Nox4 and Nox2 NADPH Oxidases Mediate Distinct Cellular Redox Signaling Responses to Agonist Stimulation

Narayana Anilkumar, Roberta Weber, Min Zhang, Alison Brewer, Ajay M. Shah

Objectives—The NADPH oxidase isoforms Nox2 and Nox4 are coexpressed in many cell types and are implicated in agonist-stimulated redox-sensitive signal transduction. We compared the involvement of Nox2 versus Nox4 in redox-sensitive protein kinase activation after agonist stimulation.

Methods and Results—We transfected HEK293 cells with Nox2 or Nox4 and compared ROS production and activation of mitogen activated protein kinases (MAPKs), Akt, and GSK3β after acute agonist stimulation. Nox4 overexpression substantially increased basal ROS generation whereas ROS generation in response to angiotensin II and tumor necrosis factor (TNF)α was enhanced in Nox2-overexpressing cells. Nox4 overexpression induced basal activation of ERK1/2 and JNK whereas Nox2-transfected cells showed a modest increase in p38MAPK activation. After angiotensin II or TNFα treatment, JNK activation was augmented in Nox2 but not Nox4-transfected cells, whereas insulin augmented phosphorylation of p38MAPK, Akt, and GSK3β specifically in Nox4-overexpressing cells and JNK specifically in Nox2-overexpressing cells.

Conclusions—These data indicate that Nox2 and Nox4 exhibit distinctive patterns of acute activation by angiotensin II, TNFα, and insulin and regulate the activation of distinct protein kinases. (Arterioscler Thromb Vasc Biol. 2008;28:1347-1354)

Key Words: NADPH oxidase ■ redox signaling ■ protein kinase ■ reactive oxygen species

ADPH oxidases (Noxs) are superoxide (O₂⁻)-generating enzymes that catalyze electron transfer from NADPH onto molecular O₂. Each Nox family member contains a distinct Nox subunit (Nox1–5). The prototypic member of the family, Nox2 oxidase, is made up of a heterodimer comprising Nox2 (also known as gp91phox) and a 22-kD subunit p22phox. It is involved in antimicrobial defense in neutrophils but is also expressed in several nonphagocytic cells. Activation of Nox2 oxidase requires the association of several regulatory subunits—namely p47phox, p67phox, p40phox, and Rac1 or Rac2—with the heterodimer. Recently, 4 other Nox oxidases expressed in a wide variety of nonphagocytic cells were identified, which are based on Nox1, Nox3, Nox4, and Nox5 catalytic subunits, respectively, but whose requirements for other components varies. In the cardiovascular system, Nox1 is expressed mainly in vascular smooth muscle cells (VSMCs)³–⁵; Nox2 in endothelial cells,⁶–⁸ cardiomyocytes,⁶–⁸ fibroblasts,⁹ and some VSMCs¹⁰; Nox4 in endothelial cells,¹¹ VSMCs,¹² cardiomyocytes,¹³,¹⁴ and fibroblasts,¹⁵ and Nox5 in human endothelial cells.¹⁶

Numerous studies have reported important roles for Nox-derived reactive oxygen species (ROS) in agonist-stimulated redox-sensitive signal transduction, eg, the activation of mitogen-activated protein kinases (MAPKs), kinases such as Akt, transcription factors (eg, NF-κB), and matrix metalloproteases. Such redox signaling is implicated in VSMCs and cardiac hypertrophy, endothelial activation, angiogenesis, and atherosclerosis. However, the specific roles of individual Noxs remain to be fully clarified. Studies undertaken to date suggest that individual Noxs may subserve distinct functions in cells that coexpress more than one isoform. For example, Nox1 mediates angiotensin II–induced VSMC hypertrophy, but Nox4 (which is also abundantly expressed in VSMC) is unable to mediate this response. Angiotensin II–induced cardiomyocyte hypertrophy or VEGF-induced endothelial cell migration are both specifically mediated by Nox2 but not Nox4 (which is also expressed in these cells). Similarly, Nox1²¹ and Nox2²² knockout mice exhibit distinct vascular phenotypes despite the presence of other Nox isoforms.

Taken together, these data suggest that individual Nox isoforms may exhibit distinct cellular effects. The mechanisms underlying such isoform-specific effects remain to be defined but could include: (1) agonist-specific activation of individual Noxs; (2) modulation of distinct downstream signaling targets by individual Noxs; or (3) distinct subcellular localization. In this regard, it is notable that Nox4 activation does not require p47phox, p67phox, or Rac unlike Nox2/Nox1 activation. Localization of Nox1/Nox2 versus Nox4 may also be different; Nox2 reportedly associates

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with the plasma membrane or perinuclear membranes whereas Nox4 may be localized to focal adhesions,19 the perinuclear endoplasmic reticulum,23 or the nucleus.25

The present study aimed to compare Nox4- versus Nox2-specific redox signaling in response to agonist stimulation in a defined cellular system.

Methods
Detailed methods are provided in the Data Supplement (available online at http://atvb.ahajournals.org).

Cell Transfection
Nox2 and Nox4 cDNA were cloned into pcDNA3.1. C-terminal c-myc–tagged Nox2 and Nox4 constructs were cloned into pCS2-Myc. Expression plasmids were transfected using Lipofectamine 2000 and experiments performed 48 hours later.

Nox4 Antibody and Immunoblotting
We generated and characterized an affinity-purified rabbit polyclonal antibody against a 12 amino acid peptide corresponding to Nox4 residues 556 to 568 (supplemental Figure I). Immunoblots for phosphorylated forms of protein kinases were performed using phosphospecific antibodies and normalized to the amount of total protein kinase detected with nonphosphospecific antibodies. For analyses of the effect of Nox2 or Nox4 overexpression, densitometric data were compared to the protein level in control transfected cells, which was arbitrarily taken as 1 U. Data shown are from at least 4 independent experiments for each condition.

Statistics
Data are mean±SE. Statistical analyses were performed by 2-way ANOVA or 2-tailed Student t test, as indicated. P<0.05 was considered significant.

Results

Nox4 Antibody Characterization
Immunoblots of Nox4-transfected HEK293 cells showed a specific 65-kDa band corresponding to the predicted molecular size of Nox4 (supplemental Figure IB, left panel). A weak band at the same size was observed in control and Nox2-transfected cells, which most likely represents endogenous Nox4. A 35-kDa band was also observed in Nox4-transfected cells which may represent a Nox4 degradation product. HEK293 cells transfected with Nox4Myc cDNA demonstrated an identical 65-kDa protein band when probed with an anti-Myc antibody (supplemental Figure IB, middle panel), whereas Nox2Myc-transfected cells showed bands at 75 to 80 kDa (supplemental Figure IB, right panel), consistent with the reported mobility of glycosylated Nox2. These data confirm that the Nox4 antibody does not cross-react with Nox2.

Nox4Myc-transfected HEK293 cells costained with anti-Nox4 and anti-Myc antibodies showed an identical perinuclear staining pattern by immunofluorescence (supplemental Figure IC). A similar staining pattern for Nox4 was found in Cos-7 cells transfected with Nox4Myc and for endogenous Nox4 in human microvascular endothelial cells. Preimmune serum used as a negative control did not show any staining (data not shown).

Nox4 and Nox2 Localization
Nox4-transfected HEK293 cells colabeled with the polyclonal anti-Nox4 antibody and a monoclonal anticalnexin antibody (as an endoplasmic reticulum [ER] marker) showed colocalization by confocal microscopy (supplemental Figure II). In contrast, in Nox2-transfected HEK293 cells, a significant proportion of Nox2 was not colocalized with calnexin but was distributed to the plasma membrane (supplemental Figure II).

Expression of Endogenous Noxs and Regulatory Subunits
Nox4, Nox2, p22phox, p47phox, and p67phox were all expressed at mRNA and protein level in HEK293 cells (supplemental Figure III). Nox2 mRNA expression was approximately 160-fold higher than Nox4 by quantitative real-time polymerase chain reaction (data not shown).

ROS Generation After Nox4 and Nox2 Transfection
Overexpression of Nox4 in HEK293 cells induced an ∼70% increase in NADPH-dependent O₂⁻ generation (lucigenin chemiluminescence) under basal conditions, whereas Nox2 overexpression did not increase basal O₂⁻ (Figure 1A). Comparable results were found in cells transfected with Myc-tagged Nox4 and Nox2 constructs, and ROS production by Nox4-overexpressing cells was unaltered by cotransfection of either constitutively active Rac1 (V12Rac1) or a dominant negative mutant (N17Rac1; data not shown). Nox4-transfected cells showed a significant increase in basal H₂O₂...
generation, but no basal increase was found in Nox2-transfected cells (Figure 1D).

Acute exposure to phorbol-12-myristate-13-acetate (PMA, 50 nmol/L; 30 minutes) induced a marked increase in ROS generation in Nox2-overexpressing but not Nox4-overexpressing cells (Figure 1B and 1C). A small PMA-induced increase in ROS generation in control (empty vector-transfected) cells may be attributable to endogenous Nox2.

**Effect of Nox2 Versus Nox4 on MAPK Activation**

Nox4 overexpression caused a significant 2.7-fold increase in ERK1/2 activation and a 3-fold increase in JNK activation compared to control cells (Figure 2A and 2B). In contrast, Nox2 overexpression was associated with modest increases in phospho-p38MAPK and phospho-JNK but no other significant changes in kinase phosphorylation. Preincubation of cells with the antioxidant butylated hydroxyanisole (BHA, 50 μmol/L; 30 minutes) significantly inhibited these increases (Figure 2C).

**Effects of Agonist Stimulation on ROS Production**

We examined the responses to angiotensin II (1 μmol/L, 30 minutes), insulin (50 nmol/L, 30 minutes) or TNFα (10 nmol/L, 30 minutes), which have each been implicated in NADPH oxidase activation in various settings but act through different receptor-mediated pathways. The HEK293 cells studied were confirmed to express angiotensin AT1 receptors by RT-PCR and immunoblotting, and it was demonstrated that angiotensin II–induced changes in ROS were inhibited by the AT1 antagonist, losartan (10 μmol/L; supplemental Figure IV).

Angiotensin II increased NADPH-dependent O2⁻ generation in control transfected cells by ~35%, whereas Nox2-transfected cells showed an ~100% increase (Figure 3A). In marked contrast, angiotensin II had no additional effect on ROS generation in Nox4-transfected cells (Figure 3A). Similar results were obtained when measuring H2O2 levels (Figure 3D).

Insulin (50 nmol/L) increased ROS generation ~1.8-fold in both control and Nox2-transfected cells (Figure 3B). In
Nox4-overexpressing cells, insulin significantly increased ROS generation such that the total level was \( \sim 30\% \) greater than in mock or Nox2-transfected cells (Figure 3B).

On the other hand, TNF\( \alpha \) induced an \( \sim 100\% \) increase in ROS generation in Nox2-transfected cells compared to \( \sim 50\% \) in mock-transfected cells but had no significant additional effect in Nox4-transfected cells (Figure 3C).

**Effects of Agonist Stimulation on Kinase Activation in Nox2- Versus Nox4-Transfected Cells**

We investigated the effects of angiotensin II (1 \( \mu \)mol/L, 30 minutes), insulin (50 nmol/L, 30 minutes), or TNF\( \alpha \) (10 nmol/L, 30 minutes) on the activation of specific protein kinases.

Angiotensin II increased phospho-ERK1/2 expression to a similar extent in control and Nox2-transfected cells, and this was inhibited by BHA in both cases (Figure 4A and 4B). In Nox4-transfected cells, the already elevated level of phospho-ERK1/2 was not further enhanced by angiotensin II but was inhibited by BHA. Angiotensin II had no effect on p38MAPK phosphorylation in control or Nox4-transfected cells (Figure 4A and 4B). In Nox2-transfected cells, the modest basal increase in phospho-p38MAPK was not further affected by angiotensin II but was inhibited by BHA. JNK phosphorylation was significantly increased by angiotensin II in Nox2-overexpressing compared to control cells and was inhibited by BHA, whereas the already elevated level of phospho-JNK in Nox4-overexpressing cells was not further increased by angiotensin II but was inhibited by BHA (Figure 4A through 4C). Angiotensin II had no effect on phospho-Akt or phospho-GSK3\( \beta \) in any group (Figure 4A and 4B).

Insulin also increased the level of phospho-ERK1/2 in control and Nox2-overexpressing cells in a BHA-sensitive fashion but did not affect the already elevated level in Nox4-overexpressing cells (supplemental Figure V). In contrast to angiotensin II, insulin significantly increased phospho-p38MAPK levels in Nox4-transfected cells, an effect inhibited by BHA (supplemental Figure V). However, insulin had no additional effect in control or Nox2-overexpressing cells. A marked BHA-inhibitable increase in phospho-JNK was observed after insulin treatment of Nox2-overexpressing cells, whereas there was no effect in control or Nox4-overexpressing cells. The most striking effect of insulin was a marked increase in phospho-GSK3\( \beta \) and phospho-Akt levels in Nox4-overexpressing cells, which was significantly greater than that found in control and Nox2-overexpressing cells (supplemental Figure V). Both these effects were inhibited by BHA.

As found with angiotensin II and insulin, TNF\( \alpha \) significantly increased phospho-ERK1/2 levels in control and Nox2-overexpressing cells, which were inhibited by BHA (supplemental Figure VI). There was no specific effect on phospho-ERK1/2 in Nox4-overexpressing cells. Phospho-JNK levels were increased by TNF\( \alpha \) only in Nox2-overexpressing cells (supplemental Figure VI). Finally, no significant TNF\( \alpha \)-induced changes in phospho-Akt levels were found in any group, whereas phospho-GSK3\( \beta \) levels increased modestly in Nox4-overexpressing cells (supplemental Figure VI).

**Effects of SOD and Catalase on Kinase Activation**

To further confirm the role of ROS in Nox-dependent kinase activation and assess the relative roles of \( \text{O}_2^- \) versus \( \text{H}_2\text{O}_2 \), we
studied the effects of polyethylene glycol (PEG)-SOD and PEG-catalase on responses to angiotensin II (Figure 5). The effects of angiotensin II on p-extracellular signal regulated kinase (ERK), p-p38MAPK, and p-JNK in Nox2- and Nox4-transfected cells were similar to those described above. Angiotensin II–induced Nox-dependent increases in kinase activation were found to be inhibited by PEG-catalase but were unaffected by PEG-SOD, suggesting that they involved generation of H2O2.

**Figure 4.** Effect of angiotensin II stimulation on kinase activation. A, Representative immunoblots. B, Relative change in phosphoprotein expression normalized by the level of pan-protein. 5 experiments per group. *P<0.05 comparing agonist-treated versus untreated for each group. #P<0.05 for significant reduction after BHA.

Nox2-Dependent Kinase Activation in Intact Tissue

Finally, to assess whether kinase activation in vascular cells in situ is also Nox isoform-selective, we studied the acute response to angiotensin II (100 nmol/L, 30 minutes) in aortic segments isolated from Nox2−/− mice and matched wild-type mice. Aortae from both groups contained abundant Nox4 (data not shown). Angiotensin II induced activation of ERK1/2 and p38MAPK in wild-type aorta but not in Nox2−/− (Figure 6A). However, these kinases could be activated by exogenous H2O2 in aorta from KO mice (Figure 6B), suggesting that it was the absence of Nox2 that was responsible for the lack of kinase activation in response to angiotensin II.

**Discussion**

NADPH oxidases are increasingly recognized as important mediators and modulators of intracellular signal transduction pathways involved in atherosclerosis, VSMCs and cardiac hypertrophy, endothelial activation, and other conditions.3,18 However, the specific effects of different Nox isoforms coexpressed in the same cell type on redox-sensitive signal transduction remain poorly defined. In particular, it remains unclear whether individual Nox isoforms are activated by distinct stimuli in the same cell type and whether they are coupled to distinct downstream signaling pathways. The key novel findings of this study are that (1) acute angiotensin II, insulin, and TNF-α differentially activate Nox2 and Nox4; (2) Nox4 versus Nox2 overexpression has distinct effects on basal MAPK activation; and (3) distinct patterns of downstream redox-sensitive kinase activation are evoked by agonist stimulation of Nox2 versus Nox4. Taken together, these results indicate a high potential for Nox isoform-specific signaling, even in the same cell type, and imply that such signaling is likely to be compartmentalized.

We used the HEK293 cell experimental system to specifically compare Nox2 and Nox4 localization, responses to agonist stimulation, and the effects of Nox2- versus Nox4-dependent ROS generation on the activation of several kinases. Using an antibody generated against a conserved Nox4 C-terminal peptide sequence, as well as Myc-tagged constructs, we found that Nox4 colocalized with the ER whereas Nox2 demonstrated a significant plasma membrane-associated as well as intracellular staining. The classical Nox2 oxidase is known to be plasma membrane–associated in phagocytic26 and nonphagocytic cells,18 although a proportion of the enzyme is also found in an intracellular perinuclear localization.5,23 In contrast, the subcellular localization of Nox4 remains ambiguous. It has variously been reported to localize to focal adhesions,19 the ER-associated perinuclear region,23,27 stress fibers,19,28 and the nucleus19,25 in different cell types. We observed a clear ER-associated localization but did not find any nuclear or plasmalemmal localization in HEK293 cells. The divergent localization of Nox2 versus Nox4 in HEK293 cells may contribute to the different signaling responses observed after activation of these isoforms.

A significant increase in ROS generation occurred after Nox4 overexpression in HEK293 cells whereas basal ROS levels were not significantly altered by Nox2 transfection, whether measured by lucigenin chemiluminescence or an HVA assay. This was unlikely to be attributable to limitation of cytosolic subunits for Nox2 activation, because these were readily detectable in the cells and Nox2-overexpressing cells displayed increased ROS generation after acute stimulation with PMA, angiotensin II, or TNFα. In contrast, none of these...
agonists increased ROS generation by Nox4-overexpressing cells. These results are consistent with other recent studies which found increased ROS generation on cellular Nox4 transfection in the absence of agonist stimulation, as well as data that Nox4 does not require either the cytosolic subunits or Rac.23,24 Interestingly, ROS generation in Nox4-overexpressing cells was increased by insulin. Although no clear mechanism for acute Nox4 oxidase activation has been defined, this result is consistent with prior data suggesting acute Nox4 activation by insulin in adipocytes.29

Figure 5. Effect of PEG-SOD and PEG-catalase on angiotensin II–induced kinase activation. A, No agonist; B, Angiotensin II alone; C, Angiotensin II plus PEG-SOD; D, Angiotensin II plus PEG-catalase. Relative changes in phosphoprotein expression, with representative immunoblots. 4 experiments per group. *P<0.05 for effect of angiotensin II. #P<0.05 for reduction after PEG-catalase.

Figure 6. Requirement of Nox2 for kinase activation in wild-type (WT) and Nox2-knockout (KO) aortae. A, Immunoblots showing effect of acute exposure to angiotensin II (Ang II) for 30 minutes. B, Effect of exogenous H2O2 in Nox2 KO aorta.

Both Nox2 and Nox4 are reported to be involved in various agonist-stimulated signal transduction pathways, but their specific roles in modulating such pathways in comparable cellular settings remain unclear. ERK1/2, p38MAPK, JNK, and the Akt/GSK3β pathway are among the redox-sensitive kinases that may be activated by NADPH oxidases, depending on cell type and agonist. MAPKs constitute an essential signal transduction cascade that plays a central role in processes such as cell proliferation, differentiation, and stress signaling. The Akt/GSK3β pathway is activated by growth factors, mechanical and other stimuli and modulates important cellular functions such as cell survival, motility, and migration. We found that enhanced ROS generation on Nox4 overexpression was accompanied specifically by ERK1/2 and JNK activation but not activation of the other kinases. Although the effects of Nox4 expression per se on acute signaling have not been specifically explored in prior studies, Nox4-dependent ERK1/2 and JNK activation was described in lipopolysaccharide (LPS)-induced CXCR6 expression in aortic VSMCs.30 The lack of major effect of Nox2 overexpression per se on MAPK activation is consistent with the lack of detectable increase in ROS generation.

Agonist stimulation evoked distinct patterns of Nox2- versus Nox4-dependent kinase activation. Angiotensin II induced specific JNK activation in Nox2-overexpressing cells, whereas no specific activation of the kinases studied
was found in Nox4-overexpressing cells. Angiotensin II-induced ERK1/2 activation was similar in control and Nox2-overexpressing cells, suggesting that although it was redox-sensitive it probably did not involve Nox2. Insulin and TNFα also induced similar redox-sensitive ERK1/2 activation in control and Nox2-overexpressing cells. Interestingly, insulin specifically stimulated JNK in Nox2-overexpressing cells whereas it stimulated p38MAPK and Akt/GSK3β phosphorylation in Nox4-overexpressing cells. Insulin could be a specific agonist for acute activation of Nox4, and it is interesting that Nox4-dependent Akt activation has been suggested in adipocytes and pancreatic cancer cells. TNFα also had distinct effects in Nox2- versus Nox4-overexpressing cells, increasing phospho-JNK in the former group and phospho-GSK3β in the latter.

The distinct patterns of redox-sensitive kinase activation after Nox2 versus Nox4 activation, notably with insulin or TNFα stimulation, suggest that these 2 isoforms are coupled to different downstream kinases in HEK293 cells. Elucidation of the molecular mechanisms responsible for such specific coupling and kinase activation was beyond the scope of the current study, but several potential mechanisms are feasible. An obvious possibility would be different localization of the Nox isoforms and the assembly of signaling complexes in close proximity or association with the active oxidase in distinct cellular compartments. For example, Nox2 oxidase subunits such as p47phox and Rac can interact with nonoxidase proteins and thereby spatially confine NADPH oxidase-derived ROS signals to the vicinity of signaling targets. This appears to be the case for VEGF-induced Nox2-dependent JNK activation and membrane ruffle formation in endothelial cells, which involved interaction of p47phox with WAVE1, an important cytoskeleton regulator. Similarly, in human microvascular ECs, TNFα-induced ERK1/2 activation required the association of phosphorylated p47phox with TRAF4. In endothelial cells migrating after VEGF stimulation, an interaction of Nox2 and Rac1 with the molecule IQGAP1 was found to be critical. Interleukin (IL)-1β stimulation of epithelial cells was recently shown to induce the formation of signaling complexes in close proximity to activated Nox2 in internalized endosomes, a mechanism involved in subsequent NFκB activation. In contrast, analogous mechanisms that might subserve spatially confined Nox4-dependent signaling remain unclear. We also considered the possibility that different ROS (ie, O2·⁻ versus H2O2) may mediate the effects of Nox2 versus Nox4. However, experiments using PEG-SOD and PEG-catalase, respectively, suggested that both Nox2- and Nox4-dependent kinase activation involve H2O2.

The present study conducted in a well-defined experimental cell system indicates that Nox2 and Nox4 exhibit distinct patterns of agonist-induced activation and downstream kinase activation, which could be attributable to specific compartmentation of redox signaling. To establish the principle that such isoform-selective effects can also occur in vascular cells in situ, we investigated acute responses to angiotensin II in aorta. These studies indicated that angiotensin II–induced ERK1/2 and p38MAPK activation was Nox2-dependent even though Nox4 is expressed in the aorta and these kinases can be activated by exogenous H2O2. This finding provides proof-of-principle for the concept of Nox isoform-specific redox signaling in whole tissue, analogous to previous cell culture studies. Such isoform-specific signaling is likely to be important for the roles of Noxs in modulating cellular and tissue pathophysiological processes.

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Disclosures

None.

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Nox4 and Nox2 NADPH oxidases mediate distinct cellular redox signaling responses to agonist stimulation

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Methods

Cells

HEK293 cells, COS-7 cells or human microvascular endothelial cells (HMEC-1) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma Chemical Co. Ltd, St Louis, MO) containing 10% heat inactivated fetal bovine serum (Invitrogen) and penicillin/streptomycin (Sigma) in a humidified 37°C incubator.

Constructs and transfection

Full length human Nox2 (kind gift of Franz Wientjies, University College London [UCL]; GenBank™ accession number NM_000397) or mouse Nox4 cDNA (kind gift of Thomas Leto, GenBank™ accession number NM_015760) were cloned into the mammalian expression vector, pcDNA3.1 (Invitrogen). In addition, full length Nox2 and Nox4 coding sequences (minus stop codons) were generated by PCR
and cloned into the vector pCS2-Myc (kind gift of John Pizzey) to generate expression constructs encoding C-terminal c-myc epitope tagged fusion proteins. 16 h before transfections, cells were seeded in six-well plates at $1 \times 10^5$ cells/well. Expression plasmids for each protein were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and experiments were carried out 48 hours post transfection. Control cells were transfected with empty vector.

**Generation of Nox4 antibody**

Investigations on the cellular actions of Nox4 have been hampered by the lack of good antibodies. A rabbit polyclonal antibody was generated (Eurogentech, Belgium) against a 12 amino acid peptide corresponding to residues 556-568 in the C-terminal of Nox4, a region that is conserved among human, mouse and rat. The generated antiserum was affinity-purified using the antigenic peptide and was tested in western blots as well as immunofluorescence staining.

**Immunoblotting and immunofluorescence**

Cell lysates were obtained by lysing the cells in modified RIPA Buffer (0.025M Tris/HCl pH 7.4, 0.15 M NaCl, 0.5% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate and protease inhibitor cocktail (Sigma). Protein content of the lysates was estimated using BioRad Protein assay reagent (BioRad) and equal amounts of proteins (40μg) were separated by SDS PAGE. Separated proteins were transferred to nitrocellulose membranes. Immunoblots for phosphorylated forms of protein kinases were performed using phospho-specific antibodies (all used at 1:1000 dilution) and were normalized to the amount of total protein kinase detected using non-
phosphospecific (ie, pan) antibodies. All immunoblots were analysed by densitometry. For analyses of the effect of Nox2 or Nox4 overexpression, densitometric data were compared to the protein level in control transfected cells. Data shown are the mean (±SE) values from at least 4 independent experiments for each condition.

To analyze Nox2-specific signalling in intact tissue, aortae from Nox2 knockout (KO) or matched wild type mice (WT) were excised and cut into 4 pieces. 2 pieces each were incubated in DMEM with or without 100nM AngII for 30 minutes and the tissues were snap frozen in liquid nitrogen. The tissue was subsequently homogenised, protein estimated and equal amounts of protein (40 μg) were loaded onto 10% SDS-PAGE gels followed by western blot using indicated antibodies. In some experiments, aortic tissue was incubated in the presence or absence of 100 μM H₂O₂.

For immunofluorescence, transfected or untransfected cells were seeded in chamber slides. After fixation and permeabilization (3.3% paraformaldehyde, 0.1% Triton X-100 in PBS respectively), cells were incubated with anti-Nox4 (1:200 dilution) or anti-Nox2 (1:100 dilution) rabbit polyclonal antibodies. Where cells were co-stained with an endoplasmic reticulum (ER marker), a mouse anti-calnexin monoclonal antibody (Chemicon, USA) was used. Bound antibodies were visualized using Alexa 488 conjugated anti-rabbit IgG and Alexa 568 conjugated anti-mouse IgG antibodies (both from Molecular Probes, 1:200 dilution). Cells were incubated with primary and secondary antibodies for 1 h each. Slides were visualized with either a Nikon Axioscope microscope with Openlab software (Improvision) or a Leica laser scanning confocal microscope (TCS-SP5). Detection of the green and red fluorescence signals was achieved using appropriate filter sets (excitation 488 nm/emission 505–530 nm or excitation 568nm/emission 620nm respectively).
Confocal images were acquired as transcellular 0.4-μm optical sections in the Z plane (15 scans/frame).

**RT-PCR**

Quantification of mRNA expression of Nox2, Nox4, p22^{phox}, p47^{phox} and p67^{phox} was performed using the Applied Biosystems 7000 sequence detection system with SYBR Green. Briefly, RNA was reverse-transcribed from total RNA extracted from HEK293 cells with the Qiagen RNEasy Kit. MLV reverse transcriptase (Promega, Southhampton, UK) was used for cDNA synthesis. SYBR Green reactions were carried out in 96-well plates to a final volume of 25 µl. All samples were tested in triplicate and actin mRNA was used for normalization. Results were analyzed by use of the standard curve method.

**ROS generation**

HEK293 cells were serum starved for 2 hours prior to exposure to agonists. NADPH-dependent ROS generation was measured in cell homogenates by lucigenin (5 µM)-enhanced chemiluminescence as described previously.\(^1\) In all experiments, the lucigenin signal was completely inhibited by diphenylene iodonium (DPI 10 µM) or the absence of added NADPH (data not shown). The area under the curve was measured over 20 min and reported as arbitrary light units.

**H\(_2\)O\(_2\) assay**

We used the homovanillic acid (HVA) assay to determine catalase-inhibitable extracellular H\(_2\)O\(_2\) production.\(^2\) Cells (70,000 cells/well) were plated on a 24 well plate (Nunc) and transfected with empty vector (mock), Nox2 or Nox4. 48 hours post-
transfection, the cells were serum starved for 2 hours and then incubated with 1 \( \mu \text{M} \) angiotensin II for 30 minutes at 37\(^\circ\)C. Subsequently, the wells were rinsed with PBS and 250\(\mu\)l 1mM HEPES with or without 750U/ml catalase was added. The plate was incubated at 37\(^\circ\)C for another 30 minutes and gently shaken every 10 minutes. Subsequently, 250\(\mu\)l 1mM HEPES containing 200\(\mu\)M HVA and 10U/ml HRP was added to every well in the absence of light. The plate was then wrapped in foil and incubated for a further 30 minutes at 37\(^\circ\)C. The 500\(\mu\)l supernatant from each of the 24 wells was pipetted into 24 eppendorf tubes and 40\(\mu\)l stop reagent (0.1M glycine, pH 10) added. 200\(\mu\)l of each sample was pipetted into a black 96 well plate in duplicate and the plate read in a flurometer (Tecan GENios, excitation at 321nm, emission 421nm) and analysed using Magellan 5 software. The total amount of protein in each well was quantified. The amount of \( \text{H}_2\text{O}_2 \) was estimated from a standard curve using the catalase-inhibitable signal.

**Reagents**

Angiotensin II, insulin, \( \beta \)-nicotinamide adenine dinucleotide phosphate sodium (NADPH), catalase, PEG-SOD, PEG-catalase, losartan and diphenyleneiodonium chloride (DPI) were purchased from Sigma-Aldrich. Human recombinant tumor necrosis factor-\( \alpha \) (TNF\( \alpha \)) was purchased from Merck. Lucigenin (bis-N-methylacridinium nitrate), Alexa 488 conjugated anti-rabbit IgG and Alexa-568 conjugated anti-mouse IgG were purchased from Molecular Probes. An affinity purified anti-human Nox2 polyclonal antibody was a kind gift from Franz Wientjies (UCL). Anti-phospho or pan antibodies for ERK\(1/2 \), p38 MAP kinase (p38MAPK), JNK, Akt and GSK3\( \beta \), all raised in rabbit were purchased from Cell Signalling. All these antibodies were used at a dilution of 1:1000. HRP-conjugated anti-rabbit IgG was
purchased from Cell Signalling and used at a dilution of 1:5000. Mouse monoclonal anti-myc antibody was purchased from Chemicon and used at a dilution of 1:1000 for western blots and 1:200 for immunofluorescence.

Statistics

Statistical analysis of data was performed using either 2-way ANOVA or 2-tailed Student t test, as indicated in the text. \( P < 0.05 \) was considered statistically significant.

References


Figures
Figure I. Nox4 antibody characterization.
A- The amino acid sequence ‘sygtrfeynkesfs’ (residues 556-568 in human Nox4) used to generate a polyclonal antibody. B- Immunoblots for Nox4, Nox2 or Myc in HEK293 cells transfected with empty vector (Mock), Nox2, Nox4, or Myc-tagged constructs. The affinity-purified Nox4 antibody or anti-Myc antibody were used. C- Immunofluorescent detection of Nox4 or Myc. Top panels: co-staining of HEK293 cells with anti-Nox4 polyclonal and anti-Myc monoclonal antibodies. Endogenous Nox4 was detected in untransfected HMEC-1.
**Figure II. Localization of Nox4 and Nox2 in HEK293 cells.** A, HEK293 cells transfected with Nox4 or Nox2 were immunostained with rabbit anti-Nox4 or Nox2 polyclonal antibodies. The cells were co-stained with a monoclonal antibody to the ER marker protein calnexin. Bound antibodies were visualized by alexa 488 conjugated anti-rabbit secondary antibody and alexa568 conjugated anti-mouse secondary antibody. Fluorescence images were captured using a Leica laser scanning confocal microscope (TCS-SP5). Images were acquired as transcellular 0.5μm optical sections in the Z plane (16 scans/frame). Arrow in the top panel points to distinct plasma membrane staining of Nox2. Bar =20 microns. B, Graphed pixel-by-pixel fluorescence intensities of the two fluorophores along an arbitrary line (thick white lines in boxed images) assessed using the Leica TCS-SP5 software. A significant overlapping pattern was found between Nox4 and calnexin whereas the Nox2 and calnexin signals showed only minor overlap, especially at the cell periphery.
Figure III. Analysis of the expression of NADPH oxidase subunits in HEK293 cells. 
A, mRNA expression of Nox2, Nox4, p22$^{phox}$, p47$^{phox}$ and p67$^{phox}$ in HEK 293 cells. B, Immunoblotting for protein expression.
Figure IV. Expression of angiotensin AT$_1$ receptors in HEK293 cells. A, AT$_1$ receptor expression analysed by RT-PCR using specific primers designed using ABI Prism software. Forward primer: 5’ AGCAACAGGAGATGAGAGTTCCA 3’ reverse Primer: 5’TGAAAACCGGCACGAAAAC 3’. Human microvascular endothelial cells (HMEC-1) were used as a positive control. B, Western blot with an anti-AT$_1$ receptor antibody (Abcam) using protein lysates from HEK293 cells and HMEC-1. C, Effect of AT$_1$ antagonist losartan (10 μM) on ROS production by angiotensin II-stimulated Nox2-overexpressing HEK293 cells. *, P<0.05 cf. basal level. #, P<0.05 for effect of losartan.
Figure V. Effect of insulin on kinase activation in Nox4 or Nox2- transfected cells. 
A, Representative immunoblots. B, Relative change in phosphoprotein expression normalized by the level of pan-protein. Means±SE of 5 experiments/group. *, P<0.05 comparing agonist-treated versus untreated for each group. #, P<0.05 for significant reduction after BHA, compared to respective sample without BHA.
Figure VI. Effect of TNFα on kinase activation in Nox4 or Nox2- transfected cells.

A, Representative immunoblots. B, Relative change in phosphoprotein expression normalized by the level of pan-protein. Means±SE of 5 experiments/group. *, P<0.05 comparing agonist-treated versus untreated for each group. #, P<0.05 for significant reduction after BHA, compared to respective sample without BHA.