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Original Contribution

Nox5 mediates PDGF-induced proliferation in human aortic smooth muscle cells

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ABSTRACT

The proliferation of vascular smooth muscle cells is important in the pathogenesis of many vascular diseases. Reactive oxygen species (ROS) produced by NADPH oxidases in smooth muscle cells have been shown to participate in signaling cascades regulating proliferation induced by platelet-derived growth factor (PDGF), a powerful smooth muscle mitogen. We sought to determine the role of Nox5 in the regulation of PDGF-stimulated human aortic smooth muscle cell (HASMC) proliferation. Cultured HASMC were found to express four isoforms of Nox5. When HASMC stimulated with PDGF were pretreated with N-acetyl cysteine (NAC), proliferation was significantly reduced. Proliferation induced by PDGF was also heavily dependent on JAK/STAT activation, as the JAK inhibitor, AG490, was able to completely abolish PDGF-stimulated HASMC growth. Specific knockdown of Nox5 with a siRNA strategy reduced PDGF-induced HASMC ROS production and proliferation. Additionally, siRNA to Nox5 inhibited PDGF-stimulated JAK2 and STAT3 phosphorylation. ROS produced by Nox5 play an important role in PDGF-induced JAK/STAT activation and HASMC proliferation.

In atherosclerosis and restenosis after percutaneous coronary intervention, cytokines elaborated by both vascular cells and cells invading the vessel wall induce vascular smooth muscle proliferation and contribute to lesion formation. In the normal vessel wall, smooth muscle cells (SMCs) maintain and regulate vascular tone. However, VSMCs change their phenotype to a synthetic, proliferative state when stimulated by a number of different growth factors.

One such growth factor, platelet-derived growth factor (PDGF), is expressed by all vascular cell types [1,2] and by invading inflammatory cells, such as monocytes and lymphocytes, in atherosclerosis [1]. PDGF has long been recognized as a powerful VSMC mitogen [3] and the induction of PDGF receptors in VSMCs during atherogenesis has been demonstrated in several studies [4,5]. Blockade of PDGF, whether by anti-PDGF antibody [6–8], PDGF receptor antisense therapy [9,10], or chimeric knockout in mice [11], reduces lesion formation after vascular injury. Ligand binding to the PDGF receptor causes tyrosine autophosphorylation and the initiation of specific signaling pathways that have been linked to growth. Calcium appears to be one of the obligate mediators of PDGF-stimulated mitogenesis, as several studies have reported that blockade of the increase in intracellular calcium caused by PDGF inhibits VSMC proliferation [12–14]. Of particular interest, overexpression of the antioxidant enzyme catalase in VSMCs also reduces PDGF-stimulated growth, indicating that reactive oxygen species (ROS) may regulate proliferation as well [15,16]. The mechanisms by which these two second messengers regulate growth are incompletely understood. One pathway that has been suggested to mediate the growth response is the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. This pathway has been implicated in a host of cellular processes such as migration, apoptosis, and proliferation [17], and importantly, in fibroblasts [18] and human pulmonary artery SMCs [19], its activation by PDGF is modulated by ROS.

ROS have long been known as the agents responsible for the antimicrobial properties of phagocytic cells. However, the discovery that non-phagocytic cells also produce ROS, albeit at low concentrations, has expanded their role to include cell signaling. The source of ROS in phagocytes is the NADPH oxidase (Nox), an enzyme complex composed of membrane-bound and cytosolic subunits. Recently, homologues of the membrane-bound catalytic subunit of the NADPH oxidase have been discovered in several different cell types. The ROS produced by these homologues in VSMCs regulate signaling cascades that are activated by cytokines, hormones, and thrombin [20]. Currently, the Nox family is composed of Nox1, Nox2 (the phagocytic NADPH oxidase), Nox3, Nox4, and Nox5, as well as the Duox enzymes [21]. These enzymes appear to have different physiological roles. For example, Nox1 is responsible for angiotensin II- and PDGF-stimulated ROS-production in rat aortic VSMCs [22], while Nox4 has been shown to be important for the maintenance of VSMC differentiation [23]. Most of the work defining the function of

Abbreviations: DCF-DA, dichlorofluorescein-DA; DHE, dihydroethidium; DPI, diphenylene iodonium; HASMCs, human aortic smooth muscle cells; NAC, N-acetylcysteine; Nox, NADPH oxidase; NSsiRNA, non-silencing siRNA; PBS, phosphate buffered saline; PEG-SOD, polyethylene glycolated superoxide dismutase; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; SEM, standard error of the mean; SMC, smooth muscle cell; SmBM, smooth muscle basal medium; VSMC, vascular smooth muscle cell.

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the NADPH oxidases in the vasculature has been performed in rodents, but human VSMCs also express Nox5 [24], a homologue unique to humans. However, the exact role of Nox5 in human VSMCs has not been defined.

With regard to PDGF signaling, Nox5 is of particular interest because it is activated by calcium and produces ROS, both of which have been shown to be required for PDGF-induced proliferation. Nox5 has been detected in several cancer cell lines [25,26] and, in a study with prostate cancer cells, appears to regulate cell growth [27]. Furthermore, the knockdown of Nox5 in human endothelial cells prevents proliferation [28]. Based on these observations, we hypothesized that Nox5 participates in PDGF-directed human VSMC proliferation via activation of the JAK/STAT pathway. Our results support this hypothesis and suggest that Nox5 may be an important regulator of a number of vascular pathologies in human disease.

Materials and Methods

Materials

PDGF-BB was purchased from R&D Systems, Inc. (Minneapolis, MN), and N-acetylcysteine (NAC) was from Sigma-Aldrich (St. Louis, MO). Non-silencing siRNA and siRNA to Nox5 (siNox5, which targets all Nox5 isoforms) were purchased from Ambion (Austin, TX). Phospho-JAK2 (Tyr1007/Tyr1008) antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA), phospho-STAT3 (Tyr705) and STAT3 antibodies were from Cell Signaling Technology, Inc (Danvers, MA), and anti-JAK2 antibody was from Millipore (Billerica, MA). AG490 and LY29004 were purchased from Calbiochem Bio-Mol (La Jolla, CA). Diphenylene iodonium (DPI) was from Alexis Biochemicals (San Diego, CA), and dihydroethidium (DHE) and BAPTA-AM were from Invitrogen (Carlsbad, CA).

Cell culture

Human aortic SMCs (HASMCs) were purchased from Cambrex (East Rutherford, NJ) and maintained in smooth muscle growth medium (SmGM: smooth muscle basal medium (SmBM) with addition of 5% FBS, 5 μg/mL insulin, 0.5 ng/mL EGF, 2 ng/mL FGF-B, gentamicin, and amphotericin B) per the company's instructions. Cells at passages 6 to 12 were used for the experiments.

Transfection

HASMCs were plated at 30% confluence, then transfected with 20 nmol/L siNox5 using oligofectamine (Invitrogen, Carlsbad, CA) as the transfection reagent in OPTI-MEM (Invitrogen). Four hours after transfection, the medium was changed to SmGM, and then to SmBM after 24 h. For the growth curve experiments, PDGF (25 ng/ml) was added to SmBM 4 h after transfection and the medium was changed daily thereafter. For the signaling experiments, the cells were left in SmBM for 2 days prior to use. For some experiments, the cells were transfected using nucleofector technology for siRNA delivery from Amaxa Biotechnologies (Gaithersburg, MD). The cells were transfected according to the manufacturer's protocol for nucleofection of HASMCs.

Cloning

Total RNA was extracted from HASMCs with RNeasy kit (Qiagen, Valencia, CA) per the manufacturer's instructions. Reverse transcription was performed with Superscript III (Invitrogen, Carlsbad, CA) oligo dT or a Nox5-specific primer (5′- GAGCTTGGAGAGGTGAGGCTAG -3′) at 55 °C. PCR using forward primers for Nox5β and δ (5′-GAGCTTGGAGAGGTGAGGCTAG-3′) or Nox5α and γ (5′-GAGCTTGGAGAGGTGAGGCTAG-3′) was performed with 1x PCR Enhancer (Epicentre, Madison, WI) and Platinum Taq HiFi (Invitrogen, Carlsbad, CA). Products were verified by sequencing.

Quantitative real-time PCR

Total RNA was extracted as above. Superscipt II (Invitrogen) and random primers were used for reverse transcription. Quantitative real-time PCR was performed with a LightCycler (Roche) real time thermocycler with primers for Nox5 (detecting all isoforms), Nox4, Nox1, and gp91phox [29]. Primers for 18 S (Classic II 18S alternate internal standards) were purchased from Ambion (Austin, TX). Results are expressed as copy number per 10^6 copies of 18S.

Cell counting

HASMCs were plated at 14,600 cells/well in a 24 well dish. After the appropriate treatment(s) and on the indicated day, cells were washed with Hank's Balanced Salt Solution (Gibco), trypsinized with trypsin/EDTA (Gibco), diluted in Isoton II (Coulter) and counted with a Z1 Coulter counter.

Dichlorofluorescein-DA assay for ROS

Cells were plated at 30% confluence in 24 well plates and transfected with 20 nmol/L non-silencing siRNA (NSsiRNA) or 20 nmol/L siNox5. After overnight incubation in SmGM, the medium was changed to SmBM + 25 ng/mL PDGF-BB and then changed daily thereafter. At day four after transfection, cells were incubated with 5 μmol/L DCF-DA (Molecular Probes) for 1 hour. Cells were washed twice with colorless HBSS and then visualized with confocal microscopy.

2-Hydroxyethidium assay for superoxide

Cells were plated at 30% confluence in 60-mm dishes and transfected with 20 nmol/L NSsiRNA or siNox5. After overnight incubation in SmGM, the medium was changed to SmBM + 25 ng/mL PDGF-BB and changed daily thereafter. Three days after transfection, polyethylene glycolated-(PEG) superoxide dismutase (SOD) was added to half the samples to a final concentration of 50 U/mL. The following day, the cells were washed twice with chilled Krebs/HEPES buffer and incubated with 1 μmol/L DHE at 37 °C for 20 min, and then scraped into amber microfuge tubes containing 300 μL methanol. Samples were stored at -80 °C until assayed by high performance liquid chromatography as previously described [30]. Results are expressed as the SOD-inhibitable signal.

Western Blotting

HASMCs at 70-90% confluence were made quiescent by incubation with SmBM containing 0.1% FBS for 24 to 48 hours. Cells were stimulated with PDGF (25 ng/mL) at 37 °C in serum-free SmBM for specified durations. After treatment, cells were washed three times with phosphate buffered saline (PBS) and lysed with ice-cold lysis buffer containing 20 mmol/L Tris-HCl at pH 7.5, 1% Triton-X, 1% Glycerol, 0.1% SDS, 1% Na-deoxycholate and 2.5 mmol/L EDTA. The lysis buffer was supplemented with Na-orthovanadate, Na-pyrophosphate, NaF, aprotinin, leupeptin and PMSF. Western analysis was performed with SDS-PAGE and subsequent transfer to nitrocellulose membranes. Incubation with the primary antibody was then carried out and detection was achieved with ECL. ImageJ software was used to analyze band intensities.

Statistical Analysis

Results are expressed as mean ± standard error of mean (SEM). For two group analyses, the unpaired t-test was used. One-way ANOVA was used to analyze data comparing three or more groups with Bonferroni's multiple comparison test for post hoc analysis (GraphPad Prism software). A value of $P<0.05$ was considered statistically significant.