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Nitric oxide affects UbcH10 levels differently in type 1 and type 2 diabetic rats

Monica P. Rodriguez, MD,[a,1] Nick D. Tsihlis, PhD,[a,1] Zachary M. Emond, MD,[a] Zheng Wang, BA,[a] Vinit N. Varu, MD,[a] Qun Jiang, MD,[a] Janet M. Vercammen, AAS,[a] and Melina R. Kibbe, MD[a,b,*]

[a] Division of Vascular Surgery, Department of Surgery, and Simpson Querrey Institute for BioNanotechnology, Northwestern University Feinberg School of Medicine, Chicago, Illinois

[b] Division of Vascular Surgery, Department of Surgery, Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois

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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 (VSMCs) 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 (±10 mg PROLI/NO), and vessels were harvested at 3 and 14 d. UbcH10 was assessed by Western blotting and immunofluorescent staining.

Results: NO more effectively reduced UbcH10 levels in LZ *versus* ZDF VSMCs; however, addition of insulin and glucose dramatically potentiated the inhibitory effect of NO on UbcH10 in ZDF VSMCs. 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 throughout the arterial wall in all animal models. NO decreased UbcH10 levels in LZ and STZ rats but not in ZDF.

Conclusions: 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.

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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 after vascular interventions to reestablish blood flow, primarily owing to excessive restenosis secondary to neointimal hyperplasia

* *Corresponding author*. Division of Vascular Surgery, Department of Surgery, Northwestern University Feinberg School of Medicine, 676 N. St. Clair Street, #650, Chicago, IL 60611. Tel.: +1 312 503 6701; fax: +1 312 503 1222.
E-mail address: mkibbe@nmh.org (M.R. Kibbe).

[1] These authors contributed equally to this article and share first authorship.

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[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 NO 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 because ubiquitination is the first step leading to recognition and subsequent degradation of a protein via the 26S proteasome. Furthermore, 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]. Furthermore, we observed increased UbcH10 staining in balloon-injured carotid artery sections, and NO decreased this staining [20]. We also showed that, after 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 after 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-wk-old male lean Zucker (LZ) and Zucker diabetic fatty (ZDF) rats (Charles River Laboratories, Wilmington, MA) were harvested and VSMCs cultured from them and maintained as previously described [27]. Cultured cells displayed a characteristic smooth muscle cell appearance ("hills and valleys") and were routinely more than 95% pure, as seen by staining for smooth muscle cell α-actin. Cultures were grown in medium containing low glucose Dulbecco's Modified Eagle Medium and Ham 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% CO_2, 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, $t_{1/2} = 20$ h at pH 7.4 in suspension) and disodium 1-[2-(carboxylato)pyrrolidin-1-iyl]diazen-1-ium-1,2-diolate (PROLI/NO, $t_{1/2} = 2$ s at pH 7.4 in suspension)

Table 1 – Description of experiments.

Experiments	Treatment	Time point	Assessment method
Cell culture			
Cell type			
LZ VSMC	±DETA/NO 250, 500, and 1000 (μM)	24 h	Westerns for E2s
ZDF VSMC	±DETA/NO 250, 500, and 1000 (μM)	24 h	Westerns for E2s
LZ VSMC	±DETA/NO 250, 500, and 1000 (μM) ± 25 mM glucose ± 24 nM insulin	24 h	Westerns for UbcH10
ZDF VSMC	±DETA/NO 250, 500, and 1000 (μM) ±25 mM glucose ± 24 nM insulin	24 h	Westerns for UbcH10
In vivo			
Animal strain			
LZ	Injury ± PROLI/NO	3 d	Lysate Westerns for UbcH10
STZ	Injury ± PROLI/NO	3 d	Lysate Westerns for UbcH10
ZDF	Injury ± PROLI/NO	3 d	Lysate Westerns for UbcH10
LZ	Injury ± PROLI/NO	14 d	Sections stained for UbcH10 by immunofluorescence
STZ	Injury ± PROLI/NO	14 d	Sections stained for UbcH10 by immunofluorescence
ZDF	Injury ± PROLI/NO	14 d	Sections stained for UbcH10 by immunofluorescence