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NOX2 and NOX4 Mediate Proliferative Response in Endothelial Cells

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ABSTRACT

Increased levels of reactive oxygen species (ROS) contribute to many cardiovascular diseases. In neutrophils, ROS are generated by a NADPH oxidase containing p22phox and NOX2. NADPH oxidases are also major sources of vascular ROS. Whereas an active NOX2-containing enzyme has been described in endothelial cells, the contribution of recently identified NOX homologues to endothelial ROS production and proliferation has been controversial. The authors, therefore, compared the role of NOX2 with NOX4 and NOX1 in endothelial EaHy926 and human microvascular endothelial cells. NOX2 and NOX4 were abundantly expressed, whereas NOX1 expression was less prominent. NOX2, NOX4, and NOX1 were simultaneously present in a single cell in a perinuclear compartment. NOX2 and NOX4 co-localized with the endoplasmic reticulum (ER) marker calreticulin. Additionally, NOX2 co-localized with F-actin at the plasma membrane. NOX2 and NOX4, which interacted with p22phox, as was shown by bimolecular fluorescent complementation, contributed equally to endothelial ROS production and proliferation, whereas NOX1 depletion did not alter ROS levels under basal conditions. These data show that endothelial cells simultaneously express NOX2, NOX4, and NOX1. NOX2 and NOX4, but not NOX1, equally contributed to ROS generation and proliferation under basal conditions, indicating that a complex relation between NOX homologues controls endothelial function. *Antioxid. Redox Signal.* 8, 1473–1484.

INTRODUCTION

Reactive oxygen species (ROS) have been originally described as cytotoxic molecules responsible for bacterial killing in host defense (16, 41). More recently, it has been appreciated that nonphagocytic cells, including vascular cells, can also produce ROS at low levels that play a role as signaling molecules in a wide spectrum of physiological and pathophysiological responses (13). In endothelial cells, low levels of ROS appear to be required for proliferation and growth and have been implicated in promoting tube formation and angiogenesis (8, 46). Exposure to higher doses of ROS may also elicit an apoptotic response, modulate vascular tone, impair the endothelial barrier functions, promote thrombosis, and mediate vascular remodeling processes (24, 34, 45). Increased and sustained production of ROS is associated with the pathogenesis of various cardiovascular diseases, including diabetes, hypertension, atherosclerosis, heart failure, and ischemia reperfusion (9, 34, 45). NADPH oxidases have now been accepted as major sources for ROS in endothelial and other vascular cells (20, 21, 33). Structure and function of the NADPH oxidase were primarily described in neutrophils, where it is composed of the two membrane-bound subunits, p22phox and NOX2—previously known as gp91phox—forming cytochrome b558 as the catalytic core of the NADPH oxidase (5, 16, 37). Other important components are the cytosolic subunits p40phox, p47phox, p67phox, and the small GTP-binding protein Rac. Dormant in resting neu-

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trophils, the cytosolic subunits translocate to and associate with cytochrome b558 after stimulation, thereby activating the oxidase and allowing the release of large amounts of superoxide in the well-characterized respiratory burst.

Recently, a NOX2-containing NADPH oxidase has been described to be functionally active in endothelial cells in which all other components of the neutrophil enzyme have also been identified (18, 35). In contrast to neutrophils, endothelial cells produce ROS already under basal conditions, but their maximal ROS levels upon stimulation are significantly lower than in neutrophils (8, 35), suggesting that important differences exist between neutrophil and endothelial NADPH oxidases. In recent years, homologues to NOX2 (NOX1–NOX5) as well as homologues to p47phox (p41nox or NOXO1) and p67phox (p51nox or NOXA1) were discovered to be expressed in different cell types, including vascular cells, suggesting the presence of multiple NADPH oxidase forms in these cells (16, 21, 32). In addition to the NOX2-containing NADPH oxidase, NOX4 has been recently found to be abundantly expressed in certain types of endothelial cells, suggesting that it may play an important role in endothelial ROS generation (3). Interestingly, high levels of NOX2 mRNA have been found in veins, whereas the amount of NOX4 mRNA was elevated in arteries, suggesting that the abundance and the potential functional role of the NOX proteins may also differ in different vascular beds (22). Only very recently, NOX1 has also been detected in certain endothelial cells (2, 44). However, to date, the relative importance of these NOX proteins for endothelial function remains ill defined.

In this study we therefore aimed to compare the contribution of NOX2 and NOX4, as well as of NOX1, to ROS production and proliferation of the endothelial cell line EaHy926 and human microvascular endothelial cells (HMEC-1). We found that proliferating endothelial cells abundantly express NOX2 and NOX4, whereas NOX1 expression is limited under these conditions. Consistently, NOX2 and NOX4, but not NOX1, contributed to basal endothelial ROS production and promoted endothelial proliferation, indicating that in proliferating endothelial cells both NOX2 as well as NOX4 are equally important, whereas NOX1 seems to play only a minor role under these conditions.

MATERIALS AND METHODS

Reagents and cell culture

N-omega-nitro-L-arginine (L-NNA) and SB202190 were from Calbiochem (Darmstadt, Germany), all other chemicals were from Sigma (Taufkirchen, Germany).

The human endothelial hybridoma cell line EaHy926 (kindly provided by Dr. U. Foerstermann, Mainz, Germany), well characterized for its endothelial phenotype and biology (14) was cultured as described (12). Human microvascular endothelial cells (HMEC-1) were purchased from CDC (Atlanta, GA) and grown in MCDB medium (Gibco, Karlsruhe, Germany) containing 10% fetal calf serum (PAN Biotech, Aidenbach, Germany), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 1 μg/ml hydrocortisone, 2 m*M* L-glutamine, and 10 ng/ml EGF.

The human cervix carcinoma cell line HeLa (ATCC CCL-2) and the human embryonic kidney cell line HEK293 (ATCC CRL-1573) were cultured in DMEM (Gibco) with 1 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were grown at 37°C and 5% CO_2 and passaged twice a week.

Plasmids

The expression vectors pcDNA3.1-NOX4sense and pcDNA3.1-p22phoxsense encoding human NOX4 and human p22phox have been described (11, 12). The plasmid pCMV-ERCFP encoded for an endoplasmatic localization sequence fused to the cyan fluorescent protein (CFP) and the pEF-ERYFP plasmid was a gift from Dr. M. Grez (Frankfurt a. M., Germany). For the p22phoxYN and the YFP-p22phox constructs, polymerase chain reaction (PCR) with primers adding appropriate restriction sites was performed on the pcDNA3.1-p22phoxsense plasmid to amplify the human p22phox coding sequence and cloned either into the pCMV-FLAG-bJunYN vector (kindly provided by Dr. T. Kerppola, Ann Arbor, MI) or pEYFP-C1 (BD Biosciences Clontech, Heidelberg, Germany), resulting in pCMV-FLAG-p22phoxYN, encoding for a FLAG-p22phoxYN chimera, and pEYFP-C1-p22phox plasmid encoding for the YFP-p22phox chimera, respectively. The pcDNA3.1-YN was created by cutting the YN-fragment from pCMV-FLAG-bJunYN with BamHI and subcloning it into pcDNA3.1. For the NOX4YC and the YFP-NOX4 fusion constructs, the cDNA encoding for human NOX4 was amplified by PCR from pcDNA3.1-NOX4sense using specific primers for adding appropriate restriction sites and cloned into the pCMV-HA-bFosYC vector (kindly provided by Dr. T. Kerppola) or pEYFP-C1 resulting in pCMV-NOX4YC encoding for NOX4YC and in pEYFP-C1-NOX4 encoding for YFP-NOX4.

The full-length human NOX2 cDNA was derived from pB-Shgp91phox and subcloned into pcDNA6.1CFP resulting in pcDNA6.1hgp91phoxCFP encoding for CFP-NOX2. For the NOX2YC fusion construct, the YC-fragment was cut from pCMV-HA-bFosYC and subcloned into pcDNA3.1, resulting in pcDNA3.1-YC. For NOX2YC and for NOX2 expression vectors, NOX2 was amplified from pcDNA6-ECFP-NOX2 by PCR using primers adding appropriate restriction sites and subcloned either in pcDNA3.1 or into pcDNA3.1-YC, resulting in pcDNA3.1-NOX2 and pcDNA3.1-NOX2YC, respectively.

Specific short hairpin RNA encoding for 19mer siRNA against NOX1 (siNOX1A and siNOX1B), NOX2 and NOX4 were designed and created using siRNA Target Designer and the siSTRIKE U6 Hairpin Cloning System (Promega, Mannheim, Germany). A random 19mer sequence was used to clone the psiSTRIKE-NONE encoding for control siRNA (siNONE). All constructs were confirmed by DNA sequencing.

Transfection

EaHy926 cells were transfected using the Effectene transfection reagent (Qiagen, Hilden, Germany) as described (12). HMEC-1 cells were transfected with SuperFect (Qiagen), according to the manufacturer's protocol. Briefly, plasmid DNA

was diluted with serum-free MCDB, mixed with SuperFect reagent (ratio 1:5) and the transfection complex was incubated for 15 min at room temperature. The growth medium of the cells was changed and the transfection complex was then added to the cells. After 2–3 h of incubation with the transfection complex, the medium was changed, and the cells were further cultured in serum-containing medium. HeLa and HEK293 cells were grown to 40–60% confluency prior to transfection. Both cell lines were transfected using the calcium phosphate method as described (17, 38).

Reverse transcriptase polymerase chain reaction

RNA was extracted as described (19, 23). First strand cDNA was synthesized with 5 μg total RNA using SuperScript III RT (Invitrogen, Karlsruhe, Germany), as per manufacturer's instructions. Polymerase chain reaction (PCR) was performed using the following primers to detect a) p22phox: 5-CAGATCGAGTGGGCCATGT-3 as forward primer and 5-TCGTCGGTCACCGGGATG-3 as reverse primer (571 bp); b) NOX1: 5-GTCTTCTGGTATACTCACCACC-3 as forward primer and 5-GAATGACCGGTGGAAGGATCCAC-3 as reverse primer (228 bp); c) NOX2: 5-CATGTTTCTGTATCTCTGTGA-3 as forward primer and 5-GTGAGGTAGATGTTGTAGCT-3 as reverse primer (614 bp); d) NOX4: 5-CCATGGCTGTGTCCTGGAGGAGCTG-3 as forward primer and 5-AGTTGAGGGCATTCACCAGATGGGC-3 as reverse primer (389 bp); and e) GAPDH: 5-TATGACAACAGCCTCAAGAT-3 as forward primer and 5-AGGTCCACCACTGACACGTT-3 as reverse primer (316 bp). The PCR was run for 36 cycles in the following cycle profile: 95°C for 45 sec, 57°C for 45 sec, and 72°C for 1 min. For NOX1 the PCR was run for 38 cycles in the following cycle profile: 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min. PCR products were separated on a 1.2% agarose gel, stained with ethidium bromide, and visualized using GelDoc software (BioRad, Munich, Germany).

Western blot analysis

Western blot analysis was performed as described (19). After blocking for 1 h in TBS containing 5% nondry milk and 0.3% Tween 20, membranes were incubated overnight at 4°C with specific antibodies raised against human p22phox (mAb 44.9), NOX2 (mAb 48) (both kindly provided by Dr. D. Roos, Amsterdam), NOX1 and NOX4 (both Santa Cruz Biotechnology, Heidelberg, Germany). After incubation with a horseradish peroxidase-conjugated secondary antibody (Calbiochem) for 1 h, proteins were visualized by performing luminol-enhanced chemiluminescence. Loading of equal amounts of proteins was confirmed by reprobing the membranes with a β-actin antibody (Santa Cruz).

Immunofluorescence and fluorescence microscopy

For immunofluorescence, EaHy926 cells were seeded on poly-L-lysine coated glass coverslips. After medium removal, cells were washed three times with PBS on ice and fixed with 50% methanol/acetone at −20°C for 5 min. After drying for 5 min and blocking in 5% BSA in PBS for 1 h, fixed cells were incubated overnight at 4°C with specific antibodies against NOX2 (Biomol GmbH, Hamburg, Germany), NOX1, NOX4 (Santa Cruz), or calreticulin (Biomol), and subsequently with Alexa Fluor488-donkey-anti-rabbit, Alexa Fluor488-donkey-anti-mouse, Alexa Fluor594-donkey-anti-goat or Alexa Fluor488-rabbit-anti-goat secondary antibodies (Mobitec, Göttingen, Germany) for 1 h. For actin staining, the cells were incubated with a rhodamine-coupled phalloidin (Mobitec) for 1 h. Cells were visualized using a fluorescence microscope (Olympus, Hamburg, Germany) and images were obtained using the Openlab Modular Software for Scientific Imaging (Improvision, Tübingen, Germany).

Immunoprecipitation

For immunoprecipitation studies, HEK293 cells were transfected with 5 μg of each plasmid and harvested in lysis buffer (50 m*M* Tris, 300 m*M* NaCl, 1% Triton X-100, 2 m*M* EDTA, 2 m*M* EGTA, 10 m*M* $Na_4P_2O_7$, 1 m*M* PMSF, 2 μg/ml each chymostatin, antipain, leupeptin, pepstatin, and aprotinin). Protein concentration was determined by a BCA Protein Assay Kit (Pierce, Rockford, IL).

After centrifugation of the cell lysate, 4 μg anti-GFP antibody (Invitrogen) and 30 μl sepharose beads (Amersham Biosciences Europe GmbH, Freiburg, Germany) were added to 400 μg protein and incubated overnight at 4°C while slowly rotating. Then after centrifugation and washing with lysis buffer, cells were mixed with 2X Laemmli buffer and applied for Western blot analysis using an antibody against human p22phox (mAB 44.9).

Subcellular cell fractionation

Microsomal membrane fraction was isolated as described with some modifications (6). Briefly, cells were harvested in lysis buffer without detergent (50 m*M* Tris, 300 m*M* NaCl, 2 m*M* EDTA, 2 m*M* EGTA, 10 m*M* $Na_4P_2O_7$, 1 m*M* PMSF, 2 μg/ml chymostatin, antipain, leupeptin, pepstatin, and aprotinin) and homogenized by sonification (5 × 10 sec burst). The cell homogenate was centrifuged at 1000 *g* for 10 min at 4°C and the supernatant was further centrifuged at 12,000 *g* for 15 min at 4°C. Subsequently, the supernatant was centrifuged at 40,000 *g* for 30 min at 4°C to receive the microsomal membrane fraction. The pellet was resuspended in 1.5X Laemmli buffer and applied for Western blot analysis using antibodies against NOX2 (mAb 48) and NOX4 (Santa Cruz), the endoplasmic reticulum protein calnexin (BD Biosciences, San Jose, CA) and against p38 MAP kinase (New England BioLabs GmbH, Frankfurt, Germany) and the transcription factor ARNT (Abcam, Cambridge, UK).

Bimolecular fluorescence complementation

For bimolecular fluorescence complementation (BiFC) (26, 27), HeLa cells were seeded on glass-bottom dishes (Willco Wells, Berlin, Germany) and cotransfected with 1.5 μg of each plasmid. The fluorescence complementation was monitored in a fluorescence microscope (Olympus) and images were obtained using the Openlab Modular Software**.**

Measurement of ROS production

ROS generation was measured using the fluoroprobe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein di-

acetate acetyl ester (CM-H_2DCFDA, Invitrogen) in a microplate reader (Tecan, Crailsheim, Germany), as previously described (12).

Proliferation measurements

Cellular proliferation was analyzed by determining DNA synthesis using 5-bromo-2′ deoxyuridine (BrdU) labeling (Roche Diagnostics GmbH, Mannheim, Germany), as described previously (12).

Statistical analysis

Values presented are means ± standard deviation (SD). Results were compared by ANOVA for repeated measurements followed by Student–Newman–Keuls *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

NOX2 and NOX4 are localized in the endoplasmic reticulum in EaHy926 cells

To determine the expression levels of NOX2 and NOX4 in EaHy926 cells, RT–PCR and Western blot analyses were performed. Both NOX2 and NOX4 were abundantly present at the mRNA and protein level (Fig. 1). In addition, EaHy926 cells also expressed p22phox mRNA and protein (Fig. 1). In contrast, in polymorphonuclear neutrophils, only NOX2 and p22phox proteins were detectable (Fig. 1B).

Co-immunofluorescence studies revealed that both NOX2 and NOX4 were present in the same cell (Fig. 2). Moreover, both proteins were localized intracellularly in a perinuclear compartment resembling the endoplasmic reticulum (ER) (Fig. 2). To further confirm that these proteins are present in the ER, EaHy926 cells were transfected with constructs where either yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) were fused to human NOX2 or NOX4. Similar to the native proteins, the fusion proteins showed a perinuclear ER-like expression pattern (Fig. 3). To verify this assumption, cells were co-transfected with constructs where an ER localization sequence was linked to either CFP or YFP. All fusion proteins co-localized with the ER-marker (Fig. 3). In addition, a fusion protein encoding for the small membrane-bound subunit p22phox linked to CFP also co-localized with the ER marker (Fig. 3). These findings were confirmed by co-immunofluorescence of NOX2 and NOX4 with calreticulin, a protein specifically expressed in the endoplasmic reticulum (ER) (Fig. 4A). Furthermore, Western blot analysis demonstrated the presence of p22phox, NOX2, and NOX4, as well as the ER protein calnexin in microsomal fractions of EaHy926 cells (Fig. 4B). In contrast, the transcription factor ARNT and the cytoplasmically located p38 MAP kinase were not present in this fraction as expected (Fig. 4B).

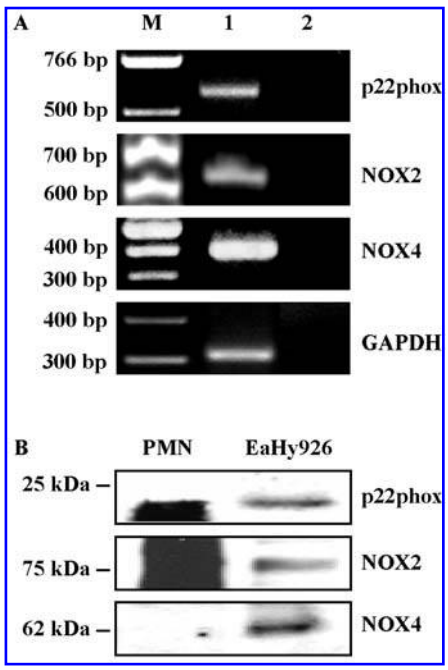

FIG. 1. NOX2, NOX4, and p22phox mRNA and protein are expressed in EaHy926 cells. (**A**) RT–PCR of mRNA derived from EaHy926 cells, using specific primers for p22phox, NOX2, and NOX4. *Lane 1*: mRNA, *lane 2*: H_2O, *M*: marker. (**B**) Western blot analysis of EaHy926 cells and polymorphonuclear neutrophils (PMN), using antibodies against human p22phox, NOX2, and NOX4.

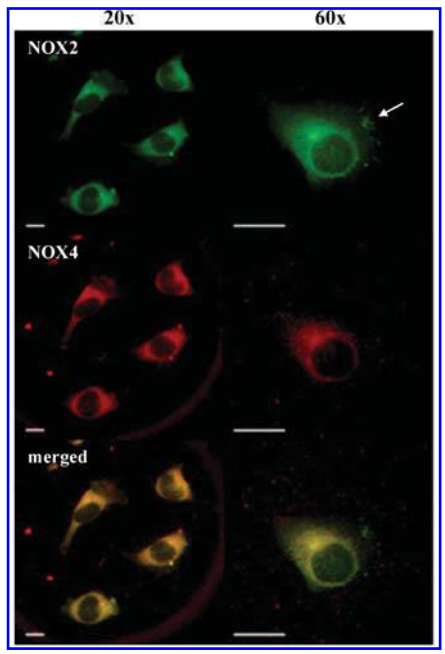

FIG. 2. NOX2 and NOX4 co-localize in an intracellular compartment in EaHy926 cells. Immunofluorescence was performed on EaHy926 cells against NOX2 (*green*) and NOX4 (*red*). Fluorescence images were taken at a 20x magnification (*left column*) and 60x magnification of a representative cell (*right column*). *Scale bars* are 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

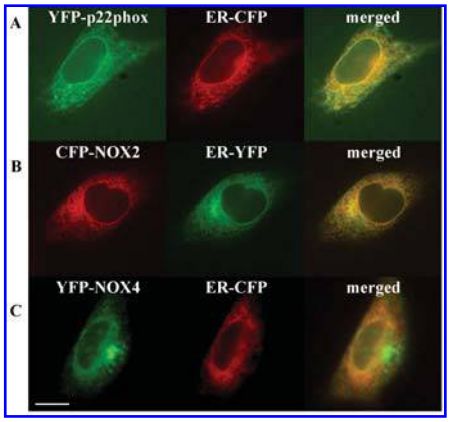

FIG. 3. p22phox, NOX2, and NOX4 are expressed in the endoplasmic reticulum in EaHy926 cells. EaHy926 cells were cotransfected with YFP-p22phox (**A**), CFP-NOX2 (**B**), or YFP-NOX4 (**C**), and constructs where the ER localization signal was linked to either CFP or YFP. *Scale bars* are 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

In addition to the localization in the ER, immunofluorescence showed a faint peripheral staining for NOX2, but not for NOX4 (Fig. 2A, arrow). Co-immunofluorescence using an Alexa Fluor488-coupled phalloidin against F-actin revealed that NOX2 partially co-localized with actin at the plasma membrane, whereas NOX4 did not show corresponding staining (Fig. 5).

NOX2 and NOX4 both interact with p22phox

Since in neutrophils dimerization of NOX2 with p22phox is required for functional activity of the NADPH oxidase, the interaction between NOX2 or NOX4 with p22phox was tested in living cells using BiFC analysis (Fig. 6A). The BiFC approach is based on the complementation of two nonfluorescent fragments of a fluorescent protein, which are brought together by an interaction between proteins fused to the fragments (26, 27).

Complementary fragments of YFP (YN and YC) were fused to the carboxyl-terminal ends of NOX2, NOX4, and p22phox. Respectively, NOX2 and NOX4 were fused to the YC-fragment (NOX2YC and NOX4YC) and p22phox was fused to the YN-fragment (p22phoxYN). The plasmid encoding for p22phoxYN was co-transfected with either the plasmid encoding for NOX2YC or NOX4YC into HeLa cells and the fluorescence was monitored by fluorescence microscopy (Fig. 6B). Both NOX2 and NOX4 formed complexes with p22phox in a perinuclear compartment resembling the ER. No fluorescence was detectable in cells transfected with plasmids encoding for NOX2YC or NOX4YC or p22phoxYN and YN or YC lacking a fusion, respectively.

To confirm that p22phox does indeed interact with both NOX2 and NOX4, co-immunoprecipitation studies of p22phox and NOX2 or NOX4 were performed (Fig. 6C). To this end, HEK293 cells were co-transfected with a plasmid encoding for human p22phox together with plasmids encoding for NOX2 or NOX4 fused to a fluorescent protein. Immunoprecipitation was performed with an antibody against GFP. Subsequent Western blot analysis with an antibody against p22phox revealed that p22phox was detectable in NOX2- and NOX4-expressing cells, but not in control cells expressing only GFP (Fig. 6C), confirming that both NOX2 and NOX4 interact with p22phox.

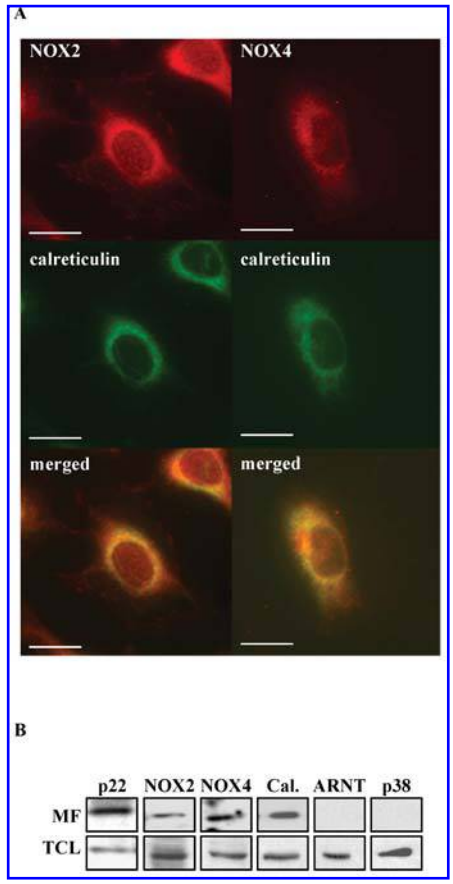

FIG. 4. NOX2 and NOX4 co-localize with calreticulin in EaHy926 cells. (**A**) Immunofluorescence was performed on EaHy926 cells against NOX2 (*left column, red*) or NOX4 (*right column, red*), and calreticulin (*green*). *Scale bars* are 20 μm. (**B**) EaHy926 cells were fractionated and Western blot analysis was performed using the microsomal fraction (MF) or total cell lysate (TCL) and antibodies against p22phox (p22), NOX2, NOX4, calnexin (Cal.), ARNT, and p38 MAP kinase (p38). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

NOX2 and NOX4 both contribute to endothelial ROS generation and proliferation

To determine the functional role of NOX2 and NOX4 in EaHy926 cells, siRNA against NOX2 (siNOX2) and NOX4 (siNOX4) was designed and cloned into a vector system encoding siRNA in the form of short hairpin RNA. A random sequence was used as control (siNONE). Transfection of siRNA against NOX2 or NOX4 diminished NOX2 and NOX4 protein levels (Fig. 7A), respectively.

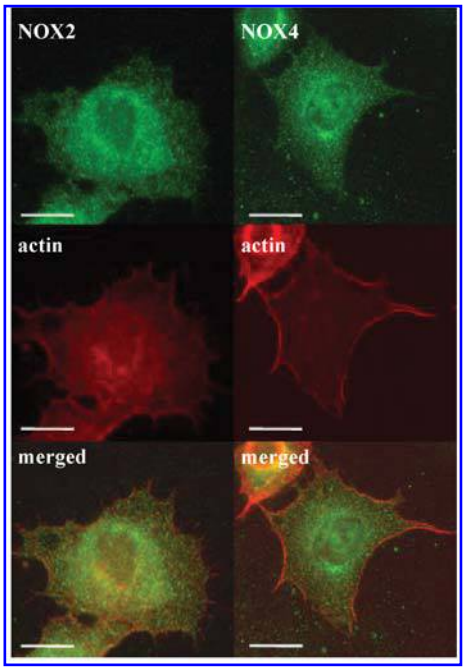

FIG. 5. NOX2, but not NOX4, co-localizes with F-actin in EaHy926 cells. Immunofluorescence of EaHy926 cells, using antibodies against NOX2 (*left column*, *green*) or NOX4 (*right column, green*) together with staining for F-actin (*red*). *Scale bars* are 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

Next, we tested the contribution of NOX2 and NOX4 to endothelial ROS production. Expression of siNOX2 as well as of siNOX4 decreased the levels of ROS in EaHy926 cells to a similar extent suggesting that both homologues are functionally active in these cells (Fig. 7B, upper graph). Moreover, overexpression of NOX2 as well as of NOX4 increased ROS levels to a similar extent (Fig. 7B, lower graph).

Since ROS and NADPH oxidases have been implicated in the regulation of endothelial proliferation, we determined the contribution of NOX2 or NOX4 to the proliferative response of EaHy926 cells by BrdU incorporation assay. Depletion of NOX2 or NOX4 reduced proliferation of EaHy926 cells to a comparable extent (Fig. 7C, upper graph). In contrast, the proliferative activity of EaHy926 cells was significantly increased when NOX2 or NOX4 were overexpressed (Fig. 7C, lower graph), further confirming that both homologues are active in EaHy926 cells and contribute equally to the proliferative response. Similarly, NOX2 and NOX4 were also present in human microvascular endothelial cells (HMEC-1) (Fig. 8). Depletion of NOX2 and NOX4 by siRNA diminished ROS production and proliferation of these cells, whereas overexpression of these proteins stimulated these responses (Fig. 8).

p38 MAP kinase contributes to the proliferative response mediated by NOX2 and NOX4

Since we previously showed that p38 MAP kinase is involved in endothelial proliferation dependent on p22phox (12), we determined the involvement of this kinase in the proliferative response mediated by NOX2 and NOX4. NOX2 and

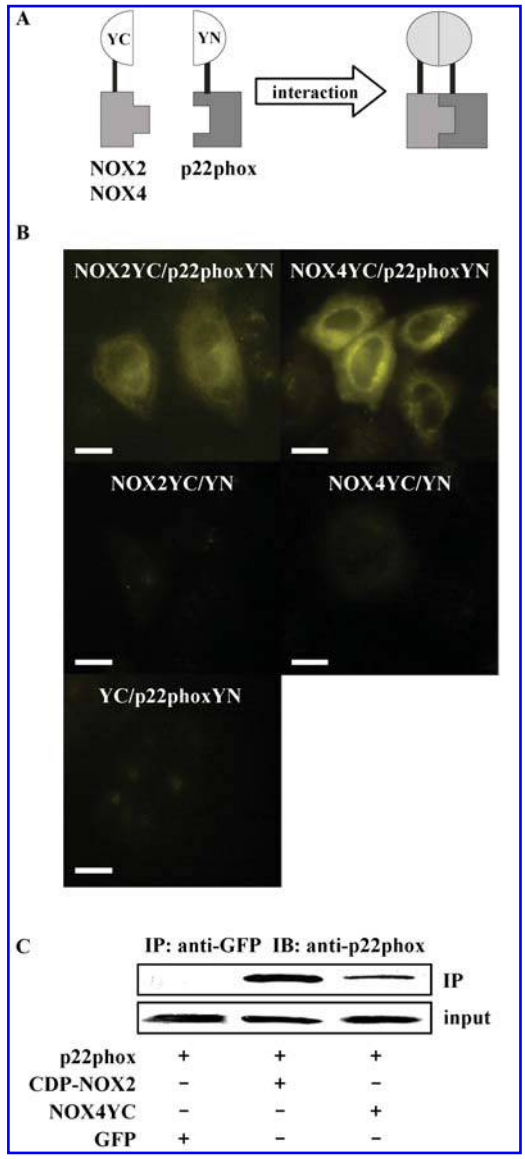

FIG. 6. NOX2 and NOX4 interact with p22phox. (**A**) Illustration of the principle of the Bimolecular Fluorescence Complementation (BiFC). The YC- or YN-fragment of YFP is fused to either NOX2, NOX4, or p22phox. After interaction and maturation of the fluorophore, its fluorescence light can be monitored using fluorescence microscopy. (**B**) BiFC analysis was performed in HeLa cells co-transfected with plasmids encoding for the indicated fusion constructs. Interaction of the proteins is shown in the fluorescence images for NOX2 and p22phox and for NOX4 and p22phox, whereas no fluorescence is observed in the negative controls. *Scale bars* are 20 μm. (**C**) HEK293 cells were co-transfected with plasmids encoding for the indicated proteins. After precipitation of CFP-NOX2, NOX4YC, or GFP with a polyclonal GFP-antibody (Molecular Probes), the immunoprecipitates were subjected to Western blot analysis, using an antibody against p22phox. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

NOX4 were expressed in EaHy926 cells. Western blot analysis showed that p38 MAP kinase was phosphorylated in the presence of either protein (Fig. 9). Treatment with the p38 MAP kinase inhibitor SB202190 decreased NOX2- and

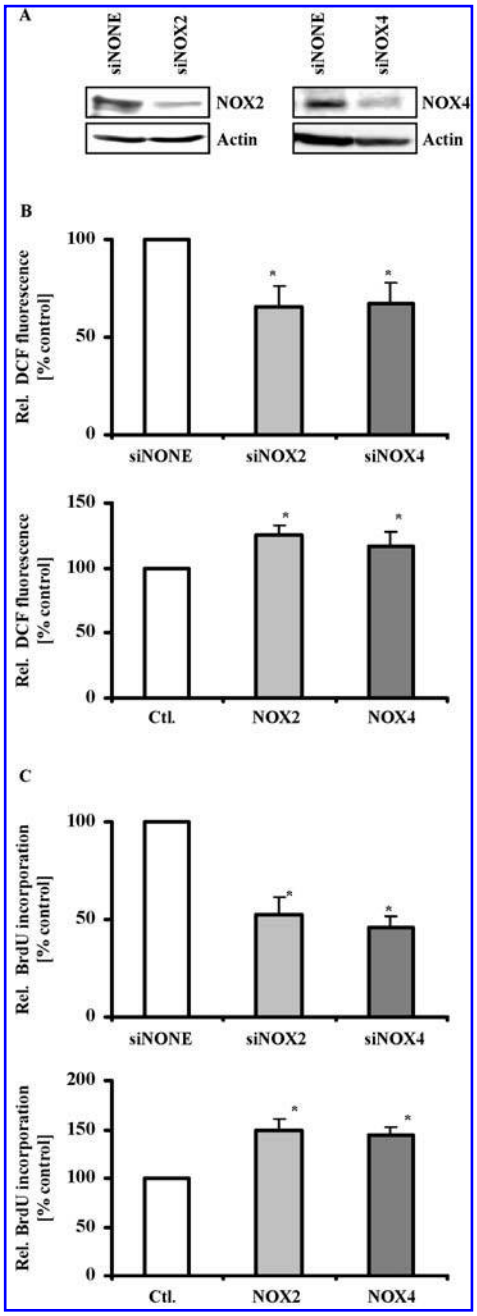

FIG. 7. NOX2 and NOX4 modulate ROS generation and proliferation of EaHy926 cells. (**A**) EaHy926 cells were transfected with constructs encoding either for a specific siRNA against NOX2 (siNOX2), NOX4 (siNOX4), or a random sequence (siNONE). Twenty four h after transfection, protein levels of NOX2 and NOX4 were analyzed in comparison with actin. (**B**) EaHy926 cells were transfected with either siNOX2, siNOX4, or siNONE, or vectors encoding for NOX2, NOX4, or with control vector (Ctl.). ROS levels were evaluated 24 h after transfection by performing DCF measurements. Data are presented as relative change compared with control (100%) [$n = 4–6$, *p < 0.05 vs. cells transfected with control vectors (Ctl. or siNONE)]. (**C**) Proliferation of EaHy926 cells was assessed 48 h after transfection by using 5-bromo-2′-deoxyuridine (BrdU) incorporation. Data are presented as relative change compared with control (100%) [$n = 4–6$, *$p < 0.05$ vs. cells transfected with control vectors (Ctl. or siNONE)].

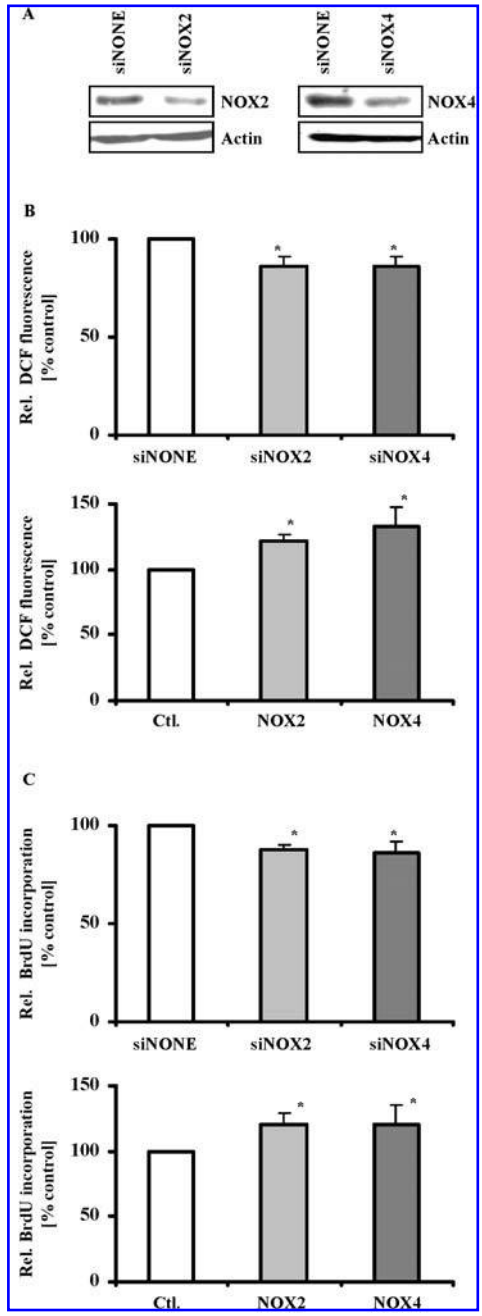

FIG. 8. NOX2 and NOX4 modulate ROS generation and proliferation of human microvascular endothelial cells (HMEC-1). (**A**) HMEC-1 cells were transfected with either siNOX2, siNOX4, or siNONE. Forty eight h after transfection protein levels of NOX2 and NOX4 were analyzed in comparison with actin. (**B**) HMEC-1 cells were transfected with either siNOX2, siNOX4, or siNONE or vectors encoding for NOX2, NOX4, or with control vector (Ctl.). ROS levels were evaluated 48 h after transfection by performing DCF fluorescence measurements. Data are presented as relative change compared with control (100%) [$n = 4–6$, *$p < 0.05$ vs. cells transfected with control vectors (Ctl. or siNONE)]. (**C**) Proliferation of HMEC-1 cells was determined 48 h after transfection by using 5-bromo-2′-deoxyuridine (BrdU) incorporation. Data are presented as relative change compared with control (100%) [$n = 4–6$, *$p < 0.05$ vs. cells transfected with control vectors (Ctl. or siNONE)].

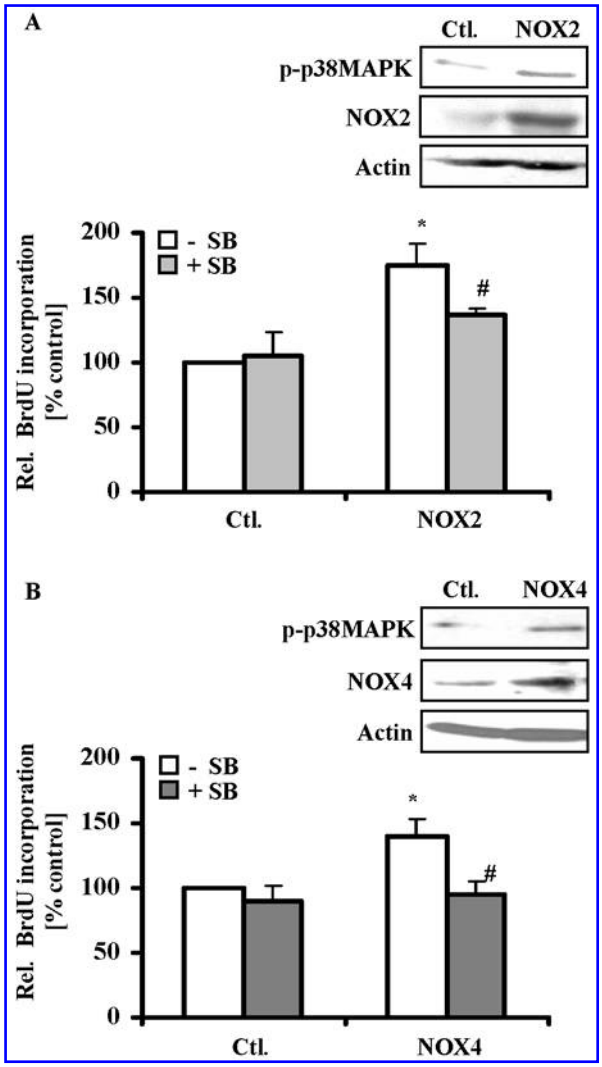

FIG. 9. The p38 MAP kinase contributes to the proliferative response mediated by NOX2 and NOX4. EaHy926 cells were transfected either with vectors encoding for NOX2 (**A**) or NOX4 (**B**) or with control vector (Ctl.). Expression of NOX2 and NOX4 and the presence of phosphorylated p38 MAP kinase were determined by Western blot analysis. Cells were treated either with solvent (DMSO, *white bars*) or with SB202190 (20 μ*M*, *gray bars*) and proliferative activity was assessed after 12 h by using 5-bromo-2´-deoxyuridine (BrdU) incorporation. Data are presented as relative change compared with control (untreated cells) (100%) ($n = 3$,*$p < 0.05$ vs. cells transfected with control vector; #$p < 0.05$ vs. DMSO-treated cells)

NOX4-mediated proliferation (Fig. 9), indicating that p38 MAP kinase contributes to the proliferative response initiated by NOX2 or NOX4.

ROS generation by NOX1 is limited in EaHy926 cells

In addition to NOX2 and NOX4, NOX1 has recently been detected in certain endothelial cell types (2, 31, 44). In EaHy926 cells, NOX1 mRNA expression determined by RT–PCR was only barely detectable, requiring an increased cycle number. In contrast, the colon carcinoma cell line CaCo2 abundantly expressed NOX1 mRNA (Fig. 10A). In addition, NOX1 protein was found in EaHy926 cells, but not in polymorphonuclear neutrophils (Fig. 10B). Immunofluorescence studies revealed that NOX1 was also present in an intracellular ER-like compartment (Fig. 10C), and Western blot analysis demonstrated the presence of NOX1 protein in the microscomal fraction (Fig. 10D).

To analyze the involvement of NOX1 in ROS generation of EaHy926 cells, two specific siRNA against NOX1 (siNOX1A, siNOX1B) were designed that were able to downregulate NOX1 protein (Fig. 10E). However, in contrast to NOX2 or NOX4, depletion of NOX1 did not significantly alter ROS levels in EaHy926 cells (Fig. 10F). Similarly, depletion of NOX1 did not significantly diminish ROS levels in HMEC-1 cells (data not shown), further emphasizing the importance of NOX2 and NOX4 for cellular function of endothelial cells.

DISCUSSION

In this study we demonstrated that both NOX2 and NOX4 are functionally active in endothelial cells since (a) mRNA and protein of NOX2 and NOX4, as well as of p22phox were detectable in EaHy926 and HMEC-1 cells; (b) NOX2 and NOX4 could be localized simultaneously in one cell; (c) both NOX4 and NOX2 interacted with p22phox; (d) overexpression of NOX2 and NOX4 was able to increase ROS production and proliferation; and (e) depletion of both NOX2 and NOX4 resulted in decreased ROS generation and proliferation of EaHy926 cells or HMEC-1 cells. In addition, although NOX1 expression was also detectable in endothelial cells, depletion of this protein did not significantly affect ROS production, further confirming that NOX2 and NOX4 both play a major role in endothelial ROS production and proliferation.

In addition to the NADPH oxidase subunit NOX2 (gp91phox), which has been initially described to be expressed and functionally active in endothelial cells (18, 35), NOX4 has been recently shown to be abundantly expressed in endothelial cells, suggesting that a NOX4-containing NADPH oxidase may play a functional role in these cells (3, 48). In this study, we showed that endothelial EaHy926 cells as well as HMEC-1 cells express both NOX2 and NOX4 at the mRNA and protein level. Interestingly, using co-immunofluorescence, we could show for the first time that both NOX2 and NOX4 are expressed simultaneously within one endothelial cell and that expression of each subunit is not limited to a subpopulation of cells. Although the functional relevance of this observation is not clear to date, previous studies suggested that NOX homologues may be present in different cellular compartments and may thus have different functions (25). However, NOX2 and NOX4 were both present in an intracellular, perinuclear compartment and co-localized with calreticulin, a marker protein for the ER. In addition, GFP-tagged NOX2 and NOX4 as well as p22phox were expressed and co-localized with co-expressed fluorescent ER marker proteins, suggesting that both NOX2 and NOX4 are localized in the ER. Similarly, GFP-tagged NOX2 and NOX4 showed an ER-like expression pattern in HUVEC (48). Furthermore,

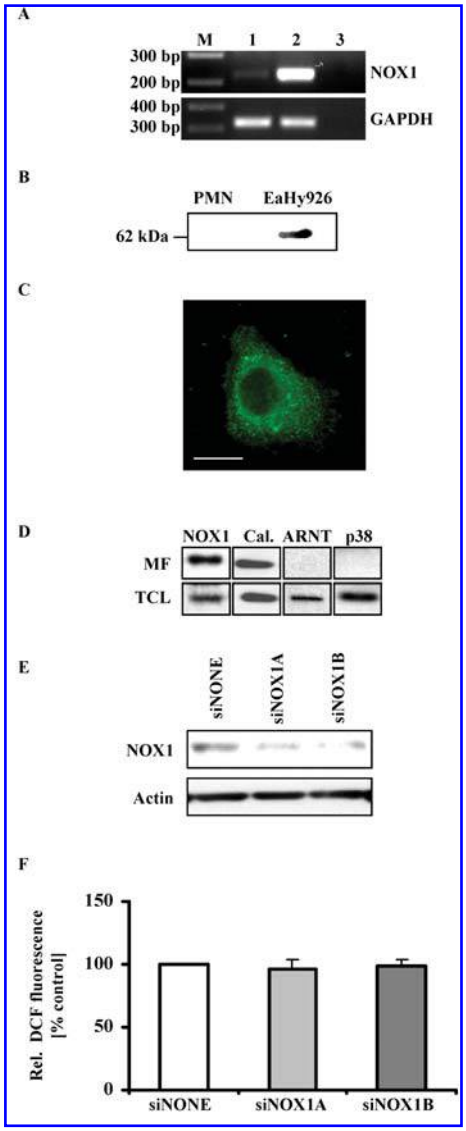

FIG. 10. ROS generation by NOX1 is limited in EaHy926 cells. (**A**) RT–PCR for NOX1 with mRNA derived either from EaHy926 cells or CaCo2 cells. The PCR products were subjected to 1.2% agarose-gel electrophoresis and stained with ethidium bromide. *Lane 1*: EaHy926; *lane 2*: CaCo2; *lane 3*: H_2O, *M*: marker. (**B**) Western blot analyses of EaHy926 cells or polymorphonuclear neutrophils (PMN) using an antibody against NOX1. (**C**) Immunocytochemistry was performed on EaHy926 cells using an antibody against NOX1. (**D**) EaHy926 cells were fractionated by differential centrifugation to receive microsomal fractions (MF). Western blot analysis of the microsomal fraction (MF) and the total cell lysate (TCL) using antibodies against NOX1, calnexin (Cal.), ARNT, and p38 MAP kinase (p38). (**E**) EaHy926 cells were transfected with plasmids encoding for siRNAs against NOX1 (siNOX1A and siNOX1B) or a random sequence (siNONE) and Western blot analysis was performed using an antibody against NOX1. (**F**) EaHy926 cells were transfected with siNOX1A, siNOX1B, or siNONE, and ROS levels were evaluated 24 h after transfection by performing DCF fluorescence measurements. Data are presented as relative change compared with control (100%) ($n = 4$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

cell fractionation experiments confirmed that NOX2 and NOX4 are present in the microsomal fraction. However, preliminary studies did not provide evidence that both proteins would interact under the experimental conditions applied. Although NOX2 was considered to be mainly localized in the plasma membrane in leukocytes, earlier studies showed that NOX2 is also present in intracellular vesicular structures in "dormant" nonactivated leukocytes (10). Consistently, immunofluorescence demonstrated that NOX2 and p22phox were expressed in an intracellular, perinuclear compartment in an ER-like manner in porcine iliac artery and bovine aortic endothelial cells (35). Similarly, NOX4 has been found in the ER in human aortic smooth muscle cells (39) and fluorescent protein-tagged NOX4 interacted with the ER marker protein disulfide isomerase in rabbit vascular smooth muscle cells (29). In contrast, NOX4 protein was found to co-localize with focal adhesions in rat vascular smooth muscle cells (25). Although the reasons for these divergent findings are not resolved to date, the functional importance of our observations was further underlined by our findings that p22phox was also present in the ER and, more importantly, that p22phox interacted with NOX2 and NOX4 in this intracellular compartment, as was determined by BiFC. This technique allows the direct visualization of the interaction of two proteins in a natural membrane context within living cells (26, 27). Importantly, although BiFC was performed in HEK293 cells, the fluorescent complexes in these cells showed a similar distribution than the fusion proteins or the "native" proteins in EaHy926 cells, indicating that these studies are representative for the situation in intact EaHy926 cells. Similarly, using FRET, a technique that allows to visualize proteins located in a very close vicinity, association of transfected rat p22phox with transfected NOX2 and NOX4 was demonstrated in HEK293 cells (4).

In addition, NOX2, but not NOX4 protein, was also present in the plasma membrane and co-localized with F-actin, whereby actin was found at the inner layer and NOX2 at the outer layer of the membrane. Intracellular co-localization of NOX2 with parts of the cytoskeleton has been previously described in endothelial cells (35, 48). In addition, a recent study showed that NOX2 can be found at the leading edge of migrating HUVEC where it co-localized with actin (28). An intact cytoskeleton and interaction with the actin-binding protein IQGAP1 were required for ROS production and migration of these cells, suggesting that in migrating and possibly also in proliferating endothelial cells, the reorganization of the actin cytoskeleton may enable NOX2 to translocate to the leading edge, assemble with the actin-binding structures, and generate ROS to promote migration and proliferation. Indeed, depletion of NOX2 by siRNA reduced ROS production of EaHy926 and HMEC-1 cells in our study. In addition, depletion of NOX4 also decreased ROS levels to a similar extent, indicating that both proteins contribute to ROS generation in these cells. Similarly, antisense DNA of NOX4 or NOX2 inhibited angiotensin-II-induced ROS generation in HUVEC (50). In addition, NOX4 antisense oligonucleotides decreased ROS levels of proliferating rat aortic endothelial cells by 50%, although the contribution of NOX2 to ROS production has not been evaluated in that study (3). In contrast, ANP-stimulated ROS production was decreased by NOX2, but not by NOX4

antisense treatment in HUVEC (15). Similarly, NOX2 antisense oligonucleotides reduced VEGF-stimulated ROS production in HUVEC (47). Although the reasons for these controversial findings are not resolved to date and may relate to the cell type used and the specific experimental conditions applied, depletion of NOX2 or NOX4 also decreased ROS production in HMEC-1 cells, further emphasizing the importance of both homologues for endothelial ROS production.

Moreover, NOX2 as well as NOX4 overexpression enhanced ROS levels in EaHy926 cells and HMEC-1 cells, further indicating that both homologues contribute to endothelial ROS production, and that the amount of NOX2 or NOX4 present in the cell is limiting the levels of ROS production. Consistently, studies in other cellular systems demonstrated that NOX4 overexpression increases ROS production with no need of concomitant expression of other subunits, as long as p22phox levels were sufficient (30, 36). In contrast, NOX2 function has been suggested to require p22phox as well as cytosolic subunits and Rac (5, 37). Thus, our findings demonstrating that NOX2 overexpression increases ROS levels suggest that sufficient cytosolic proteins are available in EaHy926 cells or HMEC-1 cells to allow functional activation of a NOX2-depending NADPH oxidase.

In addition to NOX2 and NOX4, NOX1 has also recently been suggested to play a functional role in rat basiliary and sinusoidal, as well as in mouse aortic endothelial cells (2, 31, 44), although NOX1 mRNA was not detected in HUVEC (15, 48). However, RT–PCR and Western blot analyses confirmed the presence of NOX1 in our endothelial cells, although detection by RT–PCR required an increased cycle number, indicating that only small amounts of NOX1 mRNA are present in endothelial cells. Immunofluorescence further showed that NOX1 was located in a perinuclear compartment resembling the ER, as was shown for NOX2 and NOX4, and NOX1 protein was also present in the microsomal fraction. Interestingly, NOX1 also co-localized with NOX2 within one single cell (data not shown), indicating that NOX1, NOX2, and NOX4 are co-expressed in endothelial cells. However, depletion of NOX1 by different siRNAs did not significantly reduce ROS levels in endothelial cells, indicating that NOX2 and NOX4 play a major role in endothelial ROS production.

We previously showed that low levels of H_2O_2 increase the proliferative activity of EaHy926 cells, and that modulation of p22phox levels affect the proliferative response of these cells (12). In this study, we now demonstrate that overexpression of NOX2 or NOX4, which lead to increased ROS production, also enhanced proliferative activity of EaHy926 and HMEC-1 cells, whereas depletion of NOX2 or NOX4 decreased endothelial proliferation to a similar extent. These findings indicate that NOX2-, as well as NOX4-mediated ROS production, contributes to endothelial proliferation. Consistently, depletion of NOX2 decreased VEGF-induced migration and proliferation of HUVEC (47). Although to date the role of NOX4 in endothelial proliferation has not been investigated, overexpression of NOX4 has been previously shown to decrease proliferation of NIH3T3 cells (43) and depletion of NOX4 prevented the induction of apoptosis by 7-ketocholesterol in human aortic smooth muscle cells (39). In contrast, and consistent with our study, recent studies showed that depletion of NOX4 decreased proliferation of melanoma cells (7), abrogated urotensin-II stimulated proliferation of pulmonary artery smooth muscle cells (11) and increased apoptosis of pancreatic cancer cells (49), indicating that NOX4 has also a pro-proliferative effect. Interestingly, both NOX2 and NOX4 activated p38 MAP kinase in EaHy926 cells, and inhibition of this kinase prevented the proliferative response by these NOX proteins, indicating the p38 MAP kinase contributes to the proliferative response induced by NOX2 or NOX4. Similarly, several studies showed an involvement of NADPH oxidases in the activation of p38 MAP kinase (1, 11, 28, 40, 42). Moreover, p38 MAP kinase was involved in NOX4-mediated proliferation of pulmonary artery smooth muscle cells in response to urotensin II (11) and in NOX2-mediated proliferation of hepatic stellate cells by PDGF (1).

In conclusion, we could demonstrate that endothelial cells simultaneously express NOX2 and NOX4, as well as NOX1, in an intracellular perinuclear compartment associated with the endoplasmic reticulum. Both NOX2 and NOX4 interact with p22phox in this compartment and are equally involved in basal endothelial ROS generation and proliferation, whereas NOX1 did not contribute to these responses under these conditions, indicating that a complex relation between several NOX homologues controls endothelial function and redox signaling.

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ABBREVIATIONS

ARNT, aryl hydrocarbon receptor nuclear translocator; BiFC, bimolecular fluorescence complementation; BrdU, 5-bromo-2′-deoxyuridine; CFP, cyan fluorescent protein; DCF, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco´s modified Eagle medium; DMSO, Dimethyl sulfoxide; GFP, green fluorescent protein; HAT, hypoxanthine, aminopterin, thymidin; HBSS, Hank's balanced salt solution; H_2O_2, hydrogen peroxide; L-NNA, n-omega-nitro-L-arginine; NOX, NADPH oxidase; PCR, polymerase chain reaction; Phox, phagocytic oxidase; PMN, polymorphonuclear neutrophils; Rac, ras-related C3 botulinum toxin substrate; ROS, reactive oxygen species; RT–PCR, reverse transcript polymerase chain reaction; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; YFP, yellow fluorescent protein.

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