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Nitric Oxide Differentially Affects PA28 after Arterial Injury in Type 1 and Type 2 Diabetic Rats

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Abstract

Background—Diabetic patients display aggressive restenosis following vascular interventions, likely due to pro-proliferative influences of hyperglycemia and hyperinsulinemia. We have shown that nitric oxide (NO) inhibits neointimal hyperplasia in type 2, but not type 1, diabetic rats. Here, we examined proteasome activator 28 (PA28) following arterial injury in different diabetic environments, with or without NO. We hypothesize that NO differentially affects PA28 levels based on metabolic environment.

Materials and methods—Vascular smooth muscle cell (VSMC) lysates from male, nondiabetic Lean Zucker (LZ) and Zucker Diabetic Fatty (ZDF) rats were assayed for 26S proteasome activity with or without PA28 and S-nitroso-N-acetylpenicillamine (SNAP). LZ and ZDF VSMC were treated with (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA/NO) for 24 hours. Balloon-injured carotid arteries from LZ, streptozotocin-injected LZ (STZ, type 1), and ZDF (type 2) rats treated with disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate (PROLI/NO) were harvested at 3 or 14 days. PA28 α was assessed by Western blotting and immunofluorescent staining.

Results—SNAP reversed PA28-stimulated increases in 26S proteasome activity in LZ and ZDF VSMC. Increased DETA/NO lowered PA28 α in LZ, but increased PA28 α in ZDF VSMC. At 3 days after injury, PROLI/NO potentiated injury-induced PA28 α decreases in LZ, STZ, and ZDF rats, suggesting VSMC, depleted at this early time point, are major sources of PA28 α . At 14 days

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DISCLOSURE

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

after injury, total PA28 α staining returned to baseline. However, while intimal and medial PA28 α staining increased in injured STZ rats, adventitial PA28 α staining increased in injured ZDF rats.

Conclusion—PA28 dysregulation may explain the differential ability of NO to inhibit neointimal hyperplasia in type 1 versus type 2 diabetes.

Keywords

proteasome; diabetes; nitric oxide; neointimal hyperplasia

1. INTRODUCTION

The incidence of diabetes in the United States continues to increase. According to the Centers for Disease Control and Prevention, over the last 30 years the number of people with diagnosed diabetes has risen from 5.6 to 20.9 million.^[1] In 2012, diabetic patients incurred \$176 billion in direct medical costs, and another \$69 billion in indirect costs.^[2] With respect to cardiovascular interventions, diabetic patients experience higher failure rates following vascular intervention due to more aggressive formation of neointimal hyperplasia.^[3; 4] The reasons that diabetic patients develop more aggressive neointimal hyperplasia are likely multifactorial. The ubiquitin proteasome pathway is responsible for the degradation of the majority of proteins in the cell. Proteins targeted for degradation are typically ubiquitinated then recognized and degraded by the 26S proteasome.^[5; 6] The 26S proteasome complex is formed when the 20S catalytic core combines with either the 19S or inducible 11S proteasome activator. Patients with diabetes have been shown to have derangements in the ubiquitin-proteasome pathway, such as increased protein ubiquitination due to oxidation from hyperglycemia.^[7] Hyperglycemia also stimulates the 26S proteasome, and hyperinsulinemia stimulates ubiquitination of proteins involved in insulin signaling.^[7; 8; 9] We have previously demonstrated that nitric oxide (NO) decreases neointimal hyperplasia more effectively in type 2 diabetic rats compared to nondiabetic rats, and that NO was ineffective in type 1 diabetic rats.^[10] The etiology for this different efficacy of NO in the diabetic environments remains unknown.

Interestingly, PA28, the 11S proteasome activator cap, combines with the 20S proteasome core to allow for rapid degradation of oxidized proteins without a polyubiquitin chain.^[11] The three homologous PA28 subunits — α , β , and γ — are evolutionarily conserved ^[11; 12] and combine to form the $\alpha\beta\gamma$ heteroheptamer or γ homoheptamer *in vivo*.^[13; 14] PA28 α/β are generally located in immune cells while PA28 γ is predominantly found in the brain.^[15] Specifically, PA28 α has been shown to protect against oxidative stress by degrading oxidized proteins.^[16] We have previously shown that NO inhibits PA28-induced increases in 26S proteasome activity in vascular smooth muscle cells (VSMC), and decreases PA28 subunit expression in balloon-injured arteries.^[17] More recently, we have demonstrated dysregulation of an important ubiquitin-conjugating enzyme in diabetic rats.^[18] Thus, given that NO regulates PA28 and that PA28 is involved in degrading oxidized proteins, it is possible that the PA28 activator is responsible for the differential effect of NO on neointimal hyperplasia following arterial injury in diabetic rats.

Much is known about the effects of NO and diabetes in the vasculature, especially in the context of neointimal hyperplasia.[¹⁰; ¹⁸; ¹⁹] It is also known that PA28 has complex effects on cells from different layers of the vascular wall.[⁹; ¹⁷; ²⁰] While PA28 knockout mice have been used to investigate its effects in the hepatic system of a type 2 diabetic mouse,[²¹] the role of PA28 in the formation of neointimal hyperplasia in the diabetic vasculature is unknown. Given the role of PA28 in degrading oxidized proteins, and the known interaction between diabetes and oxidative stress,[⁷] the goal of this study is to determine the role of PA28 α following arterial injury and the effect of NO on PA28 α in the diabetic vasculature. We hypothesize that NO has a differential effect on PA28 α in type 1 versus type 2 diabetic rat models.

2. MATERIALS AND METHODS

2.1. Cell culture

Primary VSMC were harvested from the abdominal aortas of 11-week-old, male Lean Zucker (LZ) and Zucker Diabetic Fatty (ZDF; Charles River Laboratories; Wilmington, MA) rats as described previously.[²²] All procedures were approved by the Northwestern University Animal Care and Use Committee and performed in accordance with protocols outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication 85-23, 1996). Cells were maintained in culture medium comprising low glucose DMEM, Ham's F12 (1:1, vol:vol), 10% fetal bovine serum (FBS; Invitrogen; Carlsbad, CA), 4 mM L-glutamine (VWR; West Chester, PA), and 100 units/mL penicillin (Invitrogen). All experiments used cells between passages 4 and 10, which were kept at 37°C, 5% CO₂, and 95% air.

2.2. S-Nitrosothiol preparation

S-nitroso-N-acetylpenicillamine (SNAP) was synthesized via stepwise addition of 240 mM sodium nitrite (Sigma; St. Louis, MO) aqueous solution to a 1.2 M solution of N-acetyl-DL-penicillamine (Sigma) in 1 part methanol, 1 part 1 N HCl, and 0.1 part concentrated H₂SO₄. The resultant green compound was filtered, washed with deionized water, allowed to dry for 7 days, and then stored in the dark at 4°C until use. Just before use, a 10 mM working solution of SNAP was prepared by dissolving in 1× phosphate-buffered saline (PBS).

2.3. Proteasome activity assays

VSMC harvested from LZ and ZDF rats were collected by scraping, transformed into whole cell lysates, and their protein concentration assessed as previously described.[¹⁷] Fluorogenic compounds specific for three different activities in the 26S proteasome (Suc-LLVY-AMC, chymotrypsin-like; Bz-VGR-AMC, trypsin-like; or Z-LLE-AMC, caspase-like; Boston Biochem; Cambridge, MA) were combined with reaction buffer (5 mM MgCl₂, 50 mM Tris [pH 7.8], 20 mM KCl, 5 mM MgOAc), 5 mM ATP, and 20 μ g of LZ or ZDF VSMC lysates in the presence or absence of SNAP (0.50 mM) and dithiothreitol (DTT, 5 mM). Activity was assayed using a fluorescent plate reader (excitation wavelength 355 nm, emission wavelength 460 nm) at a time point of 120 minutes. The proteasome inhibitor MG132 (0.05 mM) served as a negative control. For PA28-mediated enhancement of proteasome activity, reaction buffer, ATP, and 20 μ g lysate were mixed in the presence or

absence of SNAP, as above, but without addition of DTT and with addition of 1 nM recombinant PA28 (Boston Biochem).

2.4. Western blot analysis

Just before use, a 10 mM working solution of the NO donor (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA/NO, a kind gift from L. Keefer and J. Saavedra) was prepared by dissolving in culture medium. VSMC harvested from LZ and ZDF rats were exposed to DETA/NO (0.25–1.00 mM) for 24 hours, collected by scraping, and protein concentrations determined as previously described.^[17] After separation by SDS-PAGE on 10–13% polyacrylamide gels, proteins were transferred to nitrocellulose membranes. These were incubated with antibody against PA28 α (1:1000 in 1 $\times$ PBS-Tween; Cell Signaling Technologies; Danvers, MA), hybridized with goat anti-rabbit antibody (1:10000 in 1 $\times$ PBS-Tween; Pierce; Rockford, IL), and visualized via chemiluminescence reagents (SuperSignal, Pierce) and exposure to film. To ensure equal protein loading, membranes were also blotted for β -actin.

2.5. Animal surgery

To induce type I diabetes *in vivo*, male LZ rats were given a single injection of streptozotocin (60 mg/kg in saline; STZ; Sigma). To induce type 2 diabetes, male ZDF rats were fed Purina 5008 chow (Scientific Animal Feeds; Arlington Heights, IL), which caused these leptin-receptor deficient animals to develop hyperglycemia, hyperinsulinemia, hypercholesterolemia, and elevated triglycerides. Rodents with a glucose level >300 mg/dL were considered diabetic. The rat carotid artery balloon injury model was performed in all animal models (LZ, STZ, and ZDF) as previously described.^[17; 18] Treatment groups included injury and injury + disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate (PROLI/NO; 20 mg, a kind gift from L. Keefer and J. Saavedra). Since our lab previously studied the effect of NO on neointimal hyperplasia in both male and female rats, ^[23] this study was limited to a single sex to further elucidate the actions of NO in the vasculature.

2.6. Tissue processing

The carotid arteries of rats sacrificed at 3 days (n=4–7 arteries/treatment group) were explanted *en bloc*, homogenized, and protein concentration determined as previously described.^[18] These lysates were analyzed for PA28 α and β -actin by Western blotting as described above. The carotid arteries of rats sacrificed at 14 days (n=6–7/treatment group) were perfusion-fixed in 2% paraformaldehyde, preserved in 30% sucrose, and then frozen in OCT medium (Tissue Tek; Hatfield, PA). The entire injured area of the artery was then cut into 5 micron cross-sections using a CM1950 cryostat (Leica Biosystems; Buffalo Grove, IL) and stored at –80°C until stained using immunofluorescent antibodies (see below).

2.7. Immunofluorescent staining and quantitation

Carotid artery cross sections from all treatment groups in all three rat strains were fixed in 2% paraformaldehyde, permeabilized with 0.3% Triton X-100, blocked with goat serum (1:20 in BSA) for 30 minutes, and incubated in antibody against PA28 α (1:50 in 1 $\times$ PBS;

cat. #2408, Cell Signaling Technologies, Danvers, MA) overnight at 4°C. Primary antibody was omitted for negative controls. Sections were then incubated in goat anti-rabbit AlexaFluor 555 (1:100 in 1× PBS; Invitrogen, Carlsbad, CA) for 30 minutes, stained with DAPI (1:500 in 1× PBS) for 30 seconds, coverslipped with ProLong Anti Fade Reagent (Invitrogen), and allowed to dry overnight. Four quadrants of each cross section were digitally imaged using a Zeiss Imager. A2 (Hallbergmoos, Germany) and the 20× objective.

To quantitate staining, merged RGB images were used as the template to separate the intima, media, and adventitia in red channel images using the lasso tool in Adobe Photoshop CS. After the individual red layers were saved, they were opened in Photoshop and all reds were selected using the color range tool. Using the histogram window, any cached pixels were uncached and the red pixel value entered into a Microsoft Excel spreadsheet. The red selection was inverted to select all other pixels, white areas were removed from the selection using the magic wand tool (tolerance = 32), and any cached pixels seen in the histogram window were uncached. The number of black pixels was recorded in the spreadsheet and used to calculate total pixels, as well as percent red pixels.

2.8. Statistical analysis

All results are given as mean $\pm$ standard error of the mean. To analyze differences between groups, Kruskal-Wallis tests were performed using SigmaPlot (Systat Software; San Jose, CA), with the Student-Neumann-Keuls and Dunn's *post hoc* tests used for pairwise comparisons of same sized groups and different sized groups, respectively. Statistical significance was assumed when $P < 0.05$.

3. RESULTS

3.1. SNAP inhibits 26S proteasome activity in LZ and ZDF VSMC, and DTT reverses this inhibition

In order to establish baseline levels of proteasome activity in LZ and ZDF rat VSMC, an activity assay was performed using fluorogenic substrates specific for chymotrypsin-, trypsin-, and caspase-like activities in the 26S proteasome. At a time point of 120 minutes, treatment with the proteasome inhibitor MG132 (0.05 mM) significantly inhibited all three protease activities in both cell types, except for the caspase-like activity in LZ, which had low baseline activity levels (Figure 1, right). Treatment with the S-nitrosothiol SNAP (0.50 mM) significantly inhibited chymotrypsin- and trypsin-like activities in LZ and ZDF VSMC, but had no significant effect on caspase-like activity in either cell type. In the presence of the reducing agent DTT (5 mM), the SNAP-mediated inhibition of protease activities in both cell types was reversed. This is similar to what we previously showed in Sprague Dawley VSMC, where DTT alone and DTT+SNAP had similar effects on proteasome activity.^[24] It is also consistent with published literature showing that DTT can reverse oxidation-induced inhibition of proteasome activity.^[25; 26] These data suggest that SNAP regulates proteasome activity in these cell types by transnitrosation of active-site cysteines, similar to our previously published work.^[24]

3.2. PA28 stimulates 26S proteasome activity in LZ and ZDF VSMC, and SNAP reverses this stimulation

Once the baseline conditions for the proteasome activity assay were established, the assays were repeated in the presence of recombinant proteasome activator PA28 (1 nM), with or without SNAP (0.50 mM), at 120 minutes. As seen in Figure 2 (center), addition of SNAP to the reaction decreased the trypsin-like protease activity in both cell types, but not the chymotrypsin- or caspase-like activities. While PA28 significantly increased all three proteolytic activities versus control in both cell types by 1.5- to 10-fold, the addition of SNAP significantly reversed this PA28-mediated increase in activity, bringing it back down to control levels (Figure 2). Taken together, these data indicate that SNAP affects PA28-stimulated proteasome activity in LZ and ZDF VSMC by acting on a thiol group. Again, this is similar to what we previously described in Sprague Dawley rats, where addition of DTT to reactions containing PA28 and SNAP reversed the SNAP-mediated inhibition of PA28 activity.^[17]

3.3. NO has a differential effect on PA28 α levels in LZ and ZDF VSMC

To determine the effect of NO on cellular levels of PA28 α , VSMC grown from normoglycemic LZ rats and type 2 diabetic ZDF rats were exposed to various concentrations of the NO donor DETA/NO ($t_{1/2}$ = 20 hours, 0.25–1.00 mM) for 24 hours and subjected to Western blot analysis. As seen in Figure 3A, NO had a differential effect on levels of PA28 α in LZ versus ZDF VSMC. Specifically, as NO concentration increased, LZ VSMC showed lower levels of PA28 α , while ZDF VSMC showed higher levels of PA28 α . When the density of the bands in these Western blots was quantitated using ImageJ and normalized to LZ control β -actin, NO was seen to cause a 5-fold increase in ZDF PA28 α levels (Figure 3B). Since diabetic cells have increased levels of oxidative stress, these increased PA28 α levels in the presence of increased NO are not unexpected, given that increased PA28 α levels protect against oxidative stress by removing oxidized proteins.^[16]

3.4. Injury-mediated decrease in PA28 α is exacerbated by NO in LZ, STZ, and ZDF rats

To compare results seen *in vitro* with results observed *in vivo*, the rat carotid artery balloon injury model was performed in normoglycemic LZ, type 1 diabetic rats (STZ), and type 2 diabetic rats (ZDF). At 3 days after balloon injury, the carotid arteries (n=4–7) were homogenized and analyzed via Western blotting to determine levels of PA28 α . As seen in Figure 4A, STZ rats had the lowest level of PA28 α at baseline, followed by LZ rats and ZDF rats. Balloon injury decreased PA28 α levels in all three rat models, but most dramatically in diabetic ZDF and STZ rats. As shown by quantification of the PA28 α Western blots normalized to LZ control β -actin, NO further potentiated this effect in all three rat models, but most in STZ rats (Figure 4B). These data indicate that VSMC may be the primary source of PA28 activity in the vessel wall, since PA28 α staining was lost at this early time point when VSMC are depleted.

3.5. PA28 α staining is increased in type 1 and type 2 diabetic rats, and NO is unable to reverse the injury-mediated increase in PA28 α levels in type 1 rats

To determine the effects of the diabetic state, injury, and NO on the levels of PA28 α *in vivo* at a later time point when neointimal hyperplasia has developed, rats that underwent the carotid balloon injury model were sacrificed at 14 days. Cross-sections of uninjured, injury alone, and injury+NO carotid arteries from LZ, STZ, and ZDF rats (n=6–8/treatment group) were assessed for PA28 α levels using immunofluorescent staining. As seen in Figure 5A, appreciable differences were observed among the rat strains, as well as among the different vessel layers. In the intima, STZ and ZDF rats were seen to have higher baseline levels of PA28 α staining versus LZ control (Figure 5B, top left panel). Injury significantly increased this staining in LZ and STZ, but not ZDF rats. The addition of NO further increased PA28 α staining in the intima of STZ, but not LZ or ZDF rats.

In the media, both STZ and ZDF control rats again displayed significantly higher levels of PA28 α staining than LZ controls. Looking at the media in the injury group, STZ rats showed the highest level of staining, though this was not higher than control STZ media. In the ZDF injury group, the media showed significantly lower levels of staining than ZDF control. Interestingly, addition of NO did not affect levels of PA28 α staining in the media of any rat strain (Figure 5B, top right panel).

In the adventitia, ZDF rats showed significantly less PA28 α staining relative to LZ and STZ controls (Figure 5B, bottom left panel). While injury significantly increased PA28 α levels in the adventitia of LZ and ZDF rats, no effect was seen in STZ rats. Although addition of NO returned PA28 α levels in the LZ adventitia to that of control, this effect was not seen in the STZ or ZDF adventitia. Indeed, PA28 α staining in the adventitia of injury+NO STZ and ZDF were significantly higher than LZ injury+NO, and levels of PA28 α in the injury+NO ZDF adventitia were significantly higher than ZDF control.

Lastly, diabetic rats (STZ and ZDF) displayed higher levels of total PA28 α staining throughout all layers of the arterial wall at baseline versus LZ control, and higher levels in the injury+NO group versus LZ injury+NO (Figure 5B, bottom right panel). Total staining in the ZDF injury group was significantly increased versus LZ injury, whereas total STZ staining was not affected. These data suggest that the type 1 diabetic environment leads to dysregulation of the PA28-mediated protein breakdown pathway.

4. DISCUSSION

The work presented here shows that NO has a differential effect on the levels and activity of the PA28 proteasome activator in diabetic animal models. *In vitro*, we show that VSMC from type 2 diabetic rats had higher levels of PA28-induced 26S proteasome activity, and also experienced a greater drop in this activity when exposed to SNAP. While baseline levels of PA28 α in type 2 diabetic VSMC were similar to those seen in nondiabetic VSMC, addition of NO increased the levels of PA28 α in type 2 diabetic VSMC significantly. *In vivo*, type 1 diabetic rats have lower levels of PA28 α at baseline than nondiabetic or type 2 diabetic animals. At an early time point after balloon injury (*i.e.*, 3 days), we found that injury reduced PA28 α levels in all three rat models. We also show that type 2 diabetic rats

have the highest level of PA28 α , and addition of NO decreased PA28 α levels most profoundly in these rats. Lastly, at a later time point after balloon injury (*i.e.*, 14 days), we show that total PA28 α staining of the arteries returned to baseline levels, but that NO had a differential impact on PA28 α in the intima, media, and adventitia in the diabetic rat models.

Regulation of the PA28 proteasome activator is not well understood, and even less is known about its regulation in diabetic environments. While low levels of insulin in a rat model of type 1 diabetes induced by STZ were correlated with both lower PA28 levels and lower PA28 activity,^[27] samples taken from obese human type 2 diabetic patients with high levels of insulin displayed increased levels of PA28 α/β .^[21] Interestingly, when NIH-3T3 fibroblasts were exposed to hyperglycemic conditions *in vitro*, they also displayed higher levels of PA28 α/β .^[9] Given that: 1) the ZDF rat model of type 2 diabetes is characterized by profound hyperglycemia and hyperinsulinemia, whereas the streptozotocin-induced rat model of type 1 diabetes is characterized by hyperglycemia and hypoinsulinemia; 2) our data show differential regulation of PA28 in type 1 versus type 2 diabetes at baseline and following exposure to NO; and 3) the main difference in the animal models used in this study is insulin, we postulate that insulin is the likely cause for the dysregulation of PA28 in these diabetic environments. Consistent with this, we observed the highest levels of PA28 in the type 2 diabetic rat model. These data are also consistent with published studies showing that patients with insulin resistance display higher levels of PA28 α/β ,^[21] and knockdown of PA28 α abrogated the ability of cells to adapt to increased levels of ROS.^[20, 28] We further postulate that VSMC and myofibroblasts are the main source for PA28 in these diabetic environments. This is based on the fact that at 3 days following arterial injury, a time point when very few VSMC are present, we observed a dramatic decrease in PA28 levels. By 14 days after arterial injury, a time point when many VSMC and myofibroblasts are present, we observed that PA28 levels mostly returned to baseline within each animal model. Why we observed differential regulation of PA28 α among the different layers of the arterial wall remains to be determined and will be a source of further investigations.

In this study, we observed some interesting similarities and differences from our prior published work. Similar to what we previously showed in nondiabetic VSMC from Sprague Dawley rats,^[24] we show here that SNAP inhibits 26S proteasome activity in LZ and ZDF VSMC. We also show that SNAP reverses the PA28-mediated increase in proteasome activity in LZ and ZDF VSMC, which is similar to Sprague Dawley VSMC.^[17] In contrast to our previous findings in Sprague Dawley VSMC showing that NO did not affect PA28 subunit levels or localization,^[17] our results show that NO decreases PA28 α levels in LZ VSMC, but increases these levels in ZDF VSMC. We postulate that this may represent a positive feedback loop, with ZDF VSMC producing more PA28 α to attempt to overcome the increased oxidative stress caused by NO. Since it is known that NO causes oxidative stress, and overexpression of PA28 α removes oxidized proteins,^[16] these results in ZDF VSMC are in accordance with the literature. The reason for the decrease in LZ VSMC is unknown, but may be due to the fact that these cells came from a different strain than Sprague Dawley rats.

There are some limitations to this work. First, the models of type 1 and type 2 diabetes are different, with type 1 diabetes being induced acutely by STZ injection, and type 2 diabetes

being induced more gradually by diet in genetically susceptible rats. We chose these models because they are well-established diabetic rat models that have been extensively studied in the literature, and we have experience in using them. While mouse models of diabetes exist, and could prove interesting, we continued our study in rats because of our prior published findings in diabetic rats. Second, we do not show the extent of neointimal hyperplasia in these rats, as we have previously published this work.^[10; 19] Third, because PA28 α and PA28 β do not form homoheptamers *in vivo*,^[29; 30] and PA28 β acts to help facilitate formation of the α 3 β 4 heteroheptamer,^[13] we used PA28 α levels as a proxy for the PA28 11S proteasome cap. In addition, we did not perform PA28 activity assays on tissue lysates from balloon-injured rats, as the extremely small area of balloon injury would require the sacrifice of many rats to obtain enough tissue for these assays. Nor did we look at levels of oxidatively damaged proteins in diabetic rats, with or without NO treatment, as the effects of hyperglycemia and ROS on oxidized proteins are well established.^[7] Finally, we did not use MG132 in our *in vivo* studies, since others have already demonstrated the effects of proteasome inhibition on neointimal hyperplasia and oxidative stress.^[31; 32]

In conclusion, we show that PA28 α levels are different at baseline among the diabetic and non-diabetic rat models, are reduced following balloon injury, and return to baseline 14 days after injury. However, we show that NO differentially affects PA28 α levels throughout the arterial wall in the different diabetic rat models after injury. The increase in PA28 α observed in the diabetic rats is likely due to the increased oxidative environment known to be present in the diabetic milieu. Furthermore, we postulate that insulin may be partially responsible for the differential levels of PA28 α we observed. Overall, our data show that there is a link between PA28 levels, oxidative stress, and diabetic state. These data support our hypothesis, indicate a strong relationship between the proteasome and development of neointimal hyperplasia, and may account for the differential efficacy of NO in type 1 versus type 2 diabetes. 5.

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REFERENCES

1. C.f.D.C.a. Prevention. CDC - Number of Persons - Diagnosed Diabetes - Data & Trends - Diabetes DDT. 2014
2. C.f.D.C.a. Prevention. National Diabetes Statistics Report: Estimates of Diabetes and Its Burden in the United States, 2014. In: U.D.o.H.a.H. Services. , editor. Atlanta, GA: 2014.
3. Kornowski R, Mintz GS, Kent KM, Pichard AD, Satler LF, Bucher TA, Hong MK, Popma JJ, Leon MB. Increased restenosis in diabetes mellitus after coronary interventions is due to exaggerated intimal hyperplasia - A serial intravascular ultrasound study. *Circulation*. 1997; 95:1366-1369. [PubMed: 9118501]

4. Kip KE, Faxon DP, Detre KM, Yeh WL, Kelsey SF, Currier JW. Coronary angioplasty in diabetic patients - The National Heart, Lung, and Blood Institute percutaneous transluminal coronary angioplasty registry. *Circulation*. 1996; 94:1818–1825. [PubMed: 8873655]
5. Orłowski N, Wilk S. A multicatalytic protease complex from pituitary that forms enkephalin and enkephalin containing peptides. *Biochem. Biophys. Res. Commun.* 1981; 101:814–822. [PubMed: 7030330]
6. Hershko A, Ciechanover A. The ubiquitin system. *Annu. Rev. Biochem.* 1998; 67:425–479. [PubMed: 9759494]
7. Liu H, Yu S, Xu W, Xu J. Enhancement of 26S proteasome functionality connects oxidative stress and vascular endothelial inflammatory response in diabetes mellitus. *Arterioscler Thromb Vasc Biol.* 2012; 32:2131–2140. [PubMed: 22772755]
8. Rui L, Yuan M, Frantz D, Shoelson S, White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *J. Biol. Chem.* 2002; 277:42394–42398. [PubMed: 12228220]
9. Aghdam SY, Gurel Z, Ghaffarieh A, Sorenson CM, Sheibani N. High glucose and diabetes modulate cellular proteasome function: Implications in the pathogenesis of diabetes complications. *Biochem Biophys Res Commun.* 2013; 432:339–344. [PubMed: 23391566]
10. Ahanchi SS, Varu VN, Tsihlis ND, Martinez J, Pearce CG, Kapadia MR, Jiang Q, Saavedra JE, Keefer LK, Hrabie JA, Kibbe MR. Heightened efficacy of nitric oxide-based therapies in type II diabetes mellitus and metabolic syndrome. *Am. J. Physiol Heart Circ. Physiol.* 2008; 295:H2388–H2398. [PubMed: 18931034]
11. Mott JD, Pramanik BC, Moomaw CR, Afendis SJ, DeMartino GN, Slaughter CA. PA28, an activator of the 20 S proteasome, is composed of two nonidentical but homologous subunits. *J. Biol. Chem.* 1994; 269:31466–31471. [PubMed: 7989312]
12. Realini C, Jensen CC, Zhang Z, Johnston SC, Knowlton JR, Hill CP, Rechsteiner M. Characterization of recombinant REGalpha, REGbeta, and REGgamma proteasome activators. *J. Biol. Chem.* 1997; 272:25483–25492. [PubMed: 9325261]
13. Zhang Z, Krutchinsky A, Endicott S, Realini C, Rechsteiner M, Standing KG. Proteasome activator 11S REG or PA28: recombinant REG alpha/REG beta heterooligomers are heptamers. *Biochemistry.* 1999; 38:5651–5658. [PubMed: 10220354]
14. Ma CP, Slaughter CA, DeMartino GN. Identification, purification, and characterization of a protein activator (PA28) of the 20 S proteasome (macropain). *J. Biol. Chem.* 1992; 267:10515–10523. [PubMed: 1587832]
15. Rechsteiner M, Hill CP. Mobilizing the proteolytic machine: cell biological roles of proteasome activators and inhibitors. *Trends Cell Biol.* 2005; 15:27–33. [PubMed: 15653075]
16. Li J, Powell SR, Wang X. Enhancement of proteasome function by PA28 α ; overexpression protects against oxidative stress. *FASEB J.* 2011; 25:883–893. [PubMed: 21098724]
17. Tsihlis ND, Kapadia MR, Vavra AK, Jiang Q, Fu B, Martinez J, Kibbe MR. Nitric oxide decreases activity and levels of the 11S proteasome activator PA28 in the vasculature. *Nitric Oxide.* 2012; 27:50–58. [PubMed: 22561112]
18. Rodriguez MP, Tsihlis ND, Emond ZM, Wang Z, Varu VN, Jiang Q, Vercammen JM, Kibbe MR. Nitric oxide affects Ubch10 levels differently in type 1 and type 2 diabetic rats. *J Surg Res.* 2015; 196(1):180–189. [PubMed: 25801975]
19. Varu VN, Ahanchi SS, Hogg ME, Bhikapurwala 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.* 2010; 299:H772–H779. [PubMed: 20562340]
20. Pickering AM, Koop AL, Teoh CY, Ermak G, Grune T, Davies KJ. The immunoproteasome, the 20S proteasome and the PA28 α beta proteasome regulator are oxidative-stress-adaptive proteolytic complexes. *Biochem J.* 2010; 432:585–594. [PubMed: 20919990]
21. Otda T, Takamura T, Misu H, Ota T, Murata S, Hayashi H, Takayama H, Kikuchi A, Kanamori T, Shima KR, Lan F, Takeda T, Kurita S, Ishikura K, Kita Y, Iwayama K, Kato K, Uno M, Takeshita Y, Yamamoto M, Tokuyama K, Iseki S, Tanaka K, Kaneko S. Proteasome dysfunction mediates

- obesity-induced endoplasmic reticulum stress and insulin resistance in the liver. *Diabetes*. 2013; 62:811–824. [PubMed: 23209186]
22. Orekhov AN, Andreeva ER, Krushinsky AV, Smirnov VN. Primary cultures of enzyme-isolated cells from normal and atherosclerotic human aorta. *Med. Biol.* 1984; 62:255–259. [PubMed: 6513617]
23. Hogg ME, Varu VN, Vavra AK, Popowich DA, Banerjee MN, Martinez J, Jiang Q, Saavedra JE, Keefer LK, Kibbe MR. Effect of nitric oxide on neointimal hyperplasia based on sex and hormone status. *Free Radic Biol Med.* 2011; 50:1065–1074. [PubMed: 21256959]
24. Kapadia MR, Eng JW, Jiang Q, Stoyanovsky DA, Kibbe MR. Nitric oxide regulates the 26S proteasome in vascular smooth muscle cells. *Nitric. Oxide.* 2009; 20:279–288. [PubMed: 19233305]
25. Ferrington DA, Husom AD, Thompson LV. Altered proteasome structure, function, and oxidation in aged muscle. *FASEB J.* 2005; 19:644–646. [PubMed: 15677694]
26. Reinheckel T, Sitte N, Ullrich O, Kuckelkorn U, Davies KJ, Grune T. Comparative resistance of the 20S and 26S proteasome to oxidative stress. *Biochem J.* 1998; 335(Pt 3):637–642. [PubMed: 9794805]
27. Merforth S, Kuehn L, Osmer A, Dahlmann B. Alteration of 20S proteasome-subtypes and proteasome activator PA28 in skeletal muscle of rat after induction of diabetes mellitus. *Int J Biochem Cell Biol.* 2003; 35:740–748. [PubMed: 12672465]
28. Pickering AM, Linder RA, Zhang H, Forman HJ, Davies KJ. Nrf2-dependent induction of proteasome and Pa28alphabeta regulator are required for adaptation to oxidative stress. *J Biol Chem.* 2012; 287:10021–10031. [PubMed: 22308036]
29. Johnston SC, Whitby FG, Realini C, Rechsteiner M, Hill CP. The proteasome 11S regulator subunit REG alpha (PA28 alpha) is a heptamer. *Protein Sci.* 1997; 6:2469–2473. [PubMed: 9385652]
30. Wilk S, Chen WE, Magnusson RP. Properties of the beta subunit of the proteasome activator PA28 (11S REG). *Arch. Biochem. Biophys.* 2000; 384:174–180. [PubMed: 11147828]
31. Meiners S, Laule M, Rother W, Guenther C, Prauka I, Muschick P, Baumann G, Kloetzel PM, Stangl K. Ubiquitin-proteasome pathway as a new target for the prevention of restenosis. *Circulation.* 2002; 105:483–489. [PubMed: 11815432]
32. Wang S, Zhang M, Liang B, Xu J, Xie Z, Liu C, Viollet B, Yan D, Zou MH. AMPKalpha2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction in vivo: role of 26S proteasomes. *Circ Res.* 2010; 106:1117–1128. [PubMed: 20167927]

Figure 1.

SNAP inhibits 26S proteasome activity in Lean Zucker (LZ) and Zucker Diabetic Fatty (ZDF) vascular smooth muscle cells (VSMC), and dithiothreitol (DTT) reverses this inhibition. Chymotrypsin-, trypsin-, and caspase-like activities were assessed in LZ and ZDF VSMC lysates at 120 minutes, with or without addition of the S-nitrosothiol SNAP (0.50mM), and in the presence or absence of DTT (5 mM). The proteasome inhibitor MG132 (0.05 mM) was used as a negative control. Activities are expressed as % control. *P<0.05 vs. LZ control; †P<0.05 vs. LZ SNAP; #P<0.05 vs. ZDF control; **P<0.05 vs. ZDF SNAP. N=8 replicates/treatment group. Experiment repeated 3 times.

Figure 2.

PA28 stimulates 26S proteasome activity in LZ and ZDF VSMC, and SNAP reverses this stimulation. Chymotrypsin-, trypsin-, and caspase-like activities were assessed with SNAP (0.50mM) at 120 minutes, in the presence or absence of PA28 (1 nM). Activities are expressed as % control. *P<0.05 vs. LZ control; †P<0.05 vs. LZ PA28; #P<0.05 vs. ZDF control; **P<0.05 vs. ZDF PA28. N=8 replicates/treatment group. Experiment repeated 3 times.

Figure 3.

NO has a differential effect on PA28α levels in LZ and ZDF VSMC. (A) VSMC from normoglycemic LZ rats exposed to various concentrations of the NO donor DETA/NO (0.25–1.00mM) have lower levels of PA28α, while VSMC from type 2 diabetic rats (ZDF) have higher levels of PA28α at higher concentrations of NO. Western blots are representative of 2 different experiments. (B) Quantitation of 2 experiments via ImageJ, with PA28α in LZ and ZDF treatment groups normalized to LZ control β-actin levels, showed that DETA/NO increased PA28α almost 5-fold over control.

Figure 4.

Injury-mediated decrease in PA28α is exacerbated by NO in STZ and ZDF rats. (A) Western blot of homogenized carotid arteries from LZ, STZ, and ZDF rats (t = 3 days) showed that injury decreased PA28α levels. N=4–7/treatment group. Arteries for all rats in a group were pooled to increase protein concentrations and normalize rat-to-rat variability. (B) Quantification of PA28α levels in all animal models was performed and normalized to β-actin. Injury resulted in decreased PA28α levels in all models, but NO caused a further decrease from injury alone in STZ and ZDF rat models.

Figure 5.

PA28 α staining is increased in diabetic rats. (A) Representative cross-sections of control, injury, and injury+NO carotid arteries from LZ, STZ, and ZDF rats (t = 14 days) showed that diabetic rats had higher PA28 α levels. (B) Quantification of PA28 α staining in all animal models was performed using Adobe Photoshop and expressed as percent red pixels. *P<0.05 vs. LZ control; †P<0.05 vs. STZ control; #P<0.05 vs. LZ injury+NO; **P<0.05 vs. LZ injury; ††P<0.05 vs. ZDF control.