Endothelial Nitric Oxide Synthase Uncoupling and Perivascular Adipose Oxidative Stress and Inflammation Contribute to Vascular Dysfunction in a Rodent Model of Metabolic Syndrome

Chiara Marchesi, Talin Ebrahimian, Orlando Angulo, Pierre Paradis, Ernesto L. Schiffrin

Abstract—The metabolic syndrome represents a constellation of cardiovascular risk factors that promote the development of cardiovascular disease. Oxidative stress is a mediator of endothelial dysfunction and vascular remodeling. We investigated vascular dysfunction in the metabolic syndrome and the oxidant mechanisms involved. New Zealand obese (NZO) mice with metabolic syndrome and New Zealand black control mice were studied. NZO mice showed insulin resistance and increased visceral fat and blood pressure compared with New Zealand black mice. Mesenteric resistance arteries from NZO mice exhibited increased media:lumen ratio and media cross-sectional area, demonstrating hypertrophic vascular remodeling. Endothelium-dependent relaxation to acetylcholine, assessed by pressurized myography, was impaired in NZO mice, not affected by N\textsuperscript{G}-nitro-L-arginine methyl ester, inhibitor of endothelial NO synthase, and improved by the antioxidant Tempol, suggesting reduced NO bioavailability and increased oxidative stress. Dimer:monomer ratio of endothelial NO synthase was decreased in NZO mice compared with New Zealand black mice, suggesting endothelial NO synthase uncoupling. Furthermore, vascular superoxide and peroxynitrite production was increased, as well as adhesion molecule expression. Perivascular adipose tissue of NZO mice showed increased superoxide production and NADPH oxidase activity, as well as adipocyte hypertrophy, associated with inflammatory Mac-3-positive cell infiltration. Vasocostriction to norepinephrine decreased in the presence of perivascular adipose tissue in New Zealand black mice but was unaffected by perivascular adipose tissue in NZO mice, suggesting loss of perivascular adipose tissue anticontractile properties. Our data suggest that this rodent model of metabolic syndrome is associated with perivascular adipose inflammation and oxidative stress, hypertrophic resistance artery remodeling, and endothelial dysfunction, the latter a result of decreased NO and enhanced superoxide generated by uncoupled endothelial NO synthase. (Hypertension. 2009;54:1384-1392.)

Key Words: obesity ■ hypertension ■ NO ■ eNOS ■ superoxide ■ NADPH

The metabolic syndrome represents a constellation of cardiovascular risk factors of metabolic origin that promote the development of cardiovascular disease. Oxidative stress is a mediator of endothelial dysfunction and vascular remodeling. We investigated vascular dysfunction in the metabolic syndrome and the oxidant mechanisms involved. New Zealand obese (NZO) mice with metabolic syndrome and New Zealand black control mice were studied. NZO mice showed insulin resistance and increased visceral fat and blood pressure compared with New Zealand black mice. Mesenteric resistance arteries from NZO mice exhibited increased media:lumen ratio and media cross-sectional area, demonstrating hypertrophic vascular remodeling. Endothelium-dependent relaxation to acetylcholine, assessed by pressurized myography, was impaired in NZO mice, not affected by N\textsuperscript{G}-nitro-L-arginine methyl ester, inhibitor of endothelial NO synthase, and improved by the antioxidant Tempol, suggesting reduced NO bioavailability and increased oxidative stress. Dimer:monomer ratio of endothelial NO synthase was decreased in NZO mice compared with New Zealand black mice, suggesting endothelial NO synthase uncoupling. Furthermore, vascular superoxide and peroxynitrite production was increased, as well as adhesion molecule expression. Perivascular adipose tissue of NZO mice showed increased superoxide production and NADPH oxidase activity, as well as adipocyte hypertrophy, associated with inflammatory Mac-3-positive cell infiltration. Vasocostriction to norepinephrine decreased in the presence of perivascular adipose tissue in New Zealand black mice but was unaffected by perivascular adipose tissue in NZO mice, suggesting loss of perivascular adipose tissue anticontractile properties. Our data suggest that this rodent model of metabolic syndrome is associated with perivascular adipose inflammation and oxidative stress, hypertrophic resistance artery remodeling, and endothelial dysfunction, the latter a result of decreased NO and enhanced superoxide generated by uncoupled endothelial NO synthase. (Hypertension. 2009;54:1384-1392.)

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From the Lady Davis Institute for Medical Research (C.M., T.E., O.A., P.P., E.L.S.), Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montreal, Quebec, Canada; Department of Clinical Medicine (C.M.), University of Insubria, Varese, Italy.

Correspondence to Ernesto L. Schiffrin, Sir Mortimer B. Davis-Jewish General Hospital, #B-127, 3755 Cote Ste-Catherine Rd, Montreal, Quebec H3T 1E2, Canada. E-mail ernesto.schiffrin@mcgill.ca

C.M. and T.E. contributed equally to this article.

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Table 1. Physiological Variables of NZO and NZB Control Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NZB</th>
<th>NZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>32.0±1.2</td>
<td>49.8±1.6*</td>
</tr>
<tr>
<td>TL, mm</td>
<td>17.8±0.4</td>
<td>17.6±0.2</td>
</tr>
<tr>
<td>Heart weight/TL, mg/mm</td>
<td>8.5±0.3</td>
<td>11.2±0.6†</td>
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<tr>
<td>Kidney weight/TL, mg/mm</td>
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<td>33.2±0.7†</td>
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<tr>
<td>Liver weight/TL, mg/mm</td>
<td>77.0±1.6</td>
<td>136.7±6.6*</td>
</tr>
<tr>
<td>Spleen weight/TL, mg/mm</td>
<td>5.0±0.5</td>
<td>11.3±0.7*</td>
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<tr>
<td>Visceral fat weight/TL, mg/mm</td>
<td>17.2±3.5</td>
<td>147.4±6.3*</td>
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<td>Adipocyte CSA, μm²</td>
<td>1475.0±252.3</td>
<td>5704.0±372.1*</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>7.4±0.3</td>
<td>21.8±0.8*</td>
</tr>
<tr>
<td>Insulin, mg/L</td>
<td>0.9±0.2</td>
<td>4.7±0.2*</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>125.4±3.8</td>
<td>136.6±3.4‡</td>
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<tr>
<td>HR, bpm</td>
<td>603.5±24.1</td>
<td>641.8±6.8</td>
</tr>
</tbody>
</table>

Values are mean±SEM. n=8 to 10 mice per group. HR indicates heart rate; SBP, systolic BP; TL, tibia length.

*P<0.0001 vs NZB.
†P<0.001 vs NZB.
‡P<0.05 vs NZB.

reduction. Perivascular adipose tissue (PVAT) has been shown to have anticontractile effects via NO release and increasing hydrogen peroxide (H₂O₂) generation. Moreover, PVAT is a source of inflammatory cells that have been shown to contribute to vascular remodeling in different model of hypertension, in part through pro-oxidant mechanisms. There is increasing evidence that remodeling of adipose tissue may be associated with endoplasmic reticulum stress that leads to the unfolded protein reaction, which stimulates oxidative mechanisms and apoptosis and activates inflammatory mediators.

The hypothesis behind this study is that metabolic syndrome induces vascular remodeling and endothelial dysfunction in resistance arteries through oxidative stress. Here we questioned whether vascular remodeling, endothelial dysfunction, oxidative stress, and inflammation occur in a rodent model of human metabolic syndrome, investigated the vascular pro-oxidant mechanisms putatively involved, specifically NADPH oxidase and eNOS uncoupling, and determined the contribution of oxidative stress and inflammation of PVAT to vascular dysfunction.

**Methods**

Expanded materials and methods are provided in the online Data Supplement (please see http://hyper.ahajournals.org).

**Animals**

New Zealand obese (NZO/HILtJ [NZO]) and New Zealand lean (New Zealand black [NZB/BINJ [NZB]]) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The NZO mouse has been characterized by others as a model of human metabolic syndrome (please see the online Data Supplement). NZO mice exhibit polygenic obesity associated with hyperglycemia, hyperinsulinemia, increased serum cholesterol and triglyceride levels, and elevated BP, when compared with their control, the NZB mice.

**Blood Pressure**

Systolic BP and heart rate were determined by the tail-cuff technique using the Visitech systems BP-2000.

Figure 1. Endothelial function of mesenteric resistance arteries in NZO and NZB control mice. Concentration-response curves to acetylcholine (A), bradykinin (B), and sodium nitroprusside (C). Relaxation responses are percentage increase in lumen after precontraction with norepinephrine. Open and closed symbols represent NZB and NZO mice, respectively. Values are mean±SEM; n=7 mice per group. *P<0.0001; †P<0.01.
Functional Vascular Studies
Second-order branches of the mesenteric artery were dissected and studied on a pressurized myograph, as described previously.6,18 Endothelium-dependent relaxation was assessed by measuring the dilatory response to acetylcholine (10^{-9} to 10^{-6} mol/L) and bradykinin (10^{-10} to 10^{-6} mol/L) in norepinephrine precontracted vessels (5×10^{-5} mol/L). Endothelium-independent relaxation was assessed with sodium nitroprusside (10^{-8} to 10^{-3} mol/L) in precontracted vessels. NO availability and ROS production were evaluated by aacetylcholine concentration-response curve repeated after a 20-minute incubation with the NO synthase inhibitor \( \text{N}^\cdot \text{G}-\text{nitro-L-arginine methyl ester (L-NAME; 10^{-4} mol/L) and Tempol (10^{-3} mol/L), respectively. Concentration-response curves to norepinephrine (10^{-9} to 10^{-4} mol/L), endothelin-1 (10^{-10} to 10^{-7} mol/L), and angiotensin II (10^{-9} to 10^{-5} mol/L) were performed in the presence or absence of PVAT to evaluate the effect of the PVAT on vascular contractility.}

Vascular and Perivascular Adipose \( \text{O}_2^- \) Production and NADPH Oxidase Activity
Vascular \( \text{O}_2^- \) production was assessed on aorta and aortic and mesenteric PVAT with the \( \text{O}_2^- \)-sensitive fluorescent dye dihydroethidium (2 \mu mol/L). NADPH oxidase activity was assessed both in aorta and in the mesenteric artery, as well as in aortic and mesenteric PVAT, by lucigenin chemiluminescence assay, as described previously.19

Low-Temperature SDS-PAGE
Mesenteric artery extracts were prepared with sodium dodecyl sulfate sample buffer in nondenaturing conditions. Samples were loaded on 7.5% polyacrylamide gels and subjected to electrophoresis. Buffers and gels were cooled to 4°C, and the buffer tank was placed in an ice bath during electrophoresis. Endothelial eNOS monomer and dimer were detected by Western blotting analysis.

Plasma Nitrite Measurement
Plasma nitrites were evaluated with a colorimetric assay, as described previously.20

Data Analysis
Two-way repeated-measures ANOVAs were used to evaluate differences in concentration-response curves between groups, followed by Bonferroni post hoc analysis. Unpaired \( t \) test was performed to evaluate all of the other variables. \( P<0.05 \) was considered statistically significant. Results are presented as mean±SEM.
Results

Physiological Variables
Table 1 summarizes the metabolic and cardiovascular variables of NZO and NZB control mice. NZO mice had increased body weight, a greater amount of abdominal fat, and visceral adipocyte hypertrophy (please see Figure S1 in the online Data Supplement) compared with NZB mice. As well, subcutaneous adipose tissue was increased in NZO mice (data not shown). Tibia length was not different between groups. Plasma insulin was higher in NZO mice than in NZB mice. NZO mice showed increased heart, kidney, liver, and spleen weight. Systolic BP was slightly but significantly higher in NZO mice compared with NZB mice.

PVAT and Vascular Remodeling of Resistance and Conduit Vessels
Table 2 summarizes the vascular phenotype in NZO and NZB control mice. NZO mice exhibited increased PVAT and perivascular adipocyte hypertrophy in both aorta and mesenteric arteries compared with those of controls (Figure S1). Media cross-sectional area was increased in aorta of NZO mice compared with NZB mice. Mesenteric resistance arteries from NZO mice exhibited hypertrophic vascular remodeling, as shown by increased media thickness, media:lumen ratio (M/L ratio), and media cross-sectional area.

Endothelial Function of Resistance Arteries
Mesenteric arteries from NZO mice showed significantly impaired relaxation responses to acetylcholine and bradykinin compared with NZB mice (Figure 1A and 1B, respectively), indicating endothelial dysfunction. Endothelium-independent relaxation responses to sodium nitroprusside were similar in both groups, indicating integrity of the vascular smooth muscle cell layer (Figure 1C). In mesenteric arteries from NZB mice, relaxation to acetylcholine was significantly reduced by eNOS inhibition with L-NAME but unaffected by the antioxidant and \( \text{O}_2^- \) dismutase mimetic Tempol (Figure 2A and 2C, respectively). On the contrary, in mesenteric arteries from NZO mice, relaxation to acetylcholine was unaffected by L-NAME but was restored by Tempol (Figure 2B and 2D, respectively).

Mechanisms of Vascular Oxidative Stress and Inflammation in NZO and NZB Control Mice
In mesenteric arteries of NZO mice, eNOS phosphorylation and expression were increased, indicating enhanced eNOS activity in NZO mice compared with NZB mice (Figure 3A). However, the eNOS dimer:monomer ratio was decreased in NZO mice compared with NZB mice, which indicates the existence of eNOS uncoupling (Figure 3B). As a result of increased reaction between \( \text{O}_2^- \) and NO, vascular nitroty-
Perivascular Adipose Oxidative Stress and Inflammation

Perivascular adipose \(\text{O}_2^-\) production was increased in NZO mice compared with NZB mice, as well as in the vasculature (Figure 6A, left). NZO mice showed enhanced Mac-3 staining in aortic and mesenteric PVAT, suggesting macrophage infiltration (Figure 6A, right). Perivascular adipose mRNA levels of TNF-\(\alpha\) (tumor necrosis factor-\(\alpha\)) were increased in NZO mice, suggesting a proinflammatory phenotype of macrophages. As well, mRNA levels of MCP-1 (monocyte chemotactic peptide-1) were increased and adiponectin was decreased in NZO mice, indicating proinflammatory mechanisms in the PVAT (Figure 6B). NADPH oxidase activity was increased in aortic and mesenteric PVAT of NZO mice (Figure 6C). Expression of GTPase Rac1, cofactor of NADPH oxidase, was significantly greater in mesenteric PVAT of NZO mice compared with NZB mice (Figure 6D). The expression of the antioxidant \(\text{O}_2^-\) dismutase (-1, -2, and -3), which catalyzes the transformation of \(\text{O}_2^-\) in \(\text{H}_2\text{O}_2\), was lower in mesenteric PVAT of NZO mice compared with NZB mice (Figure 6D).

Discussion

Metabolic syndrome, with a prevalence of 40% in the adult population, is a critical medical issue in developed countries and requires the development of new therapies targeting more than one cardiovascular risk factor at a time. The availability of animal models allows for investigation of the pathophysiological mechanisms leading to metabolic syndrome. Here we demonstrated that the NZO mouse, a reliable model of human metabolic syndrome, presents vascular dysfunction caused by reduced NO bioavailability because of uncoupling of eNOS and increased ROS and reactive nitrogen species production. Moreover, we showed that perivascular fat contributes to vascular dysfunction, through reduced anticontractile effects, and we suggest inflammation and oxidative stress as possible mechanisms. Our findings extend recent data in the human metabolic syndrome, providing new pathophysiological mechanisms and a rodent model for future study.

In agreement with previous data, we showed that the NZO mouse exhibited visceral obesity, hyperinsulinemia, and BP increase, fulfilling the most commonly used criteria to define the metabolic syndrome in patients. Moreover, NZO mice presented increased heart, liver, and spleen weight, suggesting cardiac hypertrophy, steatohepatitis, and systemic inflammation, as described in humans.

Previous data have shown vascular dysfunction in conduit and small arteries in patients with metabolic syndrome. Similarly, we demonstrated vascular remodeling and endothelial dysfunction in NZO mice. Increased ROS production is involved in the mechanisms leading to vascular disease, reducing NO bioavailability and, therefore, promoting endothelial dysfunction, vasoconstriction, and increased vascular resistance. Indeed, vascular \(\text{O}_2^-\) production was increased in NZO mice compared with controls, and endothelium-dependent relaxation was impaired and was unaffected by inhibition of eNOS, indicating reduced bioavailability of NO. Moreover, endothelial dysfunction was improved by pretreatment with an antioxidant. This suggests a prominent role for ROS in the decreased NO bioavailability. In the vascular system of different models of hypertension and diabetes mellitus, ROS have been shown to be produced by different enzymatic sources, NADPH oxidase and uncoupled eNOS.
being the most important.\textsuperscript{23,24} Vascular NADPH did not contribute to increased ROS formation in NZO mice, suggesting different mechanisms of oxidative stress in metabolic syndrome compared with hypertension and diabetes mellitus. The dimer:monomer ratio is an indirect marker of eNOS uncoupling, because the normal function of eNOS requires its dimerization.\textsuperscript{25} We demonstrated eNOS uncoupling in our model of metabolic syndrome, suggesting its role as a source of vascular oxidative stress, in particular, NO and peroxynitrite, which result from the reaction between NO and O$_2$.\textsuperscript{-2} Recently, oral supplementation with tetrahydrobiopterin, an essential cofactor of eNOS, has been shown to lead to improvement of endothelial dysfunction in fructose-fed rats, a model of metabolic syndrome.\textsuperscript{26} However, an inverse relationship was shown recently between plasma and vascular biopterins in patients with coronary artery disease, raising concerns as to their role as biomarkers.\textsuperscript{27} Thus, it will be of interest in the future to address the role of tetrahydrobiopterin in this model and to investigate its potential as a therapeutic and diagnostic tool. Taken together, these findings suggest that uncoupling of eNOS is a major mechanism of oxidative stress in the metabolic syndrome, although we cannot exclude the contribution of other vascular sources of oxidative stress, such as xanthine oxidase, cyclooxygenase, and mitochondria, which deserve future study.

NZO mice exhibited hypertrophic vascular remodeling of both aorta and mesenteric resistance arteries. Eutrophic remodeling results from persistent vasoconstriction and extracellular matrix deposition, in the absence of growth response, whereas hypertrophic remodeling implies vascular smooth muscle cell growth and proliferation.\textsuperscript{28} In hypertension, reduction of oxidative stress has been shown to prevent hypertrophic remodeling,\textsuperscript{6} which suggests a key role for oxidative stress in vascular growth. Therefore, hypertrophic vascular remodeling in NZO mice is possibly because of both increased vasoconstriction and vascular smooth muscle cell proliferation and growth. Vascular oxidant mechanisms are likely to have contributed to remodeling of resistance arteries in NZO mice beyond the hemodynamic effects of BP increase.

Perivascular fat has been shown to regulate vascular tone through release of vasoactive mediators.\textsuperscript{10} Impairment of this

\textbf{Figure 5.} Perivascular adipose vasoactive properties in NZO and NZB control mice. Concentration-response curves to norepinephrine, endothelin-1, and angiotensin II assessed in mesenteric resistance arteries with and without PVAT in NZB mice (A, C, and E) and NZO mice (B, D, and F). Constriction responses are the percentage of maximal contraction to KCl+norepinephrine. Closed and open symbols represent with and without PVAT, respectively. Values are mean±SEM; n=7 mice per group. *P<0.0001, †P<0.001.
ability may have detrimental effects on the vascular system by increasing vascular tone in resistance arteries leading to enhanced peripheral resistance and, consequently, BP elevation. We demonstrated that, in NZO mice, the physiological anticontractile properties of PVAT were abolished. Two mechanisms are known to mediate the vasoactive effect of PVAT: an endothelium-dependent pathway involving increase of NO release and an endothelial-independent pathway involving release of H₂O₂.²⁹ In NZO mice, we suggest the presence of an impaired regulation of H₂O₂ production in PVAT, as a consequence of increased O₂⁻ production and decreased expression of SOD-1, which catalyzes the dismutation of O₂⁻ in H₂O₂. In vascular disease, O₂⁻ has been widely studied for its ability to directly attenuate the biological activity of NO. However, the short half-life and radius of diffusion of O₂⁻ drastically limit the role of this ROS as an important paracrine hormone in vascular biology. However, its metabolite, H₂O₂, is able to exert a paracrine vasoactive effect in the vasculature. Vascular H₂O₂ has been shown to elicit vasoconstriction in hypertension and cardiovascular disease, whereas PVAT-derived H₂O₂ seems to have vasorelaxing properties.⁷,²⁹,³⁰ Other than this controversy probably related to the different models and vascular beds studied, we found blunted SOD-1 expression in PVAT of NZO mice, which could be an expression of impaired regulation of H₂O₂ production that then modulates vascular tone of resistance arteries in this model of metabolic syndrome.

Inflammation has been shown recently to play a central role in the vasoactive properties of the perivascular fat.³¹ We showed the presence of hypertrophy of adipose tissue in the visceral, subcutaneous, and perivascular compartments, and PVAT in NZO mice was characterized by enhanced homing of macrophages and increased oxidative stress. Recent findings have shown that PVAT and adventitia are critical sources of vascular inflammatory cells, which, through NADPH oxidase–dependent O₂⁻ production, play a role in the pathogenesis of hypertension and vascular remodel-
ing.11,12 However, the pathophysiological mechanism by which PVAT and adventitia may mediate vascular dysfunction has not been clarified. The decreased release of adiponectin by adipocytes, induced in inflammatory pathophysiological conditions, such as metabolic syndrome, has been suggested as a potential mediator.21,31 Indeed, adiponectin was decreased in PVAT in the present model of metabolic syndrome. Another possible mechanism is that PVAT may represent a reservoir of inflammatory cells, which may migrate into the vascular wall and decrease NO bioavailability through paracrine release of ROS. Indeed, in NZO mice, we found increased vascular expression of adhesion molecules and MCP-1 (monocyte chemotactic peptide-1), which may exert a chemotactic effect, thereby inducing inflammatory cell infiltration into perivascular tissue.32 As well, inflammatory cells in the PVAT may represent a source of inducible NO synthase, which could contribute to some of the vascular pathophysiological mechanisms. Thus, it is possible to imagine that in this model of PVAT may play a role in the pathogenesis of BP increase, although the mechanisms by which perivascular adipose inflammation and oxidative stress may affect endothelial function and NO bioavailability need further investigation. In vivo approaches are required to achieve an effective manipulation of the PVAT in pathophysiological conditions, which would add insights on the mechanistic tie-in among perivascular adipose inflammation, oxidative stress, and vascular pathophysiology.

Perspectives

The present study characterizes vascular dysfunction in a rodent model of the human metabolic syndrome. The availability of animal models that present the complexity of human disease and the association of different cardiovascular risk factors, as in the metabolic syndrome, allow the study of some of the different pathophysiological mechanisms involved. The NZO mouse exhibited endothelial dysfunction attributed to eNOS uncoupling and increased ROS production. PVAT promoted vascular dysfunction, through decreased anticontractile effects, which may contribute to increased vascular tone in resistance arteries and BP values. However, the pathogenic role of adipose tissue needs to be further investigated to clearly establish the contribution of perivascular adipose proinflammatory and pro-oxidant mechanisms in vascular pathophysiology. The availability of a reliable animal model, such as the NZO mouse, may allow for future investigation and development of new therapies for subjects presenting the metabolic syndrome.

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Disclosures

None.

References


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Chiara Marchesi* MD, Talin Ebrahimian* PhD, Orlando Angulo MD, Pierre Paradis PhD and
Ernesto L. Schiffrin MD, PhD, FRSC, FRCPC
Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital,
McGill University, Montreal, Quebec, Canada (C.M., T.E., O.A., P.P., E.L.S.) and Department of
Clinical Medicine, University of Insubria, Varese, Italy (C.M.)

*The two authors contributed equally to the paper

Address for correspondence and reprints:
Ernesto L. Schiffrin MD, PhD, FRSC, FRCPC
Sir Mortimer B. Davis-Jewish General Hospital, #B-127,
3755 Cote Ste-Catherine Road,
Montreal, Quebec, Canada H3T 1E2
Ph.: (514) 340-7538; Fax: (514) 340-7539
E-mail: ernesto.schiffrin@mcgill.ca
Materials and Methods

Animals

New Zealand Obese (NZO/HILtJ) and New Zealand Lean (NZB/BINJ) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The NZO mouse has been characterized by others as a model of human metabolic syndrome (1-3). NZO mice exhibit polygenic obesity associated with hyperinsulinemia, hyperglycemia, increased serum cholesterol and triglyceride levels, and elevated BP, when compared to their control, the NZB mice. Differently from other models of obesity, in addition to obesity genes, such as a leptin receptor (Lepr) variant, the NZO mouse carries still unidentified genes that predispose to islet cell failure that, together with the insulin resistance, cause the development of overt hyperglycaemia. Also, previous data (3) have shown a good correlation between body weight, insulinemia and BP in NZO mice, suggesting that obesity, insulin resistance and hypertension are the consequence of a common genetic constellation in NZO mice, as well as in humans. All experiments were carried out in agreement with the local guidelines for the care and use of laboratory animals and in accordance to the guidelines of the McGill University Animal Care Authority. We used male mice at the age of 12±2 weeks. Animals were weighed and euthanized by decapitation. Plasma, heart, aorta, mesenteric vessels, kidneys, liver, spleen and adipose tissue were collected for experiments.

Blood pressure

Systolic blood pressure (SBP) and heart rate (HR) were determined by the tail-cuff technique using the Visitech systems BP-2000 (Apex, NC) as previously described with minor modifications (4). Experiments were performed in the morning for 3 consecutive days and the data reported are the mean of the 3 days.

Glucose and insulin

Fasting glucose was determined in whole blood by a blood glucose monitoring system (OneTouch Ultra, LifeScan Inc, Milpitas, Ca) and insulin in plasma by ELISA, according to the manufacturer instructions (Mercodia AB, Uppsala, Sweden, respectively) (Methods, page 5).

Functional and morphological vascular studies

Second order branches of mesenteric artery (~2 mm in length with internal diameter of ~150 to 250 μm) were dissected and mounted on a pressurized myograph as previously described (5;6). Briefly, mesenteric vessels were equilibrated at 45 mmHg of intraluminal pressure in warmed oxygenated (95% air and 5% CO₂) physiological saline solution (PSS, pH 7.4 containing [mmol/L]: NaCl, 120; NaHCO₃, 25; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄, 1.18; CaCl₂, 2.5; EDTA, 0.026; and glucose, 5.5.). Vessels were considered viable when they constricted >60% of their resting lumen diameter in response to extraluminal application of 125 mmol/L KCl plus 10⁻⁵ mol/L norepinephrine. Endothelium-dependent relaxation was assessed by measuring the dilatory response to acetylcholine (10⁻⁹ to 10⁻⁴ mol/L) and bradykinin (10⁻¹⁰ to 10⁻⁶ mol/L) in norepinephrine precontracted vessels (5x10⁻⁵ mol/L). Endothelium-independent relaxation was assessed with sodium nitroprusside (10⁻⁸ to 10⁻³ mol/L) in precontracted vessels. NO availability and ROS production were evaluated by
acetylcholine concentration-response curve repeated after 20-minute incubation with the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (l-NAME, 10⁻⁴ mol/L) and the anti-oxidant and superoxide dismutase mimetic Tempol (10⁻³ mol/L), respectively. Concentration response curves to norepinephrine (10⁻⁹ to 10⁻⁴ mol/L), endothelin-1 (10⁻¹⁰ to 10⁻⁶ mol/L), and angiotensin II (10⁻⁹ to 10⁻⁵ mol/L) were performed in the presence or absence of PVAT, to evaluate the effect of the PVAT on vascular contractility. As well, relaxation response curves to acetylcholine and bradykinin were performed in the presence or absence of PVAT.

To examine vascular remodeling, mesenteric resistance arteries were deactivated by perfusion with Ca²⁺-free PSS containing 10 mmol/L EGTA for 30 min. Lumen and media were measured at an intraluminal pressure at 45 mmHg, and media-to-lumen (M/L) ratio and media cross-sectional area (MCSA) were evaluated as previously described (7).

Aorta MCSA measurement was performed on hematoxylin and eosin stained 4 µm paraffin sections of the central part of the thoracic aorta and quantified with the Northern Eclipse program (EMPIX Imaging Inc, Mississauga, ON, Canada).

**Vascular superoxide (•O₂⁻) production and NAD(P)H oxidase activity**

Vascular •O₂⁻ production was assessed on 5 µm cryo-sections of aorta and aortic and mesenteric PVAT with the superoxide-sensitive fluorescent dye dihydroethidium (DHE, 2 µmol/L) in dark conditions for 5 min at 37°C. Fluorescence was visualized with a fluorescence microscope with a CY3 filter.

NAD(P)H oxidase activity was assessed both in aorta and mesenteric artery, and in aortic and mesenteric PVAT, by lucigenin chemiluminescence assay, as previously described (8). Briefly, samples were incubates for 30' at 37°C in ROS lysis buffer (pH 7.4 containing [mmol/L]: 20 KH₂PO₄, 1 EGTA) and then manually homogenized. Homogenates were re-suspended in 100 µL of ROS assay buffer (pH 7.4 containing [mmol/L]: 50 KH₂PO₄, 1 EGTA, 1 sucrose). NADPH (10⁻⁴ mol/L) was added to the suspension, containing lucigenin (5 µmol/L). Luminescence was measured every 1.8 s for 3 min in a luminometer (AutoLumat LB 953; Berthold, Wildbad, Germany) and activity was expressed as nmol •O₂⁻/min per µg protein.

**Western blotting**

Proteins were extracted from frozen mesenteric arteries, separated by electrophoresis on SDS-PAGE, transferred onto nitrocellulose membranes and incubated with anti-phospho-eNOS (Ser1177) (BD Pharmingen, San Diego, CA), anti-eNOS (Cell Signaling Technology, Inc., Danvers, MA), anti-nitrotyrosine (Upstate Biotechnology, Lake placid, NY), anti-VCAM-1, anti-PECAM-1 and anti-ICAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. As well, proteins were extracted from frozen mesenteric PVAT, and incubated with anti-Rac1 and anti-SOD-1 -2 and -3 antibodies (Santa Cruz Biotechnology). Signals were revealed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific, Rockford, IL) and Molecular Imager Chemidoc XRS system (Bio-Rad, Mississauga, ON, Canada). Membranes were subsequently stripped and reprobed with anti-β-actin antibody to verify equal loading or for normalization. Optical density of the bands was quantified using Quantity One software (Bio-Rad).

**Low-temperature SDS-PAGE**

Mesenteric artery extracts were prepared with SDS sample buffer in non-denaturing conditions (187.5 mmol/L tris-HCl [pH 6.8], 10% wt/vol SDS, 30% vol/vol glycerol, 0.002% wt/vol bromophenol blue). Samples were loaded on 7.5% polyacrylamide gels and subjected to electrophoresis. Buffers and gels were cooled to 4°C and the buffer tank placed in an ice bath.
during electrophoresis. Endothelial eNOS monomer and dimer were detected by Western blotting analysis.

**Plasma nitrite measurement**

Plasma nitrites were evaluated with a colorimetric assay as previously described (9). Briefly, samples of 50 μL plasma were incubated with cadmium overnight at 4°C in slight shaking. Plasma was mixed with 0.01% N-(1-naphthyl) ethylenediamine followed by 0.1% p-aminobenzenesulfonamide. Total nitrites were measured at 548 nm absorbance.

**Immunofluorescence**

Immunofluorescence was performed on mesenteric and aortic PVAT 4-μm paraffin sections as previously described, with minor modifications (10). Briefly, after deparaffinization, antigen retrieval was performed with proteinase K (20 μL/mL, Molecular Probes, Inc., Eugene, OR). Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide. Blocking was performed with 10% normal goat serum (NGS, Jackson ImmunoResearch, PA) in 0.1% Triton X-100 physiological basic solution, for 60 minutes at room temperature. Staining was performed using rat anti-mouse Mac-3 (BD Pharmingen) as primary antibody, incubated overnight in 1% NGS solution, followed by Alexa Fluor 647 anti-rat IgG (Molecular Probes Inc, Eugene, OR), for 60 minutes at room temperature in dark conditions the next day. Fluorescence was visualized with a fluorescence microscope with a CY5 filter.

**Quantitative RT-PCR**

TNF-α, MCP-1 and adiponectin mRNA expression levels were determined by quantitative real-time PCR (qPCR) using the TRIzol reagent (Invitrogen, Carlsbad, CA). One μg of total RNA was reversed transcribed (RT) with a Quantitect RT kit (Qiagen, Mississauga, ON, Canada). qPCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) with the Mx3005P real-time PCR cycler (Stratagene, La Jolla, CA). qPCR results were normalized with ribosomal protein S16. Primers were designed using Primer3 (11). Annealing temperature was 58°C. The following primers were used: for TNF-α 5’-ACCCTCACACTCAGATCATCTTC-3’ and 5’-AGATAGCAAATCGGCTGACG-3’, for MCP-1 5’-AGCTCTCTCTTCTACCACAC-3’ and 5’-TCTGGACCCCCATTTCTCTTG-3’, for adiponectin 5’-TCAGTGAGATCTGACGACC-3’ and 5’-CCAGAAGACCTGCATCTCC-3’, and for S16 5’-TCTGGCAAGGAGAGATTTG-3’ and 5’-CCGCCAACCTTTCTGGATTC-3’.

**Data analysis**

Two-way repeated measures ANOVA were used to evaluate differences in concentration-response curves between groups, followed Bonferroni post-hoc analysis. Unpaired t-test was performed to evaluate all other variables. P<0.05 was considered statistically significant. Results are presented as mean ± SEM.
References


Supplemental Figures

Figure S1. Morphology of visceral and perivascular adipose tissue in NZO and NZB control mice.

Representative images of visceral, aortic and mesenteric PVAT from left to right, respectively. Staining is with hematoxylin-eosin. PVAT indicates perivascular adipose tissue.
Figure S2. Endothelial NADPH oxidase activity in NZO and NZB control mice.

Vascular NAD(P)H oxidase activity in aorta (A) and mesenteric arteries (B), in the absence of PVAT. Values are means±SEM. n=5 mice per group.
Figure S3. Endothelial function of mesenteric resistance arteries in NZO and NZB mice, in presence and absence of PVAT.

Concentration-response curves to acetylcholine (A and B) and bradykinin (C and D), in presence and absence of PVAT. Relaxation responses are % increase in lumen after pre-contraction with norepinephrine. Closed and open symbols represent with and without PVAT, respectively. PVAT indicates perivascular adipose tissue.