Reduced NO-cGMP Signaling Contributes to Vascular Inflammation and Insulin Resistance Induced by High-Fat Feeding

Norma O. Rizzo, Ezekiel Maloney, Matilda Pham, Ian Luttrell, Hunter Wessells, Sanshiro Tateya, Guenter Daum, Priya Handa, Michael W. Schwartz, Francis Kim

Objective—Diet-induced obesity (DIO) in mice causes vascular inflammation and insulin resistance that are accompanied by decreased endothelial-derived NO production. We sought to determine whether reduced NO-cGMP signaling contributes to the deleterious effects of DIO on the vasculature and, if so, whether these effects can be blocked by increased vascular NO-cGMP signaling.

Methods and Results—By using an established endothelial cell culture model of insulin resistance, exposure to palmitate, 100 μmol/L, for 3 hours induced both cellular inflammation (activation of IKKβ—nuclear factor-κB) and impaired insulin signaling via the insulin receptor substrate–phosphatidylinositol 3-kinase pathway. Sensitivity to palmitate-induced endothelial inflammation and insulin resistance was increased when NO signaling was reduced using an endothelial NO synthase inhibitor, whereas endothelial responses to palmitate were blocked by pretreatment with either an NO donor or a cGMP analogue. To investigate whether endogenous NO-cGMP signaling protects against vascular responses to nutrient excess in vivo, adult male mice lacking endothelial NO synthase were studied. As predicted, both vascular inflammation (phosphorylated IkBα and intercellular adhesion molecule levels) and insulin resistance (phosphorylated Akt [pAkt] and phosphorylated eNOS [p,eNOS] levels) were increased in endothelial NO synthase−/− (eNOS−/−) mice, reminiscent of the effect of DIO in wild-type controls. Next, we asked whether the vascular response to DIO in wild-type mice can be reversed by a pharmacological increase of cGMP signaling. C57BL6 mice were either fed a high-fat diet or remained on a low-fat diet for 8 weeks. During the final 2 weeks of the study, mice on each diet received either placebo or the phosphodiesterase-5 inhibitor sildenafil, 10 mg/kg per day orally. In high-fat diet–fed mice, vascular inflammation and insulin resistance were completely prevented by sildenafil administration at a dose that had no effect in mice fed the low-fat diet.

Conclusion—Reduced signaling via the NO-cGMP pathway is a mediator of vascular inflammation and insulin resistance during overnutrition induced by high-fat feeding. Therefore, phosphodiesterase-5, soluble guanylyl cyclase, and other molecules in the NO-cGMP pathway (eg, protein kinase G) constitute potential targets for the treatment of vascular dysfunction in the setting of obesity. (Arterioscler Thromb Vasc Biol. 2010;30:758-765.)

Key Words: nitric oxide ■ eNOS ■ vascular inflammation

In conditions of nutrient excess, such as obesity and diabetes mellitus, elevated free fatty acid (FFA) levels are implicated in the pathogenesis of both inflammation and insulin resistance in a variety of tissues, including endothelial cells.1-4 At the cellular level, nutrient excess is linked to vascular insulin resistance via activation of IKKβ and, subsequently, nuclear factor (NF)-κB, a key transcriptional mediator of inflammation.1 Among the responses observed in association with obesity-induced vascular inflammation and insulin resistance is a reduction in vascular levels of NO, which in other models has been linked to endothelial inflammation, thrombosis, and vasocostriction.5,6 These observations raise the possibility that a reduced level of vascular NO, which occurs relatively early during the course of diet-induced obesity (DIO),7 underlies vascular inflammation triggered by nutritional excess. In the current studies, we sought to investigate whether interventions that reduce NO signaling predispose vascular tissues to inflammation and insulin resistance during nutrient excess and, conversely, whether an increase of endothelial-derived NO signaling might block these deleterious vascular responses.

The relationship between NO and cellular inflammation is complex because, at high concentrations, NO is cytotoxic and proinflammatory, effects that are opposite to those induced by...
much lower concentrations of NO that involve the cGMP–dependent protein kinase (PKG) pathway. Inducible NO synthase is an enzyme found in activated macrophages and other immune cells that produces NO in the micromolar range. At these concentrations, NO induces oxidative DNA damage and modifications of protein structure and function that can lead to cell death. In comparison, NO produced by endothelial NO synthase (eNOS) is typically present at nanomolar concentrations and, via increased cGMP signaling and perhaps other mechanisms, has well-documented anti-inflammatory effects. For example, NO can inhibit vascular inflammatory NF-κB activity by the induction of IkBα,8 inhibition of NF-κB–DNA binding,9 or indirect inhibitory effects on NF-κB by activation of protein kinase A (PKA).10 In addition, eNOS has been shown to regulate the expression of NF-κB11,12; and NO-mediated S-nitrosylation of the p50 subunit of NF-κB12 (resulting in inhibition of NF-κB) is suggested to limit endothelial inflammation.

Endothelial-derived NO activates guanylyl cyclase in vascular smooth muscle and other cell types, thereby increasing cGMP production. Intracellular levels of cGMP are not only governed by guanylyl cyclase activity but also by phosphodiesterase conversion of cGMP back to GMP. Sildenafil (marketed as Viagra) is a phosphodiesterase type 5 (PDE-5) inhibitor that attenuates intracellular catabolism of cGMP. In addition to its use in the management of erectile dysfunction, sildenafil’s effect of enhancing signaling via cGMP is also useful in the treatment of pulmonary hypertension and congestive heart failure; it may promote ischemia-induced angiogenesis13 and immune regulation.14 A link between cGMP signaling and insulin action was identified in a recent study15 in which long-term treatment with sildenafil improved energy balance and insulin sensitivity in mice fed a high-fat (HF) diet; sildenafil also ameliorated diabetes-induced endothelial dysfunction in both a rat model and humans.16,17 Combined with evidence that vascular NO content decreases early in the course of DIO in mice, these observations raise the possibility that reduced NO-cGMP signaling is a trigger for the development of vascular insulin resistance and inflammation during HF feeding and, therefore, that increased signaling via this pathway may have therapeutic effects in such conditions.

In this study, we demonstrate that genetic and pharmacological interventions that reduce NO-cGMP signaling predispose to vascular inflammation and insulin resistance during nutrient excess, whereas interventions that increase signaling via this pathway exert a protective effect, both in vivo and in an endothelial cell culture model. These findings collectively suggest that NO-cGMP levels play a critical role in maintaining vascular insulin sensitivity, and that reduced signaling via this pathway is a key mediator of vascular inflammation and insulin resistance that occurs during HF feeding.

Methods

Materials

Anti–phosphorylated eNOS (serine 1177), anti–phosphorylated Akt (serine 473), anti-Akt, anti–phosphorylated IkBα, anti–insulin receptor substrate (IRS)-1 rabbit polyclonal antibodies and monoclonal anti–phosphotyrosine antibody were obtained from Cell Signaling, Beverly, Mass; anti-eNOS antibody was obtained from Transduction Labs, BD Biosciences, Lexington, Ky; and anti–intercellular adhesion molecule (ICAM) antibody was purchased from R and D Systems, Minneapolis, Minn. Total Akt and pAkt (serine 473) ELISA kits were obtained from Biosource, Camarillo, Ca. (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino][diazen-1-IM1-2-diolate (DETANO) and 8-bromoguanosine-3′,5′ (8-Br)cGMP were purchased from Alexis Biochemical, Lausen, Switzerland; and the soluble guanylyl cyclase inhibitor 1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and PKG inhibitor (KT 5823) were purchased from Cayman Chemical, Ann Arbor, Mich.

Cell Culture

Human microvascular endothelial cells (HMECs) were purchased from Invitrogen–Cascade Biological (Carlsbad, Calif) and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 12 μg/mL of bovine brain extract (Clonetics, Walkersville, Md); L-glutamine, 2 mmol/L; sodium pyruvate, 1 mmol/L; and nonessential amino acids in the presence of penicillin, 100 U/mL. The HMECs were maintained at 37°C in 5% CO2. All Western blots were performed as previously described,18 using equal amounts of total protein for each condition and experiment. SDS gel electrophoresis was performed using a 4%–20% gradient gel.

Study Protocol

Adult male C57Bl6 wild-type (WT) mice and eNOS−/− mice were purchased from Jackson Laboratories (Bar Harbor, Me). Age-matched groups (6 to 12 weeks old; n=10 per group) were maintained in a temperature-controlled facility with a 12-hour light-dark cycle and were fed an equicaloric diet that was either low (10% saturated fat) or high (60% saturated fat) in fat content (Research Diets, New Brunswick, NJ; Nos. D12492 and D12450B). Body weight and food intake were measured weekly. In 1 study involving WT mice only, sildenafil tablets, 100 mg, were ground into a powder and mixed into a highly palatable “treat,” 300 mg, containing peanut butter and an HF food pellet (Research Diets) to deliver sildenafil orally at a dose of 10 mg/kg per day. Vehicle-treated controls received the same daily treat without sildenafil, which was reliably and rapidly consumed. Sildenafil or vehicle was given once daily for the final 2 weeks of an 8-week study protocol in which mice were fed either the HF or the low-fat (LF) diet.

At the conclusion of the protocol, each animal received an intraperitoneal injection of either vehicle (normal saline) or regular insulin (2 U in 300 μL of normal saline) after an overnight fast. Fifteen minutes later, mice were euthanized with an overdose of CO2 followed by cervical dislocation. Thoracic aorta and surrounding connective tissue were quickly removed and snap frozen on dry ice. Protein was subsequently extracted from tissue samples; after protein levels were quantified using a kit (Micro BCA Protein Assay Kit; Pierce, Rockford Ill), equal amounts of protein were used for each condition in each assay. Total eNOS, p-eNOS, ICAM, and phosphorylated IκBα were assessed using Western blot analysis and were quantified using software (Image J; National Institutes of Health, Bethesda, Md). All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Statistical Analysis

In all experiments, densitometry measurements were normalized to controls incubated with vehicle, and fold increase above the control condition was calculated. An analysis of the results was performed using a statistical package (STATA8). Data are expressed as mean±SEM, and P<0.05 was considered statistically significant. A 2-tailed t test was used to compare mean values in 2-group comparisons. To compare responses between sildenafil- and vehicle-treated mice that also received either vehicle or insulin, data were analyzed by 2-way analysis of variance; and the Bonferroni–post hoc comparison test was used to compare mean values between groups.
Results

Effect of Reduced NO Bioavailability on Vascular Inflammation and Insulin Signaling

To determine if basal NO signaling is required to prevent endothelial inflammation and insulin resistance, we used the eNOS inhibitor, N^6^-nitro-L-arginine methyl ester (L-NAME), to reduce basal endothelial NO levels, and asked whether this intervention increases susceptibility to palmitate-induced endothelial dysfunction. This was accomplished by exposing HMECs to palmitate complexed with BSA, 10 μmol/L, for 3 hours and then treated with BSA (C) or BSA-palmitate (F), 10 μmol/L, for 3 hours, followed by insulin stimulation (I), 100 nmol/L, for 15 minutes. Cell lysates were analyzed by Western blot, and the fold increase over, BSA control group was calculated for each experiment (n=3). A, Fold increase of phosphorylated IκBα or ICAM protein levels compared with BSA controls (Figure 1A and B). However, in HMECs pretreated with L-NAME, 50 μmol/L, incubation with this same low dose of palmitate-BSA robustly induced phosphorylated IκBα and ICAM expression (Figure 1 A and B). Similarly, exposure of HMECs to 10 μmol/L of palmitate did not induce insulin resistance at the level of IRS-1 tyrosine phosphorylation, pAkt, and peNOS protein levels compared with BSA controls (Figure 1C and D). Moreover, L-NAME did not affect any of these parameters in the absence of palmitate. These results demonstrate that although reduced NO bioavailability does not in and of itself cause endothelial inflammation, it increases the susceptibility of endothelial cells to the inflammatory effects of palmitate and subsequent endothelial insulin resistance.

Effect of Increased NO Bioavailability on Palmitate-Mediated Vascular Inflammation and Insulin Signaling in Endothelial Cells

Because eNOS inhibition predisposes to the deleterious effects of palmitate, and increased NO bioavailability decreases expression of NF-κB–dependent adhesion molecule expression in endothelial cells,10,19 we determined whether interventions that increase NO bioavailability would attenuate palmitate-mediated activation of endothelial NF-κB signaling and endothelial insulin resistance. As previously shown,1 palmitate, 100 μmol/L for 3 hours, inhibited insulin-mediated increases of IRS-1 tyrosine phosphorylation, pAkt, and peNOS protein levels in HMECs, and pretreatment with DETANO, 50 μmol/L for 12 hours, blocked each of these responses (Figure 2A–C). In HMECs, phosphorylated IκBα and interleukin 6 protein levels, markers of endothelial inflammation, were increased greater than 2-fold over BSA-treated controls in response to palmitate-BSA, 100 μmol/L, consistent with previous findings.1 However, pretreatment with the NO donor DETANO, 50 μmol/L, attenuated each of these responses to palmitate (Figure 2D and E). In the presence of soluble guanylyl cyclase inhibitor 1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 1 μmol/L, or the PKG inhibitor KT 5823, 1 μmol/L, palmitate increased interleukin 6 levels, suggesting that the anti-inflammatory effect of DETANO requires soluble guanylyl cyclase and PKG signaling elements (Figure 2E). These results demonstrate that increased NO bioavailability attenuates palmitate-mediated inflammation and its deleterious effects on endothelial insulin signaling.

Effect of Increased cGMP Levels on Inflammatory Signaling in Endothelial Cells

The inhibitory effects of NO on NF-κB–dependent adhesion protein expression reportedly involve mechanisms that are both dependent on and independent of guanylyl cyclase/cGMP activity.6,20 To investigate the role of the guanylyl cyclase pathway in palmitate-induced endothelial inflammation, we used the cell-permeable cGMP analogue, 8-BrcGMP, Pretreatment of HMECs with 8-BrcGMP, 50 μmol/L, for 12 hours attenuated increases of phosphorylated IκBα induced by either palmitate or lipopolysac-
charide (LPS); similar to palmitate, LPS induces cellular inflammation via activation of toll like receptor 4 (TLR4) (Figure 3A and B). In the presence of soluble guanylyl cyclase inhibitor 1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, increased cGMP levels are still associated with decreased inflammatory responses in the presence of palmitate (Figure 3B). Thus, increased guanylyl cyclase/cGMP signaling mimics the anti-inflammatory effects of NO in endothelial cells exposed to palmitate. Similarly, treatment with 8-Br–cGMP attenuated palmitate-mediated impairment of insulin-stimulated pAkt (Figure 3C), suggesting that the anti-inflammatory effect of increased cGMP signaling can preserve endothelial insulin signaling in the presence of palmitate.

Vascular Inflammation and Insulin Resistance in eNos−/− Mice

To test the hypothesis that the absence of vascular eNOS predisposes the vasculature to the development of inflammation and insulin resistance in vivo, we studied both eNos−/− and WT mice fed either an LF or an HF diet for 4 weeks. As expected, HF feeding in WT mice was associated with rapid weight gain compared with LF-fed controls, and this effect did not differ by genotype, suggesting that eNOS is not required for weight gain in this setting (Figure 4A). However, compared with WT controls, eNos−/− mice exhibited significant reductions in insulin-mediated activation of pAkt in thoracic aortic lysates (Figure 4B), even on the LF diet. Similarly, eNos−/− mice exhibited increased aortic phosphorylated IκBα and ICAM protein levels when fed an LF diet, whereas WT mice exhibited increased aortic phosphorylated IκBα protein levels and reduced insulin-mediated activation of pAkt only when fed the HF diet (Figure 4C and D). These results demonstrate that in vascular tissue, the absence of eNOS (and, consequently, of NO) mimics the effect of HF feeding to induce vascular inflammation and insulin resistance.

Effect of Sildenafil on Vascular Insulin Signaling and Inflammation During HF Feeding

Because reduced NO predisposes to the effect of nutrient excess to cause vascular inflammation and insulin resistance, we hypothesized that increased NO-cGMP signaling would exert a protective effect. To test this hypothesis in an in vivo model, adult male C57BL6 (WT) mice were fed either an LF
diet (10% saturated fat) or an HF diet (60% saturated fat) for 8 weeks to cause obesity, vascular inflammation, and insulin resistance. During the final 2 weeks of the HF feeding protocol, mice on each diet received daily oral administration of either vehicle or the PDE-5 inhibitor, sildenafil, 10 mg/kg. As shown in Figure 5A, daily sildenafil treatment at this dose did not affect weight gain for mice on either diet compared with the vehicle control group.

In thoracic aortic lysates from vehicle-treated mice, insulin-mediated activation of pAkt and peNOS were reduced in HF-fed compared with LF-fed mice, consistent with our previous findings. In comparison, these defective vascular responses to HF feeding were not detected in mice treated with sildenafil (Figure 5B and C). Similar results were obtained when vascular inflammation was assessed. Thus, protein levels of phosphorylated IkBα and ICAM were increased in the thoracic aorta of vehicle-treated mice in response to HF feeding (but were not observed in LF-fed controls); in sildenafil-treated mice, these adverse responses to HF feeding were not observed (Figure 5D and E). Therefore, in mice, vascular inflammation and insulin resistance induced by HF feeding can be fully blocked by pharmacological inhibition of PDE-5, which increases intracellular signaling via cGMP.

**Discussion**

During nutritional excess, inflammatory pathways are activated rapidly in both cultured endothelial cells and in vascular tissue in vivo, and growing evidence implicates these inflammatory responses in the mechanism linking cardiovascular disease to obesity and related metabolic disorders. Because endothelial NO signaling has anti-inflammatory properties, and because vascular NO levels decline early in the course of DIO, we hypothesized an etiologic role for reduced NO-cGMP signaling in the pathogenesis of vascular inflammation and insulin resistance in this setting. By using an endothelial cell culture model, we found that susceptibility to the inflammatory effects of palmitate increases when eNOS is inhibited, whereas interventions that increase bioavailability of either NO or cGMP reverse palmitate-mediated NF-κB activation and insulin resistance. Similar results were obtained using a mouse model of obesity and insulin resistance induced by HF feeding. Herein, we found that in eNOS−/− mice, genetic deficiency of vascular NO is associated with vascular inflammation and insulin resistance, even for mice on an LF diet, suggesting that endothelial NO signaling is required to restrain these responses. Conversely, the deleterious vascular effects of HF feeding were prevented in healthy mice by daily oral dosing of sildenafil, a PDE-5 inhibitor that increases signaling downstream of NO. Together, these results provide direct evidence in support of the hypothesis that vascular NO-cGMP signaling plays a physiological role in protecting against vascular inflammation and insulin resistance and suggest that DIO (when inhibiting eNOS) is a mediator of these deleterious vascular responses.

Clinical studies have demonstrated a profound effect of FFA on NO production. In healthy patient volunteers, the ingestion of a single HF meal transiently impairs endothelial function, as measured by flow-mediated brachial artery vasodilation. The infusion of high doses of intralipid plus heparin into healthy volunteers increases circulating FFA concentrations from a starting concentration of 350 μmol/L to a peak of 3800 μmol/L; methacholine-induced vasodilation was reduced by as much as 20%, indicating that elevated FFA levels induce endothelial dysfunction. In a separate
study, increasing FFA levels resulted in impairment of basal and insulin-mediated NO production. These human studies suggest that the production of NO is impaired in the presence of high circulating levels of FFA. Previously, we demonstrated that the cellular mechanism of FFA-mediated impairment of NO is dependent on NF-κB signaling.

Cellular inflammation has emerged as an important mechanism underlying insulin resistance in muscle, liver, adipose tissue, and vascular tissue; interventions that block inflammatory responses have been shown to improve insulin sensitivity in vivo. Consistent with these observations, we report that whereas reduced NO signaling (induced by pharmacological inhibition of eNOS) enhances endothelial inflammation induced by palmitate, increased NO-cGMP signaling has the opposite effect in both cultured endothelial cells and a mouse model of DIO. Specifically, we found that in mice, daily oral administration of sildenafil for 2 weeks fully reversed the vascular inflammation and insulin resistance induced by HF feeding at a dose that had no effect on body weight gain or fat mass. This observation identifies vascular NO-cGMP signaling as a potential target in the treatment of vascular inflammation associated with human obesity and related metabolic disorders.

That IKKβ–NF-κB activation mediates palmitate-induced insulin resistance in endothelial cells was established in a previous study showing that IKKβ inhibition blocks this effect, whereas IKKβ activation recapitulates it. Combined with evidence that signaling via the innate immune receptor TLR4 is required for IKKβ–NF-κB activation by palmitate in endothelial cells, these findings suggest that NO antagonizes the ability of palmitate to increase endothelial signaling via TLR4 and to subsequently activate IKKβ–NF-κB. Whether the mechanism underlying this NO effect involves an interaction directly with NF-κB or with upstream signaling molecules is unknown. Several possibilities exist. First, NO is reported to scavenge superoxide, thereby reducing the generation of hydrogen peroxide and impeding the activation of NF-κB and the subsequent expression of inflammatory mediators that promote leukocyte adhesion and macrophage recruitment. Second, NO upregulates and stabilizes IκBα, the inhibitor of NF-κB, thereby suppressing NF-κB activity; it may also inhibit DNA binding by NF-κB, thereby decreasing the transcription of genes involved in cellular inflammation.

Additional studies are warranted to identify mechanisms underlying NO-mediated inhibition of the endothelial response to palmitate, and to determine whether NO exerts similar protective effects in other cell types, as suggested by evidence that reduced eNOS activity exacerbates liver injury in response to the bacterial endotoxin, LPS (similar to palmitate, LPS induces inflammation through TLR4 activation). Conversely, increased NO signaling by either NO donor administration or eNOS overexpression can prevent liver injury in animal models of hepatoxicity.

The findings of the present study are also consistent with recent work on AMPK and NO. Although the AMPK pathway is traditionally thought of as an intracellular fuel gauge and regulator of metabolism, it is also important in the regulation and maintenance of endothelial function. AMPK and NO are integrally related because AMPK has been shown to directly activate eNOS activity; recently, NO has been shown to be an endogenous activator of AMPK, suggesting a reciprocal relationship between AMPK and eNOS activity. Furthermore, palmitate-mediated activation of NF-κB and insulin resistance can be inhibited by AMPK. Therefore, a reduction in NO bioavailability could result in reduced activation of AMPK, leading to increased palmitate-mediated activation of vascular inflammation or increased susceptibility to the inflammatory effects of palmitate. In addition, increased NO levels could activate AMPK activity; through its “anti-inflammatory” effects, AMPK could attenuate palmitate-mediated activation of NF-κB. Thus, it is possible that the protective effects of NO-cGMP could be mediated by AMPK; further studies to address this question are warranted.

Although our findings suggest a key role for NO to prevent or limit vascular inflammation induced by overnutrition, it is important to recognize that at the same time NO biosynthesis is inhibited by activation of the IKKβ–NF-κB pathway in endothelial cells. The mechanism underlying this observation involves the effect of NF-κB activation of inhibiting signal transduction via the IRS-phosphatidylinositol 3-kinase pathway, a key positive regulator of eNOS activity. Because the course of reduced NO levels during HF feeding coincides with the onset of vascular inflammation and insulin resistance, reduced NO signaling may both mediate and be a consequence of vascular inflammation in the setting of DIO. These considerations support a bidirectional model in which the effect of nutrient excess on inhibiting endothelial NO production predisposes to inflammation that, in turn, inhibits eNOS and further reduces NO synthesis and release. Thus, we propose that reduced vascular NO levels are an integral component of a vicious cycle that operates in states of nutrient excess. Consistent with this hypothesis, studies in mice have shown that although insulin-stimulated induction of vascular phospholylated eNOS is measurably reduced (by approximately 40%) within the first week of HF feeding, this response declines progressively over time such that it is entirely absent after 8 weeks on this diet. This hypothesis is also in agreement with the negative feedback loop proposed by Grumbach et al, which explains the observation that in endothelial cells, shear stress activates eNOS and increases NO signaling on the one hand, while also activating NF-κB on the other hand. To explain these seemingly paradoxical findings, the researchers hypothesized that eNOS activation by shear stress serves to prevent sustained activation of NF-κB by shear stress. Accordingly, we propose that the early decline of NO signaling in DIO predisposes endothelial cells to inflammation; this, in turn, causes insulin resistance, leading to eNOS inhibition that further reduces NO bioavailability and thereby exacerbates endothelial inflammation.

Direct evidence in support of this hypothesis stems from our observation that aortic samples from eNos−/− mice fed an LF diet display the same pattern of inflammation and insulin resistance that occurs in the vasculature of WT mice rendered obese by HF feeding (Figure 4, Supplemental Figure I, supplemental material is available at http://atvb.ahajournals.org). This vascular response is characterized by activation of the IKKβ-
NF-κB pathway and induction of the adhesion molecule ICAM, and by resistance to the ability of insulin to activate either phosphatidylinositol 3-kinase or eNOS in aortic tissue. This change in vascular inflammation does not appear to be a result of changes in blood pressure between eNOS−/− and WT mice because no difference in ICAM expression was detected when both mouse strains were fed a chow diet (Supplemental Figure II). Complementary evidence is provided by our observation that a 2-week course of the PDE-5 inhibitor sildenafil fully reversed the deleterious vascular consequences of DIO in WT mice and thereby recapitulates the protective effect exerted by deficiency of TLR4. Similarly, both TLR4 deficiency and sildenafil administration can ameliorate systemic insulin resistance in mice with DIO.

One potential advantage of a therapeutic approach based on PDE-5 inhibition is that, unlike approaches that increase NO levels, this strategy averts the risks associated with excessive NO signaling. At high concentrations, NO is cytotoxic and its release from activated macrophages and other immune cells (via activation of inducible NO synthase) plays a key role in the host defense against pathogens. Yet, excessive NO can have detrimental effects even when derived from eNOS. For example, transgenic eNOS expression paradoxically increased vascular lesion formation in atherosclerosis-prone apolipoprotein E−/− mice. One mechanism forwarded to explain this finding is that excessive NO production can act via a mechanism independent of soluble guanylyl cyclase to increase oxidative stress and favor the development of atherosclerosis. This sequence of events would not be expected to arise from PDE-5 inhibition or, alternatively, from direct activators of soluble guanylyl cyclase or the cGMP target, protein kinase G. Therefore, each of these molecules constitutes a potential target for the treatment of vascular dysfunction in the setting of obesity.

Thoracic aortic tissue lysates (Figures 4 and 5) contain endothelial cells, vascular smooth muscle cells, and adventitial tissues; changes in Akt and inflammatory signaling could reflect changes in nonendothelial tissues. To verify the inflammatory effects of palmitate on smooth muscle cells, we used a mouse smooth muscle culture model (Supplemental Figure III). As expected, palmitate increased inflammatory markers (interleukin 6 and tumor necrosis factor α) and attenuated insulin-mediated Akt phosphorylation, suggesting that changes observed in the in vivo experiments are consistent with changes in both the endothelial and smooth muscle cell population. We are unable to determine the relative contribution of the different tissue types; however, data on the changes in eNOS phosphorylation are relatively specific to endothelial tissues. The overall significance and contribution of smooth muscle cell (SMC) inflammation during HF feeding deserve further study.

In conclusion, our findings suggest that NO-cGMP signaling plays a physiological role in attenuating the vascular inflammation induced by nutrient excess both in vivo and in an endothelial cell culture model. Consequently, the effect of DIO in reducing vascular NO-cGMP signaling is implicated in the mechanism underlying vascular inflammation and insulin resistance in this setting. Therefore, therapeutic strategies designed to increase vascular NO-cGMP signaling may be valuable in the prevention and treatment of obesity-associated cardiovascular disease.

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Disclosures

None.

References


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Supplement Material

Supplementary Figure I. Effect of palmitate-mediated inflammation and insulin signaling on smooth muscle cells. Mouse carotid artery smooth muscle cells were treated with palmitate-BSA (100 µM) for either 6 or 12 h or BSA control (con) and fold increase was calculated for each experiment (n=3). A. Fold increase in IL-6 concentration in response to palmitate as determined by ELISA. B-C. Fold increase in TNF-α and ICAM. D. SMC were treated with palmitate-BSA (F) or BSA control (C) followed by insulin stimulation (I) (100 nM, 15 min). Fold increase in pAkt normalized to total Akt is shown. * p<0.05

Supplementary Figure II. Effect of daily sildenafil during HF feeding and effect of HF in eNos-/− mice on thoracic iNOS expression. A. WT mice were fed a HF or LF diet for 8 wk and during the last 2 wk of the diet received daily doses of sildenafil. B. WT or eNos -/- mice were fed both a LF or HF diet for 4 wk. Fold increase over low-fat control mice was calculated. *p<0.05, n=5 in each group.

Supplementary Figure III. Effect of chow and LF-feeding in WT and eNos-/− mice. Both WT and eNos-/− mice were fed standard chow (low saturated fat) or LF (10% saturated fat) for 4 wk. ICAM expression was measured from thoracic aortic tissues.
Supplementary figure I
Supplementary Figure II
Supplementary Figure III