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Nitric oxide decreases activity and levels of the 11S proteasome activator PA28 in the vasculature

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ABSTRACT

The 11S proteasome activator (PA28) binds to the 20S proteasome and increases its ability to degrade small peptides. Expression of PA28 subunits (α, β, γ) is induced by interferon-γ stimulation. Inflammation plays a role in the development of neointimal hyperplasia, and we have previously shown that nitric oxide (NO) reduces neointimal hyperplasia in animal models and 26S proteasome activity in rat aortic smooth muscle cells (RASMC). Here, we show that PA28 increased 26S proteasome activity in RASMC, as measured by a fluorogenic assay, and the NO donor S-nitroso N-acetylpenicillamine significantly inhibits this activation. This effect was abrogated by the reducing agents dithiothreitol and $HgCl_2$, suggesting that NO affects the activity of PA28 through S-nitrosylation. NO did not appear to affect PA28 levels or intracellular localization in RASMC *in vitro*. Three days following rat carotid artery balloon injury, levels of PA28α, β and γ subunits were decreased compared to uninjured control arteries ($n = 3$/group) *in vivo*. The NO donor proline NONOate further decreased PA28α, β and γ levels by 1.9-, 2.3- and 3.4-fold, respectively, compared to uninjured control arteries. Fourteen days following arterial injury, levels of PA28α, β and γ subunits were increased throughout the arterial wall compared to uninjured control arteries, but were greatest for the α and β subunits. NO continued to decrease the levels of all three PA28 subunits throughout the arterial wall at this time point. Since the PA28 subunits are involved in the breakdown of peptides during inflammation, PA28 inhibition may be one mechanism by which NO inhibits neointimal hyperplasia.

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Introduction

Oxidative stress and the immune response are increasingly being linked to the development of neointimal hyperplasia which occurs after interventions to treat cardiovascular disease, a leading cause of death and disability in the United States [1–4]. Oxidative stress is known to increase protein damage and accumulation, which has been implicated in a host of disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, heart disease, and atherosclerosis [5–8]. First described in 1983, the proteolytic complex known as the 20S proteasome contains the three catalytic activities (*i.e.*, trypsin-, chymotrypsin-, and caspase-like) responsible for degrading the majority of proteins in eukaryotic cells, including those damaged by oxidation [9]. This ATP-independent complex degrades small proteins and can combine with one of two activators (19S or 11S) to form a 26S complex. Combination with the former leads to formation of the 26S proteasome that is responsible for degrading ubiquitinated proteins in an ATP-dependent manner [10].

Alternatively, expression of the immune protein interferon-γ (IFNγ) induces combination of the 20S proteasome with the 11S cap also known as proteasome activator 28 (PA28) [11]. There are three PA28 subunits – α, 31 kDa; β, 29 kDa; γ, 32 kDa – which are homologous and evolutionarily conserved [11–15]. PA28α and β are mainly found in the cytoplasm, while PA28γ is mainly found in the nucleus and on some components of the cytoskeleton [16,17]. These subunits are also distinctly distributed throughout the body, with PA28α/β found in immune tissues, and PA28γ found primarily in the brain, where PA28α and β are noticeably absent [12]. While PA28α can readily form a homoheptamer *in vitro*, PA28β monomers act primarily to stabilize the PA28α/β complex [18]. Interestingly, PA28β monomers are potent inhibitors of 20S proteasome activity, but high concentrations of PA28β allow for oligomerization and proteasome activation [19].

In vivo, there are two versions of the PA28 cap, the α3β4 heteroheptamer and the γ homoheptamer, both of which are responsible

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for removing oxidatively damaged proteins from the cell, while not affecting intact proteins [20,21]. Indeed, previous work has shown that overexpression of PA28α protects against oxidative stress by clearing oxidized proteins out of cells [22]. In stark contrast, overexpression of PA28γ is observed in colorectal and thyroid cancers [23,24]. The corollary to this is that PA28γ knockout mice exhibit reduced body size, and embryonic cells lacking PA28γ are prevented from transitioning from G1 to S-phase [25,26]. More clinically relevant, decreases in proteasome activity have also been shown to reverse cardiac hypertrophy [27]. Additionally, it is known that expression of IFNγ peaks 7 days after arterial balloon injury [28]. This induction prevents apoptosis of vascular smooth muscle cells (VSMC), which contributes to the development of neointimal hyperplasia. Others have also shown, via transcriptome analysis of human atherectomy specimens, that IFNγ plays an important role in neointima formation [29]. Indeed, inhibition of IFNγ secretion by administration of a small molecule can prevent neointimal hyperplasia [28,30].

While inhibition of NO synthase has been shown to increase neointimal hyperplasia in a balloon injury model [31], our lab and others have shown that administration of exogenous nitric oxide (NO) inhibits neointima formation in a number of large and small animal arterial injury models [32–34]. We have also previously shown that NO inhibits 26S proteasome activity in rat aortic smooth muscle cells (RASMC) [35]. While it is known that arterial injury induces inflammation and production of IFNγ, neither the role of PA28 nor the effect of NO on PA28 levels or activity in the arterial injury process has been described. Thus, the goals of this study were to evaluate the effect of arterial balloon injury, with and without administration of NO, on the levels of PA28α/β/γ *in vivo*, and to determine the effect of NO on PA28 levels and activity in RASMC *in vitro*. Since balloon injury increases IFNγ, which increases PA28α/β expression and inhibits RASMC apoptosis to cause neointimal hyperplasia, and since administration of NO or inhibition of IFNγ decreases neointimal hyperplasia, we hypothesize that NO prevents neointimal hyperplasia by reducing the level and/or activity of PA28.

Materials and methods

Cell culture

Rat aortic smooth muscle cells (RASMC) were cultured from the abdominal aorta of Sprague Dawley rats (Harlan; Indianapolis, IN) using the collagenase method as described previously [36] and in accordance with protocols that conform to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication 85-23, 1996) and approved by the Northwestern University Animal Care and Use Committee. Cultured cells had the characteristic appearance of hills and valleys and were routinely more than 95% pure by smooth muscle cell α-actin staining. Cells were maintained in DMEM (low glucose)/Ham's F12, 1:1 vol:vol (JRH; Lenexa, KS) supplemented with 10% fetal bovine serum (FBS, Invitrogen; Carlsbad, CA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen) and maintained in an incubator at 37 °C, 95% air, 5% CO_2.

Whole cell lysate

RASMC were collected after media was removed from dishes and cells were rinsed with phosphate buffered saline (PBS). Cells were scraped from the dish, collected, and centrifuged at 1200 rpm for 5 min at 4 °C. After the supernatant was discarded, the pellet was resuspended in lysis buffer (1 M NaCl, 0.1 M Tris [pH 7.5], 0.5 M EDTA, Triton X-100, 1 M $MgCl_2$, 1 M sucrose, and 0.1 M ATP), incubated on ice for 15 min, and centrifuged at 20,800 rcf for 15 min at 4 °C. The supernatant was transferred to a microfuge tube and stored at −80 °C. Lysate concentration was measured by bicinchoninic acid (BCA) protein assay kit according to manufacturer's instructions (Pierce; Rockford, IL).

Proteasome activity assay and nitric oxide concentration determination

Whole cell lysate was collected from RASMC. The specific conditions for each experiment can be found in the accompanying figure legends. Reaction buffer (5 mM $MgCl_2$, 50 mM Tris [pH 7.8], 20 mM KCl, 5 mM MgOAc), ATP (5 mM), ± varying concentrations of recombinant PA28 (Boston Biochem; Cambridge, MA), and the NO-donor S-nitroso-N-acetylpenicillamine (SNAP), were added to 20 μg of lysate. This mixture was incubated at 37 °C for 10 min. To this reaction, one of three 26S proteasome-specific fluorogenic peptide substrates was added: Suc-LLVY-AMC (chymotrypsin-like), Bz-VGR-AMC (trypsin-like) or Z-LLE-AMC (caspase-like) (Boston Biochem). The final reaction volume was 200 μL. Proteasome activity was determined using a fluorogenic plate reader with excitation and emission wavelengths of 355 and 460 nm, respectively, at time points between 0 and 120 min.

To determine the concentration of NO released by SNAP in our proteasome activity assay, an Apollo 4000 Free Radical Analyzer (World Precision Instruments; Sarasota, FL) and 100-μm probe (ISO NOPF 100) were employed. After polarizing the probe, a standard curve was created using SNAP, per the manufacturer's instructions. To a vial containing 5 mL of proteasome activity assay reaction buffer, sufficient SNAP (dissolved in distilled, deionized water) was added to generate a final SNAP concentration of 10 or 50 μM. Each concentration was assessed twice. The amount of NO generated was determined by comparing the standard curve to the difference in picoamps (pA) from the baseline to the highest point of the peak.

Western blot analysis

RASMC whole cell suspension was collected following treatment ±SNAP for 24 h. Cells were rinsed with PBS, scraped, and centrifuged at 1200 rpm for 5 min. The supernatant was removed and the pellet was resuspended in buffer (20 mM Tris [pH7.4]) with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, St Louis, MO), 1 μg/mL leupeptin (Sigma) and 1 mM sodium orthovanadate (Na_3VO_4, Sigma). Protein concentration was measured via BCA assay. Samples were subjected to SDS–PAGE on 10–13% polyacrylamide gels, and transferred to nitrocellulose membranes (Schleich and Schuell; Keene, NH). Membranes were hybridized with antibodies to PA28α (1:1000), PA28β (1:1000), or PA28γ (1:1000) (Cell Signaling Technologies; Danvers, MA), followed by incubation with goat anti-rabbit antibody (1:10,000, Pierce). Proteins were visualized using chemiluminescence reagents (SuperSignal, Pierce) and exposed to film according to the manufacturer's instructions. Equal protein loading was verified via Western blotting for β-actin.

Animal surgery

All animal procedures performed were approved by the Northwestern University Animal Care and Use Committee, and according to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication 85-23, 1996). Male 11-week-old Sprague Dawley rats were anesthetized using inhaled isoflurane (0.5–3%). Atropine was administered subcutaneously (0.1 mg/kg) to decrease airway secretions. After a midline neck incision, the left common carotid artery (CCA), external carotid artery (ECA), and the internal carotid artery (ICA) were dissected and proximal and distal control obtained with microclips. A

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