Insulin enhances the effect of nitric oxide at inhibiting neointimal hyperplasia in a rat model of type 1 diabetes

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1Division of Vascular Surgery and Institute for BioNanotechnology in Medicine, Northwestern University, 2Department of Surgery, University of Illinois at Chicago, and 3Department of Surgery, University of Illinois at Mount Sinai, Chicago, Illinois; 4Basic Research Program, SAIC-Frederick, Inc., and 5Laboratory for Comparative Carcinogenesis/Center for Cancer Research, National Cancer Institute-Frederick, Frederick, MD; and the 6Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois

Submitted 23 December 2009; accepted in final form 17 June 2010

Varu VN, Ahanchi SS, Hogg ME, Bhikhapurwala HA, Chen A, Popowich DA, Vavra AK, Martinez J, Jiang Q, Saavedra JE, Hrabie JA, Keefer LK, Kibbe MR. Insulin enhances the effect of nitric oxide at inhibiting neointimal hyperplasia in a rat model of type 1 diabetes. Am J Physiol Heart Circ Physiol 299: H772–H779, 2010. First published June 18, 2010; doi:10.1152/ajpheart.01234.2009.—Diabetes confers greater restenosis from neointimal hyperplasia following vascular interventions. While localized administration of nitric oxide (NO) is known to inhibit neointimal hyperplasia, the effect of NO in type 1 diabetes is unknown. Thus the aim of this study was to determine the efficacy of NO following arterial injury, with and without exogenous insulin administration. Vascular smooth muscle cells (VSMC) from lean Zucker (LZ) rats were exposed to NO donor, DETA/NO, following treatment with different glucose and/or insulin concentrations. DETA/NO inhibited VSMC proliferation in a concentration-dependent manner to a greater extent in VSMC exposed to normal-glucose vs. high-glucose environments, and even more effectively in normal-glucose/high-insulin and high-glucose/high-insulin environments. G1/G0 cell cycle arrest and cell death were not responsible for the enhanced efficacy of NO in these environments. Next, type 1 diabetes was induced in LZ rats with streptozotocin. The rat carotid artery injury model was performed. Type 1 diabetic rats experienced no significant reduction in neointimal hyperplasia following arterial injury and treatment with the NO donor PROLI/NO. However, daily administration of insulin to type 1 diabetic rats restored the efficacy of NO at inhibiting neointimal hyperplasia (60% reduction, P < 0.05). In conclusion, these data demonstrate that NO is ineffective at inhibiting neointimal hyperplasia in an uncontrolled rat model of type 1 diabetes; however, insulin administration restores the efficacy of NO at inhibiting neointimal hyperplasia. Thus insulin may play a role in regulating the downstream beneficial effects of NO in the vasculature.

proliferation; arterial injury; vascular smooth muscle cells

Diabetes is one of the most prevalent and costly chronic diseases worldwide, primarily due to cardiovascular complications (33). It is the more aggressive and accelerated course of cardiovascular disease in the diabetic that makes diabetes a major risk factor for cardiovascular morbidity and mortality. In fact, diabetes increases the risk of developing coronary, cerebrovascular, and peripheral arterial disease up to fourfold (6). Furthermore, given the severity of atherosclerosis in diabetes, these patients have a greater likelihood of end-organ ischemia, requiring more revascularization procedures compared with the general population at an earlier point in time. Unfortunately, the diabetic population undergoing these procedures is more prone to restenosis and failure, which is secondary to a heightened formation of neointimal hyperplasia (16, 35). Furthermore, diabetic patients requiring insulin are associated with having a more severe form of the disease process secondary to less endogenous insulin reserve and having an increased cardiovascular disease burden compared with diabetic patients not requiring insulin (7).

Nitric oxide (NO) is a molecule shown to have many beneficial effects on the vasculature, particularly at inhibiting neointimal hyperplasia. Numerous studies have shown that local administration of NO reduces neointimal hyperplasia in animal models of arterial injury and bypass grafting (10, 12, 13, 17, 24, 25, 27, 28, 30–32, 36, 39). Until recently, the efficacy of NO-based therapies in a diabetic environment was unknown. Our laboratory recently showed that NO was a more effective therapy for neointimal hyperplasia in animal models of insulin resistance (i.e., type 2 diabetes and metabolic syndrome) compared with control animals (2). But to our knowledge, the efficacy of NO-based therapies in insulin-deficient type 1 diabetes remains unknown. A recent study detailing nearly 20 yr of experience of revascularization for peripheral arterial disease showed that patients with insulin-dependent diabetes demonstrated a greater systemic vascular disease burden and an increased rate of restenosis with a decreased primary patency rate vs. patients with non-insulin-dependent diabetes (4). Thus, given the more aggressive vascular disease pattern observed in patients with type 1 diabetes, and the different metabolic environment encountered in the vasculature in patients with type 1 vs. type 2 diabetes, the goal of this study was to determine whether NO would be effective at inhibiting neointimal hyperplasia in a type 1 diabetic environment, with and without insulin therapy, as this remains unknown. We hypothesized that NO would inhibit neointimal hyperplasia in a type 1 diabetic model.

Methods

Cell culture. Abdominal aortic vascular smooth muscle cells (VSMC) were isolated and cultured from lean Zucker (LZ; Crl:ZUC-Leprfa, Charles Rivers, Wilmington, MA) rats using the collagenase method described by Gunther et al. (20). Cells were maintained in media containing equal volumes of DMEM-low glucose (Invitrogen,
Carlsbad, CA) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), and 4 mM L-glutamine (VWR, West Chester, PA) and incubated at 37°C, 95% air and 5% CO₂. VSMC were used between passages 5 and 9.

Proliferation assay. Aortic VSMC plated in 12-well plates (5 x 10⁴ cells/well) were growth arrested for 24 h, after which they were exposed to media containing varying concentrations of the diazeniumdiolate NO donor (Z)-1-[(2-aminoethyl)amino]diazen-1-ium-1-2-diolate (DETA/NO), glucose, and/or bovine insulin in the presence of tritiated (³H) thymidine (5 µCi/ml, Perkin Elmer, Wellesley, MA) for an additional 24 h. ³H-thymidine incorporation into trichloroacetic acid-precipitated DNA was quantified by scintillation counting. DETA/NO was chosen because of its 20-h half-life, making it an ideal diazeniumdiolate for a 24-h in vitro assay. Sorbitol (25 mM) was used as an osmotic control for all experiments.

Cell death assay. Aortic VSMC plated in six-well plates (1 x 10⁵ cells/well) were growth arrested for 24 h, after which they were exposed to media containing varying concentrations of the NO donor DETA/NO for 24 h. Cells were trypsinized, collected, and pelleted. Cells were resuspended in 250 µl PBS, and 40 µl of this suspension were added to 160 µl of Guava ViaCount Reagent (Guava Technologies, Hayward, CA), which binds to DNA similarly to propidium iodide (PI) (Invitrogen). Cell death was assessed using a Guava Personal Cell Analysis system (Guava Technologies).

Flow cytometry. Aortic VSMC plated in six-well plates (1 x 10⁵ cells/well) were growth arrested for 24 h, after which they were exposed to media containing varying concentrations of the diazeniumdiolate NO donor DETA/NO, glucose, and/or bovine insulin for a subsequent 24 h. Cells were trypsinized, collected, and pelleted. Cells were rinsed twice with PBS (1.000 µl), resuspended in 50 µl PBS, and then fixed with 450 µl of ice-cold 70% ethanol. After ethanol fixation, cells were rinsed twice with PBS, pelleted, then resuspended in a PI staining solution (1 x PBS (pH 7.4), 50 µg/ml PI, 204 µg/ml RNase A (Sigma, St. Louis, MO), 0.1% Triton X-100 (Fisher Biotech, Fair Lakes, NJ)). Samples were incubated for 15 min at 37°C before being analyzed on a Coulter Epic XL flow cytometer. Analysis was performed using Mod Fit 3.1 LT (Verity, Topsham, ME).

Animal models. Eleven-week-old male LZ rats were obtained from Charles River Laboratories. LZ rats were used for these studies, given that they are the same strain as the animals used in our laboratory’s prior study with insulin-resistant animals (2). These rats, when fed normal chow, exhibit a normal metabolic profile and serve as the genetic control. Type 1 diabetes was induced in male 11-wk-old LZ rats using a protocol that has been previously described (2, 24, 36, 39). After injury and restoration of blood flow, 10 mg of the diazeniumdiolate NO donor disodium 1-[(2-carboxyloxy)pyrrolidin-1-yl]diazen-1-ium-1-2-diolate (PROLI/NO) was applied evenly to the external surface of the injured common carotid artery of rats in the treatment group, and the neck incision was closed. Treatment groups for control LZ, STZ, and STZ+1 (animals included 1) injury and 2) injury + PROLI/NO) (n = 5–6/treatment group). Carotid arteries were harvested at 14 days for morphometric analysis, at which time blood was collected to measure insulin, glucose, cholesterol, and triglyceride levels. PROLI/NO was used as the diazeniumdiolate for the in vivo experiments, as it is the diazeniumdiolate NO donor that we have consistently demonstrated to be superior at inhibiting neointimal hyperplasia, compared with other diazeniumdiolates, and it is the NO donor used in our laboratory’s prior study on type 2 diabetes (2, 24, 36).

Tissue processing. Carotid arteries were harvested following in situ perfusion fixation with cold PBS (250 ml) and 2% paraformaldehyde (500 ml). Vessels were placed in paraformaldehyde at 4°C for 1 h, then cryoprotected in 30% sucrose at 4°C overnight. The tissue was quick-frozen in OCT (Tissue Tek, Hatfield, PA), and 5-µm sections were cut throughout the entire injured segment of the common carotid artery.

Morphometric analysis. Carotid arteries harvested at 2 wk were examined histologically for evidence of neointimal hyperplasia using routine hematoxylin-eosin staining. Digital images of stained sections were collected with a light microscope using an Olympus BHT microscope (Melville, NY) with x4, x10, and x40 objectives. For the morphometric analysis, six evenly spaced sections through each injured carotid artery were analyzed. Intimal area (I) and media area (M) were measured using ImageJ software with uniform arbitrary units (AU) (NIH, Bethesda, MD) with subsequent calculation of the intimal-to-medial area ratio (I/M).

RESULTS

Glucose inhibits VSMC proliferation in a concentration-dependent manner in vitro. To understand the effect of glucose on VSMC proliferation, an in vitro assay was conducted using [³H]thymidine incorporation to serve as a surrogate for cellular proliferation. Exposure VSMC to low- (control, 5 mM), medium- (15 mM), and high-glucose (25 mM) concentrations for 24 h showed dose-dependent reduction in VSMC proliferation (Fig. 1A). Those exposed to the high-glucose concentration demonstrated a statistically significant reduction in proliferation compared with the control group (50%, P < 0.05). VSMC exposed to sorbitol (25 mM) as an osmotic control proliferated at rates similar to control VSMC (not shown).

Table 1. Rat insulin glargine sliding scale

<table>
<thead>
<tr>
<th>Glucose, mg/dl</th>
<th>Insulin Glargine, units</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;200</td>
<td>1</td>
</tr>
<tr>
<td>200–300</td>
<td>2–3</td>
</tr>
<tr>
<td>300–400</td>
<td>4–5</td>
</tr>
<tr>
<td>400–500</td>
<td>6–7</td>
</tr>
<tr>
<td>&gt;600</td>
<td>8–9</td>
</tr>
</tbody>
</table>

Animal surgery. Approximately 14 days following injection of STZ, animals underwent the rat carotid artery injury model. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, 1996) and approved by the Northwestern University Animal Care and Use Committee. Rats were anesthetized with inhaled isoflurane (0.5–3%). Atropine was administered subcutaneously (0.1 mg/kg) to decrease airway secretions. Weight was documented, and blood glucose was measured daily on administration of STZ. The neck was shaved and prepped with betadine and alcohol (75%). Following a midline neck incision, the rat carotid artery balloon injury model was performed using a 2F Fogarty catheter (generously provided by Edwards Lifesciences), as previously described (2, 24, 36, 39). After injury and restoration of blood flow, 10 mg of the diazeniumdiolate NO donor disodium 1-[(2-carboxyloxy)pyrrolidin-1-yl]diazen-1-ium-1-2-diolate (PROLI/NO) was used as an osmotic control for all experiments.

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**Insulin stimulates VSMC proliferation in a concentration-dependent manner in vitro.** To understand the effect of insulin on VSMC proliferation, \[^{3}H\]thymidine incorporation was assessed in the presence of increasing concentrations of insulin. Exposing the VSMC to increasing concentrations of bovine insulin (6–240 nM) for 24 h showed a dose-dependent increase in proliferation of VSMC (Fig. 1B). Those exposed to 120 and 240 nM insulin showed a statistically significant increase in proliferation compared with control (64 and 110%, respectively, *P* < 0.05).

**NO is more effective at inhibiting VSMC proliferation in a normal-glucose, high-insulin environment in vitro.** Next, the antiproliferative effects of NO were examined in different glucose and insulin environments. An environment similar to “uncontrolled type 1 diabetes” was created by exposing the VSMC to a high-glucose, low-insulin medium. A control environment consisted of normal-glucose, low-insulin media. The addition of the highest concentration of DETA/NO (1 mM) to VSMC caused a significant reduction in cell proliferation in all three environments, but the least reduction was observed in the high-glucose, low-insulin environment (58% reduction), and the greatest reduction was observed in the normal-glucose, high-insulin environment (73% reduction) vs. control (63%, *P* < 0.05, Fig. 1, C and D). Of note, VSMC exposed to high glucose and high insulin showed an even greater reduction in cellular proliferation when exposed to NO (84%, *P* < 0.05, Fig. 1, C and D) and would serve as a model for “uncontrolled type 2 diabetes.”

**NO, insulin, and glucose do not induce significant VSMC death in vitro.** Guava Personal Cell Analysis was used to determine whether NO, insulin, or glucose, or combinations thereof, induce VSMC death in vitro, in addition to inhibiting proliferation. No statistically significant impact on cell death was observed with these different treatment groups compared with the control environment (Fig. 2). Thus VSMC cell death is not responsible for the decrease in proliferation observed with the addition of NO, glucose, or insulin.
Glucose induces a G0/G1 cell cycle arrest. To further evaluate the mechanism by which NO inhibits VSMC proliferation in different glucose and insulin environments, the percentage of cells in the different stages of the cell cycle was analyzed. The majority of VSMC at baseline were in the G0/G1 phase, but high glucose resulted in a statistically significant higher percentage of cells in G0/G1 compared with normal glucose environments (65 vs. 59%, \( P = 0.024 \), Table 2). Furthermore, a statistically significant lower percentage of cells were in the S phase under high-glucose vs. normal-glucose conditions (24 vs. 30%, \( P = 0.001 \)). Taken together, these data correlate with the VSMC proliferation data observed in different glucose and insulin environments, the percentage of cells in G0/G1 compared with normal glucose (Table 3). To summarize, type 1 diabetes rats exhibited profound high glucose with reduced serum insulin levels vs. control (\( P < 0.05 \)), whereas those type 1 diabetic rats that received insulin glargine per sliding scale exhibited significantly less high glucose and increased insulin levels (\( P < 0.05 \)). The addition of the diazeniumdiolate NO donor PROLI/NO did not result in significant differences in these metabolic parameters.

Insulin does not enhance the cell cycle arrest induced by NO in vitro. The addition of DETA/NO caused an expected increase in the percentage of VSMC in the G0/G1 phase compared with control in both normal- and high-glucose environments, with no statistically significant difference noted between the two glucose groups (74 and 78%, respectively, \( P = 0.193 \), Table 2). A similar trend was seen with DETA/NO in combination with high insulin exposed to both normal- and high-glucose environments (76 and 76%, respectively, \( P = 0.771 \), Table 2). Taken together, these data do not correlate with the amount of proliferation observed in VSMC in different glucose and insulin environments when exposed to NO (Fig. 1, C and D). Thus the enhanced efficacy of NO in high-insulin environments is not due to greater enhancement of a G0/G1 cell cycle arrest.

To corroborate these findings, expression of the cyclin-dependent kinase cell cycle inhibitory proteins was assessed with Western blot analysis. No significant differences were observed in protein expression for p21 and p27 in VSMC exposed to control, high-glucose, or high-insulin conditions (data not shown).

Insulin regulates the efficacy of NO at inhibiting neointimal hyperplasia in a type 1 diabetic animal model. Next, we examined the effect of NO at inhibiting neointimal hyperplasia in rat models of uncontrolled type 1 diabetes (STZ), insulin-controlled type 1 diabetes (STZ+I), and nondiabetic controls (LZ) using the rat carotid injury model. Animal weights, serum glucose, insulin, cholesterol, and triglyceride levels were obtained throughout the experiments and confirmed the desired metabolic disease states (Table 3). To summarize, type 1 diabetes rats exhibited profound high glucose with reduced serum insulin levels vs. control (\( P < 0.05 \)), whereas those type 1 diabetic rats that received insulin glargine per sliding scale exhibited significantly less high glucose and increased insulin levels (\( P < 0.05 \)). The addition of the diazeniumdiolate NO donor PROLI/NO did not result in significant differences in these metabolic parameters.

Following balloon injury of the rat carotid artery, reproducible neointimal lesions were observed in all three rat disease states at 2 wk (Fig. 3 and Table 4). Less neointimal hyperplasia was observed in the STZ rats compared with control LZ rats (I/M 0.584 vs. 1.099, \( P < 0.05 \)), but upon insulin administration (STZ+I), an increase in the amount of neointimal hyperplasia was observed (I/M 1.32, \( P = 0.071 \)). Treatment with the NO-donor PROLI/NO caused a 37% reduction in the I/M in the LZ rats vs. injury alone (I/M 0.693 vs. 1.099, \( P < 0.05 \)). However, NO was completely ineffective at reducing neointimal hyperplasia in STZ rats that did not receive insulin (I/M 0.584 vs. 0.712, \( P = 0.312 \)). Surprisingly, with the addition of insulin to the STZ rats (STZ+I), the effectiveness of NO was restored, causing a potent 60% reduction in neointimal hyperplasia compared with injury alone (I/M 1.32 vs. 0.529, \( P < 0.05 \)).

Upon examination of the intimal and medial areas, similar trends were observed. Less intimal area was observed in the STZ rats compared with LZ rats (13,216 vs. 23,962 AU, \( P < 0.05 \), Table 4), but, upon insulin administration (STZ+I), a statistically significant increase in the intimal area was seen compared with LZ rats (41,153 vs. 23,962 AU, \( P < 0.05 \)). Treatment with the NO

Table 2. Cell cycle analysis

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Normal Glucose, %</th>
<th>High Glucose, %</th>
<th>( P ) Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starved</td>
<td>91 ± 0.5†</td>
<td>91 ± 1.2†</td>
<td>0.945</td>
</tr>
<tr>
<td>Control</td>
<td>59 ± 1.5</td>
<td>65 ± 2.0</td>
<td>0.024</td>
</tr>
<tr>
<td>DETA/NO</td>
<td>74 ± 2.8†</td>
<td>78 ± 1.1†</td>
<td>0.193</td>
</tr>
<tr>
<td>DETA/NO + I</td>
<td>76 ± 1.9†</td>
<td>76 ± 1.8†</td>
<td>0.771</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starved</td>
<td>5 ± 0.4†</td>
<td>5 ± 0.6†</td>
<td>0.853</td>
</tr>
<tr>
<td>Control</td>
<td>30 ± 1.2</td>
<td>24 ± 1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>DETA/NO</td>
<td>19 ± 2.3†</td>
<td>17 ± 0.7†</td>
<td>0.182</td>
</tr>
<tr>
<td>DETA/NO + I</td>
<td>18 ± 1.2†</td>
<td>18 ± 1.0†</td>
<td>0.917</td>
</tr>
<tr>
<td>G0/M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starved</td>
<td>4 ± 0.3†</td>
<td>4 ± 0.8†</td>
<td>0.631</td>
</tr>
<tr>
<td>Control</td>
<td>11 ± 1.0</td>
<td>11 ± 0.9</td>
<td>0.869</td>
</tr>
<tr>
<td>DETA/NO</td>
<td>7 ± 0.8†</td>
<td>6 ± 0.6†</td>
<td>0.470</td>
</tr>
<tr>
<td>DETA/NO + I</td>
<td>7 ± 1.1†</td>
<td>6 ± 0.8†</td>
<td>0.682</td>
</tr>
</tbody>
</table>

Values are means ± SE. DETA, (Z)-1-(2-aminoethyl)-N-(2-amminio-ethyl)amino]diazene-1-ium-1,2-diolate; NO, nitric oxide; I, insulin. DETA/NO, 1,000 \( \mu \)M; insulin, 200 nM; normal glucose = 5 mM glucose, high glucose = 25 mM glucose. *Low vs. high glucose. †\( P < 0.05 \) vs. control.
Injury response, and this difference accounts for the difference between the efficacies observed with the NO-based therapy in these diabetic environments.

**DISCUSSION**

In the present study, we examined the effect of NO on VSMC proliferation, cell cycle regulation, cell death, and neointimal hyperplasia in environments that mimic controlled and uncontrolled type 1 diabetes. We found that NO was most effective at inhibiting VSMC proliferation and neointimal hyperplasia in an insulin-controlled type 1 diabetic environment, while NO was ineffective in an uncontrolled high glucose type 1 diabetic state. This is a novel and important finding, as there are a vast number of patients with type 1 diabetes worldwide undergoing vascular interventions that experience significant morbidity and mortality.

Recently, a retrospective analysis by Bakken et al. (4) showed that patients with insulin-dependent diabetes tended to have a greater vascular disease burden, with increased restenosis after vascular interventions than those with type 2 diabetes.

Fig. 3. NO inhibits neointima formation following arterial injury more effectively in a rat model of type 1 diabetes controlled with insulin and is ineffective in type 1 diabetic rodents when insulin is not present. A: intima-to-media area ratios (I/M) for LZ (control), streptozotocin (STZ; uncontrolled type 1 diabetes), and STZ + insulin (I) (controlled type 1 diabetes) rats subjected to vascular injury alone and vascular injury followed by application of the NO donor disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO). Values are means ± SE; n = 5–6 per treatment group. **P < 0.05 compared with injury alone. *P < 0.05 compared with LZ. B: Representative hematoxylin-eosin-stained cross sections from LZ, STZ, and STZ + I rats: uninjured, injury alone, or injury plus application of the NO donor PROLI/NO.
nosis, decreased assisted primary patency, and worse limb salvage rates compared with non-insulin-dependent diabetics and nondiabetics following percutaneous superficial femoral artery interventions, despite having less complex lesions compared with nondiabetic patients. Moreover, studies have shown that patients receiving exogenous insulin have manifested increased rates of cardiovascular events and cardiovascular mortality compared with those not receiving insulin therapy (1, 7). The findings we have presented show that a NO-based therapy is effective at preventing neointimal hyperplasia and subsequent restenosis when insulin is present. These findings will lay the foundation for the development of designer therapeutics for patients with type 1 diabetes undergoing vascular interventions.

The endothelium, among other things, synthesizes important bioactive substances, most notably NO, which mediates vasorelaxation, inhibits platelet activation and inflammation, inhibits VSMC proliferation and migration, and stimulates endothelial cell proliferation and migration (8, 14, 19, 21, 29, 37, 42, 46). Taken together, these properties of NO inhibit neointimal hyperplasia. Over the last two decades, NO has been shown to inhibit neointimal hyperplasia, but mostly in the context of normal metabolic environments (10, 12, 13, 17, 24, 25, 27, 28, 30–32, 36, 39). However, Barbato et al. (5) examined the effect of inducible NO synthase gene transfer in rats exhibiting metabolic syndrome. They demonstrated that NO inhibited neointimal hyperplasia to a greater extent in metabolic syndrome rats, which showed a 66% reduction in the I/M after infection with an adenovirus carrying the inducible NO synthase gene, while control rats demonstrated only a 50% reduction with NO. Previously, our laboratory evaluated the effect of NO in a type 2 diabetic environment and showed that NO demonstrated enhanced efficacy in a rat model of type 2 diabetes (2). Not only was NO more effective at inhibiting VSMC proliferation in Zucker diabetic fatty rats, but there was also a 93% reduction in neointimal hyperplasia in the ZDF rats treated with NO compared with only 46% reduction in the nondiabetic LZ rats (2). These studies demonstrating greater efficacy of NO in hyperinsulinemic environments, combined with our present data, suggest a potential vital interaction between insulin and NO.

Insulin has several biological actions that may be related to the process of restenosis. Insulin exerts an adverse effect on the balance between thrombosis and fibrinolysis by modulating the plasminogen activator and inhibitor systems (34). Insulin also aggravates diabetic dyslipidemia and theoretically promotes long-term recoil of overstretched arteries (40). Although insulin and insulin-like growth factors are mitogenic, controversy remains as to the importance of insulin in promoting VSMC proliferation during formation of the neointima (35). In target tissues, insulin stimulates two major pathways, the phosphatidylinositol 3-kinase (PI3K) pathway and the above-mentioned MAPK pathway (3, 44). PI3K activation is essential for insulin-mediated glucose uptake, NO production, and cell survival, where MAPK activation stimulates cellular growth and migration, as well as prothrombotic and proinflammatory responses. In states of insulin resistance, insulin-mediated PI3K signaling is impaired, while MAPK signaling is overactive, thus promoting VSMC migration (43). To investigate the role of insulin in neointima formation, Indolfi et al. (22) also showed that, in rats with STZ-induced diabetes, balloon injury was not associated with an increase in neointima formation, similar to what we found in the present study. When the investigators administered exogenous insulin to these diabetic rats and performed islet transplantation, resulting in hyperinsulinemia in nondiabetic rats, an increase in both blood insulin levels and neointimal hyperplasia after balloon injury was observed. More recently, Jonas et al. (23) have put forth evidence that, following stent injury, the insulin-stimulated phospho-ERK (MAPK pathway)-to-phospho-AKT (PI3K pathway) ratio correlated with the neointimal response in insulin-resistant and type 1 and 2 diabetic rat models, showing that a higher phospho-ERK-to-phospho-AKT ratio correlated directly to more neointimal hyperplasia. Thus it is clear that insulin is at least in part responsible for enhanced neointimal hyperplasia secondary to enhanced MAPK activation. But with our data, we have shown that insulin is essential for NO to exert its anti-proliferative effects. Furthermore, NO has been shown to inhibit the MAPK pathway (26). Thus one potential mechanism of the heightened efficacy of NO in hyperinsulinemic environments is via NO-mediated inhibition of the MAPK pathway. This remains to be investigated.

It is possible that the heightened proliferative response observed in the animals administered insulin is responsible for the increased efficacy of NO at inhibiting neointimal hyperplasia, given that there are more cells proliferating to inhibit. However, we do not believe this heightened proliferative response accounts for all of increased efficacy of NO for several reasons. First, the STZ animals still did develop neointimal hyperplasia, just less. Thus there was still an injury response and neointimal hyperplasia to inhibit. Second, we are examining the percent reduction given each animal’s baseline, since each baseline is different. These are clearly different in each treatment group. Third, if the baseline proliferative state was the only determinant, then we should have observed different results with our earlier study in insulin resistant-animal models (2). In that study, the metabolic syndrome animals developed the greatest amount of neointima at base-

Table 4. Morphometric analysis

<table>
<thead>
<tr>
<th></th>
<th>Circumference, AU</th>
<th>Lumen Area, AU</th>
<th>Intimal Area, AU</th>
<th>Medial Area, AU</th>
<th>I/M</th>
<th>Intimal/(Intimal + Medial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LZ</td>
<td>1.518 ± 26</td>
<td>101.125 ± 6.452</td>
<td>23.962 ± 3.277</td>
<td>22.555 ± 1.251</td>
<td>1.099 ± 0.151</td>
<td>0.457 ± 0.034</td>
</tr>
<tr>
<td>LZ + PROLI/NO</td>
<td>1.467 ± 11</td>
<td>119.862 ± 4.840*</td>
<td>13.309 ± 1.614*</td>
<td>18.288 ± 5.58*</td>
<td>0.693 ± 0.070*</td>
<td>0.376 ± 0.023*</td>
</tr>
<tr>
<td>STZ</td>
<td>1.430 ± 24</td>
<td>102.001 ± 4.286</td>
<td>13.216 ± 1.558†</td>
<td>21.934 ± 1.068</td>
<td>0.584 ± 0.060†</td>
<td>0.343 ± 0.024†</td>
</tr>
<tr>
<td>STZ + PROLI/NO</td>
<td>1.447 ± 14</td>
<td>89.757 ± 8.294</td>
<td>13.764 ± 1.314</td>
<td>18.942 ± 4.89</td>
<td>0.712 ± 0.060</td>
<td>0.395 ± 0.021</td>
</tr>
<tr>
<td>STZ + I</td>
<td>1.631 ± 20†</td>
<td>118.989 ± 6.489†</td>
<td>41.153 ± 3.098†</td>
<td>30.400 ± 1.283†</td>
<td>1.32 ± 0.086</td>
<td>0.545 ± 0.018†</td>
</tr>
<tr>
<td>STZ + PROLI/NO + I</td>
<td>1.532 ± 22*</td>
<td>134.569 ± 6.655</td>
<td>13.163 ± 1.788*</td>
<td>23.790 ± 1.533*</td>
<td>0.529 ± 0.060*</td>
<td>0.305 ± 0.030*</td>
</tr>
</tbody>
</table>

Values are means ± SE. LZ, control; STZ, uncontrolled type 1 diabetic model; STZ + I, controlled type 1 diabetic model. I/M, ratio of intimal to medial area; AU, arbitrary units. *P < 0.05 compared with injury alone. †P < 0.05 compared with LZ.
line, but NO achieved only a 69% reduction. The type 2 diabetic animals developed less baseline neointima, but NO achieved 93% inhibition. Thus we really do believe that the heightened efficacy of NO has more to do with insulin levels than the baseline proliferative state.

The present study is not without limitations. First, while we and others have shown a mitogenic effect of insulin, other studies have shown anti-proliferative effects (9, 11, 22, 35, 41, 45). The etiology for this discrepancy is unclear, but a recent publication has shown interspecies variability in IGF-I receptor signaling that may be responsible for the variable mitogenic effect of insulin (15). Regardless, we did observe a direct correlation between our in vitro and in vivo experiments on VSMC proliferation and neointimal hyperplasia with insulin therapy. Second, we acknowledge that, similar to the insulin data, our glucose data are similar to and yet different from published studies. Upon review of the literature, investigators have demonstrated that glucose can both stimulate and inhibit VSMC proliferation (18, 22, 38). It is difficult to account for these observed variations in published studies, except for the fact that different strains of VSMC have been used, time of exposure to glucose was variable and under different conditions, and passage number of cells differed. In fact, we have observed both proliferative and nonproliferative effects of glucose, depending on the species and strain of cells used and the chronicity of exposure to glucose. Importantly, for this study, our in vitro data are consistent with our in vivo data, most likely due to the fact that we grew the VSMC from the same animal strains used for the in vivo studies. Thus, while these differences exist in the literature, our study is internally consistent. Third, our in vitro experiments mimicked acute insulin and glucose treatments, as opposed to the more chronic nature of the therapy with our in vivo model. While it would be prudent to assume what the outcomes of the in vitro work would show with chronic therapy, this deserves exploration. Last, we were not able to achieve normoglycemia in the animals treated with daily injections of insulin due to the fine balance between normoglycemia vs. death from hypoglycemia. We spent a considerable amount of time developing our insulin sliding scale in this rat strain before the experiment was conducted, but observed considerable variability with the responses to insulin at the lower glucose levels. Thus, to avoid mortality, we opted to achieve moderate glucose control, which we were reproducibly able to achieve. However, based on our findings from this study, as well as our laboratory’s prior study demonstrating heightened efficacy of NO in animal models of insulin resistance (2) further studies are warranted in which normoglycemia is the goal for both insulin-resistant and insulin-deficient animal models to more accurately determine whether it is the insulin and/or glucose that is the predominate factor in determining the efficacy of NO.

In conclusion, further research is required to elucidate the mechanism by which NO exerts its more robust effects in high-insulin states, as many patients with vascular disease have type 1 or type 2 diabetes and have an even greater need of therapy to reduce restenosis rates after vascular interventions. While the interaction of NO with reactive oxygen species in a diabetic milieu deserves attention, a more worthwhile avenue to explore may be found elsewhere. Given the derangement of the insulin receptor pathways in diabetes leading to a pro-proliferative and mitogenic state, coupled with the fact that NO is more effective with insulin present, further work is required to elucidate the mechanism by which NO is more efficacious in the presence of insulin. Regardless, our data have implications for the treatment of patients with type 1 diabetes undergoing cardiovascular interventions.

ACKNOWLEDGMENTS

The authors express thanks to the Northwestern University Institute for BioNanotechnology in Medicine, to Lynnette Dangerfield for administrative support, and to Edwards Lifesciences for providing the Fogarty balloon catheters.

GRANTS

This work was supported, in part, by funding from the National Institutes of Health (NIH: 1 K08HL084203 and 5 R01 EB003806-02), the Department of Veterans Affairs, Veterans Affairs Merit Review Grant, the American Vascular Association, the American Medical Association Foundation 2007 Seed Grant, the Eleanor Prince Wood Grant, the Eleanor B. Pillsbury Grant-University of Illinois, and by the generosity of Hilda Rosenbloom and Eleanor Baldwin. In addition, part of this research was supported with federal funds from the National Cancer Institute, NIH, under contract N01-CO-2008–00001 with SAIC-Frederick, Inc. and by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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