2-HO-1-transduced mice on a HF diet showed a significant increase in adiponectin levels ($P<0.01$) compared with aP2-GFP-HF mice, and this effect was reversed by administration of the HO activity inhibitor SnMP (Figure 2B). The upregulation of adiponectin levels further suggests that adipokines play an important role in improving insulin sensitivity.

Plasma glucose levels in aP2-HO-1-HF mice significantly decreased compared with aP2-GFP-HF mice, suggesting improved insulin sensitivity in response to aP2-HO-1 transduction (Figure 2C). Indeed, insulin administration to aP2-HO-1-HF mice resulted in a rapid decrease in glucose, but not in aP2-GFP-transduced mice fed a HF diet (Figure 2D). Importantly, administration of SnMP reversed the glucose-reducing effect of aP2-HO-1, indicating that this effect is mediated by increased HO activity (Figure 2C and 2D).

Effect of Adipocyte HO-1 Gene Targeting on Vascular Parameters

We studied the vascular parameters, including blood pressure and acetylcholine-induced relaxation, in mice fed a HF diet. Blood pressure was increased over the 18-week period of HF diet in mice (Figure 3A). Systolic blood pressure was 90 ± 3 mm Hg in control mice and significantly increased in mice transduced with aP2-GFP on a HF diet, to 135 ± 3 mm Hg ($P<0.01$). This elevation in systolic pressure was not observed in aP2-HO-1-transduced mice on a HF diet; SnMP negated this antihypertensive effect (Figure 3A). Vascular function, measured as the relaxation in response to acetylcholine, was impaired in mice transduced with aP2-GFP and fed a HF diet. As seen in Figure 3B, at 10^{-3} M acetylcholine, the percent of relaxation was $81.17\pm 3.05\%$ in aP2-GFP-HF mice ($P<0.05$) versus $93.41\pm 2.08\%$ in control animals. The aP2-HO-1-HF mice restored maximal relaxation to acetylcholine to levels no different from those recorded in mice fed a normal diet. Relaxation to 10^{-3} M acetylcholine

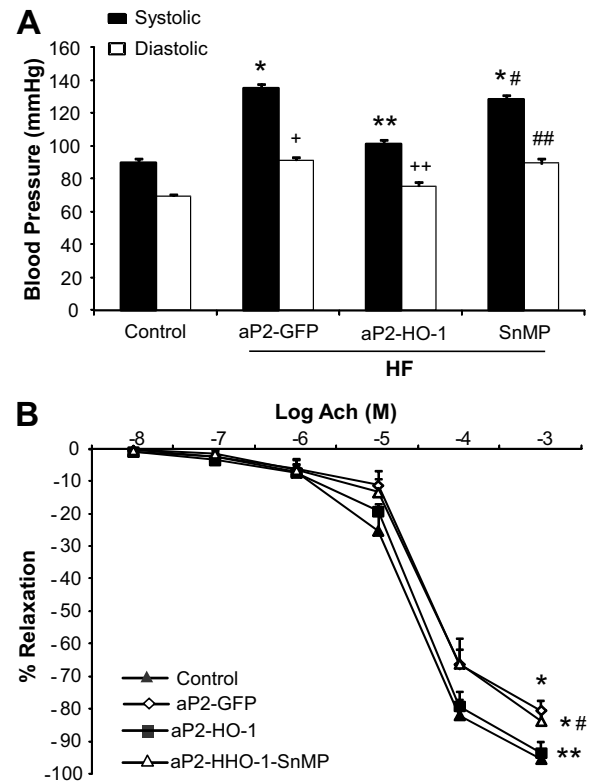


Figure 3. **A**, Blood pressure, which was measured by tail cuff method. Results are mean \pm SE (n=10/group). Systolic blood pressure; * $P<0.05$ versus control; ** $P<0.05$ versus aP2-GFP; # $P<0.05$ versus aP2-HO-1. Diastolic blood pressure; + $P<0.05$ versus control; ++ $P<0.05$ versus aP2-GFP; ## $P<0.05$ versus aP2-HO-1. **B**, Relaxation-response curves to acetylcholine in aorta. Results are mean \pm SE, n=5; * $P<0.05$ versus control; ** $P<0.05$ versus aP2-GFP; # $P<0.05$ versus aP2-HO-1.

increased ($P<0.05$) by aP2-HO-1 transduction from $81.17\pm 3.05\%$ to $92.15\pm 3.41\%$ in mice on a HF diet, and this effect was reversed by SnMP (maximal dilation, $82.55\pm 2.65\%$ versus aP2-GFP-HF; $P<0.05$; Figure 3B).

Effect of HO-1 Upregulation on Key Signaling Pathways for Adipocyte Differentiation

We examined the levels of PPAR γ , C/EBP α , adiponectin, and Shh proteins to see if they could account for the decrease in fat content in adipose tissues from mice transduced with the human HO-1 and fed a HF diet. Real time polymerase chain reaction analysis indicated that messenger ribonucleic acid (mRNA) levels of PPAR γ and C/EBP α increased by 5- to 6-fold in adipocytes from mice receiving aP2-GFP and fed a HF diet compared with mice on a normal diet (Supplemental Figure S3A and S3B). These levels decreased in adipocytes from aP2-HO-1-HF mice to levels not different from those in mice fed a normal diet. Administration of SnMP to aP2-HO-1-HF mice reversed the lowering effect of aP2-HO-1 transduction. The changes in mRNA levels correlated with changes in protein levels. As seen in Figure 4A, PPAR γ and C/EBP α protein levels increased in aP2-GFP-HF mice. HO-1 transduction (aP2-HO-1-HF) decreased the levels of both proteins, suggesting impairment in adipocyte differentiation. SnMP increased both PPAR γ and C/EBP α protein levels,

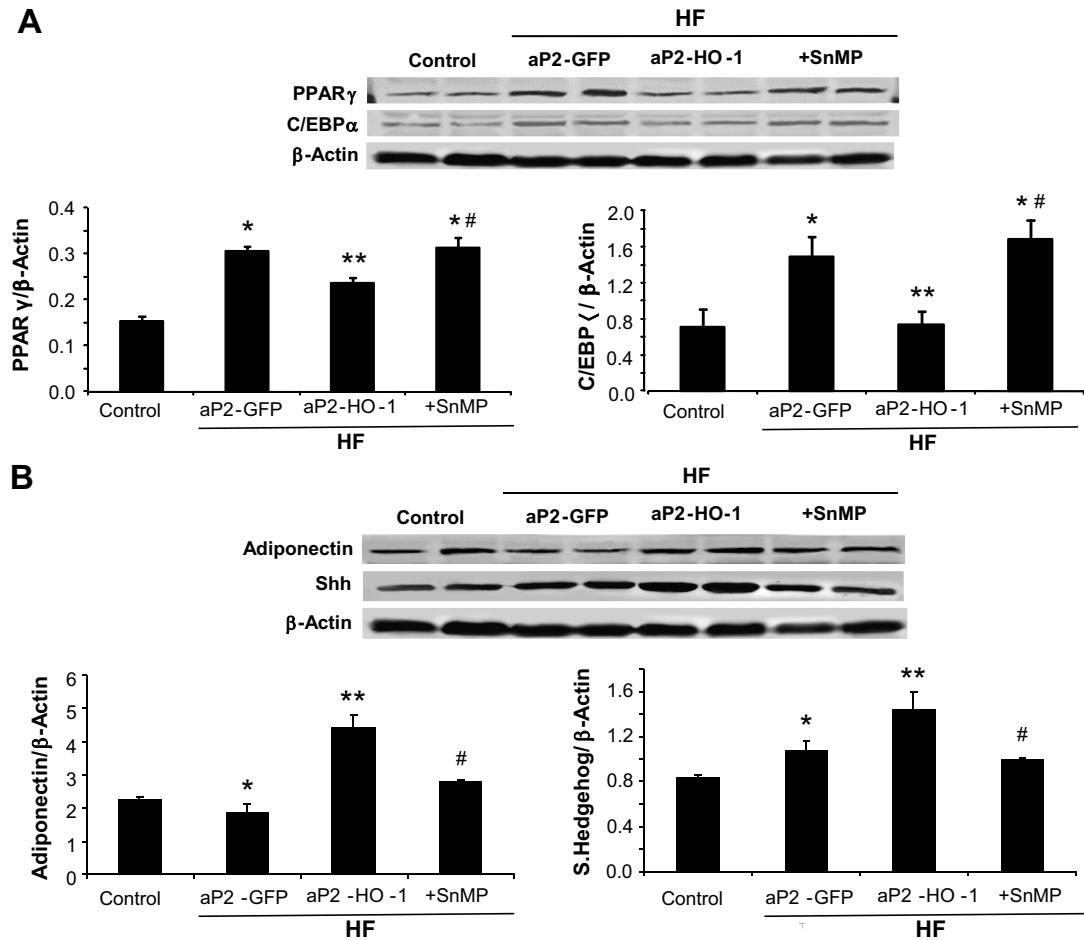


Figure 4. Western blots and densitometry analyses of **A**, PPAR γ , C/EBP α , **B**, adiponectin, and Shh proteins in adipocytes from mice fed a normal diet (control) and mice fed a HF diet and transduced with the aP2-GFP (aP2-GFP) or aP2-HO-1 untreated (aP2-HO-1) and treated with SnMP (+SnMP). Results are mean \pm SE; $n=4$; * $P<0.05$ versus control; ** $P<0.05$ versus HF+aP2-GFP; # $P<0.05$ versus HF+aP2-HO-1.

bringing these adipocyte markers to levels similar to those seen in animals fed a HF diet (Figure 4A).

In contrast, adiponectin mRNA (Supplemental Figure S3C) and protein levels (Figure 4B) increased significantly by HO-1 transduction compared with aP2-GFP-HF mice. Similarly, Shh mRNA (Supplemental Figure S3D) and protein levels (Figure 4B) were significantly ($P<0.05$) upregulated by HO-1 transduction when compared with mice receiving aP2-GFP and fed a HF diet. Administration of SnMP to aP2-HO-1-HF mice resulted in a decrease in Shh mRNA (Supplemental Figure S3D) and protein (Figure 4B) expression. These data suggest that a sustained increase in HO-1 expression improves adipocyte function and reduces adiposity through upregulation of adipocyte-protective adiponectin and through activation of a signaling pathway that increases expression of Shh.

Differential Effect of aP2-HO-1 on aP2 and Mest/Peg1 Expression

The effect of aP2-HO-1 transduction on aP2 expression is shown in Figure 5A. The aP2-GFP-HF mice expressed upregulated levels of aP2 compared with mice on a normal diet. A significant decrease of aP2 mRNA (Supplemental Figure S4A) and protein (Figure 5A) expression in fat-derived adi-

pocytes occurs following transduction of aP2-HO1, suggesting a correlation between aP-2 downregulation and a decrease of adipose tissue. Mest/Peg1, a gene that enlarges adipocytes and is a marker of adipocyte size, was upregulated in mice receiving aP2-GFP and fed a HF diet when compared with mice fed a normal diet (Figure 5B and Supplemental Figure S4B). Adipocytes from aP2-HO-1-HF mice displayed a significant decrease in both mRNA and protein levels of Mest/Peg1; this effect was reversed by administration of SnMP, resulting in an increase in Mest/Peg1 mRNA (Supplemental Figure S3D) and protein (Figure 5B) expression. The finding that increased expression of HO-1 decreased the levels of Mest/Peg1 suggests a role for HO-1 in decreasing adipocyte size.

Differential Effect of aP2-HO-1 on Wnt10b, Wnt5b, and β -Catenin Expression

Transduction of aP2-HO-1 upregulated the expression of Wnt10b in mice fed a HF diet (Figure 5C) compared with aP2-GFP-HF mice and, simultaneously, increased β -catenin levels (Figure 5E). The increases in levels of Wnt10b and β -catenin were negated by treatment of aP2-HO-1-HF mice with SnMP ($P<0.05$; Figure 5C and 5E). Contrary to the above findings, aP2-HO-1 transduction decreased the expression of

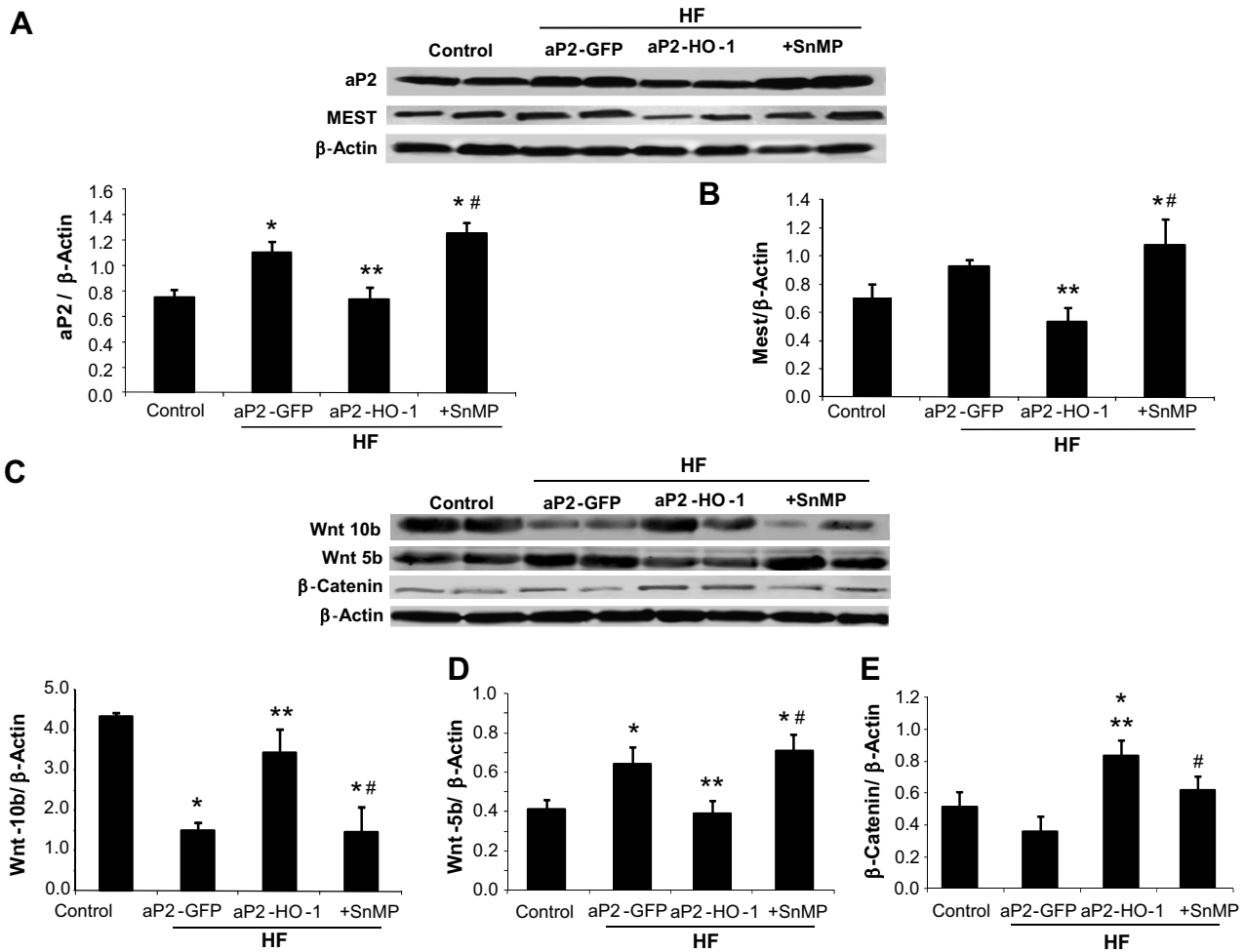


Figure 5. Western blots and densitometry analyses of **A**, aP2 and **B**, Peg1/Mest proteins in adipocytes from mice fed a normal diet (control) and mice fed a HF diet and transduced with the aP2-GFP (aP2-GFP) or aP2-HO-1 untreated (aP2-HO-1) and treated with SnMP (+SnMP). Results are mean±SE; n=4; * $P<0.05$ versus control; ** $P<0.05$ versus HF+aP2-GFP; # $P<0.05$ versus HF+aP2-HO-1. Western blots and densitometry analyses of **C**, Wnt10b, **D**, Wnt5b, and **E**, β-catenin proteins in adipocytes from mice fed a normal diet (control) and mice fed a HF diet and transduced with the control vector aP2-GFP (aP2-GFP) or with the aP2-HO-1 (aP2-HO-1) untreated and treated with SnMP (+SnMP). Results are mean±SE; n=4; * $P<0.05$ versus control; ** $P<0.05$ versus HF+aP2-GFP; # $P<0.05$ versus HF+aP2-HO-1.

Wnt5b in mice fed a HF diet compared with aP2-GFP mice fed a HF diet (Figure 5D). Additional administration of SnMP to aP2-HO-1-HF mice resulted in an increase in Wnt5b protein expression (Figure 5D).

Discussion

This is the first study to demonstrate that targeting adipocytes in mice fed a HF diet with human HO-1 gene decreased adiposity and vascular dysfunction, improved metabolic parameters, and attenuated serum levels of inflammatory cytokines. These beneficial effects are reflected by an increase in Wnt10b, a marker for preadipocytes, and a decrease in Peg1/Mest. In the present article, we demonstrate that adipocyte-specific increase in expression of HO-1 using a lentiviral construct expressing human HO-1 under the control of the aP2 promoter decreased fat content and improved insulin sensitivity and vascular function. Increased HO-1 expression also resulted in decreased levels of PPAR γ , C/EBP α , and the adipose specific protein aP2. In addition, adiponectin, manufactured only in adipocytes, was increased

after adipocyte-specific targeting of the HO-1 gene, resulting in improvement in adipocyte function.

The results reported here extend our previous findings that upregulation of HO-1 in adipocytes results in the reprogramming of adipocytes to decrease a negative signaling protein in the regulation of adiposity. In fact, the HO-1/adiponectin system is known to exert beneficial effects on obesity-induced insulin resistance.³² Previously, we reported the beneficial effects of HO-1 induction on adiponectin levels, inflammatory cytokine levels, and weight gain in rodent models of obesity.³⁵ Thus, our results confirm that HO-1 induction is capable of reprogramming adipocytes, resulting in the expression of a new phenotype with an improved ability to better insulin sensitivity. This phenomenon is identical to the positive responses of vascular tissue to HO-1 induction already reported.^{3,36–38}

Moreover, other studies suggest that adiponectin acts as a starvation signal whenever adipocyte size is small to upregulate adipose tissue with a resultant accumulation of triglycerides.^{39,40} This is in agreement with the finding of a significant decrease of mRNA and protein expression of Mest/Peg1, a gene that enlarges

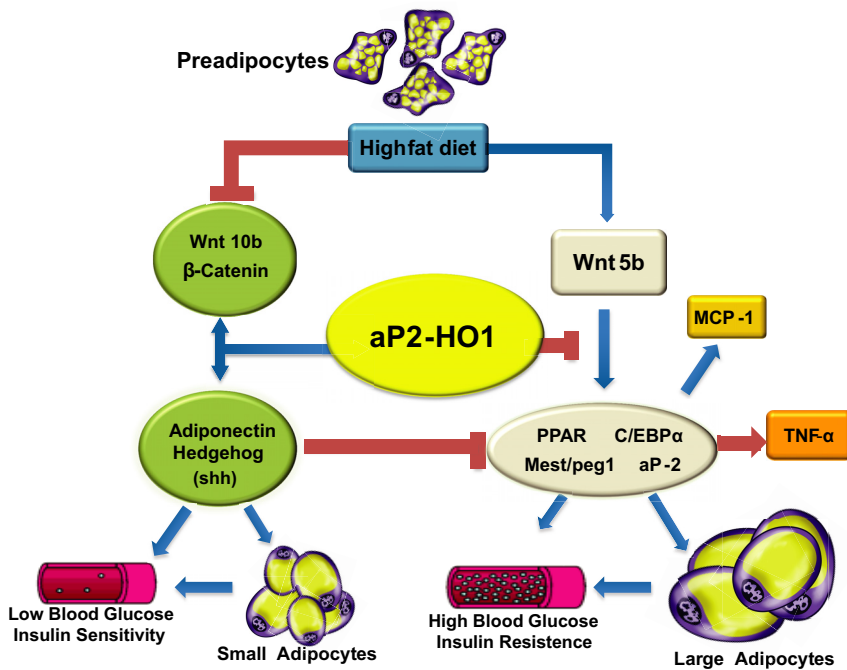


Figure 6. Proposed mechanisms underlying the adiposity-lowering effect of adipocyte-specific upregulation of HO-1. Targeted overexpression of HO-1 to the adipocyte is sufficient to counteract the detrimental effects of high fat diet on body weight, adiposity, and insulin sensitivity. Within the adipocyte, HO-1 suppresses adipocyte differentiation by decreasing expression of key regulators including PPAR γ , E/CBP α , Peg1/Mest, and aP2 leading to decreases in lipid accumulation and increases of preadipocytes and healthy adipocytes that produce cytoprotective adipokines, such as adiponectin.

adipocytes and is considered a marker of adipocyte size.⁴¹ A decrease in Peg1/Mest is beneficial in the control of adiposity, given that upregulation of Peg1/Mest occurs in obese adipose tissue in several models of obesity. Therefore, HO-1 may also control adiposity by its ability to decrease Peg1/Mest. Furthermore, a recent report has described an inverse relationship (switch) between adipocyte and osteoblast expansion by upregulation of HO-1 in MSC differentiation; in fact, increased HO-1 expression stimulated the differentiation of MCSs to osteoblasts while inhibiting adipogenic differentiation.⁴² We also examined Shh, which potentially works upstream of these known differentiation factors to induce osteogenesis in MSCs.^{11–13} Increased Hedgehog signaling promotes osteogenesis in various bone-forming cells in vitro.^{12,15,16} Conversely, Hedgehog signaling represses adipogenic differentiation in preadipocytes.^{17,18}

Mice fed a HF diet exhibited a decrease in Shh mRNA and protein expression that was reversed by HO-1 transduction, confirming its role in adipose tissue reduction. Several reports have demonstrated an association between adipogenesis and Wnt signaling,⁴³ and in the regulation of adult tissue homeostasis and remodeling.^{44,45} Wnt10b, a member of the Wnt family, maintains preadipocytes in an undifferentiated state through inhibition of the adipogenic transcription factors C/EBP and PPAR γ . It has been reported that when Wnt10b signaling in preadipocytes is prevented, these cells differentiate into adipocytes. Wnt10b is highly expressed in preadipocytes and declines rapidly after induction of adipogenic differentiation.^{19,23} Disruption of Wnt10b signaling also causes transdifferentiation of myoblasts into adipocytes in vitro, highlighting the importance of this pathway not only in adipocyte differentiation, but also in mesodermal cell fate determination.¹⁹

Studies suggest that only Wnt10b is able to stabilize free cytosolic β -catenin and block adipogenesis when ectopically expressed; it is the molecular node of the canonical Wnt signaling pathway.⁴⁶ Although Wnts inhibit adipocyte differentiation through both β -catenin-dependent and β -catenin-

independent mechanisms, current evidence supports the Wnt/ β -catenin pathway as an important regulator of adipocyte differentiation.^{47,48} In the Wnt/ β -catenin signaling pathway, β -catenin plays a central role as a transcriptional coactivator. The Wnt/ β -catenin signaling pathway affects cellular functions by regulating β -catenin levels and subcellular localization.⁴⁶ Conversely, Wnt5b, another component of the Wnt family, stimulates adipogenesis by activating PPAR γ and inhibiting the β -catenin dependent Wnt signaling pathway.⁴⁸ Consistent with these findings, we found that Wnt10b and β -catenin mRNA and protein expression were downregulated in mice fed a HF diet, and that increased HO-1 expression restored Wnt10b, β -catenin, and Shh mRNA and protein expression (Figure 5). Furthermore, we found that Wnt5b was upregulated in HF-fed animals compared with in control animals, and that increased HO-1 expression reduced Wnt5b to levels similar to those found in control animals.

Adipocyte-specific overexpression of HO-1 improved vascular function and lowered blood pressure in mice fed a HF diet. Administration of a HF diet to rodents has been used as a model of obesity-induced diabetes and hypertension.^{49,50} We have previously shown that HO-1 upregulates adiponectin expression and suppression of HO-1 expression, and HO activity decreases adiponectin production in adipocytes.^{31,51} Adiponectin has been shown to exhibit antiatherogenic, antihypertensive, and insulin-sensitizing properties, and also has the capacity to attenuate vascular disease.⁵² This study provides additional evidence for a link between adipocytes and the vascular system and suggests that this is governed by regulatory interactions between HO-1 and adiponectin at the level of the adipocytes, which was reversed by SnMP HO inhibitors.⁵³

In conclusion, we have presented novel findings indicating that adipocyte-specific targeted overexpression of HO-1 using a lentivirus construct of the human HO-1 under the control of the aP2 promoter attenuated adiposity, MSC-adipocyte stem cell

differentiation, and adipogenesis (Figure 6). HO-1 targeting of adipocytes was associated with decreases in adipocyte expansion and differentiations via signaling mechanisms that include decreases in *Peg1/Mest* and increases in *Wnt10b/β-catenin* and *Shh* proteins. HO-1 gene targeting resulted in the reprogramming of preadipocytes that express a positive network of proteins, thus maintaining adipocytes in the undifferentiated state. Hence, blocking the MSC-derived adipogenic lineage would prevent and/or treat the metabolic syndrome. In support of this conclusion, aP2-HO-1 inhibited adiposity, increased insulin sensitivity and, subsequently, improved vascular function in animals fed a HF diet. The long-term expression of HO-1 in adipocytes effectively decreased adipose tissue in mice fed a HF diet through the downregulation of the adipogenic markers *PPARγ*, *C/EBPα*, and *Peg1/Mest*. This study is the first to demonstrate that improvement of adipocyte function and increased release of adiponectin enhances insulin sensitivity and attenuates hypertension. Consequently, long-term increase of HO-1 expression may provide a therapeutic approach to address the metabolic derangements associated with the progressive nature of diabetes and the metabolic syndrome, including hypertension.

Perspectives

The epidemic of obesity continues unabated in our society with sequelae of type 2 diabetes and the metabolic syndrome, and its severe and consequent vascular diseases that range from early endothelial dysfunction and hypertension to end-stage cardiac, cerebral, and renal disease. This study is the first to demonstrate that targeting adipocytes using HO-1 gene delivery attenuates HF-mediated adiposity, increases adipocyte function, and improves insulin sensitivity and vascular function, including hypertension. The successful targeting of HO-1 expression resulted in reprogramming of adipocytes to a new phenotype as evidenced by a decrease in adipocyte size, an increase in the number of small healthy lipid droplets, and a decrease in *PPARγ*, *C/EBPα*, and aP2 along with a decrease in *Peg1/Mest* expression; the decrease in *Peg1/Mest* expression appears to be primarily responsible for increases in adipocyte differentiation and release of inflammatory cytokines. This novel adipocyte-specific targeting of HO-1 expression merits additional exploration to elucidate whether this new and very different approach can have clinical value in combating obesity-related metabolic diseases.

Acknowledgments

All authors had full access to the data and take responsibility for its integrity. All authors have read and agree with the manuscript as written.

Sources of Funding

This work was supported by National Institutes of Health grants DK068134 (NGA), HL55601 (N.G.A.), and HL34300 (M.L.S.) and by gifts from the Renfield Foundation to The Rockefeller University (A.K.).

Disclosures

None.

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Novelty and Significance

What Is New?

- Successful adipocyte specific overexpression of HO-1 in vivo using a lentiviral vector carrying the human HO-1 under the control of the aP2 promoter.
- Targeting preadipocyte during early adipocyte development with aP2-HO-1 prevents adipocyte differentiation (fat expansion) and inflammation while increasing adiponectin for vascular protection.
- Adipocyte specific HO-1 overexpression is sufficient to attenuate high-fat-diet-induced adiposity and insulin resistance.
- Improvement of high-fat-diet-induced vascular dysfunction and hypertension by adipocyte-specific HO-1 overexpression.

What Is Relevant?

- Obesity is associated with vascular diseases that range from early endothelial dysfunction and hypertension to end-stage cardiac, cerebral, and renal disease.

- A close relationship between adipocyte function and the cardiovascular system, where improvement of adipocyte function by HO-1 overexpression positively impacts vascular function and blood pressure.
- An experimental and conceptual basis for new therapeutic strategies targeting HO-1-adiponectin module to ameliorate vascular dysfunction associated with hypertension and obesity.

Summary

This is the first study showing that adipocyte-specific targeted overexpression of HO-1 under the control of the aP2 promoter attenuated adiposity, MSC-adipocyte stem cell differentiation, and adipogenesis, while increasing adiponectin for vascular protection.

Heme Oxygenase Gene Targeting to Adipocytes Attenuates Adiposity and Vascular Dysfunction in Mice Fed a High-Fat Diet

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Hypertension. 2012;60:467-475; originally published online July 2, 2012;
doi: 10.1161/HYPERTENSIONAHA.112.193805

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

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ONLINE SUPPLEMENT

HEME OXYGENASE GENE TARGETING TO ADIPOCYTE ATTENUATES ADIPOSIITY AND VASCULAR DYSFUNCTION IN MICE FED A HIGH FAT DIET

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Running Title: HO-1 gene targeting in adipocytes

Key words: adiponectin; adiposity; Wnt 10b; Peg1/Mest; diabetes; lentivirus

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MATERIALS AND METHODS

Cell culture and Animal Experimentation.

Lentiviral vectors expressing either human HO-1 or green fluorescent protein (GFP) cDNA under the control of the adipocyte aP2 specific promoter were constructed using the LentiMaxTM system (Lentigen, Baltimore, MA). Human microvessels endothelial cell line (HMEC), grown in MCDB131 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 10 ng/ml Endothelial Growth Factor (Sigma, St. Louis, MO) and 1 µg/ml hydrocortisone (Sigma). Human vascular smooth muscle cells (VSMC) were obtained from ATCC (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. The cells were incubated at 37°C in a 5% CO₂ humidified atmosphere and maintained at subconfluency by passaging with Trypsin-EDTA (GIBCO-BRL). HMEC, VSMC and adipocytes were used to evaluate functional transgene expression. Cells were infected with the lentiviral vector (2 µl of 10⁹ TU/ml) carrying either the aP2-HO-1 construct under the control of the aP2 or the aP2-GFP construct (Lentigen, Baltimore, MA). GFP and HO-1 mRNA and protein levels were measured by Western blot, RT/PCR and GFP expression was confirmed using confocal laser-scanning (Olympus Fluoview FV300) microscope.

All experimental protocols were performed following an IACUC of New York Medical College approved protocol in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Lent viral vectors expressing either HO-1 or GFP cDNA under the control of the adipocyte-specific promoter aP2 were constructed using the LentiMaxTM system (Lentigen, Baltimore, MA). Lentiviruses (50 µl, 2x10⁹ TU/ml in saline) were injected into the C57BL/6 mice by a single intracardiac injection under anesthesia with sodium pentobarbital (65 mg/kg body weight, i.p.). Two weeks later another injection (75 µl 1x10⁹ TU/ml in tail vein) was given and mice were placed on a high fat diet. Mice (C57BL/6 mice, 6-7 wks old) were divided into 4 groups (n=10 per group): Control, high fat diet (HF) mice receiving the control lentiviral vector Lenti-aP2-GFP (HF-aP2-GFP) and high fat diet mice receiving the lenti-aP2-HO-1 (HF-aP2-HO-1) with and without treatment with SnMP. Control mice were fed ad libitum a normal diet containing 11% fat, 62% carbohydrate, and 27.0% protein with total calories of 12.6 KJ/g (6, 8) while all other groups were fed a high fat diet containing 58% fat from lard, 25.6% carbohydrate, and 16.4% protein with a total calories of 23.4 KJ/g (Bio-SERV, Frenchtown, NJ) for 18 weeks in order to quantify any differences between the animals with regard to weight gain (6, 8). In the HF-SnMP group, Tin mesoporphyrin IX dichloride (SnMP, an inhibitor of HO activity, 2 mg/ 100 g body wt) was administered intraperitoneally three times a week in the last 4 weeks of high fat diets. Mice were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and blood was obtained from the tail vein for glucose measurement using a glucometer (Lifescan Inc., Miligita, CA). Mice were weighed every week and blood pressure was determined by tail cuff method. Adipose tissue, aorta and blood were collected as previously described (2, 4).

Cytokine measurements

Adiponectin, TNF α and MCP-1 were determined in serum and in cell culture media using an ELISA assay (Pierce Biotechnology Inc. Woburn, MA). Insulin was measured using an ELISA kit (Millipore, Billerica, MA) as previously described (4).

Insulin tolerance tests

After a 12-hour fast, mice were injected intraperitoneally with insulin (2.0 U/kg). Blood samples were taken at various time points (0-90min) and blood glucose levels measured as previously described (5,7).

Determination of HO-1 and adipocyte cell size

Adipose tissue was collected from all the groups and prepared for morphological analysis, as previously described (5). Samples were fixed in 4% paraformaldehyde for 24h, cut into small pieces and embedded in paraffin for histological analysis. The samples were cut by microtome (5 μ m thick), mounted on D-polyisinated glass slides, deparaffinized in xylene and either stained with haematoxylin and eosin for the evaluation of adipocyte size or processed for HO-1 immunohistochemistry. HO-1 immunostaining was carried out using a human polyclonal anti-HO-1 primary antibody as previously described (5).

Analysis of HO-1 immunostaining

HO-1 staining intensity in adipocytes was computed as integrated optical density (IOD). Digitally fixed images of the slices (n=5 per animal) at 20X magnification were analyzed using an optical microscope (Olympus, Germany) equipped with an image analyzer (Image Pro Plus, Immagine Computer, Milan, Italy). For quantitative analysis, IOD was calculated for arbitrary areas, measuring three fields with the same area for each section.

RNA extraction and real-time PCR

Total RNA was extracted from mice adipose tissue using a RNeasy Protect Mini kit (QIAGEN, Maryland, USA) according to manufacturer's instructions. Total RNA (1 μ g) was transcribed into cDNA using GeneAmp kit (Applied Biosystems, Branchburg, NJ, USA) reverse transcription reagents. Total RNA was analyzed by a quantitative real time polymerase chain reaction (qRT-PCR). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on a 7500 HT Fast Real-Time PCR System (Applied Biosystems). Specific primers for human and mouse HO-1, Mest, Sonic hedgehog (Shh), aP-2, PPAR γ , adiponectin, Wnt10b, Wnt5b and β -catenin. The human HO-1 amplification primers were Fw 5'-CAGGCAGAGAATGCTGAGTTC-3' and Rv 5'-GATGTTGAGCAGGAACGCACT-3' (1, 3), (1). The mouse HO-1 amplification primers were Fw 5'-TGAAGGAGGCCACCAAGGAG-3' Rv 5'-CCCCTGAGAGGTCACCCAGG-3'. The mouse Mest amplification primers were Fw 5'-CCAGCACATCCCGGTGCTT-3'- Rv 5'-TTCCATGAGTGACAGAGCAGG-3', the Shh amplification primers were Fw 5'-GAAGGGAGAGCCGAGCGCAA-3' – Rv 5'-GCCACGGAGTTCTCTGCTTTCACAG-3', the Fth1 amplification primer were Fw

5'CCCTCGCAAGTGCGCCAGAA-3' – Rv 5'CCAGTCATCACGGTCTGGTTTCTTT 3', the FABP-4 primers were Fw 5' GAAGTGGGAGTGGGCTTTGCCA 3'- Rv 5' CACCAGGGCCCCGCCATCTA 3', the PPAR γ , amplification primers were Fw 5' ACGGGGTCTCGGTTGAGGGG 3'- Rv 5' TCCGCCCAAACCTGATGGCATT 3', the Adiponectin amplification primers were Fw 5'- GACGACACCAAAAGGGCTCAGG -3' – Rv 5'- CATCACGGCCTGGTGTGCCA-3', the C/EBP, amplification primers were Fw 5'- CGCCATGCCGGGAGAACTCTAAC-3'- Rv 5'- TCTTGCGCACCGCGATGTTG-3', the Wnt10b amplification primers were Fw 5' GGTCTCGGCCTCCGCCCTTA 3'- Rv 5' ACGGAAACCGCGCTTGAGGA 3', the β -catenin amplification primers were Fw 5' GGCAGGATACACGGTGCCGC 3' , Rv 5' CTTCAGGTACCCTCAGGCCCGC 3', the Wnt5b amplification primers were Fw 5' CGCCAACGGTGCCGCTAAGT 3'-Rv 5' CTTATACACGGCCCCGGCGGC 3'. Each reaction was performed in triplicate as previously described (9).

Western blot analysis and HO activity

Frozen adipose tissue was pulverized under liquid nitrogen and placed in a homogenization buffer consisting of (mmol/l): 10 phosphate buffer, 250 sucrose, 1 EDTA, 0.1 PMSF and 0.1% v/v tergitol, pH 7.5. Homogenates were centrifuged at 27,000xg for 10 min at 4°C and the supernatant decanted and protein levels determined followed by immunoblotting with primary antibodies against HO-1, Wnt10b, Wnt5b, β -catenin, Peg1/Mest. HO activity was measured following previously described methods (4, 9)

Assessment of Vascular Reactivity

The aorta was removed, cleaned of fat and loose connective tissue, placed in cold Krebs-bicarbonate solution, and sectioned into 3-mm-long rings. Vasorelaxation responses of phenylephrine-constricted arteries to cumulative increments in acetylcholine (10^{-9} to 10^{-4} mol/L) were examined in the presence of indomethacin (10 μ mol/L) as described (7).

Statistical analysis

Data are expressed as means \pm S.E.M. Significance of difference in mean values was determined using one-way analysis of variance followed by the Tukey-Kramer post hoc test. $P < 0.05$ was considered to be significant.

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RESULTS

Viral vector construction and adipocyte-specific expression of the transgene in vitro.

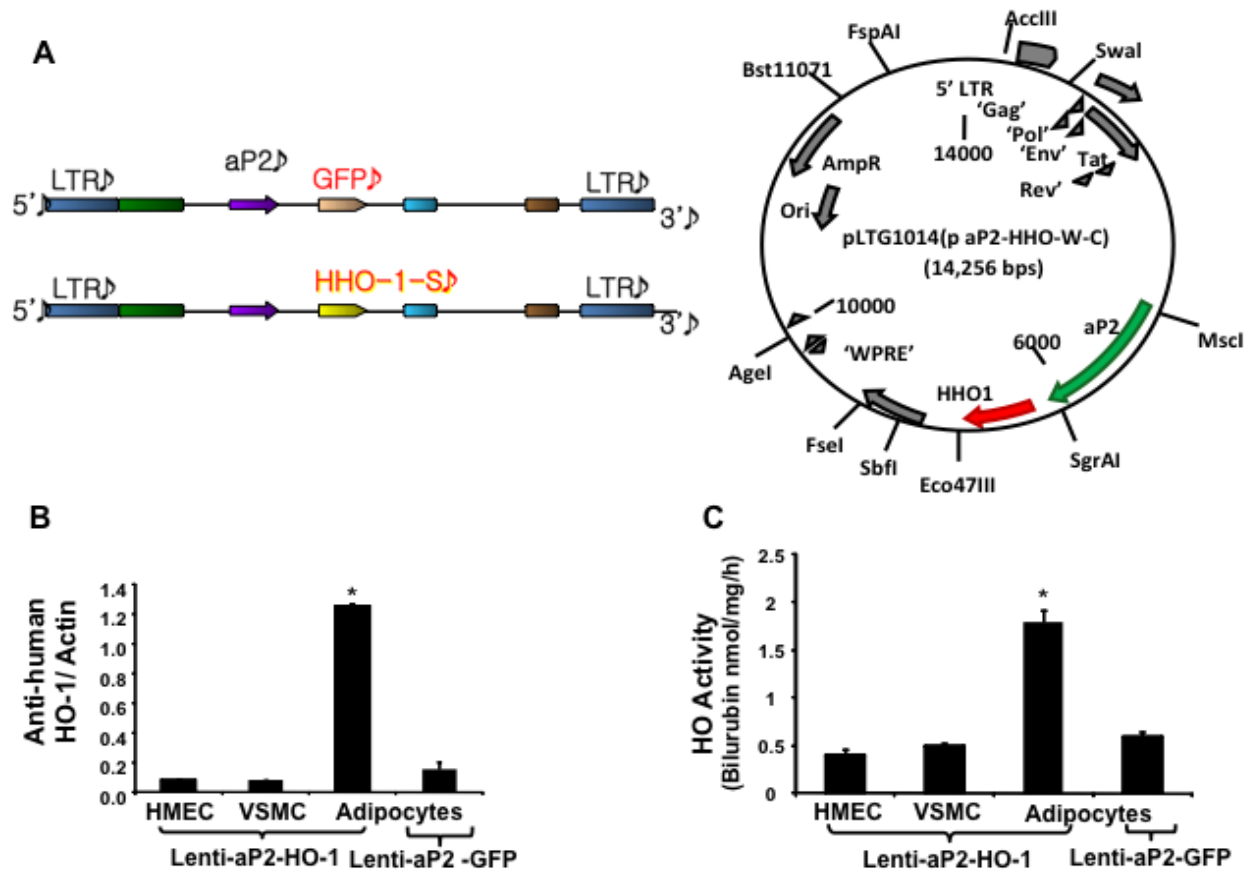


Figure S1. A) Viral construction. **B)** HO-1 protein levels in human microvessel endothelial cells (HMEC), human vascular smooth muscle cells (VSMC) and mouse adipocytes after transduction of lenti-aP2-HO-1 or lenti-aP2-GFP. Data are shown as mean band density normalized relative to β -actin (mean \pm SE; n=4; *p<0.01 vs. lenti-aP2-GFP). **C)** HO activity measured as the conversion of heme to bilirubin in cell lysates prepared from untransduced and cells transduced with the lentivirus constructs lenti-aP2-HO-1 or lenti-aP2-GFP. Results are mean \pm SE, n=3; *p<0.01 vs. lenti-aP2-GFP).

Adipocyte-specific expression of lenti-aP2-HO-1 transduction in vivo

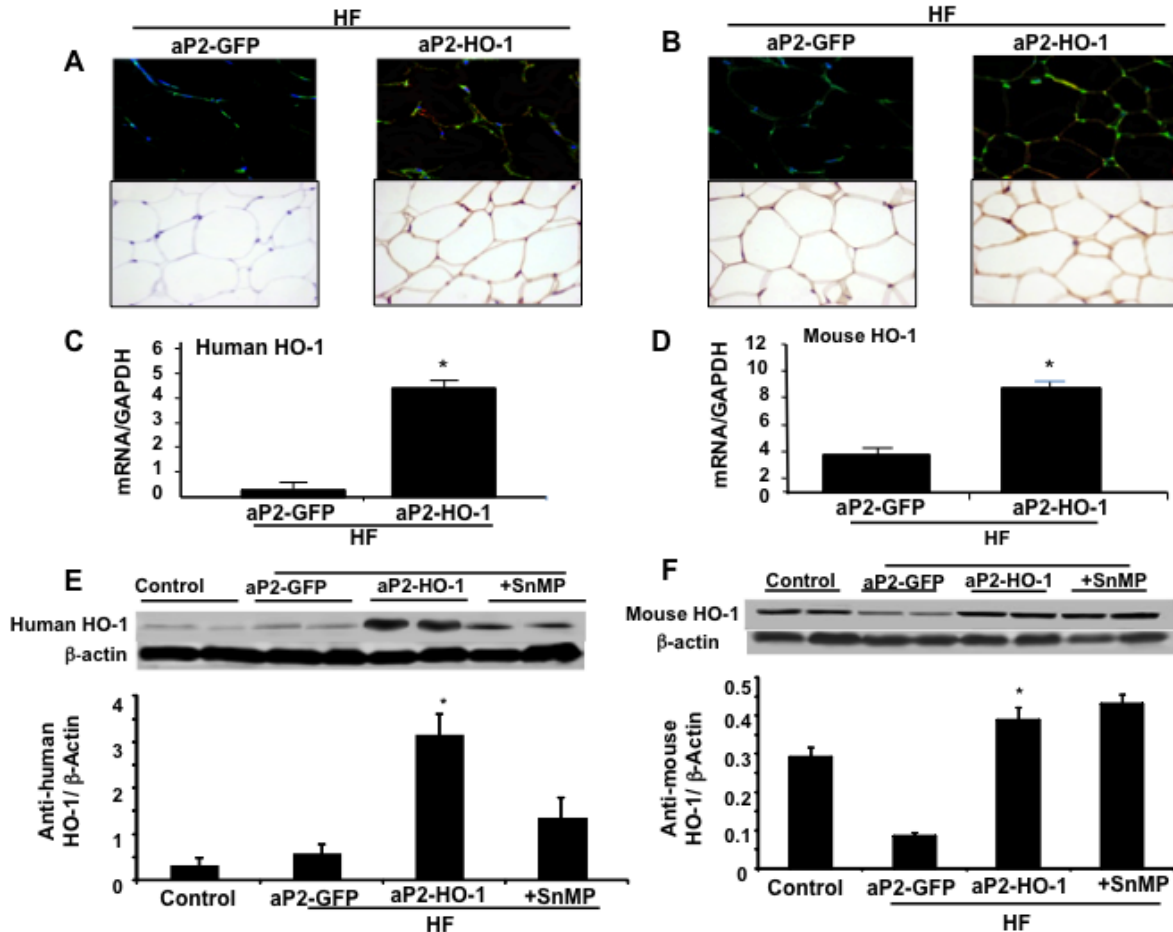


Figure S2: **A)** GFP immunofluorescence and immunostaining with antibodies against the human HO-1 (indicating that the transduction efficiency of lenti-aP2-HO-1 is approximately 80%) and **B)** the mouse HO-1 in adipocyte derived from visceral tissues surrounding aorta in mice fed HF diet and transduced with either the lenti-aP2-GFP or lenti-aP2-HO-1. **C-D)** Quantitative analysis of mRNA levels for human HO-1 and mouse HO-1 by real time PCR in adipocyte derived from visceral tissues surrounding aorta in mice fed HF diet and transduced with either the lenti-aP2-GFP or lenti-aP2-HO-1. Data are expressed as means \pm SE; *P<0.001 compared aP2-GFP. **E-F)** Western blot and densitometry analysis of human HO-1 and mice HO-1 in adipocytes from fat tissues of mice fed a normal diet (control) and mice fed a HF diet and transduced with the control vector lenti-aP2-GFP (aP2-GFP) or lenti-aP2-HO-1 untreated (aP2-HO-1) and treated with SnMP (+SnMP). Immunoblots were performed with antibodies against either human HO-1, which do not cross react with the mouse HO-1, or mice HO-1, which recognize the human HO-1 as well. Data are shown as mean band density normalized relative to β -actin (n=6, * p<0.01 vs. control).

Effect of adipocyte-specific transduction of HO-1 on mRNA expression of adipogenic markers and adipokines in adipocytes from mice fed a high fat diet.

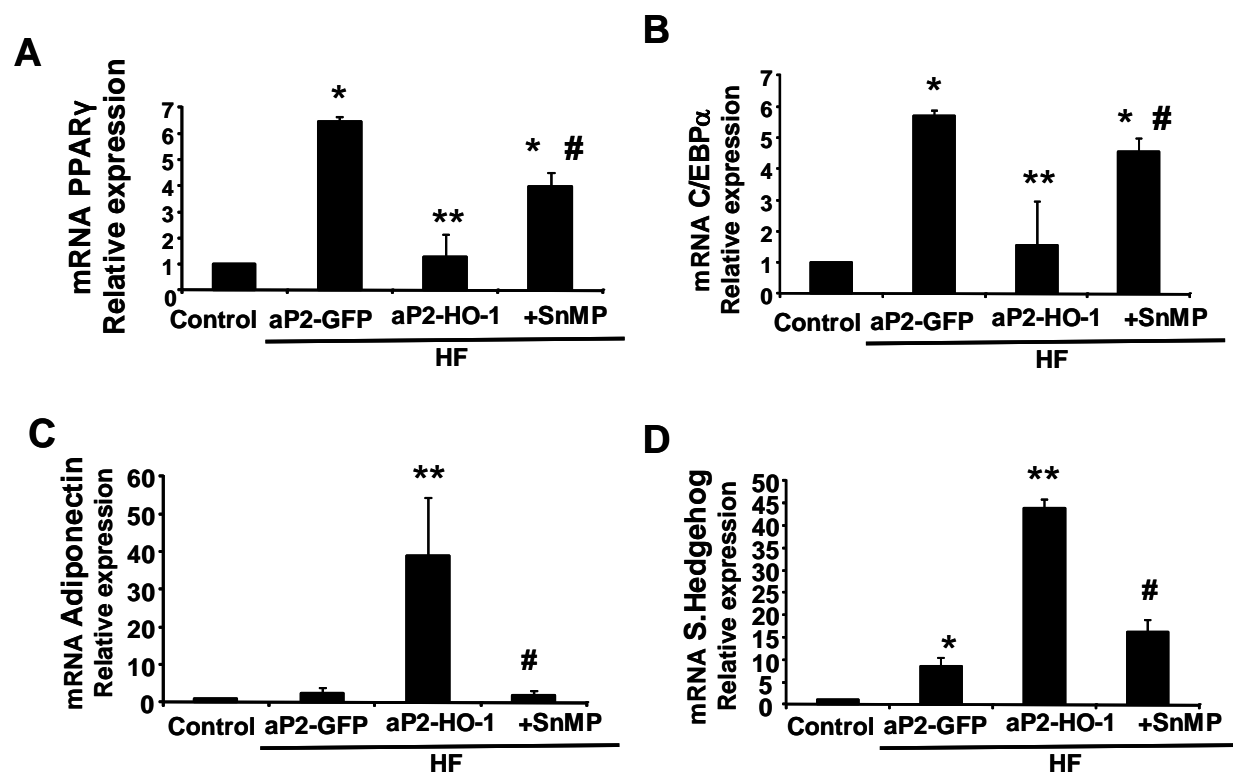


Figure S3: Real time PCR analysis of (A) PPAR γ (B)C/EBP α , (C) adiponectin and (D) Shh proteins in adipocytes from mice fed a normal diet (control) and mice fed a HF diet and transduced with the control vector lenti-aP2-GFP (aP2-GFP) or lenti-aP2-HO-1 untreated (aP2-HO-1) and treated with SnMP (+SnMP). Results are mean \pm SE; n=4; *p<0.05 vs. control; ** p<0.05 vs. HF+aP2-GFP; #p<0.05 vs. HF+aP2-HO-1.

Effect of adipocyte-specific transduction of HO-1 on mRNA expression of aP2 and Mest/peg1 in adipocytes from mice fed a high fat diet.

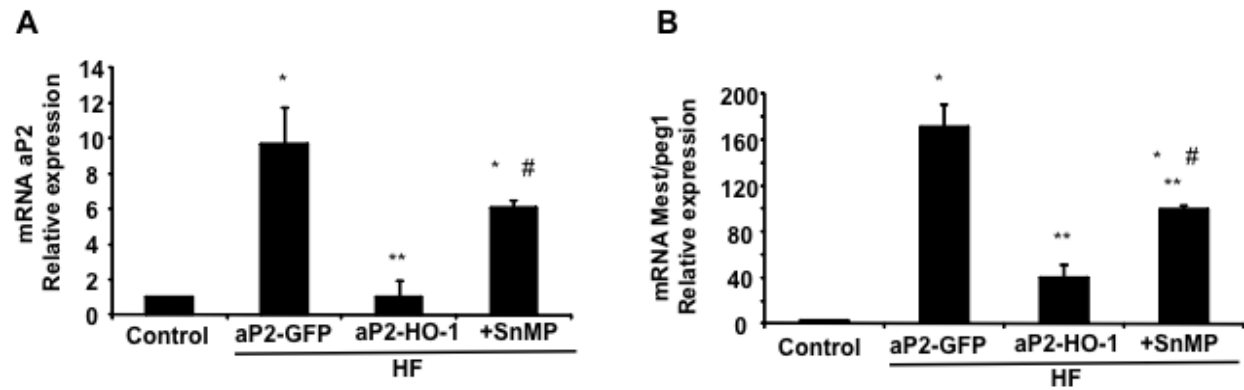


Figure S4: Real time PCR analysis of (A) aP2 and (B) Mest/peg1 proteins in adipocytes from mice fed a normal diet (control) and mice fed a HF diet and transduced with the control vector lenti-aP2-GFP (aP2-GFP) or lenti-aP2-HO-1 untreated (aP2-HO-1) and treated with SnMP (+SnMP). Results are mean \pm SE; n=4; *p<0.05 vs. control; ** p<0.05 vs. HF+aP2-GFP; #p<0.05 vs. HF+aP2-HO-1.

Effect of adipocyte-specific transduction of HO-1 on mRNA expression of Wnt-10b, Wnt-5b and β -catenin in adipocytes from mice fed a high fat diet.

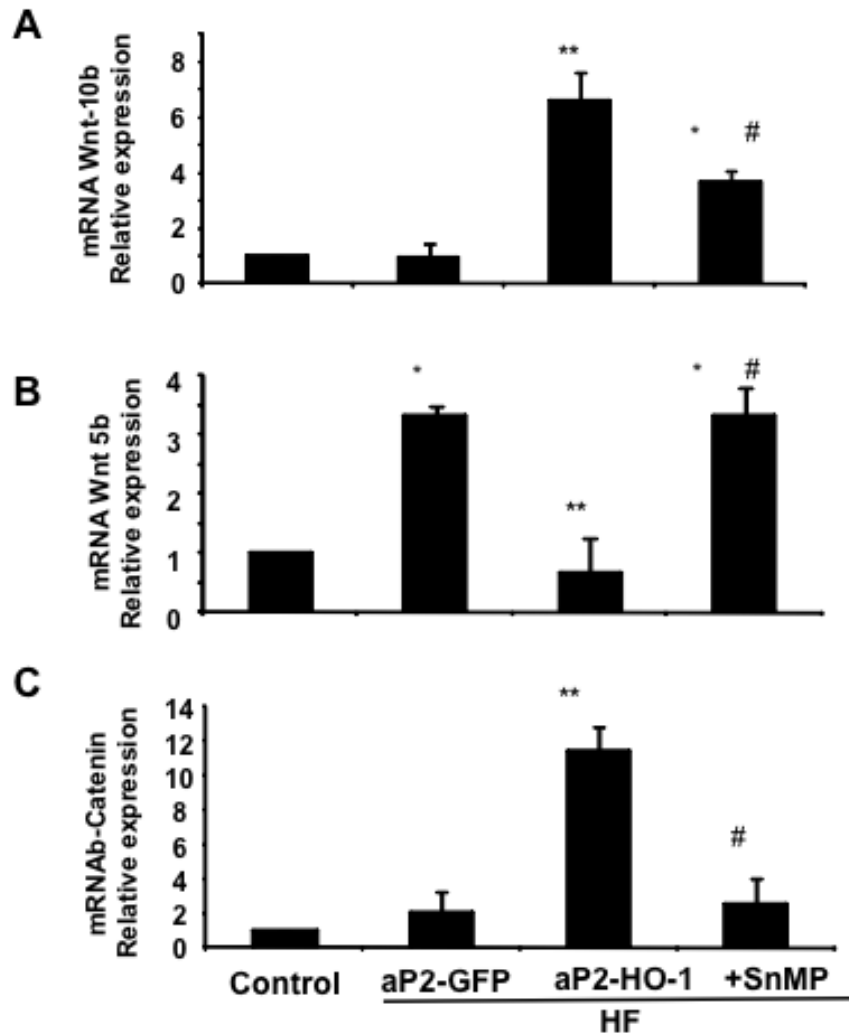


Figure S4: Real time PCR analysis of (A) Wnt10b, (B) Wnt5b and (C) β -catenin proteins in adipocytes from mice fed a normal diet (control) and mice fed a HF diet and transduced with the control vector lenti-aP2-GFP (aP2-GFP) or lenti-aP2-HO-1 untreated (aP2-HO-1) and treated with SnMP (+SnMP). Results are mean \pm SE; n=4; *p<0.05 vs. control; ** p<0.05 vs. HF+aP2-GFP; #p<0.05 vs. HF+aP2-HO-1.