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Nox2 B-loop Peptide, Nox2ds, Specifically Inhibits Nox2 Oxidase

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Abstract

In recent years, reactive oxygen species (ROS) derived from the vascular isoforms of NADPH oxidase, Nox1, Nox2 and Nox4, have been implicated in many cardiovascular pathologies. As a result, the selective inhibition of these isoforms is an area of intense current investigation. In the present study, we postulated that Nox2ds, a peptidic inhibitor that mimics a sequence in the cytosolic B loop of Nox2, would inhibit ROS production by Nox2-, but not by Nox1- and Nox4-oxidase systems. To test our hypothesis, the inhibitory activity of Nox2ds was assessed in cell-free assays using reconstituted systems expressing the Nox2-, canonical or hybrid Nox1-, or Nox4-oxidase. Our findings demonstrate that Nox2ds, but not its scrambled control, potently inhibited superoxide ($O_2^{\bullet-}$) production in the Nox2 cell-free system, as assessed by the cytochrome *c* assay. Electron paramagnetic resonance (EPR) confirmed that Nox2ds inhibits $O_2^{\bullet-}$ production by Nox2 oxidase. In contrast, Nox2ds did not inhibit ROS production in either Nox1 or Nox4 oxidase. These findings demonstrate that Nox2ds is a selective inhibitor of Nox2 oxidase and support its utility to elucidate the role of Nox2 in organ pathophysiology and its potential as a therapeutic agent.

Keywords

NADPH oxidase; reactive oxygen species; superoxide; Nox inhibitor; cardiovascular disease

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Author Disclosure Statement

None.

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Introduction

Reactive oxygen species (ROS) play an important role in the pathogenesis of cardiovascular disorders, including systemic and pulmonary hypertension, atherosclerosis, stroke and restenosis [1, 2]. ROS are often considered as highly reactive toxic byproducts of oxygen metabolism. However, it is also known that ROS contribute to a wide range of physiological processes, including regulation of vascular tone, cellular signaling, gene expression, angiogenesis, cellular senescence, and cell growth [3–6]. NADPH oxidases (Nox) are the major source of ROS in the cardiovascular system [7, 8] and strong evidence suggests that Nox proteins contribute to oxidative damage in response to a wide variety of stimuli, including cytokines, hormones, metabolic factors, and mechanical injury [1]. Therefore, the selective blockade of the undesirable actions of Nox-derived ROS is expected to be an important therapeutic strategy for treating oxidative stress-related cardiovascular pathologies.

The mechanism of production of the prototypical ROS superoxide anion ($O_2^{\bullet-}$) from NADPH oxidase has been extensively studied in phagocytes [9]. The phagocytic NADPH oxidase is an enzyme complex composed of two transmembrane “anchoring” subunits, Nox2 (a.k.a. gp91^{phox}) and p22^{phox}, three cytosolic components (p47^{phox}, p67^{phox} and p40^{phox}) and the small GTPase Rac2 [10]. Upon activation, the regulatory subunit p47^{phox} is phosphorylated and translocates to the membrane with the activator subunit p67^{phox} and p40^{phox}. Prevention of this translocation/assembly in phagocytes formed the basis for peptidic inhibitor development by our group [11, 12]. The assembled phagocyte NADPH oxidase catalyzes the transfer of one electron from NADPH through FAD and two heme groups to molecular oxygen to form $O_2^{\bullet-}$, the key determinant of nitric oxide (NO) bioavailability and the forerunner of multiple other biological ROS.

It is now known that the Nox family consists of seven members, namely Nox1, Nox2, Nox3, Nox4, Nox5, Dual oxidase (DUOX) 1 and DUOX2, which differ in tissue distribution, subcellular localization, regulation, activity and pathophysiological functions [13], but retain their ability to transfer electrons from NADPH to oxygen to form either $O_2^{\bullet-}$ or its dismuted metabolite hydrogen peroxide (H_2O_2) [10]. In the vasculature, the major family members are Nox1, Nox2 and Nox4. Nox1 is localized to caveolae in the plasma membrane and endosomes [13], while Nox4 has been identified in focal adhesions [14], the nucleus [5], and the endoplasmic reticulum [15]. Nox1 is constitutively active and requires the p47^{phox} and p67^{phox} homologues NOXO1 (Nox Organizer subunit 1) and NOXA1 (Nox Activator subunit 1), respectively, for activation. [16, 17]. Nevertheless, previous data in smooth muscle cells appear to suggest that p47^{phox} and p67^{phox} might supplant NOXO1 and NOXA1 in the Nox1 oxidase system [18]. Takeya *et al.* showed that COS cells transfected with Nox1/NOXO1/NOXA1 constitutively generate $O_2^{\bullet-}$ that can be further stimulated by phorbol myristate acetate (PMA). Moreover, $O_2^{\bullet-}$ production was significantly higher in Nox1/NOXO1/NOXA1 than in Nox1/NOXO1/p67^{phox}- or Nox1/p47^{phox}/NOXA1-transfected COS cells, while Nox1/p47^{phox}/p67^{phox}-transfected cells were inactive [17].

In contrast, Nox4 does not require the conventional subunits p47^{phox} and p67^{phox} or their homologues for activation and the possibility of a role of other cytosolic modulators is the focus of active research [19]. In addition, multiple reports suggest that Nox4 differs from other Nox isoforms in producing primarily H_2O_2 [20].

Because excessive Nox-derived ROS contribute to the progression of a wide spectrum of diseases, the Nox family of oxidases is a highly sought after therapeutic target and the selective blockade of individual Nox isoforms is an area of intense investigation [21]. To date several potential inhibitors have been identified, yet most of them appear to exhibit low

selectivity, potency and bioavailability, and none to our knowledge selectively inhibit Nox2-oxidase [21].

Our laboratory was the first to rationally design a peptidic inhibitor in its original cell-permeant chimeric form (Nox2 docking sequence-tat; Nox2ds-tat a.k.a. gp91ds-tat) targeting the assembly of Nox2; in that study, Nox2ds-tat attenuated angiotensin II (AngII)-induced vascular $O_2^{\bullet-}$ production and blood pressure elevation in mice [12]. Numerous studies demonstrated the effectiveness of this chimeric peptide inhibitor to attenuate or abolish ROS levels in normal or diseased tissue, consistent with the expression of Nox2 [12, 22–25]. However, specificity of B-loop non-chimeric peptide Nox2ds for the Nox2-oxidase has not been demonstrated to our knowledge. Recent studies suggest that certain amino acid sequences in the B-loop of Nox2 bind to the dehydrogenase (DH) domain in the C-terminal tail of Nox2 and Nox4 and that this binding is required for activity [26]. This, along with significant homology in the B-loops among isoforms raised concern for non-isoform-specific inhibition of different Noxes by Nox2ds. As the major vascular isoforms of NADPH oxidase are Nox1, Nox2 and Nox4 and since ROS derived from these isoforms have been implicated in many cardiovascular pathologies, we set out to test the inhibitory effect of Nox2ds on these isoforms and postulated that Nox2ds is specific for Nox2-, but not Nox1- and Nox4-oxidase, inhibition and its attendant ROS production.

Materials and methods

Materials

Cytochrome *c*, superoxide dismutase (SOD), lithium dodecyl sulfate (LiDS), catalase, diphenyleneiodonium chloride (DPI), horseradish peroxidase (HRP), N_{ω} -Nitro-L-arginine methyl ester (L-NAME), rotenone and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-012 was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). Febuxostat was purchased from Axon Medchem (Groningen, The Netherlands). Amplex Red was purchased from Invitrogen (Eugene, OR, USA). Protease inhibitor cocktail was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Nox2ds and scrmb Nox2ds were synthesized by the Tufts University Core Facility (Boston, MA, USA). The sequence of Nox2ds in human Nox2 is as follows: $[NH_3]-C-S-T-R-V-R-R-Q-L-[CONH_2]$. The scrmb Nox2ds sequence is as follows: $[NH_3]-C-L-R-V-T-R-Q-S-R-[CONH_2]$. In both cases, as has been the case since the original publication on these peptides, the $[NH_3]$ group represents the amino end and $[NH_2]$ represents the amide of the carboxy terminus, a consequence of synthetic procedure. The purity of Nox2ds and scrmb Nox2ds were 97.7 and 92.4 %, respectively. Since the current studies were carried out in cell-free systems, it is important to note that the peptides used in this study did not require chimeric design containing *tat* peptide for cell permeation [12, 27].

Cell lines

All cell culture reagents were obtained from Invitrogen, unless indicated otherwise. COS-22 (COS-7 cells stably expressing human p22^{phox}) and COS-Nox2 (a.k.a. COS-phox) cells (COS-7 cells stably expressing human p22^{phox}, Nox2, p47^{phox} and p67^{phox}) were kindly provided by Dr. Mary C. Dinauer (Indiana University, School of Medicine). $O_2^{\bullet-}$ production in intact COS-Nox2 cells was characterized elsewhere [28]. COS-22 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin (complete media) supplemented with 1.8 mg/ml G418 (Calbiochem/EMB Bioscience, Gibbstown, NJ). COS-Nox2 cells were maintained in complete media supplemented with 1.8 mg/ml G418, 1 µg/ml puromycin (Sigma, St Louis, MO) and 0.2 mg/ml hygromycin B (Invitrogen, Carlsbad, CA).

Plasmid preparation, amplification and purification

Plasmids encoding full-length human cDNAs for Nox1 (pcDNA3.1-hNox1), NOXO1 (pcDNA3.1-hNOXO1), NOXA1 (pCMVSPORT 6-hNOXA1), p47^{phox} (pCMV-Tag4A-hp47) and Nox4 (pcDNA3-hNox4) were kindly provided by Dr. David Lambeth (Emory University, GA) [29, 30]. Plasmids encoding Nox1, NOXO1 or NOXA1 were transformed and amplified into *Escherichia coli* strain TOP10 (Invitrogen, Carlsbad, CA). Plasmids were purified using a QIAfilter plasmid purification kit (QIAGEN Inc., Valencia, CA.). For human Nox4 expression, the BglII/NotI restriction fragment from the pcDNA3-hNox4 was subcloned into the plasmid pcDNA3.1/Hygro(−) (Invitrogen, Carlsbad, CA) to generate pcDNA3.1/Hygro-hNox4. The fragment sequence, in-frame insertion and orientation were validated by DNA sequencing after PCR amplification. pcDNA3.1/Hygro-hNox4 was amplified into *Escherichia coli* strain TOP10 and purified with a QIAfilter plasmid purification kit.

Transfection

Cell transfection was carried out using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. COS-22 cells were transiently co-transfected with pcDNA 3.1-hNox1, pCMVSPORT 6-hNOXA1 and pcDNA3.1-hNoxO1 (COS-Nox1/NOXO1/NOXA1 cells). Cells were used 24 hr after transfection. For stable transfection of Nox4, COS-22 cells were transfected with pcDNA3.1/Hygro-hNox4 (COS-Nox4 cells). COS-Nox4 cells were selected in complete media, supplemented with 0.2 mg/ml hygromycin B and 1.8 mg/ml G418. Stable transfectants were maintained in culture under the same conditions. Adherent cells were harvested by incubating with 0.05 % trypsin/EDTA for 5 min at 37 °C. Following addition of DMEM/10%FBS to neutralize the trypsin, the cells were pelleted by centrifugation at $1100 \times g$ for 5 min at 4 °C and used for the experiments.

Superoxide ($O_2^{\bullet-}$)-generating activity in COS cells

$O_2^{\bullet-}$ production was measured in intact COS-Nox1/NOXO1/NOXA1 and COS-22 cells by L-012 chemiluminescence. $O_2^{\bullet-}$ production in COS-Nox1/NOXO1/NOXA1 and COS-Nox2 cell-free systems was measured by both SOD-inhibitable cytochrome *c* reduction and electron paramagnetic resonance (EPR).

L-012 chemiluminescence—COS-Nox1/NOXO1/NOXA1 and COS-22 cells were replated into 96-well white micro-plates (Greiner-Bio One GmbH, Germany) at a density of 5×10^4 cells/well. The cells were incubated at 37 °C in PBS containing 400 μ M luminol derivative L-012 for 30 min. Luminescence was quantified over time using a Biotek Synergy 4 Hybrid Multi-Mode Microplate Reader. The specificity of L-012 for $O_2^{\bullet-}$ was confirmed by the addition of SOD (150 U/ml).

Cytochrome *c* assay—COS-Nox2, COS-Nox1/NOXO1/NOXA1 and COS-22 cells were suspended to a concentration of 5×10^7 cells/ml in ice-cold disruption buffer (8 mM potassium, sodium phosphate buffer pH 7.0, 131 mM NaCl, 340 mM sucrose, 2 mM NaN_3 , 5 mM $MgCl_2$, 1mM EGTA, 1 mM EDTA and protease inhibitor cocktail) [31]. The cells were lysed by freeze/thaw cycles (5 cycles), and passed through a 30-gauge needle 5 times to further lyse the cells. Cell disruption was confirmed by phase contrast microscopy. The cell lysate was centrifuged at $1000 \times g$ for 10 min at 4 °C to remove unbroken cells, nuclei and debris. Throughout all these procedures, extreme care was taken to maintain the lysate at a temperature close to 0 °C. Superoxide production was calculated from the initial linear rate (over 10 min) of SOD-inhibitable cytochrome *c* reduction quantified at 550 nm using the extinction coefficient of $21.1 \text{ mM}^{-1} \text{ cm}^{-1}$ (Biotek Synergy 4 Hybrid Multi-Mode

Microplate Reader). The oxidase assay buffer consisted of 65 mM sodium phosphate buffer (pH 7.0), 1 mM EGTA, 10 μ M FAD, 1 mM MgCl_2 , 2 mM NaN_3 and 0.2 mM cytochrome *c* [31]. The components of the cell-free system were added in the following order: oxidase assay buffer, cell lysate (5×10^5 cell equivalents/well) and Nox2ds/scrmb Nox2ds peptides at a final concentration of 0.1, 0.3, 1.0, 3.0 and 10 μ M. The plates were placed on an orbital shaker to mix contents for 5 min at 120 movements/min at room temperature. LiDS, an established lipid activator of phagocyte cell-free system, was added at a concentration of 130 μ M and $\text{O}_2^{\bullet-}$ production was initiated by the addition of 180 μ M NADPH. The concentration of Nox2ds peptide that caused 50% inhibition of $\text{O}_2^{\bullet-}$ production (IC_{50}) in COS-Nox2 cell lysates was calculated by Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA).

To test the effect of Nox2ds on the hybrid Nox1 cell-free system, COS-22 cells were separately transfected with Nox1, NOXO1, p47^{phox}, NOXA1 or p67^{phox} and the Nox1 membrane component, and NOXO1, p47^{phox}, NOXA1 and p67^{phox} cytosolic extracts were individually prepared. Cells were lysed as described above and cell lysates were centrifuged at $160000 \times g$ for 60 min at 4 °C to separate the membrane from the cytosol. The organizer subunit NOXO1 or p47^{phox} was preincubated with 10 μ M Nox2ds for 10 min, and then NOX1 and NOXA1 or p67^{phox} were added consecutively. After the preincubation period, LiDS (130 μ M) was added to induce the assembly of the oxidase and $\text{O}_2^{\bullet-}$ production was initiated by 180 μ M NADPH. Superoxide production was measured using cytochrome *c* as described above.

Electron paramagnetic resonance—The spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (CMH; Alexis Corp., San Diego, CA) was used to examine $\text{O}_2^{\bullet-}$ production in COS-Nox2 cell lysates using a Bruker eScan Table-Top EPR spectrometer (Bruker Biospin, USA). $\text{O}_2^{\bullet-}$ production was measured in oxidase assay buffer (65 mM sodium phosphate buffer, pH 7.0, 1 mM EGTA, 10 μ M FAD, 1 mM MgCl_2 , 2 mM NaN_3) supplemented with 50 μ M CMH. Cell lysates (5×10^5 cell equivalents) were incubated with Nox2ds and scrmb Nox2ds peptides for 5 min at room temperature. After the preincubation period, LiDS was added at a concentration of 130 μ M and $\text{O}_2^{\bullet-}$ production was initiated by the addition of 180 μ M NADPH. Analyses of the EPR spectra peak height were used to quantify the amount of $\text{O}_2^{\bullet-}$ produced by the lysates and were compared with buffer-only control spectra or spectra in the presence of 10 μ M Nox2ds, 10 μ M scrmb Nox2ds or 150 U/ml SOD. The effect of Nox2ds and scrmb Nox2ds was expressed as SOD-inhibitable formation of $\text{CM}^{\bullet}$ radical. To minimize the deleterious effects of contaminating metals, the buffers were treated with Chelex resin and contained 25 μ M deferoxamine (Noxygen Science Transfer, Germany).

In separate experiments the $\text{O}_2^{\bullet-}$ -scavenging activity of Nox2ds was determined by EPR using the xanthine/xanthine oxidase $\text{O}_2^{\bullet-}$ -generating system. Assay mixtures contained PBS, 5 mU/ml xanthine oxidase, 50 μ M CMH and 25 μ M deferoxamine in a final volume of 100 μ l. The reference samples contained 100 U/ml SOD. After addition of 25 μ M xanthine, $\text{CM}^{\bullet}$ radical formation was monitored for 10 min in the absence and presence of 10 μ M Nox2ds or scrmb Nox2ds.

Hydrogen peroxide (H_2O_2)-generating activity

H_2O_2 producing activity was quantified in intact COS-Nox4 cells by Amplex Red, according to the previously described methods [32]. H_2O_2 production was quantified in the COS-Nox4 cell-free system as described previously [32]. Briefly, COS-Nox4 cells (5×10^7 cells/ml) were disrupted in ice-cold disruption buffer (PBS containing 0.1 mM EDTA, 10 % glycerol, protease inhibitor cocktail, and 0.1 mM PMSF) by freeze/thaw cycles as we

described above. Incubation of COS-Nox4 cell lysate with Nox2ds was performed in assay buffer (25 mM Hepes, pH 7.4, containing 0.12 M NaCl, 3 mM KCl, 1 mM MgCl₂, 0.1 mM Amplex Red, and 0.32 U/ml of HRP) for 5 min at room temperature on an orbital shaker (120 movements/min), before the addition of 36 μ M NADPH, to initiate H₂O₂ production. This concentration of NADPH was used because it was found that higher concentrations interfered with Amplex Red fluorescence. Fluorescence measurements were made using a Biotek Synergy 4 Hybrid Multi-Mode Microplate Reader with a 530/25-excitation and a 590/35-emission filter. A standard curve of known H₂O₂ concentrations was developed using the Amplex Red assay (as per the manufacturer's instructions), and was used to quantify H₂O₂ production in the COS-Nox4 cell free system. Nox4 activity was obtained by subtracting non-transfected COS-22 cell lysate activity from COS-Nox4 cell lysate activity. The reaction was monitored at room temperature for 10 min, and the emission increase was linear during this interval.

Superoxide-generating activity in HEK 298 cells

In order to test the effect of Nox2ds on inducible Nox1 activity we used Nox1/NOXO1/NOXA1-transfected HEK 298 cells (hereafter referred to as HEK-Nox1) as it was shown previously that O₂^{•-} production in HEK-Nox1 cells was significantly stimulated by PMA treatment [17]. HEK-Nox1 and non-transfected HEK 298 cells were re-plated into 96-well white micro-plates at a density of 5×10^4 cells/well, and O₂^{•-} was measured in the absence and presence of PMA (1 μ M) using L-012 (400 μ M). The effect of Nox2ds on inducible Nox1 activity was tested on LiDS-stimulated O₂^{•-} in HEK-Nox1 cell-free system. HEK 298 cells were separately transfected with either Nox1, NOXO1 or NOXA1. Nox1-containing membranes, and NOXO1 and NOXA1 cytosolic extracts from each preparation were prepared as described above. The Nox1 organizer subunit NOXO1 was preincubated with 10 μ M Nox2ds for 10 min, and then NOXO1 and NOXA1 were added consecutively. After the preincubation period, LiDS (130 μ M) was added to induce the assembly of the oxidase and O₂^{•-} production was initiated by 180 μ M NADPH. Superoxide production was measured using cytochrome *c*.

Enzyme-linked immunosorbent assay (ELISA)

ELISA experiments were performed to test the mechanism by which Nox2ds could inhibit O₂^{•-} production in COS-Nox2 and not in the COS-Nox1 cell-free system. Neutravidin-coated plates (Thermo Scientific, Rockford, IL, USA) were incubated with biotinylated Nox2ds (Biotin-Nox2ds, 6 μ M) or biotinylated scrmb Nox2ds (Biotin-scrmb, 6 μ M) (Tufts University Core Facility, Boston, MA, USA) for 2 hr at room temperature. The plates were washed 3 times using wash buffer (25 mM Tris, 150 mM NaCl, 0.1% BSA, 0.05% Tween-20, pH 7.2). After 1 hr incubation at room temperature with COS-22, COS-22-p47^{phox} (COS-22 cells transfected with p47^{phox}) or COS-22-NOXO1 (COS-22 cells transfected with NOXO1) cytosolic fraction, rabbit polyclonal p47^{phox} (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit NOXO1 (1:500; Rockland Immunochemicals Inc., Gilbertsville, PA, USA) antibodies were used to detect p47^{phox} or NOXO1 bound to Nox2ds or its scrambled control, respectively. After 1 hr incubation and extensive washing, bound primary antibodies were detected by the addition of FITC-labeled goat anti-rabbit IgG antibody (1:500; Sigma-Aldrich, St. Louis, MO, USA). The fluorescence of each well was measured using a Biotek Synergy 4 Hybrid MultiMode Microplate Reader (Excitation:488 nM, Emission:518 nM- BioTek, Winooski, VT, USA).

Fluorescence Polarization

Nox4 dehydrogenase domain (amino acids 304 – 578) recombinant protein fused to GST were expressed and purified as described previously [26] from *E. coli* using Glutathione SepharoseTM 4B (GE Healthcare, Waukesha, WI, USA). The ability of the Nox2ds or scrmb

Nox2ds to compete off Nox4 B-loop from the Nox4 DH domain was measured by competition assays monitored by fluorescence polarization with a SynergyTM 2 Multi-Mode Microplate Reader and Gen5TM software package (BioTek, Winooski, VT, USA). FITC-labeled and unlabeled Nox4 B-loop peptides were synthesized and purified by the Emory University Microchemical facility. Unlabeled Nox4 B-loop peptides (amino acids 77 – 106), Nox2ds or scrmb Nox2ds peptides were titrated against a constant amount of Nox4 DH protein (60 nM) and FITC labeled Nox4 B-loop peptide (31 nM) in assay buffer containing 10 mM Hepes (pH 7.0), 75 mM NaCl, 1 μ M FAD and 0.05% Tween-20. Data were fit to a one-site competition model to obtain LogEC₅₀ values and 95% confidence intervals.

Western blot

Western blot experiments were performed to validate the expression of the catalytic membrane subunits (Nox1, Nox2 and Nox4), as well as the organizer (p47^{phox} and NOXO1) and activator (p67^{phox} and NOXA1) subunits in each reconstituted Nox system (Supplemental Fig. 1). Cell lysates were subjected to SDS-PAGE. Blots were probed with rabbit polyclonal Nox2 (1:250, Upstate Cell Signaling Solutions, Temecula, CA, USA), rabbit polyclonal Nox1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal Nox4 (1:500; Novus Biologicals, Littleton, CO, USA), rabbit polyclonal NOXO1 (1:500; Rockland Immunochemicals Inc., Gilbertsville, PA, USA), rabbit polyclonal p47^{phox} (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse polyclonal NOXA1 (1:500, abcam, Cambridge, MA, USA), or mouse monoclonal p67^{phox} 81.1 (kindly provided by Dr. Mark T. Quinn) antibody and then incubated with their respective secondary antibodies (1:10000, IRDye® antibodies, LI-COR Biotechnology, Lincoln, NE, USA). Loading of equal amounts of proteins was confirmed by re-probing the membranes with a mouse monoclonal β -actin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody. Blots were scanned using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA).

Statistical analysis

All results are expressed as means $\pm$ SEM. Comparisons between individual concentrations of Nox2ds and scrmb Nox2ds were assessed by two-way ANOVA, followed by a Bonferroni post-hoc t-test. $p < 0.05$ was considered to be statistically significant.

Results

Nox2ds dose-dependently inhibits O₂^{•−} production from Nox2-oxidase

Cytochrome c assay—To confirm that Nox2 oxidase is the major source of O₂^{•−} in the COS-Nox2 cell-free system, COS-Nox2 cell lysate was pre-treated with the nitric oxide synthase inhibitor L-NAME (100 μ M), the mitochondrial electron transport inhibitor rotenone (50 μ M), or the xanthine oxidase inhibitor febuxostat (100 nM). Preincubation with rotenone, L-NAME, or febuxostat did not decrease O₂^{•−} production significantly in COS-Nox2 cell-free system (nmol O₂^{•−}/min/10⁷ cell equivalents were: 1.69 $\pm$ 0.15, 1.95 $\pm$ 0.21, 1.64 $\pm$ 0.13 and 1.32 $\pm$ 0.1 for vehicle-, L-NAME-, rotenone- and febuxostat-treated COS-Nox2 lysates, respectively, $n = 3$). These results suggest that the major source of O₂^{•−} in the COS-Nox2 cell-free system is Nox2 NADPH oxidase.

To investigate whether B-loop peptide Nox2ds inhibits O₂^{•−} production in the Nox2 NADPH oxidase system, SOD-inhibitable cytochrome *c* reduction was measured in Nox2ds-pretreated COS-Nox2 cell lysates. Addition of LiDS to lysates derived from COS-Nox2 cells stimulated O₂^{•−} production in a reaction that was dependent on the presence of NADPH (1.33 $\pm$ 0.1 and 0.40 $\pm$ 0.1 nmol O₂^{•−}/min/10⁷ COS-Nox2 lysate-cell equivalents for LiDS- and vehicle-treated COS-Nox2 lysates, respectively, $p < 0.05$). O₂^{•−} production in

non-transfected COS-22 cell lysates (control) was 0.16 ± 0.1 nmol $O_2^{\bullet-}$ /min/ 10^7 COS-22 lysate-cell equivalents (Fig. 1). As demonstrated in Fig. 1, preincubation of COS-Nox2 cell lysates with Nox2ds (before LiDS-treatment) concentration-dependently inhibited $O_2^{\bullet-}$ production, displaying an IC_{50} of $0.74 \mu M$. In contrast, preincubation of COS-Nox2 cell lysates with scrmb Nox2ds did not inhibit Nox2-oxidase.

EPR spectroscopy confirms inhibition of Nox2-oxidase cell free assay by Nox2ds: The inhibitory effect of Nox2ds on $O_2^{\bullet-}$ production was confirmed by EPR. The reaction of hydroxylamine spin probe CMH with $O_2^{\bullet-}$ results in formation of a nitroxide radical ($CM^{\bullet}$), yielding a characteristic 3 line spectrum (Fig. 2A). $O_2^{\bullet-}$ formation was assayed as NADPH-dependent, SOD-inhibitable formation of $CM^{\bullet}$ radical. $O_2^{\bullet-}$ production in Nox2 lysates was stimulated by LiDS in the presence of NADPH and this was inhibited by SOD (150 U/ml) (Fig. 2A). Preincubation of COS-Nox2 cell lysates with Nox2ds ($10 \mu M$), but not with scrmb Nox2ds ($10 \mu M$), significantly inhibited $CM^{\bullet}$ radical formation (Fig 2B and C). Fig. 2C shows cumulative and averaged SOD-inhibitable $CM^{\bullet}$ radical intensities in vehicle-, $10 \mu M$ Nox2ds- and $10 \mu M$ scrmb Nox2ds-treated COS-Nox2 lysates.

Nox2ds does not inhibit $O_2^{\bullet-}$ production from Nox1-oxidase

L-012 chemiluminescence of transiently-transfected Nox1-oxidase—To evaluate the activity of the transiently transfected canonical Nox1-oxidase system (comprising Nox1, NOXO1 and NOXA1), whole COS-Nox1/NOXO1/NOXA1 cell activity was assessed by L-012 chemiluminescence. We confirmed previous results showing that the Nox1/NOXO1/NOXA1 reconstituted system produces $O_2^{\bullet-}$ in the absence of PMA [relative light units (RLUs) were: 52977 ± 1921 and 8734 ± 158 for COS-Nox1 and COS-22 control cells, respectively, $p < 0.05$, $n = 3$, Table 1] [17]. SOD (150 U/ml) abolished the chemiluminescent signal (177 ± 9 and 108 ± 16 RLUs for SOD-treated COS-Nox1/NOXO1/NOXA1 and COS-22 cells, respectively, $p < 0.05$, $n = 3$). PMA ($1 \mu M$) did not stimulate $O_2^{\bullet-}$ in COS-Nox1/NOXO1/NOXA1 cells (56537 ± 2741 and 5903 ± 541 , RLUs for PMA-treated COS-Nox1/NOXO1/NOXA1 and COS-22 cells, respectively, $n = 3$). These data suggested that the canonical Nox1 system in COS cells is constitutively active and cannot be further stimulated by PMA.

However, a previous study showed that $O_2^{\bullet-}$ in HEK 298 cells transfected with Nox1/NOXO1/NOXA1 was significantly enhanced by PMA stimulation [17]. We transfected HEK 298 cells with Nox1/NOXO1/NOXA1 and measured $O_2^{\bullet-}$ in the absence and presence of PMA ($1 \mu M$). PMA significantly stimulated $O_2^{\bullet-}$ in HEK-Nox1/NOXO1/NOXA1 cells (RLUs were: 12946 ± 199 and 8560 ± 337 for PMA-stimulated and -unstimulated HEK-Nox1/NOXO1/NOXA1 cells, respectively, $n = 4$, $p < 0.05$). RLUs were: 912 ± 40.5 and 755 ± 65.3 for PMA-stimulated and -unstimulated non-transfected HEK 298 cells, respectively ($n = 4$).

Cytochrome c assay of COS-Nox1 cell-free system: In contrast to the COS-Nox2 cell-free system and in line with the constitutive activity of COS-Nox1/NOXO1/NOXA1 cells, the cytochrome c assay of COS-Nox1/NOXO1/NOXA1 cell free system revealed that the canonical COS-Nox1 oxidase does not require LiDS for activation (nmol $O_2^{\bullet-}$ /min/ 10^7 cell equivalents were: 1.30 ± 0.3 and 0.16 ± 0.1 for COS-Nox1 and COS-22 cell lysates, respectively, $p < 0.05$). Preincubation of COS-Nox1 cell lysates with different concentrations of Nox2ds did not inhibit $O_2^{\bullet-}$ production (Fig. 3).

In separate experiments, we tested whether the lack of an inhibitory effect of Nox2ds on the Nox1 system is due to the stable interaction of NOXO1 and NOXA1 with Nox1. The Nox1 organizer subunit NOXO1 was preincubated with vehicle (control) or $10 \mu M$ Nox2ds for 10 min, and then NOX1 and NOXA1 were added consecutively. After the preincubation period,

LiDS was added and $O_2^{\bullet-}$ production was measured. Our results demonstrate that preincubation of NOXO1 with Nox2ds did not inhibit the canonical Nox1 oxidase (Table 1). Previous studies reported that Nox1 can be activated by not only NOXO1 and NOXA1 but also by p47^{phox} and p67^{phox} [33]. We therefore tested the effect of Nox2ds on $O_2^{\bullet-}$ production on all possible permutations of the Nox1 oxidase system. As shown in Table 1, Nox2ds did not significantly inhibit $O_2^{\bullet-}$ production in either the canonical or the hybrid Nox1 systems. This was particularly evident in the hybrid purported to exist in large vessel smooth muscle cells (Nox1/p47/NOXA1). Moreover, 1 μ M Nox2ds demonstrated no effect on LiDS-stimulated $O_2^{\bullet-}$ production in the COS-Nox1/p47^{phox}/p67^{phox} cell-free system (1.52 $\pm$ 0.18 vs. 1.59 $\pm$ 0.10 nmol $O_2^{\bullet-}$ /min/ 10^7 cell equivalents for LiDS vs. 1 μ M Nox2ds + LiDS, respectively).

Cytochrome c assay of HEK-Nox1 cell-free system—In contrast to COS-Nox1/NOXO1/NOXA1 cell free system, LiDS-treatment significantly increased $O_2^{\bullet-}$ production in HEK-Nox1/NOXO1/NOXA1 cell-free system (Table 2). In order to test the effect of Nox2ds on inducible Nox1 activity, the effect of Nox2ds was tested on LiDS-stimulated $O_2^{\bullet-}$ in HEK-Nox1/NOXO1/NOXA1 cell-free system. Our results demonstrate that preincubation of NOXO1 with 10 μ M Nox2ds did not inhibit LiDS-stimulated $O_2^{\bullet-}$ production in HEK-Nox1/NOXO1/NOXA1 cell-free system (Table 2).

Nox2ds does not inhibit H_2O_2 production from Nox4-oxidase

Amplex Red—Previous studies reported that Nox4 produces mainly H_2O_2 [20], however it is possible that Nox4 could produce $O_2^{\bullet-}$ that is rapidly converted to H_2O_2 by SOD. Prior to cell lysis, the ability of intact COS-Nox4 cells to produce H_2O_2 was confirmed by Amplex Red fluorescence. COS-Nox4 cells showed approximately 2-fold higher H_2O_2 -generating activity than non-transfected COS-22 cells (4818 $\pm$ 19 vs. 2577 $\pm$ 6.5 relative fluorescence units (RFU) for COS-Nox4 vs. COS-22 cells, respectively $p < 0.05$). 82% of the Nox4-dependent activity was inhibited with DPI (5 μ M) (2977 $\pm$ 227 RFU for DPI-treated COS-Nox4 cells).

To test whether these cells produce $O_2^{\bullet-}$, L-012 chemiluminescence was performed. Our data demonstrated that there was no difference in $O_2^{\bullet-}$ production between COS-Nox4 and non-transfected COS-22 cells (RLUs were: 1766.7 $\pm$ 41.3 and 1701.0 $\pm$ 115.8 for COS-Nox4 and COS-22 cells, respectively). Based on the results of these experiments the effect of Nox2ds was tested on H_2O_2 production in COS-Nox4 lysates by Amplex Red. Nox4-derived H_2O_2 production was calculated by subtracting non-transfected COS-22 cell lysate activity from COS-Nox4 cell lysate activity. Preincubation of COS-Nox4 cell lysates with Nox2ds did not inhibit H_2O_2 production (Fig. 4).

p47^{phox}, but not NOXO1, binds to Nox2ds

Nox2ds was designed to selectively inhibit the interaction between the cytosolic B-loop of Nox2 and p47^{phox} by binding to p47^{phox} and preventing its translocation to the membrane. To confirm this binding and to test for possible binding of Nox2ds to NOXO1, ELISA experiments were performed on lysates from p47^{phox}- or NOXO1-transfected Cos-22 cells incubated in ELISA plates with neutravidin-immobilized biotinylated Nox2ds or its scrambled control. As shown in Fig. 5A, using p47^{phox} antibody to detect binding (followed by fluorescently tagged secondary antibody), fluorescence intensity in biotinylated Nox2ds-bound wells was significantly higher when cytosolic fractions of COS-22-p47^{phox} vs. COS-22 were added. These data indicate that p47^{phox} binds to Nox2ds. Binding of p47^{phox} was sequence specific as p47^{phox} binding to the scrambled sequence produced significantly less fluorescence (Fig. 5A). In contrast, there was no difference in fluorescence intensity

when cytosolic fractions of COS-22-NOXO1 vs. COS-22 cytosol were added to plates bound with Nox2ds, followed by incubation with NOXO1 antibody (Fig. 5B).

Nox2ds does not interact with the Nox4 DH domain

Binding between Nox4 B-loop peptide and the dehydrogenase domain (DH) domain of Nox4 was previously shown and implicated in Nox4-oxidase activity [26]. Using fluorescence polarization, we tested whether Nox2ds or scrmb Nox2ds compete with the Nox4 B-loop for binding to the Nox4 DH domain. As shown in Fig. 6, the unlabeled Nox4 B-loop effectively competed off the FITC-Nox4 B-loop in the low micromolar range (LogEC_{50} : 3.0 ± 0.08 nM), however both Nox2ds (LogEC_{50} : 5.5 ± 0.5 nM) and scrmb Nox2ds were not effective at concentrations below 0.1 mM (LogEC_{50} : 5.4 ± 0.3 nM).

Nox2ds does not inhibit xanthine oxidase or directly scavenge $\text{O}_2^{\bullet-}$

To test for the possible non-specific inhibitory effect of Nox2ds on another major source of mammalian $\text{O}_2^{\bullet-}$ xanthine oxidase as well as the possibility that Nox2ds could be acting as an $\text{O}_2^{\bullet-}$ scavenger, we tested whether Nox2ds decreases $\text{O}_2^{\bullet-}$ levels produced by the classical xanthine oxidase (XO)/xanthine (X) $\text{O}_2^{\bullet-}$ generating system. Pre-incubation of Nox2ds or scrmb Nox2ds (10 μM for 10 min) did not alter XO-generated $\text{O}_2^{\bullet-}$ as assessed by EPR (100 ± 2.4 , 92.5 ± 1.8 , and 92.4 ± 1.8 % of radical intensities for XO/X control, 10 μM Nox2ds + X/XO, 10 μM scrmb Nox2ds + X/XO, respectively, Fig. 7). XO alone produced only a small signal (2.9 ± 0.4 %) and XO + X in the presence of 100 U/ml SOD yielded 15.7 ± 0.3 % radical intensity (Fig. 7).

Discussion

Previously, our laboratory and many others demonstrated the effectiveness of the chimeric peptide Nox2ds-tat (Nox2ds linked to a small portion of HIV coat named *tat* to allow cell permeation) in reducing or abolishing ROS levels in normal or diseased tissue known to contain Nox [11, 12, 25] and to attenuate progression of cardiovascular disease processes involving increased Nox activity [12, 22–24]. However, until now the specificity of inhibition of Nox2ds (non-chimeric B-loop peptide) among vascular Nox isoforms has not been investigated. To test the specificity of Nox2ds, we examined its potential inhibitory activity in cell-free assays using reconstituted systems of the major murine vascular Nox isoforms, Nox1, Nox2 and Nox4. Our data show that Nox2ds concentration-dependently inhibited $\text{O}_2^{\bullet-}$ production in a COS-Nox2 cell-free system and that Nox2ds is a potent and efficacious inhibitor of Nox2 NADPH oxidase with an IC_{50} of 0.74 μM . Furthermore, our results demonstrate that Nox2ds does not inhibit ROS production in either the COS-Nox1 or the COS-Nox4 oxidase systems. The results of the present study demonstrate selectivity of Nox2ds peptide in differentiating the contribution of Nox2- vs. Nox1- and Nox4-oxidase to Nox-derived ROS production. These findings have broad implications for distinguishing the role of Nox2 in a wide range of disease processes and support its potential use as a Nox2-targeted therapeutic agent.

There is a large body of evidence that Nox2-oxidase is a major source of ROS in vascular and cardiac tissues [7, 8] and that Nox2-derived ROS are involved in many cardiovascular disease processes, including atherosclerosis [34], hypertension [35], vascular injury [36], ischemic stroke [37], and diabetic vasculopathy [38]. Moreover, Nox2-oxidase has been implicated in a broad spectrum of other diseases [22, 24, 39, 40]. Thus, there is great interest in designing selective Nox2 inhibitors to assess the individual contribution of this enzyme to overall ROS production in cardiovascular pathologies as well as to directly modulate ROS levels and reduce oxidative stress in patients with cardiovascular and other disorders. To date, a variety of antioxidants and small molecule Nox inhibitors have been developed, yet

none of them to our knowledge selectively inhibit Nox2 [21]. Previously, the peptide-based inhibitor PR-39, which binds to the SH3 domains of p47^{phox}, was shown to prevent association of p47^{phox} with Nox2 [41, 42]. Nonetheless, as PR-39 binds to SH3 domains in general, it is not an isoform-specific inhibitor and is likely to have multiple effects in other enzyme systems that possess this domain [41]. Importantly, rationally-targeted, sequence-specific peptide-based inhibitors are likely to yield the greatest specificity among peptidic inhibitors because of their potential for being Nox-isoform specific. It thus becomes evident that, in designing isoform-specific peptidic Nox inhibitors, it is important to take advantage of differences that exist between the sequences of these isoforms. Indeed, Nox1 shares ~60 % amino acid identity with Nox2, while Nox4 shares only ~39% identity with Nox2 [43], allowing for selective targeting of these isoforms with peptide sequences unique between them. Furthermore, because of their ability to precisely model intrinsic peptide sequences in the holoenzyme and thereby compete with the proteins' interactions and activities directly, unique peptide sequences and their peptidomimetics have the potential to be among the most effective inhibitors of Nox. This, of course, does not take into account the limitations on the use of peptidic inhibitors in chronic disease prevention, which include the limited bioavailability of orally administered peptides. However, currently under intense investigation are alternative means for delivery of peptides including nanoparticle technologies, which have the potential of superseding these pharmacokinetic barriers.

Our laboratory developed a sequence-specific peptidic inhibitor, Nox2ds, which was designed to selectively target the interaction between the cytosolic B-loop of Nox2 and p47^{phox} preventing the assembly of Nox2-based enzyme complex [12]. Indeed, the results of the present study demonstrate the selectivity of Nox2ds in that Nox2ds binds to p47^{phox} but not to NOXO1, thus providing an explanation for why Nox2ds inhibits O₂^{•-} production in COS-Nox2, but not in the COS-Nox1/NOXO1/NOXA1 system. Importantly, the binding of p47^{phox} to Nox2ds was sequence specific as p47^{phox} did not bind to scrmb Nox2ds and interestingly, Nox2ds did not inhibit either COS-Nox1/p47^{phox}/NOXA1 or COS-Nox1/p47^{phox}/p67^{phox} oxidase suggesting that the interaction between the cytosolic B-loop of Nox1 and p47^{phox} is not important for Nox1 activity. Perhaps more importantly, the data presented in the present study demonstrate that Nox2ds does not inhibit inducible Nox1 activity. Moreover, since a previous study showed binding between Nox4 B-loop peptide and the DH domain of Nox4 (implicated in Nox4 activity) [26], the possibility that Nox2ds or scrmb Nox2ds could compete off Nox4 B-loop from the Nox4 DH domain was tested using fluorescence polarization. The data demonstrate that the Nox4 B-loop competed off labeled Nox4 B-loop binding to the Nox4 DH domain quite effectively, in the low micromolar range. In contrast, Nox2ds and scrmb Nox2ds demonstrated equal and poor competition with the Nox4 B-loop for binding to the Nox4 DH domain (millimolar concentrations). The very high pharmacological concentrations as well as the fact that Nox2ds and scrmb Nox2ds had equal effects suggest that Nox2ds does not interfere with the Nox4 DH domain and thus Nox4 activation.

The active sequence of Nox2ds was identified previously by random-sequence peptide phage display library analysis and had been shown to inhibit NADPH oxidase activity in a neutrophil cell-free system, which expresses Nox2 [11]. Moreover, Touyz *et al.* demonstrated that Nox2ds-tat inhibited AngII-stimulated oxidase activity in human microvascular endothelial cells, which are known to express Nox2 [18]. On the other hand, Griendling *et al.* tested the ability of Nox2ds-tat to inhibit NADPH oxidase activity in non-diseased rat aortic smooth muscle cells, which do not contain Nox2, and found no inhibitory effect (unpublished data). Consistent with these findings, multiple studies successfully applied Nox2ds-tat to attenuate ROS levels in normal and diseased tissues, implicating Nox2 in a variety of models [12, 22–25]. Data from our laboratory demonstrated cell permeation and *in vivo* effectiveness of Nox2ds-tat in an AngII-induced hypertension model in which

co-infusion of the peptide significantly decreased vascular ROS production, reduced intercellular adhesion molecule-1 (ICAM-1) expression, inhibited leukocyte infiltration and attenuated medial hypertrophy [12, 44]. Moreover, Jacobson *et al.* reported that Nox2ds-tat prevented balloon angioplasty-induced $O_2^{\bullet-}$ production and neointimal hyperplasia of the rat carotid artery [27]. Furthermore, application of Nox2ds-tat to adventitial fibroblasts, which show abundant expression of the Nox2 oxidase system [45], by adenoviral techniques attenuated Ang II-induced carotid artery medial hypertrophy and lipid peroxidation by-product 4-hydroxynonenal (4-HNE) deposition *in vivo* [46]. Until the current study, however, specificity of Nox2ds for Nox2 oxidase was not known.

Identification of isoform-specific Nox inhibitors has been a major challenge in the Nox field. Indeed, in many studies, whether Nox enzymes are the source of the ROS could not be definitively determined due to the lack of specific inhibitors. For a long time, DPI has been used to determine Nox involvement even though it is well established that DPI inhibits a multitude of flavin-containing enzymes including nitric oxide synthase (NOS), xanthine oxidase, and NADH dehydrogenase (mitochondrial complex I). [41, 47]. This is further complicated by the fact that putative Nox inhibitors could attenuate Nox-derived ROS by a number of ways other than specific inhibition of Nox activity. These include (a) acting on upstream signaling molecules, (b) interfering with activation pathways that affect the assembly of the enzyme; (c) decreasing cytosolic levels of NADPH; (d) upregulating or activating endogenous antioxidant enzymes causing a rise in cellular ROS scavenging activity; and/or (e) affecting expression of mRNA and/or protein level of different NADPH oxidase components. Although our studies have not addressed all of these potentially indirect influences of Nox2ds, use of reconstituted cell-free Nox systems was a major first step in testing for its specificity. Indeed, in the present study, Nox2ds was shown to selectively and potently inhibit the Nox2 system yielding an IC_{50} of 0.74 μM . This is in line with a previous study in phagocytes, in which the same peptide sequence inhibited $O_2^{\bullet-}$ level with an IC_{50} of 1 μM [48]. The inhibitory effect of Nox2ds was sequence specific as evident in the lack of inhibition by the scrambled sequence. Moreover, Nox2ds was highly efficacious in its inhibition of Nox2 oxidase, causing approximately 85 % inhibition of activity at 10 μM . Most importantly, Nox2ds displayed no ability to inhibit either Nox1 or Nox4 oxidase. This clear selectivity of Nox2ds for inhibition of Nox2- and not Nox1- or Nox4-oxidase suggests that Nox2ds is indeed sequence specific and that Nox2ds or its chimeras would not inhibit other Noxes or other more distinct enzyme systems that are expected to be more diverse in their sequences. Nevertheless, studies are underway to further interrogate the ability of Nox2ds to inhibit other Nox isoforms as well as to possess off-target effects.

Since the assays currently used to detect ROS have exhibited the potential for artifacts, we used EPR spectroscopy to confirm our results. In support of our initial findings with the cytochrome *c* assay, EPR experiments demonstrated that preincubation of COS-Nox2 cell lysates with Nox2ds significantly inhibited $O_2^{\bullet-}$ production. The degree of inhibition was lower with EPR than the cytochrome *c* assay (approximately 50 % vs. 85 % with EPR and cytochrome *c*, respectively). We do not have a definitive explanation for this discrepancy but we expect this to be related to the differences in the sensitivity as well as differences in background signal between the two assays. Importantly, the ineffectiveness of the scrambled control sequence of Nox2ds at inhibiting $O_2^{\bullet-}$ detection by EPR argues in favor of an isoform-specific effect of Nox2ds.

We tested the effect of Nox2ds on ROS production in Nox1 and Nox4 reconstituted systems and showed that preincubation of COS-Nox1 and COS-Nox4 lysates with Nox2ds failed to inhibit ROS production. In addition, we tested whether Nox2ds could directly alter $O_2^{\bullet-}$ levels generated by the classical xanthine oxidase/xanthine system. Our results demonstrated

that preincubation of Nox2ds with xanthine oxidase and xanthine did not alter xanthine oxidase-mediated $O_2^{\bullet-}$ levels, suggesting that Nox2ds is unable to directly inhibit this enzyme nor directly scavenge $O_2^{\bullet-}$ derived from the enzyme.

To our knowledge, no single available Nox2-specific inhibitor is ready for use in clinical trials. Ideal Nox inhibitors show specificity, absence of toxicity, lack of off-target effects, desirable pharmacokinetic profiles and *in vivo* efficacy in well-established animal models of disease. Relevant to this point, there are no reports of toxicity or any non-specific actions of Nox2ds or its chimera Nox2ds-tat, which supports its specificity for inhibiting the unique protein-protein interaction it was designed to target in Nox2. In addition, there have been no reports to date of immunosuppressive or immunogenic responses to either Nox2ds-tat or scrambled control sequences following acute administration or chronic treatment of the drug [41]. This may appear surprising since inhibition of Nox2 oxidase in phagocytes could be expected to lead to reduced microbe killing and infection. However, there are two potential reasons for this. First, our original report on the peptide chimera displayed a small effect on macrophage ROS production [12]. As proposed in that report, it is plausible that proteases on the surface of leukocytes degrade the peptide thereby reducing its effect on these cells. Second, Dinauer and coworkers have reported that even very small amounts of $O_2^{\bullet-}$ production in phagocytes from patients with chronic granulomatous disease are sufficient for microbicidal activity [49]. Thus the levels of inhibition rendered by Nox2ds in phagocytes may avert dysfunction in these cells. Nevertheless, further investigations are clearly required before toxicity, off-target effects and pharmacokinetic profiles of Nox2ds are established.

Notwithstanding the broadly-demonstrated effectiveness of Nox2ds-tat by parenteral, peritoneal, subcutaneous, and direct application to blood vessels using gene therapy [46, 50], the limitations in the use of peptides as “druggable” therapeutics are obvious. These include a very limited oral bioavailability due to peptide degradation in the gut. As mentioned earlier however, these issues are being circumvented by novel technologies including the use of nanotechnologies and the aerosolization of Nox2ds for application directly into lungs. Indeed preliminary studies by our group show that nebulization of Nox2ds-tat into mouse lungs substantially reduces right ventricular hypertrophy in pulmonary hypertension (unpublished findings).

Overall, our findings demonstrate for the first time that Nox2ds is a selective inhibitor of Nox2-oxidase and support its utility in differentiating the contribution of Nox2 vs. Nox1 (canonical or hybrid) and Nox4 NADPH oxidase to Nox-derived ROS production. These findings have broad implications ranging from the use of this inhibitor as a means to delineate the role of Nox2 in cardiovascular and other pathologies as well as support for its potential use as a therapeutic agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

AngII	angiotensin II
COS-22	COS-7 cells transfected with p22 ^{phox}
COS-NOX1	COS-22 cells transfected with Nox1, NOXO1 and NOXA1
COS-NOX2	COS-22 cells transfected with, Nox2, p47 ^{phox} and p67 ^{phox}
COS-NOX4	COS-22 cells transfected with Nox4
DPI	diphenyleneiodonium chloride
DUOX	Dual oxidase
EPR	electron paramagnetic resonance
H₂O₂	hydrogen peroxide
LiDS	lithium dodecyl sulfate
NO	nitric oxide
Nox	NADPH oxidase
Nox2ds	Nox2 docking sequence
NOXA1	Nox Activator subunit 1
NOXO1	Nox Organizer subunit 1
O₂^{•-}	superoxide anion
PMA	phorbol myristate acetate
PMSF	phenylmethanesulfonyl fluoride
ROS	reactive oxygen species
SOD	superoxide dismutase
XO	xanthine oxidase
X	xanthine

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Figure 1.

Nox2ds dose-dependently inhibits $\text{O}_2^{\bullet-}$ production from Nox2-oxidase. COS-Nox2 cell lysate was preincubated with various concentrations of Nox2ds (from 0.1 μM to 10 μM) and scrmb Nox2ds (from 0.1 μM to 10 μM) for 5 min at 25°C. After the addition of 130 μM LiDS, $\text{O}_2^{\bullet-}$ production was initiated by the addition of 180 μM NADPH and measured by the initial linear rate of SOD-inhibitable cytochrome *c* reduction. $\text{O}_2^{\bullet-}$ production is expressed as nmol $\text{O}_2^{\bullet-}/\text{min}/10^7$ cell equivalents. Data represent the mean $\pm$ SEM of 7–16 experiments. For comparison, $\text{O}_2^{\bullet-}$ production in non-transfected COS-22 cell lysate is shown. * $p < 0.05$ indicates significant differences in $\text{O}_2^{\bullet-}$ production between Nox2ds- and scrmb Nox2ds-treatment. $\dagger p < 0.05$ indicates significant difference between COS-Nox2 and COS-22 cell lysate activity.

Fig 2A

Fig 2B

Fig 2C

Figure 2.

Nox2ds inhibits $O_2^{\bullet-}$ production from Nox2-oxidase measured by EPR. A) COS-Nox2 cell lysate was incubated with 130 μ M LiDS, and $O_2^{\bullet-}$ production was initiated by the addition of 180 μ M NADPH in the absence and presence of 150 U/ml SOD. CM• radical formation was measured for 10 min at 25 °C. * $p < 0.05$ indicates significant difference in $O_2^{\bullet-}$ production between LiDS- vs. vehicle-treated and LiDS- vs. LiDS + SOD-treated COS-Nox2 lysates, respectively. B) $O_2^{\bullet-}$ production in COS-Nox2 cell lysate was assayed as LiDS-stimulated (130 μ M), NADPH-dependent (180 μ M), SOD-inhibitable (150 U/ml) formation of CM• radical. COS-Nox2 cell lysate was preincubated with Nox2ds (10 μ M) and scrmB Nox2ds (10 μ M) peptides (prior to LiDS-treatment), and assayed for $O_2^{\bullet-}$ production (representative scans). C) cumulative and averaged SOD-inhibitable CM• radical formation in vehicle-, 10 μ M Nox2ds- and 10 μ M scrmB Nox2ds-treated COS-Nox2 lysates, respectively. Data represent the mean $\pm$ SEM of 4 – 5 experiments. * $p < 0.05$ indicates significant difference in $O_2^{\bullet-}$ production between Nox2ds- and vehicle-treatment.

Figure 3.

Nox2ds does not inhibit O₂^{•-} production from Nox1-oxidase. COS-Nox1 cell lysate was preincubated with various concentrations of Nox2ds (from 0.1 μM to 10 μM) for 5 min at 25 °C. O₂^{•-} production was initiated by the addition of 180 μM NADPH and measured by the initial linear rate of SOD-inhibitable cytochrome *c* reduction. O₂^{•-} production is expressed as nmol O₂^{•-}/min/10⁷ cell equivalents. Data represent the mean ± SEM of three experiments.

Figure 4.

Nox2ds does not inhibit H₂O₂ production from Nox4-oxidase. COS-Nox4 cell lysates were preincubated with various concentrations of Nox2ds (from 0.1 μM to 10 μM) for 5 min at 25 °C and H₂O₂ production was initiated by the addition of 36 μM NADPH. The fluorescence of Amplex Red was measured for 10 min at 25 °C. Nox4 activity was tabulated by subtracting non-transfected COS-22 cell lysate activity from COS-Nox4 cell lysate activity. H₂O₂ production is expressed as nmol H₂O₂/min/10⁷ cell equivalents. Data represent the mean ± SEM of 6–10 experiments.

Fig 5A

Fig 5B

Figure 5. p47^{phox}, but not NOXO1, binds to Nox2ds. ELISA experiments were performed to test whether p47^{phox} (Fig. 5A) and NOXO1 (Fig. 5B) bind to Nox2ds and/or scrambled Nox2ds. Biotinylated Nox2ds and scrambled peptides were first bound to neutravidin-coated plates

and then incubated with COS-22, COS-22-p47^{phox} (COS-22 cells transfected with p47^{phox}) or COS-22-NOXO1 (COS-22 cells transfected with NOXO1) cytosolic extracts for 1 hr at room temperature. Rabbit polyclonal p47^{phox} or rabbit NOXO1 antibodies were used to detect Nox2ds-bound p47^{phox} or NOXO1, respectively. After 1 hr incubation and extensive washing, bound primary antibodies were detected by FITC-labeled goat anti rabbit IgG antibody. The fluorescence of each well was measured using a Biotek Synergy 4 Hybrid Multi-Mode Microplate Reader (Excitation:488 nM, Emission:518 nM). Data represent the mean $\pm$ SEM of 3–4 experiments. * p <0.05 between COS-22 and COS-22-p47^{phox} cytosolic extracts in Nox2ds wells. # p <0.05 between Nox2ds and Scrmb Nox2ds wells incubated with COS-22-p47^{phox} extracts.

Figure 6.

Nox2ds does not compete with Nox4 DH domain for B-loop binding. Binding between GST-fused recombinant Nox4 dehydrogenase domain (DH) and Nox2ds or Scrmb Nox2ds was measured by the ability of each peptide to compete with FITC-conjugated Nox4 B-loop peptide for Nox4 DH protein. Fluorescence polarization detects binding between fluorescently labeled Nox4 B-loop peptide to GST-Nox4 DH protein. Data was fit to one-site competition curves using GraphPad Prism to obtain Log EC₅₀ values. Reported are the mean Log EC₅₀ of three experiments with standard error of the mean; ■ unlabeled WT Nox4 B-loop, Log EC₅₀ = 3.0 ± 0.08 nM; ▲ Nox2ds, Log EC₅₀ = 5.5 ± 0.5 nM; ▼ Scrmb, Log EC₅₀ = 5.4 ± 0.3 nM. Difference of Log EC₅₀ of unlabeled WT Nox4 B-loop vs. Nox2ds, $p < 0.03$; WT Nox4 B-loop vs. Scrmb, $p < 0.01$; no statistically significant difference between Nox2ds and Scrmb.

Figure 7.

Nox2ds does not inhibit xanthine oxidase (XO) or directly scavenge XO-derived $O_2^{\bullet-}$. The $O_2^{\bullet-}$ -scavenging activity of Nox2ds was determined by EPR using the xanthine/xanthine oxidase $O_2^{\bullet-}$ -generating system. Assay mixtures contained PBS, 5 mU/ml XO, 50 μ M CMH and 25 μ M deferoxamine in a final volume of 100 μ l. The reference samples contained 100 U/ml SOD. After addition of 25 μ M xanthine (X), CM• radical formation was monitored for 10 min in the absence and presence of 10 μ M Nox2ds or scrmb Nox2ds. Data represent the mean $\pm$ SEM of 6 experiments. * $p < 0.05$ indicates significant differences in CM• radical intensities between XO + X vs. XO and XO + X vs. 100 U/ml SOD + XO + X.

Table 1Effect of Nox2ds on $O_2^{\bullet-}$ Generating Activity ($O_2^{\bullet-}/\text{min}/10^7$ cell equivalents) of Nox1 oxidase

Nox system	control (no LiDS)	LiDS	10 μM Nox2ds+LiDS
non-transfected COS-22	0.16 $\pm$ 0.10		
Nox1/NOXO1/NOXA1	0.53 $\pm$ 0.02*	0.49 $\pm$ 0.02	0.43 $\pm$ 0.04
Nox1/NOXO1/p67 ^{phox}	0.58 $\pm$ 0.04*	0.66 $\pm$ 0.18	0.80 $\pm$ 0.20
Nox1/p47 ^{phox} /NOXA1	0.45 $\pm$ 0.03*	0.41 $\pm$ 0.02	0.66 $\pm$ 0.20
Nox1/p47 ^{phox} /p67 ^{phox}	0.96 $\pm$ 0.03*	1.52 $\pm$ 0.18 [#]	1.29 $\pm$ 0.20

COS-22 cells were separately transfected with Nox1, NOXO1, p47^{phox}, NOXA1 or p67^{phox}, lysed and the Nox1 membrane component, as well as the NOXO1, p47^{phox}, NOXA1 and p67^{phox} cytosolic extracts were separately prepared. The respective organizer subunit (NOXO1 or p47^{phox}) was preincubated with vehicle (control) or 10 μM Nox2ds for 10 min, and then Nox1-containing membrane and NOXA1/p67^{phox} were added consecutively. After the preincubation period, LiDS (130 μM) was added to induce the assembly of the oxidase and $O_2^{\bullet-}$ production was initiated by 180 μM NADPH. $O_2^{\bullet-}$ was measured by the initial linear rate of SOD-inhibitable cytochrome *c* reduction. $O_2^{\bullet-}$ production is expressed as nmol $O_2^{\bullet-}/\text{min}/10^7$ cell equivalents. Data represent the mean $\pm$ SEM of 3–6 experiments.

* $p < 0.05$ indicates significant differences in $O_2^{\bullet-}$ production between Nox1 oxidase and non-transfected COS-22 cell lysates.

[#] $p < 0.05$ indicates significant difference between vehicle (control) and LiDS-treatment.

Table 2

Effect of Nox2ds on inducible $O_2^{\bullet-}$ production ($O_2^{\bullet-}/\text{min}/10^7$ cell equivalents) in the HEK-Nox1 cell-free system

Nox system	control (no LiDS)	LiDS	10 μM Nox2ds+LiDS
Nox1/NOXO1/NOXA1	0.53 $\pm$ 0.01	0.98 $\pm$ 0.2*	1.2 $\pm$ 0.2

HEK 298 cells were separately transfected with Nox1, NOXO1, or NOXA1, lysed and the Nox1 membrane component, as well as the NOXO1, and NOXA1 cytosolic extracts were separately prepared. NOXO1 was preincubated with vehicle (control) or 10 μM Nox2ds for 10 min, and then Nox1-containing membrane and NOXA1 were added consecutively. After the preincubation period, LiDS (130 μM) was added to induce the assembly of the oxidase and $O_2^{\bullet-}$ production was initiated by 180 μM NADPH. $O_2^{\bullet-}$ was measured by the initial linear rate of SOD-inhibitable cytochrome *c* reduction. $O_2^{\bullet-}$ production is expressed as nmol $O_2^{\bullet-}/\text{min}/10^7$ cell equivalents. Data represent the mean $\pm$ SEM of 6 experiments.

* $p < 0.05$ indicates significant differences in $O_2^{\bullet-}$ production between vehicle (control) and LiDS-treatment.