A 28-kDa Splice Variant of NADPH Oxidase-4 Is Nuclear-Localized and Involved in Redox Signaling in Vascular Cells

Narayana Anilkumar, Gorka San Jose, Iain Sawyer, Celio X.C. Santos, Claire Sand, Alison C. Brewer, Derek Warren, Ajay M. Shah

Objective—Reactive oxygen species–generating nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) proteins (Noxs) are involved in cell differentiation, migration, and apoptosis. Nox4 is unique among Noxs in being constitutively active, and its subcellular localization may therefore be particularly important. In this study, we identified and characterized a novel nuclear-localized 28-kDa splice variant of Nox4 in vascular cells.

Approach and Results—Nox4 immunoreactivity was noted in the nucleus and nucleolus of vascular smooth muscle cells and multiple other cell types by confocal microscopy. Cell fractionation, sequence analyses, and siRNA studies indicated that the nuclear-localized Nox4 is a 28-kDa splice variant, Nox4D, which lacks putative transmembrane domains. Nox4D overexpression resulted in significant NADPH-dependent reactive oxygen species production as detected by several different methods and caused increased phosphorylation of extracellular-signal-regulated kinase1/2 and the nuclear transcription factor Elk-1. Overexpression of Nox4D could also induce DNA damage as assessed by γ-H2AX phosphorylation. These effects were inhibited by a single amino acid substitution in the Nox4D NADPH-binding region.

Conclusions—Nox4D is a nuclear-localized and functionally active splice variant of Nox4 that may have important pathophysiologic effects through modulation of nuclear signaling and DNA damage. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: NADPH oxidase ➢ Nox4 ➢ Nox4D ➢ nucleus ➢ reactive oxygen species ➢ redox signaling ➢ vascular

NADPH oxidase (NADPH) family proteins (Noxs) are reactive oxygen species (ROS)-generating enzymes that are involved in diverse physiological and pathophysiological processes.7,8 Seven family members have been identified that each based on a distinct catalytic subunit, namely Nox1-5 and Duox1-2. Nox1, Nox2, Nox4, and Nox5 may be expressed in cardiovascular cells and have differing requirements for additional proteins subunits that associate with the catalytic core to form the active enzyme complex. Nox enzymes have been found to be involved in processes, such as vascular smooth muscle cell (VSMC) and endothelial cell migration, proliferation, angiogenesis, and vascular and cardiac hypertrophy, through the modulation of redox-sensitive signaling cascades.1,2 However, the specific roles of individual Noxs remain to be fully clarified. Recent studies have reported distinct functions for different Nox isoforms that are coexpressed in the same cell type. For example, Nox1, but not Nox4, mediates angiotensin II–induced VSMC hypertrophy.3,4 Endothelial cell Nox4 was found to have vasodilator effects in contrast to Nox2 expressed in the same cell type.5 In the heart, cardiomyocyte Nox4 mediated protective signaling in response to pressure overload, whereas Nox2 mediated detrimental signaling.6 Possible reasons for these differences include distinct subcellular localization and coupling to distinct signal transduction cascades for different Noxs.

Among the Nox proteins, Nox4 is unique in that it constitutively generates ROS.6,9,10 The targets of ROS generated by Nox4 and other Noxs include protein kinases and protein phosphatases that counteract the activation of kinase signaling cascades. The ROS-dependent modulation of function of kinases, phosphatases, and other proteins generally involves specific oxidative modification of amino acid residues; for example, reactive cysteines. Such signaling modifications are believed to require the spatially localized production of ROS in specific subcellular compartments. For example, Nox1- or Nox2-mediated ROS production in endosomes is involved in interleukin 1–dependent activation of nuclear factor-κB.12,13 In cardiomyocytes, Nox2-dependent ROS production at the sarcolemma was recently implicated in the regulation of calcium release from ryanodine receptors.14 Nox4-generated ROS were demonstrated to oxidize and inactivate the phosphotyrosine phosphatase, PTP1B, in the endoplasmic reticulum (ER) of endothelial cells, which served as a regulatory switch for epidermal growth factor receptor trafficking and signaling.15 The subcellular localization of Nox4 is likely to be especially

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important, given its constitutive activity, unlike isoforms, such as Nox1 or Nox2, that require agonist activation. However, its subcellular distribution remains controversial, at least in part attributable to the lack of sufficiently specific or characterized antibodies. Nox4 has been reported to be variably present in the ER,15,16 mitochondria,17 cytoskeleton,1 plasma membrane,18 and nucleus19 in different cell types. Here, we report that a 28-kDa splice variant of Nox4 that lacks transmembrane domains (Nox4D) is localized to the nucleus and nucleolus in VSMC and other cell types. Furthermore, this isoform is functionally active and can modulate nuclear signal transduction.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Nuclear-Localized Nox4 Immunoreactivity

We used a well-validated polyclonal antibody directed against the C-terminal region of Nox411,20,21 to investigate the localization of endogenous Nox4 by immunofluorescence in several cell types, including VSMC, endothelial cells, fibroblasts, and cardiomyocytes. A diffuse reticular staining pattern through the cell that colocalized with an ER marker, protein disulfide isomerase, was evident in most cell types (Figures 1A and 2) as reported previously.15,16 In addition, however, there was strong focal and granular Nox4 immunostaining within the nucleus in all cell types. EM indicated that the focal Nox4 staining was in the nucleolus (Figure 1C). We confirmed that the focal intra-nuclear staining was nucleolar by costaining with 2 different nucleolar markers, nucleophosmin and fibrillarin (Figure 1B). Preincubation of the Nox4 antibody with the antigenic peptide against which it was raised completely abolished both the perinuclear and nucleolar staining, verifying the specificity of antigen–antibody interaction (Figure 1D). Furthermore, treatment with a prevalidated Nox4 siRNA to knock down Nox4 protein expression also abolished staining as compared with a scrambled siRNA (Figure 3A).

Nuclear-Localized Nox4 Is a 28-kDa Nox4 Splice Variant

To confirm nuclear and nucleolar localization, VSMCs were fractionated into membrane and nuclear fractions. Immunoblotting for Nox4 demonstrated an ≈65-kDa band corresponding to the expected molecular size of Nox4 but also a more prominent ≈28-kDa band (Figure 4A). Both bands were depleted after treating with the Nox4 siRNA (Figure 3B). The ≈28-kDa band was the dominant band in the nuclear fraction and hardly detectable in the membrane fraction (Figure 4B). The specificity of the band was confirmed using antigenic peptide for competitive inhibition during primary antibody incubation (data not shown). We confirmed a 28-kDa Nox4 immunoreactive band in human embryonic kidney (HEK293) cells, and these cells could also purify the nucleolar fraction, which contained the same band (Figure 4C). The purity of cell fractions was confirmed using fraction-specific antigens (Figure 4C).

A previous study reported the presence of 4 Nox4 splice variants, namely Nox4B, C, D, and E, in lung tissue.22 Among these, Nox4D has a molecular size of 28 kDa. Real-time polymerase chain reaction of VSMC using primers corresponding to the 3′ and 5′ ends of full-length Nox4, followed by nucleotide sequencing of bands, revealed the presence of mRNAs corresponding to full-length Nox4 and Nox4D...
Figure 2. Localization of Nox4 to the nucleus and nucleolus in multiple cell types. Fixed and permeabilized cells were stained using Nox4 antibody together with the endoplasmic reticulum marker protein disulfide isomerase (PDI) or phalloidin for the actin cytoskeleton. Scale bars, 50 μm. HUVEC indicates human umbilical vein endothelial cells.

(Numerikumar et al. Nuclear-Localized 28-kDa Nox4 Splice Variant (Figure 4D). This suggested that the 28-kDa band in nuclear lysates was Nox4D. To distinguish between Nox4D or a cleavage product of Nox4, we used siRNAs against Nox4 exon 14 (which is expressed in Nox4D) and Nox4 Exon 3 (which is not expressed in Nox4D). The siRNA targeted against exon 14 significantly depleted the 28-kDa band and nuclear immunoreactivity as compared with the siRNA against exon 3 (Figure 4E and 5A).

We next overexpressed Nox4 and Nox4D in VSMC using adenoviral vectors, confirming protein expression by immunoblotting (Figure 4F). Immunofluorescence studies revealed that overexpressed Nox4 localized to the perinuclear region as described previously15,16 (Figure 5B, top). However, overexpressed Nox4D localized mainly to the nucleus and nucleolus, although some perinuclear staining was also evident (Figure 5B, bottom).

Nox4D Generates ROS

Although Nox4D is a truncated form of full-length Nox4, it retains NADPH- and FAD-binding domains required for electron transfer activity.23 VSMC overexpressing Nox4D showed a >2-fold increase in catalase-inhibitable H2O2 production compared with β-galactosidase (βGal)-overexpressing cells and produced comparable ROS levels with Nox4-overexpressing cells (Figure 6A). The capacity for electron transfer was further assessed by an nitroblue tetrazolium assay.24 Nox4D-overexpressing VSMC had an ≈3-fold increase in activity compared with βGal-overexpressing control cells, which was inhibited by the flavoprotein inhibitor diphenyleneiodonium (DPI, 10 μmol/L; Figure 6B). We analyzed NADPH-dependent O2− generation in whole-cell and nuclear lysates of Nox4D-overexpressing cells, using lucigenin-enhanced chemiluminescence. Both whole-cell and nuclear NADPH-dependent ROS generation was significantly higher in Nox4D-overexpressing cells compared with control cells, whereas there was little or no signal in the presence of NADH (Figure 6C). The NADPH-dependent ROS generation was almost completely abolished by DPI (10 μmol/L) but was unaffected by inhibitors of nitric oxide synthase (L-NAME),...
xanthine oxidase (oxypurinol), or the electron transport chain (rotenone; Figure 6D).

**Nox4D-Generated ROS Mediate Nuclear Redox Signaling in VSMC**

ROS generated by Nox family proteins may modulate kinase signaling cascades, such as MAPK activation, and ROS-dependent extracellular-signal-regulated kinase (ERK) activation involving the inactivation of nuclear mitogen-activated kinase phosphatase 1 has been reported. We, therefore, examined the effect of Nox4D on ERK1/2 phosphorylation. Cells infected with Nox4D adenovirus had significantly increased levels of phosphorylated ERK1/2 compared with βGal-infected control cells (Figure 7A). Immunofluorescence studies showed that there was a significant increase in nuclear phospho-ERK1/2 (Figure 8).

A downstream target of active ERK1/2 is the transcription factor Elk-1, a component of the ternary complex that binds the serum response element on target genes and promotes transcription. Levels of phosphorylated Elk-1 were significantly increased in Nox4D-overexpressing cells (Figure 7A). A moderate but significant increase in...
transcriptional activity of serum response factor was also observed in Nox4D-overexpressing cells (Figure 9).

The ROS dependence of Nox4D-mediated ERK1/2 and Elk-1 activation was further examined. The activation of both ERK1/2 and Elk-1 in Nox4D-overexpressing cells was significantly inhibited by either DPI, the ROS scavenger tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), or cell permeable polyethylene glycol-catalase (Figure 7B and 7C).

We generated a Nox4D mutant with a single amino acid substitution in the NADPH-binding domain (proline 130 to histidine, Figure 10A) that would be expected to impair its activity based on previous studies with Nox4 and Nox2. 27,28 VSMCs were transfected with wild-type or mutant Nox4D (Nox4DM) and protein expression confirmed by immunoblotting (Figure 10B). ROS production in the nuclear fraction was significantly reduced in cells transfected with the Nox4D mutant compared with wild-type Nox4D (Figure 10C). In addition, ERK1/2 activation was also significantly reduced in the Nox4DM-transfected compared with Nox4D-transfected cells (Figure 10D).

**Nox4D-Derived ROS Can Induce DNA Damage**

Oxidation-induced DNA damage leads to phosphorylation of H2AX (γ-H2AX), a member of the core histone H2A family. 29

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**Figure 6.** Reactive oxygen species (ROS) production by Nox4D. **A**, H2O2 levels in vascular smooth muscle cells (VSMCs) infected with βgal, Nox4, or Nox4D adenovirus, detected using an homovanillic acid assay. **B**, Electron transfer activity of Nox4D assessed using a nitroblue tetrazolium assay, in the presence or absence of the flavoprotein inhibitor DPI (10 μmol/L). **C**, Whole-cell (WC) or isolated nuclear fraction (NF) lysates of pcDNA (Control) or Nox4D-transfected cells were analyzed for ROS production by lucigenin assay in the presence or absence of NADPH or NADH. **D**, ROS production in whole-cell lysates of pcDNA or Nox4D-transfected cells was assayed in the presence or absence of Nox specific and other inhibitors. AU indicates arbitrary units. *P<0.05; #P<0.05 for effect of DPI.

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**Figure 7.** Nox4D-mediated extracellular-signal-regulated kinase (ERK)1/2 and Elk-1 phosphorylation. **A**, ERK1/2 and Elk-1 phosphorylation in vascular smooth muscle cells overexpressing βgal or Nox4D. Gels were blotted for phospho-ERK1/2 and total ERK1/2 (left) or phospho-Elk-1 and actin (right). *P<0.05. **B–C**, Effect of polyethylene glycol (PEG) catalase (PEG Cat, 50 U/mL, 12 hours), DPI (10 μmol/L, 12 hours), or Tiron (1 mmol/L, 12 hours) on Nox4D-dependent ERK1/2 and Elk-1 phosphorylation. AU indicates arbitrary units. #P<0.05 cf. Nox4D alone.
Nuclear γ-H2AX immunostaining was increased in VSMC by exogenous H2O2 or treatment with doxorubicin (Figure 11). Overexpression of Nox4D also significantly increased γ-H2AX levels as assessed by immunostaining and Western blot analyses of whole-cell lysates (Figure 10E, Figure 11). Cells overexpressing the Nox4D mutant showed significantly lower levels of γ-H2AX. Treatment of Nox4D-overexpressing cells with polyethylene glycol-superoxide dismutase, polyethylene glycol-catalase, or DPI all significantly reduced γ-H2AX levels (Figures 10E and 11).

Discussion

In this work, we demonstrate that a 28-kDa splice variant of Nox4, namely Nox4D, that lacks putative transmembrane domains is localized to the nucleus of multiple cell types, including VSMC. Furthermore, Nox4D is found to be functionally active in terms of ROS generation, modulates redox-sensitive nuclear transcription factor activation downstream of the phosphorylation of ERK1/2, and is capable of inducing DNA damage. These results suggest that Nox4D may have important pathophysiological effects through modulation of nuclear signal transduction and DNA damage.

Nox1-4 are predicted to have 6 transmembrane domains and are therefore considered to be membrane-associated proteins.1 The prototypical Nox oxidase, Nox2, is a plasma membrane-associated enzyme in phagocytes,1 and Nox1 is also reported to be localized to the plasma membrane.4 Both endogenous and overexpressed Nox4 has been identified in the ER of various cells,16,21 and an ER location of Nox4 was again confirmed in the current study. However, other subcellular locations have also been reported, including the nuclear envelope,30 mitochondria,17,31 and the intranuclear region.19,32 A possible intranuclear localization of Nox4 is intriguing because it is not immediately obvious how a protein with 6 putative transmembrane domains would localize within the nucleus. Nox4D, however, lacks the transmembrane domains found in full-length Nox4, although it still contains NADPH- and FAD-binding domains22 and would therefore be expected to be soluble rather than membrane-localized. Indeed, we found a prominent nuclear localization of Nox4D with both a granular distribution within the nuclear matrix and intense local staining corresponding to the nucleolus. This pattern reflected Nox4D distribution because the major isoform found in nuclear and nucleolar fractions was 28-kDa Nox4; the only splice variant that was detected by real-time polymerase chain reaction was Nox4D; transfected exogenous Nox4D localized to similar locations in the cell; and an siRNA that targeted exon 14 of Nox4, which is present in Nox4D, abolished nuclear immunostaining, whereas an siRNA targeting exon 3 (absent in Nox4D) did not. A nuclear and nucleolar localization of endogenous Nox4D was found not only in primary human and rat A7R5 VSMC but also in a variety of other primary cells and cell lines, including endothelial cells, cardiac fibroblasts, and cardiomyocytes, suggesting that multiple cell types have nuclear Nox4D localization. Previous studies have reported the presence of Nox4D in lung tissue22 and kidney cells,33 but subcellular location was not addressed in detail. In A549 cells, transfection of HA-tagged constructs suggested a mainly cytosolic location by immunostaining, but detailed analysis of location was not performed.22 We found in the present study that transfected Nox4D was located in the nucleus, as well as in extranuclear regions. Analysis of the Nox4D sequence using PSORT II localization prediction software (http://psort.hgc.jp/form2.html) indicates a 17% probability of nuclear localization, suggesting that mechanisms other than a simple nuclear localization sequence may be involved.

We found that Nox4D constitutively generated ROS (ie, without activation by agonists). The capacity for ROS
production was confirmed by 4 different assays, namely the homovanillic acid assay for H$_2$O$_2$, dihydroethidium high-performance liquid chromatography, lucigenin-enhanced chemiluminescence for O$_2^-$, and the nitroblue tetrazolium assay for electron transfer activity. In addition, it was found that ROS generation was NADPH dependent and inhibited by the potent flavoprotein inhibitor DPI but unaffected by inhibitors of other flavoprotein-containing enzymes, such as NO synthase, xanthine oxidase, and the mitochondrial electron transport chain. Furthermore, ROS production was significantly reduced in cells overexpressing a mutant construct with a single amino acid substitution in the NADPH-binding domain of Nox4D. Taken together, these data provide strong evidence that Nox4D specifically generates ROS as a result of NADPH oxidase activity. The capacity of Nox4D to generate ROS is particularly interesting in light of recent evidence from the Lambeth laboratory that the Nox4 C-terminal dehydrogenase domain, which is essentially preserved in Nox4D, is capable of constitutive electron transfer activity. This differs from the equivalent regions of Nox1, Nox2, and Nox5, which barely have any electron transfer activity. Entirely consistent with these data, we found that Nox4D has electron transfer activity as assessed by the nitroblue tetrazolium assay. In the full-length, Nox proteins, the N-terminal transmembrane portion of the enzyme, are thought to facilitate electron transfer across the membrane and thereby generate ROS on the opposite side of the membrane. The constitutive activity of Nox4D together with its lack of transmembrane domains suggests that this isoform may specifically serve to generate ROS in cytosolic compartments.

ROS generation within the nucleus may have several important effects on cellular function. ROS can inactivate nuclear-localized phosphatases and thereby enhance kinase activation by altering the balance between phosphorylation and dephosphorylation. For example, the oxidative inactivation of the nuclear phosphatase mitogen-activated kinase phosphatase 1 regulates ERK1/2 activation. We found that Nox4D specifically increased phosphorylated ERK1/2 within the nucleus of VSMC, although mitogen-activated kinase phosphatase 1 was not addressed in the current study. ROS may also alter nuclear transcription factor activation, either secondary to activation.

Figure 11. Representative images of γ-H2AX staining for DNA damage in vascular smooth muscle cell. Cells were transfected with a control pcDNA construct, Nox4D or Nox4DM, as shown. Treatment with doxorubicin (1 μmol/L) or H$_2$O$_2$ (100 μmol/L) was used as a positive control. Experiments with Nox4D transfection were repeated in the presence of DPI, polyethylene glycol (PEG) catalase (CAT), or PEG superoxide dismutase (SOD). Cells were costained with a γ-H2AX antibody (red color), the anti-Nox4 antibody (green), and DAPI (blue) to label nuclei. Scale bars, 50 μm.
of relevant kinases or via direct redox effects. We found that activation of the transcription factor Elk-1 was increased by Nox4D downstream of ERK1/2 activation, providing direct evidence for the modulation of nuclear signaling by Nox4D. Interestingly, a significant proportion of nuclear Nox4D was localized to the nucleolus. The mechanisms underlying its localization to this organelle or its specific role therein are currently unclear. However, it is of interest that the nucleolus contains several phosphatases (eg, PP1)^16 that regulate various nucleolar functions, such as ribosomal biosynthesis and nucleolar stress responses. It will be of interest in future studies to probe the roles of Nox4D in different nucleolar and nuclear functions.

Excessive production of ROS also could lead to oxidative DNA damage. To assess whether Nox4D-generated ROS can induce nuclear DNA damage, we studied nuclear γ-H2AX levels in cells transfected with Nox4D or a mutant Nox4D construct. These experiments clearly demonstrated an increase in γ-H2AX levels, which was attributable to ROS and was abrogated in cells transfected with the mutant Nox4D construct. These data indicate that Nox4D has the potential to induce nuclear DNA damage. The precise pathological circumstances under which such damage might occur will need further investigation.

In summary, we identify the presence of a ROS-generating 28-kDa splice variant of Nox4, namely Nox4D, within the nucleus of VSMC and other cell types. Nox4D modulates the nuclear activation of ERK1/2 and the Elk-1 transcription factor, and is capable of inducing nuclear DNA damage, indicating that it may have important roles in nuclear redox signaling.

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Disclosures
None.

References
NADPH oxidase proteins (Noxs) generate reactive oxygen species that are implicated in modulating intracellular signaling. Most Noxs are quiescent until acutely activated, but Nox4 is constitutively active. Its subcellular location may therefore be particularly important for its signaling functions. This study identifies a nuclear-localized splice variant of Nox4, Nox4D, which is expressed in the multiple cell types. Nox4D lacks transmembrane domains but contains the reactive oxygen species–generating dehydrogenase domain and is functionally active. In vascular smooth muscle cells, Nox4D modulates the activation of extracellular-signal-regulated kinase1/2 and its downstream transcription factor Elk-1. Overexpression of Nox4D induces DNA damage as assessed by γ-H2AX phosphorylation. These results suggest that Nox4D may have important pathophysiological effects through the modulation of nuclear signaling and DNA damage.
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MATERIALS AND METHODS

Cells
The cells used were: rat A7R5 VSMC, human aortic VSMC, human umbilical vein endothelial cells (HUVEC), H9C2 cardiomyocytes, primary rat neonatal cardiomyocytes, HEK293 cells and primary rat cardiac fibroblasts. A7R5 VSMC, primary rat neonatal cardiomyocytes and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; SigmaChemical Co. Ltd, St Louis, MO) containing 10% heat inactivated fetal bovine serum (Invitrogen) and penicillin/ streptomycin (Sigma). HUVEC were maintained in a defined endothelial cell growth medium (Invitrogen). Other primary cells were isolated in the laboratory and maintained in DMEM with 10% FCS and used within 3 passages.

Constructs and transfection
Full length human Nox4 (a kind gift of Thomas Leto, GenBank™ accession number NM_015760) or Nox4D were cloned into the mammalian expression vector, pcDNA3.1 (Invitrogen). Adenoviral vectors expressing mouse Nox4, Nox4D or β-galactosidase were generated using the AdEasy Adenoviral Vector System (Qbiogene), amplified in HEK293 cells, and isolated using the Adenopure virus purification kit (Puresyn). Nox4D mutant was generated by PCR using site-directed mutagenesis. Cells were seeded in six-well plates, 16 h before transfections, at 1 \times 10^5 cells/well. Adenoviruses were used at an MOI of 50 and βGal adenovirus was used as control. Expression plasmids were
transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Control cells were transfected with empty pcDNA3.1 vector.

Commercially available pre-validated siRNA against Nox4 were from Ambion and were transfected using siPORT transfection reagent according to the manufacturer's instructions. Experiments were carried out 48 hours post transfection unless specified otherwise. For Nox4, we used up to 20nM siRNA for knockdown and the cells were left for 72 hours after transfection.

**Immunoblotting**

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 (Roche)). Protein content of the lysates was estimated using BioRad Protein assay reagent (BioRad) and equal amounts of proteins (30μg or as indicated ) were separated by SDS PAGE. Separated proteins were transferred to nitrocellulose membranes and probed using the antibodies indicated. All immunoblots were analyzed by densitometry and normalized by the relevant protein.

**Immunofluorescence**

For immunofluorescence, transfected or untransfected cells were seeded in 8 well chamber slides. After fixation and permeabilization (3.3% paraformaldehyde, 0.1% Triton X-100 in PBS respectively), cells were incubated with anti-Nox4 (in house), anti-pERK1/2 (Cell Signaling) or anti-γH2AX (Abcam) antibodies (all at 1:200 dilution). Where cells were co-stained with an endoplasmic reticulum (ER) marker, a mouse anti-
PDI monoclonal antibody (Chemicon, USA) or mouse anti-Calnexin (Abcam) antibody was used. Bound antibodies were visualized using Alexa 488 conjugated anti-rabbit IgG and Alexa 568 conjugated anti-mouse IgG antibodies or cells were stained with Alexa 568 conjugated phalloidin (all from Invitrogen 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).

**Electron Microscopy**

Cells were fixed and cryosectioned as described in Ji et al\(^1\). Primary antibodies were all used at 1:100 and secondary at 1:40 dilution with 10 nm colloidal gold (BB International). Five grids were used per antibody pair and the experiment was repeated three times. Stained sections were examined on a FEI Tecnai T12 BioTWIN transmission electron microscope and images captured on a Gatan BioScan Model 792 MSC SI003 1 camera and analysed using Digital Micrograph 3.7.1 software. Files were exported as TIFF files at a minimum of 1024 x 1024 pixel resolution, into Adobe Photoshop CS2.

**Nuclear isolation**
Nuclear and nucleolar isolations were carried out according to the method described by Andersen et al. Briefly, cells after trypsinisation and washing were swelled in buffer A (10 mM Hepes, pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.5mM DTT) for 5 minutes on ice and the cells were broken using a Wheaton Downs homogenizer (3 strokes). The released nuclei were pelleted by centrifugation at 218g for 5 minutes. Pelleted nuclei were further purified by layering the suspension over sucrose solution (0.25 M Sucrose, 10 mM MgCl2) and centrifuging at 1430g for 5 minutes at 4°C. The supernatant after first centrifugation was centrifuged at 13000 rpm on a Biofuge (Eppendorf) for 10 min to pull down the “membrane fraction” that included plasma membrane and microsomes. The supernatant was concentrated using Amicon mini-spin columns. Proteins from each pellet were extracted using a modified RIPA buffer as above. Protein content was estimated using Bradford assay (Biorad) and equal amounts of proteins were analyzed by SDS-PAGE.

**H₂O₂ measurement**

The homovanillic acid (HVA) assay was used to determine catalase-inhibitable H₂O₂ levels according to the method described by Werner. Cells (40,000 cells/well) were plated on a 12 well plate (Nunc) and infected with βGal, Nox4 or Nox4D adenoviruses or plasmids. 48 hours post-infection, the cells were rinsed with PBS and incubated with 500μl of 1mM HEPES with or without 750U/ml catalase. The plate was incubated at 37°C for another 30 minutes and gently shaken every 10 min. Subsequently, 250μl 1mM HEPES containing 200μM 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 min at
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37°C. The 500μl supernatant from each of the 24 wells was pipetted into eppendorf tubes and 40μl stop reagent (0.1M glycine, pH 10) added. 200μl 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 H₂O₂ was estimated from a standard curve using the catalase-inhibitable signal

**NBT assay**

Nitroblue tetrazolium (NBT) assay to detect electron transfer was performed as originally described by Choi et al⁴ and later by Sturrock et al⁵. Stock NBT (25mg/ml) was prepared in methanol and stored at 4°C. Control or inhibitor treated cells in 12 well plates were washed with PBS and incubated in 1ml of serum free DMEM without phenol red. NBT (10μl) was then added to each well and cells were incubated at 37°C for one hour. The medium was removed and the cells were fixed using ice-cold methanol for 5 minutes. Cells were further washed 3 times with methanol at room temperature. After the plates were air dried, the insoluble formazan crystals within the cells were dissolved by addition of 440μl of 1M KOH to each well, incubation for 5 minutes, followed by the addition of 560 μl of DMSO. The plates were rocked gently until the crystals were completely dissolved and absorbance was determined at 630nm.

**Lucigenin-enhanced chemiluminescence**

Superoxide (O₂⁻) production in cell homogenates was measured using lucigenin-enhanced chemiluminescence assay in a microplate luminometer (Anthos Lucy 1)⁶.
Briefly, cells were detached, washed in PBS, and resuspended in 400 µL of buffer B (50 mmol/L KH$_2$PO$_4$, 1 mmol/L EGTA, 150 mmol/L sucrose, pH 7.0) with a protease inhibitor cocktail (2 µL). Cells were homogenized and distributed in triplicate (10 µg/well) onto a 96-well microplate. NADPH (300 µmol/L) and dark-adapted lucigenin (5 µmol/L) were added to wells just before they were read. O$_2^-$ production was expressed as arbitrary light units over 20 minutes. The following agents were used to assess potential sources of O$_2^-$ production: DPI, a flavoprotein inhibitor (10 µmol/L); the nitric oxide synthase inhibitor L-NAME ($N^G$-nitro-L-arginine methyl ester; 100 µmol/L); the xanthine oxidase inhibitor oxypurinol (100 µmol/L); the complex I mitochondrial electron chain inhibitor rotenone (2 µmol/L).

**HPLC-based quantification of DHE oxidation products**

HPLC-based detection of the oxidation products of dihydroethidium (DHE, Invitrogen), i.e. 2-hydroxyethidium (EOH), was performed as previously described.$^7,8$ Cells transfected with Nox4D or Nox4DM were washed twice with PBS and incubated in PBS/DTPA (0.5 ml) at a final DHE concentration of 100 µmol/L for 30 min. Cells were washed with cold PBS, harvested in cold acetonitrile (0.5 ml/well), sonicated (10 s, 1 cycle at 8 W), centrifuged (12,000 g for 10 min at 4°C) and supernatants dried under vacuum. Pellets were stored at –20°C in the dark until analysis. Samples were resuspended in 80 µl PBS/DTPA and injected (30 µL) into an HPLC system. Chromatographic separation was carried out in a C18-Kromosil column (4.6 x 250 mm, 5 µm particle size). DHE was monitored by ultraviolet absorption at 245 nm and EOH by fluorescence detection (excitation 510 nm, emission 595 nm). Quantification was
performed by comparison of peak signal between the samples and standard solutions under identical conditions. DHE-derived products were expressed as ratios of EOH generated per DHE consumed\textsuperscript{7}.

**Assessment of DNA damage**

Double stranded DNA damage caused as result of DNA oxidation was detected using mouse anti- $\gamma$H2AX antibody (Abcam)\textsuperscript{9}. A7R5 cells transfected with pcDNA3.1, Nox4D or the Nox4D mutant were fixed using 3.3% paraformaldehyde 48 hours post transfection. Fixed cells were permeabilized using 0.1% Triton X 100 and stained using anti- $\gamma$H2AX antibody together with rabbit anti-Nox4 antibody. Bound antibodies were visualised using anti mouse Alexa 568 antibody and anti-rabbit Alexa 488 and secondary antibodies.

Whole lysates of cells transfected with pcDNA3.1, Nox4D or the Nox4D mutant were separated on 12% SDS-PAGE gel and transferred to nitrocellulose. The membranes were probed using mouse anti- $\gamma$H2AX antibody and the bound antibodies detected using HRP conjugated anti-mouse secondary antibody.

**Statistics**

All data shown are the mean (±SE) values from at least 4 independent experiments for each experiment. Statistical analyses were performed by Student’s t-test when 2 groups were compared or by 1-way ANOVA when multiple groups were compared. $P<0.05$ was considered significant.

Reference List


