Phosphorylation of Nox1 Regulates Association With NoxA1 Activation Domain

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Rationale: Activation of Nox1 initiates redox-dependent signaling events crucial in the pathogenesis of vascular disease. Selective targeting of Nox1 is an attractive potential therapy, but requires a better understanding of the molecular modifications controlling its activation.

Objective: To determine whether posttranslational modifications of Nox1 regulate its activity in vascular cells.

Methods and Results: We first found evidence that Nox1 is phosphorylated in multiple models of vascular disease. Next, studies using mass spectroscopy and a pharmacological inhibitor demonstrated that protein kinase C-beta1 mediates phosphorylation of Nox1 in response to tumor necrosis factor-alpha. siRNA-mediated silencing of protein kinase C-beta1 abolished tumor necrosis factor-alpha-mediated reactive oxygen species production and vascular smooth muscle cell migration. Site-directed mutagenesis and isothermal titration calorimetry indicated that protein kinase C-beta1 phosphorylates Nox1 at threonine 429. Moreover, Nox1 threonine 429 phosphorylation facilitated the association of Nox1 with the NoxA1 activation domain and was necessary for NADPH oxidase complex assembly, reactive oxygen species production, and vascular smooth muscle cell migration.

Conclusions: We conclude that protein kinase C-beta1 phosphorylation of threonine 429 regulates activation of Nox1 NADPH oxidase. (Circ Res. 2014;115:911-918.)

Key Words: atherosclerosis ■ NADPH oxidase ■ neointima ■ PKCbeta ■ TNF-alpha ■ vascular smooth muscle cells

Nox1 serves as the catalytic core of a multi-subunit NADPH oxidase enzyme complex, which assembles in response to signaling cascades initiated by mechanical stress, cytokines, and growth factors. Nox1 is a transmembrane protein expressed in multiple tissues, including vascular smooth muscle cells (VSMCs), brain, gastrointestinal epithelium, and prostate tumor cells.1-14 Nox1 plays a critical role in the development of cardiovascular disease, amyotrophic lateral sclerosis, gastrointestinal disease, immunological disorders, and multiple forms of cancer.5-13

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Since its discovery in 1999, multiple studies have provided evidence that activation of Nox1 is a multistep process that requires assembly of a complex of proteins.14 Nox1 associates with the transmembrane protein p22phox for stability and membrane localization. The recruitment of cytosolic proteins to the membrane forms a complex, which allows electron transfer from NADPH to oxygen to form superoxide.15-17 When activated, the organizer cytosolic protein p47phox or its homolog NoxO1 tethers to p22phox.18-21 Recruitment of the activator p67phox or its homolog NoxA1 is mediated via tail-to-tail binding to the organizer protein.22,23 Mutation of the activation domain (AD) of NoxA1 abrogates Nox1-generated reactive oxygen species (ROS).24 However, the molecular interaction of Nox1 with the AD of NoxA1 is not known. Phosphorylation of NoxA1 allows for dissociation from Nox1 and is 1 mechanism to terminate enzyme activity.25 Whether post-translational modifications of Nox1 regulate its activation has not been explored.

The goal this study was to examine molecular mechanisms of Nox1 activation. Our data reveal that protein kinase C-beta1 (PKC-beta1) phosphorylation of Nox1 at threonine 429 (T429) is necessary for its interaction with NoxA1 AD, complex assembly, and generation of superoxide. Furthermore, mutation of T429 prevents Nox1-mediated VSMC migration. These findings identify a novel regulatory mechanism by which Nox1 is activated.

Methods

Phosphorylation of Nox1 and its role in Nox1 activation was assessed in cultured aortic VSMCs (C57Bl6/J mouse, Nox1-/- mouse, A7r5 rat) and in Cos7 cells that stably express p22phox or p22phox with p47phox and p67phox (CosPhox). For details on animal models, cell models, and...
transfection protocol; detection of Nox1 phosphorylation; measurement of ROS; construction of Nox1 mutants; detection of NADPH oxidase complex assembly, isothermal titration calorimetry (ITC) assays, circular dichroism analysis, and computational modeling, an expanded methods section is available in the Online Data Supplement.

**Results**

**Nox1 Phosphorylation Is Increased in Multiple Models of Vascular Disease**

Phosphorylation is a common mechanism for post-translational regulation of protein activity. Using an antibody that detects phosphorylation at serine, threonine, and tyrosine residues (anti-STY), we examined whether Nox1 is phosphorylated under conditions known to be associated with increased Nox1 activity. Specifically, we immunoprecipitated with anti-p22phox and blotted with anti-STY or anti-Nox1 in 3 models of vascular disease. First, we detected increased levels of Nox1 phosphorylation in aorta from monkeys fed an atherogenic diet as compared with normal diet (Figure 1A; Online Figure I). Next, we found elevated Nox1 phosphorylation in cultured VSMCs derived from the neointima of balloon-injured rat aorta as compared with medial VSMCs (Figure 1B). Using a murine carotid injury model known to induce neointimal hyperplasia, Nox1 phosphorylation was significantly increased as compared with contralateral noninjured arteries (Figure 1C). These results provide evidence of Nox1 phosphorylation in response to vascular injury.

**Nox1 Is Activated by Protein Kinase C-βI Phosphorylation**

We next used NetPhosK sequence analysis software to identify putative kinases that phosphorylate Nox1. Of the top 10 predicted phosphorylation sites, PKC was the predicted kinase for 7 of those sites (Online Figure II). Therefore, we examined whether inhibition of PKC with Calphostin C (CalC)26 modifies Nox1 phosphorylation after stimulation with tumor necrosis factor-α (TNF-α), which is a known activator of Nox1.27,28 Treatment of cultured A7r5 rat aortic VSMCs with TNF-α increased Nox1 phosphorylation (Figure 2A) similar to levels seen in vivo (Figure 1). CalC inhibited TNF-α-stimulated Nox1 phosphorylation. Consistent with reports that multiple agonists are capable of Nox1 activation, we observed that, in addition to TNF-α, phosphorylation of VSMC Nox1 occurs after treatment with platelet-derived growth factor-BB and phorbol myristate acetate (Online Figure III). It also seems that the timing of phosphorylation may be agonist-dependent (Online Figure III).

Next, TNF-α caused robust ROS production in wild-type (WT) but not Nox1−/− VSMCs (Figure 2B), confirming that the TNF-α-dependent generation of ROS is Nox1-dependent.27,28 CalC abrogated Nox1-dependent ROS production in WT VSMCs (Figure 2B). Analysis of VSMC membrane fractions from WT cells demonstrated that TNF-α pretreatment primes NADPH oxidase activity (Figure 2C). This effect was completely abolished by either the flavoenzyme inhibitor DPI or CalC (Figure 2C). Thus, PKC activity is required for Nox1 generation of ROS.

Nox1 has been implicated in VSMC migration to multiple agonists.29-32 Using Nox1−/− VSMCs, we first established that VSMC migration to TNF-α requires Nox1 (Figure 2D). Similar to the effects of PKC inhibition on ROS production, migration of WT VSMCs was blocked with CalC (Figure 2D). Taken together, these data demonstrate that PKC is necessary for TNF-α-mediated redox-dependent migration.

Mass spectrometry identified the interaction of PKC-βI with the Nox1-p22phox complex in response to TNF-α treatment of VSMCs (data not shown). PKC-βI and II are splice variants from the same gene and are both reported to be expressed in mice and humans.33 Western blotting demonstrated expression of PKC-βI but not PKC-βII in WT VSMCs (Online Figure IV). Using an siRNA against PKC-β (siPKC-β), we achieved significant knockdown of PKC-β expression in WT VSMCs (Figure 3A). Silencing PKC-β resulted in partial inhibition of TNF-α-induced Nox1 phosphorylation (Figure 3B) and near complete abrogation of ROS production (Figure 3C), NADPH oxidase activity (Figure 3D), and VSMC migration (Figure 3E). These findings suggest that PKC-βI is the kinase that regulates Nox1 NADPH oxidase activation.

**PKC-βI Phosphorylation of T429 Is Necessary for Nox1 Activation**

NetPhosK prediction algorithm identified several PKC consensus phosphorylation sites in Nox1 (Online Figure II). Based on the NetPhosK score, the conservation of the putative phosphorylation sites among mouse, rat, and human (Online Figure V), and their location within intracellular regions (Online Figure V and Figure 4A), we evaluated T89 and T429. T89 is located in

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**Figure 1. Nox1 phosphorylation is increased in multiple models of vascular disease.** Nox1 phosphorylation was assessed by subjecting lysates to immunoprecipitation with anti-p22phox followed by Western blotting with either anti-serine, -threonine, and -tyrosine residues (STY) or anti-Nox1. A, Aorta from monkeys fed a normal or atherogenic (Athero) diet. B, Cultured medial (Med) and neointimal (Neo) vascular smooth muscle cells derived from rat aorta 14 days after balloon injury. C, Murine carotid artery 3 days postligation. n=3 to 5 per group. *p<0.05 as compared with nondiseased.
the first intracellular loop between transmembrane domains I and II, whereas T429 is in the C-terminal region (Figure 4A).

We first mutated T89 and T429 to alanine to prevent phosphorylation and confirmed that mutation does not disrupt protein expression. Using Flag-tagged constructs (T89A, T429A, or WT Nox1), we validated expression by Western blotting and immunofluorescence in CosPhox cells that express p22phox, p47phox, and p67phox but lack Nox1 (Online Figure VI). However, functional analysis demonstrated that the C-terminal epitope tag interfered with ROS production by WT Nox1 (data not shown). Thus, subsequent studies used nontagged Nox1 mutants.

We next examined whether Nox1 phosphorylation at T89 or T429 is required for Nox1 NADPH oxidase activity after TNF-α stimulation. Expression of WT Nox1 in CosPhox (Figure 4B) or Nox1−/y VSMCs (Figure 4C) resulted in the anticipated NADPH-stimulated superoxide production as measured by lucigenin-enhanced chemiluminescence in membrane-enriched fractions. Although superoxide production in cells expressing T89A Nox1 was similar to WT levels, expression of T429A Nox1 returned superoxide to control levels. Next, we determined whether T429 is also required for TNF-α–induced VSMC migration. As with superoxide production, migration was similar in Nox1−/y VSMCs expressing either WT or T89A Nox1 (Figure 4D). By contrast, no migration was observed in cells transfected with T429A Nox1. In addition, expression of a phosphomimetic T429D Nox1 mutant resulted in superoxide production (Figure 4C) and cell migration (Figure 4D), approaching levels observed with WT Nox1. These data are consistent with a negative charge at T429 as necessary for Nox1 enzyme activity and VSMC migration after stimulation with TNF-α.

To directly evaluate whether T429 Nox1 is a bona fide PKC-β phosphorylation site, we performed an in vitro kinase assay using human recombinant PKC-β1 and a Nox1 peptide containing T429 (KLKTVKIYF). We used ITC to measure the heat generated by phosphorylation of the peptide. The slow return
to baseline that occurs after the heat of dilution confirms that
the Nox1 peptide is a substrate for PKC-β (Figure 4E). The
reduction in heat produced in subsequent reactions suggests
product inhibition, that is, inhibition of PKC-β by the phos-
phorylated Nox1 peptide (Figure 4F). This substantial product
inhibition precluded measurement of kinetic parameters. 36 In
addition, dot blot analysis of the kinase reaction using p-STY
antibody confirms phosphorylation of the Nox1 peptide within
the reaction mixture (Figure 4F). These results provide direct
evidence that PKC-β phosphorylates Nox1 at T429.

In response to various stimuli, the cytosolic subunit p47phox
or its homolog NoxO1 organizes the translocation and as-
sociation of NoxA1 with the Nox1/p22phox complex at the
membrane. We first measured agonist-stimulated superox-
dide production in intact CosPhox cells expressing p22phox,
NoxA1, and p47phox. In cells coexpressing WT Nox1, TNF-α,
platelet-derived growth factor-BB, angiotensin II, and phorbol
myristate acetate all increased cellular levels of superoxide
(Figure 5A). In the presence of the T429A Nox1 phospho-
mutable, superoxide levels in response to TNF-α, platelet-derived
growth factor-BB, and angiotensin II were markedly attenu-
ated. Measurements of agonist-dependent cellular superoxide
were repeated in CosPhox cells expressing p22phox, NoxA1,
and NoxO1. Under these conditions, activation of Nox1 was
less than that observed with NoxA1/p47phox-expressing cells
(Figure 5B), which is consistent with a previous report. 16

**T429 Phosphorylation Facilitates the Association of NoxA1 Activation Domain With Nox1**

Based on our observation that the combination of p47phox and
NoxA1 with Nox1 are dependent on phosphorylation of Nox1
T429, we studied the association of these subunits. This inter-
action at the membrane involves the association of the NoxA1
AD with the C-terminus of Nox1, 18,23,34 though the mechanism

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**Figure 4.** Phosphorylation of Nox1 T429 by protein kinase C-beta1 (PKCβ1) is required for tumor necrosis factor (TNF)-α-induced reactive oxygen species (ROS) production and vascular smooth muscle cell (VSMC) migration. A. Relative locations of T89 and T429 in Nox1. CosPhox cells (B) or Nox1−/y VSMCs (C) expressing the indicated Nox1 mutants were treated with TNF-α, followed by measurement of ROS in membrane fractions by NADPH-stimulated chemiluminescence; n=4 independent experiments. D. Migration of Nox1−/y VSMCs expressing the indicated Nox1 mutants to TNF-α. The number of cells migrating under nonstimulated conditions was subtracted from TNF-α-stimulated migration for each group; n=5 to 10 independent experiments. *P<0.05 vs mock-transfected (control) cells. #P<0.05 vs wild-type (WT)-transfected cells. E. Isothermal titration calorimetry measurement of in vitro kinase reaction of recombinant PKC-β1 with Nox1 peptide (KLKTKQYIF). F. To confirm PKC-βi phosphorylation of the Nox1 peptide, dot blot analysis of in vitro kinase assay input and product was performed using anti-p-serine, -threonine, and -tyrosine residues (STY). Phosphorylated Nox1 peptide (pNox1, KLK³T*QYIF). Total peptide levels were determined by Ponceau staining. Data were quantitated as the ratio of p-STY to total peptide using Odyssey imaging software and are indicated above the dot blot.

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**Figure 5.** Nox1 T429 is required for receptor-mediated ROS production by Nox1. CosPhox cells were transfected with p47phox and NoxA1 (A) or NoxO1 and NoxA1 (B) and transfected with wild-type (WT) or the T429A mutant Nox1. Superoxide levels were measured in intact cells immediately after stimulation with tumor necrosis factor (TNF)-α (10 ng/mL), platelet-derived growth factor (PDGF)-BB (10 ng/mL), angiotensin II (AngII, 10−7 mol/L), or phorbol myristate acetate (PMA, 10−6 mol/L). Relative light units (RLU) per second (sec) of the unstimulated sample was subtracted from the agonist-treated sample and data normalized to number of cells. n=4 independent experiments. *P<0.05 vs WT.
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is incompletely defined. Because the structure of Nox1 has not yet been solved, we used homology modeling to determine whether the position of T429 within the C-terminal domain might facilitate the interaction of Nox1 with cytosolic subunits. Using the cytochrome B5 reductase crystal structure for the FAD domain (PDB ID 2EIX) and the Nox2 crystal structure for the NADPH domain (PDB ID 3A1F) as templates for our model, we found that T429 resides in an unstructured loop on the external surface of the Nox1 cytosolic domain (Figure 6A and 6B). The position of T429 suggested a potential interaction site with NoxA1. The NoxA1 AD is also in an unstructured loop region as demonstrated by partial crystal structures of NoxA1 AD (shown as the backbone with residues labeled) was computationally docked to the Nox1 cytosolic C-terminal domain (accessible surface area in green with T429 depicted in purple).

Figure 6. Homology model of Nox1 cytosolic C-terminus computationally docked with NoxA1 activation domain. A and B. Ribbon diagram of Nox1 C-terminus modeled based on the crystal structures of the FAD domain in cytochrome B5 reductase (PDB ID 2EIX) and the NADPH domain in Nox2 (PDB ID 3A1F). Co-factors from template structures positioned within the Nox1 cytosolic C-terminal domain are noted by arrows. The N-terminus that attaches to the transmembrane domain is depicted as a blue sphere, and the extreme C-terminus of Nox1 is depicted as a red sphere. Residues involved in NADPH binding are depicted in blue, and residues involved in FAD binding are depicted in orange. The ribbon diagram in (B) is rotated 45° clockwise to reveal residues used to create the Nox1 peptide (purple) for binding experiments, including T429. C. Proposed interaction of the NoxA1 AD with Nox1 T429. The Nox1 AD peptide for the NADPH domain (PDB ID 3A1F) as templates for our model, we found that T429 resides in an unstructured loop on the external surface of the Nox1 cytosolic domain (Figure 6A and 6B). The position of T429 suggested a potential interaction site with NoxA1. The NoxA1 AD is also in an unstructured loop region as demonstrated by partial crystal structures of

Figure 7. Interaction of phosphorylated T429 of Nox1 with the NoxA1 activation domain is required for NoxA1 membrane recruitment. A, Structural analysis of NoxA1 AD and phospho-Nox1 (pNox1) peptides by circular dichroism. Peptides for pNox1 and NoxA1 AD displayed characteristic random coil signals (negative dip below 200 nm and flat, near zero shoulders in the 210–220 nm range). Complex formation of the pNox1 peptide with NoxA1 AD peptide (pNox1-NoxA1) did not induce order and the circular dichroism signal is similar to that obtained by addition of the individual peptide signals (pNox1+NoxA1). Inset shows the residual curve of the pNox1:NoxA1 complex after subtraction of the individual components, indicating no major conformational changes of these peptides on complex formation. B, Affinity and stoichiometry for the interaction of NoxA1 AD with pNox1 (top) and an unphosphorylated Nox1 peptide (middle) as measured by isothermal titration calorimetry. Lower, analyzed binding data for both pNox1 and Nox1 binding to NoxA1 AD. Kd=100 μM for unphosphorylated Nox1 peptide (stoichiometry N.D.) and 1.5±0.3 μM for pNox1 peptide (stoichiometry of 0.86±0.06; n=3 independent experiments). C, Membrane recruitment of NoxA1 was assessed in CosPhox cells expressing p22phox, NoxA1, and wild-type (WT) or T429A Nox1. After treating with TNF-α, membrane fractions were isolated and blotted for NoxA1. Whole cell lysates (before membrane isolation) were probed with anti-GAPDH. n=4 independent experiments. *P<0.05 vs Nox1 vehicle; #P<0.05 vs Nox1 TNF-α-treated.
Nox1 that contain the AD.\textsuperscript{37} Computational docking of the NoxA1 AD peptide with Nox1 consistently demonstrates its occupancy in a long groove near T429 (Figure 6C). Based on these observations, we hypothesized that phosphorylation of Nox1 at T429 is necessary for the interaction with NoxA1 AD.

Using peptides containing phosphorylated Nox1 at T429 and the NoxA1 AD, we first demonstrated by circular dichroism that these peptides are unstructured. Specifically, these peptides lack the characteristic peaks indicative of $\alpha$-helices and $\beta$-sheets (Figure 7A). The presence of a negative peak below 200 nm and near zero shoulders at longer wavelengths (210–240 nm) demonstrates unstructured peptides. Furthermore, analysis of the complex of peptide-containing phosphorylated Nox1 at T429:NoxA1 peptides suggests subtle structural changes on binding without evidence of secondary structure (Figure 7A, inset).

To determine whether Nox1 AD directly interacts with Nox1 phosphorylated at T429, we used ITC to compare the affinity of the Nox1 AD peptide with either the peptide-containing phosphorylated Nox1 at T429 peptide or a corresponding unphosphorylated Nox1 peptide (Figure 7B). NoxA1 had no measurable interaction with the unphosphorylated Nox1 peptide (affinity >100 mmol/L; stoichiometry N.D.), whereas its affinity for the peptide-containing phosphorylated Nox1 at T429 peptide was 1.5±0.3 µmol/L with a stoichiometry of 0.86±0.06 (Figure 7B; n=3). These results indicate that phosphorylation of Nox1 mediates the interaction with the AD of NoxA1.

We next validated this interaction in an intact biological system. We assessed NoxA1 localization to the membrane in cells expressing either WT or T429A Nox1. In CosPhox cells expressing p22\textsuperscript{phox}, p47\textsuperscript{phox}, and NoxA1, the expression of WT Nox1 resulted in the anticipated TNF-\(\alpha\)-induced recruitment of NoxA1 to the membrane (Figure 7C). By contrast, mutation of T429 to alanine prevented NoxA1 membrane translocation after TNF-\(\alpha\). However, the phosphomimetic Nox1 T429D mutant promoted NoxA1 membrane recruitment under non-stimulated conditions (Online Figure VII). As expected, WT but not T429A Nox1 caused a recruitment of p47\textsuperscript{phox} to the membrane in response to TNF-\(\alpha\) treatment (Online Figure VIII). These data provide additional support for NoxA1 binding to phosphorylated T429 Nox1 in the mechanism of NADPH oxidase activation.

Discussion

Activation of Nox1 NADPH oxidase requires association with cytosolic proteins that function to organize the complex and activate the enzyme to produce superoxide. In this study, we identify for the first time that Nox1 activation is regulated by post-translational modification of the C-terminal region of Nox1. Our data demonstrate that phosphorylation of Nox1 at T429 by PKC-\(\beta\) is necessary for TNF-\(\alpha\)-mediated redox signaling and migration. Homology modeling combined with ITC revealed that Nox1 T429 phosphorylation facilitates association with the AD of NoxA1. Moreover, inhibition of T429 phosphorylation prevents recruitment of the cytosolic subunits to the membrane. Together with the observation that Nox1 is phosphorylated in multiple models of vascular disease, our findings suggest that strategies to inhibit Nox1 phosphorylation may mitigate its role in the pathogenesis of vascular disease.

Nox1 NADPH oxidase complex assembly is organized by p47\textsuperscript{phox}. The phosphoinositide-binding domain of p47\textsuperscript{phox} mediates membrane association, the Src homology 3 domains interact with p22\textsuperscript{phox}, and the proline-rich domain interacts with the Src homology 3 domain of Nox1.\textsuperscript{28,34} p47\textsuperscript{phox} and Noxa1 associate in the cytosol under basal conditions. Phosphorylation of p47\textsuperscript{phox} releases binding of an auto-inhibitory domain,\textsuperscript{48,49,50} allowing translocation of the p47\textsuperscript{phox}/NoxA1 complex to the Nox1/p22\textsuperscript{phox} complex, positioning the NoxA1 AD with the C-terminus of Nox1.\textsuperscript{18,23,34} Nox1, a homolog of p47\textsuperscript{phox} lacking the auto-inhibitory domain, seems to colocalize with Nox1 in resting cells at the membrane via its phosphoinositide-binding domain.\textsuperscript{20,40} Phosphorylation of each of the cytosolic subunits has been implicated in regulating complex assembly.\textsuperscript{20,41–47}

In contrast to the cytosolic subunits, less is known about phosphorylation of the catalytic subunits. Our study provides the first evidence for the phosphorylation of Nox1. The Nox2 C-terminal domain (within residues 321–405 and 466–570) has recently been shown to be phosphorylated at serine and threonine residues.\textsuperscript{48} Similar to Nox1, phosphorylation of Nox2 was associated with increased ROS production and complex assembly in response to agents that stimulate PKC. However, there seem to be important differences in phosphorylation-mediated activation of Nox1 and Nox2. First, despite significant homology in surrounding residues, the T429 we show to be phosphorylated on Nox1 is not conserved in Nox2. Second, although the specific residue phosphorylated on Nox2 is not identified, the investigators propose that Ser333, Thr509, and Ser550 are the most likely phosphorylation sites. Interestingly, Ser333 and Thr509, but not Ser550, are conserved between Nox2 and Nox1. Third, phosphorylation of Nox5 in the FAD domain (T494/S498) has also been shown to regulate its activity;\textsuperscript{51} although the mechanism is not clear, it will be distinct from that of Nox1 and Nox2 because Nox5 does not require complex assembly for activation.

Nox1 activation is important in mediating multiple cellular pathways involved in the pathogenesis of vascular disease.\textsuperscript{14} Our data demonstrate that migration of cultured VSMCs to TNF-\(\alpha\) requires Nox1. Cell migration is also regulated by the PKC family of serine/threonine kinases that include PKC-\(\beta I\).\textsuperscript{50} suggesting a functional link between Nox1 activation and PKC phosphorylation. Providing direct evidence for PKC-\(\beta I\) phosphorylation of Nox1, we found that recombinant PKC-\(\beta I\) phosphorylates a Nox1 peptide-containing T429 in vitro. Moreover, loss of Nox1 phosphorylation at T429 or the knock down of PKC-\(\beta I\) is sufficient to inhibit ROS production and cell migration. In contrast, the knock down of PKC-\(\beta I\) only partially inhibited TNF-\(\alpha\)-induced Nox1 phosphorylation, indicative of phosphorylation of Nox1 by other kinases. In support of this interpretation, Nox1 contains residues that are homologous with proposed Nox2 phosphorylation sites.\textsuperscript{48}

Our data provide evidence that Nox1 phosphorylation is a general mechanism underlying its activation in response to multiple agonists. Specifically, we observed increased ROS production in cells expressing WT but not T429A Nox1 after stimulation with TNF-\(\alpha\), platelet-derived growth factor-BB, or angiotensin II. Agonist-dependent activation of Nox1 resulted...
in greater superoxide production in cells coexpressing NoxA1 with p47\textsuperscript{phox} as compared with its homolog NoxO1, which is consistent with previous reports.\textsuperscript{16} Although phorbol myristate acetate induces Nox1 phosphorylation, the generation of superoxide does not require phosphorylation of T429. We speculate that this observation likely results from the different activation mechanisms between the receptor-dependent agonists and phorbol myristate acetate.

Until now, the molecular mechanism whereby Nox1 interacts with the NoxA1 AD was not known. Using ITC, we provide evidence that phosphorylation of Nox1 T429 increases the association of this region to the NoxA1 AD by >100-fold. Furthermore, the T429A mutant is not able to sustain association of the p47\textsuperscript{phox}/NoxA1 complex with the Nox1/p22\textsuperscript{phox} complex, whereas expression of the phosphomimetic T429D mutant results in NoxA1 membrane recruitment under nonstimulated conditions. We propose that assembly of Nox1 to Nox1 positions its AD within a long groove adjacent to the T429 of Nox1. Taken together, our data suggest that a negative change associated with phosphorylation at T429 is the principle mechanism that stabilizes NoxA1 with the membrane complex.

In conclusion, Nox1 requires phosphorylation at T429 for complex assembly, ROS generation, and VSMC migration. Our data support a mechanism by which PKC-\textbeta phosphorylation of Nox1 T429 facilitates interaction and stabilization of the NoxA1 AD with Nox1. Furthermore, we provide the first computational model of the Nox1 C-terminus and propose that the NoxA1 AD is positioned in a long groove near T429. In combination with our findings that Nox1 is phosphorylated in atherosclerosis, VSMC dedifferentiation, and neointimal formation, we identify the phosphorylation of Nox1 as a new target for effective and directed therapy of vascular disease.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- The Nox1-containing NADPH oxidase contributes to the pathogenesis of multiple diseases, including cardiovascular disease.
- Activation of the catalytic subunit Nox1 and subsequent generation of superoxide is a multistep process that requires assembly of a complex of proteins.

**What New Information Does This Article Contribute?**

- Phosphorylation of Nox1 is increased in a variety of animal models of vascular disease.
- Phosphorylation of Nox1 by protein kinase C-β1 at threonine 429 (T429) is necessary for the interaction of Nox1 with Nox1A1 activation domain.
- In vascular smooth muscle cells, phosphorylation of Nox1 at T429 is necessary for agonist-mediated superoxide generation and cell migration.

Generation of superoxide by the Nox1 NADPH oxidase propagates redox-dependent signaling events essential to the development of hypertension, restenosis, and atherosclerosis. Activation of Nox1 requires interaction with cytosolic proteins. The molecular events necessary for Nox1 activation, in particular the mechanism of interaction of Nox1 with the Nox1A1 activation domain, remain unclear. In this study, we examined the mechanism and consequences of Nox1 phosphorylation. Site-directed mutagenesis and isothermal titration calorimetry demonstrate that protein kinase C-β1 phosphorylates Nox1 at T429, which resides in an unstructured loop on the external surface of the cytosolic domain. Phosphorylation of Nox1 at T429 is necessary for complex assembly, superoxide production, and vascular smooth muscle cell migration. Furthermore, Nox1 T429 phosphorylation facilitates the association of Nox1 with the Nox1A1 activation domain. These data suggest that post-translational modification of the C-terminal region of Nox1 regulates its activity and that protein kinase C-β1-mediated T429 phosphorylation of Nox1 may be a novel target for directed therapy of vascular disease.
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Phosphorylation of Nox1 Regulates Association with NoxA1 Activation Domain

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Detailed Materials and Methods

Materials

The following materials were obtained from Sigma-Aldrich: TNF-α (T7539, T0157), Diphenyleneiodiiodium chloride (DPI, D2926), BME Vitamins (B6891), Trypsin inhibitor (T9128), Lucigenin (M8010), NADPH (100929-71-3). From Calbiochem: CalphostinC (CalC, 208725). From Biorad: 4-20% and AnyKD Gels (456-9033S, 456-1096). From Whatman: Nitrocellulose membranes (10402468). From Invitrogen/Life Technologies: Protein G Dynabeads (10003D), Opti-MEM (11058-021), To-Pro-3 (T3605), CM-H2DCFDA (C6827), RNAiMax (13778), DH5α Max Efficiency competent cells (18258-012), Lipofectamine 2000 (11668-019). From Roche: Protease inhibitor cocktail (1873580). From Fisher Scientific: Permunt (SP15-500). From Gibco: PBS (14190), HBSS (14025), DMEM (11965), Phenol red-free DMEM (31053), Pen Strep (15140), L-Glutamine (25030), MEM (11130), HEPES (15630), Trypsin (25200). From Atlanta Biologicals: Fetal Bovine Serum (FBS, S11550). From Vector Laboratories: Vectashield (H-1000), Vectashield plus DAPI (H-1200). From Santa Cruz: siPKC-β (sc-36255). From Ambion: siControl (AM4637). From Qiagen: Taq Polymerase (201203), QIAquick Gel Extraction Kit (28704), QIAquick PCR Purification Kit (28104), Qiagen Plasmid Maxi Kit (12162). From New England Biolabs: ECORI (R0101S), Xhol (R0146S), CIP (M0290S), T4 DNA Ligase (M0202S). The following antibodies were used: pSTY (Phosphoserine/threonine/tyrosine, Abcam ab15556), anti-Nox1 (Sigma SAB2501686 and Santa Cruz sc-5821), anti-FLAG-HRP (Sigma A8592), anti-FLAG (Sigma F1804), anti-p67phox (BD 610912), anti-GAPDH (Millipore MAB374); anti-PKC-βI (Santa Cruz sc-209), PKC-βII (Santa Cruz sc-210), anti-NoxA1 (Abcam ab68523); anti-p22phox (Santa Cruz sc-11712) and anti-p47phox (generously provided by Dr. William Nauseef, University of Iowa).

Animal Models

All procedures were approved by the Institutional Animal Care and Use Committee at University of Iowa and complied with the standards stated in the National Institutes of Health Guide for the
Care and Use of Laboratory Animals.

Primate Atherosclerosis: Frozen aortas from adult male Cynomolgus monkeys fed either a normal or atherosclerotic (AS) diet (0.7% cholesterol and 43% of total calories as fat) for 45 months as previously described

were prepared for immunoprecipitation/Western blotting (IP/WB) as described below.

Rat Balloon Injury: VSMCs isolated from the medial and neointimal layers of rat aortas following balloon injury as previously described

were cultured and prepared for IP/WB as detailed below.

Mouse Carotid Ligation: Carotid ligations were performed in C57Bl6/J mice as previously described. Five or 28 days following surgery, carotids were collected and tissues were prepared for IP/WB as detailed below. For IP/WB, for the 5-day time point, tissue from two mice was combined for one ligated and one control sample. For the 28-day time point, tissue from five mice was combined into two samples each for ligated and control samples.

Cultured Cells

Rat aortic medial and neointimal VSMCs were isolated and cultured in 10% Fetal Bovine Serum (FBS) in Dulbecco’s Modified Eagles Medium (DMEM) containing 1% BME vitamins, 2 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 20 mM HEPES, and 1% MEM non-essential amino acids. Cells were kept at 37°C and 5% CO₂. Wild-type (WT) and Nox1⁻/⁻ aortic VSMCs were isolated and cultured as above. A7r5 rat aortic vascular smooth muscle cell line from ATCC was cultured as above. CosPhox cells were a generous gift from Dr. Mary Dinauer (Washington University in St. Louis). These are Cos7 cells that stably express p22phox or p22phox with p47phox and p67phox. These cells are maintained on selective 10% FBS cell culture medium containing 0.2 mg/ml hygromycin, 0.8 mg/ml geneticin, and 1μg/ml puromycin.

Tissue Preparation

Monkey aorta and mouse carotid tissues were placed in NP40 lysis buffer (1% NP-40, 150 mM NaCl₂, 50 mM Tris, 2 mM EDTA, pH 7.2, 4% protease inhibitor cocktail, 1 mM sodium vanadate
and 1mM sodium flouride are added fresh) on ice, and homogenized using a Tissue Tearor (Biospec Products, model 985370). Cultured cells were washed in PBS, lysed with NP40 lysis buffer, and scraped into microcentrifuge tubes. All samples were centrifuged at 1000g for 5 m at 4°C, sonicated twice for 10 s at 10 Watts at 4°C, centrifuged at 5000g for 10 m at 4°C, and supernatant (SNT) was transferred to new tubes on ice. Protein concentrations were measured by Bradford assay, and concentrations of samples were normalized by addition of NP40 lysis buffer. Samples were then subjected to IP as detailed below.

**Immunoprecipitation**

Equal volumes of samples were pre-cleared with Protein G Dynabeads (10 μL/1.5 mg protein in lysate) for 30 m at 4°C, end-over-end rotation, followed by a quick spin at high speed, then placed on a magnetic rack 1 m. SNT was removed to a new tube on ice. The p22\textsubscript{phox}/Nox1 complex was immunoprecipitated using anti-p22\textsubscript{phox} antibody C-17 (1 μg/500 μg protein in lysate) and rotated for 45 m at 4°C. Next, 20 μL Protein G Dynabeads was added to each sample and rotated for 20 m at 4°C, followed by a quick spin at high speed, then placed on a magnetic rack 1 m. SNT was moved to a new tube on ice and frozen for later analysis. The Dynabead pellet was washed three times with 200 μL NP40 buffer, followed by addition of 15 μL twice with sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl, pH 6.8). Samples were shaken in an orbital shaker for 30 m. Samples were then analyzed by WB.

**Western Blotting**

One quarter volume of 5X sample buffer (6 g SDS, 40% glycerol, 30% 2-mercaptoethanol, 383.3 mg dithiothreitol, 372 mg EDTA, 378.3 mg Tris, 50 mg bromphenol blue in 50 ml ddH\textsubscript{2}O, pH 6.8) was added to lysates. All samples were mixed in an orbital shaker for 30 m at 250rpm. Samples were run on 4-20% or AnyKD gels @ 200 Volts for ~30 m, transferred to nitrocellulose membranes for 45 m at 100 Volts. Membranes were blocked in 3% BSA TBS (for Odyssey imaging) or 5% BSA TBS-T (for Kodak imaging) 1 h rocking, then probed with primary
antibodies for 1 h or overnight at 4°C, then washed in 1% BSA TBS-T five times for 5 m, then probed with appropriate infrared secondary antibody, washed twice for 5 m N-TBS, washed once with TBS and imaged by Odyssey. Anti-Flag-HRP was developed with a West-Femto kit (Thermo-Scientific) and imaged on a Kodak Image Station 4000R. Phosphorylation of Nox1 residues was determined utilizing a phoshpo-serine/threonine/tyrosine (p-STY) antibody that reacts with these residues only under phosphorylated conditions. This value was normalized to total Nox1. Utilization of two infrared secondary antibodies (with emissions at 700 or 800 nm) and Odyssey imaging allowed the visualization of both probes simultaneously to confirm that the phospho-signal corresponded with Nox1 (Online Figure I).

**Nox1 Phosphorylation in VSMCs**

C57Bl6/J VSMCs were either pre-treated with Calphostin C (CalC), or an equivalent volume of DMSO (vehicle). CalC was mixed to a final concentration of 100 nM in cell culture medium lacking FBS, added to cells, light activated for 10 m, incubated at 37°C in dark for 20 m, prior to addition of agonist. Cells were then treated with 10 ng/ml mouse TNF-α for 10 m at 37°C, washed three times in cold HBSS, lysed in NP40 lysis buffer and prepared for IP/WB.

**ROS Detection by Fluorescence**

C57Bl6/J WT or Nox1-/- VSMCs were seeded on glass chamber slides at 75% confluence, serum-starved in cell culture medium containing no FBS for 24 h, washed in phenol red-free DMEM, and incubated in 10 μM CM-H2DCFDA for 30 m, pretreated with CalC or DMSO, and treated with 10 ng/ml TNF-α for 30 m. The cells were then washed 3x 100 μl cold PBS, fixed with 2% paraformaldehyde for 15 m, washed twice with PBS, incubated in 1μM To-Pro-3 for 5 m in dark, and washed twice with PBS. The chambers were removed and cells were mounted in Vectashield and imaged by confocal microscopy.

**ROS Detection by Lucigenin-Enhanced Chemiluminescence**

A7r5 rat aortic smooth muscle cells were seeded at 75% confluence in 10% FBS medium, infected with AdNox1GFP for 48 h, serum-starved in 0.2% FBS medium for 24 h. Cells were
then subjected to a 30 m pre-incubation with 100 nM DPI or 100 μM CalC or DMSO, then treated with 10 ng/ml TNF-α for 10 m, washed three times in cold PBS, and homogenized in Homogenization Buffer (0.25 M sucrose, 10 mM triethanolamine, 4% protease inhibitor cocktail, 0.1 M EDTA, pH 7.4) with a Tissue Tearor. Lucigenin was then added to a final concentration of 5 μM. Samples were analyzed by a FB12 Luminometer every 10 s for 5 m, then NADPH was added to a final concentration of 100 μM and readings were taken for another 5 m. Average baseline relative light units (RLUs) were subtracted from the average NADPH-stimulated value.

For CosPhox cells, following either mock transfection or transfection with wild-type or mutant Nox1, membrane fractions were prepared and assayed for ROS detection by lucigenin-enhanced chemiluminescence as above. For measurement of superoxide levels in intact cells, CosPhox cells were transfected as above, the following day, cells were serum starved for 24 hours, collected and resuspended in Hank’s Balanced Salt Solution containing lucigenin (25 μM) and analyzed by FB12 Luminometer every 10 s for 5 m. Following collection of unstimulated RLUs, an agonist was added to the cells and chemiluminescence measured for an additional 5 m. For data analysis, the initial 1.5 m represented a dark adaptation period and the RLUs/sec over the next 3.5 m was averaged. Unstimulated values were subtracted from the agonist stimulated values and normalized to the number of cells per sample.

**Membrane Fraction Preparation**

Following serum starvation, cells were treated with 2 ml 20 ng/ml TNF-α in FBS-free medium or treated with FBS-free medium alone at 37°C for 10 m. Plates were then placed on ice, washed three times with cold HBSS, and lysed in 150 μl NP40 lysis buffer. Cells were scraped and transferred to a tube on ice, centrifuged at 1000g for 5 m at 4°C, sonicated twice for 10 s at 10 Watts, and centrifuged at 5000g for 10 m at 4°C. Supernatants (SNT) were transferred to new tubes on ice. The SNT was centrifuged at 16,000g for 30 m at 4°C. The SNT from this spin was transferred to a Beckman polycarbonate thick-walled ultracentrifuge tube and centrifuged at
100,000g for 1 h at 4°C. The SNT from this spin was aspirated and the pellet was resuspended in 100 μl NP40 buffer.

**Migration Assay**

WT or Nox1<sup>−/−</sup> C57Bl6/J mouse aortic VSMCs were pretreated with CalC or DMSO (vehicle) for 30 m, washed once with PBS, and cells were detached with trypsin, which was quenched with trypsin inhibitor. Cells were transferred to a conical tube, centrifuged at 500g for 5 m. The SNT was aspirated, and cells were resuspended in 125 μl cell culture medium containing no FBS. Cell counts were obtained on a Beckman Z1 Coulter Particle Counter. Cells suspensions were diluted to a concentration of 1,000 cells/μl. 50 μl of the cell suspension was added to the upper chamber of a Costar Transwell Permeable Support (8.0um polycarbonate membrane, 6.5mm insert). 500 μl medium containing no FBS with or without TNF-α was added to the lower chamber. Chambers were incubated for 5 h at 37°C. Medium was aspirated, Transwells were washed twice with PBS, non-migrated cells were removed from the upper surface of the membrane with a cotton swab, followed by washing twice with PBS. Cells were fixed in 4% paraformaldehyde, membranes were removed from support with a scalpel and mounted in Vectashield plus DAPI, cover-slipped and five random fields from each membrane were imaged at 20X and the number of cells migrated to the bottom of the Transwell was counted. Data are reported as average number of cells per field after subtracting the number of cells that migrated under unstimulated conditions for each group.

**Mass Spectroscopy**

Human aortic VSMCs were treated with TNF-α, cells were lysed and the lysate was subjected to IP with anti-p22<sub>phox</sub> antibody. The IP was then subjected to SDS-PAGE to separate proteins. The gel was stained with Coomassie blue and bands were excised and subjected to MALDI-TOF mass spectroscopy.

**siPKC-β**
500 μl Opti-MEM was added to a 60 mm gelatin-coated cell culture plate. To this siPKC-β or siControl was added (final concentration 200 nM), plus 12 μl RNAiMax. Plates were rocked and incubated for 20 m. Cells were detached by use of trypsin, which was quenched in 10% FBS containing no antibiotics, centrifuged at 500g for 5 m, resuspended in 5 ml 10% FBS without antibiotics and counted. Cells were added to plate for a seeding density of 75%, and medium was added to a final volume of 3 ml. Cells were incubated for 5 h at 37°C, then medium was changed to 10% FBS. The next day, cells were serum starved in medium containing no FBS for 24 h. Cells were then prepared for analysis by WB, DCF fluorescence or migration assay.

**Nox1 Mutant Constructs**

pcDNA3.1 containing rat Nox1 (kindly provided by Dr. Kathy Griendling, Emory University) was used as a template for generating the Nox1 mutant constructs using the following primers:

- Nox1T89A-F: 5'-GCT-CAT-TTT-GCA-ACC-ACG-CGC-TGA-GAA-AGC-CAT-TG-3’
- Nox1T89A-R: 5’-CAA-TCC-CTT-TCT-CAG-CGC-GTG-GTT-GCA-AAA-TGA-GC-3’
- Nox1T429A-F: 5’-CGT-GCA-CAC-AAC-AAG-CTG-AAA-GCA-CAA-AAG-ATC-TAT-TTC-TAC-3’
- Nox1T429A-R: 5’-GTA-GAA-ATA-GAT-CTT-TTG-TGC-TTT-CAG-CTT-GTT-GTG-TGC-ACG-3’
- Nox1T429D-F: 5’-CGT-GCA-CAC-AAC-AAG-CTG-AAA-GAC-CAA-AAG-ATC-TAT-TTC-TAC-3’
- Nox1T429D-R: 5’-GTA-GAA-ATA-GAT-CTT-TTG-GTC-TTT-CAG-CTT-GTT-GTG-TGC-ACG-3’

Constructs were generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene #200523) or QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene #200514) according to manufacturer’s instructions. DNA was sequenced using the Applied Biosystems Model 3730 to ensure mutations were present at correct locations.

**Flag Tagging Constructs**

Wildtype Nox1 constructs were modified to include a C-terminal Flag tag by using PAGE purified primers, Forward-5’-GAATTCCCTGGAAAAGAGATGGACGAATTAGGCAA-3’

Reverse-5’-
CAACTCCTCGAGTCCTTATCGTCGTCATCCTTGTAATCGAACGTTTCTTTGTTGAAGTAGA
ATT-3’ which included the EcoRI and XhoI restriction sites using Taq Polymerase. The amplified
region was gel purified using the QIAquick Gel Extraction Kit. The original constructs and the
amplified region which included the Flag tag were digested with EcoRI and XhoI at 37°C for 3 h.
The original constructs that had been cut were then dephosphorylated using CIP for 1 h at
37°C. Both the dephosphorylated vector and amplified regions were cleaned up with the
QIAquick PCR Purification Kit. The two products were ligated with T4 DNA Ligase at 16°C
overnight. The reaction was transformed in to DH5α Max Efficiency competent cells, plated on
LB-Agar ampicillin plates and grown overnight at 37°C. Colonies were picked, grown in LB
overnight and the DNA was isolated the following day using the Qiagen Plasmid Maxi Kit. The
presence of the Flag tag was confirmed by sequencing using the Applied Biosystems Model
3730.

CosPhox Cell Transfection
On day 1, cells were seeded in 60mm cell culture plates at 55% confluence. Day 2, cells were
transfected with 4 μg DNA and 12 μl Lipofectamine 2000. Opti-MEM with 50 mM CaCl₂ was
used for transfections. 4 μg DNA was added to 400 μl and 12 μl Lipofectamine 2000 was diluted
in 400 μl for 10 m. These were mixed and allowed to incubate for 30 m. An additional 1.2 ml
was added for a final volume of 2 ml per transfection solution for each construct per plate. Cells
were incubated for 4 h, aspirated, washed once in 10% FBS medium, and incubated in 2 ml
10% FBS medium overnight. Day 3, cells were serum starved in cell culture medium containing
no FBS for 48 h. In some experiments, cells that contained only p22phox were co-transfected with
p47phox or NoxO1 and NoxA1 (kindly provided by Drs. Botond Banfi and William Nauseef,
University of Iowa) at the time of Nox1 transfection.

VSMC Transfection
On day 1, 4 μg DNA, 7.07 μl Plus Reagent, and 17.66 μl Lipofectamine LTX were incubated in
5.625 ml Optimem for 30 m. Meanwhile, VSMCs were trypsinized, and trypsin was quenched
with 10% FBS cell medium without antibiotics. The appropriate volume of cells was added to the DNA mixture in order to achieve 75% cell density per 60 mm cell culture plate. After 4 h, the DNA mix was aspirated and cells were serum starved in cell culture medium containing no FBS for 48 h. In some experiments (Online Figure VI), cells were co-transfected with p22<sup>phox</sup>, p47<sup>phox</sup>, and NoxA1 at the time of Nox1 transfection.

**Complex Assembly Assay**

Cells were treated with 10 ng/ml TNF-α for 10 m. Cells were then lysed, membrane fractions were prepared, and samples were analyzed for recruitment of p47<sup>phox</sup> and NoxA1 to the membrane fraction by WB.

**Isothermal Titration Calorimetry In Vitro Kinase Assay**

The ability of PKC-βI to phosphorylate threonine 429 on Nox1 was assessed by isothermal titration calorimetry (ITC) using the single injection method described by Todd and Gomez. For these experiments, the unphosphorylated Nox1 peptide was resuspended in a buffer containing 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM NaCl<sub>2</sub>, pH 7.4. The Nox1 peptide was diluted to a concentration of 1mM and the PKC-βI was diluted to a concentration of 10 fM in a buffer containing 20 mM HEPES, 10 mM MgCl<sub>2</sub>, 3 mM CaCl<sub>2</sub>, 1 mM DTT, 5 μg/ml diacylglycerol, 10 mM ATP, 150 μg/ml phosphatidylserine. The peptide and protein solutions were degassed and ITC measurements were recorded using a MicroCal VP-ITC System. 30 μL injections of Nox1 peptide into the PKC-βI solution were performed with 30 m spacing between events. The chamber was kept under constant stirring at 350 rpm and all experiments were performed at 25°C.

**Isothermal Titration Calorimetry Peptide Interaction**

The affinity of the interaction between non-phosphorylated and phosphorylated Nox1 and NoxA1 was determined by ITC. Phosphorylated or non-phosphorylated peptides corresponding to amino acids KLKTQKIYF of Nox1 and a peptide corresponding to amino acids LEPMDFLGAKV of NoxA1 peptides were purchased from ProImmune. The peptides were resuspended in 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM NaCl<sub>2</sub>, pH 7.4 to a final concentration of 800 μM.
(Nox1) and 20 μM (NoxA1). The peptides were degassed and ITC measurements recorded using a MicroCal VP-ITC System (GE Healthcare). 21 injections of Nox1 peptides into NoxA1 peptide were used, with 240 s spacing between events. The chamber was kept under constant stirring at 350 rpm and all experiments were performed at 25°C. Control experiments where Nox1 peptides were injected into buffer showed that the heats of dilution were constant across all injections. The constant heat of dilution, as determined by the average of the last 3-5 injections, was subtracted and the data are analyzed using the single site binding model provided in the ITC analysis package. The values for affinity and stoichiometry from three experiments were averaged and shown ± standard deviation.

**Dot Blot**

Nitrocellulose membranes measuring 1cm² were placed in a 96-well plate, incubated in 100 μl kinase assay input, product, or 1 mM Nox1 phospho-peptide solution in duplicate and allowed to dry overnight. One set of membranes were then incubated for 1 h in 300 μl Odyssey Blocking Buffer, 1 h in 300 μl p-STY antibody solution (1:100 in Odyssey Blocking Buffer), washed 5 times for 5 m in 300 μl TBS with 1% NP40, incubated 1 h in 300 μl secondary antibody solution (Licor infrared 680 1:1000 in Odyssey Blocking Buffer), washed 5 times for 5 m in 300 μl TBS with 1% NP40. The other set of membranes were incubated in Ponceau stain for 5 m and washed 5 times in ddH₂O. All membranes were imaged by Odyssey.

**Computer Modeling**

A homology model of the rat cytosolic C-terminal FAD-NADPH binding domain (residue 290-563 NP446135.1) was generated using the hm_build.mcr macro in Yasara Structure 13.1.25 (http://www.yasara.org). The highest scoring templates used by Yasara were the cytochrome B5 reductase crystal structure (PDB ID 2EIX) for the FAD domain and the Nox2 crystal structure (PDB ID 3A1F) for the NADPH domain. A phosphorylated T429 version of the Nox1 cytosolic C-terminal domain was also generated and both homology models energy minimized using the NOVA force field in Yasara. Docking of the NoxA1 AD peptide to the homology models
generated above was performed using the AutoDock\textsuperscript{9} derivative implemented in Yasara. Docking was restricted to a 32 Å cubic cell centered on T429 of Nox1. The docked poses were visualized and ray-traced in PyMOL 1.6 (Schrödinger).

**Circular Dichroism**

Circular dichroism (CD) analysis was performed to investigate secondary structure of a Nox1 peptide containing T429 in a non-phosphorylated (KLKTKQIYF) or phosphorylated (KLK-phospho-T(429)QKlYF) state. CD analysis was also performed on the NoxA1 AD peptide individually and in a 1:1 complex with the Nox1 peptides. The peptides were prepared in buffer containing 20 mM Na$_2$HPO$_4$, 100 mM NaCl$_2$, pH 7.4. CD spectra were collected on 100 μM samples in a 1-mm cuvette at 25°C over the range of 190-250 nm with an interval of 1 nm and a scan speed of 100 nm/m using a Jasco J-815 CD spectrophotometer.

**Statistical Analysis**

Results are expressed as mean ± SEM. Statistical comparisons were performed by a two-tailed t-test, or one-way or two-way analysis of variance (ANOVA) with appropriate post-hoc analysis. A $p$ value of < 0.05 was considered significant.
Supplemental References


Online Figure I. Immunoblotting for phospho-serine/threonine/tyrosine (p-STY). (A) Co-localization of Nox1 and p-STY signals by Odyssey imaging. Normal and atherosclerotic monkey carotid homogenates were immunoprecipitated using p22\textsuperscript{phox} antibody to pull down the Nox1/p22\textsuperscript{phox} complex. Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with Nox1 (goat) antibody and p-STY (mouse) antibody, followed by incubation in infrared secondary antibodies detected by the Odyssey imaging system: anti-mouse 800 green (upper), anti-goat 700 red (middle), and merged image (lower). (B) Specificity of p-STY antibody. HEK293 cells were treated with TNF-\(\alpha\) (10 ng/ml) for 10 minutes. The cells were lysed in the presence of phosphatase inhibitors (Lane 2) or the absence of phosphatase inhibitors and with addition of calf intestinal phosphatase (Lane 3). Lysates were incubated at 37\(^\circ\)C for 10 minutes then centrifuged to obtain membrane fractions and immunoblotted for p-STY (left blot) or Nox1 (right blot). Lane 1 shows molecular weight (MW) markers and the indicated kD.
Online Figure II. *Identification of putative Nox1 phosphorylation sites by NetPhosK*. Method: NetPhosK without ESS (Evolutionary Stable Sites) filtering. Input: Rat Nox1 sequence. Score: Output score in the range of 0.000-1.000. A higher score indicates a higher confidence of prediction. Highlighted residues (T429 and T89) were examined in this study.

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Online Figure III. Phosphorylation of Vascular Smooth Muscle Cell Nox1. A7r5 rat vascular smooth muscle cell line (A, C) and wild-type mouse aortic smooth muscle cells (B) were treated with various agonists for indicated times and cell lysates immunoprecipitated using p22phox antibody to pull down the Nox1/p22phox complex. Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted for p-STY, then the membrane stripped and blotted for total Nox1. (A) PDGF-BB (10 ng/ml), (B) TNF-α (10 ng/ml), PMA (10^{-6} M) for 10 minutes; (C) TNF-α (10 ng/ml) + IL-1β (10 ng/ml).
Online Figure IV. Analysis of PKC-βI and II expression in VSMC. (A) PKC-βI and (B) PKC-βII protein expression was assessed by Western blotting in cell lysates from CosPhox cells (Cos), C57 mouse aortic vascular smooth muscle cells (VSMCs) from wild-type (WT) and Nox1⁻/⁻ mice, and A7r5 rat vascular smooth muscle cell line. MDA MB 231 (MDA) breast cancer cell lysate was used as a positive control in (A) and (B). C57 mouse aorta and femoral artery tissue lysates were used as positive controls in (B). GAPDH, loading control.
Online Figure V. Sequence alignment of mouse, rat and human Nox1. Nox1 transmembrane domains (TM) are indicated in light gray, denoted as I-VI. Intracellular loops are indicated in green. Extracellular domains are indicated in blue. The C-terminal domain is not shaded. Predicted phosphorylation sites that were analyzed in this study are indicated in red and are conserved across mouse, rat and human Nox1.
Online Figure VI. Validation of Nox1 mutant protein expression in (A) CosPhox cells and (B) Nox1^{-/-} VSMCs. (A) Expression of flag-tagged Nox1 mutants was examined by blotting CosPhox cell lysates with anti-Flag. GAPDH, loading control. (B) Expression of flag-tagged Nox1 mutants in Nox1^{-/-} VSMCs was examined by immunofluorescence using anti-Flag primary antibody followed by anti-mouse Alexa Fluor568 secondary antibody (red); nuclei were counter-stained with ToPro3 (blue).
Online Figure VII. Association of NoxA1 with Nox1 under resting conditions. CosPhox cells were transfected with NoxA1 and p47phox. Cells were also transfected with control plasmid, or the Nox1 T429A or Nox1 T429D mutant and 48 hours later (5% serum) membrane fractions were immunoblotted for NoxA1 and for Nox1.
**Online Figure VIII.** Role for Nox1 T429 in recruitment of $p47^{phox}$ and NoxA1 to the membrane. Nox1-/- VSMCs were transfected with WT or mutant Nox1, p22$^{phox}$, $p47^{phox}$, and NoxA1. Cells were treated with TNF-α, and then membrane fractions isolated and blotted for $p47^{phox}$ or NoxA1. Whole cell lysates (prior to membrane isolation) were probed with anti-GAPDH as the loading control.