Uncoupling of Endothelial Nitric Oxide Synthase in Perivascular Adipose Tissue of Diet-Induced Obese Mice

Ning Xia, Sven Horke, Alice Habermeier, Ellen I. Closs, Gisela Reifenberg, Adrian Gericke, Yuliya Mikhed, Thomas Münzel, Andreas Daiber, Ulrich Förstermann, Huige Li

Objective—The present study was conducted to investigate the contribution of perivascular adipose tissue (PVAT) to vascular dysfunction in a mouse model of diet-induced obesity.

Approach and Results—Obesity was induced in male C57BL/6J mice with a high-fat diet for 20 weeks, and vascular function was studied by myograph. In PVAT-free aortas isolated from obese mice, the endothelium-dependent, nitric oxide–mediated vasoconstrictor response to acetylcholine remained normal. In contrast, a clear reduction in the vasoconstrictor response to acetylcholine was observed in aortas from obese mice when PVAT was left in place. Adipocytes in PVAT were clearly positive in endothelial nitric oxide synthase (eNOS) staining, and PVAT nitric oxide production was significantly reduced in obese mice. High-fat diet had no effect on eNOS expression but led to eNOS uncoupling, evidenced by diminished superoxide production in PVAT after eNOS inhibition. As mechanisms for eNOS uncoupling, arginase induction and l-arginine deficiency were observed in PVAT. Obesity-induced vascular dysfunction could be reversed by ex vivo l-arginine treatment and arginase inhibition.

Conclusions—Diet-induced obesity leads to l-arginine deficiency and eNOS uncoupling in PVAT. The combination therapy with l-arginine and arginase inhibitors may represent a novel therapeutic strategy for obesity-induced vascular disease. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI: 10.1161/ATVBAHA.115.306263.)

Key Words: eNOS uncoupling n nitric oxide n obesity n perivascular adipose tissue n vascular function

Obesity has become a global healthcare issue. The prevalence of obesity has increased exponentially in both developed and developing countries during the last decades. Such a rapid rise within this relatively short time period must result from environmental factors rather than from a change in the genome. There is clear evidence that environmental factors, such as diet and the level of physical activity, affect the risk of obesity.

Obesity is an established risk factor for diseases, such as type 2 diabetes mellitus, cardiovascular diseases, and many cancer types. A recent meta-analysis of 97 studies involving >2.88 million individuals and >270,000 deaths demonstrates that obesity is associated with higher all-cause mortality. Abdominal adiposity is strongly and positively associated with all-cause, cardiovascular disease and cancer mortality. Although major efforts are being made to attenuate such negative impacts of Western lifestyle on obesity and its sequelae, to date there are no efficient pharmacological treatment options available.

Obesity has numerous adverse effects on hemodynamics and cardiovascular structure and function. Vascular dysfunction has been demonstrated in conduit7 and small arteries7 in patients with metabolic syndrome. Obese patients are more likely to develop hypertension and cardiomyopathy and have higher stroke risk.

Despite these facts, endothelial dysfunction is not always evident in in vitro studies. When studying the vascular function of aorta isolated from diet-induced obese mice, we found that the vasodilator response to acetylcholine remained normal in the aorta from mice fed a high-fat diet (HFD) for 20 weeks. In traditional vascular physiology studies, the surrounding adipose tissue is usually cleaned from the blood vessel. Interestingly, by leaving the aortic perivascular adipose tissue intact, a clear reduction in the vasodilator response to acetylcholine was observed in the aorta of obese animals as compared with lean controls. These results indicate the importance of PVAT in regulating vascular function under this condition. In the present study, we provide evidence for a novel mechanism underlying vascular dysfunction in diet-induced obesity, namely uncoupling of the endothelial nitric oxide (NO) synthase (eNOS) in PVAT.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
Results

Role of PVAT in Vascular Dysfunction in Diet-Induced Obese Mice

Male C57BL/6j mice were fed with either HFD or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. The HFD-treated mice developed obesity (Table). Vasodilator response to acetylcholine was studied using aortas with or without PVAT in a wire myograph system. In aortas without PVAT, no significant differences in endothelium-dependent, NO-mediated vasodilator response to acetylcholine were found between NCD and HFD groups (Figure 1A). In contrast, a clear reduction in the vasodilator response to acetylcholine was observed in the aorta of obese animals compared with lean controls when PVAT was left intact (Figure 1B). The acetylcholine-induced vasodilation in the mouse aorta was NO-dependent because it could be completely blocked by the NO synthase inhibitor Nω-nitro-arginine methyl ester (l-NAME; Figure 1C). In endothelium-denuded aortic rings from control mice, acetylcholine induced a significant vasodilation if PVAT was left intact (Figure I in the online-only Data Supplement). The PVAT-mediated vasodilation was preventable with l-NAME, indicating that PVAT-derived NO contributes to acetylcholine-induced vasodilation under normal conditions.

Reduced NO Production in PVAT of Diet-Induced Obese Mice

To directly access NO production from PVAT, time-lapse fluorescence imaging was performed with aorta sections prepared from NCD or HFD-fed mice stained with the fluorescent NO probe 4,5-diaminofluorescein diacetate. As shown in Figure 1D, basal NO production could be detected in PVAT. The PVAT NO production was enhanced by acetylcholine (Figure 1D and 1E) and inhibited by eNOS inhibitor l-NAME, indicating the specificity of 4,5-diaminofluorescein diacetate staining. HFD-induced obesity significantly reduced the PVAT NO production, both under basal and acetylcholine-stimulated conditions (Figure 1D and 1E).

Uncoupling of PVAT eNOS in Diet-Induced Obese Mice

The expression of neuronal nNOS and inducible iNOS was not changed (Figure II in the online-only Data Supplement). The phosphorylation of eNOS at serine 1177 was reduced in PVAT (but not in the aorta) of HFD mice, whereas eNOS phosphorylation at threonine 495 was not changed (Figure 2D). Among the upstream kinases for eNOS serine 1177 phosphorylation, Akt was dephosphorylated at serine 473, whereas no changes in the adenosine monophosphate–activated protein kinase were found (Figure 2D).

Deficiency of l-Arginine, but Not Tetrahydrobiopterin, in PVAT of Diet-Induced Obese Mice

Major mechanisms for eNOS uncoupling include deficiency of eNOS cofactor tetrahydrobiopterin (BH₄) and substrate l-arginine.8,9 Unexpectedly, the content of BH₄ in PVAT of obese mice was not reduced but increased (Figure 4A). The reason for this increase was likely a compensatory upregulation of GTP cyclohydrolase I, the rate-limiting enzyme for BH₄ de novo biosynthesis, selectively in PVAT (Figure 4B and 4C). BH₄ can be oxidized to 7,8-dihydrobiopterin, and be regenerated back from 7,8-dihydrobiopterin by dihydrofolate reductase. The results that also 7,8-dihydrobiopterin content and dihydrofolate reductase expression were enhanced by HFD (Figure 4B and 4C) indicate an accelerated turnover of BH₄. Nevertheless, the PVAT eNOS uncoupling was unlikely to be caused by a BH₄ deficiency. Interestingly, HFD reduced the l-arginine content in PVAT (Figure 5A). Both arginase I and II, major l-arginine-consuming enzymes, were upregulated in the PVAT of obese mice (Figure 5B and 5C).

Table. Effect of Dietary Treatment on Body Weight and Adiposity

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<td>Blood glucose, mg/dL</td>
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<td>122.3±13.2†</td>
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<td>Tibia length, mm</td>
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Data are expressed as mean±SEM of 10 animals per group. HFD indicates high-fat diet; and NCD, normal control diet.

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Nonstandard Abbreviations and Acronyms

BH₄ tetrahydrobiopterin
eNOS endothelial nitric oxide synthase
HFD high-fat diet
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PVAT perivascular adipose tissue

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Upregulation of Proinflammatory Adipokines and Cytokines in PVAT of Diet-Induced Obese Mice

Treatment with HFD led to upregulation of proinflammatory adipokines/cytokines in PVAT of obese mice, including leptin, tumor necrosis factor-α, interleukin-1β, and interferon-γ. On the other hand, anti-inflammatory adipokines/cytokines, such as adiponectin and interleukin-10, were downregulated (Figure III in the online-only Data Supplement). Diet-induced obesity was also associated with upregulation of monocyte chemotactic protein 1, as well as macrophage markers (CD68 and F4/80), in PVAT (Figure III in the online-only Data Supplement).

Improvement of PVAT Function by L-Arginine and Arginase Inhibitor in Diet-Induced Obese Mice

Incubation of PVAT-containing aorta from NCD mice with a combination of L-arginine (100 μmol/L) and the arginase inhibitor Nω-hydroxy-nor-arginine (10 μmol/L) for 30 minutes ex vivo in organ chamber had no effects on acetylcholine-induced vasodilation (Figure 6). In contrast, the compounds markedly improved the vasodilator function of PVAT-containing aortas from HFD mice (Figure 6), supporting the concept of L-arginine deficiency in diet-induced obese mice.

Discussion

In the present study, we show that eNOS is expressed in PVAT, and PVAT eNOS produces NO. PVAT dysfunction represents a major mechanism for vascular dysfunction in aorta from diet-induced obese mice. We demonstrate for the first time that the PVAT eNOS is uncoupled under the condition of diet-induced obesity. The molecular mechanisms involve L-arginine deficiency because of arginase induction. Consistently, PVAT eNOS uncoupling can be reversed by a combination therapy of L-arginine supplementation and arginase inhibition.

Endothelial Dysfunction Versus PVAT Dysfunction

In aorta without PVAT, we observed no difference in acetylcholine-induced vasodilation between HFD- and NCD-treated mice (Figure 1). At a first glance, this seems surprising. Several previous studies have demonstrated that diet-induced obesity leads to endothelial dysfunction in mouse aorta without PVAT. Mice fed with a rodent diet with 45 kcal% fat for 12 weeks impaired the acetylcholine-induced, endothelium-dependent vasorelaxation. However, the effect of diet-induced obesity on endothelial function is likely to be time-dependent. Longer treatment with HFD may induce compensatory mechanisms, leading to adaptation and relative normalization of endothelial function. After treatment of male C57BL/6J mice with
HFD for 30 weeks, the acetylcholine-induced vasodilation remained completely normal in acetylcholine concentration ranges ≤100 nmol/L in the aorta. In our study, the endothelial function of the aorta from mice treated with HFD for 20 weeks was also relatively normal (Figure 1A). Thus, our experimental setting may represent an ideal model for studying the contribution of PVAT to vascular dysfunction.

PVAT has been recently recognized as a novel determinant in vascular biology. The role of PVAT in vascular function was first discovered by the observation of an endothelial nitric oxide synthase (eNOS) expression in adipose tissues (PVAT). C57BL/6J mice were put on high-fat diet (HFD) or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. Immunohistochemistry staining (Figure 2) was performed using an anti-eNOS antibody or IgG as a negative control. The pictures shown are representative for 5 independent experiments with similar results. Expression and phosphorylation of eNOS at serine 1177 and threonine 495 were analyzed at the levels of protein (Western blotting; Figure 2D). In addition, expression and phosphorylation of upstream kinases for eNOS serine 1177 phosphorylation, Akt, and AMP-activated protein kinase (AMPK) were analyzed (Figure 2D). n=8. E indicates endothelium.

Expression and Function of eNOS in PVAT

Previous studies have shown eNOS expression in adipose tissues and in adipocytes. Immunohistochemistry analysis has identified eNOS staining in adipocytes within PVAT. Incubation of epididymal fat pads with leptin leads to eNOS activation and NO release. Indeed, an enhanced relaxant response of mesenteric artery and a leptin-mediated adaptive NO overproduction from mesenteric PVAT have been demonstrated at the early phase of diet-induced obesity in C57BL/6J mice.

In the present study, we confirmed that eNOS is expressed in PVAT (Figure 2), and PVAT eNOS produces NO (Figure 1). Incubation of epididymal fat pads with leptin leads to eNOS activation and NO release. This adipocyte-derived NO is supposed to be released into the interstitial fluid and may diffuse into the capillaries and adjacent arterioles causing vasodilation. Indeed, an enhanced relaxant response of mesenteric artery and a leptin-mediated adaptive NO overproduction from mesenteric PVAT have been demonstrated at the early phase of diet-induced obesity in C57BL/6J mice.

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Uncoupling of eNOS and Vascular Pathophysiology
Under physiological conditions, eNOS produces NO, which represents a key element in the vasoprotective function of the endothelium. Under pathological conditions, however, eNOS may become uncoupled (ie, uncoupling of O₂ reduction from NO synthesis), such that it no longer produces NO, but superoxide.9,21,22

The phenomenon of eNOS uncoupling has been shown in different pathologies, including diabetes mellitus, hypertension, and atherosclerosis.8,23 To date, the scientific interest of eNOS uncoupling studies has focused on eNOS in the endothelium. The present study is the first demonstrating eNOS uncoupling in PVAT.

Numerous mechanisms have been proposed to play a role in eNOS uncoupling.21,24 Among these, depletion of BH₄, an essential cofactor for the eNOS enzyme, is likely to be a major cause for eNOS uncoupling and endothelial dysfunction. BH₄ can be oxidized by peroxynitrite and superoxide to 7,8-dihydrobiopterin, thereby leading to BH₄ deficiency.25,26

Another important cause of eNOS uncoupling is a deficiency of l-arginine because of upregulation of arginase expression/activity. In humans and mammals, there are 2 isoforms of arginases: arginase I and arginase II, which are encoded by 2 separate genes.27 Studies performed with

![Figure 4. Increased tetrahydrobiopterin (BH₄) content in perivascular adipose tissues (PVAT) of diet-induced obese mice. C57BL/6J mice were put on high-fat diet (HFD) or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. Tissue content of BH₄ and dihydriodipterin (BH₂) was measured with HPLC (A). The mRNA (B) and protein (C) expression of GTP cyclohydrolase I (GCH1, the rate-limiting enzyme for BH₄ de novo biosynthesis) and dihydrofolate reductase (DHFR, the enzyme required for BH₄ regeneration from BH₂) were studied with quantitative polymerase chain reaction (qPCR) and Western blot analyses, respectively. The blots shown are representative for 3 independent experiments with similar results. *P<0.05; **P<0.01, n=4 to 7.](http://atvb.ahajournals.org/)

![Figure 5. Reduced l-arginine content in perivascular adipose tissues (PVAT) of diet-induced obese mice. C57BL/6J mice were put on high-fat diet (HFD) or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. Tissue l-arginine content was measured with HPLC (A). The mRNA (B) and protein (C) expression of arginases were studied with quantitative polymerase chain reaction (qPCR) and Western blot analyses, respectively. The blots shown are representative for 3 independent experiments with similar results. Liver is shown as positive control. *P<0.05; **P<0.01, n=4 to 7.](http://atvb.ahajournals.org/)

![Figure 6. Improvement of perivascular adipose tissues (PVAT) function by l-arginine and arginase inhibitor in diet-induced obese mice. C57BL/6J mice were put on high-fat diet (HFD) or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. The vasodilator response to acetylcholine was performed in noradrenaline-precontracted aortic rings with PVAT, with or without preincubation with l-arginine (100 μmol/L) and arginase inhibitor N-ω-hydroxy-nor-arginine (Nor, 10 μmol/L) for 30 minutes ex vivo in organ chamber. Nor was dissolved in dimethyl sulfoxide (DMSO). The counterpart rings were treated with DMSO as solvent control (final concentration 0.1%). ***P<0.001, n=8.](http://atvb.ahajournals.org/)
vascular endothelial cells suggest that these 2 isoforms share similar functions, that is, metabolizing L-arginine to urea and L-ornithine, whereby an arginine upregulation in endothelial cells limits L-arginine bioavailability for NO production, leading to endothelial dysfunction. Uncoupling of eNOS is a crucial mechanism contributing significantly to endothelial dysfunction and atherogenesis. It not only reduces NO production, but also potentiates the preexisting oxidative stress. The overproduction of reactive oxygen species (eg, superoxide and subsequently peroxynitrite) by uncoupled eNOS in turn enhances oxidation of BH4 and upregulation of arginase expression/activity, creating a vicious circle.

Potential Mechanisms of Arginase Upregulation and L-Arginine Deficiency
The expression/activity of vascular arginasies has been shown to be enhanced by a variety of stimuli, including angiotensin II or high glucose, thrombin, and oxidized low-density lipoprotein, conditions that inevitably lead to vascular inflammation. We, therefore, investigated whether inflammation represents a cause of arginase upregulation and L-arginine deficiency in our model. It is known that PVAT produces a large variety of bioactive molecules that affect vascular function, including leptin, adiponectin, interleukin-6, and tumor necrosis factor-α. In PVAT of HFD mice, proinflammatory adipokines/cytokines (eg, leptin, interleukin-6, tumor necrosis factor-α, and interferon-γ) were upregulated, whereas anti-inflammatory adipokines/cytokines (eg, adiponectin and interleukin-10) were downregulated (Figure III in the online-only Data Supplement). This is consistent with previous studies. Diet-induced obesity also led to secretion of monocyte chemotactic protein 1 by adipocytes into the extracellular space where it induces monocyte recruitment into adipose tissue. Indeed, we observed an upregulation of monocyte chemotactic protein 1 in PVAT of HFD mice, which was associated with enhancement of macrophage markers (Figure III in the online-only Data Supplement). Thus, macrophages may also contribute to the enhanced production of proinflammatory cytokines (eg, interleukin-6 and tumor necrosis factor-α) and the resulting PVAT inflammation (Figure V in the online-only Data Supplement).

Coexistence of Uncoupled and Coupled eNOS
It is not surprising that L-NAME reduced NO production (Figure 1D), as well as superoxide production (Figure 3), from PVAT eNOS in obese animals. eNOS uncoupling is not an all-or-none phenomenon. Rather, uncoupled eNOS molecules and coupled eNOS proteins may exist in the same cell at the same time. Because of relative deficiency of BH4 or L-arginine, part of the eNOS molecules become uncoupled, whereas some others may still remain coupled. Such a situation has also been observed in the hypercholesterolemic apolipoprotein E-knockout mice, in which both superoxide and NO production by eNOS are detectable. This also applies to our model with both coupled and uncoupled eNOS molecules existing in PVAT of obese mice.

Phosphorylation is a major posttranslational modification, with phosphorylation at serine 1177 being the most important positive modulation of eNOS activity. The activation of eNOS catalytic function by serine 1177 phosphorylation is because of inhibition of calmodulin dissociation from eNOS and enhancement of the internal rate of eNOS electron transfer. This phosphorylation is required for both the NO-producing activity of the coupled eNOS and the superoxide-producing activity of the uncoupled eNOS. The detrimental effects of superoxide produced by uncoupled eNOS do not overwhelm the protective role of eNOS-derived NO. This is true in the mouse model of atherosclerosis and may also be the case in our model. Therefore, the net effect of reduced eNOS phosphorylation at serine 1177 (Figure 2D) is a reduction of NO production (Figure V in the online-only Data Supplement).

Limitation of the Study
PVAT is a conglomerate of various cell types, including adipocytes, preadipocytes, and mesenchymal stem cells, embedded in a matrix that is invested with microvessels. Although our results show clearly that eNOS dysfunction is evident in PVAT but not in the vascular wall, it is currently not possible to definitively distinguish the relative contribution of endothelial eNOS and PVAT eNOS in the present study. Moreover, microvessels present in PVAT also contain endothelial cells (Figure 2A), albeit they only represent a minority of the eNOS-positive cells in PVAT; the majority are adipocytes. The relative contribution of endothelial eNOS and PVAT eNOS will be further addressed in future studies using knockout mice with cell type–specific eNOS deletion.

Clinical Implications
There is currently no effective therapy to treat vascular complications of obesity. Results from our study indicate that L-arginine supplementation and arginase inhibition could represent a new therapeutic strategy. Promising results have been achieved with arginase inhibitors, although only limited data from clinical studies are currently available. Small-scale proof-of-concept clinical studies have shown that local administration of arginase inhibitors improves vascular function in aged humans, as well as in patients with coronary artery disease and type 2 diabetes mellitus, heart failure, or hypertension. Larger clinical studies with systemic arginase inhibition are warranted.

Conclusions
Diet-induced obesity leads to arginase induction in PVAT. The resulting L-arginine deficiency and eNOS uncoupling represents a novel mechanism underlying the vascular dysfunction in this model. Reversal of eNOS uncoupling by L-arginine and arginase inhibitors prevents obesity-induced vascular dysfunction, which may represent a novel therapeutic strategy for obesity-associated vascular disease.

Disclosures
None.

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References


The physiological role of perivascular adipose tissues (PVAT) in regulating vascular tone is well established. Through endocrine and paracrine mechanisms, factors from PVAT are known to decrease contractile responses of blood vessels to agonists. In contrast, the pathological role of PVAT is less clear. In the present study, we demonstrate for the first time that PVAT dysfunction is implicated in vascular dysfunction under condition of diet-induced obesity. In normal vessels, endothelial nitric oxide (NO) synthase expressed in PVAT contributes to vasodilation by producing NO. In diet-induced obesity, however, PVAT endothelial NO synthase becomes dysfunctional and uncoupled, such that it no longer produces NO, but superoxide. As mechanisms for endothelial NO synthase uncoupling, arginase induction and L-arginine deficiency were observed in PVAT. Obesity-induced vascular dysfunction was reversed by ex vivo L-arginine treatment and arginase inhibition. Thus, combination therapy with L-arginine and arginase inhibitors may represent a novel therapeutic strategy for obesity-induced vascular disease.
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Uncoupling of endothelial nitric oxide synthase in perivascular adipose tissue of diet-induced obese mice

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Figure I. Perivascular adipose tissue (PVAT)-dependent vasodilation in endothelium-free aorta. Thoracic aortic segments were isolated from 3-month-old male C57BL/6 mice fed normal chow. Endothelium was removed mechanically. Then, two aortic rings were made from the same endothelium-denuded segment: one with intact PVAT, while PVAT of the other was subsequently removed. The rings were contracted with noradrenaline and then relaxed with increasing concentrations of acetylcholine, in the absence or present of the NO synthase inhibitor L-NAME (500 µM). *P<0.05; **P<0.01; n= 8.
Figure II. Gene expression of nitric oxide synthase (NOS) isoforms. C57BL/6J mice were put on high-fat diet (HFD) or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. Total RNA was isolated from aorta or aortic PVAT and mRNA expression of neuronal nNOS, inducible iNOS and endothelial eNOS was analyzed with quantitative real-time PCR (qPCR). No significant difference was found between NCD and HFD; n = 10.
Figure III. Gene expression profiling in aortic PVAT. C57BL/6J mice were put on high-fat diet (HFD) or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. Total RNA was isolated from PVAT and mRNA expression was analyzed with quantitative real-time PCR (qPCR). The following genes were studied: adipokines (leptin, adiponectin, omentin, chemerin, and angiopoietin-like protein 2, ANGPTL2); cytokines (tumor necrosis factor-α, TNF-α; interleukin-1β, IL-1β; IL-6; IL-10; transforming growth factor β, TGF-β; interferon γ, IFN-γ); T cell marker (CD3); macrophage markers (CD68 and F4/80); and chemokines (monocyte chemotactic protein 1, MCP1; chemokine (C-C motif) ligand 5, RANTES; chemokine (C-C motif) ligand 3, MIP-1α).

*P < 0.05; **P < 0.01; n = 6.

Figure IV. No evidence for changes in smooth muscle cell proliferation. C57BL/6J mice were put on high-fat diet (HFD) or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. Total RNA was isolated from PVAT-free aortas and mRNA expression was analyzed with qPCR. Two proliferation marker genes were studied: Ki-67 and proliferating cell nuclear antigen, PCNA. No significant difference between NCD and HFD was found. n = 6.
Figure V. Mechanisms of reduced PVAT NO production in diet-induced obesity. Secretion of MCP1 by adipocytes leads to infiltration of monocytes that differentiate into macrophages in PVAT. Both adipocytes and macrophages contribute to the elevated levels of pro-inflammatory adipokines/cytokines resulting in PVAT inflammation. The upregulation of arginases leads to L-arginine deficiency and eNOS uncoupling. In addition, Akt inhibition leads to reduction in eNOS phosphorylation (eNOS-P) at serine 1177. The resulting inhibition of eNOS enzymatic activity represents a contributing mechanism to the reduced PVAT NO production.
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Materials and Methods

Animals

C57BL/6J mice are a well-established mouse model of diet-induced obesity. Male C57BL/6J mice were put on high-fat diet (ssniff® EF D12492 II, 60% energy from fat; Soest, Germany) for 20 weeks starting at the age of 8 weeks. Control animals were fed normal control diet (NCD; ssniff, 11% energy from fat). At the age of 28 weeks, the animals were sacrificed and analyses performed. The animal experiment was approved by the responsible regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz; 23 177-07/G 1-021) and was conducted in accordance with the German animal protection law and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Assessment of vascular function

Thoracic aortas were isolated and dissected into rings of 2-3 mm in length, with perivascular fat and connective tissues either removed or left intact. Isometric tension was recorded using a Wire Myograph system (Danish Myo Technology, Aarhus, Denmark). The rings were equilibrated for 60 minutes and contracted two times with 120 mM KCl. For assessment of vascular function, the rings were pre-contracted with norepinephrine to reach the submaximal tension (80% of that obtained with 120 mM KCl), before vasodilation was induced by acetylcholine (ACh).

Immunohistochemistry studies

Thoracic aorta samples (with PVAT) were fixed in buffered 4% formaldehyde and then embedded in paraffin. Four-micron-thick cross sections of the aorta were stained with a rabbit polyclonal antibody against eNOS (NB300-500, Novus Biologicals; Cambridge, UK) using the VECTASTAIN ABC Kit (Vector Laboratories; Burlingame, CA). Briefly, the primary eNOS antibody was biotinylated and incubated with the
tissue specimens, followed by incubation with streptavidin-peroxidase. The reaction was visualized with 3,3'-diaminobenzidine.

**Direct assessment of NO release**

NO in PVAT was detected with 4,5-diaminofluorescein diacetate (DAF-2 DA), a cell-permeable derivative of the fluorescent NO probe DAF-2 that is hydrolyzed to DAF-2 by intracellular esterases. DAF 2-DA can be used in fluorescence microscopy to measure real-time changes in NO levels.

For a better comparison, NCD and HFD cryostat sections were mounted pairwise back-to-back on the same slide. This eliminates potential artefacts otherwise caused by non-uniform staining of the sections. After an incubation with or without the NOS inhibitor N^G^-nitro-L-arginine methyl ester (L-NAME, 500 µM), the slides were loaded with DAF-2 DA (20 µM) in the absence or presence of acetylcholine (100 nM). Then, fluorescence imaging was immediately performed by real-time time-lapse imaging in a heated (37°C) incubator chamber with a Zeiss 710 confocal laser scanning microscope (Zeiss, Germany). DAF-2 DA was excited at 488 nm and fluorescence emitted between 495 nm and 550 nm was collected.

**Measurement of ROS production**

PVAT-derived ROS production was measured with two independent methods: L-012 chemiluminescence and electron paramagnetic resonance (EPR). PVAT samples from thoracic aorta were pre-incubated in the presence or absence of 500 µM L-NAME for 30 minutes. Then, 100 µM of the luminol derivate 8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt (L-012) was added, and chemiluminescence measurement was performed after a dark adaptation of 30 min. For detection of superoxide with EPR, the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethyl-pyrrolidine hydrochloride (CPH, 1 mM) was used. Paired measurements were performed with two PVAT samples from the same aorta segment, one in the absence and one in the presence of PEG-SOD (100 U/ml; Sigma-Aldrich). The SOD-inhibitable portion of EPR signal was considered as superoxide production.

**Gene expression analyses**

RNA was isolated using peqGOLD TriFast™ (PEQLAB) and cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real time RT-PCR (qPCR) reactions were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich) and 20 ng cDNA. Relative mRNA levels of target genes were quantified using comparative threshold C_T normalized to housekeeping gene TATA-binding protein (TBP). mRNA expression in control animals with NCD were set 100%. The qPCR primer sequences were as follows:
eNOS_forward: CCT TCC GCT ACC AGC CAG A, eNOS_reverse: CAG AGA TCT CA TGG CTA; GCH1_forward: TGG TGA TTG TGA AGG ACA TAG ATA TG, GCH1_reverse: GCC AAT ATG GCA CCT TCC TAC A; DHFR_forward: AAT CCT AGC GTG AAG GCT GGT A, DHFR_reverse: GGC GAC GAT GCA GTT CAA T; arginase 1_forward: GGA ACC CAG AGA GAG CA TG GA, arginase 1_reverse: TTT TTC CAG CAG ACC AGC TT; arginase 2_forward: ACC AGG AAC TGG CTG AAG TG, arginase 2_reverse: TGA GCA TCA ACC CAG ATG AC; TBP_forward: CTT CGT GCA AGA AAT GCT GAA T, TBP_reverse: CAG TTG TCC GTG GCT CTC TTA TT.

Western blot analyses

Western blot analyses were performed with total protein samples (30 µg each) from aorta or PVAT. The following primary antibodies were used: rabbit monoclonal antibody against β-tubulin I (catalog number T7816, Sigma-Aldrich; 1:200,000), mouse monoclonal antibody against eNOS (catalog number 610297, BD Transduction Laboratories; 1:2000), mouse monoclonal antibody against GCH1 (catalog number H00002643-M01, Abnova; 1:500), mouse monoclonal antibody against DHFR (catalog number 610697, BD Transduction Laboratories; 1:500), rabbit monoclonal antibody against arginase I (catalog number ab124917, Abcam; 1:2000), rabbit polyclonal antibody against arginase II (catalog number ab154422, Abcam; 1:1000). Western blot was carried out as previously described. Protein samples were separated on a Bis-Tris gel and transferred to a nitrocellulose membrane. Blots were blocked in 5% milk powder in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.1% Tween 20) for one hour at room temperature. The primary antibodies were diluted in the same solution used for blocking at 4°C overnight. Blots were then washed in TBST and incubated with a horseradish peroxidase-conjugated secondary antibody diluted in 5% milk in TBST for one hour. After washing in TBST and then in TBS, the immunocomplexes were visualized using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) according to the manufacturer’s instructions. Densitometric analysis of scanned blots was performed using the Quantity One software (Bio-Rad).

Measurement of biopterins and L-arginine

Tetrahydrobiopterin (BH₄), dihydrobiopterin (BH₂) and L-arginine were measured by using high-performance liquid chromatography (HPLC)-based methods, as previously described.

For BH₄ measurement, aorta and PVAT samples were homogenized in ice-cold lysis buffer (0.1 mM Tris-HCl, pH 7.8, containing 5 mM ethylenediamine tetraacetic acid, 0.3 mM KCl, 5 mM 1,4-dithioerythritol, 0.5 mM Pefabloc, and 0.01% saponin) with 25 nM neopterin as internal standard. Samples were oxidized under either acidic conditions (with 0.2 M HCl containing 50 mM I₂) or alkaline conditions (with 0.2 M NaOH containing 50 mM I₂). The biopterin content was assessed by HPLC with fluorescence detection (350-nm
excitation, 450-nm emission). BH4 concentration was calculated as femtomol per microgram of protein by subtracting the biopterin peak resulting from alkaline oxidation (accounting for BH2) from the biopterin peak resulting from acidic oxidation (accounting for both BH2 and BH4)3,10.

For L-arginine measurement, samples were lysed in 400 µl ice-cold ethanol 70% for 4 min at 50 Hz with a QIAGEN TissueLyser. Two nmol N6-monomethyl-L-arginine (L-NMMA) were added as internal standard, then supplemented with 0.9 ml PBS pH 7.4 and applied to an Oasis MCX ion exchange column (Waters, Eschborn, Germany). The column was washed with 1 ml each, 0.1 N HCl and methanol, and subsequently cationic amino acids (CAA) were eluted with 1 ml methanol:water:25% NH3 (5:4:1), vacuum dried and resuspended in 0.2 ml sodium borate buffer (0.5 M, pH9.6). For precolumn derivatization, 50 µl sample was supplemented with 12 µl OPA reagent (100 mg o-phthaldialdehyde), 9 ml methanol, 1 ml borate buffer (0.5 M), 100 µl 2-mercaptoethanol, and 18 µl acidic acid (1 M). Amino acid derivatives (10 µl) were separated on a Nova-Pak column (C18, 4 µm 3.9 x 300 mm, Waters, Eschborn, Germany) using a gradient of 50 mM sodium acetate (pH 6.8 supplemented with 0.044 % TEA) and acetonitril. The flow rate was 0.8 ml/min. Fluorescence (excitation wavelength, 330 nm; emission wavelength, 450 nm) was monitored with a RF-20 A fluorometer (Shimadzu) and quantified using the analysis program McDAcq (Bischoff). Recovery of CAA was about 88-92 %.

Statistics

Results are expressed as mean ± SEM (standard error of the mean). Student’s t test was used for comparison of HFD group with NCD group. Two-way ANOVA was used to compare the curves. P values < 0.05 were considered significantly different. For statistical analysis GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used.

References


