Nitric oxide suppresses NADPH oxidase-dependent superoxide production by S-nitrosylation in human endothelial cells

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Abstract

Objective: Endothelial NADPH oxidase is a major source of superoxide in blood vessels and is implicated in the oxidative stress accompanying vascular diseases, including atherosclerosis. Here we investigate the regulation of NADPH oxidase activity by nitric oxide (NO).

Methods: Human cultured microvascular endothelial cells (HMEC-1) were treated with the NO donors, diethylenetriamine (DETA)-NONOate, S-nitroso-N-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP) for 0.5–24 h. Superoxide production was measured by lucigenin chemiluminescence and dihydroethidium fluorescence, while NADPH oxidase subunit expression was measured via Western blotting. S-nitrosylation was assessed using the 2,3-diaminonapthalene (DAN) assay, and via immunoblotting with an anti-nitrosocysteine antibody.

Results: Specific siRNA reduced Nox2 and Nox4 protein expression and markedly decreased superoxide production in HMEC-1. DETA-NONOate (10–300 μmol/L) suppressed superoxide production in HMEC-1 in a concentration- and time-dependent manner, which was not entirely attributable to stoichiometric reaction with NO, for the effect was observed more than 6 h after removing DETA-NONOate from solution. Similarly, sustained attenuation of superoxide production was achieved with SNP (10–100 μmol/L) and SNAP (10–100 μmol/L). The suppressive effect of NO was not dependent on (1) the sGC/cGMP/PKG pathway, (2) peroxynitrite-formation, (3) reduced protein expression of NADPH oxidase subunits or (4) dissociation of NADPH oxidase subunits. Treatment with NO caused S-nitrosylation of the crucial organizer subunit p47phox, and de-nitrosylation with UV light restored superoxide production.

Conclusions: NO causes sustained suppression of NADPH oxidase-dependent superoxide production in human endothelial cells by S-nitrosylation of p47phox. These findings highlight a novel approach by which vascular oxidative stress might be suppressed by NO donors.

Keywords: Nitric oxide; Endothelium; NADPH oxidase; Oxygen radicals

1. Introduction

NADPH oxidases are enzymes composed of up to five protein subunits: a flavin-containing catalytic ‘Nox’ subunit, the nature of which varies with different isoforms (Nox1, 2 or 4); a smaller subunit, p22phox, which forms a stable heterodimer with the Nox in cellular membranes; a cytosolic activator protein (p67phox or NoxA1); an organiser protein (p47phox or NoxO1); and a small G-protein, Rac-1 or 2 [1]. When assembled, all isoforms of NADPH oxidase generate ROS (superoxide and/or hydrogen peroxide) by transferring electrons from NADPH to FAD to molecular oxygen, which is bound by a heme moiety on the Nox subunit [1].
Isoforms of NADPH oxidase are expressed by all major cell types of the vascular wall, with Nox2- and Nox4-utilising oxidases being major contributors to endothelial superoxide production, at least during resting conditions [2–5]. Endothelial NADPH oxidases appear to exist as preassembled complexes associated with the cytoskeleton and, when expressed at normal levels, they produce low levels of ROS to influence redox-dependent cell signalling processes including ion channel opening, protein phosphatase and kinase activity and gene expression [3,4]. However, when expression of these and other NADPH oxidase isoforms (e.g. Nox1) are inappropriately up-regulated by pathophysiologival stimuli such as oscillatory shear stress, hyperglycaemia, cytokines, growth factors, and/or oxidised lipids, they may become major initiators of oxidative stress causing reduced nitric oxide (NO) bioavailability, upregulation of adhesion molecules and chemokines, and apoptosis [1]. Indeed, we and others have shown that upregulation of endothelial NADPH oxidase-derived ROS is associated with neointima formation/atherogenesis in both animal models [1,6,7] and in humans [8]. Moreover, genetic deletion of the gene encoding p47phox markedly reduced atherosclerotic lesion development in hypercholesterolemic apolipoprotein E-deficient mice, confirming a causal role for NADPH oxidases in atherogenesis [9].

The powerful vasodilator properties of NO donors have been exploited for over a century for the acute management of angina pectoris – a major symptom of coronary artery disease. However, in addition to their effects on vascular tone, NO donors may exert numerous other protective effects on the blood vessel wall, suggesting that their therapeutic potential may extend beyond acute symptomatic treatment, to prevention, and even reversal, of artery disease [10]. These effects include inhibition of lipid oxidation, vascular smooth muscle cell proliferation, inflammatory molecule and chemokine expression and platelet activation and aggregation. More recently, several reports have highlighted that certain NO donors may also reduce superoxide levels in vascular cells both in vitro [11] and in vivo [12,13]. However, it is unclear whether these ROS lowering effects of NO donors were simply due to scavenging of superoxide by NO, inhibition of signal transduction elements upstream of NADPH oxidase, and/or to a direct effect on one/or more NADPH oxidase subunits. Therefore we set out to determine whether treatment of human cells with NO donors affects superoxide production, and if so, whether this involved a specific inhibitory effect at the level of NADPH oxidase.

2. Methods

2.1. Cell culture

Human microvascular endothelial cells (HMEC-1) were cultured in MCDB-131 media (Invitrogen) supplemented with 10% fetal bovine serum (FBS), l-glutamine (10 mmol/L), penicillin/streptomycin (all from Trace Biosciences) and hydrocortisone (50 μmol/L; Sigma) in a CO2/O2 incubator at 37 °C. Cells (passage 10–16) were seeded in a 1:8 ratio and, unless otherwise stated, used 1 day post-confluence.

2.2. siRNA transfection

HMEC-1 were seeded and allowed to grow to ~70–80% confluence in 24-well plates. On day of transfection, three transfection solutions were prepared. The first, control solution, was composed of 200 μL of serum and antibiotic-free MCDB-131. The second and third transfection solutions each contained 1 μL of lipofectamine 2000 (Invitrogen) and either 5 μL of control siRNA (250 nmol/L) or specific siRNA for Nox2 or Nox4 (250 nmol/L) in 200 μL of serum- and antibiotic-free MCDB-131. Control and transfection solutions were mixed gently for 15 min at room temperature and then added to the cells. Following incubation for 5 h, the transfection media was was removed from cells and replaced with fresh MCDB-131 with 10% FBS. At 48 h after transfection, lucigenin chemiluminescence, dihydroethidium (DHE) fluorescence or Western blotting was employed.

2.3. NO donor treatment protocols

HMEC-1 were treated with either the slow-release (i.e. half-life >20 h) NO donor, diethylenetriamine (DETA)-NONOate (3–300 μmol/L), or the solvent used to dissolve DETA-NONOate, NaOH (0.01 mol/L), for 0.5 to 24 h. In separate cells, the NO donors, S-nitroso-N-acetylpenicillamine (SNAP; 1–100 μmol/L) or sodium nitroprusside (SNP; 1–100 μmol/L) were applied for 6 h. In all but the initial studies (Fig. 2A), the possibility of stoichiometric removal of superoxide by NO at time of assay was eliminated by washing the NO donor from the cells with Krebs–HEPES (see Supplementary data) at the end of the incubation period.

2.4. Superoxide detection with lucigenin chemiluminescence and dihydroethidium (DHE) fluorescence in intact HMEC-1

Following NO donor treatment, HMEC-1 were pre-incubated for 30 min with a Krebs–HEPES-based pre-incubation solution containing NADPH (100 μmol/L), to ensure adequate substrate availability for NADPH oxidase, and diethylthioketarbamate (DETCA; 3 mmol/L), to inactivate endogenous Cu2+/Zn2+ superoxide dismutases (SOD). Some cells were also treated with inhibitor drugs for 1 h prior to exposure to the Krebs–HEPES pre-incubation solution and then for the duration of the pre-incubation. Cells were then exposed to a Krebs–HEPES-based assay solution containing lucigenin (5 μmol/L), NADPH (100 μmol/L) and the appropriate inhibitor. Photon emission, as a measure of superoxide production [14], was recorded from each well every 2 min using a TopCount (Packard) and averaged over 30 min. Cell numbers in each well were evaluated using the Cell Titer Aqueous One Solution Cell Proliferation assay.
(Promega). Individual data points for each treatment group were derived from the average of six replicates.

In some experiments, superoxide production was measured with DHE fluorescence as we have recently described [5].

2.5. Superoxide detection with lucigenin chemiluminescence in HMEC-1 homogenates

Following NO donor treatment, 100-mm dishes of confluent HMEC-1 were washed in ice-cold phosphate-buffered saline (PBS; see Supplementary data) and lysed by incubation in a high-sucrose HEPES buffer (see Supplementary data) for 20 min at 4 °C. Protein concentration was then determined using a kit (Bio-Rad). Some homogenates (500 μL aliquots) were then placed 30 cm from a 4.9-W output, UV-C lamp for 5 min at 4 °C prior to superoxide detection, to disrupt S–NO bonds. Other homogenates were exposed to antagonists in PBS buffer for 1 h prior to superoxide detection. Finally, 10 μg of protein was added to an assay solution containing lucigenin (5 μmol/L) and NADPH (100 μmol/L) and photon emission was monitored as described above.

2.6. Whole-cell protein extraction for immunoprecipitation and Western blotting

Following NO donor treatment, 60-mm dishes of confluent HMEC-1 were washed with cold PBS and incubated for 60 min at 4 °C in lysis buffer (see Supplementary data). The lysate was then centrifuged for 20 min (14,000 rpm at 4 °C), and the resulting supernatant retained.

2.7. Immunoprecipitation

Whole-cell protein (500 μg) was exposed to rabbit IgG (10 μg) for 1 h at 4 °C and then ‘precleared’ using protein-G agarose beads. Following centrifugation, the supernatant was collected and incubated overnight at 4 °C with 5 μg of either anti-p22phox antibody or anti-p47phox antibody. An equal volume of protein G agarose was then added and the samples incubated for a further 1 h at 4 °C. The suspension was then centrifuged and the subsequent pellet containing protein G agarose beads and immunoprecipitated proteins was washed with chilled PBS.

2.8. Western blotting

Whole-cell or immunoprecipitated proteins (40 μg) were separated by SDS–PAGE and immunoblotted with primary antibodies against Nox2 (rabbit polyclonal, 1:1000 dilution, Upstate), Nox4 (goat polyclonal, 1:1000 dilution, Santa Cruz), p22phox (rabbit polyclonal, 1:1000 dilution, Santa Cruz), Rac-1 (mouse monoclonal, 1:1000 dilution, Transduction Laboratories), p47phox (mouse monoclonal, 1:1000 dilution, Santa Cruz) or nitrosocysteine (mouse monoclonal, 1:1000 dilution, AG Scientific) and appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualised using ECL (Amersham).

2.9. Detection of S-nitrosylated proteins with the 2,3-diaminonaphthalene (DAN) assay

P22phox protein was immunoprecipitated from control and NO donor-treated HMEC-1. Given that p22phox is common to all NADPH oxidase isoforms (and therefore associates directly or indirectly with all known NADPH oxidase subunits), immunoprecipitation with a p22phox antibody afforded a method of separating NADPH oxidase subunits from other cellular proteins that might become S-nitrosylated. The pellet containing the antibody/antigen complex and protein G beads was washed with PBS and exposed to high glycine (pH 3) buffer to separate the protein G from the antibody/antigen complex. The supernatant obtained from the washes was pooled and then divided into two equal aliquots. All aliquots were incubated with DAN (100 μmol/L) and one aliquot from each treatment group was subsequently exposed to HgCl2 (10 μmol/L). Samples were kept on ice in the dark for 30 min before being exposed to 0.1 mol/L NaOH. Fluorescence of the triazole produced by the reaction of released NO with DAN (excitation 375 nm; emission 450 nm) was then measured from each aliquot with a Polarstar microplate reader. The fluorescence intensity generated in the absence of HgCl2 (background) was then subtracted from the fluorescence intensity generated in the presence of HgCl2. Fluorescence intensity above background was normalised to the amount of protein.

2.10. Drugs and their suppliers

See Supplementary data.

2.11. Statistical analysis

Results are expressed as mean±standard error of the mean (S.E.M.). Statistical comparisons were made using Student’s unpaired t-test or one-way analysis of variance with Tukey–Kramer’s post hoc test. P<0.05 was considered significant.

3. Results

3.1. Superoxide detection with chemiluminescence

NADPH-dependent chemiluminescence in intact HMEC-1 was attenuated by 50% and 90%, respectively, by exogenous SOD (600 U/mL) and the cell permeable superoxide scavenger, MnTMPyP (25 μmol/L; Fig. 1A). In endothelial cell homogenates, SOD appeared to be more effective at reducing NADPH-dependent chemiluminescence (i.e. by 75%), presumably due to its better access to
intracellular sites of superoxide production (Supplementary Fig. 1B), while the cell permeable ROS scavenger, tiron, abolished the signal. These observations confirm that the lucigenin-enhanced chemiluminescence signal in response to NADPH in both intact and homogenised endothelial cells is due to superoxide.

NADPH-dependent superoxide production by HMEC-1 was abolished by the flavin inhibitor, DPI (100 μmol/L), and markedly reduced by the NADPH oxidase inhibitor, apocynin (1 μmol/L, Fig. 1B; see also Supplementary Fig. 1B). In contrast, l-NAME (100 μmol/L), indomethacin (3 μmol/L), 17-OD (3 μmol/L), allopurinol (100 μmol/L) and rotenone (1 μmol/L) had no effect on superoxide production in intact HMEC-1 (Fig. 1C) or in HMEC-1 homogenates (Supplementary Fig. 2) indicating that neither eNOS, cyclooxygenase, cytochrome P450, xanthine oxidase nor the mitochondrial respiratory complex are significant sources of superoxide in endothelial cells under these conditions.

Transfection of HMEC-1 with Nox2 siRNA significantly reduced expression of Nox2 protein (Fig. 2A and B) but had no effect on Nox4 expression (data not shown). Conversely, transfection with Nox4 siRNA markedly reduced the expression of Nox4 protein (Fig. 2E and F) and had no effect on Nox2 protein expression. Importantly, transfection of HMEC-1 with a scrambled control siRNA had no effect on either Nox2 or Nox4 expression (Fig. 2A and E). Knockdown of either Nox2 or Nox4 protein by siRNA caused a significant ∼50% and ∼25% reduction in superoxide production, respectively, compared to the control siRNA (Fig. 2C, D, G and H).

3.2. NO inhibits NADPH oxidase-dependent superoxide production

Acute DETA-NONOate (100 μmol/L) exposure caused a significant ∼50% reduction in chemiluminescence compared to control cells, most likely reflecting removal of superoxide by direct reaction with NO (Fig. 3A). Longer-term incubation with DETA-NONOate caused further reductions in superoxide levels with maximum reduction achieved after 6 h of treatment (Fig. 3A). Importantly, direct stoichiometric interaction between superoxide and NO could not account entirely for the time-dependent reduction of superoxide levels caused by DETA-NONOate, for a similar time-dependent reduction in superoxide was observed when DETA-NONOate was washed from the cells prior to assay (Fig. 3B).

Treatment of HMEC-1 with DETA-NONOate (3–300 μmol/L) for 6 h followed by washout prior to assay resulted in a concentration-dependent reduction in superoxide production (Fig. 3C). Importantly, the amount of NO liberated by DETA-NONOate over this concentration range (Supplementary Fig. 2) is representative of the physiological range of NO concentrations observed in endothelial cells at rest (i.e. <50 nM) and following stimulation with endothelium-dependent relaxing agonists and statins (i.e. 250–1000 nM) [15].

Finally, SNP (1–100 μmol/L) and SNAP (1–100 μmol/L) reduced NADPH oxidase-stimulated superoxide production in HMEC-1 following a 6-h exposure period and washout prior to assay (Fig. 3D).

3.3. Sustained suppression of NADPH oxidase activity by NO

In this series of experiments, HMEC-1 were treated with DETA-NONOate (100 μmol/L) for 6 h and then assayed for superoxide 0 to 24 h later, following washout of DETA-NONOate. Superoxide production was attenuated 60–70% when measured immediately after washout of DETA-NONOate and this effect was sustained for at least 6 h (Fig. 3E). 24 h after removing DETA-NONOate from the solution, superoxide levels had returned to control levels (Fig. 3E).

To ensure that the above effects of NO donors on superoxide production were not merely an artefact of the
ROS detection technique used, the effect of DETA-NONOate and SNP on superoxide production was also assessed by DHE fluorescence. Exposure of HMEC-1 to DHE for 30 min resulted in a significant increase in fluorescence. Pretreatment of HMEC-1 with a cell membrane-permeable superoxide scavenger, MnTMPyP (25 μmol/L), for 30 min prior to DHE exposure nearly abolished the fluorescent signal, whereas exogenous native SOD (600 U/mL) had no effect (Fig. 3F). These findings confirm that DHE detects superoxide produced intracellularly [5]. A 6-h exposure of HMEC-1 to either DETA-NONOate (100–300 μmol/L) or SNP (30; 100 μmol/L), followed by washout of the NO donor for 30 min, caused a significant reduction in DHE fluorescence (Fig. 3F).

3.5. Effect of NO on expression and co-association of NADPH oxidase subunits

Chronic DETA-NONOate (100 μmol/L) exposure failed to affect protein expression of Nox2, Nox4, p47phox or the G-protein, Rac in HMEC-1 (see Supplementary Fig. 4) indicating that attenuation of NADPH oxidase activity was not due to down-regulation of expression of any of these subunits.

We next examined the effect of DETA-NONOate (100 μmol/L) on association of p47phox with p22phox as a measure of NADPH oxidase activity. Whole-cell protein was immuno-precipitated with p22phox antibody, separated by SDS–PAGE, and transferred to PVDF membranes. Immunoblotting of these membranes with p47phox antibody revealed a single protein band with a molecular weight of ~47 kDa (see Supplementary Fig. 5). Exposure of HMEC-1 to DETA-NONOate (100 μmol/L, 6 h) prior to homogenisation did not significantly affect the amount of p47phox associated with p22phox, nor did it affect the expression of p22phox (Supplementary Fig. 5). Parallel experiments were performed in which the p47phox antibody was used for

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3.4. No role for guanylate cyclase or peroxynitrite

Neither the soluble GC inhibitor, ODQ, nor the selective PKG inhibitor, KT5823, had any effect on NADPH oxidase-dependent superoxide production in HMEC-1 (data not shown). Moreover, neither inhibitor reversed the attenuation by DETA-NONOate (100 μmol/L, 6 h) of NADPH oxidase-dependent superoxide production (see Supplementary Fig. 3). Uric acid also failed to reverse the inhibition of superoxide production by DETA-NONOate thereby excluding a role for peroxynitrite in attenuation of NADPH oxidase activity.

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immunoprecipitation, and a Nox2 antibody for immunoblotting and we similarly found no effect of DETA-NONOate on association of these subunits (data not shown).

3.6. S-Nitrosylation of p47phox

To assess for a possible role of S-nitrosylation, we examined the effects of exposure of HMEC-1 homogenates to UV light, a previously used method of disrupting S–NO bonds [16], on the ability of NO donors to inhibit superoxide production. UV light exposure did not affect superoxide production in untreated cells, but completely reversed the attenuation of superoxide production following 6 h of pre-treatment with SNAP (100 μM; Fig. 4A).

Both DETA-NONOate (100 μmol/L for 24-h exposure) and SNAP (100 μmol/L for 6-h exposure) caused a significant increase in DAN fluorescence above controls (Fig. 4B), indicating that p22phox and/or its associated proteins are S-nitrosylated by chronic exposure to NO.

Finally, to examine whether p47phox is S-nitrosylated by DETA-NONOate, we measured S-nitrosocysteine immunoreactivity in blots generated from protein that was immunoprecipitated, and a Nox2 antibody for immunoblotting and we similarly found no effect of DETA-NONOate on association of these subunits (data not shown).

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Finally, to examine whether p47phox is S-nitrosylated by DETA-NONOate, we measured S-nitrosocysteine immunoreactivity in blots generated from protein that was immuno-
precipitated with the p47phox antibody (Fig. 4D). The S-nitrosocysteine antibody revealed protein bands at ∼47 kDa, the reactivity of which was significantly (∼30%) higher in samples derived from cells that were exposed to DETA-NONOate (100 μM) for 6 h and 24 h (Fig. 4C and D).

4. Discussion

Here we present the first evidence that prolonged exposure of human endothelial cells to NO donors causes sustained suppression of superoxide production via inhibition of NADPH oxidase activity through S-nitrosylation of p47phox. The implication of these findings is that NO (derived from NO donor drugs and potentially endogenous enzymatic sources) regulates NADPH oxidase activity directly, and thereby might also modulate oxidative stress in vascular disease.

Over the past decade, substantial evidence has accumulated suggesting that some NO donors, and especially those belonging to the organic nitrate class (e.g. glyceryl trinitrate), may increase vascular superoxide production and thereby exacerbate oxidative stress in diseased blood vessels [17]. The mechanism(s) by which organic nitrates elevate vascular ROS levels are yet to be fully elucidated but appear to involve upregulation of Nox2 expression, activation of endothelial protein kinase C, and uncoupling of eNOS [17]. Hence, our finding that newer generation NO donors actually decrease vascular superoxide production, highlights the fact that this (and potentially other) adverse effect only applies to certain organic nitrates (and possibly SIN-1) and not to the NO donor class per se.

Using a combination of pharmacological inhibitors to exclude other sources of vascular ROS, and gene silencing approaches to knockdown expression of NADPH oxidase subunits, we confirmed that Nox2- and Nox4-containing NADPH oxidases are major sources of superoxide in endothelial cells under basal culture conditions [2–4]. It has previously been demonstrated that NO inhibits NADPH oxidase-dependent superoxide production in neutrophils [18–20]. We have now shown that NO donor drugs similarly suppress NADPH oxidase-dependent superoxide production in endothelial cells, with maximal inhibition occurring after 6 h of treatment. On one level, this reduction of superoxide levels by NO might be expected, given that NO reacts with superoxide in a near-diffusion limited manner to produce peroxynitrite. Indeed, when HMEC-1 were exposed to DETA-NONOate immediately before superoxide detection, significantly lower levels of superoxide were measured, presumably reflecting stoichiometric removal. However, two separate observations in the present study suggest that stoichiometric removal of superoxide by NO alone cannot explain the inhibitory effects of NO donors on endothelial
ROS production. First, given that the reaction between NO and superoxide is near-diffusion limited, if stoichiometric removal of superoxide alone was responsible for the effects of DETA-NONOate on ROS levels, one would expect the time-course to maximum inhibition by DETA-NONOate, to reflect the time it takes for the NO concentration within the pre-incubation/assay solution to reach steady-state levels. Following dissolution of DETA-NONOate in physiological buffer at 37 °C, maximum concentrations of NO are achieved within 20 min [21] – a far shorter period than the 6-h time-course of inhibition by DETA-NONOate observed here. Second, we showed that a large component of the inhibitory effect of NO donors persisted even following their washout. In the case of DETA-NONOate, suppression of NADPH oxidase-dependent superoxide levels persisted for at least 6 h after the washout.

It was previously shown that chronic treatment with NO donors inhibits cytokine- and LPS-induced superoxide production and Nox2 protein expression in porcine cultured aortic endothelial cells [11]. Although the time-course of inhibition by NO donors on basal endothelial NADPH oxidase activity reported here (> 6 h) is potentially consistent with an action at the level of gene expression, we were unable to demonstrate any effect of long-term DETA-NONOate on protein levels of Nox2, or indeed any other NADPH oxidase subunits (Nox4, Rac-1, p22phox or p47phox) in HMEC-1. This suggests that while NO has no effect on the signal transduction pathways responsible for maintenance of NADPH oxidase expression under basal conditions, it may block upstream signalling elements responsible for the upregulation of NADPH oxidase during pathophysiological conditions.

An alternative explanation for how the NO donors inhibited basal superoxide production in the present study is via post-translational mechanism(s). It is now established that NO can chemically modify certain functional groups on proteins and that this represents a major mechanism by which NO modulates cellular function. The best characterised of these interactions are (1) S-nitrosylation in which NO and/or higher nitrogen oxides (N₂O₃) nitrosate (·NO) specific reactive cysteine residues; and (2) tyrosine nitration, resulting from the conversion of NO into peroxynitrite and subsequent addition of a nitro group (·NO₂⁻) onto one of the ortho carbons on the aromatic ring of tyrosine residues [22,23]. While failure of the peroxynitrite scavenger, urate [24], in the present study appears to rule out a role for peroxynitrite-dependent nitration of critical tyrosine residues on any of the NADPH oxidase subunits, a number of lines of evidence support a role for S-nitrosylation:

1. Theoretical examination of the primary amino acid structure of the human p47phox subunit reveals that this subunit contains 4 cysteine residues, namely, C98, C111, C196 and C378. The latter three of these cysteine residues are conserved across species and one of these, C378, is nested amongst an amino acid environment that is predicted to be favourable for S-nitrosylation to occur [22,23,25].
2. S-Nitrosylation-dependent protein modification by NO does not involve cGMP or the PKG pathway [22,23,25], which is consistent with our observations of a lack of effect of ODQ and KT5823 on suppression of NADPH oxidase-derived superoxide by NO.
3. In the present study, brief exposure of HMEC-1 to UV light, a commonly used procedure for disrupting S–NO bonds [16], reversed the NO-dependent suppression of superoxide production.
4. Using the DAN fluorescent assay for detection of S-nitrosylated proteins [22], we demonstrated that p22phox-associated proteins from NO treated HMEC-1 are S-nitrosylated to a greater degree than the untreated controls.
5. Finally, our data with a nitrosocysteine-specific antibody suggests that NO preferentially nitrosylates the p47phox subunit.

Given that p47phox is the major organizer protein for Nox2-containing oxidases, and that Nox4 activity is unlikely to be regulated by any of the currently identified organizer (i.e. p47phox and Nox01) or activator (p67phox and Nox01) subunits [1], our findings provide functional evidence for regulation of the constitutive endothelial Nox2-containing NADPH oxidase expressed in endothelial cells by S-nitrosylation.

It remains to be determined as to how S-nitrosylation of p47phox interferes with NADPH oxidase activity. It has previously been suggested that NO donors and related S-nitrosothiols inhibit the activity of the neutrophil NADPH oxidase complex by interacting directly with either the membrane or cytosolic components of the complex and preventing their subsequent association [18–20]. For example, Park demonstrated that when membrane fractions were pre-treated with S-nitrosothiol and then combined with a cytosolic fraction from resting neutrophils to reconstitute NADPH oxidase, both translocation of the cytosolic NADPH oxidase components (p47phox and p67phox) to the plasma membrane fraction and superoxide generation were inhibited [20]. However, such a mechanism is unlikely to explain the effects of the NO donors on resting endothelial NADPH oxidase activity described here. Whereas the neutrophil NADPH oxidase complex at rest is dissociated and generates very little superoxide, the endothelial isoforms of NADPH oxidase exist as constitutively active, pre-assembled complexes containing either Nox2 [4] or Nox4 [2] as the catalytic domain. Thus, NO generated by NO donors would not have the opportunity to prevent assembly of these pre-assembled endothelial NADPH oxidase complexes. Moreover, we provide evidence that the NO donors did not cause disassembly of the endothelial NADPH oxidase complexes by the demonstration that chronic incubation of HMEC-1 with DETA-NONOate failed to disrupt the association of p47phox to either p22phox or Nox2.
Interestingly, C378 is positioned in close proximity to one of the major phosphorylation sites on p47phox, namely Ser379, raising the possibility of significant cross-talk between these two sites. Yet while phosphorylation of p47phox appears to decrease the sensitivity of C378 to alklylation by N-ethylmaleimide [26], the converse was not true, i.e. N-ethylmaleimide treatment did not prevent phosphorylation of p47phox [27]. Whether, S-nitrosylation (as opposed to alklylation) of C378 inhibits p47phox phosphorylation remains to be determined.

In conclusion, we have demonstrated that the NO donor, DETA-NONOate, at concentrations that generate physiological levels of NO, suppresses basal NADPH oxidase-dependent superoxide production in human endothelial cells. Given that NADPH oxidases are likely to be the major source of vascular ROS during normal physiology, our findings may suggest a role for endogenous NO in regulation of basal superoxide production. Moreover, it has recently been demonstrated that upregulation of certain isoforms of NADPH oxidase in the early stages of vascular disease gives rise to ‘kindling’ radicals, that ultimately cause depletion of tetrahydrobiopterin and uncoupling of eNOS, as well as oxidative conversion of xanthine dehydrogenase into xanthine oxidase, turning these enzymes into additional sources of ROS [28]. One would predict that suppression of NADPH oxidase activity by NO donors will therefore not only reduce ROS production by this enzyme in vascular disease, but also by these other, normally dormant, oxidases. Hence our findings, together with previous reports [11–13,29], highlight the potential of newer generation NO donors (i.e. those not belonging to the organic nitrate class) as treatments, not only for the symptoms of vascular disease (e.g. angina), but also of one of the major underlying causes of it, namely oxidative stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.03.030.

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