Activation of NADPH Oxidase 1 Increases Intracellular Calcium and Migration of Smooth Muscle Cells

Matthew C. Zimmerman, Maysam Takapoo, Dammanahalli K. Jagadeesha, Bojana Stanic, Botond Banfi, Ramesh C. Bhalla, Francis J. Miller, Jr

Abstract—Redox-dependent migration and proliferation of vascular smooth muscle cells (SMCs) are central events in the development of vascular proliferative diseases; however, the underlying intracellular signaling mechanisms are not fully understood. We tested the hypothesis that activation of Nox1 NADPH oxidase modulates intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) levels. Using cultured SMCs from wild-type and Nox1 null mice, we confirmed that thrombin-dependent generation of reactive oxygen species requires Nox1. Thrombin rapidly increased [Ca\textsuperscript{2+}]\textsubscript{i}, as measured by fura-2 fluorescence ratio imaging, in wild-type but not Nox1 null SMCs. The increase in [Ca\textsuperscript{2+}]\textsubscript{i} in wild-type SMCs was inhibited by antisense to Nox1 and restored by expression of Nox1 in Nox1 null SMCs. Investigation into potential mechanisms by which Nox1 modulates [Ca\textsuperscript{2+}]\textsubscript{i} showed that thrombin-induced inositol triphosphate generation and thapsigargin-induced intracellular calcium mobilization were similar in wild-type and Nox1 null SMCs. To examine the effects of Nox1 on Ca\textsuperscript{2+} entry, cells were either bathed in Ca\textsuperscript{2+}-free medium or exposed to dihydropyridines to block L-type Ca\textsuperscript{2+} channel activity. Treatment with nifedipine or removal of extracellular Ca\textsuperscript{2+} reduced the thrombin-mediated increase of [Ca\textsuperscript{2+}]\textsubscript{i} in wild-type SMCs, whereas the response in Nox1 null SMCs was unchanged. Sodium vanadate, an inhibitor of protein tyrosine phosphatases, restored the thrombin-induced increase of [Ca\textsuperscript{2+}]\textsubscript{i} in Nox1 null SMCs. Migration of SMCs was impaired with deficiency of Nox1 and restored with expression of Nox1 or the addition of sodium vanadate. In summary, we conclude that Nox1 NADPH oxidase modulates Ca\textsuperscript{2+} mobilization in SMCs, in part through regulation of Ca\textsuperscript{2+} influx, to thereby promote cell migration. (Hypertension. 2011;58:446-453.) ● Online Data Supplement

Key Words: calcium influx • NADPH oxidase • migration • vascular disease

NADPH oxidases contribute to vascular proliferative diseases.\textsuperscript{1,2} Nox1 is the inducible catalytic subunit of NADPH oxidase in smooth muscle cells (SMCs) and is responsible for cell activation. Nox1 mediates redox-dependent signaling via regulation of gene transcription, resulting in SMC migration and proliferation.\textsuperscript{3} The mechanisms by which Nox1-derived reactive oxygen species (ROS) activate cellular signaling are complex and not completely defined. Recent studies have provided evidence linking ROS and Ca\textsuperscript{2+} signaling in the vasculature.\textsuperscript{5-9} For example, in isolated arterioles, either global inhibition of NADPH oxidase or treatment with ROS scavengers prevents angiotensin II (Ang II)–dependent increases in intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}).\textsuperscript{10} ROS control of Ca\textsuperscript{2+} signaling can occur through multiple mechanisms, including activation of Ca\textsuperscript{2+} release from intracellular stores, extracellular Ca\textsuperscript{2+} entry, or inhibition of Ca\textsuperscript{2+} reuptake.\textsuperscript{6,8} In vascular cells, ROS increases sensitivity of inositol triphosphate (IP\textsubscript{3}) receptor to IP\textsubscript{3} and promotes Ca\textsuperscript{2+} entry via activation of voltage-gated Ca\textsuperscript{2+} channels.\textsuperscript{11,12}

In this study, we used a combination of pharmacological inhibitors and genetic manipulation of Nox1 expression to examine the role of Nox1 activation in modulating [Ca\textsuperscript{2+}]\textsubscript{i}. Our data demonstrate that activation of Nox1 increases [Ca\textsuperscript{2+}]\textsubscript{i}, in part via influx of extracellular Ca\textsuperscript{2+} involving activation of L-type Ca\textsuperscript{2+} channel. In addition, the effect of Nox1 on Ca\textsuperscript{2+} mobilization is required for SMC migration. Redox control of Ca\textsuperscript{2+} handling is a novel mechanism by which Nox1 can modulate SMC signaling and function.

Materials and Methods

Vascular Smooth Muscle Cell Culture

Thoracic aortas from male Nox1 null (Nox1\textsuperscript{-/-}) mice were obtained, and SMCs were isolated and cultured as described previously.\textsuperscript{14} The cells were maintained in DMEM supplemented with 10% FBS, 2 mmol/L l-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C in a 5% CO\textsubscript{2} humidified incubator. Experiments were performed using cells between passages 4 and 10, and serum-deprived conditions were obtained by incubating 24 hours in DMEM containing 0.1% FBS. Studies conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

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Adenovirus-Mediated Gene Transfer

Experiments used the E1-deleted replication-deficient recombinant adenoviral vectors encoding Nox1 (AdNox1), 1,5 antisense Nox1 (AdNox1-AS), 1,6 green fluorescent protein (AdGFP), or empty vector (AdEmpty). Adenovirus was mixed with the cationic polymer poly-L-lysine (250 molecules per virus particle) 1,7 and added to SMCs in serum-free DMEM. 1,8 After 4 hours, medium was replaced with DMEM containing 10% FBS for 48 hours.

Detection of ROS

Thrombin-induced changes in ROS levels in Nox1 null and WT SMCs were detected by Amplex Red. SMCs were incubated with Amplex Red (20 μmol/L) and horseradish peroxidase (0.2 μU/mL) for 30 minutes, after which the fluorescence intensity of the medium was determined (excitation and emission wavelengths of 545 and 590 nm, respectively) and normalized to cell number.

Intracellular Calcium Measurement

Thrombin-stimulated changes in [Ca^{2+}], were assessed by Fura-2 fluorescence ratio imaging using a microscopic digital imaging system (Photon Technology International), as described previously,1,19,20 Briefly, WT or Nox1 null SMCs grown on 25-mm coverslips were loaded with the Ca^{2+}-specific dye Fura-2-acetoxyethyl ester (1 μmol/L, Molecular Probes/Invitrogen) for 30 minutes at 37°C. After washing with Hank’s balanced salt solution, cells were incubated for 20 minutes at 37°C in Hank’s balanced salt solution to allow complete hydrolysis of Fura-2-acetoxyethyl ester to Fura-2. Real-time shifts in Fura-2 ratio fluorescence, indicating changes in [Ca^{2+}], were recorded before, during, and after stimulating SMCs with thrombin (1 μU/mL) or H2O2 (100 μmol/L). To examine the role of NADPH oxidase, WT SMCs were pretreated with the NADPH oxidase inhibitor diphenylene iodonium (10 μmol/L, Sigma-Aldrich) for 1 hour before thrombin stimulation. In other studies, [Ca^{2+}], was examined in WT SMCs expressing antisense against Nox1 (AdNox1-AS) or Nox1 null SMCs expressing Nox1 (AdNox1). The potency of [Ca^{2+}], stores in SMCs was determined by treating cells with thapsigargin (5 μmol/L, Sigma-Aldrich). The contribution of extracellular Ca^{2+} influx on thrombin-mediated increases in [Ca^{2+}], was examined by bathing SMCs in Ca^{2+}-free Hank’s balanced salt solution or treating with nifedipine (1 μmol/L, Sigma-Aldrich) or sodium vanadate (200 μmol/L, Sigma-Aldrich) to the lower compartment. After 6 hours, nonmigrated cells were removed from the upper chamber. SMCs migrating to the lower surface of the membrane were fluorescently stained with 4',6-diamidino-2-phenylindole and quantitated microscopically.

Cell Migration

Cell proliferation was determined by measuring [3H]thymidine incorporation into SMCs infected with either AdNox1-AS or AdGFP, as described previously.7

Statistics

Data are expressed as mean ± SEM and analyzed by the Student t test when comparing only 2 groups and by ANOVA followed by Newman-Keuls correction for multiple comparisons when comparing > 2 groups. A value of P < 0.05 was defined statistical significance.

Results

Thrombin-Stimulated Increase in [Ca^{2+}] Is Dependent on Nox1

Although NADPH oxidase–derived ROS have been implicated in thrombin-mediated SMC proliferation and migration,22–24 little is known regarding the second messengers involved in ROS signaling. Therefore, we examined the role of NADPH oxidases in modulating [Ca^{2+}], in response to thrombin. In WT SMCs, thrombin caused a rapid increase in [Ca^{2+}], that was markedly attenuated when cells were pretreated with diphenylene ionium (Figure 1A and 1B), an inhibitor of thrombin-induced ROS (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Nox1 is the inducible isoform of NADPH oxidase in SMCs,25 and we have shown previously that thrombin activates Nox1 (Figure S1).15 Therefore, we next examined whether Nox1 is responsible for the thrombin-induced increases in [Ca^{2+}]. Thrombin caused a rapid and transient increase in [Ca^{2+}], peaking 25 to 30 seconds after stimulation (Figure 2B). Expression of an antisense targeting Nox1 inhibited thrombin-mediated increase in both ROS levels and in...
endoplasmic reticulum Ca\(^{2+}\)-ATPases,\(^{27}\) [Ca\(^{2+}\)] increased to similar levels in WT and Nox1 null cells (Figure 3B), suggesting no difference in IP\(_3\)-sensitive Ca\(^{2+}\) pools.

Based on these observations, we next tested whether Nox1 mediates the influx of extracellular Ca\(^{2+}\) in response to thrombin. When WT cells were bathed in Ca\(^{2+}\)-free medium, the magnitude of increase in [Ca\(^{2+}\)] after thrombin was significantly inhibited (Figure 4A), confirming influx of extracellular Ca\(^{2+}\) after stimulation with thrombin. In contrast to WT cells, the absence of extracellular Ca\(^{2+}\) had no effect on the thrombin-mediated increase in [Ca\(^{2+}\)] in Nox1 null cells (Figure 4B). These data suggest that Nox1-derived ROS modulate the influx of extracellular Ca\(^{2+}\) in response to thrombin. To extend these findings, we examined the contribution of L-type Ca\(^{2+}\) channels, because activity of voltage-gated Ca\(^{2+}\) channels is known to be regulated by redox modification of cysteine and methionine residues.\(^{27}\) Treatment with the dihydroropyridine nifedipine prevented the thrombin-stimulated increase in [Ca\(^{2+}\)] (Figure 5). Similar results were obtained with nitrendipine (Figure S2). These findings implicated L-type Ca\(^{2+}\) channel activity as the source of extracellular Ca\(^{2+}\) influx in response to thrombin and a potential target of Nox1-derived ROS.

Inhibition of protein tyrosine phosphatase (PTP) 1B by ROS has been implicated in modulating activity of Ca\(^{2+}\) entry channels,\(^{28}\) and cellular ROS are known to inactivate PTPs by the reversible oxidation of cysteine residues in the active site,\(^{29}\) thereby modifying the magnitude and duration of signaling events. Furthermore, it has been shown that PTP inactivation in response to Ang II requires Nox1-derived ROS.\(^{30}\) Treatment of Nox1 null SMCs with sodium vanadate, an inhibitor of PTPs, partially restored the thrombin-mediated increase in [Ca\(^{2+}\)] (vehicle: 0.51 ± 0.02 μmol/L; thrombin: 0.77 ± 0.03 μmol/L; sodium vanadate: 0.58 ± 0.04 μmol/L; sodium vanadate + thrombin: 1.02 ± 0.07 μmol/L; n = 26 cells; P < 0.05), identifying inactivation of PTPs as an additional potential mechanism by which Nox1 can influence Ca\(^{2+}\) signaling.

Thrombin-Induced SMC Migration and Proliferation Are Mediated by Nox1

Previous investigators have shown that thrombin mediates proliferation and migration of SMCs.\(^{31–33}\) It has also been shown that NADPH oxidase–derived ROS control thrombin-induced SMC migration.\(^{22,23}\) We examined how the Nox1 regulation of Ca\(^{2+}\) influx affects thrombin-induced migration of SMCs. As compared with control, thrombin increased the distance that WT SMCs migrated; however, thrombin-induced migration was abolished in Nox1 null SMCs and reduced in WT SMCs treated with antisense to Nox1 (Figure 6A). This approach to measure migration cannot differentiate between the changes in cell growth versus migration. Therefore, we next used the modified Boyden chamber method to further assess the role of Nox1 in migration (Figure 6B). Thrombin increased the transmigration of WT SMCs. In contrast, Nox1 null SMCs did not display a similar increase in migration to thrombin. The importance of Nox1 in this response was further verified by the loss of transmigration in WT cells pretreated with Nox1 antisense and the rescue of
migration in Nox1 null cells after exogenous expression of Nox1. With regard to proliferation, thrombin-induced DNA synthesis was increased 3-fold in WT SMCs as compared with untreated WT cells but was reduced in SMCs deficient in Nox1 and in WT SMCs expressing Nox1 antisense (Figure 6C). These findings are similar to recent reports identifying a role for Nox1 in activation of SMCs by platelet-derived growth factor3 and basic fibroblast growth factor.4 We next investigated the role of Ca\(^{2+}\)/H\(_{11001}\) in Nox1-dependent SMC migration. Consistent with the effects on Ca\(^{2+}\)/H\(_{11001}\) influx, treatment of WT SMCs with nifedipine prevented transmigration to thrombin (Figure 6D). Because sodium vanadate restored

Figure 2. Nox1 is required for the thrombin-induced increase of intracellular calcium ([Ca\(^{2+}\)]\(_{i}\)) in smooth muscle cells (SMCs). A, Wild-type (WT) and Nox1 null SMCs were infected with adenoviruses expressing antisense Nox1 (AdNox1-AS) or Nox1, respectively, and thrombin-induced changes in ROS levels were detected by Amplex Red and normalized to cell number (n=8). B, Representative Fura-2 ratiometric Ca\(^{2+}\) tracings from WT or Nox1 null SMCs infected with adenoviral vectors encoding (Ad)Empty, green fluorescent protein (GFP); AdGFP), AdNox1-AS, or AdNox1. Arrows indicate time of thrombin stimulation. C, Summary data are shown from WT cells infected with AdEmpty (n=141 cells) or AdNox1-AS (n=60 cells) or Nox1 null cells infected with AdGFP (n=57 cells) or AdNox1 (n=62 cells). Unless otherwise indicated, AdGFP was used to control for the bicistronic GFP expressed in the AdNox1 and AdNox1-AS vectors. *P<0.05 vs WT control; ‡P<0.05 vs WT + thrombin; #P<0.05 vs Nox1 null + thrombin.

Figure 3. Inositol triphosphate (IP\(_3\)) levels and intracellular calcium ([Ca\(^{2+}\)]\(_{i}\)) release are normal in Nox1 null smooth muscle cells (SMCs) after thrombin stimulation. A, Summary data of IP\(_3\) levels in wild-type (WT) and Nox1 null SMCs at 5 and 60 minutes after thrombin stimulation (n=3). *P<0.05 vs WT control. B, Summary data showing the effect of thapsigargin on [Ca\(^{2+}\)]\(_{i}\) in WT (n=103 cells) and Nox1 null SMCs (n=63 cells). *P<0.05 vs WT vehicle.
the thrombin-mediated increase in \([Ca^{2+}]_i\) in Nox1 null SMCs, we tested the hypothesis that inhibition of PTPs would restore migration of Nox1 null SMCs. There was a trend for sodium vanadate to increase migration of Nox1 null SMCs pretreated with sodium vanadate. These data suggest that PTPs may be downstream effectors of Nox1-mediated redox signaling.

**Discussion**

Previous studies have identified NADPH oxidase–derived ROS as critical signaling intermediates in SMC migration and proliferation.\(^3,4,22\) Herein we show that activation of the Nox1-based NAPDH oxidase by thrombin modulates \([Ca^{2+}]_i\) levels in SMCs in part via influx of extracellular \(Ca^{2+}\). In addition, we show that SMC migration involves Nox1-dependent increases in \([Ca^{2+}]_i\). These effects involve activation of \(l\)-type \(Ca^{2+}\) channels and inactivation of PTPs. These are the first data to identify redox control of \(Ca^{2+}\) influx as a mechanism by which Nox1 alters SMC function.

Several lines of evidence suggest that Nox1-derived ROS are critical to the development of vascular disease. We have shown recently that, in a model of hypercholesterolemia, deficiency of Nox1 reduces atherosclerotic lesion area in the aorta.\(^34\) Within days of arterial balloon injury, Nox1 expression and ROS levels are increased in neointimal SMCs and, after vascular injury, neointimal formation and proliferation are reduced in Nox1 null mice. Genetic manipulation of Nox1 confirms the role of Nox1-derived ROS in cell proliferation and SMC migration.\(^35\) Although these studies provide strong evidence for Nox1 in the pathogenesis of vascular disease, the mechanisms by which Nox1-derived ROS activate SMC are not completely understood. Our data contribute to the field by defining a mechanism by which Nox1 modulates \([Ca^{2+}]_i\) levels to induce cellular processes.

The activation of SMCs by thrombin involves activation of Nox1\(^15\) and \(Ca^{2+}\)-sensitive signaling pathways\(^26,38,39\); however, the relationship between ROS and \(Ca^{2+}\) signaling in this...
The phase was virtually absent, suggesting that thrombin induces an acute transient increase in \([\text{Ca}^{2+}]_i\). Our data demonstrate that thrombin elicited an acute transient increase in \([\text{Ca}^{2+}]_i\) in Nox1 null SMCs in the presence of Ca\textsuperscript{2+}-free medium or nifedipine, which mimicked that of WT SMCs in either Ca\textsuperscript{2+}-free medium or after treatment with nifedipine. These data suggest that Nox1-derived ROS contribute to the thrombin-mediated increase in \([\text{Ca}^{2+}]_i\) through influx of extracellular \(\text{Ca}^{2+}\). ROS have been shown to increase the sensitivity of the IP\textsubscript{3} receptor to promote IP\textsubscript{3}-mediated \(\text{Ca}^{2+}\) release.\textsuperscript{11} Our observation of no difference in total IP\textsubscript{3} levels in WT versus Nox1 null SMCs does not preclude the possibility that Nox1 activation alters the sensitivity of IP\textsubscript{3} receptor to IP\textsubscript{3}. It has also been demonstrated that ROS sustains \(\text{Ca}^{2+}\) influx via inhibition of PTPs, presumably by maintaining phosphorylation of \(\text{Ca}^{2+}\) entry channels.\textsuperscript{24} In our study, pharmacological inhibition of PTPs in Nox1 null SMCs partially restored thrombin-induced increases in \([\text{Ca}^{2+}]_i\). These findings are consistent with another study using a rat model of hypertension that found that Nox1-derived ROS is required for Ang II–dependent inactivation of Src homology region 2-containing protein tyrosine phosphatase (SHP)-2.\textsuperscript{30}

Similar to our results with thrombin, a recent study has shown that deficiency of Nox1 in SMCs reduces \([\text{Ca}^{2+}]_i\) in response to Ang II.\textsuperscript{31} These findings were attributed to abnormalities in trafficking of the Ang II type 1 receptor to the plasma membrane. In our study, activation of IP\textsubscript{3} was similar in WT and Nox1 null cells, indicating that, in contrast to the response to Ang II, the blunted increase in \([\text{Ca}^{2+}]_i\) in Nox1 null cells to thrombin is not attributed to dysregulation of thrombin receptor signaling or trafficking.

Nox1 has been shown to be integral for migration of SMCs. SMC migration involves a complicated and coordinated series of steps and is an important component of vascular remodeling. Transient changes in \([\text{Ca}^{2+}]_i\) are likely to be a key regulating signal for migration of SMCs.\textsuperscript{32} Our data demonstrate that Nox1 is required for migration of SMCs. We extend these findings to implicate a role for \(\text{Ca}^{2+}\) influx in Nox1-mediated migration. Similarly, the inhibition
of PTPs increased SMC migration in Nox1-deficient cells. This observation of increased migration in the absence of an agonist is consistent with thrombin causing Nox1-mediated inactivation of PTPs.

**Perspectives**

Increasing evidence defines a role for NADPH oxidases in the pathogenesis of vascular disease. In this study, we demonstrate that Nox1-derived ROS in SMCs are critical for changes in [Ca$^{2+}$]$_i$, via a mechanism that involves influx of extracellular Ca$^{2+}$. These data provide direct evidence that Nox1 contributes to Ca$^{2+}$ homeostasis in SMCs and identifies potential redox-sensitive mechanisms of SMC activation, which is important in regulation of vascular tone. Although treatment with antioxidants showed promise in experimental models of hypertension and restenosis, results from large clinical trials have been disappointing. Future strategies that focus on Nox1 as a potential target have the potential to reduce the morbidity and mortality associated with cardiovascular disease.

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**Disclosures**

None.

**References**


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References for Supplemental Material


Supplemental Figures

Supplemental Figure S1. Thrombin-induced ROS production in cultured SMCs is Nox1-dependent. Thrombin-induced changes in ROS levels in Nox1 null and WT SMCs were detected by the method previously described. Serum-deprived SMCs cultured on 60 mm glass-bottom dishes were treated with thrombin (1 U/ml, Sigma Aldrich) in the presence of dihydroethidium (DHE, 2 μM, Invitrogen) for 30 min at 37°C and fluorescence detected with a Bio-Rad laser scanning confocal microscope (excitation at 488 nm and detection using a 585 nm long-pass filter). (A) Representative confocal images of WT and Nox1 null SMCs incubated with DHE (2 μM) and stimulated with thrombin (1 U/ml) in the absence or presence of DPI (10 μM). Data are representative of three independent experiments. (B) Thrombin-induced changes in ROS levels in the absence or presence of DPI (10 μM) were detected DHE fluorescence as measured by flow cytometry as previously described and normalized to cell number (n=5). * P<0.05 vs. WT control; ‡ P< 0.05 vs. WT + thrombin.
Supplemental Figure S2. Thrombin-stimulated extracellular calcium influx is mediated by L-type Ca\(^{2+}\) channel activity. (A) Representative Fura-2 radiometric tracings of [Ca\(^{2+}\)]\(_i\) in WT SMCs after treatment with thrombin (1 U/ml) in the presence or absence of nitrendipine (1 µM). Arrow indicates time of thrombin addition. (B) Summary data showing the effect of nitrendipine on thrombin-stimulated [Ca\(^{2+}\)]\(_i\), (thrombin, n=30; thrombin + nitrendipine, n=25). *P<0.05 vs. thrombin.