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ORIGINAL ARTICLE

ApoA-1 mimetic restores adiponectin expression and insulin sensitivity independent of changes in body weight in female obese mice

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BACKGROUND: We examined the ability of the apolipoprotein AI mimetic peptide L-4F to improve the metabolic state of female and male *ob* mice and the mechanisms involved.
METHODS: Female and male lean and obese (*ob*) mice were administered L-4F or vehicle for 6 weeks. Body weight was measured weekly. Fat distribution, serum cytokines and markers of cardiovascular dysfunction were determined at the end of treatment.
RESULTS: L-4F significantly decreased serum interleukin (IL)-6, tumor necrosis factor-α and IL-1β. L-4F improved vascular function, and increased serum adiponectin levels and insulin sensitivity compared with untreated mice. In addition, L-4F treatment increased heme oxygenase (HO)-1, pAKT and pAMPK levels in kidneys of *ob* animals. pAKT and pAMPK levels were significantly reduced in the presence of an HO inhibitor. Interestingly, L4F did not alter body weight in female mice, but caused a significant reduction in males.
CONCLUSIONS: L-4F treatments reduced cardiovascular risk factors and improved insulin sensitivity in female *ob* mice independent of body fat changes. Reduced inflammatory cytokine levels accompanied by increased HO activity, serum adiponectin and improved insulin sensitivity suggest that L-4F may promote the conversion of visceral fat to a healthier phenotype. Therefore, L-4F appears to be a promising therapeutic strategy for treating both cardiovascular risk factors and insulin resistance in obese patients of either gender.

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Keywords: apolipoprotein mimetic peptide; insulin resistance; obesity; cardiovascular disease; female obesity; heme oxygenase

INTRODUCTION

Obesity affects over 72 million adults in the United States with a disproportionate prevalence in women.[1,2] Moderate to severe obesity is associated with increased risk for cardiovascular complications and insulin resistance in humans[3,4] and animals.[5] In particular, intra-abdominal fat correlates with insulin resistance and is an important determinant of cardiovascular risk.[6] Although men have received much attention for exhibiting such 'android' obesity, excess visceral fat deposition is present in 41% of women in Western countries (www.dh.gov.uk).

Visceral obesity creates a state of low-grade inflammation characterized by increased pro-inflammatory cytokine levels[7–9] that contributes to insulin resistance[10] and oxidative stress that impairs vascular and cardiac function.[5] Indeed, mice lacking tumor necrosis factor (TNF)-α have significantly improved insulin sensitivity and glucose homeostasis in response to diet-induced obesity.[11–13] In addition, *ob* mice overexpressing adiponectin have reduced inflammation and improved insulin sensitivity, accompanied by an increase in adipocyte number and smaller adipocyte size.[14] These data suggest that promoting the expansion of adipocyte number may prevent or stave off the negative inflammatory effects of larger adipocytes. The increase in inflammatory cytokine production observed with obesity may result from an increase in reactive oxygen species.[5,15] It has been reported that reactive oxygen species produced by adipocytes increases the expression of MCP-1, a chemoattractant for macrophages, a key producer of such cytokines.[5] This increase in reactive oxygen species contributes to progressive deterioration in vascular function.[5]

Abnormal high-density lipoprotein cholesterol (HDL-c) metabolism may also contribute to the increased cardiovascular disease risk caused by diabetes and obesity. The HDL-c particle distribution is abnormal in both types 1 and 2 diabetes, with decreases in the relative fraction of the large HDL-c particles believed to be cardioprotective.[16] Apolipoprotein A-I (apoA-1) is the major protein component of HDL-c in plasma. The level of apoA-I is more closely correlated with the reduced risk of atherosclerosis than any other marker of HDL-c.[17] ApoA-I prevents the oxidation and aggregation of pro-atherogenic low-density lipoprotein cholesterol (LDL-c) particles within the arterial wall and stimulates the mobilization of cholesterol from the same source.[18] Infusion of apoA-I halts the progression of atherosclerosis and promotes its regression, reverses endothelial dysfunction, and induces lipid and macrophage efflux from established lesions in animals.[19–21]

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Much effort has gone into designing peptide analogs of apoA-I that can promote the formation of HDL-c-like particles with improved potency or efficacy *in vivo*. L-4F, an apoA-1 mimetic peptide, improves insulin resistance in male *ob* mice, reduces inflammatory cytokines, reestablishes nitric oxide (NO)/superoxide ratios and reduces consequent cardiovascular risk.[22,23] Its enantiomer, D-4F, reduces atherosclerotic lesions,[24] causes HDL-c to become anti-inflammatory, stimulates HDL-c-mediated cholesterol efflux and increases reverse cholesterol transport from macrophages.[5,24] In a phase 1 human trial, a single oral dose of D-4F improved HDL-c anti-inflammatory function.[25] D-4F has also been shown to reduce the size of white adipose tissue stores (but not body weight) in male mice fed with a high-fat diet,[26] and to decrease visceral fat in male *ob* mice.[22,23] L-4F is currently in clinical trials and has been hailed as being especially promising as a potential treatment for obesity and the metabolic syndrome.[27] However, the actions of L-4F in female *ob* mice in addition to the efficacy of L-4F in improving markers of vascular function in obese mice remain to be examined.

Heme oxygenase 1 (HO-1) induction is known to suppress ROS production and promote increases in insulin sensitivity.[5] In this study, we evaluated the mechanism by which L-4F affected HO-1 and subsequently, visceral and subcutaneous fat distribution in female and male *ob* mice. We then examined whether reduced visceral obesity is essential for the improved metabolic profile seen in L-4F-treated animals. Male and female *ob* mice treated with L-4F had significant improvements in insulin sensitivity and reduced inflammatory cytokine levels. However, we show for the first time that administration of L-4F in *ob* female mice decreases inflammatory cytokines and improves insulin responsiveness independent of changes in body weight. Furthermore, L-4F was effective in restoring HO-1 dependent increases in NO levels in female *ob* mice, indicating improved vascular function, and restored superoxide production and systolic blood pressure in male *ob* mice. Last, L-4F treatment resulted in a marked increase in heme oxygenase (HO) activity in both male and female *ob* mice. Therefore, L-4F has the potential to have a crucial therapeutic role in improving metabolic syndrome parameters and indices of vascular function-associated poor cardiovascular health.

MATERIALS AND METHODS

Animal care and L-4F administration

Male and female *ob* mice (B6v-Lep *ob*/J) were purchased from Harlan (Chicago, IL, USA) at the age of 6 weeks. Lean mice (age-matched B6.V, lean, Harlan) were used as control. Sex-matched lean and *ob* mice were fed a normal laboratory animal diet (Research diets, New Brunswick, NJ, USA) and had free access to water. Body weight of *ob* and lean mice were 34 ± 5 g and 26 ± 3 g, respectively, and glucose levels were 229 ± 21 mg dl^{-1} and 154 ± 9 mg dl^{-1}, respectively, at the start of the experiments.

At 7 weeks of age, L-4F (2 mg kg^{-1} per day) or vehicle (ABCT: ammonium bicarbonate buffer at pH 7.4 containing 0.01% Tween-20), was administered intraperitoneally daily for 6 weeks to male and female *ob* and lean control mice. There were 10 groups of animals: (A) male lean, (B) male lean-L-4F, (C) male *ob*, (D) male *ob*-L-4F, (E) male *ob*-L-4F + SnMP (stannous mesoprophryin was given intraperitoneally 2 mg per 100 g body weight three times per week), (F) female lean, (G) female lean-L-4F, (H) female *ob*, (I) female *ob*-L-4F and (J) female *ob*-L-4F + SnMP. Systolic blood pressure was determined weekly by the tail-cuff method as previously described.[28] The Animal Care and Use Committee of New York Medical College approved all experiments.

Effect on body weight, appearance and fat content

At the time of killing, the body weight of all mice was measured. The subcutaneous and visceral fat visible in the abdomen, mesenteric fat, fat around the liver, kidney, spleen, heart, ovaries and testes were dissected free, pooled for each mouse and weighed. Subcutaneous and visceral fat were weighed separately.

Glucose levels and insulin tolerance tests

Mouse blood glucose was determined by testing 5 μl of tail-vein blood using an Accu-Chek (Roche, Indianapolis, IN, USA) active blood glucose-monitoring system. After a 12-h fast, mice were injected intraperitoneally with insulin (2.0 units kg^{-1}). Blood samples were taken at 0 (basal), 30, 60, 75 and 90 min, and used to measure glucose levels.

LDL/VLDL and cytokine measurements

Serum LDL/VLDL (very low-density lipoprotein) cholesterol levels were measured in serum collected at the time of killing (24-h post previous injection) using LDL/VLDL Quantification Kits (Biovision, Mountainview, CA, USA). Adiponectin (high molecular weight), TNF-α, interleukin (IL)-1β and IL-6 were determined in mouse serum using an enzyme-linked immunosorbent assay assay (Pierce Biotechnology, Woburn, MA, USA). The assays were performed according to the manufacturer's guidelines.

Measurement of cardiac superoxide (O_2^-) levels

The hearts were placed in plastic scintillation minivials, containing 5 μmol l^{-1} lucigenin for the detection of O_2^-, in a final volume of 1 ml of air-equilibrated Krebs solution buffered with 10 mmol M^{-1} HEPES–NaOH (pH 7.4) as previously described.[29] Lucigenin chemiluminescence was measured in a liquid scintillation counter (LS6000IC, Beckman Instruments, San Diego, CA, USA).

Determination of HO activity and NO levels

Aortic HO activity was assayed as described previously.[30] Bilirubin, the end product of heme degradation, was extracted from chloroform, and its concentration was determined using the difference in absorbance between λ460 and λ530 nm with an absorption coefficient of 40 $mM^{-1}cm^{-1}$ (dual UV/VIS beam spectrophotometer lambda 25; PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA). Under these conditions, HO activity was linear with protein concentration, time-dependent and substrate-dependent.[31]

For NO levels in whole kidney and aorta homogenate samples, 0.3 mg of protein were evaluated by measuring total nitrite and nitrate content in culture medium using the NO quantification kit and following the manufacturer's instructions (Active Motif, Carlsbad, CA, USA).

Western blot analysis of the kidney and aorta for HO-1, pAMPK and pAkt expression

At the time of killing, aorta and kidney were harvested, drained of blood and flash frozen in liquid nitrogen. Frozen aorta and kidney segments were pulverized and placed in a homogenization buffer as previously described.[32] Homogenates (20–50 μg of protein) were examined by protein immunoblot. HO-1, HO-2,[22] AMPK, pAMK, AKT and pAkt (Cell Signaling, Danvers, MA, USA) levels were determined as previously described.[22]

Statistical analyses

Statistical significance 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 analysis of variance for multiple groups or unpaired *t*-test for two groups. Data are presented as mean ± s.e.

RESULTS

Effects of L-4F on male and female body fat

L-4F treatment daily for 6 weeks prevented weight gain in *ob* male mice in accordance with previous findings (Figure 1a). Unexpectedly, *ob* female mice that received L-4F continued to gain weight at a rate similar to untreated *ob* mice (Figure 1a). Food intake in the male and female *ob* control and treated groups were comparable (*ob* male = 3.66 ± 0.35 g per day; *ob* male + L-4F = 3.6 ± 0.2 g per day; *ob* female = 3.5 ± 0.26 g per day; and *ob* female + L-4F = 3.55 ± 0.1 g per day). At the end of the 6-week treatment period, L-4F slightly decreased the ratio of

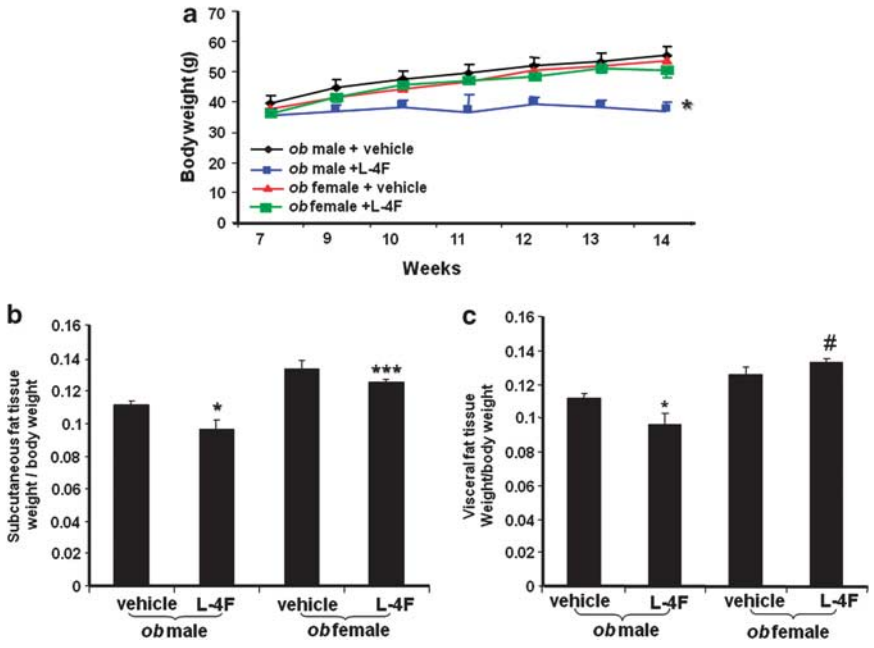

Figure 1. Effect of L-4F on body composition in *ob* mice after 6 weeks of L-4F treatment (2 mg kg^{-1} per day) or control (**a**) Effect of L-4F treatment on body weight. *ob* male and female mice, respectively, were treated and weight determined (average of two independent experiments), $n = 10$ for untreated and treated *ob* groups. Results are means ± s.e. Levels of significance: $^{*}P < 0.05$ vs *ob* male + vehicle. (**b**) Effect of L-4F on subcutaneous fat to total body weight ratio. Levels of significance: $^{*}P < 0.01$ vs *ob* male and $^{***}P < 0.05$ vs *ob* female. (**c**) Effect of L-4F on visceral fat to total body weight ratio. Levels of significance: $^{*}P < 0.05$ vs *ob* male and $^{\#}P < 0.05$ vs *ob* female.

subcutaneous fat to whole body weight in both males and females (Figure 1b). However, the ratio of visceral fat to whole body weight was decreased only in L-4F-treated males (Figure 1c). Female mice treated with L-4F showed a small but significant increase in the visceral fat to body weight ratio compared with controls (Figure 1c). The changes we measured in the fat to body weight ratio are consistent with the changes that were measured in body weight, which suggests that L-4F treatment may indeed cause sexually dimorphic responses.

Effects of L-4F on glucose levels and insulin tolerance tests

Chronic L-4F treatment reduced high fasting plasma glucose levels in *ob* males and females (Figure 2a). In addition, insulin administration to the L-4F-treated *ob* female mice resulted in a significant suppression of blood glucose levels but not in *ob* females receiving vehicle alone (Figure 2b). Last, the area under the curve determined for the data presented in Figure 2b was higher in vehicle compared with L-4F-treated *ob* females (Figure 2c). These results suggest that L-4F restores insulin sensitivity in female *ob* mice independent of changes in body weight (Figure 1a).

Effects of L-4F on adiponectin and cytokine levels

It has been shown that overexpression of adiponectin in the *ob* mouse model leads to expansion of adipose tissue.[33] We have previously shown that L-4F increases adiponectin levels in male mice.[22] We therefore examined whether male and female mice show a difference in adiponectin responses to L-4F. L-4F produced a significant increase in plasma adiponectin levels in both *ob* female and male animals (Figure 3a). However, adiponectin levels in *ob* female mice were increased compared with *ob* male mice treated with L-4F, suggesting that adipose tissue maintenance and expansion in *ob* female mice may result from increased adiponectin levels.[14]

We also examined circulating levels of cytokines that are typically associated with obesity-mediated insulin resistance and vascular dysfunction. Female *ob* animals initially exhibited increased plasma IL-6 levels when compared with age-matched *ob* male mice (Figure 3b). L-4F produced a decrease in plasma IL-6 levels in both *ob* female and male mice when compared with untreated controls. Similar results were observed for plasma TNF-α and IL-1β levels (Figures 3c and d). Thus, although visceral fat is not lost in the female *ob* mice treated with L-4F, the inflammatory cytokine state is markedly improved, suggesting that the adipose tissue is healthier.[14]

Effects of L-4F on blood pressure and vascular function

In addition to metabolic defects, *ob* mice have altered cardiac function, which is a common consequence of obesity. Figure 4a shows that *ob* male mice are hypertensive compared with lean controls. L-4F treatment was highly successful in restoring systolic blood pressure to levels matching lean controls (Figure 4a). Additionally, *ob* male mice had significantly elevated cardiac superoxide levels, an indicator of oxidative stress (Figure 4b). Following L-4F treatment, superoxide production was normalized (Figure 4b). Aortic NO contributes to endothelial function and protects against hypertension. NO release from the aorta of *ob* female mice was reduced compared with lean female mice (Figure 4c). This decrease in NO was ameliorated by L-4F treatment (Figure 4c). These results indicate that L-4F may have a role in vascular protection in obese mice.

Mice homozygous for the obese spontaneous mutation (*ob* mice) have elevated circulating free fatty acids, raised triglycerides, an increase in both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol and marked hepatic steatosis.[34–36] In these mice, L-4F was effective in reducing the LDL/VLDL cholesterol ratio in both *ob* males and females (Figure 4e).

Effects of L-4F on HO-1 expression and HO activity

Obesity has been linked with increases in adipocyte oxidative stress, while HO-1 is strongly induced by oxidant stress and helps

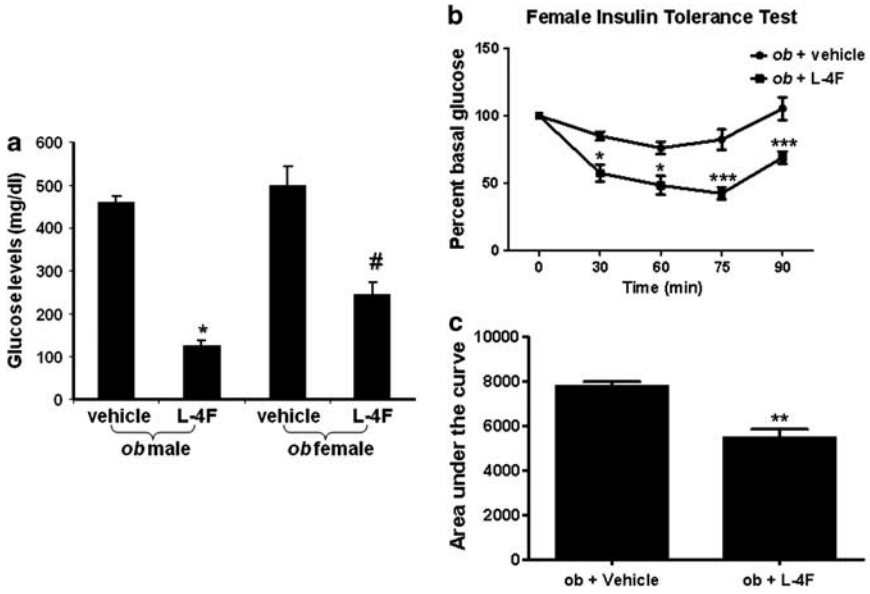

Figure 2. (**a**–**c**) Fasting glucose and insulin tolerance test. (**a**) Effect of L-4F treatment on glucose levels in *ob* male and female mice. The results are expressed as means ± s.e.m., $n = 4$. Levels of significance: $^{*}P < 0.01$ vs *ob* male and $^{\#}P < 0.05$ vs *ob* female. (**b**) Intraperitoneal insulin tolerance test (IPGTT). The results are expressed as means ± s.e.m., $n = 4$. Levels of significance: $^{*}P < 0.01$ and $^{***}P < 0.001$ vs *ob* female control. (**c**) Area under the curve for IPGTT. The results are expressed as means ± s.e.m., $n = 4$. Levels of significance: $^{**}P < 0.005$ vs *ob* female control.

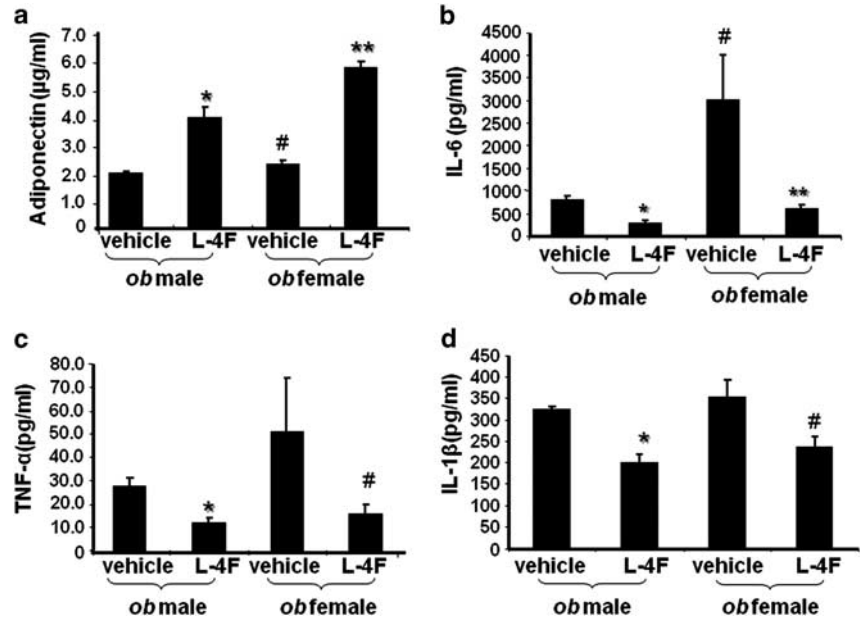

Figure 3. (**a**–**d**) Effect of L-4F on serum cytokines. L-4F was administered daily for 6 weeks and serum samples were obtained immediately before killing. (**a**) Effect of L-4F on serum adiponectin levels in *ob* male and female mice. The results are expressed as means ± s.e., $n = 8$–10. Levels of significance: $^{*}P < 0.001$ vs *ob* male, $^{\#}P < 0.05$ vs *ob* male and $^{**}P < 0.05$ vs *ob* female. (**b**) Effect of L-4F on serum IL-6 levels in *ob* male and female mice. Levels of significance: $^{*}P < 0.01$ vs *ob* male, $^{\#}P < 0.05$ vs *ob* male and $^{**}P < 0.05$ vs *ob* female. (**c**) Effect of L-4F on serum TNF-α levels in *ob* mice. Levels of significance: $^{*}P < 0.01$ vs *ob* male and $^{\#}P < 0.05$ vs *ob* female. (**d**) Effect of L-4F on IL-1β serum levels in *ob* female mice. Levels of significance: $^{*}P < 0.01$ vs *ob* male and $^{\#}P < 0.05$ vs *ob* female.

protect against oxidative insult in cardiovascular disease.[5] Given that we have previously shown that administration of an HO-1 inducer increases serum adiponectin levels,[29] we wanted to examine the possibility that L-4F increases adiponectin levels by raising HO-1 levels. Thus, we examined HO-1 levels in the kidneys and aortas of *ob* and lean mice. As in males, HO-1 in both the aortas (data not shown) and kidneys of *ob* females differed significantly from lean animals (Figures 5a and b). In addition, HO-1 levels were significantly lower in *ob* females compared with *ob* males (Figure 5b). We next compared the effect of L-4F on male and female HO-1 protein expression in renal and vascular tissue samples. L-4F increased HO-1 expression in both *ob* male and female animals (Figure 5c). In contrast to the effects on HO-1, HO-2 levels in renal and vascular tissue samples were unaffected

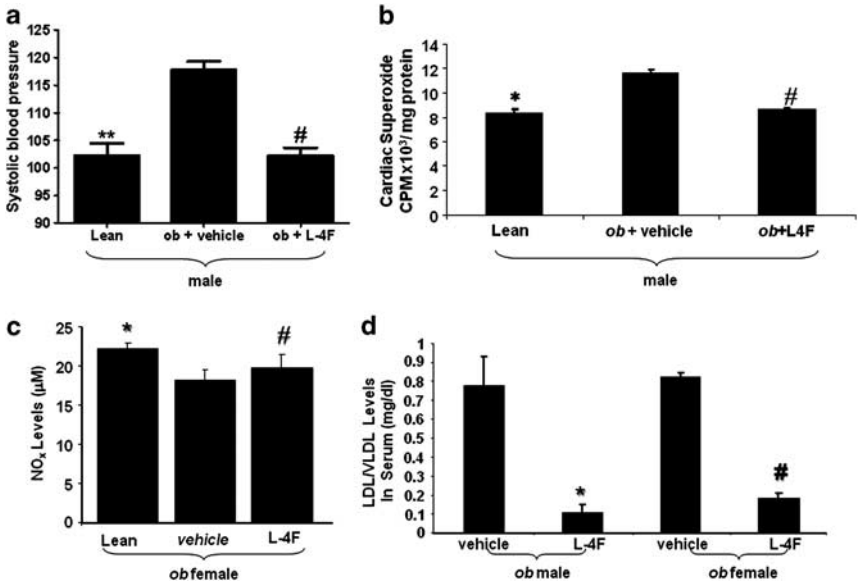

Figure 4. Effect of L-4F on blood pressure and vascular function. (**a**) Effect of L-4F on systolic blood pressure. Levels of significance: $^{**}P<0.01$ vs *ob* vehicle and $^{\#}P<0.05$ vs *ob* vehicle. (**b**) Cardiac superoxide production. Levels of significance: $^{*}P<0.05$ vs *ob* vehicle and $^{\#}P<0.05$ vs *ob* vehicle. (**c**) Effects of L-4F on NO levels. Levels of significance: $^{*}P<0.05$ vs *ob* female and $^{\#}P<0.05$ vs *ob* female. (**d**) Effect of L-4F treatment on LDL/VLDL levels. Levels of significance: $^{*}P<0.01$ vs *ob* male and $^{\#}P<0.01$ vs *ob* female.

by L-4F treatment in both male and female lean and *ob* animals (data not shown). L-4F increased HO activity in the aortic tissue samples of both *ob* male and female mice (Figure 5d). The increase in HO activity was inhibited by SnMP, an inhibitor of HO activity (Figure 5d). These results indicate that L-4F is an inducer of HO activity. Thus, the improvements in inflammatory cytokines and indices of cardiovascular function following L-4F treatment are likely mediated by L-4F-induced increases in HO activity.

To examine the contribution of vascular and renal AKT and AMPK pathways to the increase in HO-1 expression, we assessed vascular and renal expression of phosphorylated AMPK and AKT in L-4F-treated *ob* males and *ob* females. As seen in Figure 6, L-4F significantly increased basal renal AKT and AMPK phosphorylation in both *ob* males and females. Similar results were obtained with aortic tissue (data not shown). The addition of SnMP, a potent inhibitor of HO activity, to L-4F-treated animals reversed this effect in both *ob* males and females (Figures 6a–d). These results suggest that L-4F stimulation of AMPK and AKT pathways is involved in its ability to increase HO-1 expression and increase HO activity.

DISCUSSION

The metabolic syndrome is typically characterized by chronic, low-grade inflammation, excessive visceral fat, insulin resistance, abnormal cholesterol levels and increased blood pressure. The present study clearly shows for the first time that administration of the apoA-I mimetic L-4F ameliorates effects associated with the metabolic syndrome in female *ob* mice. Intriguingly, the positive effects of L-4F treatment on metabolic and vascular function in *ob* female mice were independent of changes in body weight. In fact, the visceral fat to body weight ratio increased in female *ob* mice treated with L-4F. We report that administration of L-4F to both *ob* female and male mice restored insulin sensitivity, increased serum adiponectin, decreased the LDL/VLDL cholesterol ratio and decreased serum pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β). Moreover, in *ob* male mice, L-4F improved systolic blood pressure and reduced cardiac superoxide production. In female obese mice, L4F caused an increase in HO-1-mediated NO levels. These novel findings suggest that L-4F has the potential to be a key therapeutic agent in the treatment of both vascular diseases and insulin resistance in obese patients.

We have previously shown that L-4F administration reduces fat mass in male *ob* mice.[22,23] Recent studies suggest that this effect is mediated by enhanced energy expenditure resulting from upregulation of UCP1 expression in brown fat.[26] In contrast, females treated with L-4F did not exhibit a decrease in visceral body fat over the time course measured (Figure 1c). These results are in line with the sexual dimorphism frequently observed in males and females in response to energetic challenges. Instead of primarily modulating food intake, females defend and build adipose stores by maintaining low rates of energy expenditure.[37] Indeed, when overfed with a palatable high-fat diet, female rats gain more body weight than males because of a greater conservation of energy expenditure with lower activation of thermogenesis in brown adipose tissue.[38,39]

Several factors may contribute to the observed differences in effect on body fat stores. Kim *et al.*[14] demonstrated that overexpression of adiponectin in the *ob* mouse leads to increased adipocyte number. Importantly, the adipose expansion in *ob* mice overexpressing adiponectin is associated with increases in insulin sensitivity.[14] More recently, overexpression of adiponectin was shown to promote a more metabolically flexible adipose tissue and prevented hepatic lipid accumulation in mice fed with a high-fat diet.[33] The roughly 1.5-fold elevation of adiponectin seen in L-4F-treated female *ob* mice above that seen in males could therefore promote adipose tissue expansion in *ob* female mice. Adipose tissue expansion in the L-4F-treated *ob* females is currently being investigated and could explain the increased visceral fat to body weight ratio despite increased insulin sensitivity and reduced serum TNF-α, IL-6 and IL-1β in the present study. This would suggest that L-4F treatment converts the visceral fat in female *ob* mice to a healthier phenotype, which may prevent ectopic lipid accumulation in the liver and skeletal muscle. In addition, gender differences in HO-1 and sex hormone levels may favor female adiposity, as estradiol and HO-1 induction lead to an increase in proliferation of pre-adipocytes.[40,41] It is possible that female mice require a lengthier L-4F treatment period before decreases in weight gain are observed. Nevertheless, the retention of fat mass in the female animals did not impair the ability of L-4F

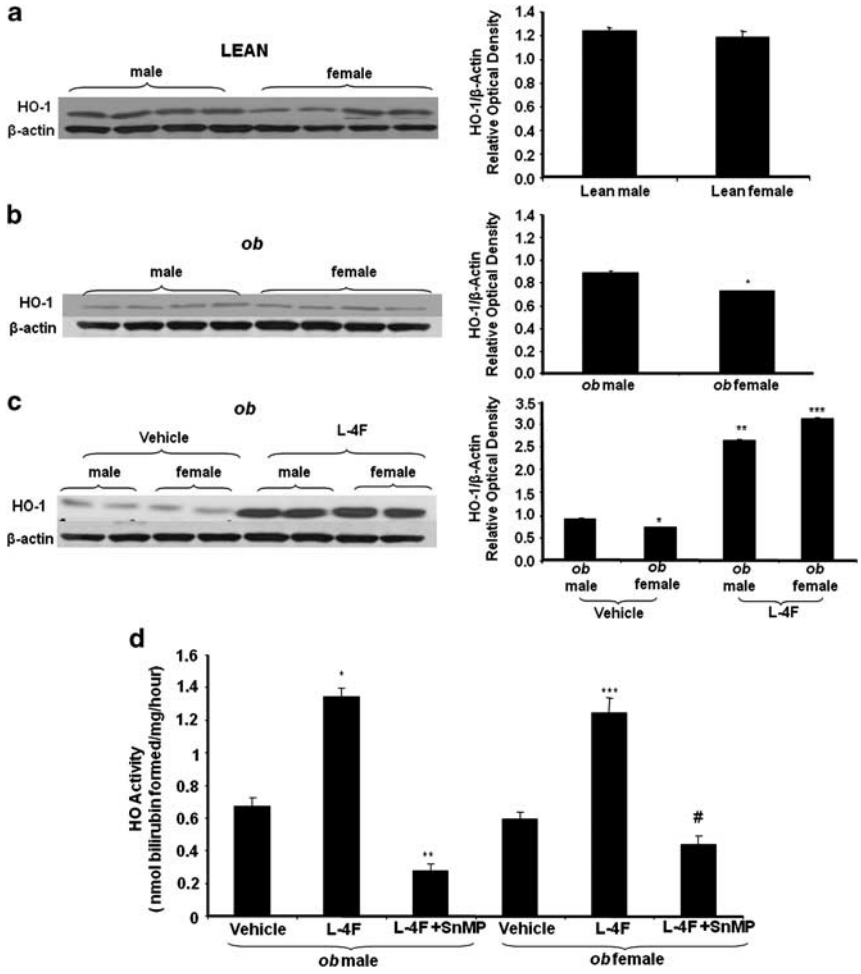

Figure 5. (**a**–**d**) Effects of L-4F on levels of HO-1 and HO activity. Kidney samples were subjected to western blotting for the determination of HO-1 protein expression and densitometry analysis of HO-1/actin ratio. (**a**, **b**) Expression of HO-1 in the kidney of lean and *ob* male and female mice. Results are means ± s.e., $n = 4$. Levels of significance: $^{*}P < 0.05$ vs *ob* male. (**c**) Effect of L-4F on HO-1 expression in the kidneys of *ob* female and male mice. Results are means ± s.e., $n = 4$. Levels of significance: $^{*}P < 0.05$ vs *ob* male, $^{**}P < 0.01$ vs *ob* male and $^{***}P < 0.01$ vs *ob* female. (**d**) Effect of L-4F and SnMP on HO activity in the aortas of *ob* male and female mice. Results are means ± s.e., $n = 4$. Levels of significance: $^{*}P < 0.001$ vs *ob* male, $^{**}P < 0.001$ vs *ob* male + L-4F, $^{***}P < 0.001$ vs *ob* female and $^{\#}P < 0.01$ vs *ob* female + L-4F.

to ameliorate the inflammatory cytokine and insulin resistant state of obese females.

HDL-c and apoA-1 have potent anti-inflammatory properties.[42,43] During atherogenesis, cholesterol accumulation in macrophage foam cells induces inflammatory responses, apoptosis and other adverse effects.[44] One of the major functions of HDL-c is to transport cholesterol from these foam cells to the liver for elimination in the bile.[45] Cells are able to export excess cholesterol to apoA-1 by virtue of specialized cell membrane transporters, such as ABCA1, that belong to a superfamily of ATP-binding cassette transporters (ABCs).[46] Mice lacking ABCA1 in all tissues or specifically in macrophages have a heightened response to treatment with the inflammatory stimulus lipopolysaccaride,[47] including increased inflammatory cytokines in the circulation, implying that macrophage ABCA1 has an anti-inflammatory function. 4F peptides can mimic apoA-I in removing cholesterol and phospholipids by the ABCA1 pathway.[48–51] We are currently perusing more in-depth studies to uncover the direct role of the ABCA1 pathway in the effects of apoA-1 mimetic treatment. In addition to this mechanism, L-4F may reduce inflammation by inhibiting cellular expression of VCAM-1 and ICAM-1 on coronary endothelial cells, reducing CD11b expression on circulating monocytes and/or reducing CD11b-dependent adhesion of leukocytes to fibrinogen.[52]

HO-1 is a stress response protein whose induction is associated with protection against oxidative stress with a concomitant increase in adiponectin. The current studies indicate that L-4F treatment decreases oxidative stress in female mice as previously seen in males.[5,22,23,32] Indeed, HO-1 was dramatically induced in male mice to levels nearly twofold above those seen in lean animals, and female increases were even larger. These effects may underlie many of the beneficial actions of L-4F. For example, treatment of obese mice with an NADPH oxidase inhibitor reduced reactive oxygen species production in adipose tissue, attenuated the dysregulation of adipocytokines, and improved diabetes, hyperlipidemia and hepatic steatosis.[53] It has been shown that induction of HO-1 serves as an intrinsic protective factor against atherosclerotic lesion formation, possibly inhibiting lipid peroxidation and influencing the NO pathway.[5] Indeed, *ob* female mice exhibited decreased NO levels and elevated LDL/VLDL cholesterol ratio compared with lean female mice. Additionally, *ob* male mice had elevated superoxide production. These findings are in line with previous data inducting that oxidative stress decreases the messenger RNA and protein expression of ABCA1 and cholesterol efflux.[54,55] L-4F administration, however, improved NO levels while decreasing the LDL/VLDL cholesterol ratio in *ob* female mice, and reduced superoxide production in *ob* male mice. Increases in HO-1 expression and HO

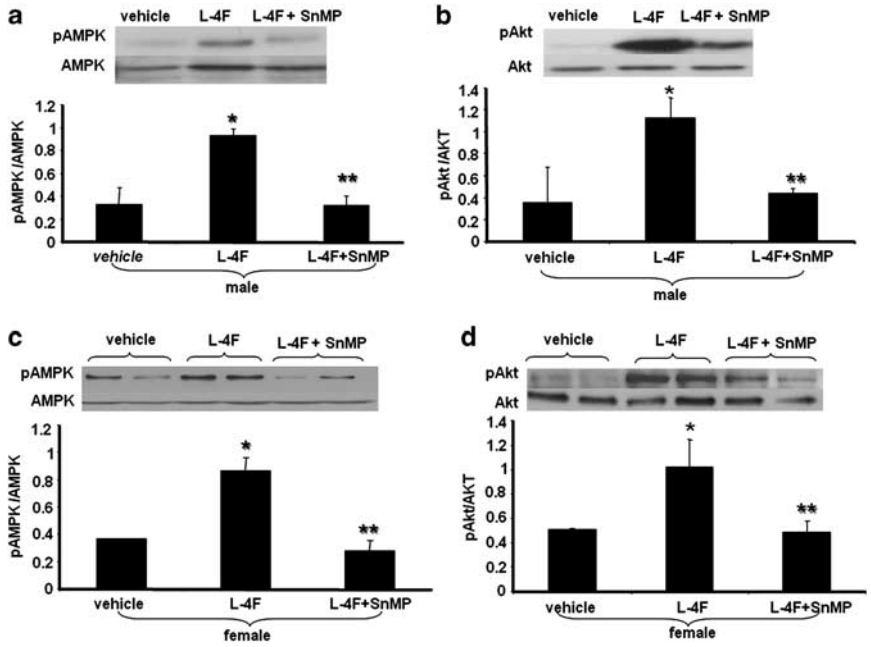

Figure 6. (**a**–**d**) Effect of L-4F and SnMP inhibition of HO activity on pAMPK, pAKT and α-actin in the kidneys of control and L-4F-treated *ob* males and females. All data are represented as phosphorylated/total. (**a**) Western blot and densitometry analysis of renal pAMPK protein in *ob* males. Results are means ± s.e., $n = 4$. *$P < 0.01$ vs *ob* male and **$P < 0.001$ vs *ob* male + L-4F. (**b**) Representative western blot and densitometry analysis of pAKT protein in *ob* male mice. Results are means ± s.e., $n = 4$. *$P < 0.05$ vs *ob* male and **$P < 0.05$ vs *ob* male + L-4F. (**c**) Representative western blot and densitometry analysis of pAMPK protein in *ob* females. Results are means ± s.e., $n = 4$. *$P <$ 0.05 vs *ob* female and **$P < 0.05$ vs *ob* female + L-4F. (**d**) Representative western blot and densitometry analysis of pAMPK protein in *ob* females. Results are means ± s.e., $n = 4$. *$P < 0.05$ vs *ob* female and **$P < 0.01$ vs *ob* female + L-4F.

activity have also been shown to suppress inflammatory cytokine production in adipose tissue.[22] Thus, HO-1 actions may also account for the reduction in plasma concentration of TNF-α, IL-6 and IL-1β in L-4F-treated mice.

L-4F-treated females also showed a remarkable improvement in parameters of glucose regulation. There is mounting evidence that removal of excess cholesterol from pancreatic beta cells via the ABCA1 has a role in protecting beta cell function and preventing diabetes.[56] Improvements in beta cell function may contribute to the beneficial effects of L4-F on glucose homeostasis. Apolipoprotein/ABCA1 interactions can also behave like a ligand/receptor system. HDL-c initiates a calcium-sensitive signaling cascade through ABCA1 that stimulates CaMKK, which phosphorylates and activates AMPK.[57,58] Thus, HDL-c has been shown to increase muscular glucose uptake in patients with type 2 diabetes mellitus by activating AMP-activated protein kinase in skeletal muscle in an ABCA1-dependent manner. The current studies confirm L-4F-induced phosphorylation of not only AMPK but also AKT in the kidney, suggesting L-4F can directly modulate insulin signaling and thereby improve glucose regulation. Finally, reductions in inflammatory cytokines and increases in adiponectin may also have a role in modulating insulin sensitivity and restoring glucose tolerance.[29,59,60]

In conclusion, we have demonstrated that targeting the apoA-I fraction of HDL-c with a mimetic not only reduces cardiovascular risk factors, but improves insulin sensitivity and vascular health in both male and female obese mice. L-4F increases pAKT and pAMPK in a manner dependent on HO activity. Furthermore, our results demonstrate that the positive effects of L-4F are independent of changes in body weight in *ob* female mice. We believe that the increase in visceral fat observed in female *ob* mice treated with L-4F is protective and may prevent the ectopic accumulation of lipids in other insulin-sensitive tissues, a hypothesis currently under investigation. Indeed, despite increased visceral fat, L-4F increased insulin sensitivity, reduced inflammatory cytokine levels and increased adiponectin levels. In addition, overexpression of adiponectin in obese mice promotes adipose tissue expansion, improved insulin and glucose tolerance and reduced hepatic lipid accumulation.[33] If extrapolated to humans, these results suggest that L-4F offers a powerful pharmacological method of treating the metabolic syndrome that may compliment weight loss therapy. L-4F appears to be a promising therapeutic strategy for treating both cardiovascular disease and insulin resistance in obese patients of either gender.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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