Published in final edited form as:

J Surg Res. 2015 June 1; 196(1): 180–189. doi:10.1016/j.jss.2015.02.012.

Nitric Oxide Affects UbcH10 Levels Differently in Type 1 and Type 2 Diabetic Rats

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Abstract

Background—Nitric oxide (NO) more effectively inhibits neointimal hyperplasia in type 2 diabetic versus nondiabetic and type 1 diabetic rodents. NO also decreases the ubiquitin-conjugating enzyme UbcH10, which is critical to cell cycle regulation. This study seeks to determine whether UbcH10 levels in the vasculature of diabetic animal models account for the differential efficacy of NO at inhibiting neointimal hyperplasia.

Materials and Methods—Vascular smooth muscle cells (VSMC) harvested from nondiabetic Lean Zucker (LZ) and type 2 diabetic Zucker Diabetic Fatty (ZDF) rats were exposed to high glucose (25 mM) and high insulin (24 nM) conditions to mimic the diabetic environment *in vitro*. LZ, streptozotocin-injected LZ (STZ, type 1 diabetic), and ZDF rats underwent carotid artery balloon injury ($\pm$ 10 mg PROLI/NO), and vessels were harvested at 3 and 14 days. UbcH10 was assessed by Western blotting and immunofluorescent staining.

Results—NO more effectively reduced UbcH10 levels in LZ versus ZDF VSMC; however, addition of insulin and glucose dramatically potentiated the inhibitory effect of NO on UbcH10 in ZDF VSMC. Three days after balloon injury, Western blotting showed NO decreased free UbcH10 and increased polyubiquitinated UbcH10 levels by 35% in both STZ and ZDF animals. Fourteen days after injury, immunofluorescent staining showed increased UbcH10 levels

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7. DISCLOSURES

None

throughout the arterial wall in all animal models. NO decreased UbcH10 levels in LZ and STZ rats, but not in ZDF.

Conclusion—These data suggest a disconnect between UbcH10 levels and neointimal hyperplasia formation in type 2 diabetic models, and contribute valuable insight regarding differential efficacy of NO in these models.

Keywords

diabetes; neointimal hyperplasia; UbcH10; ubiquitin; artery

1. INTRODUCTION

Diabetic patients exhibit significantly greater risk for developing peripheral vascular disease, coronary artery disease, and cerebrovascular disease. Unfortunately, this population is also subject to increased rates of failure following vascular interventions to re-establish blood flow, primarily owing to excessive restenosis secondary to neointimal hyperplasia.[1-7] Neointimal hyperplasia arises via an inflammatory response to arterial injury in which platelets, macrophages, and leukocytes aggregate, become activated, and secrete various cytokines and growth factors that induce vascular smooth muscle cell (VSMC) and adventitial fibroblast proliferation and migration.[8-10] Nitric oxide (NO) is a gasotransmitter with many vasoprotective properties, including inhibition of platelet aggregation, adherence of leukocytes, and proliferation of VSMC.[11] We and others have previously demonstrated the effectiveness of NO as an inhibitor of the hyperplastic response.[7;12-15] We have also reported that nitric oxide more effectively inhibits neointimal hyperplasia in a rat model of uncontrolled type 2 diabetes than in nondiabetic controls.[16] Furthermore, we reported that NO did not effectively inhibit neointimal hyperplasia in a type 1 diabetic animal model without insulin control, but that insulin administration restored the ability of NO to inhibit neointimal hyperplasia.[16-19] These data suggest that the efficacy of NO may be dependent, in part, on the metabolic environment.

To investigate the etiology of the markedly different efficacy of NO in type 1 versus type 2 diabetes, we directed our attention to the protein ubiquitination, since ubiquitination is the first step leading to recognition and subsequent degradation of a protein via the 26S proteasome. Further, proteins involved in cell cycle progression, namely the cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors are tightly regulated through protein ubiquitination and degradation. We have previously shown that NO affects UbcH10, an E2 enzyme responsible for the ubiquitination and subsequent degradation of cyclin A and B.[20] Specifically, we showed that UbcH10 levels in VSMC correlated directly with proliferation levels, with overexpression causing increased proliferation and knockdown causing decreased proliferation, and that NO exposure led to reduced levels of UbcH10 and decreased proliferation.[20] Further, we observed increased UbcH10 staining in balloon-injured carotid artery sections, and NO decreased this staining.[20] We also showed that, following injury, NO increased ubiquitination through lysine 48, thereby favoring proteasomal degradation of proteins.[21] Recent data suggest that there is a complex interplay between diabetes and protein ubiquitination and degradation.[22-26] The effect of

diabetes on UbcH10 is not known. We hypothesize that baseline levels of UbcH10 differ in type 1 and type 2 diabetic arteries, and respond differently to NO following arterial injury. These differences in UbcH10 may account for the differential efficacy of NO in the different metabolic environments, given the prominent role UbcH10 has in regulating cell cycle progression, cellular proliferation, and neointimal hyperplasia. To investigate our hypothesis, we assessed the role of UbcH10 in arteries from animals with type 1 and 2 diabetes, *in vitro* and *in vivo*, with and without exposure to NO.

2. MATERIALS AND METHODS

2.1. Cell culture

The abdominal aortas of 11-week-old male Lean Zucker (LZ) and Zucker Diabetic Fatty (ZDF) rats (Charles River Laboratories, Wilmington, MA) were harvested, and vascular smooth muscle cells (VSMC) cultured from them and maintained as previously described. [27] Cultured cells displayed a characteristic SMC appearance (“hills and valleys”) and were routinely more than 95% pure, as seen by staining for SMC α -actin. Cultures were grown in medium containing low glucose DMEM and Ham’s F12 (1:1, vol:vol), as well as fetal bovine serum (10%, Invitrogen, Carlsbad, CA), L-glutamine (4 mM, VWR, West Chester, PA), and penicillin (100 units/mL, Invitrogen). VSMC were incubated at 37°C, 5% CO₂, and 95% air, and all experiments used cells between passages 4 and 8. See Table 1 for experimental overview.

2.2. Diazeniumdiolate preparation

(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO, $t_{1/2}$ = 20 hours at pH 7.4 in suspension) and disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO, $t_{1/2}$ = 2 seconds at pH 7.4 in suspension) were synthesized by Keefer and Saavedra, as described.[28;29] Working solutions of DETA/NO (10 mM) were prepared just before use by dissolving in the appropriate complete culture medium. Since all NO donors we have tested lowered levels of UbcH10, DETA/NO was chosen for cell culture experiments because the $t_{1/2}$ of 20 hours works well for the 24 hour time points used here.

2.3. Western blot analysis

Suspensions of VSMC treated with DETA/NO (250-1000 μ M), in the presence or absence of glucose (25 mM) or insulin (24nM), as well as untreated controls, were prepared as previously described.[30] Total protein concentrations were obtained using bicinchoninic acid (BCA) assays performed per the manufacturer’s protocol (Pierce, Rockford, IL). Suspensions of whole cells underwent acrylamide gel electrophoresis on 13% gels, followed by transfer to nitrocellulose. Levels of UbcH1, 2, 3, 5, 6, 7, 9, 10, and 12 were determined using commercially available antibodies for these proteins (1:500 to 1:2000; Boston Biochem, Boston, MA; Santa Cruz Biotechnology, Santa Cruz, CA). Equal loading was verified by incubation with β -actin antibody (Sigma-Aldrich, St. Louis, MO).

2.4. Diabetic animal models

Type 1 diabetes was induced in 11-week-old male LZ rats (Charles River Laboratories) via single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg in saline, Sigma). Type 2 diabetes was assessed using the ZDF strain of rats (Charles River Laboratories), which contains a homozygous mutation in the leptin receptor. When inbred ZDF rats are fed a high-fat, high-carbohydrate diet (Purina 5008, Scientific Animal Feeds, Arlington Heights, IL), they display several characteristics of type 2 diabetes, such as elevated levels of insulin, glucose, cholesterol, and triglycerides. For both strains, a glucometer was used to obtain non-fasting daily serum glucose levels via tail vein puncture. Rats considered diabetic and included in the study displayed glucose concentrations of ≥ 300 mg/dL. Animals remained in their treatment group assignments for 3 weeks prior to surgery and throughout the entire post-operative period.

2.5. Animal surgery

All animal procedures were approved by the Northwestern University Animal Care and Use Committee and 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). Inhaled isoflurane (0.5-3%) was used to anesthetize rats, and subcutaneously administered atropine (0.1 mg/kg) was used to decrease airway secretions. After administration of STZ, animals were weighed and their blood glucose measured daily. The neck was prepared by shaving, followed by swabbing with betadine and 70% ethanol. Following exposure of the carotid arteries via midline neck incision, a 2F Fogarty catheter (provided by Edwards Lifesciences) was used to perform the balloon injury model. Blood flow was then restored, followed by even application of 10 mg of the NO donor PROLI/NO to the external surface of the injured common carotid artery of rats in the treatment group, as has been previously described.[15-20] PROLI/NO was chosen for the *in vivo* experiments because our laboratory has shown in prior studies that this agent more effectively inhibits neointimal hyperplasia than other NO donors.[15] Finally, the incision in the neck was closed. LZ, STZ, and ZDF treatment groups included injury and injury+PROLI/NO. Morphometric analysis was performed on carotid arteries harvested 14 days after injury. Insulin levels in blood were determined by ELISA-based kit (SPI-Bio, Bertin Pharma, France).

2.6. Tissue processing for Western blot analysis

Carotid arteries were harvested 3 days after balloon injury (n = 4-7/treatment group) and lysed as follows. The rat carotid artery balloon injury model was performed as described above, except tissues were not perfused with saline and fixed with paraformaldehyde. Injured and control arteries were ligated at the aortic arch, and then explanted *en bloc* as quickly as possible. Carotid arteries were separated from the surrounding tissue, washed with cold 1X phosphate-buffered saline (PBS), and opened *en face*. Next, the injured area of the common carotid artery was isolated, cut into small sections, and kept under liquid nitrogen until lysed via ceramic mortar and pestle (CoorsTek; Golden, CO). The crushed tissue was resuspended in a lysis buffer comprising 50 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol (volume/volume), 10 mM Na₄P₂O₇, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 50 mM NaF, 1

mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride. The BCA assay was used to determine protein concentration, and samples stored at -80°C until undergoing electrophoresis and Western blotting, as described above.

2.7. Tissue processing for histology

Carotid arteries harvested 14 days after balloon injury were prepared for sectioning and staining as follows (n = 6-7/treatment group). Rats anesthetized with inhaled isoflurane were euthanized via injection of 0.5 mL Euthasol (Virbac Animal Health, Virbac Corporation, Ft. Worth, TX) and bilateral thoracotomies. Both carotid arteries were exposed via a long midline incision, perfused *in situ* using cold 1X PBS (250 mL), fixed using 2% paraformaldehyde (weight/volume in PBS, 500 mL), and then removed *en bloc* from the carotid bifurcation to the aortic arch. Vessels were separated from the surrounding tissue, soaked in 2% paraformaldehyde for 1 hour at 4°C, and then incubated in 30% sucrose overnight at 4°C. The vessels were then snap-frozen in OCT (Tissue Tek, Hatfield, PA) and the entire injured area cut into 5-μm sections, as previously described.[12]

2.8. Immunofluorescent staining

Sections of carotid arteries from uninjured control, injury, and injury+NO rats were stained for Ubch10 as follows. After fixation in 2% paraformaldehyde, sections were permeabilized for 10 minutes in 0.3 % Triton X-100 (volume/volume in PBS). Following 30 minutes of blocking with donkey serum (5% volume/volume in bovine serum albumin [BSA]), sections were incubated for 1 hour at 4°C with an antibody to Ubch10 (1:50 in BSA, Santa Cruz). Sections incubated without primary antibody served as negative controls. After 30 minutes of incubation in goat anti-mouse AlexaFluor 555 secondary antibody (1:100 in PBS, Invitrogen), the nuclear stain DAPI (1:500 in PBS) was added for 30 seconds. Finally, coverslips were placed on sections using ProLong Anti Fade Reagent (Invitrogen), which was allowed to dry overnight. Spot Advanced software (Diagnostic Instruments; Sterling Heights, MI) was used to acquire digital micrographs of sections using the 40X objective of an Eclipse 50i Microscope (Nikon Instruments, Inc.; Melville, NY), and intensity of Ubch10 staining was quantified on a scale of 0-3 by 3 blinded graders.

2.9. Statistical analysis

Results are given as mean ± the standard error of the mean (SEM). Differences between multiple groups were assessed using one-way analysis of variance, and the Student-Newman-Keuls *post hoc* test was employed for all pair-wise comparisons (SigmaStat; SPSS, Chicago, IL). Results were assumed to be statistically significantly different when P<0.05.

3. RESULTS

3.1. Nitric oxide treatment reduces Ubch10 levels more in LZ than ZDF VSMC

We began our evaluation by identifying whether differences existed in ubiquitin-conjugating enzyme levels in LZ and ZDF VSMC. We performed Western blot analysis on whole-cell suspensions of VSMC exposed to increasing concentrations of NO donor (DETA/NO, 250-1000 μM) for 24 hours. As seen in Figure 1, NO treatment had little effect on levels of Ubch2, 3, and 6 in either LZ or ZDF VSMC. Although levels of Ubch1, 5, and 7 were

slightly decreased in LZ VSMC, they were unaffected in ZDF VSMC (Figure 1). On the other hand, UbchH9 and 12 were unaffected in LZ VSMC, but decreased in ZDF VSMC (Figure 1). Though we did observe slight changes in levels of other E2s, the responses were not the same in both cell types, nor were these changes very large. Importantly, levels of UbchH10 were decreased by NO treatment in both cell types, and 500 μ M DETA/NO was more effective at lowering UbchH10 levels in LZ vs. ZDF VSMC (Figure 1). Since we have previously shown that changes in UbchH10 levels correlate directly with proliferation, the remainder of the experiments presented here focus on investigating the role of this E2 in the diabetic milieu.

3.2. Environments containing high glucose and insulin potentiate the effect of NO treatment on UbchH10 levels in LZ and ZDF VSMC

To better recapitulate the *in vivo* metabolic environment, we evaluated the effect of NO on VSMC in different glucose and insulin conditions *in vitro*. While NO reduced UbchH10 levels in LZ VSMC exposed to control media, NO exposure in high glucose (25 mM) media caused a decrease in UbchH10 levels at 500 μ M of DETA/NO (Figure 2A). NO exposure in high insulin media (24 nM) markedly decreased UbchH10 levels in LZ VSMC (Figure 2A). NO had less of an inhibitory effect on UbchH10 levels in ZDF VSMC in control media compared to LZ VSMC (Figure 2B). Compared to baseline levels of UbchH10 in LZ VSMC plated in control media, ZDF VSMC had higher levels of UbchH10 (Figure 2A control vs. Figure 2B control). When ZDF VSMC were treated with high glucose or high insulin concentrations, the inhibitory effect of NO on UbchH10 levels in ZDF cells was also much more dramatic (Figure 2B). There was a near complete inhibition of UbchH10 levels by NO in both the high insulin and high glucose environments, but the greatest inhibitory effect was observed with high glucose (Figure 2B). Of note, UbchH10 levels were very low at baseline (*i.e.*, without NO) in ZDF VSMC exposed to insulin compared to media- and glucose-exposed VSMC.

3.3. Metabolic characteristics of the rodent models

In order to assess the effect of NO on UbchH10 levels *in vivo*, we created and evaluated different rodent models of diabetes: normal control (LZ), type 1 diabetic (STZ), and type 2 diabetic (ZDF) rats. The desired control or diabetic state of all animals in this study was confirmed by measurements of blood glucose and insulin (Table 2). LZ rats had normal levels of insulin and glucose, and weighed between 320-330 grams. LZ rats given STZ required 3-5 days to display elevated glucose levels and were found to be appropriately hypoinsulinemic at sacrifice. STZ insulin levels were significantly less for injury alone and injury+NO treatment groups (22 ± 4 and 17 ± 6 pmol/L, respectively) compared to diabetic ZDF rats (Table 2, $P<0.05$). In addition, STZ glucose levels were significantly increased and their weights were significantly decreased compared to those of LZ rats, further confirming our model. Compared to LZ controls, ZDF rats exhibited significantly higher weights at surgery and at harvest time (Table 2, $P<0.05$), as well as significantly increased blood glucose levels.

3.4. Nitric oxide affects free and polyubiquitinated UbchH10 levels differently in diabetic versus nondiabetic rat arteries following arterial injury

In order to determine the effects of NO on UbchH10 levels *in vivo*, Western blot analysis was performed on lysates of carotid arteries harvested 3 days following balloon injury, with and without NO treatment (PROLI/NO 10 mg; Figure 3). At baseline, LZ rats had low levels of free UbchH10. Injury increased free UbchH10 levels by 24%, and NO treatment caused a further increase to 61% higher than baseline (Figure 3B, left panel). Evaluation of polyubiquitinated-UbchH10 (polyUb-UbchH10) showed increased levels in uninjured control LZ arteries and a subsequent 16% decrease with balloon injury (Figure 3B, center panel). NO applied after injury increased levels of polyUb-UbchH10 in LZ arteries 13% compared to uninjured controls (Figure 3B, center panel).

Interestingly, STZ and ZDF diabetic rats were different from LZ rats but similar to each other. In uninjured control diabetic arteries, more free UbchH10 was present at baseline, without injury or NO treatment (Figure 3A). Balloon injury to the carotid arteries decreased free UbchH10 levels in both diabetic models. Similarly, with the addition of NO, free UbchH10 remained lower than baseline levels in both STZ (33% reduction) and ZDF (40% reduction) diabetic models (Figure 3B, left panel). Of note, these data are consistent with the *in vitro* data, which showed a greater reduction of UbchH10 when NO was given with glucose and insulin. Injury alone decreased polyUb-UbchH10 levels in both STZ (21%) and ZDF (30%) rats, whereas the addition of NO increased polyUb-UbchH10 levels similar to those seen in uninjured controls (Figure 3B, center panel). Remarkably, the increase in polyUb-UbchH10 levels in the two diabetic models occurred more discretely at higher molecular weight bands of polyUb-UbchH10 (Figure 3A).

Since UbchH10 levels are regulated by auto-ubiquitination, we determined the polyUb-UbchH10 to free UbchH10 ratio (polyUb:free UbchH10) using densitometry. Notably, in LZ rats the ratio decreased with injury and injury+NO by around 30% (Figure 3B, right panel). However, in both STZ and ZDF rats, the ratio stayed relatively the same with injury but increased 35% above baseline for the injury+NO treatment groups (Figure 3B, right panel).

3.5. Nitric oxide treatment affects UbchH10 levels differently in nondiabetic, type 1, and type 2 diabetic rats at 14 days

Next, we used immunofluorescent staining to analyze UbchH10 levels in balloon-injured rat carotid arteries 14 days after injury (Figure 4A). At this time point, significant neointimal hyperplasia has formed, along with medial remodeling. The 14-day time point allows for analysis within the intimal, medial, and adventitial layers of the arterial wall, which cannot be performed at the 3-day time point. In the intima, UbchH10 levels were minimal in uninjured control arteries in all three rat groups. Injury significantly increased UbchH10 levels in all three groups (LZ: 0.0 vs. 0.8, $P<0.001$; STZ: 0.1 vs. 1.7, $P<0.001$; ZDF: 0.2 vs. 2.0, $P<0.001$; Figure 4B). With NO treatment, while there was a trend toward decreased UbchH10 levels in the intima of LZ rats at 14 days, UbchH10 levels in STZ rats decreased significantly (from 1.7 to 0.9, $P<0.001$, Figure 4B). However, NO treatment in ZDF rats resulted in no change in UbchH10 levels compared to injury alone (Figure 4B).

In the media of ZDF rats, we observed increased UbchH10 levels in uninjured control arteries compared to LZ and STZ rats ($P<0.001$, Figure 4B). In all three animal groups, there was a significant increase in UbchH10 levels in the media with injury compared to uninjured controls ($P<0.001$, Figure 4B). In the NO treatment group of both LZ and STZ rats, there was a significant reduction in UbchH10 levels (52% and 68%, respectively, $P<0.05$). NO treatment resulted in no significant change in UbchH10 levels in ZDF arteries (Figure 4B).

The adventitial layer of uninjured control arteries showed minimal levels of UbchH10 in LZ and STZ rats, while arteries from ZDF rats expressed some baseline UbchH10 staining (Figure 4B). Balloon injury significantly increased UbchH10 levels in all three animal groups ($P<0.001$), but the addition of NO treatment caused no change in UbchH10 levels in LZ and STZ animals (Figure 4B). Interestingly, NO treatment resulted in a trend towards an increase in the UbchH10 level in the ZDF animals compared to injury alone (Figure 4B).

Lastly, we combined the UbchH10 levels throughout all arterial layers in balloon-injured control, type 1, and type 2 diabetic rats after 14 days, with and without NO treatment. While injury increased overall UbchH10 levels in all three rodent models ($P<0.001$), we observed that NO treatment following arterial injury resulted in a trend toward decreased UbchH10 levels in LZ and STZ rats compared to injury alone ($P=0.057$), but had no effect in ZDF rats (Figure 4B).

4. DISCUSSION

In this manuscript, we present data on the status of UbchH10 in the vasculature in the different metabolic environments of diabetes *in vitro* and *in vivo*. *In vitro*, we show that NO reduced UbchH10 levels in LZ VSMC to a much greater extent than in ZDF VSMC. However, addition of exogenous glucose or insulin potentiated the inhibitory effects of NO on UbchH10 levels, especially in ZDF VSMC. *In vivo*, we found differential effects of NO on UbchH10 levels at early (3 days) and late (14 days) time points following injury. At 3 days, NO decreased free UbchH10 levels in type 1 and type 2 diabetic arteries compared to uninjured controls, and diabetic animals had higher polyUb-UbchH10 levels. At 14 days, injured LZ and STZ rats showed increased UbchH10 levels, which were reduced with the addition of NO; however, NO did not reduce UbchH10 levels in ZDF rats, and there was a trend towards an increase. Thus, our current results reveal strong dysregulation of UbchH10 in type 1 and 2 diabetic environments at baseline, with distinct differences noted following injury and NO exposure. We focus here on UbchH10, as it has been shown to directly correlate with development of neointimal hyperplasia in Sprague Dawley rats.[20] In addition, inhibition of UbchH10 by NO is associated with less VSMC proliferation *in vitro* and a reduction in neointimal hyperplasia *in vivo*.[20]

Protein ubiquitination and subsequent degradation affects many biological functions, including inflammation, proliferation, and apoptosis.[31] With respect to the development of neointimal hyperplasia, inhibition of the 26S proteasome via a single dose of MG-132 significantly reduced neointimal formation by 74% after balloon arterial injury in the rat. [32] Many of the genes involved in protein ubiquitination and degradation were found to be altered in anastomotic intimal hyperplasia.[33] Also, ubiquitin-conjugating enzymes are

likely involved in the formation of foam cells in atherosclerosis.[34] While modulation of protein ubiquitination offers encouraging prospects for targeted therapies to prevent restenosis and atherosclerosis, only a few studies have investigated the role of protein ubiquitination in diabetic environments. For instance, hyperinsulinemia caused differential modulation of insulin-like growth factor 1 via increased ubiquitination and subsequent degradation.[35] Hyperglycemic patients were shown to have significantly higher levels of ubiquitin within the carotid plaque.[36] In addition, ubiquitin-conjugating enzymes were involved in the response of pancreatic β -cells to hyperglycemia, and inhibition of the proteasome enhanced insulin release in response to glucose.[37] Finally, hyperglycemia increased ubiquitination and degradation of guanosine 5' triphosphate cyclohydrolase I, which leads to deficiency of tetrahydro-L-biopterin[24], an essential co-factor for eNOS. Together, these studies suggest that protein ubiquitination and subsequent degradation are differentially affected by diabetic environments.

Our results correlate well with these studies. *In vitro*, higher levels of the E2 enzyme UbcH10 are found in diabetic versus nondiabetic VSMC, and UbcH10 is less responsive to NO inhibition than in nondiabetic LZ VSMC. *In vivo*, both diabetic animal strains had higher levels of free UbcH10 at baseline, likely due to the hyperproliferative state caused by uncontrolled glucose. Since UbcH10 levels are regulated by auto-ubiquitination, we evaluated both free and polyubiquitinated UbcH10. Polyubiquitination of UbcH10 increased with NO treatment 3 days after arterial injury in hyperglycemic diabetic animals. Furthermore, 14 days after injury and NO treatment, type 2 diabetic ZDF rats had increased levels of UbcH10 at baseline and responded differently to NO treatment, with NO failing to diminish UbcH10 levels as it did in the type 1 and nondiabetic rats. It is possible that the increased polyUb-UbcH10 observed at 3 days persisted at 14 days in the type 2 diabetic animals, but the insulin-deficient type 1 diabetic animals reacted oppositely. We know hyperinsulinemia affects protein ubiquitination, as increased IRS-1 ubiquitination and degradation are mediated by chronic insulin treatment.[25] We further show that not only are UbcH10 levels increased at baseline in diabetic rats, but there are also significant differences in the response of UbcH10 to NO in type 1 versus type 2 diabetic environments. The increased efficacy of NO at inhibiting neointimal hyperplasia in ZDF rats may be consistent with the changes we observed in overall UbcH10 levels on immunofluorescence, since the antibody used cannot differentiate free from polyubiquitinated UbcH10. It may be that NO decreased free UbcH10 and increased polyUb-UbcH10, favoring inhibition of the cell cycle and, thus, proliferation. Overall, we postulate that NO is more effective in type 2 diabetic rats by reducing levels of free UbcH10 and increasing levels of polyUb-UbcH10. This mechanism would lead to less functional UbcH10 in NO-treated ZDF rats, resulting in more effective inhibition of neointimal hyperplasia. In the insulin-deficient type 1 diabetic rats, other factors may come into play. Finally, we have seen no evidence that elevated UbcH10 is protective against neointimal hyperplasia at any time point.

This study is not without limitations. First, the different methods of arterial processing used at 3 (lysate collection) and 14 days (sectioning) after arterial injury do not allow for direct comparisons between time points, but do provide useful and meaningful data. Second, the results obtained in control LZ animals at 3 days were paradoxical to prior results observed in

Sprague Dawley rats.[20] This is likely due to the fact that LZ and Sprague Dawley rats are substantially different strains. Finally, regarding the doses of NO given in this study, we used ~25 mg/kg of PROLI/NO powder (220 g/mol) to locally treat injured vessels periadventitially. Assuming an animal weight of 0.400 kg gives 45 μ moles of PROLI/NO for this dose, which releases 90 μ moles of NO. For cell culture experiments, VSMC were exposed to a maximum dose of 1 mM DETA/NO (163 g/mol), which equates to 4 μ moles DETA/NO and releases 8 μ moles of NO. So, while our *in vivo* experiments are performed at supraphysiological levels, our *in vitro* dose is actually much lower than that used *in vivo*.

Future directions for our laboratory consist of actively assessing the mechanism by which NO exerts its effects on UbcH10. Based on our results to date, NO appears to encourage ubiquitination of UbcH10 via a post-translational effect. As endothelial dysfunction is an early sign of diabetic vascular disease, we are currently working to modify levels of UbcH10 *in vivo* using siRNA. This would bypass NO and allow for determination of the effects of modulating UbcH10 levels on endothelial cells in the absence of a potent vasodilator. Since we have previously shown that decreasing UbcH10 via exogenous NO decreases proliferation in a variety of cell types, including endothelial cells and fibroblasts (unpublished data), we will also assess the effects of this siRNA treatment on VSMC and fibroblasts, as well as on neointimal hyperplasia. Additional future directions include testing the effects of delayed NO administration on UbcH10 levels and neointimal hyperplasia in order to verify our previous findings that the most effective time to deliver NO is at the time of angioplasty.[38] We will also examine the effects of inflammation and apoptosis in these diabetic models, since ubiquitination is implicated in both of these processes. Finally, it may be possible to use UbcH10 as a biomarker by measuring it via ELISA on serum collected following balloon angioplasty. While the relevance of systemic UbcH10 level changes to the local arterial environment and neointimal hyperplasia is unknown, we may be able to correlate UbcH10 levels with restenosis.

In conclusion, diabetic vascular disease negatively affects the lives of millions of patients daily. Bypass or angioplasty failure and need for multiple re-interventions increases the morbidity and lessens the quality of life for this patient population. Here, we have shown a differential relationship between NO and levels of the ubiquitin-conjugating enzyme UbcH10 in diabetic vascular environments. Our work contributes to the understanding of diabetic vascular biology yet calls for more investigations in the future.

5. ACKNOWLEDGMENTS

The authors would like to express their thanks to Lynnette Dangerfield for her administrative support, and to Edwards Lifesciences for providing the Fogarty balloon catheters.

6. GRANTS

This work was supported in part by funding from the National Institutes of Health (T32HL094293), the Department of Veterans Affairs (VA Merit Review Grant I01 BX000409), Society of University Surgeons (Ethicon Resident Research Grant), the American Medical Association Foundation Seed Grant program, the University of Illinois (Eleanor B. Pillsbury Grant), and by the generosity of Mrs. Hilda Rosenbloom and Mrs. Eleanor Baldwin.

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Figure 1.

Nitric oxide (NO) treatment reduces UbcH10 levels (arrow) more in Lean Zucker (LZ) than Zucker Diabetic Fatty (ZDF) vascular smooth muscle cells (VSMC). VSMC were treated with the NO donor DETA/NO (250-1000 μM), then subjected to Western blot analysis using antibodies to the E2 ubiquitin-conjugating enzymes shown on the left. Treatment time = 24 hours. Images are representative of three separate experiments.

Figure 2.

Environments containing high glucose and insulin potentiate the effect of nitric oxide (NO) treatment on UbcH10 levels in Lean Zucker (LZ) and Zucker Diabetic Fatty (ZDF) vascular smooth muscle cells (VSMC). VSMC were treated with the NO donor DETA/NO (250-1000 μM) in the presence of increasing concentrations of either glucose (25 mM) or insulin (24 nM), then subjected to Western blot analysis. Quantitation of UbcH10 and β-actin was performed using ImageJ. UbcH10 was adjusted by β-actin loading, and the ratio normalized to controls within each group. Graphical representation of the densitometry data showed glucose and insulin potentiated the inhibitory effects of NO on UbcH10 levels in A) LZ VSMC and B) ZDF VSMC, but the greatest effect was observed in the ZDF VSMC with glucose. Treatment time = 24 hours. Images and densitometry are representative of two experiments. *P<0.05 vs. controls.

Figure 3.

Nitric oxide (NO) affects free and polyubiquitinated UbH10 levels differently in diabetic versus nondiabetic rat arteries following arterial injury. A) Following balloon injury and treatment with or without PROLI/NO (10 mg, t = 3 days), rat carotid arteries were homogenized and subjected to Western blot analysis (n = 4-7 rats/treatment group). B) Densitometry of the Western blots showed low baseline levels of UbH10 in Lean Zucker (LZ) rats that increased following balloon injury (24%) and injury+NO treatment (61%). Diabetic rats had higher baseline levels of UbH10, which decreased slightly with injury and more markedly with injury+NO treatment (STZ: 33%, ZDF: 40%). Balloon injury decreased levels of polyubiquitinated UbH10 in all three rat models (LZ: 16%, STZ: 21%, and ZDF: 30%). Contrary to LZ rats, NO treatment increased the ratio of polyubiquitinated UbH10 to free UbH10 in streptozotocin-injected LZ (STZ) and Zucker Diabetic Fatty (ZDF) rats by 35%.

Figure 4.

Nitric oxide (NO) treatment affects UbCH10 levels differently in nondiabetic, type 1, and type 2 diabetic rats at 14 days. A) Carotid arteries harvested 14 days after balloon injury (n = 6-7/treatment group) with or without the NO donor PROLI/NO (10 mg) were sectioned and subjected to immunofluorescent staining for UbCH10 (red). Green is autofluorescence of the internal elastic lamina and blue is nuclear DAPI staining. Overall, while uninjured control arteries show little UbCH10 staining, UbCH10 levels significantly increased with injury in the intima and media in all three rat models. NO treatment decreased UbCH10 staining in the intima and media in LZ and STZ rats, but had no effect in ZDF rats. B) Staining was assessed by 3 blinded graders on a scale of 0-3 in the intima, media, adventitia, and total artery. * $P < 0.001$ vs. uninjured control; ** $P < 0.001$ vs. ZDF uninjured control; # $P = 0.001$ vs. injury; † $P < 0.05$ vs. injury.

Table 1

Description of experiments.

Cell culture			
Cell type	Treatment	Time point	Assessment method
LZ VSMC	± DETA/NO 250, 500, 1000 (µM)	24 hours	Westerns for E2s
ZDF VSMC	± DETA/NO 250, 500, 1000 (µM)	24 hours	Westerns for E2s
LZ VSMC	± DETA/NO 250, 500, 1000 (µM)	24 hours	Westerns for Ubch 10
	± 25 mM Glucose		
	± 24 nM Insulin		
ZDF VSMC	± DETA/NO 250, 500, 1000 (µM)	24 hours	Westerns for Ubch 10
	± 25 mM Glucose		
	± 24 nM Insulin		
<i>In vivo</i>			
Animal strain	Treatment	Time point	Assessment method
LZ	Injury ± PROLI/NO	3 days	Lysate Westerns for Ubch10
STZ	Injury ± PROLI/NO	3 days	Lysate Westerns for Ubch10
ZDF	Injury ± PROLI/NO	3 days	Lysate Westerns for Ubch10
LZ	Injury ± PROLI/NO	14 days	Sections stained for Ubch10 by IF
STZ	Injury ± PROLI/NO	14 days	Sections stained for Ubch10 by IF
ZDF	Injury ± PROLI/NO	14 days	Sections stained for Ubch10 by IF

Table 2

Metabolic characteristics of the rodent models.

	LZ	LZ + NO	STZ	STZ + NO	ZDF	ZDF + NO
<i>N</i>	10	11	9	12	11	14
Post-injury weight (g)	320±7	320±4	333±17	322±12	364±7 *	365±7 *
Harvest weight (g)	329±7	332±6	291±7 *	295±6 *	364±6 *	350±10 *
Insulin (pmol/L)	62±28	23±8	22±4 **	17±6 **	87±19	101±29
Glucose (mg/dL)	108±7	111±4	548±16 *	471±28 *	462±24 *	459±24 *

Values are means ± SE for all rats used in the 3-day and 14-day experiments. N is number of rats; LZ, Lean Zucker rats; STZ, streptozotocin-injected LZ rats; ZDF, Zucker Diabetic Fatty rats; NO, PROLI/NO. Blood tests were performed on non-fasted rats.

* P<0.05 compared to LZ rats.

** P<0.05 compared to ZDF rats.