Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase

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Abstract

Objective: A poorly characterized phagocyte-type NADPH oxidase, which is reportedly NADH- rather than NADPH-dependent, is a major source of endothelial reactive oxygen species (ROS) production. We investigated the molecular nature of this oxidase and the characteristics of NADPH- versus NADH-dependent O₂ production in endothelial cells of three different species. Methods: NADPH oxidase expression in human, bovine and porcine endothelial cells was studied by RT-PCR and immunoblotting. O₂ production was assessed by lucigenin chemiluminescence and cytochrome c reduction assay. Results: The NADPH oxidase subunits p47-phox, p67-phox, p22-phox, gp91-phox, and rac1 were all expressed in endothelial cells. NADPH-dependent O₂ production by endothelial cells was readily detectable using lucigenin 5 μmol/l, was minimally affected by increasing lucigenin dose up to 400 μmol/l, and was abolished by diphenyleneiodonium. In contrast, NADH-dependent O₂ production was only detectable with lucigenin ≤50 μmol/l, increased substantially with higher lucigenin dose, and was unaffected by diphenyleneiodonium. Predominance of NADPH- over NADH-dependent O₂ production was confirmed in cell homogenates and by cytochrome c reduction assay. Conclusion: Endothelial cells express all components of a phagocyte-type NADPH oxidase. Like the neutrophil enzyme, the endothelial oxidase is preferentially NADPH- rather than NADH-dependent. NADH-dependent O₂ production appears to be an artefact related to the use of lucigenin doses ≥50 μmol/l.

Keywords: Cell culture/isolation; Endothelial function; Free radicals; Gene expression

1. Introduction

Endothelial cell (EC) production of reactive oxygen species (ROS), e.g., O₂ and H₂O₂, is involved in the pathophysiology of disorders such as hypercholesterolemia and atherosclerosis [1,2]. ROS production is stimulated by factors including cytokines, pulsatile stretch, hypoxia-reoxygenation, and ischemia [1–5]. Low level endothelial ROS production rapidly inactivates nitric oxide, and modulates intracellular signaling pathways and gene expression [1–3]. Potential endothelial ROS sources include xanthine oxidase, dysfunctional nitric oxide synthases (NOSs), NAD(P)H-dependent electron transport chains, cyclooxygenases and EDHF synthase [2,3,6]. Several recent studies suggest that a phagocyte-type NADPH oxidase is a major source of endothelial ROS [2–5,7–13]. ROS generated by this oxidase are implicated in the pathophysiology of several conditions, including endothelial dysfunction associated with hypertension and hypercholesterolemia [2,3]; hypoxia-reoxygenation injury [4,5]; and endothelial cell proliferation [11].

The neutrophil NADPH oxidase comprises a membrane-associated cytochrome b₅₅₅, composed of one p22-phox and one gp91-phox subunit, and several cytosolic subunits (p47-phox, p67-phox, and rac1 or rac2) [14]. Similar oxidases are reportedly expressed in non-phagocytic cells, including adventitial fibroblasts, vascular smooth muscle (VSM), and renal mesangial cells [2,3,15]. The phagocyte-type NADPH oxidase expressed in EC (and VSM) appears to differ from the neutrophil enzyme in at least two important respects [2,3]. Firstly, it apparently prefers

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NADH rather than NADPH as electron donor. Greater NADH- than NADPH-dependent $O_2^-$ production was reported in cultured bovine coronary endothelium [7], human umbilical vein endothelial cells (HUVEC) [8], rat coronary microvascular EC [9], and fresh porcine coronary artery endothelium [10]. Secondly, the endothelial oxidase is continuously active at a low level even in the absence of stimulation by specific agonists, whereas the neutrophil oxidase is inactive in unstimulated cells. The reasons underlying these differences are unclear. One possibility is that the molecular structure of the endothelial NADPH oxidase may be different. In cultured VSM, the gp91-phox subunit is replaced by a novel homologue, termed mox (for mitogenic oxidase), which is ~60% homologous to gp91-phox [16]. However, gp91-phox is present at both mRNA and protein level in EC, for example in HUVEC and rat coronary microvascular EC [8,9,12,13]. In rat EC, the p22-phox cDNA has also been shown to be highly homologous (or identical) to the neutrophil sequence [12]. However, it remains unclear whether other NADPH oxidase subunits (i.e., p47-phox, p67-phox and rac1) are similarly expressed in EC and neutrophils.

Most studies that reported greater NADH- than NADPH-dependent endothelial $O_2^-$ production used the method of lucigenin-enhanced chemiluminescence [7–10]. However, recent work has emphasized that lucigenin dose itself is an important experimental variable, and that in certain settings high lucigenin concentrations can lead to artefactual $O_2$ production based on redox cycling of the probe [17]. Previous studies used high lucigenin doses (>200 μM/l), and the issue of relative NADPH- versus NADH-dependent endothelial $O_2^-$ production was not rigorously investigated using non-redox cycling doses of lucigenin (5–10 μM/l) or with alternative methods of $O_2^-$ detection.

In view of the likely importance of the endothelial NADPH oxidase in cardiovascular pathophysiology (see above), it is important to clearly determine the molecular structure and biochemical properties of oxidase. In the present study, we investigated in parallel in human, bovine and porcine EC: (a) the mRNA and protein expression of NADPH oxidase subunits, in particular p47-phox, p67-phox and rac1; and (b) relative NADPH- versus NADH-dependent $O_2^-$ production using a range of doses of lucigenin in chemiluminescence studies, as well as cytochrome c reduction assays.

2. Experimental procedures

2.1. Reagents

Cell culture media, fetal calf serum (FCS), glutamine and antibiotics were purchased from Gibco (UK). EC growth supplement, recombinant human epidermal growth factor (r-hEGF), gelatin, diphenyllethiodonium (DPI), and all other chemicals were from Sigma (UK).

2.2. Cell culture

HUVEC were prepared from fresh umbilical cords and used at passage 2–4 [18]. HMEC-1 (a transformed human microvascular EC line) [19] were obtained from the Centers for Disease Control and Prevention (Atlanta, GA). ECV304, a transformed HUVEC cell line, was obtained from the American Tissue Culture Cell Collection (Rockville, MD). Primary human dermal microvascular endothelial cells (HDFM) were purchased from Clonetics. PIEC (porcine iliac arterial EC) and BAEC (bovine aortic EC) were kindly provided by Professor J Fabre (King’s College London, UK) [20]. HUVEC, HMEC-1 and HDFM were grown in medium 199 supplemented with 20% FCS, heparin (5 IU/ml), hydrocortisone (1 μg/ml), l-glutamine (2 mmol/l), streptomycin (50 μg/ml), penicillin (50 U/ml), endothelial cell growth supplement (50 μg/ml), and r-hEGF (10 ng/ml). BAEC and PIEC were maintained in RPMI 1640 with 10% FCS, l-glutamine (2 mmol/l), streptomycin (100 μg/ml), and penicillin (100 U/ml). ECV304 were grown in medium 199 with supplements identical to BAEC. Cells were cultured in 0.1% (w/v) gelatin-coated flasks until confluent. Some experiments were also undertaken with proliferating cells (~70% confluent). EC identity was confirmed by a cobblestone appearance, von Willebrand factor expression and uptake of fluorescently labeled Dil-Ac-LDL.

2.3. RT-PCR

Primers for amplification of p22-phox, gp91-phox, p47-phox and p67-phox, were designed from published human phagocyte sequences [8], to amplify fragments of 316 bp, 767 bp and 746 bp, respectively. Primer sequences were: (a) p22-phox: sense 5'-GTTTGTGCTCGTGGAGT-3'; antisense 5'-TGGGCGGCTGCTGTAGTG-3'; (b) gp91-phox: sense 5'-GCTGTTCAATGCTTGCTG-3'; antisense 5'-TCTCCTCATCATGGTGCACA-3'; (c) p47-phox: sense 5'-ACCAGCCAGCACATGTGT-3'; antisense 5'-AGTAGGTTGACACGTCCT-3'; (d) p67-phox: sense 5'-CGAGGAAACCAGCTGATAG-3'; antisense 5'-CATGGGAAACACTGAGCTTC-3'; β-actin cDNA as an internal control was amplified using sense: 5'-GGCATGAGTGCTAGAAGATT-3'; and antisense, 5'-TACATGGCTGGGTTCTGA -3' primers. PCR reactions were performed in 50 μl final volumes containing 2–5 μl of RT reaction (100–250 ng total RNA), 10 pmol/l sense and antisense primers each, 100 μmol/l dNTPs, and 1.5 U Taq polymerase. Cycles comprised 94°C for 55 s, 60°C for 55 s, and 72°C for 80 s, followed by extension at 72°C for 10 min. A total of 25 cycles were used for β-actin and p22-phox, and 35 cycles for gp91-phox, p47-phox and p67-phox.
Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>p22-phox (%)</th>
<th>p47-phox (%)</th>
<th>p67-phox (%)</th>
<th>Rac1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>74.8±13.2</td>
<td>46.8±16.0</td>
<td>57.8±5.5</td>
<td>61.2±6.5</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>55.2±13.0</td>
<td>41.0±5.5</td>
<td>42.5±4.9</td>
<td>67.5±9.2</td>
</tr>
<tr>
<td>BAEC</td>
<td>83.1±7.8</td>
<td>23.9±3.9</td>
<td>43.2±10.9</td>
<td>43.8±5.1</td>
</tr>
<tr>
<td>PIEC</td>
<td>79.2±9.9</td>
<td>13.7±1.3</td>
<td>20.2±7.6</td>
<td>32.3±9.0</td>
</tr>
</tbody>
</table>

Equal amounts of protein (25 μg/per sample) were loaded on gels. Gels were scanned densitometrically and protein expression expressed relative to the level detected in PMA-stimulated U937 cells run on the same gel (100%). Data are mean±S.D. of three independent experiments. gp91-phox was not quantitated because of the multiple band pattern obtained with this subunit.

2.4. Immunoblotting

EC (5×10⁶/ml) were homogenized in buffer containing Tris–HCl (50 mmol/l), NaCl (150 mmol/l), PMSF (1 mmol/l), EDTA (0.1 mmol/l), leupeptin (2 μmol/l) and pepstatin (2 μmol/l), pH 7.2, and the homogenate was extracted with 0.5% triton X-100. Equal amounts (25 μg) of protein were separated on 10% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk/PBS/0.2% TWEEN 20. Specific polyclonal antibodies against the human neutrophil NADPH oxidase subunits p22-phox, gp91-phox, p47-phox, p67-phox and rac1 were kind gifts from F. Wientjes (University College London, UK) and M. Quinn (Montana State University, MT) [21,22]. Membrane protein from activated human neutrophils (a gift from F. Wientjes) was used as a positive control for p22-phox and gp91-phox; and protein extract from human phagocytic U937 cells, after PMA stimulation to induce differentiation, as a positive control for other subunits.

2.5. Chemiluminescent measurement of \(O_2^-\)

\(O_2^-\) production by intact cells was measured by lucigenin-enhanced chemiluminescence [17]. Briefly, EC in culture flasks were detached using 0.25% trypsin/EDTA (1 mmol/l), washed with PBS, and resuspended at 10⁶/ml in modified HEPES buffer containing (mmol/l) NaCl 140, KCl 5, MgCl₂ 0.8, CaCl₂ 1.8, Na₂HPO₄ 1, HEPES 25 and 1% glucose, pH 7. Cells were distributed at 5×10⁴/well on a 96-well microplate luminometer (Model Lucy 1, Rosys Anthos, Austria). Immediately before recording chemiluminescence, NADH or NADPH (final concentration 100 μmol/l) were added [23], and dark-adapted lucigenin (5–400 μmol/l) was added via an auto-dispenser. Light emission was recorded and expressed as mean arbitrary light units/min over 20 min. Each experiment was performed in triplicate.

In some experiments, cells were pre-incubated with superoxide dismutase (SOD, 200 units/ml), tiron (5 mmol/l), DPI (10–250 μmol/l), N-o-nitro-L-arginine methyl ester (L-NAME, 100 μmol/l), rotenone (50 μmol/l) or...
oxypurinol (100 μmol/l) before measurement of chemiluminescence.

O$_2$ production was also studied using total EC homogenate rather than intact cells [9]. Apart from addition of endothelial homogenate (100 μg protein/well) instead of EC, the protocol was as described above.

2.6. Cytochrome c reduction assay

These experiments were performed using EC suspensions in a similar manner to the lucigenin studies [24]. EC were resuspended in DMEM without phenol red, and incubated in 96-well flat-bottom cell culture plates (2×10$^5$ cells/well) for 10 min at 37°C in a humidified CO$_2$ incubator. Cytochrome c (250–2000 μmol/l final concentration) and NADPH or NADH (100 μmol/l each) were added to cells in the presence or absence of SOD (200 units/ml) and were allowed to incubate for 30 min. Cytochrome c reduction was measured by reading absorbance at 550 nm on a microplate reader. O$_2$ production in nmol/2×10$^5$ cells was calculated from the difference between absorbance of samples with or without SOD and the extinction coefficient for change of ferricytochrome c to ferrocytochrome c, i.e., 21.0 mmol/l$^{-1}$·cm$^{-1}$.

2.7. Calibration of chemiluminescence

To estimate the amount of O$_2^-$ equivalent to measured chemiluminescence signals, O$_2$ was generated in parallel in the lucigenin assay (10 μmol/l lucigenin) and the cytochrome c reduction assay (250 μmol/l) using a range of xanthine/xanthine oxidase concentrations [10]. Amounts of O$_2^-$ measured by cytochrome c reduction were then related to chemiluminescence readings obtained under identical conditions. An excellent linear correlation ($r$ > 0.99) was obtained between calculated O$_2^-$ production (cytochrome c assay) and measured chemiluminescence.

2.8. Statistics

Data from chemiluminescence and cytochrome c assays are presented as mean±S.D. of at least three different culture experiments for each cell type. Comparisons were made by unpaired $t$-test, with Bonferroni correction for

![Graphs showing O$_2$ production by different cell types](https://example.com/graphs)

**Fig. 3.** NADPH- versus NADH-dependent O$_2$ production by intact endothelial cells. O$_2$ production by intact HUVEC, HMEC-1, BAEC, and PIEC was measured by lucigenin (10 μmol/l)-enhanced chemiluminescence. Results are expressed as mean arbitrary light units (±S.D.) averaged over 20 min. Cells=lucigenin chemiluminescence in the absence of added NADPH or NADH. The insets in the bottom panels show the kinetics of O$_2$ production by HMEC-1 and PIEC over a 20 min period. *$P$<0.05 compared to cells alone.
multiple testing as appropriate. \( P<0.05 \) was considered statistically significant.

3. Results

3.1. Expression of phagocyte-type NADPH oxidase subunits in endothelial cells

mRNAs for p22-phox, p47-phox, p67-phox and gp91-phox were all detected in HUVEC and BAEC by RT-PCR (Fig. 1). In PIEC, only p22-phox mRNA was detected using human primers. PMA-stimulated U937 cell RNA was used as a positive control, and samples without RT were used as negative controls.

NADPH oxidase subunit expression was confirmed at protein level using anti-human neutrophil polyclonal antibodies. Specific bands were detected at \( \sim 47, \sim 67 \) and \( \sim 21 \) kDa with the anti-p47-phox, p67-phox and anti-rac1 antibodies, respectively, in all EC and in U937 cells (Fig. 2A). The anti-p22-phox polyclonal detected two bands in all endothelial samples and in U937 cells, one at \( \sim 22 \) kDa and an additional band at \( \sim 28 \) kDa (Fig. 2C). In human neutrophil membrane, a single band was detected at \( \sim 22 \) kDa. Table 1 provides a densitometric quantification of these subunits in the different EC relative to the level in PMA-stimulated U937 cells.

The anti-gp91-phox antibody detected a major band at \( \sim 75 \) kDa and a weaker band at \( \sim 50 \) kDa in HUVEC, HMEC-1, BAEC, and U937 cells (Fig. 2B). In PIEC, a major band was detected at \( \sim 50 \) kDa with a weaker band at \( \sim 75 \) kDa. In human neutrophil membrane, in addition to the \( \sim 75 \) and \( \sim 50 \) kDa bands, a strong band was detected at \( \sim 90 \) kDa, a pattern believed to represent the presence of variably glycosylated forms of gp91-phox [25,26].

3.2. NADPH- versus NADH-dependent \( \text{O}_2^- \) production by lucigenin chemiluminescence

A low level of chemiluminescence was detected with EC and lucigenin (10 \( \mu \text{mol/l} \)) alone, in the absence of added NADH or NADPH, compared to buffer and lucigenin alone (expressed as zero) — Fig. 3. Addition of NADPH (100 \( \mu \text{mol/l} \)) progressively increased \( \text{O}_2^- \) production, with a plateau achieved after \( \sim 10 \) min (Fig. 3, lower panel insets). In contrast, addition of NADH (100 \( \mu \text{mol/l} \)) had no significant effect on \( \text{O}_2^- \) production in any of the EC studied. Similar results were obtained with a lucigenin dose of 5 \( \mu \text{mol/l} \). Compared with HUVEC, NADPH-dependent \( \text{O}_2^- \) production by BAEC was of a similar level, by HMEC-1 was \( \sim 2 \) fold higher \((P<0.05)\), and by PIEC was \( \sim 6 \) fold greater \((P<0.05)\). To estimate maximal enzyme activity, chemiluminescence data were calibrated as described in the Methods. Maximal NADPH-dependent oxidase activity was \( 0.14 \pm 0.01 \text{ nmol} \text{O}_2^- / 10^6 \) cells/min in HUVEC, and was \( 0.776 \pm 0.104 \text{ nmol} \text{O}_2^- / 10^6 \) cells/min in PIEC.

NADPH- versus NADH-dependent \( \text{O}_2^- \) generation was also assessed in proliferating EC. Experiments were performed in parallel using HDMVEC that were \( \sim 70\% \) confluent (“proliferating”) or fully confluent (“quiescent”) — Fig. 4A. Under both conditions, \( \text{O}_2^- \) generation was only detected in the presence of NADPH but not NADH. The magnitude of \( \text{O}_2^- \) generation was significantly greater in proliferating cells. Similar results were obtained with proliferating and quiescent HUVEC.

We also assessed NADPH- versus NADH-dependent \( \text{O}_2^- \) generation in HUVEC that were pre-stimulated for 10 min with angiotensin II (100 nM), which is reported to increase \( \text{O}_2^- \) generation by phagocyte-type NAD(P)H oxidases in the vasculature [3]. As shown in Fig. 4B, NADPH-dependent \( \text{O}_2^- \) generation was significantly increased by angiotensin II. Of note, there was no detectable increase in Fig. 4. \( \text{O}_2^- \) production by proliferating endothelial cells, and in response to angiotensin II. \( \text{O}_2^- \) production was measured by lucigenin (10 \( \mu \text{mol/l} \))-enhanced chemiluminescence, and results are expressed as mean arbitrary light units (\( \pm \text{S.D.} \)) averaged over 20 min. Cells+lucigenin chemiluminescence in the absence of added NADPH or NADH. (A) Confluent compared to proliferating HDMVEC. (B) Effect of pretreatment of HUVEC with angiotensin II (Ang II, 100 nM) for 10 min. *\( P<0.05 \) compared to cells alone. \#\( P<0.05 \) between groups.
NADH-dependent $O_2^-$ generation even after angiotensin II stimulation.

3.3. Influence of lucigenin dose on NADPH- versus NADH-dependent $O_2^-$ production

Lucigenin doses between 10 and 400 μmol/l were assessed in parallel studies. NADPH-dependent chemiluminescence did not vary greatly with an increase in lucigenin from 10 to 400 μmol/l, apart from a modest increase in PIEC (Fig. 5). In contrast, NADH-dependent chemiluminescence was only detectable using lucigenin ≥50 μmol/l, and the level increased steeply with further increases in lucigenin dose. NADPH-dependent chemiluminescence predominated over NADH up to a lucigenin dose of 50 μmol/l in HUVEC and BAEC, and up to >200 μmol/l in HMEC-1 and PIEC.

Both NADPH- and NADH-dependent chemiluminescence were significantly and similarly inhibited by SOD (200 units/ml), e.g., −54±2.9% for the NADPH- and −61±5.1% for the NADH-dependent signal in HUVEC at a lucigenin dose of 100 μmol/l (Fig. 6). The cell-permeable $O_2^-$ scavenger, tiron (5 mmol/l), virtually abolished the chemiluminescence signal evoked either by NADPH or NADH; e.g., by −91±1.9 and −90±2.6%, respectively, in HUVEC (Fig. 6). Similar results were obtained with HMEC-1, HDMVEC, BAEC and PIEC.

3.4. Effect of specific enzyme inhibitors on NADPH- versus NADH-dependent $O_2^-$ production

Neither NADPH- nor NADH-dependent $O_2^-$ production were significantly altered by a NOS inhibitor (L-NAME 100 μmol/l), a mitochondrial oxidase inhibitor (rotenone 100 μmol/l), or a xanthine oxidase inhibitor (oxypurinol 100 μmol/l) in any cell type — data for HUVEC are shown in Fig. 6. The flavoprotein inhibitor, DPI (≥10 μmol/l) [27], virtually abolished NADPH-dependent $O_2^-$ production but had no significant effect on NADH-dependent chemiluminescence, even at concentrations up to 250 μmol/l. DPI (50 μmol/l) inhibited NADPH-dependent $O_2^-$ production by HUVEC by 87±3% ($P<0.01$) whereas NADH-dependent $O_2^-$ production was 115±14% of con-

![Fig. 5. Effect of lucigenin dose on NADPH- versus NADH-dependent $O_2^-$ production by endothelial cells. Lucigenin was used at 0, 10, 50, 100, 200 and 400 μmol/l in parallel experiments with HUVEC, HMEC-1, BAEC, and PIEC, exactly as for the experiments presented in Fig. 3. Filled circles (●) show NADPH-dependent $O_2^-$ production, and open circles (○) NADH-dependent $O_2^-$ production. Results are expressed as mean arbitrary light units (±S.D.) averaged over 20 min. * and #, $P<0.05$ in the presence of NADPH or NADH respectively compared to the basal level.](https://academic.oup.com/cardiovascres/article-abstract/52/3/477/349245/58?cookieSet=true&redirected=true)
3.5. NADPH- versus NADH-dependent $O_2^-$ production by EC homogenates

Using BAEC homogenate, significant NADPH-dependent chemiluminescence was detected with a lucigenin dose down to 5 μmol/l (Fig. 7). A modest increase in chemiluminescence occurred with increasing lucigenin dose up to 20 μmol/l, with no further increase in signal thereafter. In marked contrast, no NADH-dependent chemiluminescence was detectable with a lucigenin dose below 20 μmol/l. However, increasing lucigenin dose from 50 to 400 μmol/l caused a substantial increase in NADH-dependent chemiluminescence. Comparison of Fig. 7 (BAEC homogenate) and Fig. 5 top right panel (BAEC suspensions) shows that relative NADPH- versus NADH-dependent $O_2^-$ production was similar in both settings.

Both NADPH- and NADH-dependent chemiluminescence by EC homogenate were abolished by tiron at all lucigenin doses (data for lucigenin 100 μmol/l is shown) — Fig. 8. NADPH-dependent $O_2^-$ production was abolished by DPI (100 μmol/l) and was also slightly but significantly reduced by l-NAME (100 μmol/l), but was unaffected by rotenone or oxypurinol. In contrast, NADH-dependent $O_2^-$ production was only reduced by ~40% by DPI (P=NS), and was also reduced to a similar extent by oxypurinol.

3.6. NADPH- versus NADH-dependent $O_2^-$ production measured by cytochrome c reduction

Both in human and porcine EC, a low level of basal $O_2^-$ production was detectable by cytochrome c reduction (Fig. 9). Addition of NADPH significantly increased $O_2^-$ production in both cell types. In contrast, addition of NADH did not alter $O_2^-$ production relative to baseline.

4. Discussion

The present study shows that: (1) The NADPH oxidase expressed in EC is highly similar to the neutrophil enzyme in terms of expression of major subunits (i.e., p47- phox, p67-phox, p22-phox, gp91-phox and rac1). The failure to
least 10–50-fold higher [28]. The reasons underlying this dramatic difference in maximal enzymatic activity between EC and neutrophils despite apparently similar subunit composition and substrate preference requires further investigation. Possible reasons include a reduced level of subunit expression and/or altered stoichiometry in EC, or differences in post-translational modification of oxidase subunits.

The use of lucigenin-enhanced chemiluminescence for detecting \( \text{O}_2^\cdot \) production by biological tissues has recently come under scrutiny because of the potential for redox cycling and artefactual \( \text{O}_2^\cdot \) generation when high doses of lucigenin are used [17]. Li et al. [17] showed that such redox cycling only occurred at lucigenin concentrations substantially higher than 5 \( \mu \text{mol/l} \), and that the potential for redox cycling was influenced by the precise system under study. The present study significantly extends those findings by demonstrating that, both in intact EC suspensions and EC homogenates, the propensity for lucigenin-driven \( \text{O}_2^\cdot \) generation is considerably greater in the presence of NADH than NADPH. Although the reasons for this remain unclear, experiments with DPI suggest that whereas NADPH-dependent \( \text{O}_2^\cdot \) production is predominantly attributable to a flavoprotein, virtually none of the NADH-dependent chemiluminescence in intact EC and only a small proportion in homogenates involves a flavoprotein. Interestingly, we found differences between \( \text{O}_2^\cdot \) production assessed in intact cell suspensions versus homogenates. In homogenates but not intact EC, a small production may involve dysfunctional NOS. This finding suggests that cell homogenization itself might potentially influence the characteristics of \( \text{O}_2^\cdot \) production.

As in previous studies [10,13,23,29–31], we found that extracellular addition of NADPH significantly increased EC \( \text{O}_2^\cdot \) production. The mechanism underlying this effect remains uncertain. It is speculated that extracellular NADPH may directly bind to and stimulate NADPH oxidase localized on the plasma membrane [3,29]. Alternatively, extracellular addition of NADPH may elevate intracellular NADPH content, either through transfer of NADPH itself into the cell or possibly via an indirect shuttle mechanism analogous to mitochondrial shuttles. It was notable in the present study that the characteristics and magnitude of NADPH-dependent \( \text{O}_2^\cdot \) production by cell suspensions was very similar to that in homogenates, suggesting that both approaches provided an equivalent measure of cellular NADPH oxidase activity. Likewise, Guzik et al. [31] recently reported a very high correlation between NADPH-dependent \( \text{O}_2^\cdot \) production measured in parallel in paired intact human vascular rings and vascular homogenates from the same patients.

An interesting observation in the present study was that anti-neutrophil gp91-phox antibodies mainly detected \(~75\) and \(~50\) kDa bands on SDS–PAGE in EC, with a 91-kDa

detect p47-phox and p67-phox mRNA in PIEC, using primers based on human sequences, probably reflects a species–specific difference. (2) As in neutrophils, at a biochemical level endothelial \( \text{O}_2^\cdot \) production is predominantly NADPH- rather than NADH-dependent, whether assessed in intact cell suspensions or in cellular homogenates using two different methods (i.e., lucigenin chemiluminescence and cytochrome c reduction). This NADPH predominance was also true for proliferating EC or for cells acutely stimulated with angiotensin II. Previous reports of predominantly NADH-dependent endothelial ROS production were almost certainly an artefact related to the use of high lucigenin concentrations (~200 \( \mu \text{mol/l} \)) [7–10], which substantially increased NADH- but not NADPH-dependent ROS production under our experimental conditions. In the present study, NADPH- and NADH-dependent ROS production could also be clearly disassociated in the cytochrome c assay and by the differential effects of DPI. (3) Maximal NADPH oxidase activity is ~0.14 nmol \( \text{O}_2^\cdot /10^6 \) cells/min in HUVEC and ~0.776 nmol \( \text{O}_2^\cdot /10^6 \) cells/min in PIEC. Comparative values for neutrophil NADPH oxidase activity in the literature are at


**Fig. 8.** Effect of specific enzyme inhibitors on NADPH- versus NADH-dependent \( \text{O}_2^\cdot \) production by BAEC homogenates. Results are expressed relative to NADPH- or NADH-dependent \( \text{O}_2^\cdot \) production in the absence of inhibitors. *\( P<0.05 \).
Fig. 9. NADPH- versus NADH-dependent \( \text{O}_2 \) production by endothelial cells measured by cytochrome \( c \) reduction assay. SOD-inhibitable \( \text{O}_2 \) production by HUVEC or PIEC alone (Cells) or in the presence of added NADPH or NADH (100 \( \mu \text{mol/l} \) each) is shown. Experiments were undertaken in parallel using different concentrations of cytochrome \( c \). Results are mean±S.D. of at least three separate experiments. \( *P<0.01 \) compared to cells alone.

In summary, this study shows that: (a) all the major phagocyte-type NADPH oxidase subunits, including p47-\( \text{phox}, \) p67-\( \text{phox} \) and \( \text{rac}1, \) are expressed in EC; (b) \( \text{O}_2 \) production by EC or homogenate is predominantly NADPH- rather than NADH-dependent. These results are of potential relevance to several cardiovascular conditions in which ROS generated by a phagocyte-type EC oxidase are implicated, including endothelial dysfunction associated with hypercholesterolemia and hypertension [2,3]; hypoxia-reoxygenation injury [4,5]; and endothelial cell proliferation [11]. However, it should be noted that all the experiments in the current study were undertaken using cultured EC, so that the results may not necessarily be extrapolated to EC in situ which are subject to different hormonal and mechanical stimuli, notably shear stress.

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References