Overexpression of Endothelial Nitric Oxide Synthase Prevents Diet-Induced Obesity and Regulates Adipocyte Phenotype


Rationale: Endothelial dysfunction is a characteristic feature of diabetes and obesity in animal models and humans. Deficits in nitric oxide production by endothelial nitric oxide synthase (eNOS) are associated with insulin resistance, which is exacerbated by high-fat diet. Nevertheless, the metabolic effects of increasing eNOS levels have not been studied.

Objective: The current study was designed to test whether overexpression of eNOS would prevent diet-induced obesity and insulin resistance.

Methods and Results: In db/db mice and in high-fat diet-fed wild-type C57BL/6J mice, the abundance of eNOS protein in adipose tissue was decreased without significant changes in eNOS levels in skeletal muscle or aorta. Mice overexpressing eNOS (eNOS transgenic mice) were resistant to diet-induced obesity and hyperinsulinemia, although systemic glucose intolerance remained largely unaffected. In comparison with wild-type mice, high-fat diet-fed eNOS transgenic mice displayed a higher metabolic rate and attenuated hypertrophy of white adipocytes. Overexpression of eNOS did not affect food consumption or diet-induced changes in plasma cholesterol or leptin levels, yet plasma triglycerides and fatty acids were decreased. Metabolomic analysis of adipose tissue indicated that eNOS overexpression primarily affected amino acid and lipid metabolism; subpathway analysis suggested changes in fatty acid oxidation. In agreement with these findings, adipose tissue from eNOS transgenic mice showed higher levels of PPAR-α and PPAR-γ gene expression, elevated abundance of mitochondrial proteins, and a higher rate of oxygen consumption.

Conclusions: These findings demonstrate that increased eNOS activity prevents the obesogenic effects of high-fat diet without affecting systemic insulin resistance, in part, by stimulating metabolic activity in adipose tissue. (Circ Res. 2012;111:1176-1189.)

Key Words: adipose tissue ■ cardiovascular disease ■ diabetes ■ endothelial nitric oxide synthase ■ metabolism ■ mitochondria ■ obesity

Obesity and type 2 diabetes have become major health challenges worldwide. Current data show that approximately 1.5 billion adults aged 20 years or older are overweight, and 10% are obese. In the United States, one-third of the population meets the criteria for metabolic syndrome. Although lifestyle changes and lack of exercise are important risk factors for weight gain, excessive caloric intake appears to be one key factor fueling the epidemic of obesity. Poor dietary habits negatively affect a broad range of cardiovascular functions and promote the onset of type 2 diabetes.
to stem the tide of the epidemics of type 2 diabetes and obesity, it is important to understand the relationship between obesity and insulin resistance, as well as the physiological processes that regulate their development.

Accumulating evidence suggests that the vascular endothelium regulates insulin action. In humans, states of obesity and insulin resistance are characterized by endothelial dysfunction, impaired vasodilation, and insulin resistance;9 in rats, inhibition of endothelial nitric oxide (NO) synthase (eNOS) decreases insulin-stimulated uptake of glucose by skeletal muscle, suggesting that eNOS may be a key regulator of metabolic homeostasis. This role of eNOS is further corroborated by the observations that in mice deletion of the eNOS gene induces insulin resistance10,11 and impairs fatty acid oxidation.12 Nevertheless, the role of eNOS in regulating metabolic changes that contribute to obesity under conditions of nutrient excess is not well-understood. In particular, it is unclear whether eNOS could prevent or attenuate diet-induced adiposity and insulin resistance.

To understand the metabolic role of eNOS, we studied the effects of high-fat diet in mice overexpressing eNOS. Our hypothesis was that increasing eNOS levels mitigates the effects of high-fat feeding by regulating adipose tissue metabolism. We found that eNOS transgenic (eNOS-TG) mice were resistant to diet-induced weight gain, but not glucose intolerance. These findings reveal a new antiobesogenic role of eNOS and its favorable influence on adipose tissue metabolism.

**Methods**

**Animal Studies**

The B6.BKS(D)-Leprdb/db (db/db) mice and C57BL/6J (wild-type; WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The eNOS-TG mice, which express bovine eNOS under the control of the preproendothelin-1 promoter,13 were maintained on the C57BL/6J background. At 8 weeks of age, male mice were placed on either a 10% low-fat diet (#D12450B; Research Diets) or a 60% high-fat diet (#D12492; Research Diets) and maintained for 6 to 15 additional weeks. Water and diet were provided ad libitum. Body weights were recorded weekly. During the weeks 7 and 13 of feeding, glucose and insulin tolerance tests were performed. Pyruvate tolerance tests were performed only after week 13 of feeding; all other parameters were evaluated after euthanasia. All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

**Expression Analyses**

Tissue homogenates were prepared and used for Western blot protein expression analysis. For quantitative reverse-transcription polymerase chain reaction, RNA extracted from tissues was used to assess pgc1a, cyt6b, gapdh, ppara, and pparγ expression using commercially available primers (SABiosciences, Valencia, CA).

**Glucose, Insulin, and Pyruvate Tolerance Tests**

As described before,13 glucose tolerance tests were performed after a 6-hour fast by injection (intraperitoneally) of D-glucose (1 mg/g) in sterile saline. Insulin tolerance tests were performed on nonfasted animals by interperitoneal injection of 1.5 U/kg Humulin R (Eli Lilly, Indianapolis, IN). After a 6-hour fast, pyruvate tolerance tests were performed as described.15

### Non-standard Abbreviations and Acronyms

- **AA**: antimycin A
- **ALDH2**: aldehyde dehydrogenase 2
- **ALT**: alanine aminotransferase
- **AST**: aspartate aminotransferase
- **ATP5B**: ATP synthase beta subunit
- **BSTFA**: bistrimethylsilyl-trifluoroacetamide
- **CK**: creatine kinase
- **CoA**: coenzyme A
- **COX4I1**: cytochrome c oxidase subunit 4 isoform 1
- **Cytb6**: cytochrome B6
- **DAPI**: 4',6-diamidino-2-phenylindole
- **Dexscan**: dual-energy X-ray absorptiometry
- **ECAR**: extracellular acidification rate
- **eNOS**: endothelial nitric oxide synthase
- **eNOS-TG**: endothelial nitric oxide synthase transgenic
- **ESI**: electrospray ionization
- **FDR**: false discovery rate
- **FT-ICR**: Fourier transform ion cyclotron resonance
- **GAPDH**: glyceraldehyde 3-phosphate dehydrogenase
- **HbA1c**: hemoglobin A1c
- **HFD**: high-fat diet
- **HPLC**: high-performance liquid chromatography
- **HRP**: horseradish peroxidase
- **IB**: immunoblot
- **IHC**: immunohistochemistry
- **LDH**: lactate dehydrogenase
- **LFD**: low-fat diet
- **LIMS**: Laboratory Information Management Systems
- **LIT**: linear ion-trap
- **OCR**: oxygen consumption rate
- **PBS**: phosphate-buffered saline
- **PGC1α**: peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
- **Ppara**: peroxisome proliferator-activated receptor alpha
- **Pparg**: peroxisome proliferator-activated receptor gamma
- **PTT**: pyruvate tolerance test
- **QA/QC**: quality assurance/quality control
- **RER**: respiratory exchange ratio
- **Rot**: rotenone
- **SAM**: S-adenosyl methionine
- **Sirt3**: sirtuin 3
- **TG**: transgenic
- **T2D**: type 2 diabetes
- **VGCO**: carbon dioxide production
- **VDAC**: voltage-dependent anion channel
- **VEGFR2**: vascular endothelial growth factor receptor 2
- **VO2**: oxygen consumption
- **WT**: wild-type
- **XF**: extracellular flux
Biochemical Analyses
Plasma lipids, proteins, and metabolites were measured using a Cobas Mira Plus 5600 Autoanalyzer (Roche, Indianapolis, IN) or Luminex kits (Millipore, Billerica, MA). Plasma levels of nonesterified free fatty acids and glycerol were measured by enzyme-linked immunosorbent assay (Wako Chemicals, Richmond, VA, and Cayman Chemical, Ann Arbor, MI, respectively). Nitrite and nitrate levels were measured as described.16

Adipocyte Size Measurements
Adipose tissue excised at the time of euthanasia was either snap-frozen at −80°C or fixed in 10% formalin (Leica), paraffin-embedded, and sectioned. The sections were stained in hematoxylin and eosin. Adipocyte cross-sectional area was measured using the Nikon Elements software. To assess relative mitochondrial abundance, the sections were stained with MitoID Red (Enzo Life Sciences, Farmingdale, NY). Crown-like structures and inflammatory cells indicative of adipose tissue inflammation were measured as described previously.17,18

Body Composition and Calorimetry
Body composition was measured by dual-energy X-ray absorptiometry using a mouse densitometer (PIXImus2; Lunar, Madison, WI). Whole-body energy expenditure, respiratory exchange ratio, food consumption, and locomotion, ambulatory, and fine movements were measured using a physiological/metabolic cage system (TSE PhenoMaster System, Bad Homberg, Germany).

Immunostaining of Adipose Tissue
Capillary density was quantified in paraffin-embedded sections using fluorescently labeled lectin B4 as described.19 Nitrotyrosine adducts were measured in paraffin-embedded tissues using antinitrotyrosine and goat-antirabbit IgG-Cy3 antibodies.

Adipose Tissue Bioenergetic Measurements
The oxygen consumption rate and extracellular acidification rate of intact adipose tissue explants were measured using a Seahorse XF24 analyzer (Seahorse Bioscience, Billerica, MA). Briefly, freshly isolated epididymal adipose tissue was rinsed with unbuffered Dulbecco modified Eagle medium (pH 7.4). The adipose tissue was cut into sections, and 10 mg was placed in each well of an XF 24 Islet Capture Microplate (Seahorse Bioscience, Billerica, MA). The tissue was then covered with a screen, which allowed free perfusion while minimizing tissue movement. Unbuffered Dulbecco modified Eagle medium (500 μL) supplemented with 50 μmol/L bovine serum albumin-conjugated palmitic acid, 200 μmol/L L-carnitine, and 2.5 mmol/L D-glucose were then added to each well. At least two replicates from each animal were used for the assay, and each tissue section was examined to ensure absence of large vessels (which can skew oxygen consumption measurements). The plate was incubated at 37°C in a non-CO2 incubator for 1 hour before extracellular flux analysis. After three baseline measurements, a mixture of antimycin A (10 μmol/L) and rotenone (1 μmol/L) was injected. After injection, the oxygen consumption rate was closely monitored until the rates stabilized, and then the experiment was terminated.

Metabolomic Analysis of Adipose Tissue
Epididymal adipose tissue was used for metabolomic analysis. After tissue harvest, the metabolites were extracted in methanol and subjected to metabolic profiling by ultra-high-performance liquid chromatography/tandem mass spectrometry and gas chromatography mass spectrometry.20,21 Detailed Methods are provided in the Online Supplement.

Statistical Analyses
Data are mean ± standard error of the mean. Multiple groups were compared using one-way or two-way analysis of variance, followed by Bonferroni posttests. Unpaired Student t test was used for direct comparisons. Statistical analysis of metabolic profiling is described in the Online Supplement; P<0.05 was considered significant.

Results

Nutrient Excess Alters eNOS Abundance
To study the effects of obesity and diabetes on eNOS protein levels, C57BL/6J mice were placed on a high-fat diet,22 and db/db mice were used as a model of type 2 diabetes.23 High-fat feeding for 6 and 12 weeks resulted in a profound decrease in eNOS levels in adipose tissue (Figure 1A, B), with no statistically significant changes in the aorta (Figure 1A, C) or skeletal muscle (Online Figure IA, B). Similar changes were observed in 20-week-old db/db mice, in which eNOS in the adipose tissue was undetectable despite a lack of change in eNOS levels in most other tissues. Interestingly, eNOS expression was increased in the hearts of db/db mice (Online Figure IA, C), which might be a compensatory change in response to an increase in NO demand. These data show that both obesity and diabetes result in tissue-specific changes in eNOS expression, with a profound and selective decrease in eNOS levels in the adipose tissue. This decrease in eNOS in adipose tissue is consistent with previous reports in obese humans24,25 and in mouse models of obesity,26 indicating that the expansion of adipose tissue establishes a state of chronic eNOS deficiency.

Overexpression of eNOS Prevents Diet-Induced Obesity
To examine the role of eNOS, we used eNOS-TG mice.13 Previous studies have shown that these mice reproduce in a Mendelian fashion, maintain normal growth characteristics, and are protected from numerous pathologies, including myocardial,27 hepatic,28 lung,29 and vascular injury,30 as well as sepsis.31 In comparison with WT mice, hemizygous mice showed a four-fold increase in eNOS levels in the aorta, with no significant change in eNOS levels in the adipose tissue (Figure 1D, E). In contrast, in homozygous mice there was a two-fold increase in eNOS in the adipose tissue and a six-fold increase in the aorta. The eNOS in TG animals localized exclusively with isolectin staining (Online Figure II), indicating that the transgene was expressed only in the vasculature.13,32 Plasma from eNOS-TG mice showed increased L-citrulline and nitrite levels when compared with WT mice (Online Figure IIIA, C), and adipose tissue from eNOS-TG mice demonstrated an increase in L-citrulline (Online Figure IIIB). Because of high variability, there were no significant differences in nitrate or nitrite in adipose tissue (Online Figure IIID), perhaps because of other confounding factors, such as nitrite/nitrate found in the diet or reduction of nitrite to NO.

When placed on a high-fat diet for 6 weeks, the homozygous eNOS-TG mice gained 50% less weight than WT mice, and this effect persisted for 12 weeks (Figure 2B, C). Food intake was not different between WT and eNOS-TG mice (Figure 2D). A more modest resistance to weight gain also was observed in hemizygous eNOS-TG mice (Online Figure ID), perhaps because of lower adipose tissue eNOS levels in these mice compared with homozygous eNOS-TG mice. Hence, for all subsequent studies, only eNOS homozygous mice were used. Measurements of body composition by dual-energy...
X-ray absorptiometry (Dexascan) showed that after 6 weeks of high-fat feeding, the body fat content was much lower in eNOS-TG mice than in nontransgenic mice (Figure 2E, F). The transgenic mice maintained a higher percent of lean mass (Figure 2G), although the tibia length in transgenic mice was only slightly smaller than in WT mice (Figure 2H). These observations indicate that overexpression of eNOS decreases adiposity and prevents weight gain induced by high-fat diet.

**eNOS Overexpression Increases Whole-Body Metabolism**

To determine how eNOS overexpression affected whole-body metabolism, we measured oxygen consumption (V\text{O}_2), carbon dioxide production (V\text{CO}_2), and activity in high-fat-fed WT and eNOS-TG mice over the course of a 12-hour dark period and a 4.5-hour light period. The fact that food intake was not different between WT and TG mice (Figure 2D) indicates that the lean phenotype of eNOS-TG mice is not attributable to a decrease in food consumption. This view is reinforced by the observation that high-fat feeding increased plasma cholesterol and leptin to similar levels in WT and eNOS-TG mice (Table). In comparison with WT mice, eNOS-TG mice showed higher mean V\text{O}_2 and V\text{CO}_2 rates throughout the dark and light periods (Figure 2I, J), with no change in the respiratory exchange ratio (Figure 2K). Activity levels assessed by horizontal activity count (beam breaks) showed similar patterns and levels of activity, and total ambulatory activity was not significantly different (Figure 2L). Taken together, these observations suggest that on a high-fat diet, eNOS-TG mice maintain a higher metabolic rate than WT mice. This increase in systemic metabolism, however, cannot be attributed to thyroid hormones, because plasma levels of triiodothyronine and thyroxine in WT and eNOS-TG mice were not significantly different (Online Figure IV).

**Effect of eNOS on Diet-Induced Insulin Resistance**

Because we found that eNOS overexpression decreased diet-induced weight gain, we expected concurrent changes in insulin resistance. We found that overexpression of eNOS completely prevented diet-induced hyperinsulinemia (Table 1), although plasma levels of adiponectin and resistin were not affected. This was associated with a remarkably lower homeostasis model of assessment-insulin resistance score (WT low-fat, 1.45±0.65; WT high-fat, 34.4±5.3; P<0.05 vs WT low-fat; TG low-fat, 6.9±3.1; TG high-fat, 8.2±2.9; P<0.05 vs WT high-fat). Moreover, even though 6 weeks of high-fat feeding did not significantly increase triglycerides or plasma nonesterified free fatty acids, both of these were decreased by 50% in the TG mice compared with WT mice (Table 1). We did not find significant differences in plasma glycerol between the groups (Table 1), suggesting that adipose tissue lipolysis was not affected. Other parameters measured in the plasma are shown in Online Table I. Collectively, these data indicate that overexpression of eNOS prevents high-fat diet-induced hyperinsulinemia and decreases plasma triglycerides and fatty acids.

To examine how eNOS overexpression affects systemic glucose disposal, WT and eNOS-TG mice were placed on a high-fat diet for 6 weeks, and glucose and insulin
tolerance tests were performed. There was no significant difference in the basal blood glucose levels in nonfasted WT and eNOS-TG mice (Figure 3A). After a fast of 6 hours, the plasma glucose levels of both high-fat groups were significantly increased compared with the WT low-fat-fed group. Fasting for 16 hours resulted in near-normalization of blood glucose in WT mice; however, glucose levels in the eNOS-TG mice remained slightly, but significantly, elevated (Figure 3A). There were no significant differences in plasma HbA1c in any group (Figure 3B). Measurements of insulin resistance by glucose tolerance test indicated that, in both WT and eNOS-TG mice, high-fat diet led to an increase in the glucose tolerance test area under the curve, indicating the onset of insulin resistance (Figure 3C, D); however, there were no significant differences when area under the curve was compared (Figure 3E). Measurements of insulin tolerance (Figure 3F–H) showed no significant difference in glucose disposition in any of the groups.

To test whether the effects of the transgene would manifest after prolonged feeding, we placed WT and eNOS-TG mice on high-fat diet for 12 weeks and assessed insulin resistance. At completion of the feeding protocol, the glucose tolerance test and insulin tolerance test curves were superimposable, suggesting that eNOS overexpression does not affect diet-induced insulin resistance even after prolonged nutrient excess (Online Figure VA–D). Although, plasma glucose levels in nonfasted and 6-hour fasted mice were not statistically different, a 16-hour fast led to a greater decrease in blood glucose in WT mice (Figure 3C, D); however, there were no significant differences when area under the curve was compared (Figure 3E). Measurements of insulin tolerance (Figure 3F–H) showed no significant difference in glucose disposition in any of the groups.
VE), indicating that the TG mice were more resistant to starvation-induced hypoglycemia, which could be attributable to increased gluconeogenesis in the liver. To test this, we performed pyruvate tolerance tests, which did not show remarkable differences between WT and TG mice (Online Figure VF, G), indicating that resistance to hypoglycemia in TG mice may not be attributable to increased hepatic production of glucose. Collectively, these data suggest that eNOS

Table. Parameters Measured From Plasma of Low-Fat Diet-Fed and High-Fat Diet-Fed Wild-Type and Endothelial Nitric Oxide Synthase Transgenic Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT LFD</th>
<th>WT HFD</th>
<th>eNOS-TG LFD</th>
<th>eNOS-TG HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Insulin (pg/mL)</td>
<td>116.2±40.7</td>
<td>301.6±537.3†</td>
<td>480.8±95.4¶</td>
<td>568.1±175.6</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>22.0±3.8</td>
<td>25.7±4.0</td>
<td>27.8±3.0</td>
<td>30.1±3.8</td>
</tr>
<tr>
<td>Resistin (pg/mL)</td>
<td>2449.8±156.1</td>
<td>7894.0±1155.0†</td>
<td>3547.3±387.2¶</td>
<td>6251.3±497.9§**</td>
</tr>
<tr>
<td>Leptin (pg/mL)</td>
<td>209.0±38.3</td>
<td>5099.0±1265.0†</td>
<td>997.4±203.4¶</td>
<td>4874.4±783.1§**</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>96.4±2.7</td>
<td>117.8±5.9†</td>
<td>92.1±4.4¶</td>
<td>124.0±6.8§**</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>37.8±2.5</td>
<td>46.2±4.8</td>
<td>26.2±3.0¶</td>
<td>23.8±2.8§</td>
</tr>
<tr>
<td>NEFA (mEq/L)</td>
<td>0.39±0.05</td>
<td>0.38±0.06</td>
<td>0.19±0.04¶</td>
<td>0.16±0.02§</td>
</tr>
<tr>
<td>*Glycerol (mg/L)</td>
<td>11.2±1.2</td>
<td>14.7±1.9</td>
<td>12.0±1.4</td>
<td>12.7±0.5</td>
</tr>
</tbody>
</table>

*eNOS indicates endothelial nitric oxide synthase; HFD, high-fat diet; LFD, low-fat diet; NEFA, nonesterified free fatty acids; TG, transgenic; WT, wild-type.

WT and eNOS-TG mice were fed LFD or HFD for 6 wk. Plasma from the mice was used to measure the indicated parameters.

*n=6 to 7 mice per group. For all other parameters, the groups contained 13 to 14 mice per group; †WT LFD vs WT HFD; ‡WT LFD vs eNOS-TG LFD; §WT LFD vs eNOS-TG HFD; ¶WT HFD vs eNOS-TG LFD; WT HFD vs eNOS TG HFD; **eNOS-TG LFD vs eNOS-TG HFD.

**Figure 3. Effect of endothelial nitric oxide synthase (eNOS) overexpression on indices of insulin resistance.** After 6 weeks of a low-fat diet (LFD) or high-fat diet (HFD), glucose tolerance and insulin sensitivity were examined in wild-type (WT) and eNOS transgenic (eNOS-TG) mice. A. Nonfasting and fasting glucose levels; white bars, WT LFD; blue bars, eNOS-TG LFD; white hatched bars, WT HFD; blue hatched bars, eNOS-TG HFD. B, HbA1c. Glucose tolerance tests (C–E) and insulin tolerance tests (F–H). n=14 per group. *P<0.05 vs WT LFD or otherwise indicated groups.
overexpression does not significantly affect diet-induced insulin resistance or glucose intolerance, but maintains glucose homeostasis during starvation.

**Effect of eNOS on Adipose Tissue**

Given our observations that obesity and diabetes were associated with a selective decrease of eNOS levels in adipose tissue and that eNOS-TG mice were resistant to diet-induced weight gain, we measured changes in adipocyte area and size in epididymal fat pads. These measurements revealed that high-fat diet induced adipocyte hypertrophy, leading to a three-fold to four-fold increase in mean adipocyte area (Figure 4A, B). Moreover, the high-fat diet promoted size heterogeneity in WT, but not eNOS-TG mice (Figure 4C), indicating that eNOS overexpression prevents diet-induced adipocyte hypertrophy and size dispersion.

In murine models of diet-induced obesity, adipocyte hypertrophy is associated with inflammation and accumulation of macrophages in adipose tissue.\(^{18,33,34}\) This is commonly recognized by the presence of crown-like structures that appear between adipocytes.\(^{18,33,34}\) In humans, obesity is similarly associated with adipose tissue inflammation, and weight loss interventions such as bariatric surgery improve endothelial function.\(^{35,36}\) Therefore, we examined adipose tissue inflammation in WT and eNOS-TG mice after 6 weeks of high-fat diet. Analysis of adipose tissue showed no significant difference in the abundance of crown-like structures between WT and TG mice (Figure 4A), and analysis of the adipose tissue stromal vascular fractions showed no difference in total F480* cells or changes in macrophage subtypes (Online Figure VI). These results are in accordance with studies showing that macrophage accumulation and insulin resistance occur only with prolonged high-fat feeding (>10 weeks)\(^{22,17}\) and suggest that the antihypertrophic effects of eNOS are not associated with significant changes in adipose tissue inflammation, but are likely to be related to favorable changes in metabolism that prevent lipid accumulation and adipocyte expansion.

**Metabolic Changes in Adipose Tissues of eNOS-Overexpressing Mice**

The lean phenotype of eNOS-TG mice and their resistance to diet-induced weight gain and adipocyte expansion clearly indicated to us that eNOS overexpression has a significant impact on adipocyte metabolism. Therefore, to assess this impact, we measured metabolite levels in epididymal adipose tissue of high-fat-fed WT and eNOS-TG mice using ultra-high-performance liquid chromatography/tandem mass spectrometry and gas chromatography mass spectrometry. Spectral data were identified, searched against a standard library, and quantified (see Online Supplement and Online Figure VII for details). Internal standards, including injection standards, process standards, and alignment standards were used for quality control and to control for experimental and instrument variability. This analysis led to the identification of 192 metabolites, of which 37 were significantly different between WT and eNOS-TG mice (Figure 5A and Online Table II). Although intermediates in the glycolytic pathway and the citric acid cycle were not affected, there were significant increases in propionylcarnitine, acetylcar

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**Figure 4. Endothelial nitric oxide synthase (eNOS) overexpression decreases diet-induced adipocyte hypertrophy.** Adipocyte size measurements from wild-type (WT) and eNOS transgenic (eNOS-TG) mice fed low-fat diet (LFD) or high-fat diet (HFD) for 6 weeks. A, Representative hematoxylin and eosin-stained images of adipose tissue from the epididymal fat pad (×20 magnification; scale bar=100 μm). B, Mean adipocyte area. C, Distribution of adipocyte sizes from mice fed a LFD (top) and a HFD (bottom). n=5 per group. *P<0.05 vs WT LFD; †P<0.05 vs WT HFD.
indicated that overexpression of eNOS stimulates amino acid and fatty acid metabolism in adipose tissue.

**Adipose Tissue Mitochondria Are Increased in eNOS-TG Mice**

Favorable changes in branched chain amino acid and fatty acid metabolism are indicative of increased mitochondrial activity. Previous studies have shown that branched chain amino acids increase mitochondrial biogenesis, and that this is attenuated in eNOS-null mice. In addition, it has been reported that NO triggers mitochondrial biogenesis in adipocytes and that deletion of eNOS decreases mitochondrial content in adipose tissue. Based on this evidence, we hypothesized that the change in branched chain amino acids and fatty acid metabolism in the adipose tissue of eNOS-TG mice may be related to greater mitochondrial content. Adipose tissue, but not skeletal muscle, from eNOS-TG mice showed significant increases in key mitochondrial proteins such as COX4I1, Sirt3, and ALDH2 (Figure 7A, C). The increase in mitochondrial proteins in TG adipose tissue could be attributable to remodeling of the mitochondria or an increase in mitochondrial abundance. To distinguish between these possibilities, sections of adipose tissue were stained with a nonmembrane potential-dependent mitochondrial stain, mitoID-Red. As shown in Figure 7E, adipose tissue isolated from high-fat-fed eNOS-TG mice stained more strongly than that from WT mice, indicating that the adipose tissue mitochondrial content was higher in TG than in WT mice. Adipocytes isolated from high-fat-fed eNOS-TG mice were more brown than those isolated from WT mice (Figure 7F), suggesting an increase in mitochondrial cytochromes. In addition to increased abundance of COX4I1 (Figure 7A, C), the expression of the mitochondrial gene cytochrome, cytochrome b6 (cytb6), was elevated two-fold in eNOS-TG mice (cytb6:gapdh ratios, fold change: WT, 1.0±0.1; eNOS-TG, 2.0±0.3; n=4–7/group; P<0.05). That this increase in mitochondrial content may be attributable to increased biogenesis is supported by our observation that in comparison with WT mice, TG mice had higher adipose levels of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha, as well as an increase in ppara and pparγ (Figure 7G), factors that are important activators of mitochondrial biogenesis.

**Effect of eNOS on Adipose Tissue Metabolic Flux**

To assess the functional implications of our observations, we measured oxygen consumption in adipose tissue explants using extracellular flux technology. As shown in Figure 8B, adipose tissue from eNOS-TG mice showed a significantly higher oxygen consumption rate compared with adipose tissue from WT mice. To determine the contribution of mitochondria to the oxygen consumption rate, we treated the explants with the electron transport chain inhibitors antimycin A and rotenone. The stabilized rate measured thereafter was used to calculate the mitochondria-derived oxygen consumption rate, which was two-fold higher in eNOS-TG compared with WT adipose tissue (Figure 8C). No statistically significant difference was observed in the extracellular acidification rate, a surrogate index of glycolysis (Figure 8D). Collectively, these observations corroborate our metabolic, biochemical, and

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**Figure 5. Metabolomic analyses of adipose tissues from high fat-fed mice.**

Metabolomic analyses of epididymal adipose tissue metabolites from wild-type (WT) and endothelial nitric oxide synthase transgenic (eNOS-TG) mice fed high-fat diet (HFD) for 6 weeks. A, Univariate analysis: t tests of compounds from adipose tissues. All metabolites above the dotted line were found to be significantly different between WT and eNOS-TG mice (P<0.05). A table of these metabolites can be found in the Data Supplement (Online Table II). B, Multivariate analysis: partial least-squares discriminant analysis (PLS-DA). C, Hierarchical clustering: heatmap and dendogram using the most significantly different metabolites. D, The significant metabolites were subjected to pathway impact analysis using Metaboanalyst MetPA and the *Mus musculus* pathway library. Fisher exact test was used for overrepresentation analysis, and relative between-ness centrality was used for pathway topology analysis. n=14 animals: 7 WT HFD, 7 eNOS-TG HFD.
anatomic measurements by demonstrating directly that the adipose tissue of eNOS-TG mice maintains a hypermetabolic state that could at least partially account for their increase in whole-body oxygen consumption and resistance to obesity.

**Discussion**

The major findings of this study are that high-fat diet results in the downregulation of eNOS in adipose tissue and that overexpression of eNOS prevents diet-induced obesity. These findings support a causal role of eNOS in regulating obesity and whole-body metabolism. Our results suggest that the mechanism of this antiobesogenic effect of eNOS is related to an increase in whole-body oxygen consumption associated with increased mitochondrial abundance and activity in the adipose tissue. Collectively, these observations support the notion that NO is an important regulator of adipocyte metabolism and therefore weight gain attributable to a high-fat diet. Although it has been shown before that deletion of eNOS gives rise to features of metabolic syndrome, the rescue of the obese phenotype by increasing eNOS indicates that enhancing eNOS expression can overcome the metabolic changes caused by consumption of high-fat diet.

Several lines of evidence gathered during this study support the view that the antiobesogenic effects of eNOS are attributable to favorable changes in adipocyte metabolism. Although on the basis of current results we cannot rule out or even fully assess all potential systemic effects, our observations that food consumption, activity, plasma levels of cholesterol, leptin, and thyroid hormones were not different between WT and TG mice argue against a global systemic change that could completely account for the lean phenotype of the TG mice. Both insulin resistance and obesity are complex phenotypes that are regulated by multiple interactions between several tissues, some or all of which might be affected in a manner not captured by our current analysis. Nevertheless, in regulating obesity, the adipose tissue appears to be a major target of eNOS. Our gene–dosage studies show that despite a decrease in eNOS expression in adipose tissue, some or all of which might be affected in a manner not captured by our current analysis. Nevertheless, in regulating obesity, the adipose tissue appears to be a major target of eNOS. Our gene–dosage studies show that despite a decrease in eNOS expression in adipose tissue, an increase in eNOS in adipose tissue. Only in homozgyous mice, in which eNOS was increased both in adipose tissue and aorta, did the antiobesogenic effects of eNOS become apparent. This association of the lean phenotype with eNOS expression in adipose tissue supports the view that an increase in NO in adipose depots may be required for the manifestation of the antiobesogenic effects of eNOS.

**How does eNOS regulate adipose tissue metabolism?**

Our results suggest that eNOS supports both mitochondrial

**Figure 6. Overexpression of endothelial nitric oxide synthase (eNOS) regulates intermediary metabolism in adipose tissue.** Metabolite analysis from adipose tissues of wild-type (WT) and eNOS transgenic (eNOS-TG) mice fed high-fat diet (HFD) for 6 weeks. A, The z-score plots of significantly changed metabolites. B, Correlation analysis was assessed using the Spearman rank correlation test, and the metabolites that correlated with citrulline were then examined. C, Superpathway and subpathway distribution of adipose tissue metabolites found to be significantly different between WT and eNOS-TG mice. n=14 animals: 7 WT HFD and 7 eNOS-TG HFD.
biogenesis and metabolic activity. Previous observations showing that β-oxidation is impaired in eNOS-null mice and that dietary supplementation with the NO precursor nitrite reverses features of metabolic syndrome in eNOS-null mice are supportive of this concept. Although AMP kinase has been shown to relate with NO levels, we did not find an increase in the phosphorylation state of AMP kinase in adipose tissue (Online Figure VIII). However, we did find elevated levels of several metabolites such as branched chain amino acids and short-chain acylcarnitines (eg, acetylcarnitine, propionylcarnitine) in the adipose tissue of TG mice that were indicative of high metabolic activity. Interestingly, oral supplementation with propionylcarnitine reduces obesity and hyperinsulinemia in obese rats, which at least partially recapitulates the phenotype of eNOS-TG mice. We also found in the adipose tissue of TG mice elevated levels of proteins such as peroxisome proliferator-activated receptor-γ coactivator 1 alpha (PGC1α), ALDH2, COX4I1, and Sirt3. That the increase in these proteins was functionally significant is reflected by our observations that mitochondrial abundance and the rates of fatty acid oxidation were higher in the adipose tissue from eNOS-TG mice. On the basis of these observations, we propose that high levels of eNOS lead to an increase in mitochondrial biogenesis and stimulation of fatty acid oxidation. This establishes a state of heightened metabolism that attenuates the obesogenic effects of high fat consumption.

Although our results show that eNOS overexpression increases adipose tissue metabolism by increasing mitochondrial content and activity, metabolic activity also could be affected by eNOS-dependent changes in oxygen distribution. Hence, it is possible that adipocytes of eNOS-TG mice are better-perfused than those of WT mice. Such an increase in tissue perfusion could be attributable to either regulatory effects on vascular tone and O₂ consumption or an increase in angiogenesis. Nevertheless, we found that capillary density was unaffected by eNOS overexpression, because isolectin B4 staining per adipocyte and VEGFR2 expression were similar between the groups (Online Figure IX), suggesting that an increase in angiogenesis is unlikely to be a reason underlying the lean phenotype of eNOS-TG mice.

The metabolic role of eNOS, however, appears to be tissue-specific. We found that high-fat feeding decreased eNOS in the adipose tissue but not in the heart or the skeletal muscle. Hence, we expected that overexpression of eNOS would ameliorate adipose tissue hypertrophy without...
not skeletal muscle, and therefore elevated levels of eNOS in the adipose tissue prevent obesity without affecting systemic insulin resistance.

Results showing that overexpression of eNOS prevents obesity without affecting insulin resistance also suggest that the two symptoms of metabolic syndrome could be dissociated from one another. Similar segregation between obesity and insulin resistance has been described previously. For instance, it has been shown that overexpression of adiponectin completely rescues the diabetic phenotype of ob/ob mice while promoting morbid obesity. Moreover, the observations that decreasing inflammation does not result in lower adiposity but improves insulin sensitivity, and that PPARγ agonists decrease insulin resistance but increase weight gain provide additional support to the concept that obesity and diabetes are disconnected and, in some cases, even conflicting events in the etiology of metabolic disease. However, it remains to be established how eNOS prevents hyperinsulinemia and impacts other processes that are associated with insulin resistance, such as inflammation. It is currently believed that because of excessive adipocyte expansion, hypoxia and necrosis occur in adipose tissue, which in turn leads to the recruitment of inflammatory cells. The resultant low-grade chronic inflammation is proposed to establish a state of insulin resistance. However, the eNOS-TG mice develop the antiobesogenic phenotype far before macrophage infiltration, inflammation, and insulin resistance in adipose tissue occur.22,37

It is important to note that the eNOS-TG mice did not display a lipodystrophic phenotype. Lipodystrophy in humans and animal models generally results in severe hypertriglyceridemia, hyperinsulinemia, and insulin resistance. The eNOS-TG mice, however, show decreased triglycerides and were protected from hyperinsulinemia despite development of diet-induced glucose intolerance. The prevention of hyperinsulinemia does not appear to be attributable to a pancreatic defect; baseline insulin levels were not significantly different from WT mice (Table 1), the glucose tolerance test showed a normal profile (Figure 3 and Online Figure V), and the pancreatic islets from eNOS-TG mice appeared unremarkable (Online Figure X). These observations raise the interesting possibility that hyperinsulinemia in response to systemic insulin resistance may be partly regulated by the adipose tissue, although additional work is required to fully understand this relationship.

Additional investigations also will be required to assess how high-fat diet affects eNOS activity and expression. Although it has been shown that eNOS levels are suppressed in high-fat diet in part because of tumor necrosis factor–dependent mechanisms, the effects of diet on eNOS protein and activity are less clear. The eNOS protein is subject to several posttranslational modifications, including phosphorylation, O-GlyNAcylation, S-glutathiolation, and acylation. In addition, the enzyme also could be uncoupled and therefore generate superoxide instead of synthesizing NO. Interestingly, we found that whereas eNOS monomer abundance was maintained in eNOS-TG mice (Online Figure XI), the phosphorylation of eNOS at Ser1177 and abundance of the eNOS dimer were significantly decreased in both WT and TG mice fed a high-fat diet (Online Figure XI). Although these changes in the eNOS-TG mice...
might be compensated by continually elevated levels of eNOS protein, as evidenced by persistently elevated citrulline levels (Online Figure IIIA, B), such changes in WT mice might result in a chronic state of NO deficiency. Moreover, uncoupling of the enzyme could lead to increased superoxide production and the formation of the toxic metabolite peroxynitrite. We found increased nitrotyrosine formation in adipose tissue of high-fat-fed mice (Online Figure XII), although this was not significantly affected by eNOS overexpression. Hence, in future studies it will be important to identify the processes that regulate eNOS activity and how they might be involved in the development of diet-induced obesity and insulin resistance.

In conclusion, the present study shows that preventing eNOS depletion by forced expression of the eNOS transgene attenuates diet-induced obesity in mice, without ameliorating systemic insulin resistance. These findings reveal a novel antiobesogenic role of eNOS and are consistent with the notion that eNOS prevents weight gain in high-fat-fed mice by stimulating mitochondrial biogenesis and activity in adipose tissues. Further understanding of this role of eNOS could lead to the development of new therapeutic modalities for preventing obesity and weight gain in human populations.

Acknowledgments

The authors thank Chris Kevil (Louisiana State University) for providing the eNOS-TG mice, as well as Dan Conklin, Don Mosley, and Emily Steinmetz for their help in animal handling and metabolic chamber studies.

Sources of Funding

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Disclosures

None.

References


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60. Musicki B, Kramer MF, Becker RE, Burnett AL. Inactivation of phosphorylated endothelial nitric oxide synthase (Ser-1177) by O-GlcNAcNA
What Is Known?

- Obesity is positively and robustly associated with the risk of development of cardiovascular disease and diabetes.
- Vascular dysfunction and, in particular, deficits in endothelial-derived nitric oxide (NO) production and bioavailability are associated with insulin resistance, adiposity, and deleterious changes in metabolism.

What New Information Does This Article Contribute?

- The expression of endothelial NO synthase (eNOS) in adipose tissue is decreased in mouse models of obesity and type 2 diabetes.
- Transgenic overexpression of eNOS in mice decreases circulating fatty acids and prevents obesity and hyperinsulinemia induced by a high-fat diet.
- Overexpression of eNOS prevents adipocyte hypertrophy, increases mitochondrial abundance and activity, and regulates branched chain amino acid metabolism.

Vascular dysfunction and decreased NO bioavailability are associated with metabolic syndrome; however, the therapeutic effects of increasing endogenous NO production have not been tested, and the effects of NO on metabolism are poorly understood. We show here that consumption of a high-fat diet decreases the abundance of eNOS in adipose tissue and that increasing eNOS expression prevents diet-induced obesity. The antiobesity effect of eNOS was associated with enhanced mitochondrial activity and significant changes in amino acid and lipid metabolism in adipose tissue. These findings suggest that eNOS prevents obesity by increasing the ability of adipocytes to burn excess fat. This novel regulation of adipocyte phenotype adds to our growing knowledge of the plasticity of adipose tissue and suggests that eNOS could regulate brown or beige-like gene programs. Thus, increasing NO availability or production may be an important therapeutic strategy for preventing obesity and its cardiovascular complications.
Overexpression of Endothelial Nitric Oxide Synthase Prevents Diet-Induced Obesity and Regulates Adipocyte Phenotype


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SUPPLEMENTAL MATERIAL

OVEREXPRESSION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE PREVENTS DIET-INDUCED OBESITY AND REGULATES ADIPOCYTE PHENOTYPE

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A. Detailed Methods

Animal studies: The B6.BKS(D)-Leprdb/J (db/db) mice and C57BL/6J (wild-type; WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The eNOS-TG mice, which express bovine eNOS under the control of the preproendothelin-1 promoter1, were maintained on the C57BL/6J background. At 8 weeks of age, male mice were placed on either a 10% low fat diet (LFD; Research Diets, Inc., #D12450B) or a 60% high fat diet (HFD; Research Diets Inc., #D12492) and maintained for 6–15 additional weeks. Water and diet were provided ad libitum. Body weights were recorded weekly. During the 7th and 13th weeks of feeding, glucose and insulin tolerance tests were performed. Pyruvate tolerance tests were performed only after the 13th week of feeding; all other parameters were evaluated after euthanasia. All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

Expression analyses: Tissue homogenates were prepared exactly as described in Horrillo et al.2. Equal amounts of protein were separated by SDS-PAGE, electroblotted to PVDF membranes, and probed using primary antibodies according to the manufacturers’ protocol. All antibodies used in this study, their manufacturer, dilutions and diluents are shown in Online Table III. For HRP, Western blots were developed using ECL Plus followed by luminescence detection using a Typhoon 9400 variable mode imager (GE Healthcare). Quantification of band intensities was performed using ImageQuant TL software.

For quantitative RT-PCR, RNA was extracted from tissues using the RNeasy lipid tissue kit (Qiagen), followed by cDNA synthesis. Real-time PCR amplification was performed with SYBR Green qPCR Master Mix (SA Biosciences) using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and commercially available primers for pgc1α, cyt b6, gapdh, ppara, and ppary (SA Biosciences). Relative expression was determined by the 2−ΔΔCT method after internal normalization to hprt.
**Glucose, insulin, and pyruvate tolerance tests:** Glucose tolerance tests were performed following a 6 h fast by injection (i.p.) of D-glucose (1 mg/g) in sterile saline. Insulin tolerance tests were performed on nonfasted animals by i.p. injection of 1.5 U/kg Humulin R (Eli Lilly, Indianapolis, IN). After a 6 h fast, pyruvate tolerance tests were performed as described. Blood samples were obtained from the tail and glucose levels were measured at indicated time points using an Aviva Accu-Chek glucometer. The homeostatic model assessment of insulin resistance (HOMA-IR) score was calculated based on the formula: glucose (mmol) x insulin (mU/ml)/22.5.

**Biochemical analyses:** Total plasma cholesterol, high-density lipoproteins, low-density lipoproteins, triglycerides, total protein, albumin (cholesterol Cl II enzymatic kit; L-type TG-H kit; Bradford reagent, bromocresol green; Wako, Richmond, VA), alanine aminotransferase (ALT), creatine kinase (CK), lactate dehydrogenase (LDH), creatinine, hemoglobin A1c (HbA1c), aspartate aminotransferase (AST; Infinity, ThermoElectron) levels were measured using commercially available assay reagents as indicated. Assays were performed using a Cobas Mira Plus 5600 Autoanalyzer (Roche, Indianapolis, IN). Plasma insulin, adiponectin, and resistin were measured using commercially available Luminex kits (Millipore, Billerica, MA, USA) according to the manufacturer's guidelines. Plasma levels of non-esterified free fatty acids and glycerol were measured by ELISA (Wako Chemicals, Richmond, VA and Cayman Chemical, Ann Arbor, MI, respectively).

**Adipocyte size measurements and indices of inflammation:** Adipose tissue was excised at the time of euthanasia, and wet weight was recorded. All adipose tissue was either snap-frozen at −80°C or fixed in 10% formalin (Leica), paraffin embedded, and sectioned. The sections were stained in hematoxylin and eosin. Adipocyte cross-sectional area was determined using Nikon Elements. Some sections were stained with MitoID Red (Enzo Life Sciences, Farmingdale, NY) for qualitative observations of mitochondrial mass. Macrophages subpopulations in adipose tissue stromal vascular fractions were measured by flow cytometry as described before.

**Body composition and calorimetry:** Body composition was measured on anesthetized mice by dual-energy X-ray absorptiometry using a mouse densitometer (PIXImus2; Lunar, Madison, WI). Whole body energy expenditure; respiratory exchange ratio; food consumption; and locomotion, ambulatory and fine movements were measured using a physiological/metabolic cage system (TSE PhenoMaster System, Bad Homberg, Germany).

**Capillary density:** Capillary and blood vessel densities were quantified in paraffin-embedded adipose tissue section using fluorescently labeled isolectin B4 as described. Additionally, the expression of VEGFR2 was examined by Western blotting.

**Nitrite and nitrate measurements:** Plasma and adipose nitrite and nitrate were measured after methanol extraction of tissues by the Griess reaction coupled to HPLC separation using the ENO-20 (EiCom, Japan).

**Nitrotyrosine staining:** Adipose tissues were formalin-fixed, paraffin-embedded, and sectioned. To visualize nitrotyrosine adducts by immunofluorescence, the following steps were performed: (1) after antigen retrieval, the sections were blocked with Rodent Block M Blocking Reagent (Biocare, Concord, CA) for 1 hour at room temperature, and incubated with the primary anti-nitrotyrosine antibody (1:50) (Millipore, Temecula, CA) in Antibody Diluent with Background Reducing Components (Dako, Carpinteria, CA) overnight at 4°C. The sections were then incubated with a secondary antibody ECL Plex goat-α-rabbit IgG-Cy3 (1:500) (Amersham,
Pittsburgh, PA) for 1 hour at room temperature in the dark. Slides were mounted with SlowFade® Gold antifade reagent containing DAPI (Molecular Probes, Eugene, OR). Fluorescent photographs were obtained using an EVOS fluorescence microscope (Advanced Microscopy Group, Bothell, WA). Nitrotyrosine positive area (pixel) was quantified in five random fields (×20) per animal using MetaMorph software (Molecular Devices, Sunnyvale, CA). Negative controls were prepared by incubating the primary antibody with 10 mM nitrotyrosine (Sigma) in PBS for 1 hour at room temperature. Positive controls were prepared by incubated deparaffinized slides with 1 mM sodium nitrite and 1 mM hydrogen peroxide (in 100 mM sodium acetate, pH 5.0) for 20 min at room temperature. The slides were then stained as described above.

Adipose tissue bioenergetic measurements: The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of intact adipose tissue explants were measured using a Seahorse XF24 analyzer (Seahorse Bioscience, Billerica, MA). Briefly, freshly isolated epididymal adipose tissue was rinsed with unbuffered DMEM (Dulbecco’s modified Eagle’s medium, pH 7.4). The adipose tissue was cut into sections, and 10 mg were placed in each well of an XF 24 Islet Capture Microplate (Seahorse Bioscience, Billerica, MA). The tissue was then covered with a screen, which allows free perfusion while minimizing tissue movement. Unbuffered DMEM (500 μl) supplemented with 50 μM BSA-conjugated palmitic acid, 200 μM L-carnitine, and 2.5 mM D-glucose was then added to each well. At least two replicates from each animal were used for the assay, and each tissue section was examined to ensure absence of large vessels (which can skew oxygen consumption measurements). The plate was incubated at 37°C in a non-CO₂ incubator for 1 h prior to extracellular flux analysis. After three baseline measurements, a mixture of antimycin A (10 μM) and rotenone (1 μM) was injected. Following injection, the OCR was closely monitored until the rates stabilized, and then the experiment was terminated.

Adipose tissue metabolite profiling: White adipose tissue from the epididymal fat pad of fasted mice (16 h fast) was collected and snap-frozen in liquid nitrogen. At the time of analysis, sample metabolites were extracted with methanol. A recovery standard was introduced at the beginning of the extraction process. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS/MS platforms. Also included were several technical replicate samples created from a homogeneous pool containing a small amount of all study samples. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS.

LC/MS, LC/MS²: The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS² scans using dynamic exclusion.

GC/MS: The samples destined for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-
trifouroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramp was from 40° to 300° C in a 16 minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis. The information output from the raw data files was automatically extracted as discussed below.

Accurate mass determination and MS/MS fragmentation: In addition to the LIT front end, the LC/MS portion of the platform had a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer backend. For ions with counts greater than 2 million, an accurate mass measurement could be performed. Accurate mass measurements could be made on the parent ion as well as fragments. The typical mass error was less than 5 ppm. Fragmentation spectra (MS/MS) were typically generated in data-dependent manner, but if necessary, targeted MS/MS could be employed, such as in the case of lower level signals.

QA/QC: Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the samples, which are technical replicates of pooled samples. Values for instrument and total process variability were 5% for internal standards and 15% for endogenous biochemicals, respectively. For QA/QC purposes, a number of additional samples are included with each day’s analysis. Furthermore, a selection of QC compounds was added to every sample, including those under test. These compounds were carefully chosen so as not to interfere with the measurement of the endogenous compounds.

Metabolite identification: Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. More than 1000 commercially available purified standard compounds had been acquired registered into the Metabolon Laboratory Information Management System (LIMS) for distribution to both the LC and GC platforms for determination of their analytical characteristics. The combination of chromatographic properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity.

Curation: A variety of curation procedures were carried out to ensure that a high quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, mis-assignments, and background noise. Visualization and interpretation software were used to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected if necessary.

Bioinformatics: The bioinformatics system consisted of four major components, the LIMS system, data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools. The purpose of the LIMS system was to enable fully auditable laboratory automation through a secure, easy to use, and highly specialized system. The scope of the LIMS system encompasses sample accessioning, sample preparation and instrumental analysis and reporting and advanced data analysis. Some of the subsequent software systems were grounded in the LIMS data structures, which have been modified to leverage and interface with
the Metabolon information extraction and data visualization systems, as well as other data analysis software such as Metaboanalyst (http://www.metaboanalyst.ca/).

Metabolomic analysis: The general outline for how metabolomic data were analyzed is shown in Online Fig. VII. Metabolites with missing values were imputed by replacing missing values with half of the minimum positive value in the original data. Metabolites with greater than 57% of the values missing were omitted from the analysis. The data were then quantile normalized within replicates after log transformation. This step was performed to transform the intensity values so that the distribution was more Gaussian. T-test statistical comparisons were then performed. Further univariate and multivariate analysis, such as correlation analysis, principal component analysis and partial least squares discriminant analysis was then performed using the Metaboanalyst 2.0 software (http://www.metaboanalyst.ca/).8, 9.

Statistical analyses: Data are expressed as mean ± SEM. Multiple groups were compared using one-way or two-way ANOVA, followed by Bonferroni post-tests. Unpaired Student’s t test was used for direct comparisons. Statistical analyses were performed with the program “R” http://cran.r-project.org/, Metaboanalyst (http://www.metaboanalyst.ca/), and/or GraphPad 5.0. A P value less than 0.05 was considered significant.

B. Supplementary References


8. Xia J, Wishart DS. Metabolomic data processing, analysis, and interpretation using metaboanalyst. *Curr Protoc Bioinformatics*. 2011;Chapter 14:Unit 14 10

C. Online Tables

Online Table I. Parameters measured from plasma of low fat-fed and high fat-fed WT and eNOS-TG mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT LFD</th>
<th>WT HFD</th>
<th>eNOS-TG LFD</th>
<th>eNOS-TG HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL (mg/dl)</td>
<td>74.1±1.6</td>
<td>92.1±4.1\textsuperscript{†}</td>
<td>71.5±3.6\textsuperscript{†}</td>
<td>99.6±4.9\textsuperscript{§,@}</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>14.4±1.1</td>
<td>14.9±0.8</td>
<td>14.9±0.9</td>
<td>17.3±1.3</td>
</tr>
<tr>
<td>*Total protein (g/dl)</td>
<td>4.7±0.05</td>
<td>4.9±0.04</td>
<td>4.1±0.06\textsuperscript{†,†}</td>
<td>4.5±0.13\textsuperscript{§,@}</td>
</tr>
<tr>
<td>*Albumin (g/dl)</td>
<td>3.2±0.06</td>
<td>3.2±0.05</td>
<td>2.9±0.06\textsuperscript{†,†}</td>
<td>3.1±0.07</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>34.1±3.5</td>
<td>38.7±1.2</td>
<td>31.5±1.1</td>
<td>30.7±0.8\textsuperscript{#}</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>66.0±6.1</td>
<td>71.8±4.7</td>
<td>73.1±2.9</td>
<td>66.4±3.4</td>
</tr>
<tr>
<td>CK (U/l)</td>
<td>124.9±19.0</td>
<td>100.0±28.8</td>
<td>236.5±25.9\textsuperscript{†,†}</td>
<td>176.1±12.9</td>
</tr>
<tr>
<td>*LDH (U/l)</td>
<td>225.9±20.3</td>
<td>190.6±6.3</td>
<td>191.1±49.2</td>
<td>166.4±11.5</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.18±0.02</td>
<td>0.19±0.02</td>
<td>0.23±0.02</td>
<td>0.21±0.02</td>
</tr>
</tbody>
</table>

Wild-type (WT) and eNOS-TG mice were fed a low fat diet (LFD) or high fat diet (HFD) for 6 weeks. Plasma from the mice was used to measure the indicated parameters. *n = 6–7 mice per group; for all other parameters, the groups contained 13–14 mice per group.

\textsuperscript{†}WT LFD vs. WT HFD
\textsuperscript{‡}WT LFD vs. eNOS-TG LFD
\textsuperscript{§}WT LFD vs. eNOS-TG HFD
\textsuperscript{‖}WT HFD vs. eNOS-TG LFD
\textsuperscript{#}WT HFD vs. eNOS-TG HFD
\textsuperscript{@}eNOS-TG LFD vs. eNOS-TG HFD
Wild-type (WT) and eNOS-TG mice were fed a high fat diet (HFD) for 6 weeks. Epididymal adipose tissue was then subjected to LC or GC mass spectrometric analysis. Those metabolites found to be significantly different by t-test are listed above. The (-) indicates no KEGG identification number; FDR, false discovery rate. n = 7 mice per group.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>KEGG</th>
<th>Super pathway</th>
<th>Sub-pathway metabolism</th>
<th>p-value</th>
<th>–log(10)p</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-dehydrocarnitine</td>
<td>C02636</td>
<td>Lipid</td>
<td>Carnitine</td>
<td>1.0e-5</td>
<td>4.99</td>
<td>0.001</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>C00079</td>
<td>Amino acid</td>
<td>Phe/Tyr</td>
<td>1.2e-5</td>
<td>4.91</td>
<td>0.001</td>
</tr>
<tr>
<td>Histamine</td>
<td>C00388</td>
<td>Amino acid</td>
<td>His</td>
<td>2.3e-5</td>
<td>3.64</td>
<td>0.013</td>
</tr>
<tr>
<td>Citrulline</td>
<td>C00327</td>
<td>Amino acid</td>
<td>Urea/Arg/Pro</td>
<td>2.9e-5</td>
<td>3.53</td>
<td>0.013</td>
</tr>
<tr>
<td>Creatine</td>
<td>C00300</td>
<td>Amino acid</td>
<td>Creatine</td>
<td>4.8e-5</td>
<td>3.32</td>
<td>0.016</td>
</tr>
<tr>
<td>2-amino adipate</td>
<td>C00956</td>
<td>Amino acid</td>
<td>Lys</td>
<td>5.7e-5</td>
<td>3.24</td>
<td>0.016</td>
</tr>
<tr>
<td>Serine</td>
<td>C00065</td>
<td>Amino acid</td>
<td>Gly/Ser/Thr</td>
<td>6.4e-5</td>
<td>3.19</td>
<td>0.016</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>C00346</td>
<td>Lipid</td>
<td>Glycerolipid</td>
<td>8.6e-5</td>
<td>3.06</td>
<td>0.018</td>
</tr>
<tr>
<td>Pantothenate</td>
<td>C00864</td>
<td>Cofactors/Vitamins</td>
<td>Pantothenate/CoA</td>
<td>0.001</td>
<td>2.99</td>
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<td>0.192</td>
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Online Table III. Antibodies used in this study.

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<th>Target Antigen</th>
<th>Isotype</th>
<th>Source</th>
<th>Catalog No.</th>
<th>Application</th>
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<td>IB</td>
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<td>2°</td>
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Online Fig. I: Effects of nutrient excess on eNOS levels, and body weight gain of mice expressing different levels of eNOS. Panels A–C: Immunoblot analysis of eNOS expression in skeletal muscle and heart in C57BL/6J mice fed a low fat diet (LFD) or high fat diet (HFD) for 6 or 12 weeks; db/db mice age-matched to the 12 week feeding group were included as an additional model of metabolic syndrome. (A) Representative Western blots of eNOS expression in skeletal muscle and heart; (B) Quantification of skeletal muscle eNOS expression; and (C) Quantification of heart eNOS expression. n = 3–4 per group; ***p<0.001 vs. WT LFD. Panel D: Weight gain of wild-type, littermate eNOS hemizygous, and eNOS homozygous mice fed HFD over the course of 12 weeks. n = 4–8 per group.
Online Fig. II: The eNOS transgene localizes to the vasculature in adipose tissue. Immunofluorescence images of epididymal adipose tissue from eNOS-TG mice: Adipose tissues were fixed, sectioned, and stained with DAPI (blue), isoelectin (green), and eNOS antibody (red). The overlay shows the co-localization of the eNOS and isoelectin signals.
Online Fig. III: Measurements of eNOS and NO metabolites in plasma and adipose tissue. Mice were fed a LFD or HFD for 6 weeks and citrulline, nitrite, and nitrate levels in the plasma and adipose tissue were analyzed by LC/MS or HPLC. Panels A and B: Relative levels of L-citrulline from (A) plasma and (B) adipose tissue; Panels C and D: Measurements of nitrite from (C) plasma and (D) adipose tissue; Panels E and F: Measurements of nitrate from (E) plasma and (F) adipose tissue. n = 6–7 per group; *p<0.05 vs. WT LFD; #p<0.05 vs. TG LFD; $p<0.05 vs. WT HFD.
Online Fig. IV: Diet and genotype do not affect circulating free T3 or T4 levels. Free triiodothyronine (T3) and thyroxine (T4) were measured in plasma from WT and eNOS-TG mice that were fed LF or HF diets for 6 weeks. n = 4–6 per group.
Online Fig. V: Measures of insulin resistance and gluconeogenesis in WT and eNOS-TG mice fed a high fat diet for 12 weeks. After 12 weeks of HFD, glucose tolerance and insulin sensitivity were examined in WT and eNOS-TG mice: (A) Glucose tolerance test (GTT); (B) Insulin tolerance test (ITT); (C) GTT area under the curve (AUC); (D) ITT AUC; (E) Blood glucose under non-fasted, 6-h-fasted and 16-h-fasted conditions; (F) Pyruvate tolerance test (PTT) was used to determine differences in gluconeogenesis between the mice; and (G) PTT AUC. n = 4 per group; *p<0.05 vs. WT.
Online Fig. VI: Effects of high fat diet on macrophage subtypes in WT and eNOS-TG mice. 
Macrophage subpopulations measured in epididymal adipose tissues after 6 weeks of LFD or HFD:
(A–D) Representative flow cytometry dot plots of F4/80+ adipose tissue macrophages from WT and eNOS-TG mice. (E) Quantification of M1 macrophage subpopulations; (F) Quantification of M2 macrophage subpopulations; and (G) Quantification of macrophages doubly positive for M1 and M2 macrophage markers. n = 6 per group.
Online Fig. VII: Flow chart illustrating procedure for metabolomic profiling of adipose tissues. Mice were fed a HFD for 6 weeks. The adipose tissue was then procured, and metabolites were extracted. The samples were divided for GC/MS or LC/MS analysis. Following spectral analysis, the data were imputed, normalized, and analyzed using Metaboanalyst 2.0 software.
Online Fig. VIII: Western blot analysis of AMPK activation status. Mice were fed a LFD or HFD for 6 weeks and P-AMPK and total AMPK abundance were measured by western blotting. n = 3–4 per group.
Online Fig. IX: Overexpression of eNOS does not affect capillary density in adipose tissue. Fluorescence images and markers of capillary density in sections of epididymal adipose tissue isolated from WT or eNOS-TG mice fed a LFD or HFD for 6 weeks: (A) Representative images of isolectin B4 (green) staining. (B) Isolectin B4 staining quantified per adipocyte. n = 9 per group. (C) VEGFR2 expression in adipose tissue. Density of the VEGFR2 bands were normalized to amido black stain. n = 6 per group. Note: the apparent decrease in isolectin staining in HFD groups from panel A relates to an increase in adipocyte size relative to the LFD group.
Online Fig. X: Hematoxylin and eosin-stained images of pancreas from WT and eNOS-TG mice. Representative photomicrographs of pancreas isolated from WT and eNOS-TG mice fed a HFD for 6 weeks; ×20 magnification.
**Online Fig. XI: Analysis of eNOS expression and modification.** Immunoblotting of eNOS enzyme states that reflect eNOS activity state: WT and eNOS-TG (TG) mice were fed a LFD or HFD for 6 weeks and eNOS abundance and phosphorylation status were examined by immunoblotting. (A) Representative Western blots of eNOS dimer, Ser^1177^ phosphorylation of eNOS (P-eNOS), and the eNOS monomer; (B) Quantification of P-eNOS; (C) Quantification of the eNOS dimer; n = 3–4 per group; *p<0.05 vs WT LFD; #p<0.05 vs. TG LFD.
Online Fig. XII: High fat feeding increases protein-nitrotyrosine adducts in adipose tissue. Immunofluorescence images and quantification of nitrotyrosine adducts in adipose tissue: WT and eNOS-TG mice were fed a LFD or HFD for 6 weeks. The adipose tissue was stained for nitrotyrosine adducts, and the adducts were visualized by fluorescence microscopy. (A) Negative (–) and positive (+) controls for nitrotyrosine staining. (B) Representative images of nitrotyrosine staining in WT and eNOS-TG mice fed a LFD or HFD. (C) Quantification of nitrotyrosine adducts from adipose tissues. n = 3 per group; *p<0.05 vs. indicated group.