Nitric oxide modulates vascular inflammation and intimal hyperplasia in insulin resistance and the metabolic syndrome

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Barbato, Joel E., Brian S. Zuckerbraun, Marcus Overhaus, Kathleen G. Raman, and Edith Tzeng. Nitric oxide modulates vascular inflammation and intimal hyperplasia in insulin resistance and the metabolic syndrome. Am J Physiol Heart Circ Physiol 289: H228–H236, 2005. First published February 25, 2005; doi:10.1152/ajpheart.00982.2004.—Type 2 diabetes mellitus (DM) is associated with an increased incidence of many comorbid conditions in people of all ages (3). Conservative estimates place the number of patients suffering from DM worldwide at 150 million (13), a number that could exceed half a billion within a few decades (27). Of those patients with DM and vasoocclusive disease, ~90% have Type 2 DM (3), which is characterized by increased fasting serum glucose levels, insulin resistance, and hyperlipidemia. Type 2 DM is increasingly associated with obesity, both as a risk factor and as a causal agent in the pathology of the disease (38, 46). Obesity and insulin resistance are also key components of the metabolic syndrome, affecting more than 40 million Americans (43). The metabolic syndrome has often been considered a prediabetic state but is also considered a disease state in itself (17).

Patients with Type 2 DM and the metabolic syndrome have been shown in numerous studies to have an aggressive form of vascular disease characterized by accelerated atherosclerosis and proinflammatory changes (19, 37). In addition, these patients have significantly worse outcomes following vascular interventions such as angioplasty and stenting due to pronounced intimal hyperplasia and progression of atherosclerosis (24, 28). Interestingly, patients with insulin resistance and hyperglycemia in association with obesity in the form of the metabolic syndrome have also been shown to have a similar propensity toward aggressive cardiovascular disease (17, 37). Undiagnosed and unrecognized “diabetic” patients with insulin resistance and elevated oral glucose tolerance tests have also been shown to have worse outcomes following vascular interventions (44).

The etiology of this enhanced vascular disease and poor response to interventions in insulin-resistant states appears to be associated with increased inflammatory and proliferative activities that may be driven by increased serum levels of insulin and glucose or by other biochemical aberrancies. There may also be a role for increased NADPH and reactive oxygen species (ROS) production as well as the deposition of advanced glycation end products (AGE) and oxidized low-density lipoprotein (LDL) in the vascular wall. Investigators (7, 59) have reported that the interaction between the receptor for AGE (RAGE) and AGEs contributes to neointima formation. Also, the interaction of oxidized LDL with its receptor LOX-1 can stimulate several signaling events in endothelial cells as well as downregulate nitric oxide (NO) production, which can contribute to cardiovascular complications (10). Earlier studies into diabetic vascular responses clearly demonstrated that blood vessels from patients with DM have reduced NO bioavailability (56). The combination of all these perturbations may result in the propensity of patients with insulin resistance to develop atherosclerosis and vascular complications.

Potential therapies aimed at the prevention of intimal hyperplasia have largely been studied in animal models where normal blood vessels were subjected to injury. It is unclear how the increased oxidative stress and inflammatory phenotype associated with obesity and insulin resistance would impact on the efficacy of such potential therapies. It is possible that these therapies may be ineffective given the enhanced proliferative and inflammatory activity. On the basis of studies (20, 42) that suggest that the vascular injury response in the insulin-resistant obese Zucker rat is similar to that seen in patients with the metabolic syndrome, affecting more than 40 million Americans (43).

Diabetes mellitus (DM) is associated with an increased incidence of many comorbid conditions in people of all ages (3). Conservative estimates place the number of patients suffering from DM worldwide at 150 million (13), a number that could exceed half a billion within a few decades (27). Of those patients with DM and vasoocclusive disease, ~90% have Type 2 DM (3), which is characterized by increased fasting serum glucose levels, insulin resistance, and hyperlipidemia. Type 2 DM is increasingly associated with obesity, both as a risk factor and as a causal agent in the pathology of the disease (38, 46). Obesity and insulin resistance are also key components of the metabolic syndrome, affecting more than 40 million Americans (43). The metabolic syndrome has often been considered a prediabetic state but is also considered a disease state in itself (17). Patients with Type 2 DM and the metabolic syndrome have been shown in numerous studies to have an aggressive form of vascular disease characterized by accelerated atherosclerosis and proinflammatory changes (19, 37). In addition, these patients have significantly worse outcomes following vascular interventions such as angioplasty and stenting due to pronounced intimal hyperplasia and progression of atherosclerosis (24, 28). Interestingly, patients with insulin resistance and hyperglycemia in association with obesity in the form of the metabolic syndrome have also been shown to have a similar propensity toward aggressive cardiovascular disease (17, 37). Undiagnosed and unrecognized “diabetic” patients with insulin resistance and elevated oral glucose tolerance tests have also been shown to have worse outcomes following vascular interventions (44).

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metabolic syndrome and DM, we further examined the vascular injury response in these animals, focusing on the inflammatory events induced by injury and the resultant development of intimal hyperplasia. We also examined the therapeutic effect of increasing local NO bioavailability, using adenovirus-mediated inducible NO synthase (iNOS) gene transfer, on local inflammation and on neointima formation in the setting of similar to that of the metabolic syndrome or Type 2 DM.

MATERIALS AND METHODS

Virus. First generation E1 and E3 replication-deficient adenoviral vectors were utilized for these experiments. The preparation of a recombinant adenoviral vector carrying the human hepatocyte iNOS cDNA (AdiNOS) has been previously described (16, 50). Control vectors carried the β-galactosidase (AdLacZ) marker gene. Viral stocks were diluted to the appropriate concentration using serum-free Optimem (GIBCO; Grand Island, NY).

Surgical procedure. All animal procedures were performed under aseptic conditions in accordance with the Institutional Animal Care and Use Committee at the University of Pittsburgh. Obese Zucker rats [homozygous recessive mutation [fa/fa] for the leptin receptor (61)] and their genetic controls (lean Zucker rats) were purchased from Harlan Laboratories (Indianapolis, IN). They underwent surgery between 9 and 12 wk of age and were anesthetized with intraperitoneal pentobarbital sodium (45 mg/kg). The left common carotid artery was exposed and subjected to balloon catheter injury through the external carotid artery as previously described (50). Briefly, an arteriotomy was made in the external carotid artery, and a 2-Fr Fogarty catheter (Edwards Lifesciences; Irvine, CA) was inserted retrograde into the carotid artery where the balloon had the greatest diameter (47). The area of greatest injury was located in the midportion of the common carotid artery at 200−m intervals were utilized for morphometry measure-

Tissue processing. Animals were euthanized with CO2 asphyxia-

NO production and iNOS expression after iNOS gene transfer. Rats underwent carotid artery injury followed by treatment with AdiNOS, AdLacZ, or medium alone as described in Surgical procedure. After transfection, animals were immediately euthanized, and the carotid arteries were placed in culture in Dulbecco’s modified Eagle’s medium-Ham’s F12 nutrient (1:1) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mMol/l l-glutamine, and 10 μM BH4. Tissues were maintained in a 37°C, 95% air-5% CO2 incubator for 72 h. Nitrite accumulation in the culture medium was quantified by using the Griess reaction (26). The carotid arteries were then homogenized in lysis buffer, and the cytosol was collected as previously described (25). Protein concentrations of the cytosol were quantified using the BCA kit (Pierce). Proteins were visualized using chemiluminescence reagents according to the manufacturer’s instructions (Supersignal Substrate; Pierce).

Immunohistochemistry. Frozen sections of carotid arteries were utilized for immunofluorescent staining as previously described (26). Primary antibodies were ED1 against tissue macrophages (1:100, mouse anti-rat monoclonal; Serotec no. MCA341), Ki-67 against actively dividing nuclei (1:50, mouse anti-rat monoclonal; Pharmingen no. 550609), CD45 against leukocytes (1:100, mouse anti-rat monoclonal; Cymbus Biotechnology no. CBL-1502), P-selectin (1:100, rabbit anti-human polyclonal; Pharmingen no. 553716), LOX-1 receptor (1:100, goat anti-mouse polyclonal; Santa Cruz no. SC11653), and ICAM-1 (1:50, mouse anti-rat monoclonal; Pharmingen no. 550302). Secondary antibodies were goat (or rat for the case of the goat primary) antibodies against their respective primary antibody labeled with CY3 (Jackson Immunoresearch) or Alexa488 (Molecular Probes). Nuclear staining was performed with Hoechst stain (Sigma Chemical). Sections were visualized with Olympus Provis fluorescent microscopes. Fluorescence was quantified with Metamorph software. CD45 and ED-1 positive cells were quantified per high-powered field (with equal total areas counted for different groups). Mean fluorescence intensity of adhesion marker (ICAM-1 and P-selectin) and LOX-1 expression was quantified within the media and intima. This was done by obtaining fluorescent images of the vessels at the same optical intensity and outlining these areas on the computer software. The mean fluorescent intensity was then calculated per unit area with arbitrary values of 0 to 256 possible. Six sections from a minimum of three animals were utilized for all quantitation.

Proliferative index. Proliferative index was calculated using Meta-

RESULTS

A total of 80 animals underwent carotid artery injury and were euthanized at 3, 7, or 21 days or for baseline analysis. Baseline weights and serum levels of glucose, insulin, triglycerides, and cholesterol from fed animals are summarized in Table 1. These data demonstrate that the obese Zucker rats exhibit biochemical profiles that are similar to those seen in humans suffering from Type 2 DM as well as the metabolic syndrome with obesity, elevated glucose, insulin, and cholesterol levels as well as significant increases in triglycerides (1, 43).

Table 1. Baseline characteristics of Zucker rats

<table>
<thead>
<tr>
<th></th>
<th>Obese</th>
<th>Lean</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>506±41</td>
<td>355±16</td>
<td>0.003</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>314±35</td>
<td>187±13</td>
<td>0.002</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>125±7</td>
<td>74±2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>566±100</td>
<td>91±9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>21±4</td>
<td>9.9±2</td>
<td>0.013</td>
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Values are means ± SE; n, number of rats. Student’s t-test was used for P values.
Obese Zucker rat demonstrates increased inflammatory response to balloon injury. Carotid arteries from lean and obese Zucker rats were examined for inflammatory cell infiltration and adhesion molecule expression (Figs. 1 and 2). Uninjured contralateral carotid arteries and baseline preinjury vessels from both lean and obese rats showed no detectable staining for inflammatory cells (ED1-positive macrophages or CD45-positive leukocytes) in the vessel wall or in the perivascular tissues. Similarly, there was no appreciable adhesion molecule expression (data not shown). At 3 days after injury, vessels from the lean rats now showed the presence of infiltrating ED1-positive macrophages/monocytes (Fig. 1, A and B). However, vessels from obese Zucker rats had significantly greater staining for these inflammatory cells compared with lean rats (Fig. 1B). These macrophages were located predominantly in the periadventitial tissue with some infiltration of the media and intima. The expression of the cell-surface adhesion molecules ICAM-1 (3 days postinjury) and P-selectin (7 days) were also induced by injury in the lean rats (Fig. 2, A and B) but were dramatically upregulated in injured vessels from obese rats. ICAM-1 expression was induced throughout the media and intima, whereas P-selectin expression was confined to the regenerating endothelium. There was essentially no adhesion molecule expression detected in the contralateral uninjured vessel in any of the animals (data not shown), indicating that the upregulation of these elements represented a local response to injury and not a systemic inflammatory response. There was also no evidence of baseline upregulation of adhesion molecules in the obese animals that could explain the augmented inflammatory response. Unlike the adhesion molecules, there was detectable LOX-1 expression in the medial cells of uninjured carotid arteries from the obese animals but minimal expression in lean animals (Fig. 1C). Injury increased the expression of LOX-1 in both lean and obese animals but to a much greater extent in obese Zucker rats.

Generation of NO following injury. Transfection of rat carotid arteries with iNOS led to more than a 17-fold increase in nitrite production compared with AdLacZ-treated controls as well as uninjured vessels (Fig. 3A; P < 0.01). This corresponded to an increase in iNOS protein expression in the AdiNOS-transfected vessels compared with control vessels that had no detectable protein expression (Fig. 3B). These data confirm the successful expression of iNOS and NO production in the carotid arteries after adenoviral infection.

Adenovirus-mediated iNOS gene transfer inhibits the inflammatory response after injury. We evaluated the ability of iNOS gene transfer to reduce vascular inflammation after injury in the setting of insulin resistance and obesity. Injured vessels from obese rats treated with AdLacZ had significantly greater numbers of ED1-positive macrophages and CD45 leukocytes at 3 and 7 days, respectively (Fig. 1, A and B) compared with their lean counterparts after infection with the control vector AdLacZ. These levels of inflammatory cell infiltration were similar to those seen in animals not treated with adenoviral vector. After iNOS gene transfer, however, both lean and obese vessels demonstrated a substantial reduction in the number of ED1- (42% and 35%, respectively; P < 0.05) and CD45-positive cells (55% and 58%, respectively; P < 0.01). In addition, both lean and obese animals showed significant upregulation of LOX-1 21 days postinjury within the endothelium, the intimal, and medial cells (Fig. 1, A and C). LOX-1 expression was unaltered by AdLacZ. However, iNOS gene transfer greatly reduced LOX-1 expression, demonstrating that supplemental NO may block oxidized LDL deposition and subsequently downregulate the expression of its receptor.

iNOS gene transfer decreases adhesion molecule expression after injury. Similar to the levels of ICAM-1 and P-selectin expression detected in injured vessels from lean and obese rats, AdLacZ-treated vessels from lean and obese animals (Fig. 2, A and B) showed increased adhesion molecule expression after balloon injury. However, iNOS gene transfer at the time of injury reduced ICAM-1 expression dramatically within the media and intima in both lean and obese animals. P-selectin expression was also markedly diminished by iNOS gene transfer in both lean and obese rats at 7 days.

iNOS gene transfer inhibits proliferation. Frozen sections of carotid arteries were stained using immunofluorescent techniques for Ki-67 at 3 and 7 days postinjury (Fig. 4). Proliferative index was calculated by dividing the number of Ki-67-positive nuclei by the total number of nuclei. At day 3, vessels from lean rats had a proliferative index of 0.196 ± 0.017, whereas those from their obese counterparts had a significantly higher proliferative index of 0.299 ± 0.021 (P < 0.001, Fig. 4C). By day 7, the proliferative index was reduced to 0.047 ± 0.008 in the lean animals and 0.051 ± 0.013 in obese animals (P < 0.05 vs. day 3). iNOS gene transfer decreased proliferation by 54% on day 3 in lean rats (proliferative index of 0.091 ± 0.011, P < 0.001 vs. untreated and AdLacZ-treated lean rats). Interestingly, iNOS gene transfer had an even greater antiproliferative effect in vessels from the obese Zucker rats, reducing proliferation by 73% to a proliferative index of 0.079 ± 0.045 on day 3 (P < 0.001). By day 7, there was no difference in proliferative activity between AdLacZ- or AdiNOS-treated vessels.

iNOS gene transfer decreases neointimal formation. The effect of iNOS gene transfer on intimal hyperplasia in the setting of obesity and insulin resistance was examined (Fig. 5; Table 2). Lean animals displayed a mean intima-media (I/M) ratio at 3 wk of 0.52 ± 0.07, whereas the obese Zucker rats had a mean I/M ratio of 0.91 ± 0.06, representing a 75% increase in intimal hyperplasia in the presence of insulin resistance over controls (P = 0.001). Despite the larger neointimal lesions in the obese animals, there was no difference in total luminal area between the lean and obese animals (Table 1). There appeared to be adaptation of the vessels from the obese animals with increases in the areas encompassed by the internal elastic lamina and the external elastic lamina. Treatment of injured carotid arteries with AdLacZ (Fig. 5A) did not change neointima formation at 3 wk compared with untreated injured vessels in either the lean or the obese animals. Lean rats had a 53% reduction in neointima lesion size 3 wk after treatment with AdiNOS compared with AdLacZ-treated vessels and a 50% reduction over uninfected vessels (P < 0.05). In obese animals, iNOS gene transfer decreased the I/M ratio by 67% compared with those treated with AdLacZ and 66% compared with uninfected vessels (P < 0.001). After iNOS gene transfer, the amount of intimal hyperplasia that developed in response to carotid injury was similar between the obese and the lean animals, indicating that NO has enhanced efficacy in the presence of metabolic derangements like that seen in the metabolic syndrome and DM.
Fig. 1. Inflammatory response to balloon injury with inducible nitric oxide (NO) synthase (iNOS) gene transfer. Immunofluorescent staining of carotid arteries from lean and obese Zucker rats with gene transfer of iNOS. ED1-positive macrophages at 3 days postinjury and CD45-positive leukocytes detected with CD45 at 7 days (representative sections shown in A; cells staining red, as indicated by white arrows, are positive; ×400). The expression of the receptor for oxidized LDL (LOX-1) was examined at 21 days postinjury (red staining as indicated by white arrows is positive; ×1,000). Quantitation for macrophages and leukocytes (B) and LOX-1 (C; day 21 and baseline) was performed utilizing Metamorph software (baseline studies for other inflammatory markers was either negligible or absent). A total of six sections from a minimum of three animals were used for each data point. HPF, high-power field.
DISCUSSION

The fact that Type 2 DM and the metabolic syndrome have a negative impact on the progression of atherosclerosis as well as on the sequelae of reconstructive vascular interventions is well documented (3, 24, 28, 44). There is still a very limited understanding of the pathophysiology of diabetic vascular disease because of the complexity of the local and systemic changes associated with diabetes and, more generally, conditions of insulin resistance. Obesity and insulin resistance are associated with an enhanced inflammatory phenotype and increased oxidative stress contributing to endothelial dysfunction (8). It has long been recognized that blood vessels from patients suffering from DM are less vasoreactive, being less able to vasodilate after treatment with endothelium-dependent agonists stemming from reduced NO bioavailability (8). NO has a variety of vasoprotective properties. It can inhibit platelet adherence, decrease smooth muscle cell proliferation and migration, promote endothelial regeneration, and decrease leukocyte chemotaxis (15, 30, 45, 60). Inhibition of NOS activity can also lead to inflammatory changes in the absence of vascular injury with increased mononuclear infiltration and monocyte chemoattractant protein-1 (MCP-1) expression (54). This decreased NO availability may leave diabetic patients without physiological protection not only for the normal day-to-day rigors of vascular activity but also for derangements inherent in vascular interventions.

In our study, we confirm that obese Zucker rats biochemically resemble patients with the metabolic syndrome as well as Type 2 DM with increased serum glucose and insulin levels, hyperlipidemia, and obesity. This model has been used successfully by other groups to represent both conditions (35, 39, 42, 43). Also similar to the human condition, these rats respond to vascular injury with heightened inflammation characterized by the upregulation of ICAM-1 and P-selectin (8). This heightened inflammatory response is associated with enhanced proliferative activity and with greater neointima formation. We also show that vascular

Fig. 2. Adhesion molecule expression in diabetic vascular injury. ICAM-1 expression (red; white arrow indicates positive area of staining) at 3 days postinjury and P-selectin (green; white arrow indicates positive area of staining) at 7 days postinjury in Zucker rat carotid arteries (×400) (A). Obese diabetic animals have increased adhesion molecule expression at both time points, which is abrogated in the setting of iNOS transfection. Quantitation of ICAM-1 and P-selectin (B) was performed using Metamorph software and plotted as a function of mean intensity of fluorescence within media and intima. A total of six sections from a minimum of three animals were used for each data point.
injury in the obese Zucker rats induces LOX-1 expression, which may lead to increased atherogenesis at the site of injury. Other investigators have also demonstrated increased thrombospondin-1 expression, MCP-1 expression, and AGE accumulation after vascular injury in the setting of insulin resistance and diabetes (5, 53, 59), all of which have been shown to contribute to neointima formation.

Our findings suggest a discrepancy in the early events that occur in the obese and lean animals. After endothelial denudation and disruption of the elastic laminae, there is upregulation of cell surface markers such as ICAM-1 and P-selectin. These assist and enable platelet and leukocyte adherence and migration into the underlying interstitium of the vessel (6). Whereas these adhesion molecules may be expressed as early as 3–6 h after injury, we and other investigators (29) showed that the expression of selectins and the CAM family of adhesion molecules may persist for several days following injury, leading to a prolonged recruitment and influx of inflammatory cells. Furthermore, the finding that the obese Zucker rats had increased expression of ICAM-1, P-selectin, and inflammatory cell infiltration after injury is consistent with findings in human vessels, which exhibit increased baseline levels as well as injury-induced expression of these molecules (36).

Previous studies have demonstrated the beneficial effect of iNOS gene transfer on the inhibition of intimal hyperplasia (12, 50). Like the vast majority of preclinical studies, however, these studies were conducted in otherwise normal vessels subjected to injury. Our current studies show that, in the setting of increased inflammation and oxidative stress present in a diabetic blood vessel, iNOS gene transfer decreased intimal hyperplasia by 67% in the obese Zucker rats compared with 53% in their nondiabetic counterparts. This NO delivery reduced adhesion molecule expression and leukocyte recruitment to the site of injury. Whereas our method of gene transfer to an isolated segment of artery results in uptake largely confined to the vessel wall, NO is a diffusible molecule and may therefore have effects within any layer of the vessel wall as well as on infiltrating inflammatory cells residing within the wall (26).

![Fig. 3. Increased NO production after iNOS gene transfer. Vessels were injured, transfected either with AdiNOS, AdLacZ, or received no virus and cultured ex vivo. A: nitrite, a stable breakdown product of NO, was measured using the Griess reaction after 3 days of culture and normalized to protein content. Values represent the mean of three animals per group. B: Western blot of pooled carotid tissue homogenate demonstrating iNOS protein expression in injured vessels at 72 h postinjury with loading controls.](http://ajpheart.physiology.org/)

![Fig. 4. Effect of iNOS gene transfer on proliferation in vivo. Immunofluorescence for Ki-67 (red, indicated by white arrows) and nuclei (blue) was performed 3 and 7 days following injury. Representative sections from obese (fa/fa) Zucker rat carotid artery 3 days following injury and AdLacZ (A; ×400) and AdiNOS treatment (B). Proliferative index (number of Ki67-stained nuclei divided by total number of nuclei) was calculated and plotted (C). There were no statistical differences among the various 7-day time points.](http://ajpheart.physiology.org/)
Interestingly, the proliferating cells at the site of vascular injury were located predominantly in the adventitia, suggesting that the cells that form the neointima may be derived from extravascular precursor cells or from infiltrating myofibroblasts (41). NO may influence the ability of such cells to infiltrate the vessel wall or differentiate into smooth muscle cells by affecting the expression of adhesion molecules or more fundamentally by altering the phenotype of the cells.

Another effect of iNOS gene transfer was the reduction of LOX-1 expression. Type 2 DM is associated with increased vascular deposition of oxidized LDL (57). We confirmed this in the obese Zucker rats that had baseline increases in LOX-1 expression in the vascular media compared with their lean counterparts. LOX-1 staining was slightly upregulated in the lean animals after injury but was dramatically increased in the obese animals. Oxidized LDL and its interaction with LOX-1 has been identified in endothelial cells, smooth muscle, and macrophages (32). Increased LOX-1 expression may lead to enhanced oxidized LDL deposition and subsequent athrogenesis. iNOS gene transfer to vessels from insulin-resistant rats significantly reduced LOX-1 expression following injury, and this might represent another mechanism by which NO has a more profound protective effect on diabetic vasculature than in normal blood vessels.

The mechanisms by which NO inhibits the expression of adhesion molecules and LOX-1 have not been fully elucidated. NO has been reported to downregulate ICAM expression in endothelial cells stimulated by hydrogen peroxide (58) or by IL-1β (4). The regulation appears to be at the transcriptional level and involves transcriptional factors Sp1 and AP-1, both redox-sensitive factors. The involvement of NO in LOX-1 regulation has not been as well established. Smirnova et al. (51) reported a twofold increase of LOX-1 expression in human monocytes primed by NO synthesis inhibition, suggesting that NO downregulates LOX-1 and is anti-atherogenic. LOX-1 expression is regulated by proinflammatory cytokines (22) and nuclear factor-κB (33). The ability of NO to down-regulate LOX-1 may be linked to the inhibition of nuclear factor-κB activation by NO (23). These pathways may all center around the redox environment created by NO.

Whereas our studies on the effect of iNOS gene transfer on vascular injury-induced intimal hyperplasia (26, 49, 50, 55) have all shown this modality to be effective at inhibiting this

Table 2. Morphometric analysis of Zucker rat carotid arteries 21 days after balloon injury

<table>
<thead>
<tr>
<th>Zucker Obese Rats</th>
<th>Zucker Lean Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No virus</strong></td>
<td><strong>Ad.LacZ</strong></td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>Intimal area</strong></td>
<td>7.6±0.5</td>
</tr>
<tr>
<td><strong>Medial area</strong></td>
<td>8.4±0.2</td>
</tr>
<tr>
<td><strong>Luminal area</strong></td>
<td>38.0±0.6</td>
</tr>
<tr>
<td><strong>I/M ratio</strong></td>
<td>0.91±0.06</td>
</tr>
<tr>
<td><strong>EEL</strong></td>
<td>54.0±1.1</td>
</tr>
<tr>
<td><strong>IEL</strong></td>
<td>45.6±1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. Areas in 10⁴ μm²; I/M, intima/media; EEL, external elastic lamina; IEL, internal elastic lamina; EIL, external elastic lamina; IIL, internal elastic lamina. *P < 0.05 vs. no virus and AdLacZ; †P < 0.01 vs. no virus and AdLacZ; ‡P < 0.05 vs. obese.

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process without detriment to the blood vessel, it is important to remember that NO has potential cytotoxic actions as well. NO derived from iNOS has been associated with organ damage and cellular necrosis and/or apoptosis (2, 14). It can also lead to the formation of other reactive nitrogen species with greater cytotoxic potential such as peroxynitrite. Systemically, NO can lead to vasodilation and refractory hypotension. Finally, it is still highly debated what the role of iNOS is in atherogenesis with data to support both pro- and anti-atherogenic effects (21, 31). These aspects of NO biology underly the importance of localizing iNOS gene transfer and fully evaluating the consequences of this treatment before clinical application.

Our current study does not identify the causative factor(s) for the inflammatory phenotype and increased intimal hyperplasia response observed in the setting of insulin resistance and obesity. There is no clear evidence that increased insulin or other physiological changes associated with insulin resistance are individually linked to these changes, although this has been suggested by some human studies (34, 40), nor is the role of hyperglycemia fully understood. Whereas other investigators have suggested that diabetic patients have an impaired response to NO and that this impairment in NO production can increase inflammation following injury (18, 54, 56), our study does not suggest or prove that the primary insult in DM is decreased NO bioavailability. Instead, the purpose of our studies was to determine whether iNOS gene transfer, which is effective at blocking intimal hyperplasia in normal blood vessels, would be effective in the setting of insulin resistance where inflammation and proliferative activities are greatly increased. Our study demonstrates that not only does gene transfer of iNOS result in decreased intimal hyperplasia following angioplasty in the obese Zucker rats, this effect is enhanced in the setting of insulin resistance and obesity. These findings also suggest that the causative mechanisms for this exaggerated injury response in insulin resistance and diabetes may include increased adhesion molecule expression, augmentation of the inflammatory cell influx, as well as upregulation of the oxidized LDL receptor. Further studies are required to understand the changes associated with insulin resistance and obesity that prime these animals for this enhanced inflammatory response. Such information may lead to therapies that can suppress this “primed” state.

In conclusion, the vascular injury response in the insulin-resistant states, such as the metabolic syndrome and possibly Type 2 DM, is characterized by increased adhesion molecule expression, LOX-1 expression, inflammatory cell infiltration, and neointima formation. iNOS gene transfer was able to significantly inhibit all these events. The actual role of NO in the pathophysiology of intimal hyperplasia and atherogenesis in the metabolic syndrome and Type 2 DM remains only partially understood. Our findings, however, indicate that the augmented inflammatory response and oxidative stress in insulin resistance can be reversed by increasing NO bioavailability.

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GRANTS

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REFERENCES


30. Phillips SA, Sylvester FA, and Frisbee JC. Oxidant stress and constric-


