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NADPH Oxidase 4 is required for interleukin-1 β -mediated activation of protein kinase C δ and downstream activation of c-Jun N-terminal kinase signaling in smooth muscle

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Abstract

Reactive oxygen species (ROS) are generated in the vascular wall upon stimulation by pro-inflammatory cytokines and are important mediators of diverse cellular responses that occur as a result of vascular injury. Member of the NADPH oxidase (NOX) family of proteins have been identified in vascular smooth muscle cells (VSM) as important sources of ROS. In this study, we tested the hypothesis that NOX4 is a proximal mediator of IL-1 β -dependent activation of PKC δ and increases IL-1 β stimulated c-Jun kinase (JNK) signaling in primary rat aortic VSM cells. We found that stimulation of VSM cells with IL-1 β increased PKC δ activity and intracellular ROS generation. siRNA silencing of NOX4 but not NOX1 ablated the IL-1 β -dependent increase in ROS production. Pharmacological inhibition of PKC δ activity as well as siRNA depletion of PKC δ or NOX4 blocked the IL-1 β -dependent activation of JNK. Further studies showed that the IL-1 β -dependent upregulation of iNOS expression was inhibited through JNK inhibition and NOX4 silencing. Taken together, these results indicate that IL-1 β -dependent activation of PKC δ is modulated by NOX4-derived ROS. Our study positions PKC δ as an important redox sensitive mediator of IL-1 β -dependent signaling and downstream activation of inflammatory mediators in VSM cells.

Keywords

Smooth muscle; NADPH oxidase; Protein kinase C; MAP kinase; JNK; NOX4; NOX1; Interleukin-1 β ; Reactive oxygen species; iNOS

Introduction

Vascular diseases such as atherosclerosis, post-angioplasty restenosis, post-transplant coronary arteriopathy and vascular hyporesponsiveness after septic shock all have an inflammatory component associated with their etiologies [1]. In the vessel wall, the inflammatory response leads to the production of cytokines and growth factors which induce the proliferation and migration of the underlying smooth muscle resulting in thickening of the medial layer, matrix deposition, and formation of a neointima [2]. Mice deficient in the

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IL-1 receptor IL-1R1 present reduced neointima/media formation after injury [3] while animals deficient in the IL1 receptor antagonist display increased neointimal formation upon injury [4]. IL-1 β is expressed by vascular smooth muscle (VSM) ¹ cells and other cell types in advanced stages of atherosclerotic plaques as well as non-atherosclerotic vascular injury models where it regulates VSM cell gene expression and proliferation [5-8].

The prototypical signaling pathway associated with IL-1 β requires the adaptor protein MyD88 and the recruitment of the IL-1 receptor associated kinase (IRAK) and TNF α receptor associated factor 6 (TRAF6) [9, 10]. These in turn activate downstream kinases and regulate the transcriptional activation of inflammatory genes. We also identified PLC- γ and PKC δ as important components of IL-1 β signaling in cultured rat aortic VSM cells [11]. Most if not all of these signaling elements are sensitive to changes in intracellular redox status, although the significance of these effects have not always been demonstrated in smooth muscle.

A common feature of vascular diseases is the dysregulation of reactive oxygen species (ROS) production in the vascular wall. In this context, some of the most significant sources of ROS are NADPH oxidases (NOXs) because their activation allow for temporal and spatial regulation of intracellular signaling pathways [12]. NOXs are composed of one or two transmembrane proteins (such as NOX, DUOX, and p22phox) and in some cases include regulatory subunits such as p47phox, NOXO1, p40phox, p67phox, or NOXO1. In MCF-7 cells stimulated with IL-1 β , NOX2 is required for the recruitment of TRAF6 to the IL-1 receptor complex [13]. The contribution of NOX to IL-1 β signaling in the vasculature and in VSM cells has not been well characterized although IL-1 β -induced ROS production had been previously established in VSM cells [14-16]. In the present study, we show that the IL-1 β -stimulated production of ROS involves NOX4 and that this results in PKC δ -dependent activation of c-Jun N-terminal kinase (JNK) downstream signaling in rat aortic VSM cells.

Materials and Methods

Materials

Antibodies for PKC- δ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), antibody for JNK was from Cell Signaling Technologies (Beverly, MA), and antibodies for iNOS, NOX4, and β -actin were from Millipore (Billerica, MA), Proteintech (Chicago, IL), and Sigma (St. Louis, MO), respectively. Inhibitors of PKC- δ , SP00125 and Protease Inhibitor Cocktail III were purchased from Calbiochem (La Jolla, CA). All tissue media were purchased from GICO-BRL Life technologies (Carlsbad, CA) unless specifically stated. All other chemicals were purchased from Sigma (St Louis, MO).

All adenovirus stocks were propagated by the addition of small amounts of virus to human embryonic kidney-293 cells. When cells were ~50% lysed, cells and media were collected, subjected to 3x freeze/thaw cycles, aliquoted, and stored at -80 °C. Titer assays were performed using an adenovirus containing β -galactosidase as a control at matching multiplicity of infection.

¹Abbreviations: DPI, diphenyliodonium chloride; DUOX, dual oxidase; HE, hydroethidine; IL-1 β , interleukin-1 β ; IRAK, IL-1 receptor associated kinase; JNK, c-Jun kinase; MAPK, mitogen activated protein kinase; MKP-1, MAPK phosphatase 1; NOX, NADPH oxidase; PKC δ , protein kinase C δ ; PLC- γ , phospholipase C- γ ; phox, phagocytic oxidase; PTP1B, protein-tyrosine phosphatase 1B; ROS, reactive oxygen species; TGF- β 1, transforming growth factor β 1; TLR1, Toll-like receptor 1; TRAF6, tumor necrosis factor α receptor associated factor 6; VSM, vascular smooth muscle;

Generation of adenoviral siRNA constructs

Adenoviral delivery of siRNA was achieved using the methods developed by Zhao, et al. [17]. Target sequences were identified using the Dharmacon website [18]. Adenoviral vectors were designed to express siRNA hairpins targeted to rat Nox4 (GenBank Acc# NM_053524) or rat Nox1 (GenBank Acc# NM_053683). The target sequences were chemically synthesized as complementary oligonucleotides (Integrated DNA Technologies), annealed and ligated into the pAdTrack-HP vector. The pAd-HP/siNox4 and pAd-HP/siNox1 constructs were sequenced for verification (Center for Functional Genomics, Rensselaer, NY). These constructs were then linearized with *PmeI* and electroporated into AdEasy-1 cells which contain the AdEasy-1 plasmid. Adenoviruses were generated using the methods developed by He, et al. [19]. Successful recombinants were cloned and verified by restriction enzyme analysis. Rat aortic vascular smooth muscle cells (VSMC) were infected at an MOI of 50. Infection efficiency was monitored by following the expression of green fluorescent protein (GFP).

Cell culture

Rat VSMCs were enzymatically digested from the medial layer of thoracic aortas of Sprague-Dawley rats as described previously [20] and grown in Dulbecco's modified Eagle's medium/ Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum (FBS), antibiotic/antimycotic mixture and L-glutamine. Cells were used between passages 4 and 12. At confluence, cells were maintained in DMEM/F12 with 0.4% FBS for 16-24 hrs before use.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR reactions were carried out on a Cepheid Smartcycler with the primers: Nox1 sense 5' - TTATGCGGCCCTCCGA-3', and antisense, 5' - TGAGTACCGCCGACAGCA-3'; Nox4 sense 5' - AGTCCTGGCTTACCTTCGCG-3', and antisense, 5' - GCTGCATTTCAGTGCAAGGAA-3'; β -actin sense 5' - AGGCCCTCTGAACCCTAAG-3', and antisense 5' - CATGGGCACAGTGTGGGTG-3'. Standard curves were generated from each of the amplicons by PCR cloning. Stocks of each amplicon were maintained in transformed DH5 α cells and plasmid DNA. DNA concentration was determined by spectrophotometry of a 1:50 dilution, and copy number was determined.

RNA was extracted and 2.5 μ g of total RNA was loaded onto cDNA synthesis reactions. Quantitative PCR reactions were performed for Nox1, Nox4 and β actin using 2 μ l of the cDNA synthesis reaction. C_t s were compared to standard curves for each gene to yield a copy number. Nox1 and Nox4 levels in each sample were corrected for synthesis variability using the β actin level, which did not change significantly ($6.26 \times 10^6 \pm 1.82 \times 10^6$ in the unstimulated, $5.46 \times 10^6 \pm 0.7 \times 10^6$ in the IL-1 β stimulated samples, for Nox1 and Nox4, *P < 0.05 vs. unstimulated sample).

Immunoblotting

Following experimental treatments, cells in 6-well plates were lysed by scraping in radioimmunoprecipitation assay (RIPA) buffer (50mM Tris base, 150mM NaCl, 0.5% deoxycholate, 1mM NaF, 2mM NaVO₄, 1% Nonidet P-40, 0.1% sodium dodecylsulphate). After separation by SDS-PAGE and transfer to nitrocellulose membranes, proteins and/or their phosphorylated forms were visualized by enhanced chemiluminescence, and quantified by densitometry.

Confocal microscopy

VSMC were seeded on 25-mm round glass coverslips coated with gelatin and grown to near confluence. Cells were stained for 20 min with 10 μ M of hydroethidine (HE) at 37 °C in the dark and were treated for an additional 30 min with IL-1 β . Coverslips were mounted in a chamber containing Hanks Balanced Salt Solution (HBSS) and imaged with a Zeiss LSM 510 META Confocal Microscope, excitation 514nm with a 590nm LP filter to monitor fluorescence. HE and its intermediates react with multiple ROS such as superoxide and hydrogen peroxide to form different products including ethidium and 2-hydroxyethidium, which have similar fluorescence characteristics [21]. Thus, HE staining combined with confocal fluorescence and molecular approaches to modulate specific sources of ROS may be used as a convenient probe to evaluate changes in intracellular production of ROS associated with specific sources.

Nitrite determination

Nitrite was detected by chemiluminescence with a nitric oxide analyzer (Sievers). Cell culture media was injected into the reaction vessel containing acetic acid and potassium iodide as previously described [22]. Chemiluminescence signals derived from nitrite reduction to NO were integrated and nitrite concentrations were calculated using a nitrite standard curve.

Statistical analysis

All data are represented as mean $\pm$ SEM. Student's t test was used for comparisons between two groups. For multiple comparisons, a one way analysis of variance (ANOVA) was performed followed by Newman-Keuls post hoc comparisons to determine significant differences between several groups.

Results

IL-1 β -induced C-Jun N-terminal kinase (JNK) activation is ROS-dependent in VSM cells

Past studies support a role for c-jun N-terminal kinase (JNK) in mediating pro-inflammatory signaling events in VSM cells [23-26]. A time course of JNK activation over 3 hours following IL-1 β stimulation (10 ng/ml) revealed that JNK was maximally activated 10 min after stimulation and quickly diminished thereafter (Figure 1A). Given the sensitivity of JNK activity to activation by exogenous and endogenous sources of ROS [26-31], we determined whether IL-1 β -mediated activation of JNK in VSM cells required the production of ROS. To this end, we pre-incubated VSM cells with the H₂O₂ scavenger catalase or flavoprotein inhibitor diphenyliodonium chloride (DPI) and we observed the effect of these treatments on IL-1 β induced JNK activation. We found that both catalase and DPI attenuated IL-1 β induced JNK activity (Figure 1B and C) suggesting that IL-1 β -dependent activation of JNK required the production of H₂O₂ and the activity of a flavoprotein.

NOX4 is required for IL-1 β -induced ROS generation and JNK activity

We and others previously showed that IL-1 β stimulated the generation of ROS in cultured rat aortic VSM cells [14, 15]. In the next experiments, we sought to determine the source of ROS. In this context, the NOXs were attractive candidates to study because they have been shown to produce ROS in response to agonists which control MAP kinase signaling pathways in various VSM preparations [32, 33]. Based on RT-PCR, *Duox1*, *Nox1*, and *Nox4* were expressed in our preparation with no evident expression of *Duox2*, *Nox2*, and *Nox3* (Figure 2A). Analysis of *Nox1* and *Nox4* mRNA expressions using quantitative RT-PCR revealed that the number of *Nox4* mRNA molecules was approximately 1,000 fold greater than *Nox1* in rat VSM cells (Figure 2B).

In light of the abundance of *Nox4* in rat aortic VSM cells, we investigated its role in mediating the generation of ROS upon IL-1 β stimulation. To carry out these studies, we used the reactive dye hydroethidine (HE) that generates oxidative red fluorescent products including 2-hydroxyethidium (2-OH-E⁺; maximum emission at 567 nm) and the edithium cation (E⁺; maximum emission at 590 nm). The product E⁺ may be formed from the oxidation of HE by H₂O₂ with low molecular weight complexes of iron or heme proteins in the intracellular environment [34]. Because the primary product of NOX4 activity is believed to be H₂O₂ [35], we maximized selectivity for E⁺ fluorescence by exciting at 514 nm and collecting emission fluorescence at 590 nm or higher [36]. In addition, VSM cells take up the H₂O₂-scavenging enzyme catalase providing a convenient way to verify the contribution of intracellular H₂O₂ to HE-derived red fluorescence [32]. To establish whether HE-derived red fluorescence could be used as an intracellular H₂O₂ detector independent of cytokine stimulation, we exposed VSM cells to a 30 min incubation with 40 μ M menadione, a quinone which generates superoxide and hydrogen peroxide upon reduction by intracellular reductase [37]. As shown in Figure 3, menadione caused an increase in the red fluorescence over basal levels, an effect which was almost completely inhibited in cells pretreated with catalase. Similarly, we found that IL-1 β stimulated an increase in HE fluorescence and catalase inhibited this effect. These results are consistent with past studies using different fluorescent probes [15] and indicate that changes in intracellular H₂O₂ levels in IL-1 β -stimulated VSM cells may be adequately monitored by following HE-derived red fluorescence.

Next, we decreased *Nox4* expression using adenoviral delivery of siRNA duplexes. Advantages of these constructs are high efficiency infection (>90%) and the ability to identify and track individual cells through co-expression of GFP encoded in the adenovirus. Rat aortic VSM cells were infected with the virus overnight and growth media was restored the following day. The infected cells were then serum starved overnight and treated the following day with IL-1 β or starvation media. At an MOI of 50, nearly all the cells were infected (Figure 4A), NOX4 protein levels were decreased compared to siCon (Figure 4B), and *Nox4* mRNA expression was decreased by approximately 70% (Figure 4B). In contrast, *Nox1* mRNA expression was not affected by *Nox4* silencing (Figure 5A).

We found that basal HE fluorescence was not affected by si*Nox4* (Figure 3C and D). IL-1 β stimulated an increase in HE red fluorescence after 30 minutes treatment in vector-infected cells, and this effect was inhibited by si*Nox4* (Figure 4C and 4D). In order to determine the specificity of the requirement of IL-1 β -induced ROS production for NOX4, we tested the effect of adenoviral delivery of a short hairpin siRNA targeting *Nox1*. Treatment resulted in an approximately 80% decrease in *Nox1* at the transcriptional level (Figure 5B). Under these conditions, we found no statistical difference in IL-1 β mediated increase in HE fluorescence (Figure 5C). Overall our results indicate that NOX4 but not NOX1 is required for IL-1 β induced ROS production in cultured rat aortic vascular smooth muscle cells. The catalase sensitivity of the IL-1 β stimulated increase in HE red fluorescence is consistent with H₂O₂ as the primary product of NOX4 activity [35].

Next, to determine if the NOX4-dependent increase in ROS generation mediated JNK activity, we stimulated *Nox4* deficient VSM cells (Si*Nox4*) with IL-1 β and measured JNK activity. As illustrated in Figure 6, IL-1 β -dependent activation of JNK was significantly attenuated in VSM upon silencing of *Nox4*. These results indicate that NOX4-dependent ROS generation is required for IL- β -mediated JNK activation in rat aortic VSM cells.

PKC δ mediates the IL-1 β -dependent activation of JNK

We showed previously that PKC δ is an important component of the IL-1 β -mediated activation of MAPK in rat aortic VSM cells [11]. Because PKC δ has been reported to

mediate increases of JNK activity in other cell types [38, 39], we wanted to determine whether PKC δ is an upstream mediator of IL-1 β - and NOX4-dependent JNK activity in VSM cells. We found that treatment with the PKC δ selective inhibitor rottlerin attenuated IL-1 β induced JNK activity (Figure 7A). Treatment with the PKC δ selective inhibitor Go6976 failed to inhibit IL-1 β -mediated increase in JNK activity in VSM cells indicating that PKC δ plays no role in activating JNK (data not shown). In another experiment, we tested the effect of temporary loss of PKC δ expression upon treatment with the phorbol ester 12,13-dibutyrate (PDBu). As shown in Figure 7B, treatment of VSM cells with PDBu resulted in the loss of PKC δ . Under these conditions, IL-1 β stimulation was unable to induce JNK activation (Figure 7B). To support these pharmacological approaches, we suppressed PKC δ expression with adenoviral particles containing short hairpin siRNA targeted to PKC δ . Under these conditions of reduced PKC δ expression, IL-1 β was unable to induce JNK activity (Figure 7C). Taken together, these results indicate that PKC δ mediate the IL-1 β activation of JNK in rat aortic VSMCs.

Nox4 mediates the IL-1 β -dependent activation of PKC δ

Having ascertained that PKC δ is upstream of JNK, we determined whether NOX4 regulated the activation of PKC δ . As previously shown [11], stimulation of VSM cells with IL-1 β resulted in an increase in PKC δ activity based on serine phosphorylation and kinase activity (Figure 8A and B). Pretreatment of VSMCs with catalase (Figure 8A) or DPI (Figure 8B) significantly decreased IL-1 β -induced activation of PKC δ . Suppression of NOX4 expression with the adenoviral particles containing siRNA targeted to NOX4 blocked IL-1 β -dependent phosphorylation of PKC δ (Figure 8C and D). We also observed a significant increase in PKC δ phosphorylation in non-stimulated cells upon silencing of NOX4 (Figure 8D). Taken together, these results indicate that PKC δ activation is regulated by endogenous ROS derived from the activity of NOX4.

NOX4 and JNK regulate the IL-1 β -dependent activation of inducible nitric oxide synthase (iNOS)

In order to examine the functional significance of NOX4-dependent activation of JNK, we examined the expression of iNOS upon IL-1 β stimulation. To this end, we pretreated VSM cells with increasing concentrations of the JNK inhibitor SP600125 and examined iNOS expression by immunoblotting and nitrite accumulation after 24 h stimulation with IL-1 β . Consistent with past studies, IL-1 β increased iNOS protein expression and nitrite accumulation (Figure 9). Pharmacological inhibition of JNK inhibited iNOS protein expression and nitrite accumulation, indicating that IL-1 β mediated upregulation of iNOS was JNK-dependent (Figure 9 A and B). Depletion of NOX4 using pAd/siNox4 resulted in a decrease in both iNOS protein expression and nitrite accumulation (Figure 9C and D) indicating that IL-1 β mediated upregulation of iNOS required NOX4.

Discussion

NADPH Oxidase 4 (NOX4) is required for the maintenance of the differentiated smooth muscle phenotype in the vascular wall [40] and regulates the differentiation of embryonic stem cells into smooth muscle cells [41]. At the same time, NOX4 facilitates the proliferation of human pulmonary artery smooth muscle cells [42] and promotes vascular restenosis after carotid injury in obese Zucker rats [43]. These results indicate that the role of NOX4 in the vascular wall is context-dependent and mediates distinct functions that might rely on the coupling of NOX4-mediated ROS production to different signaling pathways. In the present study, we report for the first time a role for NOX4 in mediating IL-1 β -dependent activation of JNK. We provide evidence that PKC δ is activated in an IL-1 β - and NOX4-dependent manner and is an important modulator of JNK activity in VSM

cells. Overall, our results indicate that activation of JNK by IL-1 β is regulated by NOX4 at or upstream of PKC δ .

Past studies indicated that IL-1 β stimulates the production of ROS in rat aortic and pulmonary microvascular VSM cells although the source had not been defined [14, 15]. IL-1 β activates NOX2 in the epithelial cell line MCF-7 [13] but NOX2 is not expressed to any significant amounts in rodent VSM cells. Among the five *Nox* and two *Duox* examined, we found that rat aortic VSM cells expressed only *Duox1*, *Nox1*, and 4 (Figure 2). To our knowledge, the expression of DUOX1 in VSM cells has not been documented and this observation will require further study. The high levels of *Nox4* mRNA compared to *Nox1* in VSM cells has been documented by others although estimates vary widely (between approximately 50 and 0.5 % *Nox1* relative to *Nox4*) [33, 44, 45]. The reasons for these discrepancies are not clear, but may arise from a difference in technique to isolate VSM cells and perform PCR or differences in the genetic background of the animals or species studied.

We found that NOX4 silencing through adenoviral delivery of a short hairpin specifically targeting NOX4 was sufficient to inhibit IL-1 β mediated ROS production. In contrast, NOX1 silencing did not have any effect. Past studies had already indicated that insulin [46], 7-ketocholesterol [47], lipopolysaccharide [25], intercellular adhesion molecule-1 (ICAM-1) [48], insulin-like growth factor-1 (IGF-1), urotensin II [49], transforming growth factor- β (TGF- β) [43, 50-52], and oxygen [53] increase NOX4 activity in various tissues and cells. Increase in NOX4 activity is usually considered to be regulated by an increase in NOX4 protein levels [43, 50-52]. However, acute regulation of NOX4 activity has been observed previously, for example upon stimulation of adipose cells with insulin [46]. Similarly, our results would point to the rapid engagement of a signaling pathway leading to NOX4 activation following IL-1 β stimulation. The short-term stimulation (30 min or less) with IL-1 β limited any long term changes in NOX4 levels that might have altered ROS production. In addition in the present study, the basal levels of ROS production were not altered upon NOX4 silencing. This is in contradiction with past studies that have indicated that NOX4 regulates basal levels of cellular ROS [54]. The reason for this discrepancy is unknown. It is possible that the fluorescent detector used in the present study was not sensitive enough to establish differences at low fluorescence. Alternatively, culture conditions or the level of silencing applied to the present experiments may not have been conducive to the detection of a NOX4-sensitive signal under basal conditions.

Regulation of NOX4 activity through direct posttranslational protein modification has yet to be established. NOX4 requires p22phox for activity [55] and Lyle and coworkers demonstrated that polymerase delta-interacting protein 2 (Poldip2) interacts with the NOX4/p22phox complex to regulate ROS production [56], suggesting a role for protein-protein interactions in the regulation of Nox4 activity. A direct interaction between NOX4 and toll-like receptor 4 (TLR-4) is also required for LPS-induced generation of ROS [57]. Because the TLR family of receptor is related to the IL-1 superfamily, this could suggest a possible regulation of NOX4 activity through direct interaction between the IL-1 receptor and NOX4. Whatever the mechanism, these studies would be consistent with alternate routes of NOX4 regulation other than changes in mRNA and/or protein levels that may be operative during IL-1 β -mediated NOX4-dependent production of ROS. Future studies will require more detailed molecular approaches to elucidate this issue.

It has been long recognized that ROS activate JNK [27] and in some cases, a requirement for NOX including NOX1, 2, 3, and 4 was demonstrated [58-60]. In the present study, we found that silencing of NOX4 attenuated IL-1 β -induced activation of JNK, strongly supporting a role for NOX4 in this signaling pathway in cultured rat aortic VSM cells. Past studies

indicated that NOX4 contributes to the differentiated state not only of VSM cells but also of cardiomyocytes [40, 41, 61, 62]. In this case, NOX4 activation is strongly linked to the activation of p38MAPK [51, 61]. We reported previously that inhibition of IL-1 β -mediated ROS production did not impact IL-1 β stimulated p38MAPK activity [15], an observation which we confirmed using the flavoprotein inhibitor DPI (data not shown). This result further support the concept that compartmentalization of NOX4 activation is a critical determinant of the signaling pathways that are initiated and their ultimate functional effects.

The mechanism by which NOX4 regulates MAPK such as JNK is poorly understood. Mahadev et al. showed that activation of insulin signaling in 3T3-L1 adipocytes occurred via NOX4-dependent inhibition of the protein-tyrosine phosphatase PTP1B [46]. Liu et al. also showed that TGF- β 1-induced activation of Nox4 inhibits MAPK phosphatase 1 (MKP-1) and leads to the sustained activation of JNK and p38MAPK [63]. In the present study, we positioned PKC δ upstream of JNK activation but downstream of NOX4-derived ROS. PKC δ is unique among the PKC family in that it contains multiple tyrosine residues that impact its intracellular location and activation [64]. The activation of PKC has also been shown to be modulated through direct oxidation of cysteine residues [65]. For example, treatment with low levels of oxidants modify the regulatory domain of PKC resulting in loss of activity while higher concentrations modify the catalytic subunit and increase PKC activity [66]. It is still possible that NOX4 may not directly change the oxidation state of PKC δ and instead regulate upstream signaling elements that impact PKC δ phosphorylation. Interestingly, we found that NOX4 silencing also increased basal PKC δ phosphorylation (Figure 8). Although one may only be able to speculate on the reasons for this observation, this might be due to a change in local ROS production that was not detected upon silencing of NOX4. Alternatively, NOX4 may have scaffolding functions that are independent of ROS production such that NOX4 loss may have resulted in increase basal activity [67]. Whatever the mechanism, ROS-dependent regulation of PKC δ activity coupled with PKC δ 's ability to mediate multiple signaling pathways with diverse functional outcomes make PKC δ an ideal integrator of NOX4-dependent processes in VSM cells.

In summary, we found that (i) IL-1 β induces the production of ROS in cultured rat aortic VSM cells via NOX4; (ii) NOX4 is required for IL-1 β -stimulate JNK activation and downstream upregulation of iNOS; (iii) PKC δ mediates the IL-1 β dependent activation of JNK; and (iv) NOX4-dependent regulation of IL-1 β mediated activation of JNK occurs at or upstream of PKC δ . Because IL-1 β is a key component of the inflammatory response in the vasculature, our results support the hypothesis that NOX4 contributes to vascular injury.

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Figure 1. IL-1 β -induced JNK activation is ROS-dependent in rat aortic VSM cells

(A) Time course of IL-1 β induced c-jun kinase (JNK) in VSM cells. VSM cells were stimulated with 10 ng/ml IL-1 β for the indicated times. Whole cells lysates were then resolved using SDS-PAGE and, transferred to nitrocellulose membrane as described in the "Materials and Methods" section. The nitrocellulose membranes were then immunoblotted (IB) with antibody for active c-jun kinase (P-JNK) and total c-jun kinase (JNK) to insure equivalent loading of protein in each well. (B) VSM cells were treated with 3000 units/ml catalase 16 h prior to stimulation with 10 ng/ml IL-1 β for 10 min. JNK activation was determined as described in (A). (C) VSM cells were treated with 10 μ M DPI for 30 min

prior to stimulation with 10 ng/ml IL-1 β for 10 min. JNK activation was determined as described in (A). Right panels for each immunoblots represent the quantitation of 3 to 4 separate experiments.

Figure 2. *Nox4* is the predominant NADPH oxidase in cultured rat VSM cells

(A) Total RNA was prepared using TRIzol, and RT-PCR was performed with primers for rat *Duox1* and 2, and *Nox1*, 2, 3, and 4 in rat aortic vascular smooth muscle cells as described in the “Materials and Methods” section. (B) Determination of messenger RNA levels of *Nox1* and 4 using QPCR. *Nox1* and *Nox4* levels in each sample were corrected for synthesis variability using β -actin as described in the “Materials and Methods” section (n= 3).

Figure 3. IL-1 β -induced hydroethidine fluorescence in VSM cells

(A) VSM cells were grown on gelatin coated 25 mm coverslips and incubated for 24 h with or without catalase (Cat). Cells were rinsed twice with HBSS and treated for 30 min with either 40 μ M menadione (Men) or 10 ng/ml IL-1 β , rinsed again, then incubated with 10 μ M hydroethidine for 20 min, and imaged on a Zeiss LSM 510 META. Confocal images are enhanced by pseudocolor (WCIF Image J “Fire” lookup table, scale shown at bottom, darker colors indicate areas of low fluorescence and brighter colors indicate areas of high fluorescence); result presented is a representative experiment of 3 separate experiments. (B) Quantification of (A). *P < 0.05 vs. vector control, #P < 0.05 vs. same treatment but no catalase (n=3).

Figure 4. NOX4 is required for IL-1 β -induced ROS generation

(A) VSM cells were infected with either the control (siCon) or siNox4 adenoviral vectors and incubated for 24 hours. GFP fluorescence was monitored to determine the efficiency of infection with the adenoviruses. Phase contrast microscopy shows the total cells (bottom panels). (B) Western blot (upper panel; approximately 65 kDa band) and qRT-PCR of VSM cells infected with siCon or siNox4 (lower panel). RNA was extracted and analyzed by quantitative RT-PCR for Nox4 levels which were normalized to β -actin levels (* $P < 0.05$, $n=3$). The immunoblot shown is representative of three experiments. (C) VSM cells grown on gelatin coated glass coverslips were treated for 30 min with IL-1 β , then incubated with 10 μ M hydroethidine for 20 min, and imaged on a Zeiss LSM 510 META Confocal Microscope. Images are vector control unstimulated (*top left*), vector control stimulated (*top right*), siNox4 expressing unstimulated (*bottom left*) and siNox4 expressing stimulated

(*bottom right*). Images are enhanced by pseudocolor (WCIF Image J “Fire” lookup table, scale shown at bottom, darker colors indicate areas of low fluorescence and brighter colors indicate areas of high fluorescence); result presented is a representative experiment of 3 separate experiments. **(D)** Quantification of (C). * $P < 0.01$ vs. vector unstimulated control, # $P < 0.01$ vs. vector stimulated control (n=3).

Figure 5. NOX1 is not involved in IL-1 β -induced ROS generation

qRT-PCR of VSM cells infected with siCon and siNox4 (**A**) or siNox1(**B**). RNA was extracted and analyzed by quantitative RT-PCR for *Nox1* mRNA levels, which were normalized to β -actin levels (* $P < 0.05$ $n=3$ compared to siCon). (**B**) VSM cells grown on gelatin coated glass coverslips were treated for 30 minutes with IL-1 β , then incubated with 10 μ M hydroethidine for 20 minutes, imaged as described under Materials and Methods, and mean hydroethidine was quantified. * $P < 0.01$ vs. vector unstimulated control, ($n=3$). There was no statistically significant difference between siCon and siNox1 (stimulated or unstimulated conditions).

Figure 6. NOX4 is required for IL-1 β -induced JNK activation

(A) VSM cells infected with control (SiCon) or siNox4 vectors as described in the “Materials and Methods” section were incubated in the presence or absence of IL-1 β (10 ng/ml) for 10 min. The cell lysates were then immunoblotted for active JNK (P-JNK) and β -Actin as a loading control. These immunoblots are representative of three separate experiments. (B) Quantitation of (A). *P < 0.05 vs. IL-1 β stimulated siCon (n=3).

Figure 7. PKCδ mediates IL-1β-induced JNK activation

(A) VSM cells were treated with 3 μ M Rott for 30 min prior to stimulation with 10 ng/ml IL-1 β for 10 min. Lysates were immunoblotted for active JNK (P-JNK) and β actin to insure equal protein loading. (B) VSM cells were treated with 300 nM PDBu for 16 hours prior to stimulation with 10 ng/ml IL-1 β for 10 min. Lysates were immunoblotted for active JNK (P-JNK), β actin to insure equal protein loading, and total PKC δ to determine extent of PKC δ protein expression. (C) VSM cells infected with control (SiCon) or siPKC δ adenoviruses as described in the "Materials and Methods" section were incubated in the presence or absence of IL-1 β (10 ng/ml) for 10 min. The cell lysates were then immunoblotted for active JNK (P-JNK), total PKC δ (P-PKC δ) to determine the extent of PKC δ "silencing" and β -Actin as

a loading control. Right panels for each immunoblot represent the quantitation of three separate experiments.

Figure 8. NOX4 mediates IL-1β-dependent PKCδ activity

(A) VSM cells were treated with 3000 u/ml catalase 16 h prior to stimulation with 10 ng/ml IL-1β for 10 min. The VSM cell lysates were immunoblotted for active PKCδ (P-PKCδ) and total PKCδ (PKCδ) to insure equal protein loading. (B) VSM cells were treated with 10 μM DPI for 30 min prior to stimulation with 10 ng/ml IL-1β for 10 min. PKCδ activity was determined by its ability to phosphorylate a specific substrate as described in the “Materials and Methods” section.). *P < 0.05 vs. vector unstimulated control, **P < 0.05 vs. IL-1β alone (n=3). (C) VSM cells infected with control (SiCon) or siNox4 adenoviruses were incubated in the presence or absence of IL-1β (10 ng/ml) for 10 min. The cell lysates were then immunoblotted for active PKCδ (P-PKCδ) and β-Actin as a loading control. (D)

Quantitation of 3 separate experiments as described in (C). (*P<0.05 vs. SiCon unstimulated, #P< 0.05 vs. SiNox4 unstimulated, **P < 0.05 vs. siCon stimulated).

Figure 9. JNK inhibition and NOX4 silencing suppress IL-1 β -dependent iNOS expression
(A) VSM cells were pretreated with various concentrations of the JNK inhibitor SP600125, followed by 24 h stimulation with IL-1 β (10 ng/ml). Cell lysates were immunoblotted with beta actin and iNOS antibodies. Immunoblot is representative of 3 experiments. **(B)** Conditions were similar to (B) and nitrite levels from cell supernatant were determined by chemiluminescence. (* P <0.05 vs. untreated controls, # P <0.05 vs IL-1 treated, no SP600125, n =3). **(C)** VSM cells infected with control (SiCon) or siNox4 adenoviruses were incubated in the presence or absence of IL-1 β (10 ng/ml) for 24 h. The cell lysates were then immunoblotted for iNOS and β -Actin as a loading control. Immunoblot is representative of 3 separate experiments **(D)**. Conditions were similar to (C) and nitrite levels from cell supernatant were determined by chemiluminescence (* P <0.01 vs. SiCon unstimulated, # P <0.01 vs. siCon stimulated).