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Role of Metabolic Environment on Nitric Oxide Mediated Inhibition of Neointimal Hyperplasia in Type 1 and Type 2 Diabetes

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Abstract

Nitric oxide (NO) is well known to inhibit neointimal hyperplasia following arterial injury. Previously, we reported that NO was more effective at inhibiting neointimal hyperplasia in a type 2 diabetic environment than control. We also found that NO was ineffective in an uncontrolled type 1 diabetic environment; however, insulin restored the efficacy of NO. Thus, the goal of this study was to more closely evaluate the effect of insulin and glucose on the efficacy of NO at inhibiting neointimal hyperplasia in both type 1 and type 2 diabetic environments using different doses of insulin as well as pioglitazone. Type 1 diabetes was induced in male Lean Zucker (LZ) rats with streptozotocin (60mg/kg IP). Groups included control, moderate glucose control, and tight glucose control. Zucker Diabetic Fatty (ZDF) rats fed Purina 5008 chow were used as a type 2 diabetic model. Groups included no therapy, insulin therapy, or pioglitazone therapy. After 4 weeks of maintaining group assignments, the carotid artery injury model was performed. Treatment groups included: control, injury, and injury plus NO. 2 weeks following arterial injury, in the type 1 diabetic rats, NO most effectively reduced the neointimal area in the moderate and tightly controlled groups (81% and 88% vs. 33%, respectively, $p=0.01$). In type 2 diabetic rats, the metabolic environment had no impact on the efficacy of NO (81%–82% reduction for all groups). Thus, in this study, we show NO is effective at inhibiting neointimal hyperplasia in both type 1 and type 2 diabetic environments. A greater understanding of how the metabolic environment may impact the efficacy of NO may lead to the development of more effective NO-based therapies for patients with diabetes.

Keywords

nitric oxide; neointimal hyperplasia; insulin; diabetes

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1. Introduction

Diabetes continues to be a major public health problem in the United States. In 2010, the Centers for Disease Control estimated that 26.9% of Americans over the age of 65 have diabetes.[1] Diabetes is the leading cause of nontraumatic lower limb amputation in the United States.[1] Significant strides have been made in the reduction of macrovascular complications of the disease, but unfortunately, patients with type 1 and 2 diabetes suffer worse prognosis for the treatment of macrovascular disease, such as coronary artery, cerebrovascular, and peripheral vascular interventions, due to a greater rate of restenosis.[2–6]

Nitric oxide (NO) is a small, naturally occurring molecule that has many beneficial effects in the vasculature, such as inhibition of vascular smooth muscle cell migration and proliferation, endothelial cell apoptosis, and leukocyte chemotaxis.[7,8] We and others have shown that NO effectively inhibits the formation of neointimal hyperplasia.[8–19] We have also shown that NO is more effective in uncontrolled type 2 compared to type 1 diabetic and control rats.[20] However, with the addition of insulin, the efficacy of NO was restored in type 1 diabetic rats.[21] Yet, with these studies, the effect of NO in a controlled type 1 or type 2 diabetic environment was not determined. It is unclear whether the hyperinsulinemia and/or hyperglycemia are responsible for regulating the efficacy of NO in these diabetic environments. Also, the effect of pioglitazone, an insulin sensitizer that is known to regulate the proliferation and migration of vascular smooth muscle cells, improve endothelial cell function, and reduce inflammation, on the efficacy of NO has not been determined.[22–24] Thus, the goal of this study is to more closely evaluate the effect of insulin and glucose on the efficacy of NO at inhibiting neointimal hyperplasia in both type 1 and type 2 diabetic environments through the use of sustained release insulin pellets, and pioglitazone therapy. This study differs from our earlier work as our goal here is to produce metabolic environments with more controlled insulin and glucose levels, to discern the individual contribution of each on the efficacy of NO. With our prior work, the animals remained hyperglycemic, making it impossible to discern if the restored efficacy was due to the insulin therapy, or the lower glucose level. We hypothesized that NO is more effective in a normoglycemic, hyperinsulinemic environment in both type 1 and type 2 diabetic rats *in vivo*.

2. MATERIALS AND METHODS

2.1 Type 1 Diabetic Animal Model

11-week-old male Lean Zucker (LZ) rats were obtained from Charles Rivers Laboratories (Wilmington, MA). Type 1 diabetes was induced in LZ rats with a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg). Daily serum glucose levels were assessed with a glucometer via tail vein puncture and animals with glucose concentrations of 300 mg/dL or above were considered diabetic and included in the study. Blood glucose concentrations were maintained by the use of subcutaneous insulin pellets (Linplant, Canada) which deliver 2U insulin every 24 hours for up to 40 days. Three subsets of type 1 diabetic rats were created (Figure 1). The first subset, consisted of uncontrolled type 1 diabetic rats that received no insulin therapy, was referred to as “STZ No Control.” The second subset, which received subcutaneous insulin pellets to maintain blood glucose concentrations between 200–300 mg/dL, was referred to as “STZ Moderate Control.” The third subset, received subcutaneous insulin pellets to maintain blood glucose levels of less than 200 mg/dL and, was referred to as “STZ Tight Control.” Insulin therapy commenced approximately 7 days after STZ injection, when the hyperglycemia was detected and stable for several days. Insulin therapy continued for 21 days before and 14 days after surgery was performed. The non-fasting daily blood glucose concentration was recorded.

2.2 Type 2 Diabetic Animal Model

Zucker Diabetic Fatty (ZDF) rats were obtained from Charles Rivers Laboratories. The ZDF strain has a homozygous leptin receptor mutation pre-disposing the rats to type 2 diabetes. When the inbred ZDF males were fed the Purina 5008 diet, which is manufactured high in carbohydrates and fats, they exhibited hyperinsulinemia, hyperglycemia, hypercholesterolemia, and hypertriglyceridemia, mimicking a type 2 diabetic state. Three subsets of type 2 diabetic rats were created (Figure 1). The first group received no supplemental insulin therapy and was referred to as “ZDF No Control.” The second subset received subcutaneously implanted insulin pellets to maintain insulin levels below 300 mg/dl and was referred to as “ZDF Insulin-treated.” The third subset received pioglitazone-treated chow obtained from Science Diets (New Brunswick, NJ) and was referred to as “ZDF Pioglitazone-treated.” The pioglitazone dose delivered per rat was determined by the average amount of chow consumed by a 400g rat, and was estimated to be 10 mg/kg per day. Rats were kept in their respective group assignments for 3 weeks prior to and 2 weeks following surgery.

2.3 Animal Surgery

All animal procedures were performed in accordance with the Guide for 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 upon administration of STZ. The neck was shaved and prepped with betadine and alcohol (70%). 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.[8,20] After injury and restoration of blood flow, 10 mg of the diazeniumdiolate NO donor disodium 1-[(2-carboxylato)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. This is the same dose of PROLI/NO that was used in our prior studies.[20,21] Treatment groups for all STZ and ZDF group assignments included: 1) injury, and 2) injury+PROLI/NO (n=6–7/treatment group). Carotid arteries were harvested 14 days after injury for morphometric analysis. Blood was collected to measure insulin and glucose levels. Insulin levels were determined using an ELISA-based insulin assay kit (SPI-Bio, Bertin Pharma, France). 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 to other diazeniumdiolates, and is the NO donor used in our prior studies on diabetes.[20,21]

2.4 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 hour, 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.

2.5 Morphometric Analysis

Carotid arteries harvested at 2 weeks were examined histologically using routine hematoxylineosin staining. Digital images of sections were collected with light microscopy using Zeiss Axio Imager.A2 (Jena, Germany) with 4, 10, and 40X objectives. For morphometric analysis, six evenly spaced sections through control and injured carotid

arteries were obtained. Intimal area (I), medial area (M), luminal area, and circumference were measured using Image J software with uniform arbitrary units (AU) (NIH, Bethesda, MD).

2.6 Immunohistochemistry

Masson's Trichrome staining was performed by using the Trichrome Stain kit (Masson; Sigma-Aldrich cat. no. HT15), according to the manufacturer's instructions. Nuclei were stained black, cytoplasm and muscle red, and collagen blue. Cross-sections were imaged with light microscopy, and blinded grading was performed at 200X with 4 quadrants per slide. Smooth muscle cells, macrophages, and SOD-1 levels were assessed immunohistochemically with primary antibodies against α -smooth muscle cell (SMC) actin (1:200, Abcam: #ab5694), CD68 (ED1, 1:2000, Santa Cruz: #sc-59103), and SOD-1 (1:100, Abcam: #ab13498). ED1 and SOD-1 were imaged using fluorescent microscopy, and blinded grading was performed at 50X magnification. α -SMC actin was imaged using light microscopy, and cells in the media staining positive for α -SMC actin were counted at 200X with 4 quadrants per slide. All analysis was performed including 4–8 rats per treatment group.

2.7 Arginase Activity and GTP Cyclohydrolase 1 Levels

Arginase activity was assessed in the plasma from the animals in all treatment groups using the Quantichrom arginase assay kit (BioAssay Systems, #DARG-200) according to manufacturer's instructions. This assay measured the conversion of arginine to urea by arginase. Plasma samples were desalted using Amicon Ultra-0.5 mL Centrifugal Filters (Millipore). GTP cyclohydrolase 1 plasma concentrations were analyzed using a rat GCH1 Elisa Kit (MyBioSource, #mbs924637) according to manufacturer's instructions.

2.8 Statistical Analysis

Results are expressed as mean $\pm$ the standard error of the mean (SEM). Differences between multiple groups were analyzed using one-way analysis of variance with the Student-Newman-Keuls post hoc test for all pair-wise comparisons (SigmaStat; SPSS, Chicago, IL). Statistical significance was assumed when $P < 0.05$.

3. RESULTS

3.1 Metabolic Characteristics

Type 1 diabetic rat group assignments included no glucose control, moderate glucose control, and tight glucose control. Type 2 diabetic rat group assignments included no therapy, insulin-treated, and pioglitazone-treated. Animal weight, number of insulin pellets required, serum glucose, and insulin levels were obtained throughout the experiments and confirmed the metabolic states desired (Table 1). STZ moderate control and STZ tight control rats were kept in their respective groups with additional insulin pellet therapy administered as necessary. Blood glucose levels usually stabilized within 24 hours of subcutaneous implantation. Glucose levels were approximately 3- and 2-fold lower in the tight and moderate control groups compared to the untreated STZ group ($p < 0.05$). Insulin levels in the untreated STZ group were the lowest, as expected. The average total number of insulin pellets used was 1.3 and 1.6 pellets per rat in the moderate and tight control groups, respectively. Untreated STZ rats exhibited an expected decrease in body mass compared to insulin-treated STZ rats.

The metabolic characteristics of the ZDF rats in the study are also included in Table 1. The blood glucose levels in the ZDF rats were not stringently controlled as in the STZ moderate control and STZ tight control rats. Groups were based on the mechanism of insulin delivery

rather than the level of glucose control provided. Thus, glucose levels in the ZDF insulin-treated group averaged 238 mg/gL and in the ZDF pioglitazone-treated group averaged 481 mg/gL. The average total number of insulin pellets required to achieve a blood glucose level <300 mg/dL was 4 per ZDF rat. Additionally, the measured insulin levels at sacrifice were 18-fold greater than ZDF controls ($p<0.05$). Pioglitazone promoted insulin uptake in peripheral tissues, as the blood insulin levels were 70% lower compared to the ZDF no control rats, though this did not reach statistical significance. ZDF insulin- and ZDF pioglitazone-treated animals weighed significantly more (23% and 24% respectively) compared to ZDF no control rats ($p<0.05$). These data confirm that the rat models exhibited a biochemical profile in accordance with their intended subgroups.

3.2 Ability of NO to Inhibit Neointimal Hyperplasia Type 1 Diabetic Rats

After the balloon carotid artery injury model was performed, we examined the effect of PROLI/NO on the development of neointimal hyperplasia. Neointimal lesions were reproduced in all groups in both STZ and ZDF rats (Table 2, Figures 2 and 3). Of note, additional staining with Masson's Trichrome did not change our morphometric data from the H&E stained samples (not shown). In the STZ rats, insulin treatment resulted in an increase in the development of neointimal hyperplasia. In the STZ moderate and STZ tight control groups, there was a 2.8- and 3.9-fold increase, respectively, in the intimal area ($p<0.05$ compared to STZ no control) (Figure 2A and 3A). With PROLI/NO therapy, there was only a modest reduction in the intimal area of STZ no control rats. In the STZ moderate and STZ tight control groups, PROLI/NO significantly reduced the intimal area by 81% and 88%, respectively ($p<0.05$ compared to injury alone) (Figure 3A).

The extent of vascular remodeling also correlated with insulin therapy in type 1 diabetic STZ rats. Without insulin therapy, PROLI/NO caused a 12% reduction in medial area compared to injury alone, but with insulin therapy the percent reduction in medial area more than doubled (26% in both STZ moderate and STZ tight control groups) (Figure 3B). Luminal area and circumference trended toward a decrease in the STZ moderate and STZ tight groups compared to STZ no control. NO slightly increased the circumference and luminal area, but statistical significance was not reached. To determine if the effect of NO on the media was due to inhibition of vascular smooth muscle cell hypertrophy versus hyperplasia, we stained sections for α -SMC actin. Consistent with our prior publications evaluating cellular proliferation with BrdU incorporation or Ki67 staining, we found that NO significantly inhibited α -SMC actin staining in the STZ moderate group (84% reduction, $P<0.05$). [12,18] There was a trend in the other two groups toward inhibition of NO, but this did not reach statistical significance.

The intima/media (I/M) area ratio was evaluated as another surrogate for neointimal hyperplasia. The development and inhibition of neointimal hyperplasia was again directly proportional to the extent of insulin therapy in type 1 diabetic rats. The STZ no control rats benefited the least amount from NO therapy, with only a 33% reduction in the I/M area ratio (Figure 3C). NO resulted in a significantly greater reduction in neointimal hyperplasia in STZ moderate and STZ tight control rats (75% and 83% reduction, respectively, $p<0.05$) (Figure 3C).

To evaluate the inflammatory response, infiltration of macrophages was evaluated with ED1 staining. At 2 weeks, there were no statistically significant differences between the treatment groups in all three type 1 diabetic models (Figure 4).

To evaluate the role of oxidative stress, we evaluated SOD-1 levels, as SOD-1 metabolizes superoxide to hydrogen peroxide. Evaluation of total SOD-1 levels throughout the entire

arterial wall showed a significant decrease in the STZ control group with the addition of NO (39% reduction) (Figure 5).

Next, we assessed arginase activity to determine if the metabolic environment or NO impacts arginase activity. In the type 1 diabetic groups, there was a trend for decreasing arginase activity with increasing glucose control. From the STZ no control group to the STZ tight control group, there was a 91% reduction in injury alone and an 81% reduction with the addition of NO, however this did not reach statistical significance (Figure 6).

Lastly, there were no significant differences in any of the type 1 diabetic groups in GTP cyclohydrolase 1 level (Figure 6).

3.3 Ability of NO to Inhibit Neointimal Hyperplasia in Type 2 Diabetic Rats

Neointimal development in ZDF insulin-treated rats was 12% less compared to ZDF no treatment, whereas ZDF pioglitazone-treated exhibited 21% more neointimal hyperplasia (Table 1, Figures 2 and 3). However, neither alteration reached statistical significance. The addition of PROLI/NO resulted in a significant reduction in the intimal area by 84–88% in the three subgroups ($p < 0.05$) (Figure 3A). The greatest difference among these subgroups was appreciated in the degree of medial area remodeling. In the ZDF no control rats, PROLI/NO caused a 31% reduction in the medial area ($p < 0.05$) (Figure 3B). In the ZDF insulin-treated group, PROLI/NO was least effective at reducing the medial area by only 10% compared to injury alone (Figure 3B). The circumference and luminal area trended towards an increase in size with insulin and pioglitazone therapy compared to control animals. Again, NO slightly increased the circumference and luminal area, but statistical significance was not reached. In the ZDF pioglitazone-treated group, PROLI/NO reduced the medial area by 28%, although significance was not achieved (Figure 3B). The I/M area ratio was significantly reduced by NO in all type 2 diabetic subgroups by 81–82% ($p < 0.05$ compared to injury alone) (Figure 3C). Masson's trichrome stain was used to confirm our morphometric analysis (not shown).

As in our type 1 diabetic groups, α -SMC actin stain was used to assess vascular smooth muscle cell hypertrophy versus hyperplasia within the media in our type 2 diabetic groups. All three groups had a decrease in α -SMC actin staining after the addition of NO, but only ZDF no control and the ZDF pioglitazone-treated groups were significantly different (74% and 69% reduction, respectively, $P < 0.05$).

Macrophage infiltration was assessed in the different layers in the type 2 diabetic groups at 2 weeks. Significant differences were present in only the ZDF no control group for both the medial layer and the total quantitation throughout all 3 layers (Figure 4).

Interestingly, SOD-1 staining showed a statistically significant decrease in the intimal layer with all three type 2 diabetic groups (Figure 5). Furthermore, there was a significant decrease in total SOD-1 in the ZDF insulin-treated group (Figure 5).

In the type 2 diabetic groups, there was no significant difference in the arginase activity (Figure 6). Similarly, there were no significant findings in any of the type 2 diabetic groups for GTP cyclohydrolase 1 level (Figure 6).

4. DISCUSSION

In this study we investigated the role of different metabolic environments in type 1 and type 2 diabetic rats on the development of neointimal hyperplasia as well as the ability of NO to inhibit neointimal hyperplasia in these environments. We found that NO was effective at

inhibiting neointimal hyperplasia in all of the type 1 and type 2 diabetic models. In the type 1 diabetic rats, we found that the percent reduction of neointimal hyperplasia following arterial injury by NO was greater in the hyperinsulinemic, normoglycemic environment. In the type 2 diabetic rats, we found no difference in the percent reduction of neointimal hyperplasia by NO following arterial injury in the different metabolic environments (i.e., with insulin or pioglitazone). Of note, the percent reduction of neointimal hyperplasia by NO was greatest in the animal treatment groups with the largest proliferative drive following balloon injury (i.e., all three type 2 diabetic groups and the type 1 diabetic with tight glucose control). These data may have clinical implications for the eventual implementation of NO-based therapies as diabetes is a rising health problem around the world. Accordingly, the World Diabetes Foundation has estimated that 7.8% of the world's population will have diabetes in 2030.[1]

We have previously shown that insulin promotes a proliferative state in type 1 diabetic rats. [21] In our prior publication, we found that in an insulin-deficient environment, type 1 diabetic rats developed approximately half as much neointimal hyperplasia compared to LZ control rats. Upon the administration of insulin, there was a dramatic increase in the amount of neointimal development observed. This prior study also elucidated the difference in efficacy of NO in controlled and uncontrolled type 1 diabetes by demonstrating heightened efficacy of NO in the setting of insulin administration. However, in this prior study, blood glucose levels averaged 295 – 300 mg/dl, still exhibiting hyperglycemia. Thus, one of the main goals of the current study was to discern the contribution of insulin and glucose toward the efficacy of NO in the vasculature. We strove to create a type 1 diabetic animal model in which insulin therapy achieved euglycemia. We also wanted to evaluate the separate contributions of insulin and glucose to the efficacy of NO in the type 2 diabetic animal model, so that we could make comparisons between the different insulin and glucose metabolic environments. In the current study, we reproduced the results of our earlier work with insulin in the type 1 diabetic animal model, but in a more robust manner. We showed that insulin administration produced a dose-dependent increase in neointimal hyperplasia in rodents. With respect to the metabolic environment, in this study we maintained strict adherence to blood glucose control groups, maintaining glucose between 200–300 mg/dL in STZ moderate control and 100–200 mg/dL (euglycemic) in STZ tight control. We were also able to demonstrate that NO is increasingly efficacious as euglycemia was achieved with higher insulin dosing. Thus, insulin and glucose may have an impact on the efficacy of NO in the type 1 diabetic environment.

Regarding the type 2 diabetic environment, we draw several different conclusions. First, in our type 2 diabetic rats, where hyperinsulinemia is the pathologic root of the disease, further hyperinsulinemia via exogenous administration induced no further development of neointimal hyperplasia. The reason for this is unclear, but perhaps due to a threshold effect of insulin. Baseline insulin production in this type 2 diabetic rat model appears to induce a pro-proliferative state above which no further stimulation of proliferation with insulin is possible. In fact, Desouza, et al. has shown that insulin resistance is key to the development of neointimal hyperplasia.[25] Second, we ventured to determine if insulin levels were instrumental to the efficacy of NO in preventing the development of neointimal hyperplasia and found equivocal results with supplemental insulin and pioglitazone therapy. The three type 2 diabetic animal groups had vastly different mean blood insulin levels (1.5, 27.8, and 0.4 ng/ml for the no control, insulin-treated, and pioglitazone-treated animals, respectively). Yet, no significant differences existed in the efficacy of NO between these animal models. These data suggest that insulin may not be a determinate of the efficacy of NO in the type 2 diabetic environment. We can also conclude that glucose does not appear to impact the efficacy of NO in this animal model, since the type 2 diabetic rats treated with supplemental insulin had mean glucose levels that were less than half those of the untreated type 2

diabetic rats (238 vs. 542 mg/dL, respectively) yet the efficacy of NO was no different between the two animal models. Thus, we conclude that in the type 2 diabetic animal model, insulin and glucose may have no impact on the efficacy of NO at inhibiting neointimal hyperplasia.

The well-established insulin-signaling pathway may play a role in these interesting findings. Two primary pathways are activated by insulin binding to the insulin receptor and activating the insulin receptor substrate-1 (IRS-1): the MAP kinase pathway and the PI3K/Akt pathway, resulting in proliferation or survival, respectively. Studies have shown that hyperinsulinemia and hyperglycemia result in preferential phosphorylation of IRS-1 serine/threonine residues and over-stimulation of the MAPK pathway.[26] This derangement results in diminution of the Akt pathway and creation of a less vasoprotective environment.[27] The relationship between insulin and neointimal hyperplasia in type 1 diabetic environments has been recently investigated. Jonas et al. has reported that following stent injury, the phospho-ERK (MAPK pathway)-to-phospho-AKT (PI3K pathway) ratio was proportional to the development of neointimal hyperplasia. Ratios favoring the MAPK pathway were associated with increased neointimal hyperplasia in type 1 and 2 diabetic rats.[28] Concurrent with this theory, *in vitro* studies by Varu et al. demonstrated that vascular smooth muscle cell proliferation was promoted in a dose-dependent fashion with increasing concentrations of insulin. Thus, it is possible that preferential activation of the Akt pathway by NO is the mechanism by which NO is more effective at inhibiting neointimal hyperplasia in tightly-controlled type 1 diabetic rats.

Neointimal hyperplasia is a multifactorial process and additional studies were undertaken to help clarify this complicated process. In an effort to indirectly assess metabolism of superoxide, which readily reacts with NO to form peroxynitrite, we assessed SOD-1 levels in our samples. In our type 1 diabetic rats we saw an interesting, but not significant trend: with increasing glucose control, total SOD-1 levels decreased with injury but increased with the addition of NO. Although interesting, this is not significant, but further studies may help to interpret this finding. Next, we found that with our type 2 diabetic animals there was a significant decrease in SOD-1 within the intima with the addition of NO. Only the type 2 diabetic group with exogenous insulin and NO was significantly different for total SOD-1 levels. The other groups had a decreasing trend of SOD-1 with the addition of NO. It seems as though the differences within the layers may be more important than total SOD-1 level. Lastly, we evaluated arginase activity and GTP cyclohydrolase 1 level in the plasma from all the treatment groups. No significant differences were seen. We did observe a trend toward decreased GTP cyclohydrolase 1 with NO in the type 2 diabetic animals. While this was not significant, the decrease of GTP cyclohydrolase 1 may be due to negative feedback from the periadventitial application of NO or its substrates. Further studies will be needed.

The discrepancy between type 1 and type 2 diabetic vascular complications have been investigated in human studies as well. Results from the Diabetes Control and Complication Trial (DCCT) in type 1 diabetics and the Action in Diabetes and Vascular Disease (ADVANCE) and the Veterans Affairs Diabetes Trial (VADT) in type 2 diabetics demonstrated that intense glycemic control resulted in a reduction of microvascular complications.[5,29,30] Furthermore, macrovascular complications in type 1 diabetic patients in the Epidemiology of Diabetes Intervention and Complications (EDIC/DCCT) were reduced by 42% in combined cardiovascular outcomes.[5] Unfortunately, results in type 2 diabetics were less definitive. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial was halted prematurely due to an increase in all-cause mortality in the intensive-therapy group compared to a standard-therapy group of type 2 diabetic patients. Furthermore, in the ADVANCE trial, type 2 diabetics had no significant reduction of

macrovascular complications with intensive-therapy. Thus, studies determining the benefit of glucose control in type 2 diabetic environments are needed to elucidate this discrepancy.

PPAR- γ agonists like thiazolidinediones are being used clinically to increase insulin sensitivity, favorably affect blood glucose and lipid profiles, and reduce cardiovascular risk factors such as hyperglycemia, hyperinsulinemia, and hyperlipidemia.[31] Law et al showed that PPAR- γ receptors are expressed on all major cells in the vessel wall, and PPAR- γ agonist activation inhibits vascular smooth muscle cell migration and proliferation.[32] Desouza et al. demonstrated that PPAR- γ agonists cause a decrease in intimal hyperplasia following endothelial injury in normoglycemic, insulin-resistant, obese fatty rats.[25] More recently, they determined that endothelial regrowth takes approximately 6 months.[25,31] These findings have also been translated into human studies. Takagi, et al. found significantly less neointimal volume in type 2 diabetic arteries with pioglitazone therapy compared to control type 2 diabetic arteries following coronary stent implantation 6 months after treatment.[33] Furthermore, it has been shown that angiographic restenosis rate was reduced in patients treated with post-procedural pioglitazone (17%) vs. untreated controls (35%).[33] Thus, we were surprised to find that pioglitazone did not confer protection from the development of neointimal hyperplasia, as reported by Desouza and Takagi et al.[25,33] On the contrary, in our study pioglitazone treatment resulted in a trend towards an increase in neointimal hyperplasia. These differences may be due to the fact that our rats exhibit a diabetic physiology, where both insulin-resistance and hyperglycemia impact cellular signaling. Furthermore, the rats in our study were sacrificed at 2 weeks and may explain the difference in outcome compared to Desouza et al. in which rats were sacrificed at 6 months. Clearly, while pioglitazone has been reported to reduce neointimal hyperplasia at 6 months in non-diabetic animal and human studies, we found that the short-term effect of the drug in diabetic rodent models is the opposite, trending towards increasing neointimal hyperplasia.

Our study is not without limitations. In this study, the diabetic states were induced acutely. Unfortunately, this is a limitation of diabetic animal models. Maintenance of the diabetic state for prolonged periods of time results in significant animal mortality due to the profound hyperglycemia. In addition, blood glucose control was not achieved equally in the type 1 and type 2 diabetic rats. While we achieved excellent adherence to subgroups in the type 1 diabetes rats, the type 2 diabetic rats were less stringently controlled. Type 2 diabetic rats receiving insulin or pioglitazone therapy were moderately and poorly controlled, respectively. Due to the insulin-resistant nature of the type 2 diabetic rats, obtaining tight glucose control was expectedly very difficult. As it stands, insulin levels were 18 times greater in the ZDF insulin-treated group than the ZDF no control group. In the ZDF pioglitazone-treated group, we used doses reported in the literature, despite continued hyperglycemia. Additionally, our rats may have not been treated for an adequate amount of time with the pioglitazone to see a significant difference. Further studies with a longer time course may be needed to sufficiently answer this question. Moreover, the evaluation of macrophages with ED1 staining also may need further evaluation at earlier time points, as our samples were 2 weeks post injury. Direct assessment of superoxide may be beneficial to further investigate the role of NO bioavailability. Polyamine synthesis may play a role in the development of neointimal hyperplasia. Although not measured in our study, previous work by Conover et al. showed that in insulin deficient rats treated with STZ, ornithine decarboxylase (the rate limiting enzyme in polyamine synthesis) was diminished as compared to those with exogenous insulin administration.[34] This may be an important area for further research. Regardless, we have obtained valuable information regarding the effect of glucose control on the efficacy of NO to inhibit neointimal hyperplasia.

In conclusion, improvement in outcomes for diabetic patients undergoing vascular interventions is of extreme importance considering diabetes is a major risk factor for

vascular morbidity. We have determined that the metabolic environment impacts the proliferation drive of neointimal hyperplasia. Further, NO is effective at inhibiting neointimal hyperplasia in different type 1 and type 2 diabetic environments, but the percent reduction of neointimal hyperplasia by NO appeared related to the proliferative drive following balloon angioplasty. These findings further the understanding of diabetic vascular disease and NO and have implications for future targeted therapies.

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Highlights

- Insulin induces more neointimal hyperplasia in type 1 diabetic rats
- Metabolic environment effects the efficacy of NO in type 1 diabetic rats
- Metabolic environment has no impact on the efficacy of NO in type 2 diabetic rats

Figure 1.

Study timeline. Type 1 diabetes was induced in LZ rats with a single injection of streptozotocin (STZ). Group assignments included: I - STZ No Control; II - STZ Moderate Control; and III - STZ Tight Control. Type 2 diabetes was induced in ZDF rats fed Purina 5008 chow. Group assignments included: IV - ZDF No Control; V - ZDF Insulin-Treated; and VI - ZDF Pioglitazone-treated.

Figure 2.

The efficacy of nitric oxide (NO) at inhibiting neointimal formation following arterial injury correlates with insulin levels in type 1 but not type 2 diabetic rats. Representative hematoxylin and eosin stained cross sections from (A) STZ No Control (STZ), STZ Moderate Control, and STZ Tight Control; and (B) ZDF No Control (ZDF), ZDF Insulin-Treated, and ZDF Pioglitazone-Treated. For each group assignment, treatment groups included injury alone and injury plus the application of NO donor PROLI/NO.

Figure 3.

Graphical representation of the morphometric analysis of the efficacy of nitric oxide (NO) at inhibiting neointimal hyperplasia in type 1 and type 2 diabetic rats. (A) Intimal area, (B) Medial area, (C) Intima-to-Media (I/M) area ratio. Group assignments include: STZ No Control (blood glucose > 300 gm/dl); STZ Moderate Control (glucose 200–300 gm/dl); STZ Tight Control (blood glucose <200); ZDF No Control; ZDF Insulin-Treated; and ZDF Pioglitazone-Treated. Treatment groups for all group assignments include: injury and injury plus the application of the NO donor PROLI/NO. * $p < 0.05$ vs. injury alone. ** $p < 0.05$ vs. reduction with NO compared to injury alone in STZ No Control.

Figure 4.

Graphical representation of ED1 staining in type 1 and type 2 diabetic rats. (A) intimal ED1 staining, (B) medial ED1 staining, (C) adventitial ED1 staining, and (D) total ED1 staining throughout all three arterial layers. * $P < 0.05$.

Figure 5. Graphical representation of SOD-1 staining in type 1 and type 2 diabetic rats. (A) intimal SOD-1 levels, (B) medial SOD-1 levels, (C) adventitial SOD-1 levels, and (D) total SOD-1 levels throughout all three arterial layers. * $P < 0.05$.

Figure 6.

(A) Arginase activity in type 1 and type 2 diabetic rats measured from the plasma samples collected at sacrifice at 14 days. (B) GTP cyclohydrolase 1 levels in type 1 and type 2 diabetic rats measured from the plasma samples collected at sacrifice at 14 days.

Table 1

Metabolic Characteristics.

	STZ No Control	STZ Moderate-Control	STZ Tight-Control	ZDF No Control	ZDF Insulin-Treated	ZDF Pioglitazone-Treated
<i>N</i>	14	14	14	14	13	14
Weight (Pre-Tx), g	303±6	307±7	305±7	379±8	462±4	462±10
Weight (Post-Tx), g	275±6	333±8	355±12	366±6	472±9*	481±16*
Glucose (Pre-Tx), mg/dL	503±17	532±24	510±15	537±19	419±11*	398±35
Glucose (Post-Tx), mg/dL	543±18	280±33*	173±6*	542±19	238±9*	481±16*
Insulin, ng/mL	0.2±0.1	2.8±1.2*	3.6±1.2*	1.5±0.3	27.8±7.5*	0.4±0.1*
Pellets	0	1.33±0.1	1.65±0.2	0	4.07±0.3	0

Values are means ± SE; n, no. of rats; STZ, streptozotocin; ZDF, Zucker diabetic fatty;

* p<0.05 vs. No Control, STZ, and ZDF, respectively.

Table 2

Morphometric analysis.

	Circumference	Lumen Area	Intimal Area	Medial Area	I/M Area	I/(I+M)
STZ	1420±62	128212±5015	7588±1630	22218±2869	0.38±0.08	0.26±0.04
STZ + PROLI/NO	1424±65	139791±13424	4994±993	19426±2026	0.25±0.04	0.19±0.02
STZ Moderate	1280±45	109135±8608	21808±5906	25416±1906	0.89±0.16	0.43±0.05
STZ Moderate + PROLI/NO	1348±43	123606±7045	4064±3484	18692±695*	0.22±0.02*	0.18±0.02*
STZ Tight	1333±42	114498±7705	29834±3351	26706±2997	1.11±0.11	0.51±0.03
STZ Tight + PROLI/NO	1434±23	136556±5346	3521±6096	19712±1767*	0.18±0.02*	0.15±0.02*
ZDF	1290±49	103241±9254	22876±993	28346±2611	0.80±0.08	0.44±0.02
ZDF + PROLI/NO	1403±22	132170±4143	2760±450	19552±2210*	0.15±0.01*	0.13±0.01*
ZDF Insulin	1380±32	122505±9571	20118±603	21142±1250	0.93±0.16	0.47±0.04
ZDF Insulin + PROLI/NO	1479±36	144949±9352	3187±253	19002±1214	0.18±0.01*	0.14±0.01*
ZDF Pio	1418±87	122924±14961	28885±228	28613±4840	0.99±0.05	0.49±0.01
ZDF Pio + PROLI/NO	1433±30	126733±9908	3419±328	20532±1819*	0.17±0.01*	0.14±0.01*

Values are means ± SE; n, no. of rats; I = intimal area; M = medial area; STZ = streptozotocin injected Lean Zucker rats (type 1 diabetes); ZDF = type 2 diabetic rats;

* p<0.05 vs. None, STZ and ZDF, respectively. PROLI/NO = NO donor disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate; Pio = Pioglitazone