Role of Vascular Oxidative Stress in Obesity and Metabolic Syndrome

Running title: Vascular oxidative stress and obesity

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Word Count: 5,953
Abstract: 200
Number of Figures: 9
Number of Supplemental Figures: 0
Number of Tables: 2
ABSTRACT

Obesity is associated with vascular diseases that are often attributed to vascular oxidative stress. We tested the hypothesis that vascular oxidative stress could induce obesity. We previously developed mice that overexpress p22phox in vascular smooth muscle, \( \text{tg}^{\text{sm/p22phox}} \), which have increased vascular ROS production. At baseline, \( \text{tg}^{\text{sm/p22phox}} \) mice have a modest increase in body weight. With high-fat feeding, \( \text{tg}^{\text{sm/p22phox}} \) mice developed exaggerated obesity and increased fat mass. Body weight increased from 32.16±2.34 g to 43.03±1.44 g in \( \text{tg}^{\text{sm/p22phox}} \) mice (vs. 30.81±0.71 g to 37.89±1.16 g in the WT mice). This was associated with development of glucose intolerance, reduced HDL-C, and increased levels of leptin and MCP-1. \( \text{Tg}^{\text{sm/p22phox}} \) mice displayed impaired spontaneous activity, and increased mitochondrial ROS production and mitochondrial dysfunction in skeletal muscle. In mice with vascular smooth muscle-targeted deletion of p22phox (\( \text{p22phox}^{\text{loxp/loxp/tg}^{\text{smmhc/cre}}} \) mice), high-fat feeding did not induce weight gain or leptin resistance. These mice also had reduced T cell infiltration of perivascular fat. In conclusion, these data indicate that vascular oxidative stress induces obesity and metabolic syndrome, accompanied by and likely due to exercise intolerance, vascular inflammation and augmented adipogenesis. These data implicate that vascular ROS may play a causal role in the development of obesity and metabolic syndrome.

**Key Words:** vascular oxidative stress, obesity, insulin resistance, leptin resistance, metabolic syndrome, NADPH oxidase, p22phox, reactive oxygen species, exercise intolerance, inflammation, adipogenesis
INTRODUCTION

Obesity is recognized as the leading public health problem in Western Societies. Approximately one-third of American men and women over 20 years of age are obese (1). In addition to excessive energy intake, obese animals and human display reduced spontaneous activity and energy expenditure. The mechanisms for this remain unclear, but impairments in skeletal muscle perfusion and insulin uptake are present in humans with diabetes and obesity (2). Likewise, obesity and metabolic syndrome are commonly associated with oxidative stress (3), which in turn likely contributes to perturbations of tissue perfusion.

Obesity is also commonly associated with vascular diseases including hypertension and atherosclerosis (4). A major source of reactive oxygen species (ROS) in vascular cells is the NADPH oxidases, or NOX enzymes (5). These enzymes are activated by various hormones, cytokines and altered mechanical forces. ROS produced by the NOX enzymes can activate downstream enzymatic sources of ROS, such as uncoupled nitric oxide synthase and mitochondria (6). In experimental hypertension, atherosclerosis and diabetes, the NOX enzymes are activated to contribute to vascular dysfunction. Mice lacking components of the NOXs are protected against hypertension and when crossed to the apoE⁻/⁻ background have reduce atherosclerotic lesion formation (7; 8).

In the present study, we tested the hypothesis that excessive vascular ROS produced by the NOX enzymes play a causal role in obesity, by promoting inflammation, adipogenesis and exercise intolerance. To perform these studies, we employed mice that we previously generated in which the NOX subunit p22phox is overexpressed in smooth muscle cells (tg⁡sm/p22phox) (9). As a docking subunit for all NOX proteins in rodents, p22phox stabilizes these proteins and is essential for their function. Tg⁡sm/p22phox mice have increased vascular smooth muscle NOX1 (9), and increased vascular superoxide and hydrogen peroxide production at baseline. When given angiotensin II,
these animals develop augmented hypertension (10). We found that tg^{sm/p22phox} mice develop marked obesity, insulin resistance, leptin resistance, and parameters of the metabolic syndrome upon high-fat feeding. These mice also had impaired spontaneous activity and skeletal muscle mitochondrial dysfunction. Studies of mice lacking p22phox in vascular smooth muscle confirmed a role of this protein in modulation of weight gain. Taken together; these studies identify a previously unidentified role for vascular ROS, as a causal factor for obesity and its associated metabolic consequences.

RESULTS

Augmented obesity, leptin resistance, and adipogenesis in high-fat fed tg^{sm/p22phox} mice: High-fat feeding induced a significantly greater increase in body weight in tg^{sm/p22phox} mice compared to WT controls (Fig. 1A-1B). The composition of high-fat diet is provided in suppl. Table 1. As shown in Table 2, resting levels of body weight, food intake, water intake, energy intake, leptin, cholesterol, insulin and glucose were not different among all groups. Figure 1A illustrates the appearance of representative WT or tg^{sm/p22phox} mice fed a normal or high-fat diet for 6 weeks. Whereas body weight of 6 months old WT mice increased from 30.81±0.71 g to 37.89±1.16 g after high-fat feeding for 6 wks, body weight of tg^{sm/p22phox} mice increased from 32.16±2.34 g to 43.03±1.44 g (Figs. 1B). The percentage of body weight increase was 34% vs. 23% for tg^{sm/p22phox} vs. WT mice, indicating 50% more weight gain in the tg^{sm/p22phox} animals. Of note, the augmented weight gain in tg^{sm/p22phox} mice was accompanied by increased abdominal white fat (Fig. 1C), and liver size (Fig. 1D). There were no any noticeable increases in intake of water, food and calculated energy in tg^{sm/p22phox} mice compared to their WT controls, when fed high-fat diet (Figs. 2A-2C). Although water intake was transiently reduced in tg^{sm/p22phox} mice at 3 weeks of high-fat feeding, it did not affect energy intake.

In a subgroup of animals, NMR analysis of tissue subtype revealed that tg^{sm/p22phox} mice had slightly greater skeletal muscle mass than the WT mice at baseline, and this didn’t change in either group
with fat feeding (Fig. 3C). In contrast, adipose tissue markedly increased in the \(tg^{sm/p22phox}\) mice as compared to the WT mice (Fig. 3B), corresponding to an increased body weight as assessed by NMR as well (Fig. 3A).

Plasma leptin levels were markedly elevated in high-fat fed \(tg^{sm/p22phox}\) mice compared to that of WT mice (Fig. 4A). Given that leptin is a key adipocyte-derived hormone in controlling body weight and energy balance via regulation of food intake, the parallel increase in body weight and plasma leptin levels seems to indicate a leptin resistant phenotype. Although total cholesterol levels were similar between the groups (Fig. 4B), HDL-cholesterol was significantly reduced in high-fat fed \(tg^{sm/p22phox}\) mice (Fig. 4C, 66.47±19.35 to 64.05±11.34 for WT vs. 88.87±31.06 to 27.18±1.92 for \(tg^{sm/p22phox}\) respectively). Of note, even at baseline of 6 month old, the \(tg^{sm/p22phox}\) mice had modestly increased body weight comparing to age-matched WT controls (33.91±0.96 g vs. 30.34±0.58 g for \(tg^{sm/p22phox}\) vs. WT, \(n=25, p<0.05\)). This was not noted before, but the animals studied previously were 6 weeks old (9). In addition, circulating level of monocyte chemoattractant protein-1 (MCP-1), a marker of inflammation that is often elevated in obesity, was significantly increased in high-fat fed \(tg^{sm/p22phox}\) mice (Fig. 4D), which is positively correlated with leptin level (Fig. 4E).

**Insulin resistance and augmented glucose intolerance in high-fat fed \(tg^{sm/p22phox}\) mice:** High-fat feeding slightly increased fasting plasma glucose levels in both WT and \(tg^{sm/p22phox}\) mice (Fig. 5A). However, plasma insulin levels were elevated in a time dependent manner in high-fat fed \(tg^{sm/p22phox}\) mice (Fig. 5B). As is obvious in Fig. 5A and Fig. 5B, \(tg^{sm/p22phox}\) mice developed glucose intolerance as assessed by glucose tolerance tests. Glucose intolerance was observed in high-fat fed \(tg^{sm/p22phox}\) mice at week 3 (Fig. 6A), and this was significantly aggravated by high-fat feeding at week 5 in \(tg^{sm/p22phox}\) mice (Fig. 6B).

**Reduced spontaneous activity in high-fat fed \(tg^{sm/p22phox}\) mice:** Because \(tg^{sm/p22phox}\) and WT mice had similar energy intake during high-fat feeding, we considered the possibility that excessive weight gain in \(tg^{sm/p22phox}\) mice is due to alterations in energy utilization. To examine this, we monitored
nocturnal spontaneous activity using a video monitoring system. As shown in Fig. 7A, the spontaneous activity was similar between \textit{tg}^{\text{sm/p22phox}} and WT mice before high-fat feeding. Whereas high-fat feeding did not change spontaneous activity in WT mice, it induced a significant and graduate decline in spontaneous activity in high-fat fed \textit{tg}^{\text{sm/p22phox}} animals.

**Mitochondrial dysfunction and ROS production in skeletal muscle of high-fat fed \textit{tg}^{\text{sm/p22phox}} mice:** Mitochondrial function is critical for skeletal myocyte ATP supply. We have previously shown that ROS produced by the NOX enzymes can impair mitochondrial function, and therefore considered the hypothesis that ROS produced by the vascular NOX might affect skeletal muscle mitochondrial function (11; 12). Interestingly, high-fat feeding induced a near 3-fold increase in mitochondrial superoxide production in \textit{tg}^{\text{sm/p22phox}} mice (Fig. 7B), which was accompanied with markedly impaired mitochondrial function as assessed by calcium induced swelling assay (Fig. 7C).

**Prevention of high-fat induced obesity and leptin resistance in p22phox VSMC conditional KO mice:** To further examine the role of vascular ROS in the development of obesity and leptin resistance, VSMC p22phox conditional KO mice were made using a Cre-LoxP approach (p22phox^{loxp/loxp}/tg^{smmhc/cre}). As is obvious in Fig. 8A, activation of Cre recombinase by Tamoixfen injection decreased p22phox protein expression. Importantly, the weight gain caused by fat feeding was virtually absent in mice lacking vascular p22phox (Fig. 8B). Plasma leptin levels were markedly attenuated in these animals in response to high-fat diet (Fig. 8C). In contrast, leptin levels were elevated in WT animals treated with corn oil as a control.

**Prevention of high-fat induced perivascular inflammation in p22phox VSMC conditional KO mice:** In addition to enhanced adipogenesis and exercise intolerance, vascular ROS might induce obesity by augmenting inflammation in perivascular fat tissues. This process has been previously shown to mediate vascular dysfunction in hypertension (13-15). Therefore we analyzed leukocytes and T cell subpopulations in perivascular fat of high-fat fed p22phox^{loxp/loxp}/tg^{smmhc/cre} mice. As is
obvious in Fig. 9, both leukocyte and T cell subtypes were markedly reduced in the perivascular tissues of high-fat fed p22phox<sup>loxp/loxp</sup>/tg<sup>smmhc/cre</sup> mice.

**DISCUSSION**

The most significant finding of the present study is that vascular ROS play an important role in the development of obesity and metabolic syndrome as characterized by dyslipidemia, leptin resistance, inflammation, insulin resistance and glucose intolerance. High-fat feeding of genetically altered mice with elevated vascular ROS resulted in exaggerated obesity and a phenotype characteristic of the metabolic syndrome. Notably, this phenotype is associated with increased fat mass, impaired spontaneous activity and skeletal muscle mitochondrial dysfunction, as well as enhanced inflammation of perivascular fat. Additional experiments demonstrated that these phenotypes were attenuated in mice lacking vascular p22phox.

Epidemiologically, obesity is commonly associated with diseases like hypertension, hypercholesterolemia and diabetes (16). Moreover, experimental studies have shown that these diseases promote vascular reactive oxygen species (ROS) production (17). It has been thought that obesity is often causal in these conditions (18-20). However, our present study suggests that vascular ROS overproduction might instead precede and predispose to the development of obesity and the metabolic syndrome. Fat feeding induced greater weight gain, glucose intolerance and leptin intolerance in tg<sup>sm/p22phox</sup> mice than in WT mice. It is important to note that these animals had taken similar amount of calculated energy, implicating that weight gain was not caused by increased appetite or energy intake. In additional experiments we found that these animals had reduced spontaneous activity and skeletal muscle mitochondrial dysfunction, implicating reduced energy expenditure.

Many obese patients habitually consume a high-fat diet. Our data suggest that co-existing conditions associated with increased vascular ROS production such as hypertension or
hypercholesterolemia, might serve as a second stimulus in addition to dietary indiscretion, together contributing to development of obesity and metabolic syndrome. Intriguingly, plasma leptin levels were markedly increased in fat fed \( \text{tg}^{\text{sm/p22phox}} \) mice, while the body weight was still much elevated. These data establish an important role of vascular ROS in inducing leptin resistance. In normal conditions, insulin stimulates leptin secretion from adipocytes, which in turn inhibits insulin synthesis and secretion from pancreatic beta cells. In leptin resistance, however, this regulation is disrupted, creating a feed forward cycle leading to further weight gain (21). In fat fed \( \text{tg}^{\text{sm/p22phox}} \) mice, leptin resistance occurred 2 weeks after initiation of high-fat diet, and this was followed by the development of glucose intolerance at 3 weeks of fat feeding, implicating a deleterious contribution of vascular ROS to the axis of leptin-insulin regulation.

The impaired spontaneous activity in the fat fed \( \text{tg}^{\text{sm/p22phox}} \) mice is linked to increased ROS production in the skeletal muscle. Yokota and colleagues described exercise intolerance and mitochondrial complex I and II deficiencies in fat feeding induced diabetes, which were improved by administration of apocynin, an inhibitor of flavin containing oxidases (22). These findings suggest a role of ROS in regulating skeletal muscle mitochondrial function and exercise capacity (23). Prior studies from our group and others have shown that ROS generated by the NOX enzymes can diffuse to the mitochondria to stimulate ROS production. Based on this concept of ROS-dependent ROS production (24), we hypothesize that vascular ROS is capable of diffusing to adjacent skeletal muscle cells to activate ROS in these cells. Recently, it was also found that angiotensin II-induced oxidative stress in skeletal muscle limits exercise capacity while inducing skeletal muscle mitochondrial dysfunction, both of which were attenuated by apocynin administration (25). Consistent with this, mice deficient in Mn-SOD developed severe exercise disturbance (26). In the present study, we found that Mn-SOD inhabitable superoxide is substantially increased in the skeletal muscle of \( \text{tg}^{\text{sm/p22phox}} \) mice. Taken together, vascular oxidative stress may induce skeletal muscle dysfunction via 1) activation of skeletal muscle ROS production; and 2) perturbation of perfusion to skeletal muscle due to ROS scavenging of the vasodilatation factor nitric oxide (NO).
Our data also suggest a possible role of inflammation in the modulation of obesity. We found a significant increase in T cells in the mesenteric fat of fat-fed WT mice, and this was prevented in mice lacking the vascular NADPH oxidase. A similar infiltration of T cells to perivascular adipose tissue occurs in angiotensin II infused mice (13-15). It has been suggested that perivascular adipose tissue functions as an endocrine organ, releasing bioactive factors that regulate vascular function (27). It has been unclear as to whether inflammation of the perivascular adipose tissue contributes to obesity. Our data indicate that in mice deficient in vascular ROS production, T cell infiltration of perivascular adipose tissue is markedly reduced, likely contributing to the reduction in obesity observed in these animals. Conversely, elevated MCP-1 was found in high fat fed \( \text{tg}^{\text{sm/p22phox}} \), which correlated well with an elevation in leptin levels. Given that MCP-1 expression is upregulated in obese patients and that MCP-1 is inducible by leptin (28) or high glucose (29) via ROS dependent pathway, our data further demonstrate that vascular ROS may contribute to the development of obesity via regulation of inflammation.

In conclusion, our present study for the first time defines an important causal role of vascular oxidative stress in development of obesity and the metabolic syndrome, likely due to exercise intolerance, vascular inflammation and augmented adipogenesis. These findings maybe paradigm shifting in revealing that vascular oxidative stress can be a cause, rather than a mere consequence, of obesity and metabolic syndrome. Thus, targeting vascular dysfunction and oxidative stress might prove to be an effective approach to prevent and/or treat obesity.

**MATERIALS AND METHODS**

**Animals and experimental model:** Male C57BL/6 mice (6 mo old) were purchased from Charles River Laboratories (Hollister, CA, USA) to serve as wild-type (WT) control. Age-matched mice overexpressing p22phox in smooth muscle (\( \text{tg}^{\text{p22amc}} \)) have been previously described (30) and were bred in house at the University of California Los Angeles (UCLA) and Vanderbilt University. The
p22phox$^{\text{loxp/loxp}}$/tg$^{\text{smmhc/cre}}$ mice were bred at Vanderbilt University. The transgenic mice with Tamoxifen inducible Cre recombinase driven by the smooth muscle myosin heavy chain (tg$^{\text{smmhc/cre}}$ mice) were generous gifts from Dr. Stephan Offermanns, University of Heidelberg and were crossed with mice containing loxP sites flanking the coding region of p22phox, described previously (31). For Cre-inducible deletion of p22phox in the vascular smooth muscle, p22phox$^{\text{loxp/loxp}}$/tg$^{\text{smmhc/cre}}$ mice received Tamoxifen injections (3 mg/20 grams, i.p., every other day for 10 days) prior to high fat diet feeding for 6 weeks.

Animals were maintained in a temperature controlled environment (22°C) on a 12 hr light-dark cycle. Mice were randomly divided into two dietary groups and were fed either a high fat diet (42 % fat, Harlan Laboratories, Madison, USA) or a standard diet for 6 weeks (Table 1). Mice were provided with 200 g of food and 400 ml of water and their weekly intake monitored. Energy intake, calculated as kcal per gram of food was 3.1 kcal/g for the control diet and 4.5 kcal/g for the high fat diet, based on information provided by the supplier. Activity was monitored using infrared webcams for 8 wks and analyzed using motion detection software. The Institutional Animal Care and Use Committees at UCLA and Vanderbilt approved all experimental procedures.

**Analysis of fasting glucose, insulin, leptin, MCP-1, and lipids:** Blood glucose was determined at baseline and weekly thereafter using the One Touch Ultra® blood glucose meter (Lifescan). Plasma insulin levels were analyzed using an ELISA for rat insulin (Ultra Sensitive Rat Insulin ELISA; Crystal Chem Inc., USA). Plasma leptin levels were determined using a mouse leptin ELISA kit (Crystal Chem Inc.). Quantitative determination of mouse MCP-1 levels in plasma was performed by using a ELISA kit (R&D systems Inc, USA). Plasma cholesterol was determined using a cholesterol reagent colorimetric assay kit (Roche Diagnostics). Triglyceride levels were measured colorimetrically at 540 nm using the triglyceride (glycerol phosphate oxidase) reagent set (Pointe Scientific, Canton, MI). To determine plasma HDL cholesterol levels, plasma was incubated with an HDL cholesterol precipitating reagent (Pointe Scientific, Canton, MI) followed by separation of HDL by centrifugation.
HDL was then quantified using an enzymatic cholesterol detection kit (Roche diagnostics).

**Glucose tolerance test:** Following an 8 hour fast, mice were injected intraperitoneally with glucose (2 g/kg body weight in 0.9 % saline). Whole-blood samples were collected from the tail vein at baseline and 15, 30, 60 and 120 min following glucose injection.

**Mitochondrial swelling assay:** Mitochondria from skeletal muscle were isolated by differential centrifugation as described previously (32). Freshly isolated mitochondria were incubated with a buffer containing 250 mM sucrose, 10 mM Tris (pH 7.4) and 5 mM succinate for 1 min at room temperature before swelling is initiated by the addition of 250 µM CaCl$_2$. Mitochondrial swelling was measured by monitoring the decrease in absorbance at 540 nm.

**Electron spin resonance measurement of mitochondrial superoxide production:** Freshly isolated skeletal muscle tissues were grounded with 3 volume of mitochondrial isolation buffer I (250 mM sucrose, 10 mM HEPES, 10 mM Tris, 1 mM EGTA, pH 7.4) in a glass tissue grinder by 15 strokes. Homogenates were centrifuged at 800 g for 7 min at 4°C. Supernatants were further centrifuged at 4,000 g for 15 min at 4°C. Pellet containing mitochondria was rinsed by resuspension with mitochondrial isolation buffer II (250 mM sucrose, 10 mM HEPES, 10 mM Tris, pH 7.4) and centrifugation at 4,000 g for 15 min. After centrifugation, pellet was resuspended with 100 µl of mitochondrial isolation buffer II and then used for superoxide measurement. Freshly prepared mitochondrial fraction of skeletal muscle were incubated with spin trap solution in the presence and absence of 100 U/ml of Mn-SOD for 5 min prior to be loaded into glass capillary (Fisher Scientific) for analysis of $O_2^*$ signal using e-Scan electron spin resonance (ESR) spectrophotometer (Bruker Biospin) as we previously published (22; 33-38).

**Isolation and analysis of T cell populations in perivascular fat:** Mesenteric vascular arcade with its attached perivascular fat were isolated and digested with collagenase and hyalurindase as
previously described (13-15). The single cell suspensions were subjected to FACS for detection of CD45+ cells (total leukocytes), CD3+ cells (T cells), CD4+ and CD8+ cells and macrophages (with CD11b and F4/80) in fat (13-15).

**Statistical analysis:** Differences among different groups of means were compared with ANOVA for multiple means with a Tukey's multiple comparison as a post-hoc. For comparisons of mean values among groups over time, two-way ANOVA followed by Bonferroni post-test was performed. Before data analysis, resting levels at baseline were subtracted from the data by using the function of “remove baseline and column math” of Graphpad Prism version 6.0 software. The resting levels were presented in suppl. Table 2, while the analyzed data after subtraction were presented in Figs. 1-5. Correlation between levels of leptin and MCP-1 was assessed using Pearson's correlation analysis. Statistical significance was considered present for p<0.05. All data are presented as Mean±SEM.
AUTHOR CONTRIBUTIONS

J.Y.Y. research data, wrote manuscript; K.L.S. research data; H.L. research data; H.I. research data; D.G.H. created mice, research design, reviewed/edited manuscript; H.C. research design, wrote/edited manuscript

ACKNOWLEDGEMENT

The authors work was supported by National Heart, Lung and Blood Institute (NHLBI) Grants HL077440 (HC), HL088975 (HC), HL108701 (HC, DGH), HL119968 (HC), and an American Heart Association Established Investigator Award 12EIA8990025 (HC). Dr. Hua Cai is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICTS OF INTERESTS

None of the authors have conflicts of interests to disclose.
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FIGURE LEGENDS

**Figure 1:** Augmented obesity in high-fat diet fed \textit{tg}^{sm/p22phox} mice. \textbf{A):} Mice of representation from WT and \textit{tg}^{sm/p22phox} groups fed high-fat diet for 6 weeks. \textbf{B):} Body weight gain in WT and \textit{tg}^{sm/p22phox} mice fed with control or high-fat diet for 6 weeks. (*p<0.05, **p<0.01, ***p<0.001 vs control diet fed WT, #p<0.05, ## p<0.01, ###p<0.001 vs control diet fed \textit{tg}^{sm/p22phox}). \textbf{C):} White fat mass (*p<0.05, **p<0.01 vs control diet fed WT, #p<0.05 vs control diet fed \textit{tg}^{sm/p22phox} mice), \textbf{D):} Liver weight, in WT and \textit{tg}^{sm/p22phox} mice fed with control or high-fat diet for 6 weeks (*p<0.05 vs. control diet fed WT, ##p<0.01 vs control diet fed \textit{tg}^{sm/p22phox} mice). Data are presented as Mean±SEM, n=10-14 for A-D)

**Figure 2:** Changes in water intake, food intake, and energy intake in WT and \textit{tg}^{sm/p22phox} mice fed with control or high-fat diet for 6 weeks. \textbf{A):} Water intake was measured weekly and there were no significant changes among the 4 different groups except week 3 to 5 (*p<0.05, **p<0.01, ***p<0.001 vs. control diet fed WT, $$p<0.01 vs. high-fat fed WT mice). \textbf{B):} Weekly food intake was decreased in WT mice after high-fat feeding for 2 weeks (**p<0.01 vs. control diet fed WT). \textbf{C):} Energy intake was calculated into kcal from gram of food taken as described in the Methods Section. Data are presented as Mean±SEM, n=7-11 for A-C)

**Figure 3:** NMR analysis of body weight, fat mass and muscle mass in WT and \textit{tg}^{sm/p22phox} mice fed with control or high-fat diet for 6 weeks. \textbf{A):} Body weight was measured weekly. High fat diet feeding induced an exaggerated body weight gain in \textit{tg}^{sm/p22phox} mice (*p<0.05, **p<0.01, ***p<0.001 vs control diet fed WT, ## p<0.01, ###p<0.001 vs control diet fed \textit{tg}^{sm/p22phox}, $$p<0.01, $$$p<0.001 vs. high-fat diet fed WT). \textbf{B):} Total fat mass was measured weekly and found substantially more increased by high fat diet feeding in \textit{tg}^{sm/p22phox} mice. (*p<0.05 vs control diet fed WT, #p<0.05, ## p<0.01, ###p<0.001 vs control diet fed \textit{tg}^{sm/p22phox}, $$$p<0.001 vs. high-fat diet fed WT). \textbf{C):} Total muscle mass was monitored weekly and found not different either at baseline or 6 weeks after high
fat diet-feeding between WT and $\text{tg}^{\text{sm/p22phox}}$ mice (**$p<0.01$ vs control diet fed WT, $p<0.05$, $$p<0.01$, $$$p<0.001$ vs. high-fat diet fed WT). Data are presented as Mean±SEM, n=5 for A-C)

**Figure 4:** Leptin resistance and dyslipidemia in high-fat diet fed $\text{tg}^{\text{sm/p22phox}}$ mice. **A:** Plasma leptin levels were measured weekly as described in the Methods Section. A remarkable increase in plasma leptin levels was observed in high-fat diet fed $\text{tg}^{\text{sm/p22phox}}$ mice while it did not occur in the WT mice fed with high-fat diet (*$p<0.05$, ***$p<0.001$ vs. control diet fed WT, ##$p<0.01$, ###$p<0.001$ vs. control diet fed $\text{tg}^{\text{sm/p22phox}}$, $$p<0.01$, $$$p<0.001$ vs. high-fat fed WT). These data implicate a leptin resistance phenotype. **B:** Total cholesterol levels were increased in both WT and $\text{tg}^{\text{sm/p22phox}}$ mice fed with high-fat diet (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control diet fed WT, #p<0.05, ###$p<0.001$ vs control diet fed $\text{tg}^{\text{sm/p22phox}}$). **C:** High-fat diet feeding induced a significant reduction in HDL cholesterol in $\text{tg}^{\text{sm/p22phox}}$ mice (*$p<0.05$ vs. high-fat fed WT). **D:** Plasma MCP-1 levels at 6 weeks of high fat feeding were markedly increased in $\text{tg}^{\text{sm/p22phox}}$ mice (*$p<0.05$ vs. high-fat diet fed $\text{tg}^{\text{sm/p22phox}}$). Data are presented as Mean±SEM, n=7-11 for A-C, n=6-7 for D). **E:** Plasma MCP-1 levels were positively correlated with plasma leptin levels (n=27 of 4 groups).

**Figure 5:** Insulin resistance in high-fat diet fed $\text{tg}^{\text{sm/p22phox}}$ mice. **A:** Fasting glucose levels were measured weekly over 6 weeks (###$p<0.001$ vs. control diet fed $\text{tg}^{\text{sm/p22phox}}$, $$p<0.01$ vs. high-fat fed WT mice). **B:** Weekly circulating insulin levels were determined by ELISA. Insulin levels were elevated in a time dependent manner in high-fat fed $\text{tg}^{\text{sm/p22phox}}$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs control diet fed WT, #p<0.05, ###$p<0.001$ vs. control diet fed $\text{tg}^{\text{sm/p22phox}}$). Data are presented as Mean±SEM, n=7-11 for A-B)

**Figure 6:** Impaired glucose tolerance in high-fat fed $\text{tg}^{\text{sm/p22phox}}$ mice. Intraperitoneal glucose tolerance test was performed at week 3 and 5. **A:** Glucose intolerance was observed in high-fat fed $\text{tg}^{\text{sm/p22phox}}$ mice at week 3 (***$p<0.001$ vs. control diet fed WT, ##$p<0.01$, ###$p<0.001$ vs. control diet fed $\text{tg}^{\text{sm/p22phox}}$). **B:** Glucose intolerance was aggravated by high-fat feeding at week 5 in $\text{tg}^{\text{sm/p22phox}}$ mice (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control diet fed WT, #p<0.05, ##$p<0.01$, ###$p<0.001$ vs. $\text{tg}^{\text{sm/p22phox}}$. **C:** Plasma MCP-1 levels at 6 weeks of high fat feeding were markedly increased in $\text{tg}^{\text{sm/p22phox}}$ mice (*$p<0.05$ vs. high-fat diet fed $\text{tg}^{\text{sm/p22phox}}$). Data are presented as Mean±SEM, n=7-11 for A-C, n=6-7 for D). **E:** Plasma MCP-1 levels were positively correlated with plasma leptin levels (n=27 of 4 groups).
control diet fed Tg_{sm/p22phox}, $p<0.05, $$$p<0.01$ vs high-fat fed WT). Data are presented as Mean±SEM, n=7-11 for A-B)

**Figure 7:** Decreased spontaneous activity accompanied by mitochondrial dysfunction in skeletal muscle of high-fat fed Tg_{sm/p22phox} mice. **A:** Spontaneous activity was monitored over 8 weeks of high-fat diet feeding, and progressively declined in the Tg_{sm/p22phox} mice, while remained constant in the WT mice (**p<0.01, ***p<0.01 vs. WT at 0 week, n=6-7).** **B:** Mitochondrial fraction from skeletal muscle was prepared as described in the Methods Section, and subjected to superoxide detection using electron spin resonance (ESR). Mitochondrial superoxide production from high-fat fed Tg_{sm/p22phox} mice was increased more than 3 fold compared to WT controls fed high-fat diet (**p<0.01 vs WT, n=6-7). **C:** Calcium induced swelling of skeletal muscle mitochondria was significantly augmented in high-fat fed Tg_{sm/p22phox} mice compared to WT controls fed high-fat diet. (*p<0.05, **p<0.01, ***p<0.001 vs. WT, n=11-13). Data are presented as Mean±SEM.

**Figure 8:** Prevention of obesity induction in p22phox knockout mice. p22phox^{loxp/loxp} crossed with mice expression Cre recombinase driven by the tamoxifen-inducible smooth muscle myosin heavy chain promoter, Tg_{smmmhc/cre}. **A:** Expression of p22phox was decreased upon tamoxifen introduction. **B:** High-fat feeding for 6 weeks failed to induce body weight gain in p22phox knockout mice (**p<0.001, n=6). **C:** Leptin level was attenuated in high-fat fed p22phox knockout mice while it was increased in vehicle corn oil treated mice with high-fat diet feeding or cre negative mice (**p<0.01, ***p<0.001, ****p<0.0001, n=5-6). Data are presented as Mean±SEM.

**Figure 9:** Effect of high-fat feeding on mesenteric fat leukocytes in presence and absence of VSM p22phox: Mice were fed either a control diet or a high-fat diet for 6 weeks. Mice fed a high-fat diet were p22phox/LoxP/LoxP and half had the VSMC specific Cre transgene induced by tamoxifen injection (grey bars). As control, p22phoxLoxP/LoxP Cre negative mice were also fed a high-fat diet and were treated with tamoxifen (black bars). The mesenteric vasculature with all adjacent fat was removed en-bloc, digested and subjected to FACS analysis for measurement of total leukocytes and
T cell subtypes. **A)**: Populations of leukocytes were analyzed by FACS (**p<0.01, ***p<0.001, ****p<0.0001). **B)**: T cell subtypes were also analyzed by FACS (**p<0.01, ****p<0.0001). Data are presented as Mean±SEM, n=4 for A-B).
Figure 1A

0 week vs. 6 weeks high-fat diet

Diabetes

WT

Tg^{sm/p22phox}
Figure 1

B

![Graph showing body weight gain over time (weeks)](image)

- **WT + control diet**
- **Tg^{sm/p22phox} + control diet**
- **WT + high-fat diet**
- **Tg^{sm/p22phox} + high-fat diet**

* p<0.05, ** p<0.01, *** p<0.001 vs. WT + control diet
# p<0.05, ## p<0.01, ### p<0.001 vs. Tg^{sm/p22phox} + control diet

C

* p<0.05, ** p<0.01 vs. WT + control diet
# p<0.05 vs. Tg^{sm/p22phox} + control diet

D

* p<0.05 vs. WT + control diet
## p<0.01 vs. Tg^{sm/p22phox} + control diet
Figure 2

A

Water intake change (ml/day/mouse)

WT + control diet

Tg^{sm/p22phox} + control diet

WT + high-fat diet

Tg^{sm/p22phox} + high-fat diet

Time (weeks)

**p<0.01 vs. WT + control diet

#p<0.05 vs. Tg^{sm/p22phox} + control diet

B

Food intake change (g/day/mouse)

WT + control diet

Tg^{sm/p22phox} + control diet

WT + high-fat diet

Tg^{sm/p22phox} + high-fat diet

Time (weeks)

**p<0.01 vs. WT + control diet

#p<0.05 vs. Tg^{sm/p22phox} + control diet
Figure 2

C

Energy intake change (Kcal/day/mouse) vs. Time (weeks)

- WT + control diet
- Tg\textsuperscript{sm/p22phox} + control diet
- WT + high-fat diet
- Tg\textsuperscript{sm/p22phox} + high-fat diet
**Figure 3**

**A**

Body weight (g)

![Graph showing body weight changes over time for different diet groups](image)

- **WT + control diet**
- **Tg^{sm/p22phox} + control diet**
- **WT + high-fat diet**
- **Tg^{sm/p22phox} + high-fat diet**

* *p<0.05, **p<0.01, ***p<0.001 vs WT + control diet*

## **p<0.01, ###p<0.001 vs Tg^{sm/p22phox} + control diet**

$$ **p<0.01, $$$p<0.001 vs WT + high-fat diet$$

**B**

Fat (g)

![Graph showing fat changes over time for different diet groups](image)

- **WT + control diet**
- **Tg^{sm/p22phox} + control diet**
- **WT + high-fat diet**
- **Tg^{sm/p22phox} + high-fat diet**

* *p<0.05, ***p<0.001 vs WT + control diet*

# p<0.05, ## p<0.01, ###p<0.001 vs Tg^{sm/p22phox} + control diet

$$ $$$p<0.001 vs WT + high-fat diet$$
**Figure 3**

**C**

![Graph showing muscle (g) vs. time (weeks) for different dietary groups.]

- **WT + control diet**
- **Tg<sup>sm/p22phox</sup> + control diet**
- **WT + high-fat diet**
- **Tg<sup>sm/p22phox</sup> + high-fat diet**

**Legends:**
- *p<0.05, **p<0.01, ***p<0.001 vs WT+ control diet*
- $p<0.05, $$p<0.01, $$$p<0.001 vs WT + high-fat diet
Figure 4

A

Leptin (pg/ml)

WT + control diet

Tg\textsuperscript{sm/p22phox} + control diet

WT + high-fat diet

Tg\textsuperscript{sm/p22phox} + high-fat diet

*\(p<0.05\), ***\(p<0.001\) vs. WT+control diet

##\(p<0.01\), ###\(p<0.001\) vs. Tg

\textit{sm/p22phox} + control diet

$$$\(p<0.01\), $$$\(p<0.001\) vs. WT + high-fat diet

B

Cholesterol (\(\mu g/ml\))

WT + control diet

Tg\textsuperscript{sm/p22phox} + control diet

WT + high-fat diet

Tg\textsuperscript{sm/p22phox} + high-fat diet

*\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) vs. WT+control diet

#\(p<0.05\), ##\(p<0.01\) vs. Tg\textsuperscript{sm/p22phox} + control diet

WT + high-fat diet

$\$\(p<0.01\), $$$\(p<0.001\) vs. WT + high-fat diet
Figure 4

C

![Graph showing HDLc (mg/dL) levels over 0 and 5 weeks for WT and Tg^sm/p22phox^ genotypes.]

- 0 week
- 5 week

*p<0.05 vs. WT+ high-fat diet

D

![Graph showing MCP-1 (pg/ml) levels for WT, Tg^sm/p22phox^, WT, and Tg^sm/p22phox^ genotypes on control diet and high-fat diet.]

- *p<0.05 vs. Tg^sm/p22phox^ + control diet

E

![Scatter plot showing the correlation between Leptin (pg/ml) and MCP-1 (pg/ml) levels.]

- R=0.5878
- p=0.0013
Figure 5

A

Blood glucose (mg/dL)

WT + control diet
Tg^{sm/p22phox} + control diet
WT + high-fat diet
Tg^{sm/p22phox} + high-fat diet

###p<0.001 vs. tg^{sm/p22phox} + control diet
$$p<0.01$$ vs WT + high-fat diet

B

Insulin (pg/ml)

WT + control diet
Tg^{sm/p22phox} + control diet
WT + high-fat diet
Tg^{sm/p22phox} + high-fat diet

*p<0.05, **p<0.01, ***p<0.001 vs WT + control diet
#p<0.05, ###p<0.001 vs tg^{sm/p22phox} + control diet
Figure 6

A

WT + control diet  
WT + high-fat diet  
Tg^{sm/p22phox} + control diet  
Tg^{sm/p22phox} + high-fat diet

i.p. injection of 2g/kg of glucose

**p<0.01, ***p<0.001 vs WT+ control diet
##p<0.01, ###p<0.001 vs Tg^{sm/p22phox} + control diet
$p<0.05, $$p<0.01 vs WT+ high-fat diet

B

i.p. injection of 2g/kg of glucose

**p<0.01, ***p<0.001 vs WT+ control diet
##p<0.01, ###p<0.001 vs Tg^{sm/p22phox} + control diet
$p<0.05, $$p<0.01 vs WT+ high-fat diet
Figure 7

**A**

![Graph showing % Activity over weeks after high-fat diet for WT and Tg^{sm/p22phox}](image)

Weeks after high-fat diet

**B**

![Bar graph showing Superoxide production (Fold Inc.) for WT and Tg^{sm/p22phox}](image)

**C**

![Graph showing Δ A_{540} over time for WT and Tg^{sm/p22phox}](image)

Diabetes

*p<0.05, **p<0.01, ***p<0.001 vs. WT

**p<0.01 vs. WT

***p<0.001 vs. 0 week
Figure 8

A

Vehicle
Tamoxifen

p22phox
β-actin

B

Baseline 6 weeks high-fat diet

***p<0.001

C

**p<0.01, ***p<0.001, ****p<0.0001

Body Weight (g)

Cre(-)  Cre(+)

Leptin (pg/ml)

Cre(-)/Tam  Cre(+)/Tam  Cre(+)/Corn-oil
Figure 9

**A**

![Graph showing cell count per mesenteric bed X 10^3 for CD45 and CD3 under different diets.](image)

- Control diet
- High-fat diet
- High-fat diet VSM p22phox KO

**p<0.01, ***p<0.001, ****p<0.0001**

**B**

![Graph showing cell count per mesenteric bed X 10^3 for CD4 and CD8 under different diets.](image)

- Control diet
- High-fat diet
- High-fat diet VSM p22phox KO

**p<0.01, ****p<0.0001**
Suppl. TABLE 1. Diet composition

<table>
<thead>
<tr>
<th>Selected Nutrient Information</th>
<th>% Kcal from</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control diet</td>
</tr>
<tr>
<td>Protein</td>
<td>33</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>53</td>
</tr>
<tr>
<td>Fat</td>
<td>14</td>
</tr>
</tbody>
</table>
**Suppl. TABLE 2.** Resting levels of body weight, food intake, water intake, energy intake, leptin, cholesterol, insulin and glucose

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>High Fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Tg^em/p22phox</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>31.36 ± 0.924</td>
<td>32.58 ± 0.420</td>
</tr>
<tr>
<td>Food Intake (g/day/mouse)</td>
<td>4.20 ± 0.300</td>
<td>3.88 ± 0.375</td>
</tr>
<tr>
<td>Water Intake (ml/day/mouse)</td>
<td>3.93 ± 0.325</td>
<td>4.81 ± 1.107</td>
</tr>
<tr>
<td>Energy Intake (Kcal/day/mouse)</td>
<td>13.02 ± 0.930</td>
<td>12.01 ± 1.162</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>4640 ± 1031</td>
<td>3762 ± 1161</td>
</tr>
<tr>
<td>Cholesterol (µg/ml)</td>
<td>1.11 ± 0.038</td>
<td>0.93 ± 0.038</td>
</tr>
<tr>
<td>Insulin (pg/ml)</td>
<td>618.5 ± 43.03</td>
<td>533.4 ± 125.80</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>135.9 ± 8.58</td>
<td>147.1 ± 7.96</td>
</tr>
</tbody>
</table>