Intracellular Localization and Preassembly of the NADPH Oxidase Complex in Cultured Endothelial Cells*

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The phagocyte-type NADPH oxidase expressed in endothelial cells differs from the neutrophil enzyme in that it exhibits low level activity even in the absence of agonist stimulation, and it generates intracellular reactive oxygen species. The mechanisms underlying these differences are unknown. We studied the subcellular location of (a) oxidase subunits and (b) functionally active enzyme in unstimulated endothelial cells. Confocal microscopy revealed co-localization of the major oxidase subunits, i.e. gp91phox, p22phox, p47phox, and p67phox, in a mainly perinuclear distribution. Plasma membrane biotinylation experiments confirmed the predominantly (>90%) intracellular distribution of gp91phox and p22phox. After subcellular protein fractionation, ~50% of the gp91phox (91-kDa band), p22phox, p67phox, and p40phox pools and ~30% of the p47phox were present in the 1475 × g (“nucleus-rich”) fraction. Likewise, ~50% of total NADPH-dependent O2 production (assessed by lucigenin (5 μM) chemiluminescence) was found in the 1475 × g fraction. Co-immunoprecipitation studies and measurement of NADPH-dependent reactive oxygen species production (cytochrome c reduction assay) demonstrated that p22phox, gp91phox, p47phox, p67phox, and p40phox existed as a functional complex in the cytoskeletal fraction. These results indicate that, in contrast to the neutrophil enzyme, a substantial proportion of the NADPH oxidase in unstimulated endothelial cells exists as a preassembled intracellular complex associated with the cytoskeleton.

Endothelial cells generate reactive oxygen species (ROS) such as O2− and H2O2, which may inactivate nitric oxide, may modulate redox-sensitive signaling pathways and gene expression, and are implicated in the pathophysiology of disorders such as hypercholesterolemia and atherosclerosis (1, 2). A major source of ROS production in endothelial cells has recently been found to be a phagocyte-type NADPH oxidase (2–8). Several studies have suggested that, at a molecular level, the endothelial NADPH oxidase is analogous to the phagocyte NADPH oxidase complex in that all the main components of the phagocytic enzyme (i.e. gp91phox, p22phox, p47phox, p67phox, and Rac1) are detectable at both mRNA and protein level in endothelial cells (5, 7–12). Furthermore, we and others have shown that the endothelial cell p22phox and gp91phox cDNA sequences are highly (>90%) homologous to published neutrophil sequences (8, 12).

Despite this apparent similarity at a molecular level between the neutrophil and endothelial NADPH oxidase, the latter exhibits a number of striking differences in terms of its activity. First, several studies have suggested that NADPH-dependent ROS production in endothelial cells occurs continuously at a low level even in the absence of cell stimulation by extrinsic agonists (3–10). In contrast, the neutrophil oxidase is inactive in quiescent cells, and the continuous “basal” activity observed in endothelial cells has no obvious functional correlate in neutrophils (13). Second, a substantial proportion of the ROS generated in endothelial cells appears to be intracellular (6–8, 14, 15), whereas neutrophil O2− generation during phagocytosis is thought to occur in the extracellular (phagosomal) compartment. The mechanisms underlying these differences between endothelial and neutrophil NADPH oxidase are unknown. However, an obvious possibility is that the structural assembly and subcellular location of the enzyme in endothelial cells may be different from that reported for neutrophils.

The neutrophil NADPH oxidase comprises a plasma membrane-bound cytochrome b558 (which is a heterodimer of one p22phox and one gp91phox subunit) and at least four cytosolic subunits, namely p47phox, p67phox, p40phox, and Rac1 (13). During neutrophil activation in response to various agonists, the cytosolic subunits translocate to and associate with the cytochrome b558, a process that results in oxidative activation. In cell-free assays, both cytochrome b558 and the cytosolic subunits are required for oxidative activity. Based upon the above information, it has been assumed but not proven that the endothelial NADPH oxidase should also comprise a predominantly plasma membrane-bound cytochrome b558 with the other units present in the cell cytosol. Accordingly, NADPH-dependent O2− production (or “NADPH oxidase activity”) is detectable in crude membrane preparations such as particulate cell fractions spun down at 29,000 × g (16). However, these data do not provide direct evidence regarding the precise location of functional enzyme complexes or which subunits comprise these complexes. Furthermore, they do not explain why “constitutive” activity should exist in unstimulated cells. In a recent study where p22phox and gp91phox location was assessed by confocal immunofluorescence microscopy in rat endothelial cells, we found that both subunits appeared to have a predominantly intracellular localization, contrary to the above speculations regarding a mainly plasma membrane location (12).

In the present study, we used a range of complementary methods including confocal microscopy, plasma membrane pro-

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The abbreviations used are: ROS, reactive oxygen species; PIEC, porcine iliac arterial endothelial cells; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; t-NNAME, Nω-nitro-L-arginine methyl ester; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline.
tein biotinylation, subcellular fractionation, and co-immuno-
precipitation to investigate (a) the subcellular location of all of the main components of the NADPH oxidase in cultured endo-
thelial cells and (b) where the functional O$_2^-$-generating oxidase is located. The results indicate that a substantial proportion of the NADPH oxidase in unstimulated endothelial cells exists as a functional intracellular complex associated mainly with the cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Culture media, fetal calf serum (FCS), glutamine, and antibiotics were purchased from Invitrogen. Endothelial cell growth supplement, recombinant human epidermal growth factor, and gelatin were from Sigma. Porcine iliac arterial endothelial cells (PIEC) and bovine aortic endothelial cells (BAEC) were kindly provided by J. Fahre’s research group (King’s College London) (17). BAEC and PIEC were maintained in RPMI 1640 with 10% FCS, 2 mM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) were prepared according to established methods (18). HUVEC were cultured in 0.1% (w/v) gelatin-coated flasks in Medium 199 supplemented with 20% fetal calf serum, heparin (5 IU/ml), hydrocortisone (1 μg/ml), t-glutamine (2 mM), streptomycin (50 μg/ml), penicillin (50 units/ml), endothelial cell growth supplement (50 μg/ml), and human epidermal growth factor (10 ng/ml). HUVEC were used at passages 2–4. Endothelial cell identity was confirmed by their characteristic cobblestone appearance, positive expression of von Willebrand factor, and Dil-As-LDL uptake (18). All cells were studied at the stage when they had just achieved confluence (i.e. were not proliferating).

HL-60 cells were from the American Type Culture Collection. They were differentiated to neutrophils by incubation with 1.3% MeSO for 7 days (19). Human polymorphonuclear leukocytes were isolated from peripheral blood using a Percoll gradient (Amersham Biosciences) according to the manufacturer’s instructions.

**Antibodies and Immunoblotting**—Antibodies directed against NADPH oxidase subunits and their sources were as follows. An anti-p22$^{phox}$ monoclonal antibody (mAb449) was a kind gift from Dr. A. Verhoeven (Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands) (20). Rabbit polyclonal antibodies against recombinant p47$^{phox}$, p40$^{cox}$, p67$^{asa}$, and Rac1 and against a 30-amino acid C-terminal fragment of gp91$^{phox}$ (Pep37) were kindly provided by Dr. F. Wientjes (University College London) (21). Separate rabbit polyclonal antibodies against recombinant p47$^{phox}$ (R360) and p67$^{asa}$ (R1469), p22$^{phox}$ holoprotein (R3179), and a 12-amino acid C-terminal fragment of gp91$^{phox}$ (R2085) were kindly provided by Dr. M. T. Quinn (Montana State University) (22, 23). Anti-p47$^{cox}$ and anti-p67$^{asa}$ monoclonal antibodies were purchased from Transduction Laboratories. The goat anti-CD31 (PECAM-1) and rabbit anti-extracellular signal-regulated kinase 1/2 polyclonal and the anti-VE-cadherin monoclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-alpha-tubulin monoclonal antibody was from Sigma.

For immunoblotting, equal amounts (25 μg) of protein were separated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk, PBS, 0.2% Tween 20. Membrane protein extract from activated human neutrophils (a gift from F. Wientjes) was used as a positive control for p22$^{phox}$ and gp91$^{phox}$ expression. The protein extract from human phagocytic U937 cells, after phorbol 12-myristate 13-acetate stimulation, was used as a positive control for the other subunits.

**Immunofluorescence Microscopy**—Cell samples were prepared as described previously (24). Briefly, endothelial cells were cultured onto four-chamber slides precoated with 1% gelatin until nearly confluent. Cells were washed twice with PBS, permeabilized, and fixed with methanol/acetone (50% each, v/v). For staining with antibody to α-tubulin (Fig. 8), cells were fixed with 3.7% paraformaldehyde plus 10% Me$_2$SO, 1% glucose, and 0.02% Triton X-100 in PBS for 20 min at 37 °C. Slides were blocked with 20% fetal calf serum in PBS for 30 min at room temperature. Cells were washed with 0.1% bovine serum albumin/PBS three times with gentle shaking and then incubated with primary antibodies diluted (1:50 to 1:250) in PBS with 0.1% bovine serum albumin for 30 min at room temperature. Biotin-conjugated anti-rabbit IgG or anti-mouse IgG (1:500 dilution) were used as the secondary antibody and incubated for 30 min. Specific antibody binding was detected by fluorescein isothiocyanate-labeled (green fluorescence) or TRITC-labeled (red fluorescence) extravidin. Normal rabbit or mouse IgG (5 μg/ml) was used instead of primary antibody as negative control in each case. Confocal microscopy was performed using a Bio-Rad 1024 system. Optical sections were taken at 0.5-μm intervals, and images were captured and stored digitally for analysis. Fluorescein isothiocyanate was excited with a 488-nm argon ion laser line, and TRITC was excited with a 543-nm green helium/neon laser line. The emission for fluorescein and TRITC were both set at 515/530 and 580/540, respectively. To avoid bleed-through, while allowing optimal emission filters to be used, sequential acquisitions were performed (i.e. fluorescein isothiocyanate first followed by TRITC).

**Cell Surface Membrane Protein Biotinylation**—Cell surface protein biotinylation and streptavidin-agarose purification of biotylated protein was performed previously (25). PIEC membranes that had just reached confluence in 60-mm culture dishes were washed twice with ice-cold PBS (pH 8.0), and EZ-link$^{	ext{TM}}$ sulfo-NHS-LC-biotin (0.5 mg/ml in PBS, pH 8.0; Pierce) was added for 20 min on ice. The solution was removed, and the cross-linking procedure was repeated. After aspiration, the remaining biotin was blocked with 50 mM NH$_4$Cl in PBS (pH 8.0) for 10 min on ice with occasional agitation. Cells were washed twice with PBS and scratched into 1 ml of ice-cold buffer A containing 20 μM Hesper (pH 7.2), 150 mM NaCl, 100 μg/ml phenyl-
methylsulfonyl fluoride, and 1 μg/ml leupeptin. The cells were pelleted, rinsed, and resuspended in buffer A. Cells were sonicated for 2 × 15 s, and 1% Triton X-100 was added into the solution and extracted on ice for 20 min. Solubilized proteins were diluted 1:1 in buffer B (20 mM Tris–HCl, 1% (w/v) SDS, 0.5% Nonidet P-40, and protease inhibitors as described above) and incubated with 25 μl of prewashed streptavidin-agarose for 2 h at 4 °C. The supernatant containing nonbiotinylated proteins was removed into a new tube. The beads were washed four times with buffer B and once with PBS before resuspending in Laemmli sample buffer with 0.6% β-mercaptoethanol. Samples were boiled for 5 min to release bound, biotinylated proteins for immunodetection.

To ensure that p22$^{phox}$ and gp91$^{phox}$ expressed on the plasma membrane were amenable to biotinylation, differentiated HL-60 cells and freshly isolated human polymorphonuclear leukocytes (2 × 10$^6$/ml) were stimulated with phorbol 12-myristate 13-acetate (100 ng/ml for 30 min), and then underwent surface protein biotinylation in the same way as described for PIEC.

**Subcellular Fractionation**—Differential centrifugation based on standard protocols was used for isolation of subcellular fractions (26, 27). Confluent endothelial cells were washed twice with ice-cold PBS, and scratched into a 12-ml Falcon tube. Cells were pelleted and resuspended (20 × 10$^6$/ml) in MOPS-KOH buffer (20 mM MOPS-KOH, 250 mM sucrose, pH 7.4) containing phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μg/ml), and pepstatin (2 μg/ml). Cells were disrupted by quick freezing in liquid nitrogen followed by two cycles (20 s each) of homogenization (Polytron PT 2100) and two cycles of sonication at 100 W for 15 s on ice. The homogenate was quickly centrifuged at 200 × g for 5 min to remove any unbroken cells. The nuclei-enriched fraction (N fraction) was pelleted by centrifugation at 12,000 × g for 15 min. The resulting supernatant was then centrifuged for 15 min at 10,000 × g to obtain primary mitochondria and other large organelles (C fraction). The supernatant of C fraction was centrifuged for 15 min at 29,000 × g to obtain a pellet of submitochondrial particles, smaller organelles, and some microsomes (D fraction). The supernatant of D fraction was centrifuged for 60 min at 100,000 × g to sediment a pellet of microsomes, microperoxisomes, and membrane fractions (E fraction). The supernatant of E fraction includes the soluble cytoplasmic protein (S fraction). All pellets were resuspended in homogenization buffer and washed once again using the original centrifugation conditions before resuspending in 100 μl of MOPS-KOH buffer. The protein concentration was determined using a Bio-Rad kit. Proteins from each fraction (100 μg) were analyzed for NDPH-dependent oxidase activity by lucigenin chemiluminescence (see below). The rest of the protein fractions were dissolved in SDS buffer and used for immunoblotting.

To evaluate the degree of “purity” of the different subcellular fractions, especially with regard to partitioning of the plasma membrane, a number of marker enzyme activities were assayed in each fraction. As a plasma membrane marker, 5-nucleotidase activity was determined using a Sigma Diagnostic Kit (Sigma) according to the manufacturer’s recommendations with minor modifications of sample volumes. Pamamurase activity (EC 4.2.1.2) and lactate dehydrogenase activity (EC 1.1.1.27) were used as marker enzymes for mitochondria and the cytosol, respectively. Their activities were measured spectrophotometrically according to the methods of Stitt (28) for fumarase and Vassault (29) for lactate dehydrogenase using 25–50 μl aliquots of each fraction. The results of these assays are shown in Table 1. It is evident that 5-nucleotidase
activity was enriched in the E fraction, with little activity evident in the N fraction. Mitochondrial fumarase activity was enriched in the C fraction, whereas lactate dehydrogenase activity was significantly enriched in the S fraction.

Separation of Whole Cell Protein into Triton X-100-soluble and -insoluble (Cytoskeleton) Fractions—Extracting cell protein into Triton X-100-soluble and -insoluble (cytoskeleton) fractions was performed as described previously (23, 30) with some modifications. Confluent PIEC were washed twice in ice-cold PBS and detached by scraping. Cells were washed twice in ice-cold PBS and detached by scratching. Cells were ruptured by quick freezing in liquid nitrogen followed by two cycles of sonication at 100 W for 15 s on ice. The whole cell homogenate was extracted on ice for 20 min. A proportion of the whole cell homogenate at this stage was kept for measurement of NADPH oxidase activity by cytochrome c reduction assay. The Triton X-100-soluble and -insoluble fractions were separated by centrifugation at 14,000 × g for 15 min. Supernatant (Triton X-100-soluble fraction) was removed to a new tube, and the pellet (Triton X-100-insoluble cytoskeleton fraction) was washed with Triton buffer to eliminate the residual soluble element (the supernatant from wash was reserved for the assay of residual NADPH activity). The cytoskeleton fraction was then resuspended in 0.5 ml of Triton buffer, and the soluble protein concentration was determined using a Bio-Rad kit. Aliquots of protein samples were boiled in SDS buffer for immunoblotting.

Measurement of O2•− Production—O2•− production by endothelial cell protein fractions was measured using either lucigenin (5 μM) chemiluminescence or cytochrome c reduction assay as described previously (9, 31, 32). For chemiluminescence studies, we used a 96-well microplate luminometer (model Lucy 1; Rosys Anthos, Wals, Austria). Briefly, protein fractions were diluted in modified HEPES buffer containing 140 mM NaCl, 5 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 1 mM Na2HPO4, 25 mM HEPES, and 1% glucose (pH 7) and distributed (100 μg/well) onto the microplate. NADPH (100 μM) was added into the well just before reading. Light emission was recorded and expressed as mean arbitrary light units/min over 20 min. The specificity of O2•− thus measured was confirmed either by adding superoxide dismutase (200 units/ml) or Tiron (10 μM), a nonenzymatic scavenger of O2•− to quench the O2•−-dependent chemiluminescence. In some experiments, cell homogenates were preincubated with one of the following agents: (a) the flavoprotein inhibitor, diphenyleneiodonium (100 μM); (b) an NO synthase inhibitor, N-ω-nitro-L-arginine methyl ester (L-NAME, 100 μM); (c) a xanthine oxidase inhibitor, oxyurin (100 μM); or (d) a mitochondrial inhibitor, rotenone (50 μM). Each experiment was performed in triplicate.

For cytochrome c reduction assays, cell protein (final concentration 100 μg/ml) diluted in Dulbecco’s modified Eagle’s medium without phenol red was distributed in 96-well flat bottom culture plates (final volume 200 μl/well). Cytochrome c (500 μM) and NADPH (100 μM) were added in the presence or absence of Tiron (10 μM) and incubated at 37 °C for 30 min. Cytochrome c reduction was measured by reading absorbance at 550 nm on a microplate reader. O2•− production in nmol/mg protein was calculated from the difference between absorbance with or without Tiron and the extinction coefficient for change of ferrocyanochrome c to ferrocytochrome c (i.e. 21.0 mmol-liter−1-cm−1).

Co-immunoprecipitation for Co-Localization of NADPH Oxidase Subunits—Co-immunoprecipitation studies were performed using methods described previously (33). The whole cell protein extract and Triton X-100-insoluble protein fractions extracted from PIEC were used for the experiments. Protein samples (250 μg in a final volume of 750 μl) were diluted in immunoprecipitation buffer containing 0.05 M Tris-HCl (pH 7.4), 0.55 M NaCl, 0.1% Nonidet P-40 (v/v), 50 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Proteins were immunoprecipitated down with appropriate antibodies coupled to protein G-agarose beads (Sigma) overnight at 4 °C. Normal rabbit IgG-coupled protein G-agarose beads were used as negative controls. Immunocomplex-bound beads were washed four times with immunoprecipitation buffer and resuspended in 25 μl of 2× Laemmli buffer. Samples were boiled for 3 min, and proteins were separated by 10% SDS-PAGE for immunoblotting.

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 multiple testing as appropriate. p < 0.05 was considered statistically significant.

RESULTS

NADPH Oxidase Subunit Localization in Endothelial Cells by Immunofluorescence Microscopy—Confocal immunofluorescence microscopy of BAEC labeled with specific NADPH oxidase antibodies demonstrated a similar labeling pattern for all four subunits examined (i.e. p22phox, gp91phox, p47phox, and

FIG. 1. Immunofluorescent detection of NADPH oxidase subunits in unstimulated BAEC by confocal microscopy. BAEC cultured onto glass chamber slides were studied, using polyclonal antibodies against p22phox (R3179) and gp91phox (Pep37) and monoclonal antibodies against p47phox and p67phox. A similar perinuclear, slightly eccentric distribution with also a more diffuse reticular staining pattern was observed for each of the four subunits. Negative controls using mouse and rabbit nonspecific IgG are shown in the bottom panels.
NADPH Oxidase Localization and Assembly in Endothelial Cells

**Fig. 3. Detection of p22phox and gp91phox in the biotinylated cell surface protein and nonbiotinylated intracellular protein pool of PIEC, HL-60 cells, and human polymorphonuclear cells (PMN).** A, immunoblotting for p22phox and gp91phox in the biotinylated cell surface and nonbiotinylated intracellular protein pools of PIEC. CD31 and VE-cadherin were used as endothelial cell surface markers, and tubulin and extracellular signal-regulated kinase 1/2 (ERK1/2) were used as intracellular protein markers. B, Coomassie Blue staining of the polyvinylidene difluoride membrane of the gp91phox immunoblot shown in A, to demonstrate equal loading as well as the overall differences in pattern of protein distribution between plasma membrane and intracellular protein pools. C, immunoblotting for p22phox and gp91phox in the biotinylated cell surface and nonbiotinylated intracellular protein pools of differentiated HL-60 cells and polymorphonuclear cells.

p67phox subunits) (Fig. 1). In each case, there was a predominantly perinuclear and slightly eccentric distribution with also a more diffuse reticular staining extending toward the cell membrane. Also shown in Fig. 1 are the background controls using rabbit or mouse nonspecific IgG. Identical results were shown in Fig. 1 with HUVEC and PIEC (not shown).

To confirm that the putative "cytosolic" subunits, p47phox and p67phox, were present in the same location as cytochrome b558 in resting cells, dual labeling studies were performed. In Fig. 2 (left panels), BAEC were dual labeled with anti-p22phox mAb448 (Fig. 2A) and an anti-p47phox polyclonal antibody (Fig. 2B). In Fig. 2 (right panels), BAEC were dual labeled with anti-p22phox mAb448 (Fig. 2D) and an anti-p67phox polyclonal antibody (Fig. 2E). In each case, both subunits were co-located, as evident from the yellow color in the superimposed images (Fig. 2, C and F). These results suggest that the "cytosolic" subunits p67phox and p47phox are co-located with the cytochrome b558 in unstimulated endothelial cells, contrary to the traditional concepts of NADPH oxidase subunit distribution in resting cells.

**Detection of p22phox and gp91phox in the Biotinylated Plasma Membrane and Nonbiotinylated Intracellular Protein Pools—**

From the literature, a substantial proportion of p22phox and gp91phox is considered to be plasma membrane-bound in neutrophils, and the same has been assumed to be the case for vascular cells. To substantiate the results of the immunofluorescence studies, which suggested a mainly intracellular location for these subunits, the plasma membrane proteins of intact PIEC in culture were labeled with biotin, and then the whole cell proteins were separated into biotinylated and nonbiotinylated pools. Fig. 3 shows the results of immunoblotting of these protein pools. Immunoblotting for gp91phox with either of two different polyclonal antibodies (Pep37 and R2085, respectively) revealed clear bands at ~90 and at ~75 kDa, similar to the bands observed with neutrophil membrane. For p22phox, a single band at ~22 kDa was obtained with mAb448, similar to the band observed for neutrophil membrane (Fig. 3A). At least 90% of the total gp91phox and p22phox protein were detected in the nonbiotinylated intracellular pools with only weak bands detectable in the biotinylated plasma membrane pool. Stripping and reprobing of the p22phox immunoblot with an anti-CD31 (PECAM-1) antibody or an anti-VE-cadherin antibody, two established endothelial cell plasma membrane markers, demonstrated that CD31 and VE-cadherin were present predominantly in the biotinylated plasma membrane protein pool (Fig. 3A). Stripping and reprobing of the gp91phox immunoblot with a mouse monoclonal antibody against α-tubulin as a cytoskeleton marker, demonstrated the presence of α-tubulin mostly in the nonbiotinylated intracellular pool. As another control, extracellular signal-regulated kinase 1/2 was also detected mainly in the nonbiotinylated intracellular protein pool. Equal loading of proteins in each lane was confirmed by Coomassie Blue staining of the polyvinylidene difluoride membrane used for gp91phox detection (Fig. 3B). These results indicate that the vast majority of p22phox and gp91phox in endothelial cells is located intracellularly.

To confirm that p22phox and gp91phox expressed on the plasma membrane of neutrophils were amenable to biotinylation, we also undertook studies on differentiated HL-60 cells and human polymorphonuclear leukocytes (Fig. 3C). In this case, substantial amounts of p22phox and gp91phox were detected in the biotinylated pool for both cell types, consistent with a predominantly plasma membrane location.

**Detection of NADPH Oxidase Subunits in Subcellular Protein Fractions of Endothelial Cells—**

Next, we investigated which subcellular protein fraction of HUVEC, BAEC, and PIEC the NADPH oxidase components were present in. Fig. 4A shows that both the R2085 and the Pep37 antibody detected ~90-kDa and an ~75-kDa band in the total cell protein of all three endothelial cell types and in the neutrophil membrane positive control. This panel also demonstrates the specificity of the Pep37 antibody in that adsorption with an N-terminal gp91phox fusion protein fully abolished all labeling of endothelial cell and neutrophil protein. Fig. 4B is a representative
5'-nucleotidase activity was low and was instead enriched in the E fraction (Table I).

Fig. 5 shows the results for the distribution of p40\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac1 in subcellular fractions of PIEC. Interestingly, the major proportions of p40\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac1 bands were still found in the N fraction relative to the other fractions. However, for p47\textsuperscript{phox} equal amounts were detected in the N fraction and the E fraction (the pellet spun down at 100,000 \( \times g \)). Quantitative densitometric analysis (Fig. 5B) showed that the N fraction contained 54 \( \pm \) 4% of p40\textsuperscript{phox}, 32 \( \pm \) 3% of p47\textsuperscript{phox}, 39 \( \pm \) 1% of p67\textsuperscript{phox}, and 38 \( \pm \) 6% of Rac1.

Assessment of NADPH Oxidase Activity in Subcellular Fractions of Endothelial Cells—Aliquots (100 \( \mu \)g of protein) of the subcellular fractions obtained above were assayed for NADPH-dependent \( \Delta \Omega \) generation by lucigenin (5 \( \mu \)M) chemiluminescence. Fig. 6A shows that the highest level of NADPH-dependent \( \Delta \Omega \) generation was detected in the N fraction. Fig. 6B shows the relative NADPH oxidase activity in each fraction, expressed as a percentage of the total oxidase activity of whole cell homogenate. 53 \( \pm \) 1% of the NADPH oxidase activity was found in the N fraction; 20 \( \pm \) 2% was in the C fraction; 9 \( \pm \) 2% was in the D fraction; and 11 \( \pm \) 2% was in the E fraction. No \( \Delta \Omega \) generation (~2%) was detected in the S fraction. The oxidase activity in the N fraction was completely inhibited by diphenyleneiodonium but was unaffected by i-NAME, oxypurinol, or rotenone (Fig. 6C), consistent with NADPH oxidase as the source of \( \Delta \Omega \) generation. The \( \Delta \Omega \) scavenger, Tiron, completely abolished the lucigenin chemiluminescence signal.

Expression and Activity of NADPH Oxidase in Triton X-100-soluble and -insoluble (Cytoskeleton) Fractions—It has been reported in neutrophils that NADPH oxidase activity is associated with the cytoskeleton (23, 30). In view of the data presented so far indicating an intracellular co-location of NADPH oxidase subunits as functional complexes, we examined the possibility that the NADPH oxidase subunits may be associated with the cytoskeleton. Whole cell protein of PIEC was separated into Triton X-100-soluble and -insoluble (cytoskeleton) fractions and was probed for the presence of NADPH oxidase subunits (Fig. 7A). A neutrophil membrane preparation was used as the positive control for detection of p22\textsuperscript{phox} and gp91\textsuperscript{phox}, and U937 cell protein was used as the positive control for detection of p40\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac1. Surprisingly, almost the entire proportion of all the subunits was detected in the Triton X-100-insoluble (cytoskeleton) fraction, with only very weak bands detected in the Triton X-100-soluble fraction.

The NADPH-dependent \( \Delta \Omega \)-generating activity of the Triton X-100-soluble and -insoluble (cytoskeleton) fractions was examined by cytochrome c reduction assay (rather than by the lucigenin assay because high concentrations of Triton X-100 induced artifacts in the latter assay in our hands). In keeping with the immunoblot data, virtually all of the NADPH oxidase activity was present in the Triton X-100-insoluble fraction (Fig. 7B). The NADPH-dependent \( \Delta \Omega \) Association of NADPH Oxidase with Cytoskeletal Microtubes—To further investigate the relationship between oxidase subunit distribution and cytoskeletal elements, additional confocal immunofluorescence studies were undertaken in PIEC that were dual labeled for gp91\textsuperscript{phox} and \( \alpha \)-tubulin. To preserve the integrity of cytoskeletal microtubes, cells were fixed with paraformaldehyde in the presence of Me\textsubscript{4}SO and Triton X-100. Fig. 8 demonstrates that there was a significant overlap between the tubulin and gp91\textsuperscript{phox} distributions, particularly in the perinuclear region. It is notable that cytoskeletal microtubes extended from the perinuclear region to the cell periphery, whereas gp91\textsuperscript{phox} labeling was significantly less nearer the plasma membrane.
Co-immunoprecipitation of NADPH Oxidase Subunits in Endothelial Cells—All of the results presented so far suggest that a substantial proportion of the total cellular pool of NADPH oxidase in endothelial cells exists as a preassembled enzyme complex. To confirm the association of oxidase subunits into complexes in unstimulated endothelial cells, we performed co-immunoprecipitation experiments. The Triton X-100-insoluble protein fraction of confluent PIEC was used for these studies. Immunoprecipitation was undertaken using polyclonal antibodies against the NADPH oxidase subunits p22\textsuperscript{phox} (R3179), gp91\textsuperscript{phox} (R2085), p47\textsuperscript{phox} (R360), p67\textsuperscript{phox} (R1469), p40\textsuperscript{phox}, and Rac1. Subsequent immunodetection was performed using different antibodies, namely the anti-p22\textsuperscript{phox} mAb448, anti-gp91\textsuperscript{phox} antibody (Pep37), and an anti-p47\textsuperscript{phox} antibody from a different source (F. Wientjes). Fig. 9 demonstrates that p22\textsuperscript{phox} was readily detected in the immunoprecipitates of p67\textsuperscript{phox}, p47\textsuperscript{phox}, p40\textsuperscript{phox}, and gp91\textsuperscript{phox} as a single band running at the same molecular weight as the positive control. Likewise, an additional band running at \(-105\) kDa was observed in all lanes including the negative control and represents a nonspecific band. The p47\textsuperscript{phox} subunit was also co-immunoprecipitated down with all of the subunits of NADPH oxidase (Fig. 9).

Using the anti-tubulin antibody for immunoprecipitation, we were unable to detect any of the NADPH oxidase components (data not shown), suggesting that although the NADPH oxidase appears to be associated with microtubules as a whole, there does not seem to be strong binding to \(\alpha\)-tubulin specifically.

### Table I

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<tr>
<th>Subcellular fractions</th>
<th>Enzyme activity</th>
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<tr>
<td></td>
<td>5'-Nucleotidase</td>
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<td>Whole cell homogenate</td>
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<td>N: Nuclei-enriched</td>
<td>9.08 ± 3.68</td>
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<tr>
<td>C: Mitochondria and large organelles</td>
<td>31.80 ± 3.37</td>
</tr>
<tr>
<td>D: Mitochondria and small organelles</td>
<td>20.62 ± 2.18</td>
</tr>
<tr>
<td>E: Membrane and microsomes</td>
<td>50.68 ± 5.38*</td>
</tr>
<tr>
<td>S: Cytoplasmic protein</td>
<td>17.82 ± 4.80</td>
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Co-immunoprecipitation of NADPH Oxidase Subunits in Endothelial Cells—All of the results presented so far suggest that a substantial proportion of the total cellular pool of NADPH oxidase in endothelial cells exists as a preassembled enzyme complex. To confirm the association of oxidase subunits into complexes in unstimulated endothelial cells, we performed co-immunoprecipitation experiments. The Triton X-100-insoluble protein fraction of confluent PIEC was used for these studies. Immunoprecipitation was undertaken using polyclonal antibodies against the NADPH oxidase subunits p22\textsuperscript{phox} (R3179), gp91\textsuperscript{phox} (R2085), p47\textsuperscript{phox} (R360), p67\textsuperscript{phox} (R1469), p40\textsuperscript{phox}, and Rac1. Subsequent immunodetection was performed using different antibodies, namely the anti-p22\textsuperscript{phox} mAb448, anti-gp91\textsuperscript{phox} antibody (Pep37), and an anti-p47\textsuperscript{phox} antibody from a different source (F. Wientjes). Fig. 9 demonstrates that p22\textsuperscript{phox} was readily detected in the immunoprecipitates of p67\textsuperscript{phox}, p47\textsuperscript{phox}, p40\textsuperscript{phox}, and gp91\textsuperscript{phox} as a single band running at the same molecular weight as the positive control. Likewise, an additional band running at \(-105\) kDa was observed in all lanes including the negative control and represents a nonspecific band. The p47\textsuperscript{phox} subunit was also co-immunoprecipitated down with all of the subunits of NADPH oxidase (Fig. 9).

Using the anti-tubulin antibody for immunoprecipitation, we were unable to detect any of the NADPH oxidase components (data not shown), suggesting that although the NADPH oxidase appears to be associated with microtubules as a whole, there does not seem to be strong binding to \(\alpha\)-tubulin specifically.
type NADPH oxidase are expressed in endothelial cells (5, 7–12) in contrast to vascular smooth muscle cells, where homologues of the gp91phox subunit have been described (34–36). However, despite the apparent similarity between endothelial and neutrophil oxidases at a molecular level, there are a number of puzzling features central to the biological functions of the endothelial enzyme complex that have remained unexplained. In particular, it has not been clear why the NADPH oxidase seemingly generates a continuous low level of ROS even in the absence of agonist stimulation or why a substantial proportion of the ROS generation is intracellular rather than extracellular. The results of the present study help to address both of these questions. Using a range of complementary methods, we show that (a) the vast majority of NADPH oxidase subunit expression and functional activity in endothelial cells is intracellular rather than plasma membrane-bound; (b) a significant proportion of the NADPH oxidase subunits in unstimulated cells are present as fully assembled functional ROS-generating complexes; and (c) the functional oxidase is associated with the intracellular cytoskeleton, particularly in a perinuclear distribution.

Subcellular Location and Association of NADPH Oxidase Subunits—In the neutrophil, it is generally accepted that the majority of the gp91phox-p22phox heterodimer that makes up cytochrome b558 and is the core component responsible for enzyme activity is located on the plasma membrane (13). The “cytosolic” components p47phox, p67phox, p40phox, and Rac1 translocate to the membrane and associate with the cytochrome upon cell activation. However, in the present study, we not only found that by confocal immunofluorescence microscopy both p22phox and gp91phox were predominantly intracellular in endothelial cells, but more importantly confirmed this distribution by cell surface protein biotinylation experiments. In addition, confocal microscopy with dual labeling demonstrated that the “cytosolic” subunits were in fact also largely co-located with p22phox and gp91phox in this distribution. The validity of the latter result was strengthened by the observation that following subcellular fractionation, a major proportion of each subunit was present in the same fraction (the N fraction). Furthermore, direct evidence of a functional association of the subunits was obtained in co-immunoprecipitation experiments.

Functional Activity of the Endothelial NADPH Oxidase—Since the association of “cytosolic” subunits with cytochrome b558 is thought to initiate oxidase activity in neutrophils, the finding of apparently preassembled NADPH oxidase complexes in endothelial cells is likely to account for the low level ROS-generating activity observed in unstimulated nonproliferating endothelial cells. In support of this possibility, NADPH-dependent O2 production in subcellular fractions correlated well

FIG. 7. Expression and activity of NADPH oxidase in Triton X-100-soluble and -insoluble (cytoskeleton) fractions of PIEC. A, immunoblotting for subunits of NADPH oxidase. Lane 1, whole cell homogenate; lane 2, Triton X-100-soluble fraction; lane 3, Triton X-100-insoluble (cytoskeleton) fraction. A neutrophil membrane preparation was used as positive control for the detection of p22phox (mAb448) and gp91phox (Pep57), and U937 cell protein was used as positive control for the detection of other subunits. Exp, experiment. B, O2 generation by whole cell homogenate and Triton X-100-soluble and -insoluble fractions as measured by cytochrome c reduction assay.

FIG. 8. Confocal microscopic analysis of gp91phox and cytoskeletal microtube distribution in PIEC. PIEC were co-labeled with the anti-gp91phox polyclonal antibody Pep37 (A) and a monoclonal anti-α-tubulin antibody (B). In the superimposed images (C), the yellow color indicates areas of association. D is the negative control treated with nonspecific IgG.

FIG. 9. Co-immunoprecipitation of NADPH oxidase subunits in PIEC. The Triton X-100-insoluble fraction was used. NADPH oxidase subunits were immunoprecipitated using polyclonal antibodies as labeled below each lane. Subsequent immunodetection for co-existence of other subunits was performed with antibodies to p22phox (mAb448), gp91phox (Pep37), and p47phox (F. Wientjes). The protein precipitated down with normal rabbit IgG was used as a negative control for immunoprecipitation. A neutrophil membrane preparation was used as the positive control for detection of p22phox and gp91phox.
with the distribution of gp91phox, p22phox, and the other subunits. Thus, the highest NADPH oxidase activity was detected in the N fraction, which was also the fraction with the highest expression of oxidase subunits. This distribution is consistent with previous reports that endothelial NADPH oxidase activity was found in particulate cellular fractions spun down at \(-29,000 \times g\) (16). In the present study, comparison of Triton-soluble and -insoluble fractions also showed that oxidase activity paralleled the distribution of oxidase subunits.

The endothelial cell NADPH oxidase is not only "constitutively" active but responds to stimulation by various agonists (e.g. angiotensin II, phorbol ester, and cytokines) (34). An important question that therefore arises is the nature of the mechanisms that could be involved include an increase in the total number of fully assembled complexes, the translocation of subunits such as p47phox or Rac1 to partially assembled complexes, changes in the phosphorylation state (or other modification) of subunits that are already part of the oxidase complex, and/or changes in the amount of NADPH available to the enzyme. The present study clarifies the basis of endothelial NADPH oxidase activity in relatively quiescent endothelial cells (i.e. confluent cells in the absence of added agonist), which is an important prerequisite to further investigation of these potential mechanisms. Although analysis of these possibilities was not part of the current study, it was of interest that a significant expression of oxidase subunits, notably p47phox, was also found in fractions other than the N fraction that contained the majority of functional activity.

An interesting finding in relation to the gp91phox subunit distribution in the subcellular fractionation experiments was that although the \(-90-kDa\) band was most abundant in the N fraction, the \(-75-kDa\) band was more evenly distributed among all the fractions. In neutrophil membrane preparations, gp91phox typically migrates as multiple bands or a smear between \(-65\) and \(-100\) kDa on SDS-PAGE because of variable post-translational protein glycosylation (37–39). In the present study, two different anti-gp91phox antibodies both detected bands between \(-75\) and \(-90\) kDa in endothelial cell protein as well as neutrophil membrane, suggesting that authentic gp91phox with variable glycosylation was being detected. Furthermore, the specificity of the Pep37 anti-gp91phox antibody with respect to these bands was confirmed by the competitive inhibition experiments performed in the presence of recombinant gp91phox N-terminal fusion protein. The observation that only the \(-90-kDa\) band was found preferentially in the N fraction with the highest functional activity might suggest that the less glycosylated forms of gp91phox possibly do not contribute to oxidative activity to the same extent as the fully glycosylated form. In support of this possibility, in the co-immunoprecipitation experiments (Fig. 9), the \(-90-kDa\) band was the only one clearly detected. However, confirmation of this hypothesis will require additional appropriately designed direct studies.

Association of Functional Complexes with the Cytoskeleton—In neutrophils, an interaction between the cytoskeleton and oxidase complexes has been reported, and the active oxidase is reported to be found in the cytoskeleton fraction (23, 30, 40). The microfilament network, in particular, may play an important role in localizing and stabilizing the NADPH oxidase complex (23, 40, 41). In endothelial cells, the reticular pattern of labeling observed by confocal microscopy (Figs. 1 and 2) suggested that the oxidase may also associate with intracellular filaments or cytoskeletal elements. In fact, both the expression of oxidase subunits and oxidative activity were detected predominantly in Triton X-100-insoluble fractions. Furthermore, confocal dual labeling studies demonstrated an association of gp91phox with cytoskeletal microtubules, mainly in the perinuclear region (Fig. 9). Collectively, these results suggest that cytoskeletal elements may provide a scaffold upon which the oxidase components can assemble readily and where the stability of the complex can be maintained. These data are also consistent with previous observations that a substantial proportion (at least) of the ROS generated by NADPH oxidase in endothelial cells is intracellular (6–8, 14, 15).

Interestingly, previous studies have reported that remodeling of the cytoskeleton is a prerequisite for endothelial cell motility and that there is a close relationship between actin polymerization and ROS generation after wounding of endothelial cell monolayers (15). In an experimental model of endothelial cell hypoxia, the reorganization of actin microfilament structure was modulated by the redox state of the cells, and the incorporation of actin into filaments could be inhibited by diphenyleneiodonium, suggesting a role for NADPH oxidase in microfilament formation (42). Although these interactions were not the focus of the present study, the localization of functional NADPH complexes that we report here may be relevant to a role of cell redox state and ROS generation in modulating cellular structure and motility.

Conclusion—In summary, this study has shown that a substantial proportion of the NADPH oxidase in unstimulated cultured endothelial cells exists as a preassembled, functional intracellular complex associated with the cytoskeleton in a mainly perinuclear distribution. Although this might be considered an unexpected finding, it would appear to be logical in view of the postulated role of the enzyme product of the oxidase in the modulation of intracellular signal transduction pathways, especially those that influence gene expression in endothelial cells (1, 6, 43). However, extrapolation of these findings to endothelial cells in vivo must be made with caution because of the likely differences in physical and biological microenvironment that these cells are subject to.

REFERENCES

23. Woodman, R. C., Ruedi, J. M., Jesatsis, A. J., Okamura, N., Quinn, M. T.,


