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 ([Ca2+]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 [Ca2+]i, as measured by fura-2 fluorescence ratio imaging, in wild-type but not Nox1 null SMCs. The increase in [Ca2+]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 [Ca2+]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 Ca2+ entry, cells were either bathed in Ca2+-free medium or exposed to dihydropyridines to block L-type Ca2+ channel activity. Treatment with nifedipine or removal of extracellular Ca2+ reduced the thrombin-mediated increase of [Ca2+]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 [Ca2+]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 [Ca2+]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 Ca2+ mobilization in SMCs, in part through regulation of Ca2+ 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.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.3,4 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 Ca2+ signaling in the vasculature.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 ([Ca2+]i).10 ROS control of Ca2+ signaling can occur through multiple mechanisms, including activation of Ca2+ release from intracellular stores, extracellular Ca2+ entry, or inhibition of Ca2+ reuptake.6,8 In vascular cells, ROS increases sensitivity of inositol triphosphate (IP3) receptor to IP3 and promotes Ca2+ entry via activation of voltage-gated Ca2+ channels.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 [Ca2+]i. Our data demonstrate that activation of Nox1 increases [Ca2+]i, in part via influx of extracellular Ca2+ involving activation of L-type Ca2+ channel. In addition, the effect of Nox1 on Ca2+ mobilization is required for SMC migration. Redox control of Ca2+ 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−/−) and control littermate wild-type (WT; Nox1+/+) mice were obtained, and SMCs were isolated and cultured as described previously.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% CO2 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 Medicine.

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Health and were approved by the University of Iowa Institutional Animal Care and Use Committee.

Adenovirus-Mediated Gene Transfer

Experiments used the EI-deleted replication-deficient recombinant adenoviral vectors encoding Nox1 (AdNox1),13 antisense Nox1 (AdNox1-AS),16 green fluorescent protein (AdGFP), or empty vector (AdEmpty). Adenovirus was mixed with the cationic polymer poly-L-lysine (250 molecules per virus particle)17 and added to SMCs in serum-free DMEM. 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\textsuperscript{2+}], were assessed by Fura-2 fluorescence ratio imaging using a microscopic digital imaging system (Photon Technology International), as described previously. Briefly, WT or Nox1 null SMCs grown on 25-mm coverslips were loaded with the Ca\textsuperscript{2+}-specific dye Fura-2-acetoxymethyl 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-acetoxymethyl ester to Fura-2. Real-time shifts in Fura-2 ratio fluorescence, indicating changes in [Ca\textsuperscript{2+}], were recorded before, during, and after stimulation with SMCs with thrombin (1 U/mL) or H\textsubscript{2}O\textsubscript{2} (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\textsuperscript{2+}], was examined in WT SMCs expressing antisense against Nox1 (AdNox1-AS) or Nox1 null SMCs expressing Nox1 (AdNox1). The potency of [Ca\textsuperscript{2+}], stores in SMCs was determined by treating cells with thapsigargin (5 μg/mL, Sigma-Aldrich). The contribution of extracellular Ca\textsuperscript{2+} influx on thrombin-mediated increases in [Ca\textsuperscript{2+}], was examined by bathing SMCs in Ca\textsuperscript{2+}-free Hank’s balanced salt solution or treating with nifedipine (1 μmol/L) during thrombin stimulation. Summary data represent the average difference in the basal and peak increase in [Ca\textsuperscript{2+}], except for the dihydropyridine experiments, in which the change in [Ca\textsuperscript{2+}], was determined at all of the time points, and the lowest value was subtracted from the highest value.

IP\textsubscript{3} Levels

Cells were grown in 6-well plates to 80% to 90% confluence, washed with the assay media (inositol-free DMEM containing 20 mmol/L of HEPES, 2 mmol/L of glucose, 10 μg/mL of streptomycin, 10 U/mL of penicillin, and 0.1% BSA) and then incubated in the assay medium containing 4 μCi/mL of [2-3H]myo-inositol (NEN Life Science Products) for 18 to 24 hours at 37°C. At the end of the labeling period, the cells were incubated with assay medium containing 20 mmol/L of LiCl for 15 minutes at 37°C, followed by the addition of thrombin (1 U/mL) for 5 minutes. Cells were placed on ice, and the medium was quickly aspirated and replaced with equal volumes of cold 1.5% perchoric acid and 0.5 mol/L of HClO\textsubscript{4}. After a 30-minute incubation on ice, the extracts were collected, centrifuged, and the supernatants were neutralized by the addition of 0.72 mol/L of KOH/0.6 mol/L of KHCO\textsubscript{3}. The precipitated KClO\textsubscript{4} salt was removed by centrifugation, and the supernatants were mixed with 100 mmol/L of inositol and water. These samples were then used for the assay of inositol phosphates. An ion-exchange resin AG-1-X8 (200 to 400 mesh, formate, Bio-Rad) was rehydrated with water, poured onto 0.5 x 3.0-cm chromatography columns, and washed once with water and twice with 10 mmol/L of myo-inositol. The cell extracts were then applied to the columns, followed by several washing steps, including 10 mmol/L of myo-inositol and 5 mmol/L of sodium borate/60 mmol/L of sodium formate solution. The last elution step was performed with 0.1 mol/L of formic acid/1.0 mol/L of ammonium formate solution. This fraction contains inositol phosphates. Samples were counted in a liquid scintillation counter.

Cell Migration

The migration of SMCs was determined by scratch wound assay and by modified Boyden chamber method. Where indicated, cells were infected with AdNox1-AS, AdNox1, or AdGFP for 24 hours, followed by serum starvation for an additional 24 hours. For scratch wound assays, the serum-starved SMC monolayer was disrupted with a sterile cell scraper to create a cell-free zone. Cells were then washed with medium and treated with or without thrombin (1 U/mL) in DMEM containing 0.1% FBS, and images were taken 24 hours after injury using a microscope equipped with a digital camera. For the modified Boyden chamber method, SMC migration was determined in Transwell cell-culture chambers with collagen polycarbonate membrane with 8-μm pores. SMCs were grown to ~80% confluence and then made quiescent in 0.1% serum for 48 hours before migration. SMCs (10\textsuperscript{5} cells per milliliter) were added to the upper chamber of the transwell and allowed to attach to the membrane for 30 minutes. Chambers contained medium with 0.1% serum. Migration of SMCs was induced by the addition of thrombin (1 U/mL) in the presence or absence of 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 Proliferation

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

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\textsuperscript{2+}], Is Dependent on Nox1

Although NADPH oxidase-derived ROS have been implicated in thrombin-mediated SMC proliferation and migration, little is known regarding the second messengers involved in ROS signaling. Therefore, we examined the role of NADPH oxidases in modulating [Ca\textsuperscript{2+}], in response to thrombin. In WT SMCs, thrombin caused a rapid increase in [Ca\textsuperscript{2+}], that was markedly attenuated when cells were pretreated with diphenylene iodonium (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, and we have shown previously that thrombin activates Nox1 (Figure S1). Therefore, we next examined whether Nox1 is responsible for the thrombin-induced increases in [Ca\textsuperscript{2+}],. Thrombin caused a rapid and transient increase in [Ca\textsuperscript{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,7 [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.7 Treatment with the dihydropyridine nifedipine prevented the thrombin-stimulated increase in [Ca^{2+}], (Figure 5). Similar results were obtained with nitrendipine (Figure S2). These findings implicate 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

[S Ca^{2+}], in WT SMCs (Figure 2). Similarly, the peak increase in [Ca^{2+}], in Nox1 null SMCs was markedly reduced as compared with WT cells. Heterologous expression of Nox1 in Nox1 null SMCs restored the thrombin-mediated increase in ROS and in [Ca^{2+}], (Figure 2). The effects that we observe are specific to the absence of Nox1-derived ROS, because the addition of H_{2}O_{2} to WT and Nox1 null SMCs resulted in a similar increase in [Ca^{2+}], (WT: 32±12%; Nox1 null: 40±13%; n=6). Taken together, these data indicate that Nox1 initiates [Ca^{2+}], mobilization in response to thrombin in SMCs.

**Nox1 Mediates Influx of Extracellular Ca^{2+} in Thrombin-Stimulated SMCs**
It has been shown previously that the thrombin-mediated increase in [Ca^{2+}], is secondary to the activation of phospholipase C, with a subsequent increase in IP_{3}.26 We assessed whether the observed differences in [Ca^{2+}], between WT and Nox1 null SMCs in response to thrombin resulted from differences in IP_{3} generation. WT and Nox1 null SMCs demonstrated a similar increase of IP_{3} levels at 5 and 60 minutes after thrombin (Figure 3A), indicating that IP_{3} levels are independent of Nox1-derived ROS. Furthermore, in response to thapsigargin, an inhibitor of the sarco-

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**Figure 1.** Thrombin-induced increase in intracellular calcium ([Ca^{2+}]) depends on NADPH oxidase. A, Representative Fura-2 ratiometric images of [Ca^{2+}], showing inhibitory effects of diphenylene iodonium (DPI) on thrombin-induced increase in [Ca^{2+}], in wild-type (WT) smooth muscle cells (SMCs). B, Summary data of thrombin-induced Ca^{2+} response in WT SMCs pretreated with vehicle (n=150 cells) or DPI (n=129 cells), followed by stimulation with thrombin. *P<0.05 vs control; †P<0.05 vs vehicle + thrombin.

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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 factor and basic fibroblast growth factor. We next investigated the role of Ca\(^{2+}\) in Nox1-dependent SMC migration. Consistent with the effects on Ca\(^{2+}\) 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 \([\text{Ca}^{2+}]_i\) 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 in the absence of thrombin (Figure 6E). The addition of thrombin did not further 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 \([\text{Ca}^{2+}]_i\) in SMCs in part via influx of extracellular \(\text{Ca}^{2+}\). In addition, we show that SMC migration involves Nox1-dependent increases in \([\text{Ca}^{2+}]_i\). These effects involve activation of \(\text{Ca}^{2+}\)-sensitive signaling pathways26,38,39; however, the relationship between ROS and \(\text{Ca}^{2+}\) signaling in this

![Figure 4. Nox1 activation mediates influx of extracellular \(\text{Ca}^{2+}\) in smooth muscle cells (SMCs).](image)

A. Representative Fura-2 ratiometric tracings of thrombin-induced increase in intracellular calcium ([Ca\(^{2+}\)]) in wild-type (WT; left) and Nox1 null (right) SMCs. Arrow indicates time of thrombin addition. B. Summary data of thrombin-mediated increase in [Ca\(^{2+}\)]\(_i\) in WT SMCs bathed in Ca\(^{2+}\)-containing (WT: n=89 cells) or Ca\(^{2+}\)-free medium (Nox1 null: n=179 cells) and Nox1 null SMCs bathed in Ca\(^{2+}\)-containing (n=88 cells) or Ca\(^{2+}\)-free medium (n=89 cells). *P<0.05 vs WT control; ‡P<0.05 vs WT + thrombin in Ca\(^{2+}\)-containing medium.

![Figure 5. Thrombin-stimulated extracellular calcium influx is mediated by \(\text{Ca}^{2+}\) channel activity.](image)

A. Representative Fura-2 radiometric tracings of intracellular calcium ([Ca\(^{2+}\)]) in wild-type (WT) smooth muscle cells (SMCs) after treatment with thrombin in the presence or absence of nifedipine. Arrow indicates time of thrombin addition. B. Summary data showing the effect of nifedipine on thrombin-stimulated [Ca\(^{2+}\)]. (thrombin: n=14 cells; nifedipine + thrombin: n=30 cells). *P<0.05 vs thrombin.

are the first data to identify redox control of \(\text{Ca}^{2+}\) 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,35 and, after vascular injury, neointimal formation and proliferation34 are reduced in Nox1 null mice. Genetic manipulation of Nox1 confirms the role of Nox1-derived ROS in cell proliferation36,37 and SMC migration.3,4 Although these studies provide strong evidence for Nox1 in the pathogenesis of vascular disease, the mechanisms by which Nox1-derived ROS activate SMC is not completely understood. Our data contribute to the field by defining a mechanism by which Nox1 modulates \([\text{Ca}^{2+}]_i\) levels to induce cellular processes.

The activation of SMCs by thrombin involves activation of Nox115 and \(\text{Ca}^{2+}\)-sensitive signaling pathways26,38,39; however, the relationship between ROS and \(\text{Ca}^{2+}\) signaling in this
context remains unclear. The multiple mechanisms by which ROS regulate intracellular calcium homeostasis, such as activation of Ca^{2+} release and entry channels and inhibition of Ca^{2+} reuptake, have been extensively reviewed. Voltage-gated Ca^{2+} channels contain many cysteine and methionine residues susceptible to redox modification that could influence channel function. In addition to direct effects of ROS on calcium channels, oxidation of regulatory proteins, such as calmodulin, may also affect channel function. Changes in the cellular redox state can increase Ca^{2+} by inducing Ca^{2+} influx through voltage-dependent Ca^{2+} channels, by stimulating IP3-mediated Ca^{2+} mobilization from intracellular stores, by stimulating ryanodine receptors, and by inhibiting activity of sarcoplasmic reticulum calcium ATPase or plasma membrane Ca^{2+} ATPase.

Multiple integrated mechanisms regulate [Ca^{2+}], levels. Our data demonstrate that thrombin elicited an acute transient increase in [Ca^{2+}], followed by sustained phase of elevated [Ca^{2+}]. However, in Ca^{2+}-free medium, the peak increase in [Ca^{2+}] was significantly inhibited, and the sustained plateau phase was virtually absent, suggesting that thrombin induces an influx of extracellular Ca^{2+}. The transient changes in [Ca^{2+}], in Nox1 null SMCs in the presence of Ca^{2+}-mimicked that of WT SMCs in either Ca^{2+}-free medium or after treatment with nifedipine. These data suggest that Nox1-derived ROS contribute to the thrombin-mediated increase in [Ca^{2+}], through influx of extracellular Ca^{2+}. ROS have been shown to increase the sensitivity of the IP3 receptor to promote IP3-mediated Ca^{2+} release. Our observation of no difference in total IP3 levels in WT versus Nox1 null SMCs does not preclude the possibility that Nox1 activation alters the sensitivity of IP3 receptor to IP3. It has also been demonstrated that ROS sustains Ca^{2+} influx via inhibition of PTPs, presumably by maintaining phosphorylation of Ca^{2+} entry channels. Similar to our results with thrombin, a recent study has shown that deficiency of Nox1 in SMCs reduces [Ca^{2+}], in response to Ang II. 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.

Similar to our results with thrombin, a recent study has shown that deficiency of Nox1 in SMCs reduces [Ca^{2+}], in response to Ang II. These findings were attributed to abnormalities in trafficking of the Ang II type 1 receptor to the plasma membrane. In our study, activation of IP3 was similar in WT and Nox1 null cells, indicating that, in contrast to the response to Ang II, the blunted increase in [Ca^{2+}] 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 [Ca^{2+}] are likely to be a key regulating signal for migration of SMCs. Our data demonstrate that Nox1 is required for migration of SMCs. We extend these findings to implicate a role for 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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Activation of NADPH Oxidase 1 Increases Intracellular Calcium and Migration of Smooth Muscle Cells

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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.