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

Desmond B. Jay, Christopher A. Papaharalambus, Bonnie Seidel-Rogol, Anna E. Dikalova, Bernard Lassègue, and Kathy K. Griendling

Division of Cardiology, Department of Medicine, Emory University, Atlanta, GA 30322

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.

Keywords

NADPH oxidase; reactive oxygen species; Nox5; vascular smooth muscle cells; 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

Address correspondence to: Kathy K. Griendling, Emory University, Division of Cardiology, 319 WMB, 1639 Pierce Dr., Atlanta, GA 30322, Telephone: 404-727-3364, Fax: 404-727-3585, e-mail: kgriend@emory.edu.

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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 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 μ g/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 Nox5 β and δ (5'-GAGCTTGGAGAGGTGAGGCTAG-3') or Nox5 α 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. Superscript 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 18S (Classic II 18S alternate internal standards) were purchased from Ambion (Austin, TX). Results are expressed as copy number per 10⁶ 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 μ mol/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 μ mol/L DHE at 37°C for 20 min, and then scraped into amber microfuge tubes containing 300 μ L 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 $\pm$ 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.

Results

HASMCs predominantly express Nox4 and all isoforms of Nox5

Real time PCR with primers to Nox family members revealed that proliferating HASMCs express Nox4 and Nox5, with Nox4 mRNA more abundant than Nox5 mRNA ($23,895 \pm 7,150$ vs. 395 ± 56 copies per 10^8 18S). Using primers to Nox1 and Nox2, we were able to measure copy numbers as low as 10 per reaction, but detected minimal Nox1 and no Nox2 mRNA in HASMCs. Five variants of Nox5 have been reported: α , β , γ , δ , and a short form that is missing exon 6 [24,31,32]. We used reverse transcriptase PCR using primers specific to the α/γ or β/δ isoforms to amplify products that were then cloned and sequenced. The sequencing of several clones revealed that HASMCs express all four isoforms (data not shown). The presence of the short form of Nox5 was not investigated. Because of the difference in the relative expression levels of Nox1, 2, 4, and 5, and the data demonstrating the importance of Nox4 in differentiation [23] and Nox5 in human cell growth [27,28], we concentrated on examining the role of Nox5 in HASMC cell proliferation.

PDGF-directed HASMC proliferation is ROS-dependent

A previous study revealed that HASMC growth induced by 10% FBS is arrested by the addition of the antioxidant NAC [33]. We first determined if HASMC growth in response to PDGF is also ROS sensitive, as previous studies have only demonstrated this property in other cell types or in rat VSMCs [15,16]. As demonstrated in Figure 1, PDGF strongly stimulates HASMC proliferation. NAC alone slightly decreases cell number in the absence of PDGF, but inhibits

PDGF-induced HASMC proliferation by $41 \pm 3\%$. This finding indicates that ROS are important mediators of PDGF-induced HASMC proliferation.

Nox5 produces ROS in HASMCs in response to PDGF stimulation

The previous experiments indicate that ROS are required for proliferation, but do not provide insight into the source of ROS production. We hypothesized that the calcium-activated homologue Nox5 may mediate PDGF-induced ROS formation. To determine if Nox5 participates in PDGF-induced ROS production, we first tested its calcium sensitivity. As shown in Figure 2, pretreatment of HASMC with the intracellular calcium chelator BAPTA-AM (30 min, 10 $\mu\text{mol/L}$) essentially abolished superoxide production induced by PDGF (20 ng/mL). We next used siRNA to specifically downregulate all isoforms of Nox5 (Figure 3A), and used two assays to determine ROS production. The first was a qualitative measurement by DCF-DA staining. Figure 3B reveals that ROS production in cells treated with NSsiRNA or siNox5 alone is similar to that of control cells. However, cells treated with siNox5 failed to increase ROS production when treated with PDGF, while PDGF was able to increase ROS in cells treated with NSsiRNA. Figure 3C shows a quantitative decrease in superoxide production by $45 \pm 14\%$ as measured by hydroxyethidium in cells treated with siNox5 and PDGF compared to cells treated with NSsiRNA and PDGF. In contrast, siNox4, under conditions that we have previously shown to be effective [23], had no effect on PDGF-induced superoxide production (Figure 3D). These findings demonstrate that Nox5 is a significant source of ROS in PDGF-stimulated HASMC.

Nox5 plays a role in PDGF-stimulated HASMC growth

To determine whether Nox5 participates in PDGF-induced HASMC proliferation, we used cells in which Nox5 was downregulated with siRNA, treated them with PDGF-BB, and counted them on a Coulter counter at predetermined time points. Viability of these cells was unaffected by PDGF treatment. As shown in Figure 4, non-transfected control cells and cells treated with the transfection reagent, oligofectamine, grow at similar rates. At day 5, cells treated with PDGF are increased in number by $134 \pm 5\%$ when compared to untreated cells. The NSsiRNA does have a small effect on cell growth at day 5, because cell number increased by $91 \pm 4\%$ under these conditions. However, on day 5 the cells treated with siNox5 were only able to increase their number by $31 \pm 6\%$ when compared to the control cells. This result demonstrates that Nox5 is an important participant in PDGF-directed HASMC growth.

The JAK/STAT pathway mediates PDGF-induced HASMC proliferation

We next sought to identify effectors that depend on Nox5 for their activation and mediate PDGF-induced growth. One candidate is the JAK/STAT pathway, which has been shown to be ROS-sensitive in several different cell types including fibroblasts [18] and pulmonary artery SMCs [19]. As seen in Figure 5, pretreatment with the JAK inhibitor AG490 (10 $\mu\text{mol/L}$) completely inhibits HASMC proliferation in response to PDGF stimulation.

PDGF activation of JAK is flavin-containing enzyme-dependent in HASMC

To verify that activation of JAK/STAT in HASMC is ROS sensitive and that AG490 effectively inhibits JAK, we pretreated cells with either AG490 or the flavin-containing enzyme inhibitor DPI and then stimulated them with PDGF. Figure 6A demonstrates the prevention of PDGF-induced JAK2 phosphorylation with AG490 or DPI. The phosphorylation of STAT3 also appears to be ROS-sensitive (Figure 6B), but is not completely abolished with DPI treatment, suggesting that there may be another non-Nox-dependent, PDGF-activated JAK that can induce its phosphorylation.

Nox5 is required for PDGF-induced JAK/STAT activation

To investigate the participation of Nox5 in PDGF-stimulated JAK/STAT phosphorylation, we transfected HASMCs with siNox5 and NSsiRNA then stimulated them with PDGF. As shown in Figure 7A, the transfection of HASMCs with siNox5, but not NSsiRNA, was able to significantly decrease Jak2 phosphorylation. Figure 7B demonstrates that the phosphorylation of STAT3 is also prevented by the knockdown of Nox5. These data demonstrate the importance of Nox5 for PDGF-induced JAK/STAT activation.

Discussion

The NADPH oxidases have emerged as important elements of signaling pathways in VSMCs. There is ample evidence that more than one Nox exists in this cell type and that the different homologues are located in different compartments [34] and serve different functions. In rat VSMCs, Nox1 appears to be required for angiotensin II- and PDGF-induced ROS production and growth [22], and Nox4 has been shown to be important for the maintenance of a contractile, non-proliferative phenotype [23]. However, human VSMCs appear to possess a different population of Noxes than rodent cells, with extremely low expression of Nox1 and much higher expression of Nox4 and Nox5. In this study, we show that Nox5 is a major modulator of PDGF-directed ROS production and HASMC growth. We also provide the first characterization of Nox5 in HASMCs, and identify one of its downstream targets.

Our data clearly demonstrate that Nox5 participates in PDGF-induced ROS production in HASMCs. Both the DCF-DA staining and the hydroxyethidium assay reveal that cells transfected with siNox5 produce significantly less ROS when treated with PDGF. Previous studies had shown that VSMCs produce ROS in response to PDGF stimulation. While in rat VSMC the source is likely Nox1 [22], we show that Nox5 plays an important role in ROS production in PDGF-treated HASMCs.

We found that HASMCs express more than one isoform of Nox5. Multiple isoforms of Nox5 have been found in other cell types such as Caco2 cells, endothelial cells, and SMCs isolated from pulmonary arteries [28]. We have also found Nox5 in human umbilical vein endothelial cells and human aortic endothelial cells (unpublished data). The cDNA sequences of Nox5 α and γ consist of exons 3–19, while those of Nox5 β and δ consist of exons 1, 2, and 4–19. The majority of the differences in the isoforms are in the 5' UTR. An alternative splice site at the end of exon 3 gives Nox5 γ , a slightly longer transcript than Nox5 α . The same is true for Nox5 δ , which contains a longer exon 2 sequence than Nox5 β . Our siNox5 targets all isoforms of Nox5, and thus we are not able to determine which isoform or isoforms are responsible for the response to PDGF. However, given the tight regulation of ROS needed in signaling pathways, it is plausible that the different isoforms in HASMC may regulate specific pathways and thus different biological functions. For example, PDGF has not only been shown to mediate VSMC proliferation, but also migration and apoptosis. It is also possible that one or more isoforms are important for the PDGF-mediated response at different steps along a single pathway, depending on their subcellular distribution. We show in this study that at least one isoform of Nox5 is responsible for PDGF-induced HASMC proliferation. This finding is in concert with studies of Nox5 in several cancer cell lines [25–27] and in human microvascular endothelial cells [28]. Further work will be necessary to investigate the participation of Nox5 in other cellular functions and to characterize the exact role of each of its isoforms.

One novel aspect of our findings is the identification of a signaling pathway that is dependent on Nox5 for its activation. A number of growth-related pathways have been shown to be regulated by ROS, including extracellular signal regulated kinases (Erk1/2), p38 mitogen-activated protein kinase and Akt [20]. However, none of these pathways is ROS-sensitive in PDGF-stimulated HASMCs (unpublished observations). The JAK/STAT pathway has also

been shown to be activated by ROS [18,19], and is interesting because it regulates PDGF-regulated cell growth in various cell types such as pancreatic stellate cells [35] and in human pulmonary SMCs [19]. As seen in Figures 6A and 6B, ROS are also important for PDGF-induced JAK/STAT activation in HASMCs. Furthermore, the source for these ROS appears to be Nox5, as cells transfected with siNox5 demonstrated reduced phosphorylation of both JAK2 and STAT3 (Figures 7A and 7B).

The studies published on Nox5 have thus far concentrated on its role in cell growth. However, its unique property of direct calcium activation that is not a feature of Nox1, 2, 3, or 4 provides it with potential to be involved in a number of different and important cellular processes. Moreover, the fact that it exists in different isoforms that may differ in location and mode of modulation (by phosphorylation [36] and/or calmodulin binding [37]) endows Nox5 with the complexity needed to participate in a variety of signaling pathways. If these postulations are true, Nox5 could prove to be a key regulator of calcium and ROS-dependent biological functions. The characterization of Nox5 activation has not yet been performed in HASMC. To date, the studies that have investigated the mechanism of Nox5 activation have been performed in cells transfected with Nox5 expression plasmids. The mRNA transcripts of the isoforms of Nox5 present in HASMC all contain calcium-binding EF hands, however, further studies would be needed to show that the native Nox5 isoforms in HASMC indeed bind calcium and produce superoxide as they do when heterologously expressed in other cells.

In summary, we have shown that HASMCs express several isoforms of Nox5, and that PDGF exerts its mitogenic properties on these cells by acting via Nox5 to stimulate the JAK/STAT pathway. This is the first evidence linking a specific Nox to JAK/STAT activation in this cell type. Furthermore, these findings provide a basis for understanding inter-species differences in Nox signaling and insight into a process important for several vascular diseases. Additional studies will be needed to determine which isoform(s) are responsible for mediating PDGF-induced HASMC proliferation and to discover if Nox5 plays a role in other PDGF-directed cellular processes.

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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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Fig 1. PDGF-induced HASMC growth is ROS-sensitive

HASMCs were treated with 10 mmol/L NAC, 25 ng/mL PDGF-BB or both. Cells were counted at specified time points. A control group was maintained in SmBM + 0.1% FBS. Data are the mean $\pm$ SEM of 3 experiments. * $P < 0.001$ compared to PDGF alone. Some error bars are smaller than the symbols.

Figure 2. PDGF-induced ROS formation is calcium-sensitive

HASMCs were untreated or treated with 10 μmol/L BAPTA-AM for 30 min prior to addition of 20 ng/mL PDGF for 5 hours. Superoxide was measured using DH, followed by high performance liquid chromatography. Data are the mean ± SEM of 4 experiments. * $p < 0.02$ compared to PDGF alone, † $p < 0.05$ PDGF vs. PDGF + BAPTA.

Figure 3. siNox5 downregulates Nox5 and inhibits PDGF-induced ROS production

A. HASMCs were transfected with 20 nmol/L siNox5 and Nox4 and Nox5 mRNA were measured by quantitative real-time PCR. Data are expressed as copies per 10^6 copies of 18S cDNA and are the mean $\pm$ SEM of 3 independent experiments. * $p=0.008$. **B.** HASMCs were not transfected, or transfected with non-silencing siRNA (NSsiRNA) or siNox5 (both at 20 nmol/L), and then treated with 25 ng/mL PDGF for 4 hours. DCF-DA staining for ROS was visualized with confocal microscopy. The response to H_2O_2 (100 μ mol/L) is shown for comparison. Results are representative of 3 independent experiments. **C.** Superoxide measurement using DH, followed by high performance liquid chromatography was performed on HASMCs transfected with NSsiRNA or siNox5 (20 nmol/L) and stimulated with 25 ng/mL PDGF for 4 hours. Data are representative of 6 independent experiments and are expressed as mean $\pm$ SEM. * indicates $P=0.007$. **D.** HASMCs were treated with NSsiRNA or siNox4 (25 nmol/L) and stimulated with 20 ng/mL PDGF for 5 hours prior to measurement of superoxide. Data are the mean of 6 independent experiments and are expressed as mean $\pm$ SEM. * $p=0.02$ of PDGF vs. appropriate control.

Fig 4. Nox5 regulates PDGF-induced HASM proliferation

Cells were plated at 30% confluence, transfected with 20 nmol/L NSsiRNA or siNox5, and treated with or without 25 ng/mL PDGF-BB. At the indicated times, cells were trypsinized and counted with a Coulter counter. Data are mean $\pm$ SEM of 3 separate experiments performed in triplicate. * P <0.001 compared to NSsiRNA. Some error bars are smaller than the symbols. Oligof= oligofectamine.

Fig 5. PDGF-induced HASMC proliferation is inhibited by the JAK/STAT inhibitor AG490
 HASMCs were made quiescent, preincubated with AG490 (10 μ mol/L) overnight and then stimulated with PDGF (25ng/ml). The cells were counted at specific time points. Values are mean $\pm$ SEM of 3 independent experiments performed in triplicate. Some error bars are smaller than the symbols. * $P=0.002$, † $P<0.001$ compared to cells treated with PDGF.

Fig 6. PDGF-induced JAK2 and STAT3 phosphorylation is modulated by ROS and flavin-containing enzymes

HASMCs were pretreated with the JAK inhibitor AG490 (100 μ mol/L, 1h) or with the flavin-containing enzyme inhibitor DPI (10 μ mol/L, 30min) before stimulation with PDGF (25 ng/

mL) for 5 min. Phosphorylated JAK2 (**A**) or STAT3 (**B**) was analyzed by Western analysis. Total JAK2 or STAT3 was used as a loading control. Values are mean±SEM of 5 to 8 independent experiments. (A) * $P<0.05$ vs. unstimulated cells, † $P<0.05$ vs. cells treated with PDGF. (B) * $P<0.001$ vs. non-stimulated cells, † $P<0.001$ vs. cells stimulated with PDGF.

Fig 7. PDGF-induced JAK/STAT activation is mediated by Nox5

HASMCs were transfected with siNox5 (20 nmol/L) or NSsiRNA and 72h later stimulated with PDGF (25ng/mL) for 5min. Western blotting was performed using primary antibody to phosphoJAK2 (**A**) or phosphoSTAT3 (**B**). Total JAK or STAT3 was used as loading control. Values are mean $\pm$ SEM of 5 independent experiments performed in duplicate. (A) * $P < 0.001$ vs. unstimulated cells with the indicated intervention, † $P < 0.001$ vs. cells transfected with NSsiRNA and stimulated with PDGF. (B) * $P < 0.001$ vs. unstimulated cells with the indicated intervention, † $P < 0.001$ vs. cells transfected with NSsiRNA and stimulated with PDGF.