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Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase

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Abstract

All methods used for quantitation of superoxide have limitations when it comes to differentiating between extracellular and intracellular sites of superoxide production. In the present study, we monitored dihydroethidium (DHE)-derived fluorescence at 570 nm, which indicates hydroxyethidium derived from reaction with superoxide produced by human leukemia cells (HL-60) and microvascular endothelial cells (HMEC-1). Phorbol-12-myristate 13-acetate (PMA; 100 ng/ml) caused an increase in fluorescence and lucigenin chemiluminescence in HL-60, which was abolished by superoxide dismutase (SOD; 600 U/ml) indicating that DHE detects extracellular superoxide. Furthermore, both HL-60 cells and HMEC-1 generated a fluorescence signal in the presence of DHE under resting conditions, which was unaffected by SOD, but abolished by polyethylene glycosylated-SOD (PEG-SOD) (100 U/ml) and MnTmPyP (25 μM), indicating that DHE also detects superoxide produced intracellularly. In HMEC-1, silencing of either Nox2 or Nox4 components of NADPH oxidase with small interference RNA (siRNA) resulted in a significant reduction in superoxide detected by both DHE fluorescence (Nox2 siRNA; 71 ± 6% and Nox4 siRNA 83 ± 7% of control) and lucigenin chemiluminescence (Nox2; 54 ± 6% and Nox4 74 ± 4% of control). In conclusion, DHE-derived fluorescence at 570 nm is a convenient method for detection of intracellular and extracellular superoxide produced by phagocytic and vascular NADPH oxidase.

Keywords: *Dihydroethidium fluorescence, NADPH oxidase, Nox2, Nox4, endothelium, superoxide*

Abbreviations: *H_2-DCF-DA, dichlorodihydrofluorescein-diacetate; DHE, dihydroethidium; HMEC-1, human microvascular endothelial cells; MnTmPyP, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin; PMA, Phorbol-12-myristate 13-acetate; ROS, reactive oxygen species; siRNA, small interference RNA; SOD, superoxide dismutase*

Introduction

Reactive oxygen species (ROS) are a family of highly reactive molecules formed by stepwise, enzymatic, one-electron reductions of molecular oxygen, yielding superoxide anions and other species. Superoxide and its derivatives influence important physiological processes ranging from oxygen sensing and vasodilatation to smooth muscle cell proliferation and migration in angiogenesis. However, in excessive amounts ROS can cause vascular dysfunction and inflammation, which often are associated with cardiovascular disease states such as hypertension, atherosclerosis and myocardial infarction, as well as neurodegenerative diseases [8,19].

The detection of superoxide in biological systems has been difficult for several reasons. First, in cellular systems such as vascular cells, superoxide is generated

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in such small amounts that are often not detectable by many probes. This is attributed to the fact that superoxide is highly reactive in nature with an estimated steady-state intracellular concentration in the high picomolar to low nanomolar range. This results from tight regulation of its production both by enzymatic and non-enzymatic means, as well as diffusion-limited inactivation by cellular superoxide scavenging enzymes such as superoxide dismutases (SOD), nitric oxide (NO) and other low molecular weight scavengers. A second problem with currently available probes for superoxide detection is that they are not specific for the superoxide radical itself or may themselves act as radical generators. For example, lucigenin-enhanced chemiluminescence is arguably the most sensitive assay for superoxide detection, but it has been suggested that lucigenin may generate superoxide, which could overestimate the amount of superoxide generated by the responsible source. The dichlorodihydrofluorescein-diacetate (H_2DCF-DA) fluorescent probe is also commonly employed, but this may react with several ROS including hydrogen peroxide (H_2O_2), hydroxyl radicals and peroxynitrite [18,32]. One particular pitfall of electron spin resonance spectroscopy and cytochrome *c* for superoxide detection is their inability to detect superoxide when it is generated intracellularly [27,39]. A sensitive method for detection of intracellular superoxide would be very useful for quantifying (patho) physiologically relevant superoxide produced by vascular cells.

One assay for the detection of superoxide produced intracellularly is dihydroethidium (DHE) fluorescence. Although the chemical reaction between superoxide and DHE has not been precisely defined, it is assumed that DHE becomes oxidized by superoxide to generate ethidium, which then binds to DNA and fluoresces at excitation wavelengths in the range of 500 to 530 and emission ranging from 590 to 620 nm. However, recent evidence by Zhao et al. indicates that DHE reacts with superoxide to form a different product identified as hydroxyethidium, which has a distinct fluorescence spectrum [40]. Unlike ethidium, hydroxyethidium has a fluorescence emission peak at 567 nm, is stable intracellularly and is not produced by other reactive oxygen and nitrogen species such as H_2O_2, hydroxyl radical or peroxynitrite [13,40]. Fink et al. (2004) subsequently demonstrated that chronic angiotensin II treatment of bovine aortic endothelial cells caused an increase in hydroxyethidium fluorescence which was reversed by polyethylene glycosylated-SOD (PEG-SOD) [13]. However, they did not address whether the signal from cells at rest was attributed to superoxide. Therefore, one aim of the present study was to determine whether hydroxyethidium fluorescence is an indicator of basal intracellular superoxide production in endothelial cells and to elucidate the enzymatic source of this radical. Furthermore, given the potential advantages of hydroxyethidium as a fluorescence marker for superoxide detection, we also sought to examine whether this analysis could be used to detect superoxide produced extracellularly by NADPH oxidase in phagocytic type HL-60 cells.

Materials and methods

Materials

MCDB-131, RPMI 1640 cell culture media and fetal bovine serum (FBS) were purchased from Gibco-BRL (California, USA). L-glutamine and penicillin/streptomycin were purchased from Trace Biosciences (Australia). Gp91phox siRNA (aka Nox2), control siRNA and anti-Nox4 goat polyclonal antibody were purchased from Santa Cruz Biotechnology (USA). Specific Nox4 (product ID 118807) and control siRNA were purchased from Ambion. Anti gp91phox rabbit polyclonal antibody was purchased from Upstate Biotechnology (USA). DHE and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Molecular Probes (Eugene, OR, USA). Complete cocktail of protease inhibitors were obtained from Roche. N^G-nitro-L-arginine methyl ester (L-NAME), allopurinol, indomethacin, 17-octadecynoic acid (17-ODYA), rotenone, 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), diethyldithiocarbamate (DETCA), superoxide dismutase (SOD; bovine erythrocytes), polyethylene glycol superoxide dismutase (PEG-SOD; bovine erythrocyte), xanthine, xanthine oxidase, acetovanillone (apocynin) and lucigenin were obtained from Sigma Chemical Co. (MO, USA). The SOD mimetic MnTmPyP was obtained from Cayman Chemicals Co. (MI., USA). Gp91 ds-tat peptide sequence as described by Rey et al., H-RKKRRQRRRCSTRIRRQL-NH2 [31] was synthesized by Auspep Pty. Ltd (Vic., Australia).

Cell culture

HL-60 (originally from ATCC; a gift from Dr Jenny Leung, Department of Surgery, St Vincent's Hospital, Melbourne, Australia) cells were cultured in RPMI 1640 with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). To induce differentiation to granulocyte-like cells, HL-60 cell suspensions were incubated with 1.5% dimethylsulphoxide (DMSO) for 3 days [15]. Superoxide production from differentiated HL-60 cells (10^6 cells/well) was quantified by either DHE fluorescence or lucigenin-enhanced chemiluminescence.

Human microvascular endothelial cells (HMEC-1 originally from National Center for Infectious disease and Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia, USA; a gift from Prof Philip Hogg, University of New South Wales, Sydney, Australia) were cultured in MCDB-131 media

supplemented with 10% FBS, L-glutamine (1 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml). HMEC-1 were seeded (40,000 cells/well) in either white (chemiluminescence) or black (fluorescence) 96-well opti-plates for 48 h, after which time, cells became confluent. Superoxide production was then quantified by either hydroxyethidium fluorescence or lucigenin-enhanced chemiluminescence, whereas total ROS production was quantified by H_2DCF-DA fluorescence. In some experiments HMEC-1 homogenates were prepared by lysis of cells with a high sucrose HEPES buffer (pH 7.4, 250 mM sucrose, 10 mM sodium HEPES and a cocktail of protease inhibitors).

Extracellular and intracellular detection of superoxide in HL-60 cells by hydroxyethidium fluorescence

DMSO-differentiated HL-60 cells were washed with pre-warmed (37°C) HBSS and re-suspended in Krebs HEPES buffer (composition in mM: Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127.8, HCO_3^- 25.0, SO_4^{2-} 1.2, H_2PO_4-1.2, HEPES 20, and glucose 11.0) containing DHE (25 μM). Two hundred microliters ($\sim 10^6$ cells/well) of the cell suspension were dispensed into each well of a 96-well black view plate. Some cells were then stimulated with PMA (100 ng/ml) and fluorescence intensity was quantified using a Polarstar microplate reader (BMG laboratories, Australia) with excitation and emission wavelengths of 480 ± 10 and 570 ± 10 nm, respectively to optimally monitor hydroxyethidium [27,39]. In some cases HL-60 cells were exposed to either the cell impermeable SOD protein (600 U/ml), cell permeable SOD mimetic MnTmPyP (25 μM), apocynin (1 mM for 30 min) or gp91ds-tat peptide (10 μM for 30 min) before PMA application. The component of the fluorescence inhibited by SOD is attributed to the release of superoxide into the extracellular space. Each treatment group was performed in triplicates and fluorescence was recorded from each well every 2 min and averaged over 2 h. To minimize any potential artifactual fluorescence due to visible light the entire procedure was performed under dark conditions. In preliminary experiments we found that cells produce no background fluorescence at the excitation and emission wavelengths used for superoxide detection. We also measured background fluorescence caused by DHE in the absence of cells. These background values were then subtracted from the total fluorescence caused by cells to determine the amount of fluorescence attributed to cells only.

Intracellular detection of superoxide in HMEC-1 by hydroxyethidium fluorescence

Confluent HMEC-1 were incubated with pre-warmed (37°C) Krebs-HEPES buffer in the absence or presence of either SOD (600 U/ml) or MnTmPyP (25 μM) or inhibitors of potential enzymatic and non-enzymatic sources of superoxide for 45 min. These included the nitric oxide synthase (NOS) inhibitor L-NAME (100 μM; $IC_{50} \sim 0.1-0.5$ μM), the xanthine oxidase inhibitor allopurinol (100 μM; IC_{50} 0.8 μM), the electron transport chain inhibitor rotenone (3 μM; $IC_{50} \sim 8-20$ nM), the inhibitor of cytochrome P_{450} 17-ODYA (100 μM; $IC_{50} <$ 100 nM) or the cyclo-oxygenase inhibitor, indomethacin (3 μM; IC_{50} 0.008 μM for COX1 and 0.04 μM for COX2), DMSO (0.1%, the solvent control for rotenone) or ethanol (0.1%, the solvent control for indomethacin). In some cases, HMEC-1 were incubated with PEG-SOD (100 U/ml) for 4 h in cell culture media. Cells were then washed with Krebs-HEPES buffer and incubated with DHE (25 μM) in the continued presence of the appropriate inhibitor or solvent for 30 min and then washed with Krebs-HEPES to remove any unreacted DHE from the surrounding incubant and consequently the extracellular space. Fluorescence intensity was then quantified as described above. Each treatment group was performed in triplicates and fluorescence was recorded from each well every 2 min and averaged over 20 min.

Measurement of superoxide by lucigenin-enhanced chemiluminescence

Lucigenin-enhanced chemiluminescence was also used to measure superoxide produced by DMSO-differentiated HL-60 cells and HMEC-1. Li et al. have previously shown that lucigenin at 5 μM does not undergo redox cycling to generate superoxide [24]. HL-60 cells were incubated with a Krebs-HEPES-based assay solution containing lucigenin (5 μM). Two hundred microlitres ($\sim 10^6$ cells/well) of cell suspension were dispensed into 96-well opti-plates for luminescence reading. In some experiments cells were incubated with either SOD (600 U/ml), MnTmPyP (25 μM), apocynin (1 mM for 30 min) or gp91 ds-tat peptide (10 μM for 30 min) before PMA (100 ng/ml) addition.

Adherent HMEC-1 were incubated in pre-warmed (37°C) Krebs-HEPES solution for 45 min containing NADPH (100 μM) and diethyldithiocarbamate (DETCA; 3 mM) the latter to inactivate endogenous SOD. Some cells were also treated with different inhibitors of enzymatic and non-enzymatic sources of superoxide or superoxide scavengers in the manner specified above for DHE fluorescence experiments. All cells were then incubated with a Krebs-HEPES-based assay solution containing lucigenin (5 μM), NADPH (100 μM) and the appropriate inhibitor or superoxide scavenger. For both HL-60 cells and HMEC-1 experiments each treatment group was performed in triplicate and photon emission was

recorded from each well every 2 min using a Polarstar microplate reader and averaged over 20 min for HMEC-1 or 2 h for HL-60 cells.

Measurement of total ROS using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) fluorescence

H_2DCF-DA fluorescence was used to measure total ROS production in HMEC-1 cultured in 24-well (~80,000 cells/well) opti-plates. HMEC-1 were untreated or treated with the superoxide scavenger tiron (10 mM) for 1 h, washed with warm (37°C) HBSS to remove any residual tiron from the incubant and then exposed to H_2DCF-DA (10 μM). Also H_2DCF-DA was added to separate wells in the absence of cells to establish the background fluorescence caused by H_2DCF-DA. Fluorescence was then measured with excitation and emission wavelengths of 480 ± 10 and 530 ± 20 nm, respectively, using a Polarstar microplate reader at 37°C over a period of 1 h. Each treatment group was conducted in triplicates.

Knockdown of Nox subunits with siRNA

An siRNA sequence of 22 nucleotides specific for either the human *Nox2* or *Nox4* genes were used to silence the expression of Nox2 and Nox4 protein in HMEC-1. Briefly, for Nox2 siRNA experiments, HMEC-1 were seeded in 24-well plates before the day of transfection to obtain ~70–80% confluence. We have preliminary evidence (Peshavariya et al., unpublished observations), which indicates that Nox4 expression is higher in proliferating cells compared to confluent cells and thus we seeded cells to obtain 40–50% confluence for Nox4 siRNA transfection studies. These findings are supported by the study of Bayraktutan, which demonstrate that p22phox expression is also markedly higher in proliferating endothelial cells compared with confluent cells [5]. On the day of transfection, 1 μl of Lipofectamine 2000 solution (1 mg/ml) and 5 μl of either Nox2 (10 μM), 1 μl of Nox4 (50 μM) or 1 μl of control siRNA (50 μM) were mixed in 100 μl of Opti-MEM at room temperature for 15 min. Cells were washed with PBS and 400 μl of Opti-MEM were added to each well, followed by addition of 100 μl of Opti-MEM containing lipofectamine–siRNA solution to the respective wells. The final concentration of Nox2, Nox4 and control siRNA was 100 nM/well. Control cells contained only 500 μl of Opti-MEM. The control and siRNA transfection solutions were incubated at 37°C and 5% CO_2 for 6 h. Using a fluorescein isothiocyanate (FITC) conjugated siRNA the transfection efficiency was quantified as ~90–95% following 6 h incubation. Following incubation the transfection media was removed from cells and replaced with fresh MCDB-131 with 10% FBS. Forty-eight hours after transfection, DHE and H_2DCF-DA fluorescence assays were employed to quantify superoxide and total ROS production in adherent HMEC-1. For lucigenin-enhanced chemiluminescence, cell suspensions were prepared from HMEC-1 grown in 24-well plates. Confluent HMEC-1 were detached by trypsinization (Trypsin–EDTA; 0.25%), collected by centrifugation (300 g for 5 min) and then resuspended in a pre-incubation solution containing NADPH (100 μM) and DETCA (3 mM). After 45 min of pre-incubation, cells were sedimented by centrifugation at 300 g for 5 min and then resuspended in white 96-well opti-plates in a Krebs-HEPES-based assay solution containing lucigenin (5 μM) and NADPH (100 μM). Finally, chemiluminescence was measured every 2 min from each well using the Polarstar microplate reader and averaged over 20 min. We also performed additional experiments using HMEC-1 homogenates. Homogenates (10μg of protein) were exposed to NADPH (100 μM) and lucigenin (5 μM) for 20 min and chemiluminescence was expressed as counts per seconds per microgram of protein.

Hydroxyethidium fluorescence and lucigenin chemiluminescence detection of superoxide by xanthine/xanthine oxidase

DHE (25 μM) or lucigenin (5 μM) were incubated with xanthine (100 μM) in PBS (0.1 M, pH 7.4) in the absence or presence of SOD (600 U/ml) or MnTmPyP (25 μM) [20]. After addition of xanthine oxidase (0.03 U/ml), fluorescence or chemiluminescence was measured for 1 h. Each treatment group was conducted in triplicates.

Gel electrophoresis and Western blotting

Following siRNA transfection, HMEC-1 were washed with cold (4°C) PBS and lysed with 100 μl of cell lysis buffer (composition; 1% Triton X-100, 10 mM Tris, 2 mM EDTA, 10 mM sodium fluoride, 10 mM glycerophosphate, 0.2 mg/ml benzamidine HCl, 100 mM NaCl and a protease inhibitor cocktail). Protein concentrations were determined with a commercially available kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Aliquots of whole cell protein were mixed with an appropriate volume of sample laemelli buffer (6X) containing SDS (4%), Tris (125 mM) and β-mercaptoethanol prior to being boiled at 95°C for 5 min. Equal amounts of protein were then separated by electrophoresis using 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham). After blocking with 5% non-fat milk in a buffer containing Tris–HCl (20 mM, pH 7.5), NaCl (100 mM) and Tween 20 (0.1%), respective membranes were incubated at 4°C overnight with either primary antibodies against Nox2 (rabbit polyclonal, 1:1000 dilution) or Nox4

(goat polyclonal, 1:200 dilution). Membranes were then washed (20 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.1% Tween 20) for 30 min and incubated for 2 h at room temperature with appropriate secondary antibodies conjugated to horseradish peroxidase (1:2000 dilution, Bio-Rad). Immunoreactive bands were then visualized using the ECL detection kit (Amersham). After Nox2 and Nox4 band visualization, membranes were washed and incubated with a primary antibody against β-actin (mouse monoclonal 1:5000 dilution) for 1 h. Following a 30 min wash period, membranes were incubated for 1 h at room temperature with a rabbit anti-mouse secondary antibody conjugated to horseradish peroxidase (1:2000 dilution, Chemicon). Immunoreactive bands of β-actin were then visualized using the ECL detection kit. The Gene Genius Bio imaging system was used to capture the images and densitometry analysis. The proteins extracted from human HL-60 cells and rat kidney cortex were used as positive controls and as a test for the specificity of Nox2 and Nox4 antibody.

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made using one-way analysis of variance with Tukey–Kramer *post hoc* tests. $P < 0.05$ was considered significant.

Results

Measurement of superoxide generated by xanthine/xanthine oxidase cell free system using hydroxyethidium fluorescence and lucigenin-enhanced chemiluminescence

Background fluorescence caused by addition of DHE to a solution containing PBS and xanthine oxidase (0.03 U/ml) measured 670 ± 56 RFU ($n = 4$). Subsequent addition of xanthine (100 μM) to a PBS solution containing xanthine oxidase (0.03 U/ml) and DHE caused a substantial increase in fluorescence above background, which was markedly reduced by either SOD (600 U/ml) or MnTmPyP (25 μM; Figure 1A). This confirms that most of the DHE-dependent fluorescent signal, which has previously been attributed to hydroxyethidium formation, was due to superoxide. Also, both SOD (600 U/ml) and MnTmPyP (25 μM) abolished the xanthine/xanthine oxidase-dependent lucigenin chemiluminescent signal (Figure 1B).

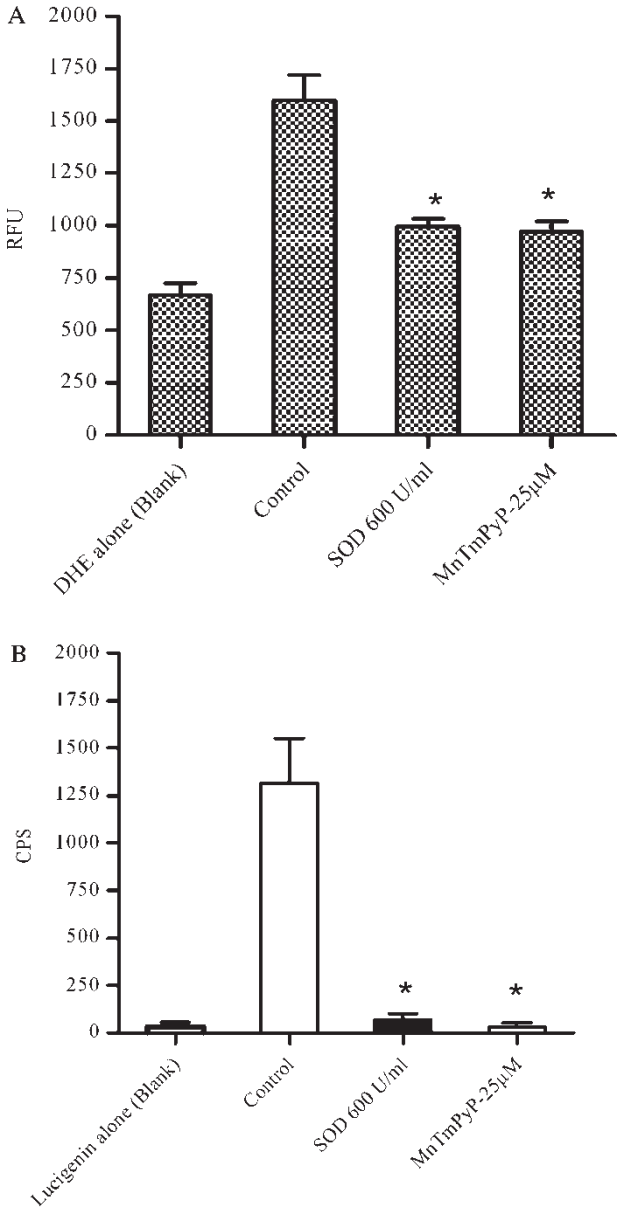

Figure 1. Detection of superoxide generated by a xanthine/xanthine oxidase cell-free system using DHE fluorescence (A) and lucigenin-enhanced chemiluminescence (B). DHE (25 μM) or lucigenin (5 μM) were incubated with xanthine (100 μM) and xanthine oxidase (0.03 U/ml) in PBS solution (pH 7.4) in the absence or presence of SOD (600 U/ml) or the SOD mimetic MnTmPyP (25 μM). Also shown are background DHE fluorescence (DHE alone; blank) and lucigenin chemiluminescence signals (lucigenin alone; blank) in the absence of superoxide. Values (mean ± SEM from 3 to 4 experiments) represent relative fluorescence units (RFU, A) and counts per second (CPS, B). The asterisk denotes a $P < 0.05$ value following a one-way ANOVA with Tukey–Kramer *post hoc* analysis.

Hydroxyethidium fluorescence detects extracellular and intracellular production of superoxide in HL-60 cells

Exposure of HL-60 cells to DHE resulted in a significant increase in fluorescence, which was unaffected by addition of extracellular SOD protein (600 U/ml) but significantly reduced by the cell permeable SOD mimetic MnTmPyP (25 μM; Figure 2A) and PEG-SOD (100 U/ml; 15 ± 3% of the control; $n = 3$). These findings indicate that DHE detects basal superoxide produced intracellularly by HL-60 cells. In the presence of PMA (100 ng/ml), there was a significant ~2-fold increase in fluorescence above basal, which was abolished by extracellular SOD

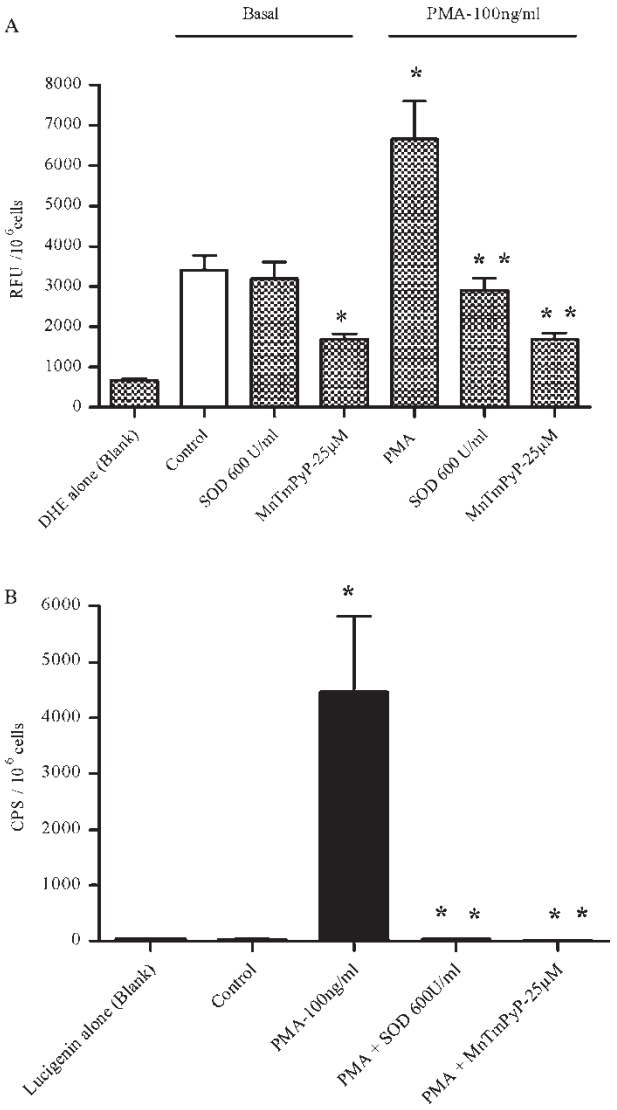

Figure 2. Measurement of intracellular and extracellular superoxide using DMSO-differentiated HL-60 cells using DHE fluorescence (25 μM, A) or lucigenin (5 μM, B) enhanced chemiluminescence. To determine the intracellular or extracellular production of superoxide in response to PMA (100 ng/ml), HL-60 cells were incubated with either SOD (600 U/ml) or the cell permeable SOD mimetic MnTmPyP (25 μM). Also shown are background DHE fluorescence (DHE alone; blank) and lucigenin chemiluminescence signals (lucigenin alone; blank) in the absence of cells. Values (mean ± SEM from 3 to 5 experiments) represent relative fluorescence units (RFU, A) or counts per second (CPS, B) per 10^6 cells. Asterisk (*) denotes a $P < 0.05$ value following a one-way ANOVA with Tukey–Kramer *post hoc* analysis. A single asterisk (*) shows significance between control vs. PMA-stimulated, whereas double asterisks (**) show significance between PMA stimulated vs. PMA in the presence of either SOD or SOD mimetic MnTmPyP.

(600 U/ml) indicating that DHE detects extracellular release of superoxide by PMA (Figure 2A). MnTmPyP (25 μM) also abolished the PMA-dependent increase in hydroxyethidium fluorescence (Figure 2A). To examine whether the extracellular component of the hydroxyethidium fluorescence was attributed to NADPH oxidase, HL-60 cells were pre-incubated with NADPH oxidase inhibitors apocynin (1 mM) or gp91ds-tat peptide (10 μM for 30 min). The basal DHE signal caused by HL-60 cells was unaffected by either of these inhibitors whereas the PMA-stimulated DHE fluorescence (136 ± 6.1% of basal) was significantly inhibited by both apocynin (112 ± 4.3% of basal) and gp91ds-tat (117 ± 3.7% of basal; Figure 3A).

We next compared our hydroxyethidium fluorescence observations with lucigenin-enhanced chemiluminescence. In the absence of PMA, HL-60 cells failed to produce detectable chemiluminescence, however, this signal increased substantially following activation

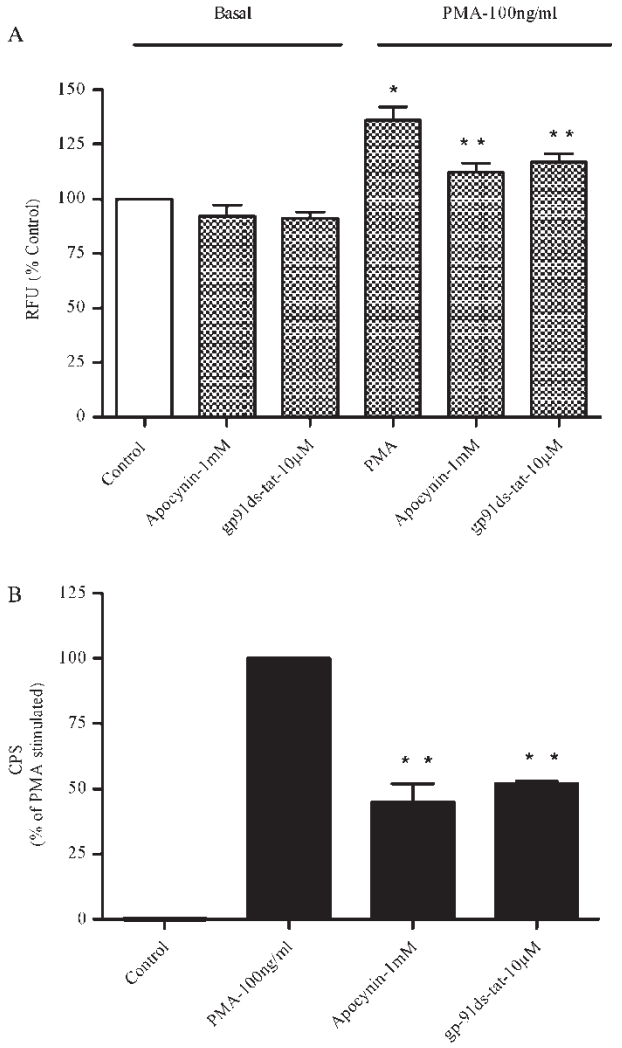

Figure 3. Effect of NADPH oxidase inhibitors apocynin and gp91 ds-tat peptide on PMA-stimulated HL-60 cells. To determine whether the PMA-stimulated DHE fluorescence (A) and lucigenin chemiluminescence signal (B) is due to NADPH oxidase-derived superoxide, DMSO-differentiated HL-60 cells were incubated for 30 min with either apocynin (1 mM) or gp91 ds-tat peptide (10 μM) before PMA stimulation. Values (mean ± SEM from 3 experiments) represent relative fluorescence units (RFU, A) or counts per second (CPS, B), and are expressed as percentage control in Figure 3A or percentage of PMA induced response in Figure 3B. A single asterisk (*) shows significance between control vs. PMA stimulated, whereas double asterisks (**) show significance between PMA stimulated vs. PMA in the presence of either apocynin or gp91ds-tat.

with PMA (Figure 2B). Importantly, the PMA-dependent increase in lucigenin chemiluminescence was completely abolished by either SOD (600 U/ml) or MnTmPyP (25 μM; Figure 2B). Furthermore, the PMA-stimulated lucigenin chemiluminescence signal was due to NADPH oxidase as both NADPH oxidase inhibitors apocynin (1 mM) and gp91ds-tat peptide (10 μM) significantly inhibited this signal (Figure 3B).

Intracellular detection of superoxide production in HMEC-1

In endothelial cells it has been shown that the formation of hydroxyethidium from DHE is proportional to the rate of superoxide formation [13]. We wanted to determine whether basal, intracellular production of superoxide could be detected by hydroxyethidium fluorescence. Exposure of HMEC-1 to DHE for 30 min followed by removal of DHE from the surrounding incubant, resulted in a significant increase in fluorescence (Figure 4A). Pre-treatment of HMEC-1 either with MnTmPyP (25 μM) for 45 min or with PEG-SOD (100 U/ml) for 4 h, abolished this increase in basal fluorescence whereas addition of SOD (600 U/ml) protein to the extracellular space had no effect (Figure 4A). The lack of effect of extracellular SOD supports the view that DHE detects superoxide produced intracellularly in endothelial cells. The protocol of DHE addition and subsequent removal from the supernatant allows for the specific detection of superoxide produced intracellularly.

We and others have previously shown that, in the absence of NADPH, vascular cells fail to produce detectable signal with the lucigenin (5 μM) chemiluminescence assay [12,21]. Exposure of resting HMEC-1 to lucigenin failed to produce a detectable chemiluminescence signal above background (data not shown), but concomitant addition of NADPH (100 μM) to HMEC-1 markedly increased the signal

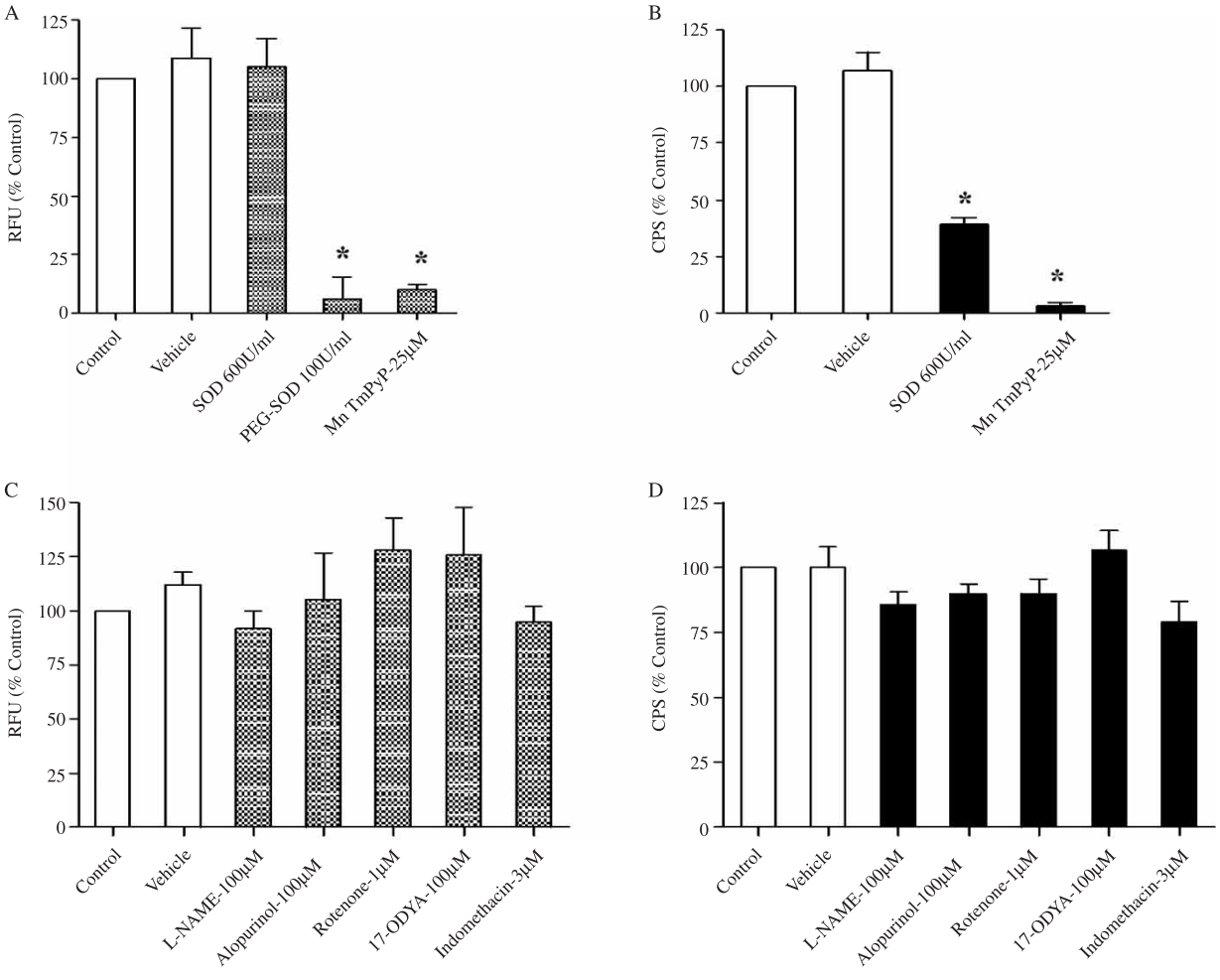

Figure 4. Effects of superoxide scavengers and inhibitors of potential enzymatic and mitochondrial sources of superoxide on basal superoxide production or NADPH-dependent superoxide production in HMEC-1 as measured by dihydroethidium fluorescence (DHE; 25 μM A and C) or lucigenin-enhanced chemiluminescence (5 μM, B and D), respectively. HMEC-1 were either untreated (control) or treated with vehicles (0.1% DMSO or 0.1% ethanol), SOD (600 U/ml), PEG-SOD (100 U/ml), the SOD mimetic MnTmPyP (25 μM), the NOS inhibitor L-NAME (100 μM), the xanthine oxidase inhibitor allopurinol (100 μM), the electron transport chain inhibitor rotenone (1 μM), the cytochrome P450 inhibitor 17-ODYA (100 μM) or cyclo-oxygenase inhibitor indomethacin (3 μM). Values (mean ± SEM from 3 to 5 experiments) represent relative fluorescence units (RFU, A and C) or counts per second (CPS, B and D), and are expressed as a percentage of the control. The asterisk denotes a $P < 0.05$ value following a one-way ANOVA with Tukey–Kramer *post hoc* analysis.

(Figure 4B). The NADPH-dependent lucigenin chemiluminescent signal was attributed to superoxide for treatment with MnTmPyP (25 μM) at the time of assay abolished the signal (Figure 4B). Moreover, the application of exogenous SOD protein caused a ~60% reduction in the signal compared to control indicating that more than half of the signal is attributed to extracellular superoxide.

These data indicate that HMEC-1 produce superoxide at rest. We also tested the effect of inhibitors of eNOS (L-NAME), xanthine oxidase (allopurinol), the electron transport chain (rotenone), cytochrome P_{450} (17-ODYA) and cyclo-oxygenase (indomethacin) to determine whether these potential sources of superoxide contribute to the basal DHE fluorescent signal. Exposure of HMEC-1 for 45 min to L-NAME (100 μM), allopurinol (100 μM), rotenone (1 μM), 17-ODYA (100 μM) or indomethacin (3 μM) had no effect on the DHE fluorescent signal (Figure 4C) compared to controls (0.1% DMSO or ethanol). Similar to the findings with DHE fluorescence analysis, none of L-NAME, allopurinol, rotenone, 17-ODYA or indomethacin significantly affected the NADPH-dependent lucigenin enhanced chemiluminescence (Figure 4D).

Silencing of Nox subunits reduced basal and NADPH-dependent superoxide production in HMEC-1

In endothelial cells, major sources of superoxide are Nox2 and Nox4 containing NADPH oxidases. Due to the lack of selective inhibitors of NADPH oxidases, we used specific siRNA to silence either Nox2 or Nox4 expression. HMEC-1 were transfected with either specific Nox2 or Nox4 siRNA or control siRNA and subsequent expression of Nox2 and Nox4 were analyzed by Western blotting with commercially available Nox2 and Nox4 antibodies. Using cell lysates from control HMEC-1, we detected strong protein bands with an estimated molecular weight of 75 and 65 kDa of Nox2 and Nox4 proteins, respectively (Figure 5A,B). As validation of the specificity of the polyclonal Nox2 and Nox4 antibodies, we demonstrated similar protein bands at ~75 kDa (Nox2) from HL-60 cells and ~65 kDa (Nox4) from rat kidney cortex protein lysates, respectively (Figure 5A,B). Transfection of HMEC-1 with the control siRNA did not significantly alter Nox2 or Nox4 expression compared to untreated HMEC-1 (Figure 5A,B; $n = 4–5$). However, specific Nox2 and Nox4 siRNA significantly reduced the expression of Nox2 (53 ± 8% of controls) and Nox4 (32 ± 18% of controls) protein in HMEC-1, respectively, compared to control siRNA (Figure 5C,D). Also, shown in Figures 6A and 7A, the hydroxyethidium fluorescent signal was reduced to 71 ± 6% of control ($n = 7$) and 83 ± 7% of control ($n = 4$) in the presence of Nox2 and Nox4 siRNA, respectively, compared to control siRNA. We also assessed the effect of Nox silencing on the NADPH-dependent lucigenin-enhanced chemiluminescence signal. As with hydroxyethidium fluorescence, the control siRNA had no effect on NADPH-dependent chemiluminescence compared to control (Figures 6B and 7B). However, Nox2 and Nox4 siRNA caused a significant reduction to 54 ± 6% of control ($n = 7$) and 74 ± 4% of control ($n = 4$) respectively, in the NADPH-dependent chemiluminescence compared to control siRNA (Figures 6B and 7B). Thus, the silencing of catalytic subunits Nox2 and Nox4 inhibits both basal and NADPH-stimulated superoxide production in endothelial cells.

Lucigenin-enhanced chemiluminescence can only be detected when NADPH is applied exogenously to HMEC-1 in culture. This approach has been queried because it is unclear how exogenous NADPH increases NADPH oxidase activity in intact cells given that NADPH is unlikely to penetrate cell membranes and that the binding regions for NADPH on the oxidase are intracellular. We therefore conducted experiments with HMEC-1 homogenates. HMEC-1 were transfected with Nox2 and Nox4 siRNA and after 48 h homogenates were prepared as described in the "Methods" section and superoxide measured with NADPH-dependent chemiluminescence. Superoxide production was reduced by ~30% following transfection with Nox2 siRNA and ~15% with Nox4 siRNA (Figures 6C and 7C). Moreover, this signal was abolished by either MnTmPyP (25 μM) or SOD (300 U/ml).

Nox subunits contribute to total ROS

Finally, we examined the contribution of Nox2 and Nox4 protein to resting intracellular ROS generation using H_2DCF-DA fluorescence analysis in HMEC-1. The silencing of Nox2 and Nox4 with siRNA caused a significant 22 ± 3.5 and 36 ± 4.5% decrease in total ROS production, respectively, compared to controls (Figure 8A,B). The inhibition of total ROS obtained by specific silencing of Nox subunits was similar to the magnitude of inhibition caused by the superoxide scavenger tiron (27 ± 3%; Figure 8A).

Discussion

Here we have demonstrated that hydroxyethidium fluorescence analysis can be used for quantification of intracellular superoxide production in endothelial and HL-60 cells, as well as extracellular release of superoxide by HL-60 cells. In addition, the silencing of Nox catalytic subunits (Nox2 and Nox4) with specific siRNA has revealed that both Nox2 and Nox4 containing NADPH oxidases are important intracellular sources of constitutive superoxide and ROS production in human endothelial cells.

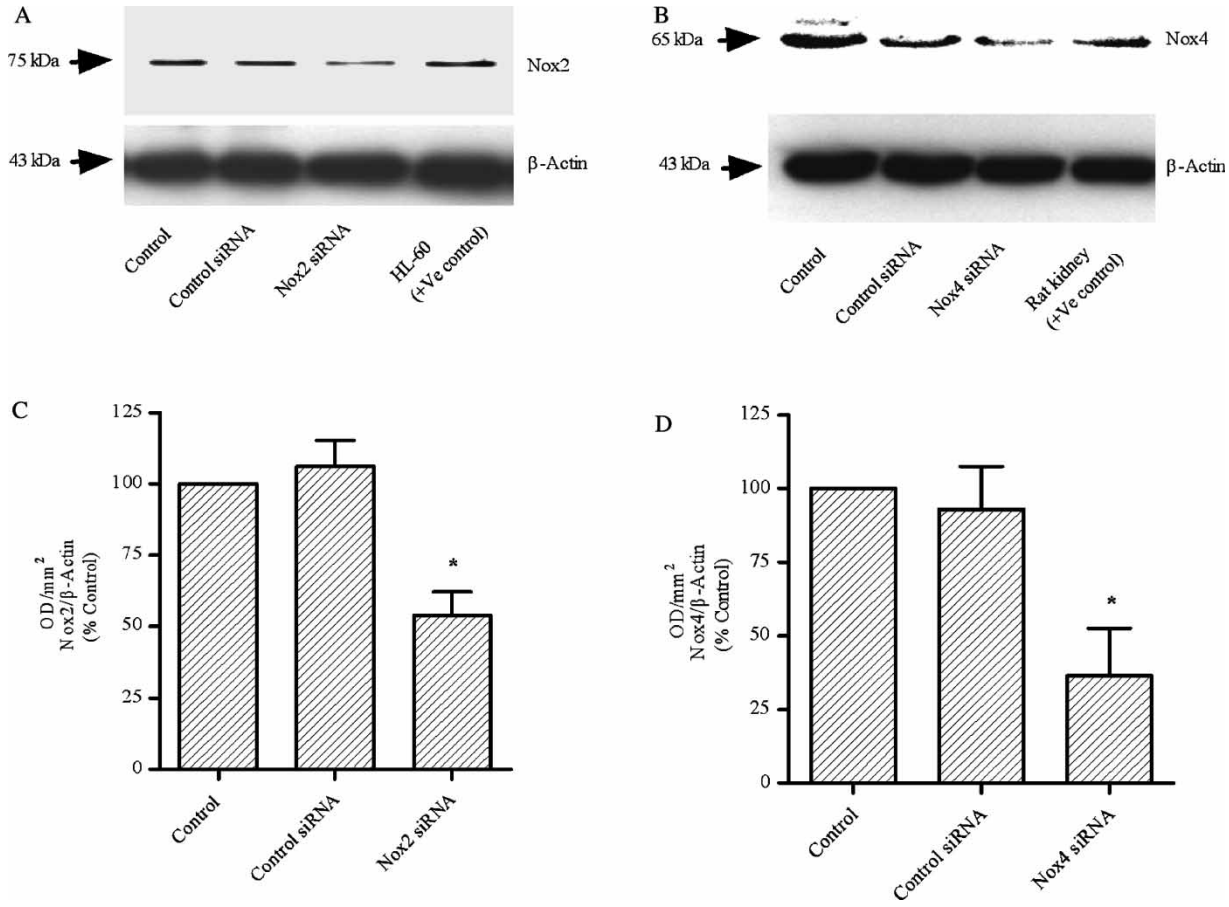

Figure 5. Western blot analysis showing the silencing of Nox2 and Nox4 protein expression with siRNA molecules in HMEC-1. HMEC-1 were untreated (control) or treated with either control siRNA, Nox2 (A) or Nox4 (B) specific siRNA molecules for 48 h. C and D shows densitometry analysis from five separate experiments of Nox2 and Nox4 proteins, respectively. HL-60 cells or rat kidney cortex were used as a positive control for Nox2 and Nox4 proteins, respectively. The amount of protein loaded was determined in each blot by subsequent immunoblotting of β-actin. Values (mean ± SEM from 4 to 5 experiments) are represented as OD/mm^2 after normalization to the respective β-Actin level and are expressed as a percentage of the control. The asterisk denotes a $P < 0.05$ value following a one-way ANOVA with Tukey–Kramer *post hoc* analysis.

DHE reacts with superoxide to form hydroxyethidium, which has an emission fluorescence peak at 567 nm [40]. Although, this reaction also generates ethidium, by using specific excitation (480 nm) and emission (570 nm) filters it is possible to detect hydroxyethidium with minimal spectral interference from ethidium. In fact, the contribution of ethidium to the emission peak at 567 nm has been estimated as ~20% of the total emission with equimolar concentrations of ethidium and hydroxyethidium [39]. However, the contribution of ethidium is likely to be even smaller given that the concentration of ethidium generated by reaction of DHE with superoxide is significantly lower than the concentration of hydroxyethidium. The study by Fink et al. demonstrates that with a given amount of superoxide (using xanthine oxidase/xanthine as a generator of this radical), the amount of hydroxyethidium generated from DHE (30 μM) was significantly (~10-fold) higher than ethidium [13]. Therefore, the contribution of ethidium to the fluorescence at 570 nm is likely to be negligible [40]. We therefore set out to monitor hydroxyethidium fluorescence emission at 570 nm to quantify superoxide produced extracellularly by HL-60 cells. Similar to phagocytic cells, HL-60 cells possess all the components of NADPH oxidase [11] to cause an extracellular release of superoxide upon activation by PMA and other stimuli [28,35]. In the present study, PMA caused a marked increase in fluorescence, which was abolished by the presence of SOD protein in the extracellular space. These findings indicate that DHE could be used to detect superoxide generated extracellularly by HL-60 and other phagocytic-like cells. We verified these findings with lucigenin-enhanced chemiluminescence, where a similar SOD-sensitive PMA-dependent increase in chemiluminescence was observed in HL-60 cells. The enzymatic source of superoxide was indeed NADPH oxidase, as both the increase in hydroxyethidium fluorescence and lucigenin chemiluminescence was reduced by the NADPH oxidase inhibitors apocynin and gp-91ds-tat peptide.

We also found that whilst hydroxyethidium fluorescence is selective for the detection of superoxide

released extracellularly, this indicator appears to be less sensitive than lucigenin-enhanced chemiluminescence. For instance, the PMA-dependent signal produced above basal levels by the respiratory burst in HL-60 cells was markedly higher with lucigenin chemiluminescence than it was with DHE. These differences between the lucigenin chemiluminescence and the DHE fluorescence methods might result from the difference in the rates of reaction between superoxide and each of these detector molecules. Thus, the rate constant of reaction of superoxide and lucigenin has been estimated to be $\sim 10^8\,M^{-1}\,s^{-1}$ whereas DHE reacts considerably slower with superoxide with an estimated rate constant of $2.6 \times 10^5\,M^{-1}\,s^{-1}$ [2,3,40]. Both detector molecules would need to compete against endogenous scavengers and superoxide inactivating enzymes such as SOD, which reacts with superoxide at a rate constant of $1.6 \times 10^9\,M^{-1}\,s^{-1}$ [14]. This final point may offer an explanation as to why DHE underestimates the level of superoxide.

Our findings in HL-60 cells demonstrate the utility of DHE as a probe for the detection of superoxide produced extracellularly. However, DHE may also detect superoxide produced intracellularly by endothelial cells and vascular tissues with a slightly modified protocol. With these experiments we exposed HMEC-1 to DHE for 30 min and then removed it from the extracellular space prior to measuring fluorescence. This protocol has several advantages over other methods for the detection of intracellular superoxide produced in small amounts by endothelial cells as it allows for the continuous formation and accumulation of hydroxyethidium, which is stable in cells for prolonged periods of time. Using a similar method Fink et al. (2004) showed an increase in basal DHE fluorescence in bovine aortic endothelial cells, which was not attributed to ethidium but to formation of hydroxyethidium. Despite these observations, Fink et al. did not address whether or not superoxide accounted for this signal [13], rather they demonstrated that the increases in hydroxyethidium production caused by Ang II and menadione were due to superoxide. In the present study, using this protocol of DHE addition and subsequent removal from the supernatant, we showed a significant increase in basal fluorescence in resting endothelial cells, which was nullified by the cell membrane permeable SOD mimetic MnTmPyP, but unaffected by application of extra-cellular SOD. As Mn porphyrin compounds

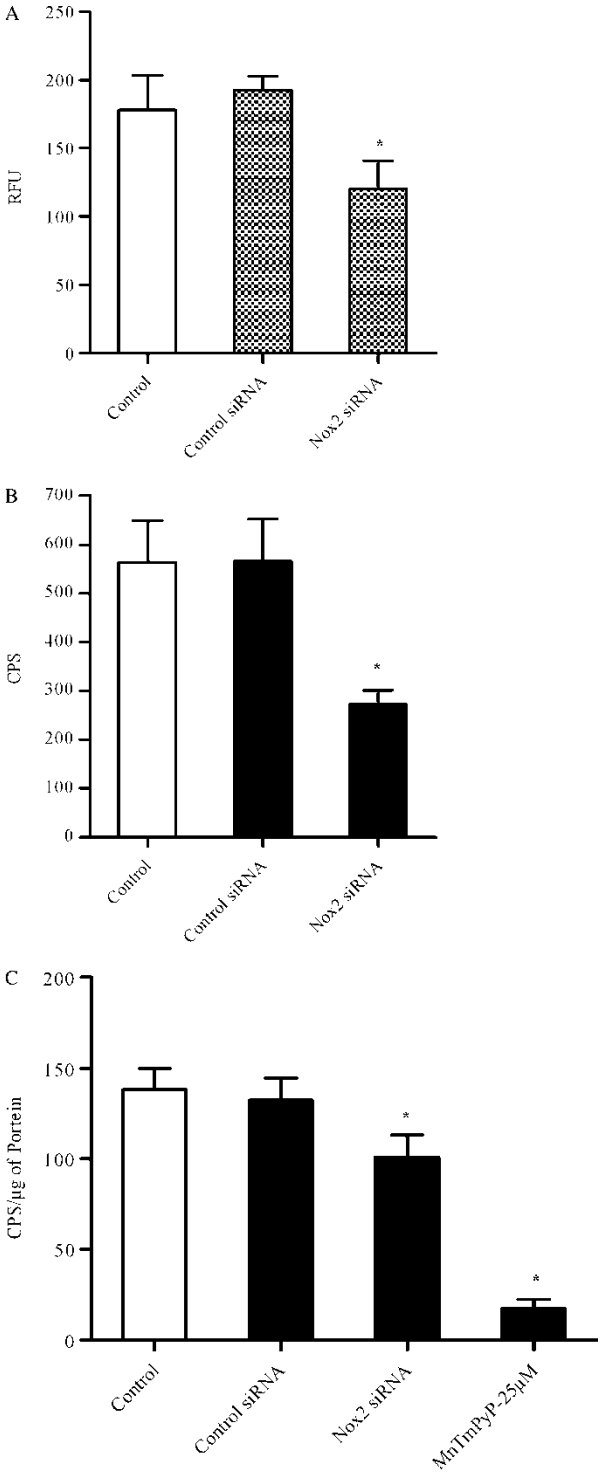

Figure 6. Effect of Nox2 protein silencing on both basal and NADPH-dependent superoxide production in HMEC-1. HMEC-1 were untreated (control) or treated with either control siRNA or specific Nox2 siRNA molecules for 48 h prior to being exposed to either dihydroethidium (DHE; 25 μM, A) or lucigenin (5 μM, B). Values (mean ± SEM from 7 experiments) are expressed as relative fluorescence units (RFU, A) and counts per second (CPS, B). RFU and CPS were normalized per 2×10^5 cells. (C) HMEC-1 homogenates were prepared from control, control siRNA and Nox2 siRNA treated cells (48 h of transfection). Superoxide was measured with lucigenin-enhanced chemiluminescence in the presence of NADPH (100 μM). In some cases cell homogenates were treated with SOD mimetic MnTmPyP (25 μM). Counts per second (CPS) were measured for 20 min and values (mean ± SEM from 3 to 4 experiments) were normalized per microgram of protein. The asterisk denotes a $P < 0.05$ value following a one-way ANOVA with Tukey–Kramer *post hoc* analysis.

could react with DHE and have non-specific effects, we used PEG-SOD as an alternative scavenger of superoxide. In these experiments it was essential to incubate cells with PEG-SOD for at least 4 h to ensure sufficient time to allow permeation of the plasma membrane as shown previously [6]. As with MnTmPyP, PEG-SOD abolished the basal hydroxyethidium fluorescence signal in endothelial cells. Therefore, our findings highlight that DHE is sufficiently sensitive to detect small amounts of superoxide generated constitutively and intracellularly by resting endothelial cells and HL-60.

In the present study, we demonstrated that in contrast to DHE fluorescence, lucigenin-enhanced chemiluminescence fails to detect superoxide produced constitutively by vascular cells. In fact, several studies have provided evidence that when vascular cells are supplemented with exogenous NADPH, superoxide production is increased [7,16,17,21,29,30]. However, it is unclear how exogenous NADPH, being cell impermeable, activates the oxidase, as the binding regions for NADPH on the oxidase are intracellular. Despite this, our previous [12] and present study would suggest that the NADPH-dependent, lucigenin-enhanced chemiluminescence signal is caused by superoxide produced predominantly by NADPH oxidase. However, the quantification of superoxide by this method should be taken with caution for two reasons. First, exogenous NADPH may target NADPH oxidases selectively over other enzymatic sources of superoxide, and second this may not represent quantitatively the relative sources of superoxide derived from endogenous NADPH. Given that exogenous NADPH is not necessary in DHE fluorescence analyses of superoxide production in vascular cells, we suggest that this fluorescence method provides a more reliable measure of basal superoxide generation and its enzymatic source.

Clearly, from our DHE fluorescence studies the source of intracellular superoxide in endothelial cells is unlikely to be NOS, xanthine oxidase, cyclo-oxygenase, cytochrome P_{450} or the mitochondrial respiratory chain since selective inhibitors of these enzymes failed to influence superoxide production. As mentioned the most likely enzymatic source is NADPH oxidase. Certainly, NADPH oxidase complexes in endothelial cells have the capacity to produce low levels of superoxide in a continuous fashion [14]. The main catalytic component of NADPH oxidase is the FAD and heme containing Nox(s), of which there are

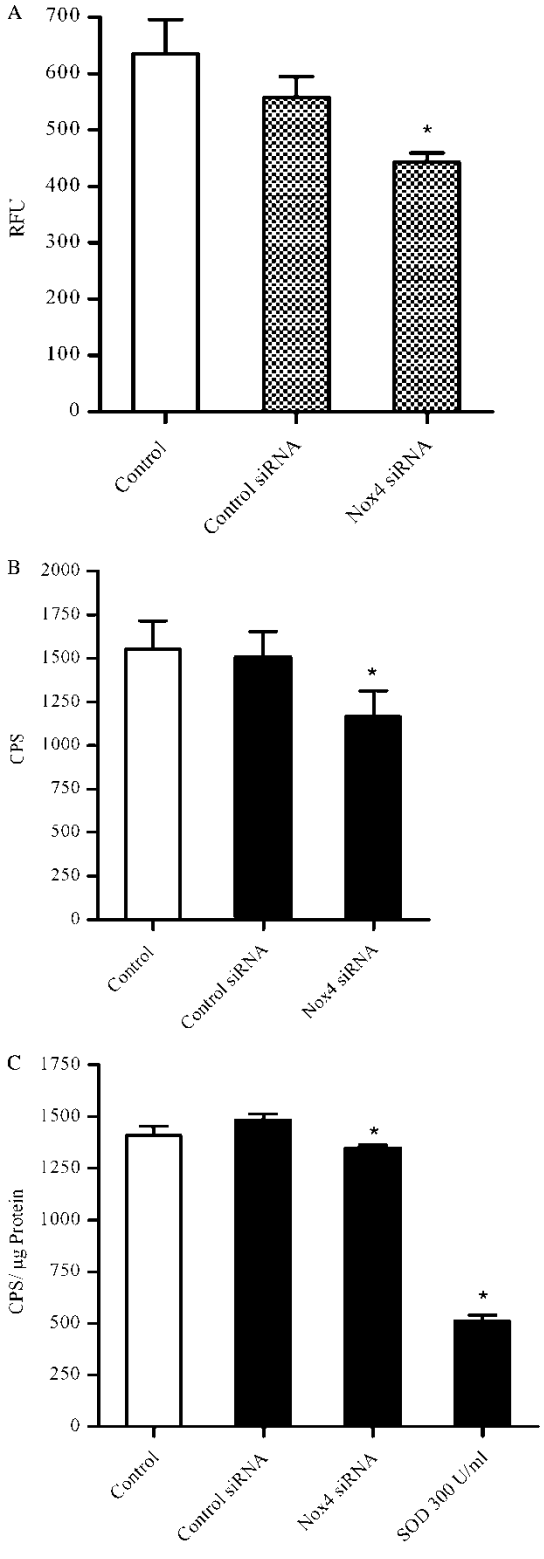

Figure 7. Effect of Nox4 protein silencing on both basal and NADPH-dependent superoxide production in HMEC-1. HMEC-1 were untreated (control) or treated with either control siRNA or specific Nox4 siRNA molecules for 48 h prior to being exposed to either dihydroethidium (DHE; 25 μM, A) or lucigenin (5 μM, B). Values (mean ± SEM from 4 experiments) are expressed as relative fluorescence units (RFU, A) and counts per second (CPS, B). RFU and CPS were normalized with per 2×10^5 cells. (C) HMEC-1 homogenates were prepared from control, control siRNA and Nox4 siRNA treated cells (48 h of transfection). Superoxide was measured with lucigenin-enhanced chemiluminescence in the presence of NADPH (100 μM). In some cases cell homogenates were treated with SOD (300 U/ml). Counts per second (CPS) were measured for 20 min and values (mean ± SEM from 3 experiments) were normalized per microgram of protein. The asterisk denotes a $P < 0.05$ value following a one-way ANOVA with Tukey–Kramer *post hoc* analysis.

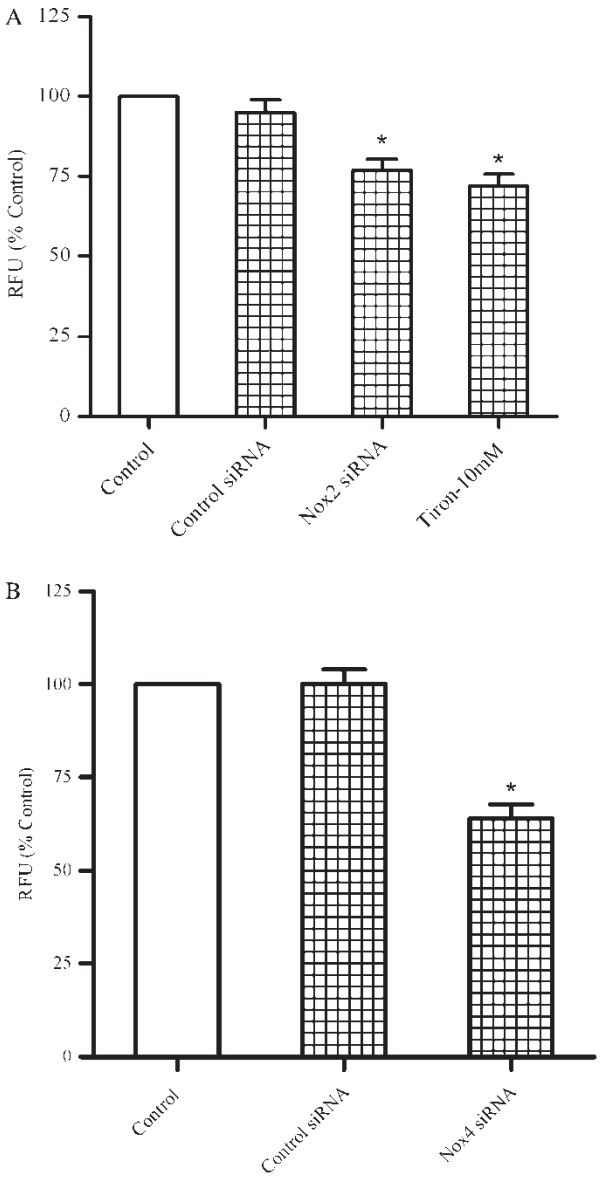

Figure 8. Effect of silencing Nox2 and Nox4 with siRNA on total intracellular ROS production in HMEC-1 as assessed with H_2DCF-DA fluorescence. HMEC-1 were untreated (control) or treated with either control siRNA molecules, specific siRNA molecules for Nox2 (Figure 8A) and Nox4 (Figure 8B) for 48 h, or to the superoxide scavenger, tiron (10 mM) for 30 min prior to being exposed to H_2DCF-DA (10 μM). Values (mean ± SEM from 3 to 5 experiments) are represented as relative fluorescence units (RFU) and expressed as a percentage of the control. The asterisk denotes a $P < 0.05$ value following a one-way ANOVA with Tukey–Kramer *post hoc* analysis.

currently five identified homologues, called Nox1 through to Nox5. The major catalytic domains expressed in endothelial cells are Nox2 and Nox4 [4,23]. To test directly whether constitutively active Nox2 and Nox4 containing NADPH oxidase complexes account for basal superoxide production in HMEC-1 we employed a siRNA approach to downregulate expression of Nox2 and Nox4 protein. Transfection of HMEC-1 with a sequence specific oligonucleotide against Nox2 caused a significant 50% reduction of Nox2 protein, which resulted in a ~30% reduction in superoxide production as measured by both DHE fluorescence and lucigenin chemiluminescence as well as a ~20% reduction in total ROS levels. The transfection process with Nox2 siRNA was specific for Nox2 for it did not affect Nox4 protein expression (data not shown). We similarly silenced Nox4 protein expression with specific siRNA and demonstrated a significant reduction in both superoxide and total ROS levels with no change in Nox2 expression. Interestingly the reduction of Nox4 protein caused a disproportionately greater decrease in total ROS production (~35%) compared with superoxide production (~20–25%), which suggests that this protein may produce ROS in addition to those directly derived from superoxide. Indeed, recent studies by Martyn et al. (2006) support this idea where they demonstrate that cell lines stably expressing Nox4 are capable of directly producing high levels of hydrogen peroxide in a constitutive fashion [25].

Our study demonstrates that both Nox2 and Nox4 containing NADPH oxidases are crucial sources of ROS produced constitutively by human endothelial cells perhaps localized within different subcellular compartments and serving different cell signalling functions. It is important to mention that an additional family of NAD(P)H oxidases called ECTO-NOX could be sources of extracellular superoxide and ROS production as their activity can be inhibited by DPI [26,33]. However, from our study it would appear that these enzymes are not major sources of ROS in endothelial cells but could account for residual ROS generated following our siRNA treatments. Future studies would need to determine where in the vasculature ECTO-Nox is expressed and into which compartment (i.e. extracellular or intracellular) ECTO-Nox produces ROS.

Both Nox2- and Nox4-containing oxidases have been shown to influence a variety of physiological functions pervading the vascular wall. For example, Nox2-containing NADPH oxidases expressed in the endothelium influence cell proliferation and migration induced by VEGF and may play a key role in angiogenesis [37]. Furthermore, several studies have shown that MAP kinases including ERK1/2, p38MAP kinase, JNK and ERK5 mediate the downstream cellular signalling actions of ROS, particularly for H_2O_2 [1,9,34,36,38]. Although ROS are physiological signalling molecules, a dysregulated and persistent increase in ROS levels will lead to oxidative stress, which can have major consequences for cell and artery function. It is also well established that superoxide inactivates NO and compromises its essential vasodilator and protective actions on the blood vessel wall, including its ability to suppress lipoprotein oxidation, vascular smooth muscle migration and platelet aggregation. Moreover, proinflammatory mediators like TNF-α and angiotensin-II

activate PKC to phosphorylate the p47phox subunit of NADPH oxidase (see reviews [10,19,22]). These mechanisms are undoubtedly involved in atherosclerosis, restenosis and hypertension.

In summary, we suggest that DHE fluorescence can be used as a specific tool to quantify superoxide produced both extracellularly by HL-60 cells and intracellularly by endothelial cells. We have also demonstrated that both Nox2- and Nox4-containing NADPH oxidases are constitutively active sources of superoxide and ROS in endothelial cells using three different methods of detection. We are now in a better position to elucidate how these enzymes generate autocrine and paracrine acting ROS and influence physiological functions such as proliferation and apoptosis, and how these are altered in vascular disease.

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