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Expression and Localization of NOX2 and NOX4 in Primary Human Endothelial Cells

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ABSTRACT

Reactive oxygen species (ROS) control the integrity of the vascular endothelium. Our laboratory has recently shown that transduction of human umbilical vein endothelial cells (HUVECs) with an active variant of the small GTPase Rac promotes the production of ROS, ROS-dependent activation of p38 mitogen-activated protein kinase, and loss of vascular/endothelial-cadherin-mediated cell–cell adhesion. Here we show that HUVECs express mRNAs for NOX2 as well as NOX4 mRNA, but not for NOX1 or NOX3. Interestingly, NOX4 was expressed at ~100-fold higher levels compared with NOX2. NOX4–green fluorescent protein largely localizes to an intracellular compartment that costained with a marker for the endoplasmic reticulum, and its distribution did not overlap with lysosomes, Weibel–Palade bodies, or mitochondria. The NOX2-regulatory proteins $p47^{phox}$ and $p67^{phox}$ associated with the actin cytoskeleton and were found in cell protrusions and membrane ruffles, colocalizing with Rac1. This translocation to the cell periphery was promoted by tumor necrosis factor (TNF)-α. Finally, scavenging of ROS was found to impair TNF-α-induced cytoskeletal rearrangements and the formation of a confluent endothelial monolayer. Together, these data prove the differential mRNA expression of NOX family members in human endothelium and indicate that these NOX proteins and their regulators may be involved in the control of endothelial cell spreading, motility, and cell–cell adhesion. *Antioxid. Redox Signal.* 7: 308–317.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) are well known for their role in killing engulfed pathogens by phagocytic leukocytes. ROS are generated in the cells through the NADPH oxidase system, which is a complex of multiple membrane-associated and cytosolic components (37). This multimolecular complex uses electrons, derived from intracellular NADPH, to generate superoxide anions, which subsequently dismutate to hydrogen peroxide (4).

However, next to their role as "pathogen killer," it has been shown that low levels of ROS function as second messengers and are involved in intracellular signaling in a variety of cell types. Irani and colleagues showed previously that ROS were required for Ras-mediated cell growth in fibroblasts (18). Since then, numerous reports have been published on the signaling capacities of low levels of ROS (for reviews, see 5, 8, 23), in particular in the vascular endothelium. At sites of inflammation and infection, the local environment is enriched with cytokines, such as tumor necrosis factor (TNF)-α, interleukin-1, and interferon-γ. These cytokines induce a dose- and time-dependent increase in ROS in endothelium (23). Also, shear stress and vasoactive peptides such as bradykinin induce production of ROS in endothelial cells (16, 40).

The generation of ROS requires the activation of the small GTPase Rac. Studies with Rac2-deficient cells have shown clearly that the NADPH oxidase system in neutrophils depends mainly on Rac2 (12, 15). However, in other cell types such as endothelium and monocytes, it has recently been shown that the NADPH oxidase complex favors Rac1 over

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Rac2 for the generation of ROS, albeit that the amounts of ROS generated in endothelial cells are almost 100-fold lower than in neutrophils (29, 41).

Recent literature indicates that the NADPH oxidase system, as it is present in neutrophils, is also expressed in endothelial cells (13, 21). We recently reported that transduction of an active form of Rac1 (RacV12) into endothelial cells induces ROS production and ROS-mediated loss of endothelial cell–cell adhesion (34). Moreover, endothelial cell migration also appeared to be dependent on ROS (34). However, the mechanism and signaling pathways that drive Rac-dependent ROS production in endothelial cells are currently unclear.

In the present study, we analyzed the expression of the leukocyte NADPH oxidase components gp91^{phox} (phagocytic oxidase), also called NOX2 (NADPH oxidase 2), p67^{phox}, p47^{phox}, and p22^{phox} in endothelial cells. We found, using real-time PCR, relatively high expression of NOX4, a NOX2 homologue, in endothelial cells. Using microinjection of a NOX4–green fluorescent protein (GFP) fusion construct into endothelial cells, we detected NOX4-GFP at the endoplasmatic reticulum. Moreover, GFP fusion proteins of p47^{phox} and p67^{phox} also localize to the endoplasmatic reticulum and translocate to the tips of TNF-α-induced membrane ruffles, together with the small GTPase Rac1. These findings confirm expression of multiple oxidase-generating enzymes in endothelial cells that play a role in endothelial cell–cell adhesion and motility.

MATERIALS AND METHODS

Materials

Monoclonal antibodies (mAb) to β-catenin were obtained from Transduction Laboratories (Becton Dickinson, Amsterdam, The Netherlands). Polyclonal antibodies (Abs) to p67^{phox} and p47^{phox} were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). mAb to NOX2 (7D5) was a kind gift of Dr. Nakamura (Nagasaki University, Nagasaki, Japan), mAb to p67^{phox} was a kind gift of Dr. Quinn (Montana State University, Bozeman, MT, U.S.A.), and mAb to p47^{phox} was a kind gift of Dr. Heyworth (Scripps Research Institute, La Jolla, CA, U.S.A.). mAb to p22^{phox} was kindly provided by Dr. D. Roos (Sanquin Research at CLB, Amsterdam, The Netherlands). mAb to vascular/endothelial (VE)-cadherin (7H1) was obtained from Pharmingen (Becton Dickinson). Texas Red phalloidin, Alexa-488-labeled GαM-Ig, Alexa-568-labeled GαM-Ig, Alexa-568-labeled GαR-Ig secondary Abs, and Mitotracker were purchased from Molecular Probes (Leiden, The Netherlands). Ab to calreticulin was obtained from Affinity Bioreagents (Golden, CO, U.S.A.). CD63 mAb was obtained from Sanquin. TNF-α, *N*-acetylcysteine (N-AC) and Tiron were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

Primary human umbilical vein endothelial cells (pHUVECs) were isolated from fresh umbilical veins as described previously (6) and used for passage 2–4. pHUVECs, immortalized HUVECs, and immortalized human microvascular endothelial cells (HMECs) were cultured at 37°C at 5% CO_2 in fibronectin (FN)-coated flasks in M199 medium (GibcoBRL, Life Technologies, Paisley, Scotland, U.K.), supplemented with L-glutamine (300 μg/ml, GIBCO), 20% heat-inactivated fetal calf serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were passaged by treatment with trypsin/EDTA solution (GIBCO). Jurkat and Ramos cells and stroma cell line 88.7 were cultured at 37°C at 5% CO_2 in Iscove's modified Dulbecco medium (GIBCO) supplemented with L-glutamine (300 μg/ml, GIBCO), 10% heat-inactivated fetal calf serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin. Stroma cells were passaged by treatment with trypsin/EDTA solution (GIBCO). Neutrophils were isolated according to Roos and de Boer (28).

Constructs

The full-length cDNA encoding NOX4 was a kind gift of Dr. J.D. Lambeth (Emory University Medical School, Atlanta, GA, U.S.A.). The NOX4 cDNA was subcloned as a KpnI-ApaI PCR fragment into the pE-GFP N1 vector, resulting in a NOX4-GFP fusion product (Clontech, Palo Alto, CA, U.S.A.). The full-length cDNAs encoding NOX2, p67^{phox}, and p47^{phox} were a kind gift of Dr. D. Roos (Sanquin Research at CLB). The NOX2 cDNA was subcloned as a BamHI-HindIII PCR fragment into the pE-GFP-C1 vector (Clontech), resulting in a GFP-NOX2 fusion construct. The p67^{phox} cDNA was subcloned into the pE-GFP C1 vector as described (32). The p47^{phox} cDNA was subcloned as an EcoRI-ApaI PCR fragment into the pE-GFP C1 vector (Clontech). The constructs were microinjected into the nuclei of primary endothelial cells as described under *Microinjection*.

GFP-Rac–expressing endothelial cells

The full-length cDNA encoding Rac1 was a kind gift of Dr. J.G. Collard (Netherlands Cancer Institute, Amsterdam, The Netherlands). The Rac1 cDNA was subcloned as an XhoSnaBI PCR fragment and swapped with the actin cDNA from the modified LZRS-GFP-actin-IRES-zeocin vector (39). The resulting construct, LZRS-GFP-Rac-IRES-zeocin, was transfected into amphotropic Phoenix packaging cells (20) by means of the calcium phosphate transfection system (Life Technologies) to produce retroviruses. HUVECs were transduced with virus-containing supernatant in the presence of 10 μg/ml DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate) (Boehringer). After 6 h, the supernatant was replaced with fresh medium, and the cells were allowed to recover overnight. This procedure was repeated twice on two consecutive days. Transduced cells were sorted for GFP expression by FACStar (Becton Dickinson, Heidelberg, Germany).

Real-time RT-PCR

Total RNAs were extracted from confluent HUVECs. The PCR reaction contained Pwo polymerase (Roche, Basel, Switzerland), buffer, dNTPs, 2 μg of total RNA, and the primers in a final volume of 20 μl and was performed at 42°C

for 60 min. After a final denaturation step at 95°C for 2 min, 6 μl of cDNA was subjected to PCR consisting of denaturation at 95°C for 10 s, followed by 30 s of annealing at 61.5°C and 2 min of elongation at 72°C for 35 cycles. The PCR product was purified over QiaSpin-PCR purification columns (Qiagen, Valencia, CA, U.S.A.). The following primers for real-time PCR were used: forward primer for NOX1, GACAATGGGAAACTGGGTGGTTAAC; reverse primer for NOX1, GGGAAGTTTGAATCCCGCGGATGTCA; forward primer for NOX2, CTGGACAGGAATCTCACCTTTCAT; reverse primer for NOX2, AATTTATCTACACGTTACCACACTTAG; forward primer for NOX3, TATCATGATGGGGTGCTGGATTTTG; reverse primer for NOX3, ACAAGGAGAATATTCTCAATTCTTCA; forward primer for NOX4, GCCAACGAAGGGGTTAAACA; reverse primer for NOX4, CGGGAACCAATATGTTCGTTCTTC. The housekeeping gene Gus was used as a control to generate a standard curve, and the relative expression of NOX2 and NOX4 was calculated by means of this standard curve.

Microinjection

Confluent monolayers of HUVECs were injected with cDNA of GFP-NOX2, NOX4-GFP, p67^{phox}-GFP, or p47^{phox}-GFP (50–60 cells/glass cover slip) by means of an Eppendorf Microinjection Unit (Microinjector model 5242, Micromanipulator model 5170, CO_2 Controller model 3700, and Heat Controller model 3700) attached to a microscope (Axiovert 135m; Zeiss, Inc., Thornwood, NY, U.S.A.). After microinjection, the samples were processed for confocal imaging microscopy as described in the next section.

Immunocytochemistry

HUVECs were cultured on FN-coated glass cover slips and were fixed and immunostained as described (17) with antibodies (5 μg/ml) to the various proteins as indicated. The antibodies were subsequently visualized with fluorescently labeled secondary anti-mouse IgG or anti-rabbit IgG antibodies (5 μg/ml). F-actin was visualized by Texas Red phalloidin (1 U/ml). In some experiments, the cells were pretreated 30 min at 37°C with TNF-α (10 ng/ml), or overnight with N-AC (5 m*M*) or Tiron (5 m*M*), as indicated. Images were recorded with a Zeiss LSM510 confocal microscope with appropriate filter settings. Cross-talk between the green and red channel was avoided by use of sequential scanning.

Western blotting

Cells were grown to confluency on FN-coated 50-cm^2 Petri dishes, washed twice gently with ice-cold Ca^{2+}- and Mg^{2+}-containing phosphate-buffered saline and lysed in boiled sodium dodecyl sulfate (SDS) sample buffer containing 4% (wt/vol) 2-mercaptoethanol (Bio-Rad). The samples were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to 0.45-μm nitrocellulose (Schleicher and Schuell Inc., Keene, NH, U.S.A.), and the blots were blocked with blocking buffer [1% (wt/vol) low-fat milk in Tris-buffered saline with Tween-20 (TBST)] for 1 h, subsequently incubated at room temperature with the appropriate Abs for 1 h, followed by incubation with RαM-Ig-horseradish peroxidase for 1 h at room temperature. Between the various incubation steps, the blots were washed three times with TBST and finally developed with an enhanced chemiluminescence detection system (Amersham).

Measurement of ROS

To measure generation of ROS in endothelial cells, pHUVECs cultured on FN-coated glass cover slips were loaded with dihydrorhodamine-123 (DHR; 30 μ*M*; Molecular Probes) for 30 min, washed, and subsequently treated at 37°C with TNF-α (10 ng/ml), control Ab IgG, or medium. Fluorescence of DHR was quantitated by time-lapse confocal microscopy. Intensity values are shown as the percentage increase relative to the basal DHR values at the start of the experiment.

Statistics

Student's *t* test for paired samples (two-tailed) was used for statistical analysis. Student's *t* test for independent samples was used when indicated.

RESULTS

To analyze expression of NOX2 homologues in pHUVECs, we used real-time quantitative PCR with specific primers for NOX1, 2, 3, and 4 mRNAs. We included leukemic and T-cell lines in the analysis to serve as positive controls for each NOX variant. Specificity of the primers was confirmed by PCR amplification of the cloned cDNAs, which showed that the respective primers did not cross-hybridize to other NOX cDNAs (data not shown).

The results of the real-time quantitative PCR analysis are shown in Table 1. Primary human endothelial cells were found to express NOX2 and NOX4 mRNA, but not NOX1 or NOX3 mRNA. The expression of NOX2 in endothelium had been suggested previously, based on immunocytochemical data and western blot analysis (21). The levels of NOX4 mRNA were found to be at least 100 times higher than those of NOX2 (Table 1). This might indicate that NOX4 is the most abundant member of this protein family in human endothelium. NOX4 mRNA was also expressed in the erythroleukemic cell line K562 and in the T-lymphocyte cell line Jurkat, but not in the B-lymphocyte cell line Ramos. In contrast, NOX2 mRNA was expressed in Ramos, but not in K562 and Jurkat. NOX3 mRNA was found only in Jurkat T-cells, and NOX1 mRNA was detectable only in Jurkat and Ramos lines.

The NOX2 protein is known to form a multimolecular complex with a series of regulatory proteins, including p22^{phox}, p47^{phox}, p67^{phox}, and the small GTPase Rac in activated phagocytes (37). Western blot analysis was used to confirm the expression of the phagocytic NADPH oxidase components NOX2 (gp91^{phox}), p47^{phox}, and p22^{phox} in HUVECs (Fig. 1). Neutrophil lysates were used as a positive control, and a stromal cell line served as a negative control. NOX2 is highly glycosylated in neutrophils, and migrated as a smear rather than a distinct band, as was also shown by others (26, 36). However, in endothelial cells, two distinct bands ap-

TABLE 1. EXPRESSION OF NOX MRNAS IN HUVECS

	pHUVECs	*K562*	*Jurkat*	*Ramos*	*Relative expression of pHUVECs*
NOX1	−	−	+	+	
NOX2	+	−	−	+	3.3
NOX3	−	−	+	−	
NOX4	+	+	+	−	402

mRNA was isolated from pHUVECs, K562, Jurkat cells, or Ramos cells, and primers specific to NOX1, 2, 3 and 4 were used to quantify mRNA expression by real-time PCR as described in Materials and Methods. Samples that did not express the indicated mRNAs are indicated with −. Positive samples are indicated with +. Relative expression levels of NOX2 and NOX4 mRNA were calculated as described in Materials and Methods. Results are means ± SD of three independent experiments.

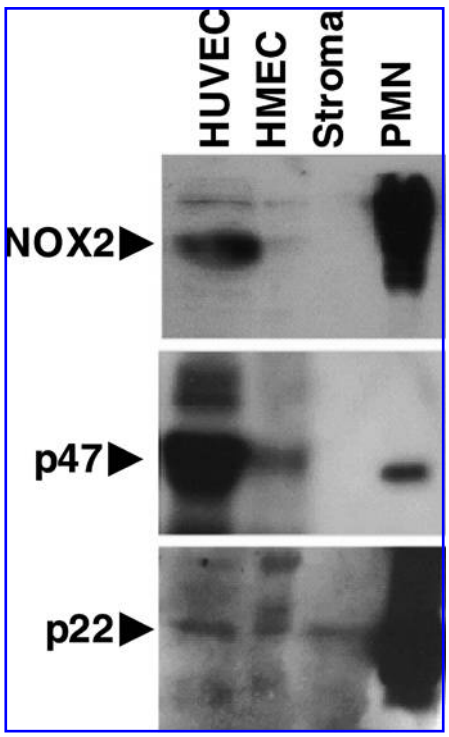

FIG. 1. Expression of the NADPH oxidase complex in human endothelial cells. Cells were cultured as described in Materials and Methods, and cell lysates were analyzed by western blotting for expression of the indicated components of the NADPH oxidase complex. Detection of NOX2 revealed two proteins with an apparent molecular mass around 90 and 75 kDa (arrowheads) in lysates of HUVECs, as well as of HMECs, although the expression in HMECs was less than in HUVECs. The stromal cell line 88.7 (Stroma) did not show any detectable expression of NOX2, whereas NOX2 in polymorphonuclear (PMN) cell lysates appeared as a smear, due to the high glycosylation of NOX2 in PMN. $p47^{phox}$ is expressed in HUVECs, HMECs, and PMN, but not in stroma (arrowhead). $p22^{phox}$ is expressed in all four cell types (arrowhead). Experiments were performed at least three times.

peared, one at ~90 kDa and one band at ~75 kDa, possibly due to differential glycosylation. The detection by western blot analysis of a doublet for NOX2 in the endothelial cell lysates has also been reported by others (21). $p47^{phox}$ is highly expressed in endothelial cells, suggestive of a prominent role in endothelial ROS formation. The stromal cells expressed only $p22^{phox}$ to a detectable level, but were devoid of the other components analyzed, including NOX2 (3). Unfortunately, we were unable to confirm expression of $p67^{phox}$ protein, probably due to the fact that the available Abs were not appropriate for blotting. Also detection of NOX4 protein by western blot failed, because blotting Abs are not available at this time.

To visualize the intracellular localization of the NOX2 and NOX4 proteins, we made use of microinjection into pHUVECs of expression constructs encoding GFP-fused NOX proteins. The GFP-NOX2 fusion protein showed a nonhomogeneous intracellular distribution, suggesting that the protein associated with intracellular membranes (Fig. 2Aa). GFP-NOX2 did not show prominent association with the plasma membrane or with F-actin in these experiments. The distribution of the GFP-NOX2 fusion protein was comparable to the staining obtained with a mAb to NOX2 (Fig. 2Aa and d).

The NOX4-GFP fusion protein showed a comparable distribution as NOX2, localizing to intracellular membranes, in particular in the perinuclear region (Fig. 2Ba, d, f, and h). The use of different markers indicated that NOX4-GFP does not colocalize to the actin cytoskeleton, lysosomes, or Weibel–Palade bodies, because CD63, as a marker for these organelles (38), did not colocalize with NOX4-GFP, nor did the mitochondrial marker Mitotracker (Fig. 2Be and g). However, NOX4-GFP colocalized with an Ab to calreticulin, a marker for the endoplasmic reticulum (Fig. 2Bi and j). Comparable to NOX4-GFP, the GFP-NOX4 fusion protein was expressed similarly following microinjection (data not shown). NOX2 was also found at the endoplasmic reticulum, because NOX2 colocalized with anti-calreticulin, similar to NOX4 (data not shown).

To determine the intracellular distribution of NOX2-associating proteins $p47^{phox}$, $p67^{phox}$, and Rac1, we used both immunocytochemistry and microinjection of GFP fusion constructs. Immunostaining for the $p47^{phox}$ showed that the protein localized on actin stress fibers (Fig. 3Aa). Moreover, $p47^{phox}$ also distributed in part to a perinuclear compartment

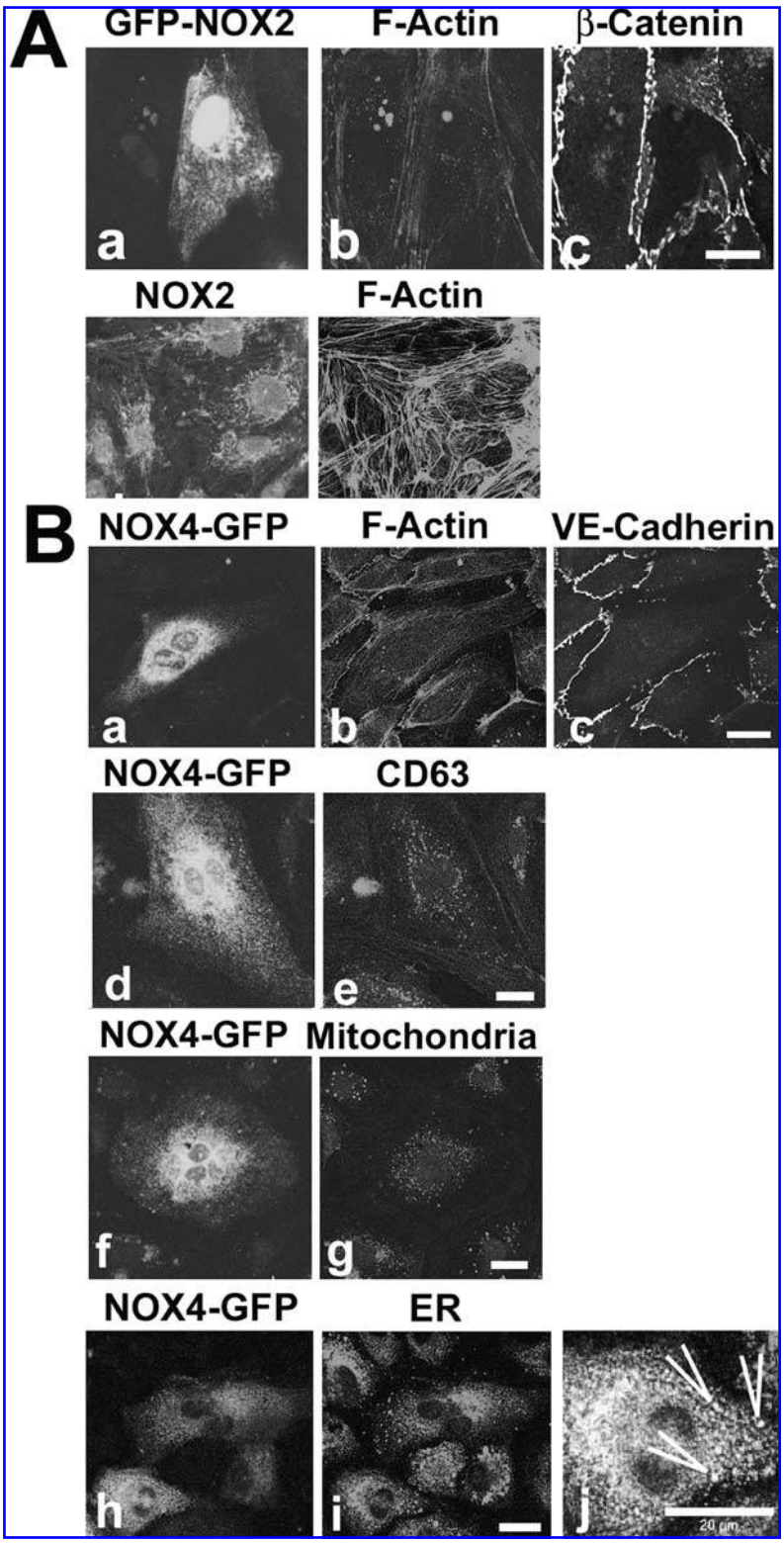

FIG. 2. Localization of NOX2 and NOX4 in human endothelial cells. Endothelial cells were cultured on FN-coated glass cover slips, fixed, permeabilized, and stained as described in Materials and Methods. (**A**) NOX2 localization was studied by antibody staining, as well as microinjection of a GFP-NOX2 fusion construct. Confocal microscopy revealed that GFP-NOX2 is distributed in a perinuclear region (**a**). The green nucleus is explained by the coinjection of GFP-histone-2B to determine the microinjected cells (a). F-actin is shown in image (**b**) and β-catenin, as a marker for the cell border, in (**c**). Bar = 20 μm. Antibody staining of NOX2 is shown in (**d**) and F-actin in (**e**). Bar = 50 μm. (**B**) NOX4-GFP fusion proteins were expressed following microinjection revealing a perinuclear distribution (**a, d, f,** and **h**). F-actin is shown in (**b**) and VE-cadherin, as a marker for cell borders, is shown in (**c**). CD63 is a marker for lysosomes and Weibel–Palade bodies in endothelial cells and is shown in image (**e**). Mitochondria are visualized by Mitotracker and are shown in image (**g**). Endoplasmatic reticulum (ER), visualized by calreticulin, is shown in (**i**). Image (**j**) is a magnification of image (i) and shows colocalization in detail, indicated by the arrowheads (j). Bar = 20 μm.

(Fig. 3Ad), similar to the NOX2 and NOX4 proteins. Expression of a $p47^{phox}$-GFP fusion protein revealed that $p47^{phox}$ localized perinuclearly and associated with actin stress fibers (Fig. 3Ac). In single, nonassociated endothelial cells, $p47^{phox}$-GFP localized to the tips of membrane ruffles (Fig. 3Ac). Detailed confocal microscopy analysis revealed that $p47^{phox}$ colocalized at least in part with the ER (Fig. 3Ae and f).

Ab staining for $p67^{phox}$ and microinjection of $p67^{phox}$-GFP revealed that $p67^{phox}$ is localized in a perinuclear region (Fig. 3Ba, f, g, and j). Moreover, detailed confocal microscopy

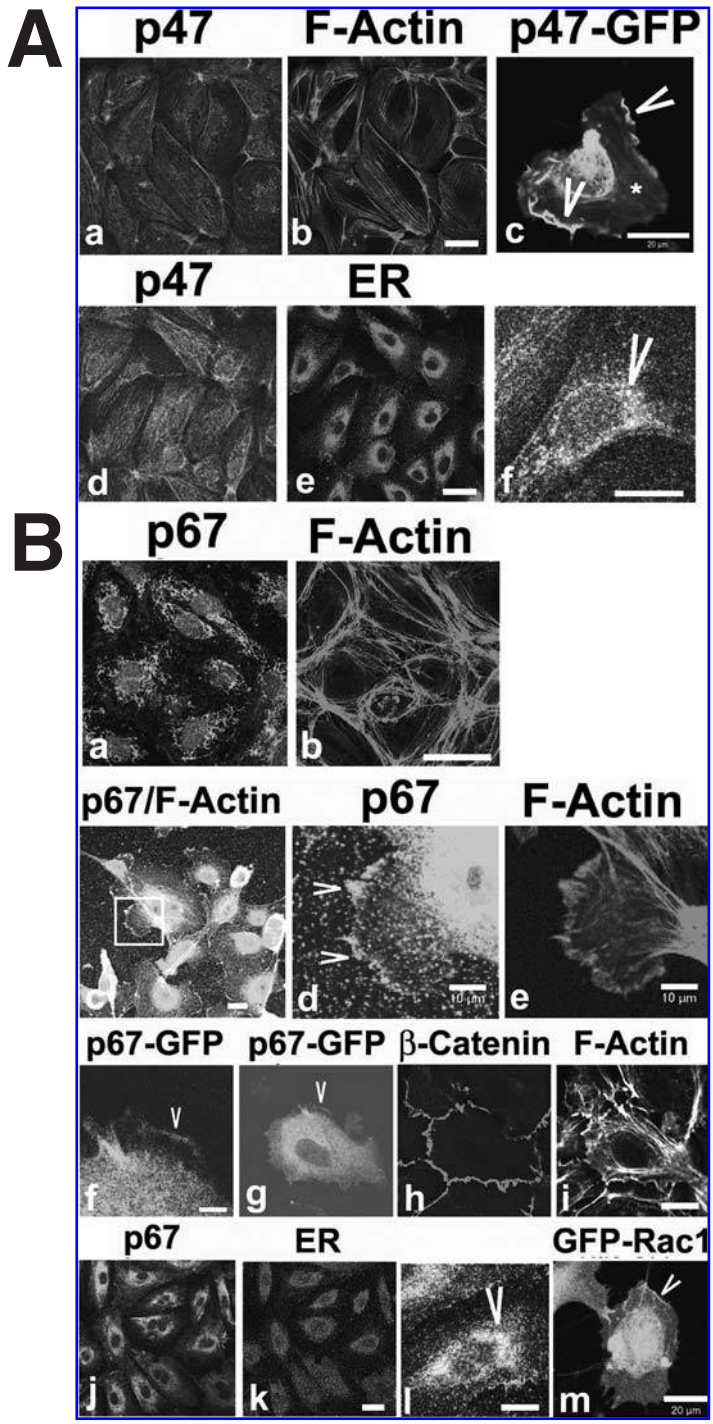

FIG. 3. Localization of p47^{phox} and p67^{phox} in human endothelial cells. (**A**) Endothelial cells were cultured on FN-coated glass cover slips, fixed, permeabilized, and stained as described in Materials and Methods. p47^{phox} is detected in a perinuclear region, on actin stress fibers, and at the cell periphery, at the tips of membrane ruffles. Antibody staining of p47^{phox} is shown in image (**a**), F-actin is indicated in (**b**), and image (**c**) shows a single endothelial cell that displayed extensive membrane ruffling and expresses p47^{phox}-GFP at the tips (open arrowheads). Moreover, p47^{phox}-GFP is also found perinuclear and at stress fibers (asterisk; c). Bar = 20 μm. Antibody staining of p47^{phox} is shown in (**d**) and the endoplasmatic reticulum (ER), visualized by calreticulin, is shown in (**e**). Image (**f**) is a magnification of image (e) and shows colocalization in detail (open arrowhead). Bar = 20 μm. (**B**) Analysis of p67^{phox} immunostaining by a mAB and microinjection of the p67^{phox}-GFP fusion construct showed perinuclear expression and expression at membrane ruffles. Antibody staining of p67^{phox} is shown in (**a**) and F-actin is represented in (**b**). Image in (**c**) represents p67^{phox} antibody staining and F-actin. Bar = 50 μm. Marked area is enlarged in images (**d–f**). Image (d) shows p67^{phox} staining at the tips of membrane ruffles (open arrowheads), and F-actin in (e). Image (f) shows p67^{phox}-GFP, localized to membrane ruffle [magnification of image (**g**); open arrowhead]. Bar = 5 μm. p67^{phox}-GFP microinjection is shown in (g), β-catenin in (**h**), and F-actin in (**i**). Bar = 10 μm. Image (**j**) represents antibody staining of p67^{phox}, and the endoplasmic reticulum (ER), visualized by calreticulin, is shown in (**k**). Image (**l**) is a magnification of image (k) and shows colocalization in detail (open arrowhead). Bar = 20 μm. Image (**m**) shows GFP-Rac localization at membrane ruffles (open arrowhead) in green. Bar = 20 μm.

analysis showed p67^{phox} expression at the tips of membrane ruffles, where F-actin also is localized (Fig. 3Bc–g, and i). Also, p67^{phox} localized at least in part to the endoplasmic reticulum (Fig. 3Bk and l). The small GTPase Rac is also an important regulator of the NADPH oxidase complex (1). Confocal microscopy imaging of living endothelial cells, cultured at low density, showed that GFP-Rac1 localized to membrane ruffles, similar to p47^{phox} and p67^{phox} (Fig. 3Bm). We were unable to show p22^{phox} staining using confocal microscopy, because the available Ab was not appropriate for immunocytochemistry in endothelial cells.

TNF-α is an inflammatory cytokine, which is a potent activator of endothelial cells, resulting in increased endothelial permeability, up-regulation of adhesion molecules, production of chemokines, and induction of gene expression (9, 19). Therefore, TNF-α might play an important role in early as well as later stages of transendothelial migration. Our lab has recently shown that production of endothelial ROS is in-

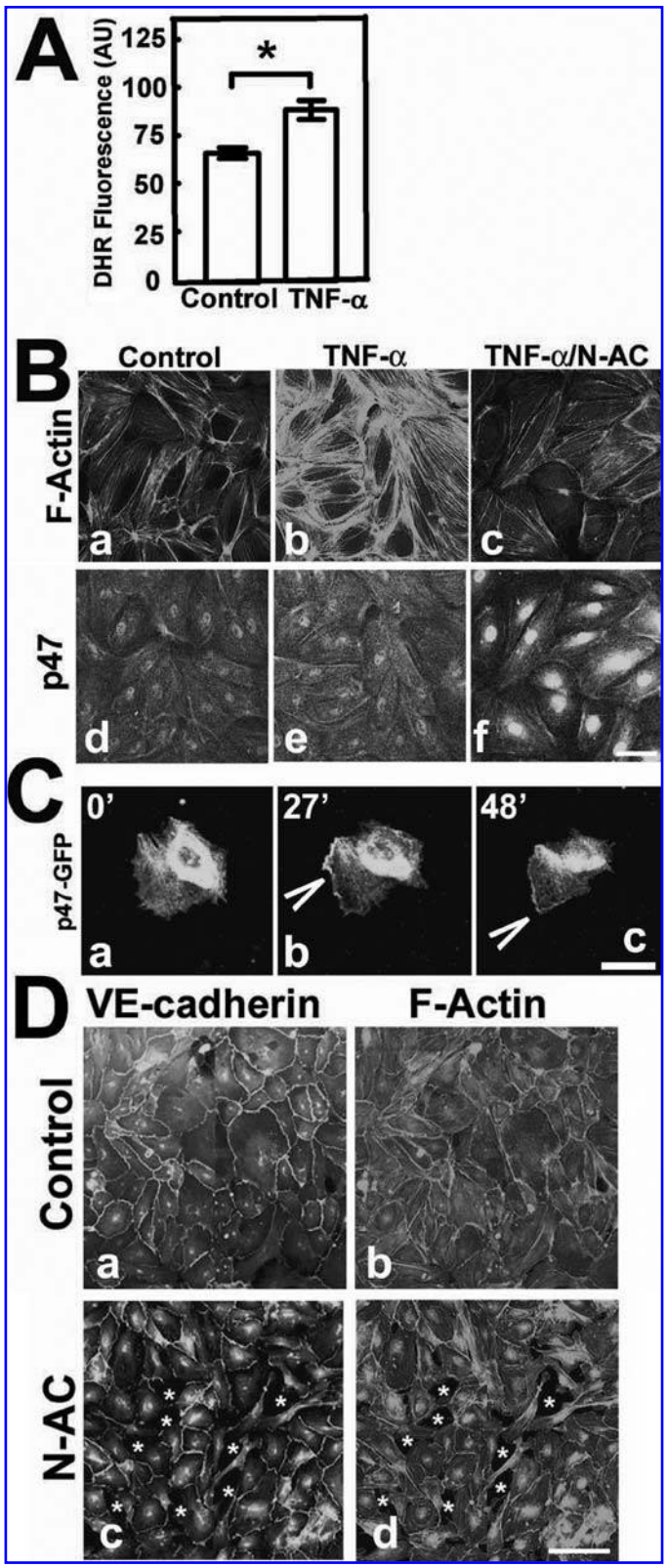

FIG. 4. TNF-α stimulation induces stress fibers and ROS production in human endothelium. (**A**) Endothelial cells (HUVECs) were cultured on FN-coated glass cover slips, grown to confluency, preincubated with DHR as indicated in Materials and Methods, and then treated for 30 min with TNF-α (10 ng/ml) or medium, and DHR intensity was measured by confocal microscopy. Control bar represents basal levels of ROS in resting endothelial cells. TNF-α treatment increased ROS production significantly by 30%. This experiment was repeated three times in duplicate. *$p < 0.05$. (**B**) Endothelial cells (HUVECs) were cultured on FN-coated glass cover slips, pretreated overnight with 5 m*M* N-AC, and subsequently stimulated for 30 min with TNF-α or not (control), fixed, permeabilized, and stained. F-actin staining showed a massive increase in stress fiber formation, which was blocked by ROS scavenging. F-actin is represented in (**a–c**), and (**d–f**) shows $p47^{phox}$ staining. Bar = 20 μm. This experiment was performed twice in duplicate. (**C**) Image (**a**) is a still image of a $p47^{phox}$-GFP-expressing endothelial cell at time point 0. Image (**b**) (after 27 min, indicated in upper left corner) and image (**c**) (after 48 min) are still images taken from a 60-min recording of TNF-α-treated $p47^{phox}$-GFP-transduced endothelial cells, available as a quick-time movie (*Figure 4C p47GFP*), and show induction of membrane ruffles (open arrowhead). Bar = 20 μm. (**D**) Endothelial cells were treated with medium (control) or 5 m*M* N-AC, as indicated in Materials and Methods, in suspension and subsequently plated on FN-coated glass cover slips. After 2 h, the cells were processed for confocal microscopy analysis, and VE-cadherin is shown in (**a** and **c**), F-actin is shown in red (**b** and **d**). Scavenging ROS resulted in an impaired monolayer, illustrated by the asterisks. Bar = 100 μm.

volved in transendothelial migration of leukocytes (33). We tested whether TNF-α induced ROS production in endothelial cells and found that 30 min of TNF-α treatment significantly increased ROS production (Fig. 4A). Moreover, F-actin staining showed that TNF-α induced stress fibers, which is in agreement with its effects on endothelial cell morphology and endothelial monolayer permeability (25) (Fig. 4Ba and b). Importantly, scavenging ROS with N-AC prevented TNF-α-induced actin rearrangements, indicating that ROS are required for this effect (Fig. 4Bc). We next tested whether

TNF-α would affect the localization of $p47^{phox}$ by immunostaining. These experiments showed that in confluent monolayers, TNF-α does not induce a prominent change in the intracellular distribution of $p47^{phox}$ (Fig. 4Bdf). However, TNF-α treatment of cells that were pretreated with ROS scavengers resulted in an, as yet unexplained, increased $p47^{phox}$ staining in the nucleus (Fig. 4B-f). Surprisingly, we found that TNF-α treatment of $p47^{phox}$-GFP-expressing endothelial cells that were cultured at low density induced formation of membrane ruffles to which p47-GFP was recruited (Fig. 4C; also available as Quick Time movie: *Figure4C p47GFP*). These findings indicate that short-term TNF-α treatment induces membrane ruffles in single-cell cultures and that these membrane ruffles contain the oxidase components Rac1, $p47^{phox}$, and $p67^{phox}$.

The presence of Rac1, $p47^{phox}$, and $p67^{phox}$ at the cell periphery of nonconfluent, stimulated cells suggests that these proteins might be involved in cell spreading and migration or in the formation of endothelial cell–cell contact, or in both. Indeed, we have previously shown that scavenging ROS blocks endothelial cell migration in a Transwell assay (34). To analyze this further, suspended endothelial cells were incubated with ROS scavengers and formation of cell–cell junctions was analyzed by confocal microscopy. The results showed that the formation of a proper monolayer is dependent on endothelial ROS, because treatment of the cells with the ROS scavengers N-AC or Tiron (data not shown) resulted in an impaired formation of endothelial cell–cell junctions, as concluded from the presence of multiple intercellular gaps (Fig. 4D). These findings indicate that endothelial ROS are involved in the formation of the endothelial monolayer and suggest that ROS are important determinants of endothelial integrity.

DISCUSSION

The role for ROS in the control of endothelial cell function is well established. However, only recently insight in the mechanism of action of these signaling molecules and their source has increased. Endothelial ROS are produced upon stimulation by various agonists, including cytokines, fluid shear stress, ischemia–reperfusion, and leukocyte adhesion (16, 23, 40). ROS-mediated signaling is known in endothelial cells to mediate both short-term [*e.g.,* p38 mitogen-activated protein (MAP) kinase activation] and long-term effects (induction of gene expression) (5). Moreover, we have recently shown that endothelial ROS negatively control endothelial cell–cell adhesion, directly implicating ROS in endothelial integrity (33–35).

The sources of ROS in endothelial cells are not well defined. We have shown that production of endothelial ROS depends on the action of the small GTPase Rac, suggesting that the ROS are produced by a member of the family of NOX2-related proteins (34). Our real-time PCR analysis has shown that primary human endothelial cells express a limited amount of NOX2 mRNA (*i.e.,* gp91) and relatively high levels of NOX4, although the levels of ROS produced in endothelial cells remain 40–200 times less compared with those produced in neutrophils. These data may also explain why chronic granulomatous disease (CGD) patients that carry mutations in their NOX2 gene do not have vascular defects, as the role for NOX2 in the vasculature appears very limited. Our findings are in agreement with earlier reports on the expression of the NOX2 homologue NOX4 mRNA in vascular cells in human atherosclerosis and restenosis (30, 31).

The regulatory components that are involved in control of the NOX2 protein in phagocytes all appear to be expressed in endothelial cells as well, in line with data from other laboratories (13, 21). The $p47^{phox}$ protein is apparently expressed at high levels compared with its expression in phagocytes, which may indicate that it has a role in control of NOX4 activation. However, although $p47^{phox}$ and also $p67^{phox}$ are highly expressed in endothelial cells, CGD patients that carry mutations in their $p47^{phox}$ or $p67^{phox}$ gene do not show any vascular defects either. Recently, two new p47 and p67 homologues have been identified, termed NOXO1 and NOXA1, that control the activity of NOX1 (2, 10). Although the expression of these proteins appears to be restricted to colon epithelium, their expression at low levels in endothelial cells cannot be excluded. In addition, these proteins might replace the mutated $p47^{phox}$ or $p67^{phox}$ proteins, as described for CGD patients, in endothelial cells. However, even if these proteins are expressed in endothelial cells, their possible preference for activating one NOX protein over another remains to be established.

The small GTPase Rac is important for ROS production in granulocytes, as well as in other cell types (16, 37). The regulation of the NADPH oxidase complex in neutrophils is mainly controlled by Rac2 (12), although Rac1 is also able to activate the NADPH oxidase system (27). More recently, Zhao and colleagues showed that monocytes prefer Rac1 over Rac2 to generate ROS (41). Our lab has shown that transduction of active Rac1 into endothelial cells increased ROS production (34). Rac1 was now found in the tips of membrane ruffles, together with $p47^{phox}$ and $p67^{phox}$. The assembly of these proteins in the tips of membrane ruffles suggests involvement in the restoration of cell–cell junctions, underlined by the fact that scavenging ROS results in impaired cell–cell contacts.

The expression studies using the various GFP fusion proteins showed that the NOX2 and NOX4 proteins in endothelial cells reside primarily in the endoplasmic reticulum. Also, in neutrophils, the NOX2 protein complex is localized at intracellular vesicles, and plasma membrane expression is detectable after activation (7, 11). It is unknown whether the NOX proteins in HUVECs can localize to the plasma membrane. However, this is not very likely because currently available data suggest that endothelial cells produce low levels of ROS that are primarily involved in intracellular signaling and do not have a role outside the cell. Our current observations show that in low-density cultures, in the absence of cell–cell contact, $p47^{phox}$ and $p67^{phox}$ are localized to the cellular periphery, which suggests that under certain conditions a ROS-generating system may be present at or near the plasma membrane. Given our earlier data that have shown a role for endothelial ROS in Rac1-mediated modulation of cell–cell adhesion, the localization of the $p47^{phox}$ and $p67^{phox}$ proteins may well reflect a state of the cell in which ROS are required for cellular spreading and control of cell–cell adhesion. This is supported by the observation that TNF-α, which increases ROS production and endothelial permeability, also promotes localization of $p47^{phox}$-GFP to membrane ruffles. Moreover, formation of a tight monolayer was prevented in cells that were pretreated with ROS scavengers, suggesting

that ROS are required for cell spreading and formation of cell–cell contact. A role for ROS in cell spreading may also explain the defect in cell motility in endothelial cells treated with N-AC (34).

In endothelial monolayers, Rac-mediated ROS production leads to the activation of p38 MAP kinase and loss of integrity through the inactivation of VE-cadherin-mediated cell–cell adhesion. This pathway is also activated upon leukocyte adhesion and is important for efficient leukocyte transendothelial migration. Conversely, in isolated or freshly seeded endothelial cells, ROS are required for spreading and formation of cell–cell contact. This latter finding may also relate to inefficient inactivation of Rho, for which ROS are also required, in the N-AC-treated cells (24). The emerging picture is that ROS production is required for the dynamics of endothelial cell–cell adhesion and that it is the state of cell–cell contact that determines which effect the ROS will exert.

In conclusion, the current study defines the proteins and their localization that are likely to be involved in the generation of ROS in endothelial cells. The possibility of NOX4 as one of the major NOX2 homologues in endothelial cells will focus studies toward its activation, its regulation by associating proteins, and its role in the control of endothelial integrity in health and disease.

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ABBREVIATIONS

AB, antibody; CGD, chronic granulomatous disease; DHR, dihydrorhodamine-123; FN, fibronectin; GFP, green fluorescent protein; HMEC, human microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; mAB, monoclonal antibody; MAP, mitogen-activated protein; N-AC, *N*-acetylcysteine; NOX, NADPH oxidase; phox, phagocyte oxidase; pHUVEC, primary human umbilical vein endothelial cell; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween 20; TNF-α, tumor necrosis factor-α; VE, vascular/endothelial.

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