ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 10, 2008

DOI: 10.1089/ars.2008.2035

Original Research Communication

NOX4 Regulates ROS Levels Under Normoxic and Hypoxic Conditions, Triggers Proliferation, and Inhibits Apoptosis in Pulmonary Artery Adventitial Fibroblasts

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Abstract

The NADPH oxidases are involved in vascular remodeling processes and oxygen sensing. Hypoxia-induced pulmonary arterial remodeling results in thickening of the vessel wall and reduction of the area of vessel lumen, leading to pulmonary hypertension and cor pulmonale. The proliferation of pulmonary artery adventitial fibroblasts (PAFB) is critically involved in this process. In this study, we analyzed the role of the nonphagocytic NADPH oxidase subunits NOX1 and NOX4 in PAFB. NOX4 was predominantly expressed in comparison to NOX1 at mRNA levels. Under hypoxic conditions, NOX4 was significantly upregulated at mRNA and protein levels. Silencing of NOX4 by siRNA caused reduction of ROS levels under both normoxic and hypoxic (24 h) conditions and suppressed the significant hypoxic-induced ROS increase. PAFB proliferation was significantly decreased in cells transfected with NOX4 siRNA, whereas apoptosis was enhanced. Also, the expression of NOX4 was studied in PAFB isolated from the lungs of patients with idiopathic pulmonary arterial hypertension (IPAH). Interestingly, a significant increase of NOX4 mRNA expression was observed under hypoxic conditions in PAFB from the lungs with IPAH compared to healthy donors. In conclusion, NOX4 maintains ROS levels under normoxic and hypoxic conditions and enhances proliferation and inhibits apoptosis of PAFB. *Antioxid. Redox Signal*. 10, 1687–1697.

Introduction

NADPH oxidases generate reactive oxygen species (ROS), namely superoxide, by electron transfer to oxygen. Superoxide can be further converted to hydrogen peroxide by cellular superoxide dismutases. These ROS are involved in cell signaling and host defense. NADPH oxidases consist of membrane-associated and cytosolic subunits (for review, see ref. 6). The most thoroughly investigated NADPH oxidase is the phagocytic gp91phox (NOX2)-containing subunit that interacts with a second membrane-bound subunit, p22phox, and several cytosolic and regulatory subunits including p47phox, p67phox, and Rac. Both NOX1 and NOX4 are homologs of NOX2, and are expressed in vascular and other nonphagocytic cells. The activity of NOX4 also depends on association with p22phox; however, the assembly of the cytosolic subunits appears to be irrelevant for its activity. Activation of NOX1 strongly depends on interaction with two additional subunits, NOXO1 and NOXA1, homologs of p67phox and p47phox.

The nonphagocytic NADPH oxidases in particular appear to be involved in signal transduction and oxygen sensing (28). The family of NOX enzymes has been implicated in vascular diseases including hypertension, aortic media hypertrophy, and arteriosclerosis (19, 23, 29). The altered generation of ROS is believed to underlie the aberrant vascular responses observed under hypoxic conditions (10, 17, 39, 53). Both NOX1 and NOX4 are expressed in vascular cells of all vessel layers (6). A recent study demonstrated a role of NOX4 in the pathogenesis of hypoxia-induced pulmonary arterial

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remodeling and pulmonary hypertension, as well as idiopathic pulmonary arterial hypertension (IPAH) (33). Pulmonary hypertension is characterized by narrowing of the vessel lumen and increased pulmonary artery pressure, leading to pulmonary hypertension and cor pulmonale (22, 44, 45). The different vessel layers of the pulmonary artery can contribute to this process. Endothelial cells from the intima exhibit atypical proliferation and form plexiform lesions (49). Smooth muscle cells (SMC) from the medial layer exhibit hyperplasia and enhanced contractility (8, 11). The adventitial layer is thickened by hypoxia-induced proliferation of adventitial fibroblasts (PAFB) (8, 40, 45). Furthermore, transdifferentiation of PAFB into myofibroblasts or SMC contribute to this process (43).

In this study, we initially analyzed the expression of NOX1 and NOX4 mRNA in PAFB by real-time RT-PCR. We observed a higher level of NOX4 expression than NOX1 in PAFB. Furthermore, we observed that NOX4 expression was significantly upregulated under hypoxic conditions. The siRNA-mediated knockdown of NOX4 expression demonstrated that NOX4 contributed to the increase in ROS generation under hypoxic conditions, and stimulated proliferation and inhibited apoptosis of PAFB.

Materials and Methods

Preparation and culture of human pulmonary artery adventitial fibroblasts (PAFB)

The PAFB were isolated from human pulmonary arteries as described previously (40). Lungs from healthy donors and patients with idiopathic pulmonary arterial hypertension (IPAH) were used in the experiments. This protocol was approved by the Justus–Liebig University Giessen Ethics Committee. The PAFB were cultured with fibroblast growth medium (Promocell, Heidelberg, Germany). Cells between passages two and five were used for experiments. Real time RT-PCR analyses showed no significant differences in NOX4 mRNA expression between cells from these passages. Hypoxic incubation was performed in a water-saturated atmosphere with an adjusted gas mixture of 1% O_2 and 5% CO_2 at 37°C. Control cells were cultured under normoxic conditions in a water-saturated atmosphere (room air) supplemented with 5% CO_2 at 37°C.

Total RNA and protein extraction

Both RNA and protein were extracted and separated from the same samples (NucleoSpin RNA/Protein, Macherey–Nagel, Germany).

Reverse-transcription and PCR

Total RNA (1 μg) was denatured at 65°C for 5 min. After cooling on ice, the following components were added to the samples: 4 μl of 5× first strand buffer, 2 μl of 40 m*M* deoxynucleotide mixture, 1 μl of random-hexamer primer, 1 μl of 0.1 *M* dithiothreitol, 1 μl RNase inhibitor (peQlab, Erlangen, Germany), 1 μl Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) (Invitrogen, Karlsruhe, Germany). After 60 min at 39°C, reverse transcriptase was inactivated by heating the mixture at 96°C for 2 min. For the negative control, MMLV-RT was omitted. Real-time PCR was performed using the ABI Prism 7300 Detection System (Applied Biosystems, Lincoln, CA) with SYBR-Green as fluorescent dye, enabling real-time detection of PCR products according to the manufacturer's protocol. The cDNA was submitted to real-time PCR using the primer pairs listed below. Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 94°C for 10 s, 54°C for 10 s, 72°C for 30 s. For quantification, the target gene was normalized to porphobilinogen deaminase (PBGD) mRNA. Real-time data are presented as ΔCt ($\Delta Ct = CT_T - CT_R$; CT_T: threshold cycle of target gene, CT_R: threshold cycle of PBGD), or as the relative expression ratio to PBGD mRNA. The following primer sets (+, forward; –, reverse) derived from the GenBank sequences were used: PBGD (Accession No.: NM_000190) PBGD+: 5′-TGT CTG GTA ACG GCA ATG CG-3′, PBGD–: 5′-CCC ACG CGA ATC ACT CTC AT-3′; NOX4 (Accession No.: NM_016931) NOX4+: 5′-AAA CTT CTC TTC ACA ACT GTT CCT G-3′, NOX4-: 5′-TGG TAA GGA AAT ATT CTG AGA GCT G-3′; NOX1 (Accession No.: NM_007052) NOX1+: 5′-CTC TCT CCT GGA ATG GCA TC-3′, NOX1–: 5′-TGG AAA ACA TCC TCA CTG GC-3′; PDK1 (Accession No.: NM_002610) PDK1+: 5′-CAA TTG GTA CAA AGC TGG TAT ATC C-3′, PDK1–: 5′-TAA CTT CAA GTA CAT TGC AGT TTG G-3′.

Western blot analysis

For the detection of NOX4 by Western blot analysis, a custom-made polyclonal anti-NOX4 antibody raised in rabbits was employed (15). For the detection of β-actin, a monoclonal β-actin antibody (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was employed. After determination of the protein concentration (BCA protein assay, Pierce, Rockford, Il), 15 μg of protein was resolved on an 8% SDS-PAGE gel. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA) by semi-dry electroblotting. After the membrane was blocked in NET-gelatin buffer (150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris-HCl, 0.25% gelatin, and 0.05% Triton X-100) for 2 h at room temperature, the NOX4 antibody (dilution 1:2,000) was added and incubated overnight at 4°C. After washing the membranes in NET buffer, specific immunoreactive signals were detected by enhanced chemiluminescence (ECL, Amersham, Freiburg, Germany) using a secondary antibody coupled to horseradish peroxidase. For detection of β-actin, the membrane was stripped and β-actin antibody added with a dilution of 1:20,000.

Measurement of ROS

DCFH technique. Intracellular ROS generation was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, Saint Louis, MO) as described previously (14). After cellular uptake of DCFH-DA, it is cleaved by cellular esterases to 2′,7′-dichlorofluorescin (DCFH). The intracellular ROS cause oxidation of DCFH to the fluorescent product 2′,7′-dichlorofluorescein (DCF). Cells plated on 6-well cell culture plates (Corning Inc., Corning, NY) were maintained under normoxic conditions (21% O_2, 5% CO_2, 94% N_2) or hypoxic conditions (1% O_2, 5% CO_2, balanced N_2) for 23 h, after which cells were washed and incubated with DCFH-DA (30 μM) in serum-free culture medium under normoxic or hypoxic conditions (employing a hypoxic bag) for 1 additional hour. The medium was removed; cells were washed three times with serum-free medium, lysed in

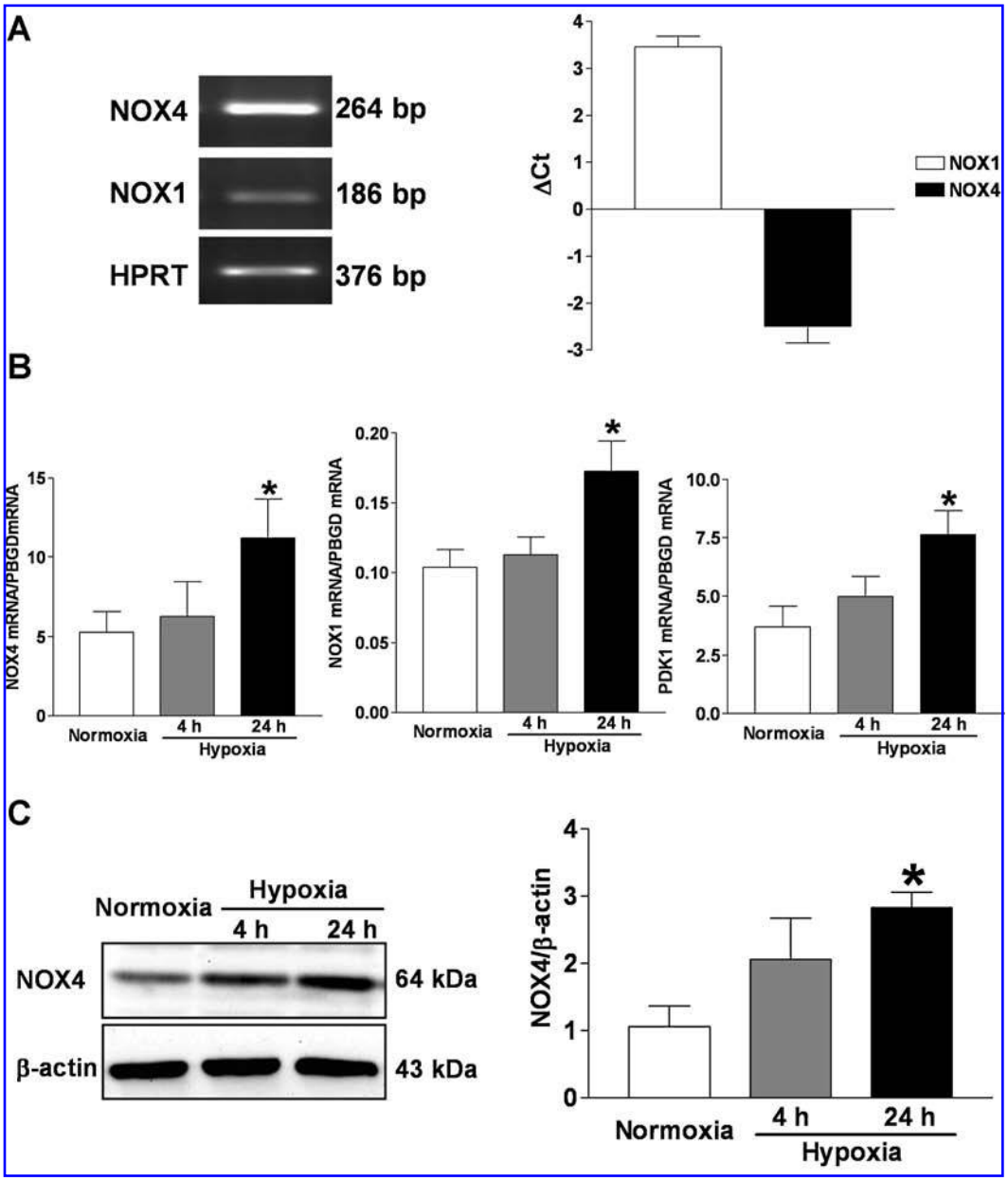

FIG. 1. Expression of NOX1 and NOX4 in PAFB. (**A**) *Left*: NOX1 and NOX4 mRNA detection by RT-PCR on an ethidium bromide-stained agarose gel. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a reference gene. *Right*: Real-time RT-PCR analysis of NOX1 and NOX4 mRNA levels. The ΔC_T values are illustrated relative to porphobilinogen deaminase (PBGD) mRNA. The differences of ΔC_T values of NOX4 and NOX1 ($\Delta\Delta C_T \sim 6$) suggest an approximate 60-fold higher expression level of NOX4 mRNA than NOX1 mRNA ($n = 7$). (**B**) Regulation of NOX4 mRNA and NOX1 mRNA under normoxic and hypoxic conditions in comparison to pyruvate dehydrogenase kinase I (PDK1) mRNA, a well-established hypoxia-dependent target gene. The values represent the relative induction as measured by real-time RT-PCR relative to PBGD mRNA levels ($n = 7$). (**C**) *Left*: Western blot analysis of NOX4 and β-actin under normoxic and hypoxic conditions (4 h, 24 h). *Right*: Densitometric analysis of NOX4 normalized to β-actin ($n = 6$). *Significant differences compared with normoxia ($p < 0.05$).

liquid nitrogen in the presence of serum-free medium, and centrifuged (1,200 *g*, 5 min, 4°C). The fluorescence of the supernatant was measured at 535 nm with an excitation wavelength of 485 nm using a spectrofluorometer (FL-600, BioTek Instruments, Inc., Winooski, VT). Values are given as fluorescence at 535 nm from wells containing cells from which background (fluoresence from wells without cells) was subtracted.

Amplex Red technique. Intracellular reactive oxygen species (ROS) generation was measured by the Amplex Red Hydrogen Peroxide/Peroxidase Assay (Invitrogen, Molecular Probes, Eugene, OR) under normoxic or hypoxic conditions. The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), in the presence of horseradish peroxidase, reacts with H_2O_2 from samples and generates a fluorescent product. The assay was carried out according to the manu-

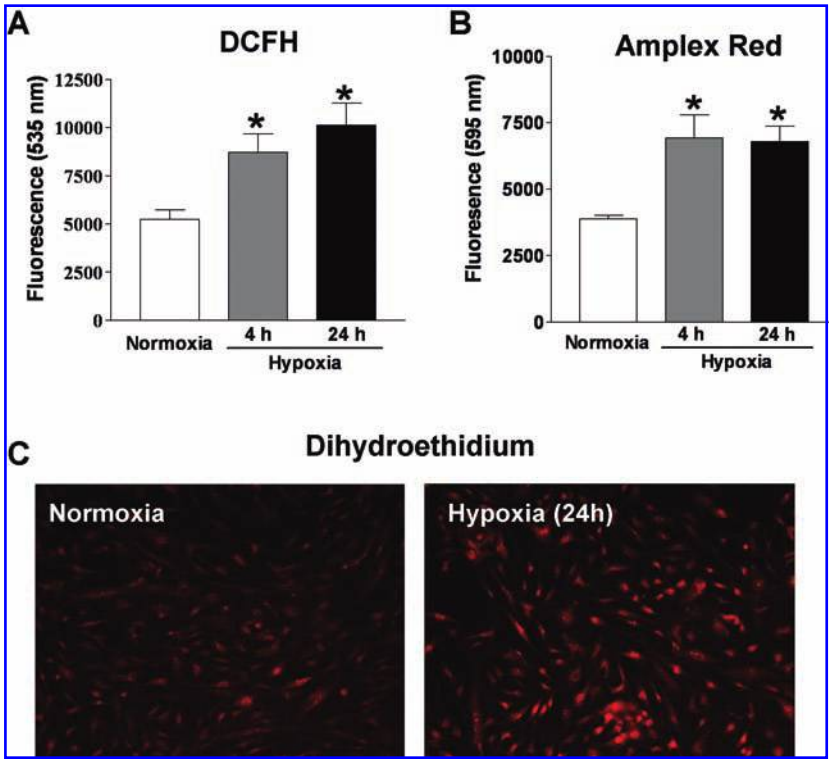

FIG. 2. Effect of hypoxia on ROS levels as measured by different techniques in PAFB. Intracellular reactive oxygen species (ROS) generation was measured by the (**A**) dichlorofluorescein (DCFH) and (**B**) Amplex Red techniques after incubation under normoxic or hypoxic (4 h, 24 h) conditions ($n = 7$). (**C**) Representative cytofluorescent image employing the dihydroethidium (DHE) technique for ROS detection under normoxic and hypoxic (24 h) conditions. (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).

facturer's protocol with minor modifications. After culturing of the cells in normoxic or hypoxic conditions, the medium was removed; cells were washed once with serum-free medium, and lysed in liquid nitrogen in the presence of 50 μl of 1×Reaction Buffer. Fifty μl of the Amplex Red Reagent/HRP working solution was added and incubated at room temperature for 30 min protected from light. The fluorescence was measured at 595 nm with an excitation wavelength of 530 nm using a spectrofluorometer (FL-600, BioTek Instruments, Inc.). Values are given as fluorescence at 595 nm from wells containing samples from which background (fluoresence from wells containing reaction buffer) was subtracted.

DHE cytofluorescence. Cells plated on 8-well chamber slides were cultured under normoxic or hypoxic conditions (23 h). Then the cells were washed. Dihydroethidium (DHE, 5 μM) was added in serum-free medium and cells were incubated under normoxic or hypoxic conditions (employing a hypoxic bag) for 1 h. Slides were washed three times with phosphate-buffered saline and fixed in acetone and methanol (1:1) for 5 min. After washing with phosphate-buffered saline, fluorescent signals were captured by fluorescent microscope.

Inhibition of NOX4 by RNA interference

For the NOX4 RNA interference experiments, a siRNA specific for human NOX4, 5′-CCU CUU CUU UGU CUU CUA C dTdT-3′ corresponding to nucleotides 585–603 and its complementary sequence, were employed. As a control, a random nontargeting siRNA: 5′-UAA GGC UAU GAA GAG AUA C dTdT-3′ and its complement (Dharmacon, Lafayette, CO), were employed. Transfection of siRNA was carried out at a final concentration of 200 nM with Oligofectamine^{TM} (Invitrogen, Carlsbad, CA). After an incubation period of 24 h under normoxic conditions, the cells were exposed to normoxic or hypoxic conditions for an additional 24 h.

Detection of proliferation by BrdU incorporation

Cellular DNA synthesis (cell cycle S-phase) was assessed by incorporation of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) into the DNA of replicating cells using a colorimetric immunoassay (Roche, Mannheim, Germany). Before BrdU incorporation, PAFB were incubated with low-serum medium (DMEM with 0.1% fetal calf serum) for 24 h. After BrdU labeling for 4 h, cells were fixed and incorporated BrdU was measured by ELISA using a specific anti-BrdU antibody. The values given in the figures represent the optical density of spectrophotometric measurements at 450 nm from which the control values had been subtracted.

Detection of apoptosis by caspase-3 immunocytochemistry

Cells were cultured on 8-well chamber slides (Nunc, Wiesbaden, Germany). Slides were washed and fixed in acetone and methanol (1:1) for 5 min. After incubation with 3% bovine serum albumin, cells were incubated with a polyclonal affinity purified antibody directed against a peptide from the p18 fragment of human caspase-3 (Promega: Anti-ACTIVE Caspase-3 antibody). It specifically stains apoptotic cells without staining of nonapoptotic cells as confirmed by functional tests. The caspase-3 antibody (Promega, Madison, WI) diluted 1:200 in phosphate-buffered saline, followed by an Alexa 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Leiden, Netherlands) diluted 1:1,000 in phosphate-buffered saline. Nuclei were detected with 1 μM of 4′,6-diamidino-2-phenylindol (DAPI, Sigma-Aldrich Chemie GmbH). For quantification, the ratio of the number

of caspase-3 positive cells to the number of DAPI stained cells was determined.

Statistical analysis

Results are expressed as mean ± SEM (standard error of the mean) from at least three independent experiments, if not indicated differently. Statistical analysis was done using Student¸s *t*-test. A probability value of < 0.05 was considered significant.

Results

The expression of nonphagocytic NADPH oxidase subunits varies among different cell types with distinct and tissue-restricted expression patterns. Initially, we analyzed the expression of the gp91phox homologs NOX1 and NOX4 in PAFB by RT-PCR (Fig. 1A). Both NOX1 and NOX4 were detected in these cells. Comparison of expression levels by real time RT-PCR demonstrated a considerable higher expression of NOX4 mRNA than of NOX1 mRNA. The expression of NOX4 mRNA was upregulated by more than twofold under hypoxic conditions. This upregulation was similar to that of pyruvate dehydrogenase kinase 1 (PDK1), an established hypoxia-dependent gene. NOX1-mRNA was upregulated by ~1.6-fold (Fig. 1B). The upregulation of NOX4 expression was also observed at the protein level as measured by Western blot analysis (Fig. 1C).

We examined the generation of ROS under hypoxic conditions, where the induction of ROS generation in PAFB was observed after both a 4 and 24 h exposure to hypoxia employing DCFH and Amplex Red techniques (Fig. 2A and B). Furthermore, we performed DHE cytofluorescence analyses, revealing that the signal intensity per cell (indicating ROS) is increased (Fig. 2C). This was accompanied by increased proliferation as analyzed by BrdU incorporation (Fig. 3A) and reduced apoptosis as analyzed by caspase-3 detection (Fig. 3B). To assess whether NOX4 contributed to the increased ROS generation and altered proliferation and apoptosis profiles of PAFB observed under hypoxic conditions, we impaired NOX4 activity by siRNA-mediated knockdown of NOX4 expression. The NOX4 mRNA levels in PAFB were significantly reduced after treatment with NOX4 siRNA as compared with random siRNA (Fig. 4A). The siRNA-mediated knockdown of NOX4 was further validated by Western blot analysis (Fig. 4B). We observed a reduction of ROS generation in cells treated with NOX4 siRNA both under normoxic and hypoxic conditions. Particularly, the hypoxia-induced upregulation of ROS was prevented by NOX4 siRNA (Fig. 5A and B). Furthermore, knockdown of NOX4 expression resulted in downregulation of PAFB proliferation (Fig. 5C). On the other hand, treatment of cells with siRNA against NOX4 increased apoptosis (Fig. 5D).

To further confirm the importance of ROS levels as mediators of proliferation and apoptosis, we treated cells with catalase. Addition of catalase reduced intracellular ROS levels identifying hydrogen peroxide as the relevant ROS (Fig. 6A and B). Furthermore, catalase decreased proliferation (Fig. 6C) and increased apoptosis (Fig. 6D), observations which were consistent with our data obtained from NOX4 siRNA-treated PAFB.

Finally, we compared the expression of NOX4 mRNA in PAFB from healthy donor lungs and patients with IPAH. We observed significantly increased levels of NOX4 mRNA under hypoxic conditions in PAFB from IPAH patients when compared to PAFB from donor lungs (Fig. 7).

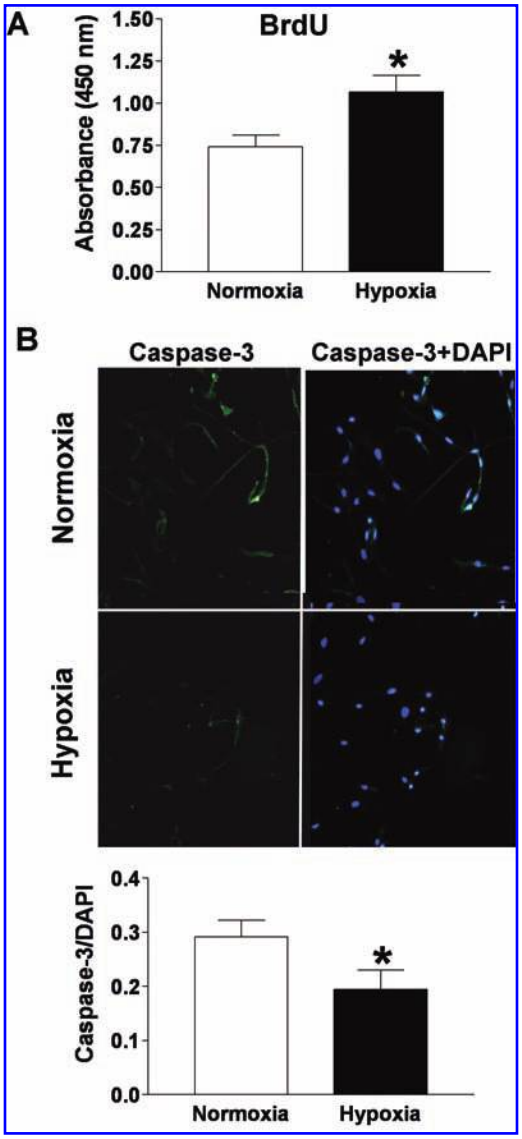

FIG. 3. Effect of hypoxia on proliferation and apoptosis in PAFB. (**A**) Proliferation as measured by BrdU incorporation assay after incubation of cells under normoxic or hypoxic (24 h) conditions. Data are derived from duplicate measurements of four independent experiments. (**B**) Apoptosis as measured by caspase-3 detection of cells incubated under normoxic or hypoxic (24 h) conditions. *Top*: Representative cytoimmunofluorescent image of caspase-3 (*green*) detection and the merged image of caspase-3 (*green*) and DAPI (*blue*) for nuclear cell staining. *Bottom*: Quantification of caspase-3 staining by calculation of the caspase-3/DAPI ratio (triplicate measurements of five independent experiments). *Significant differences compared with normoxia ($p < 0.05$). (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).

Discussion

In this study, we analyzed the nonphagocytic NADPH oxidases NOX1 and NOX4 in pulmonary artery adventitial fi-

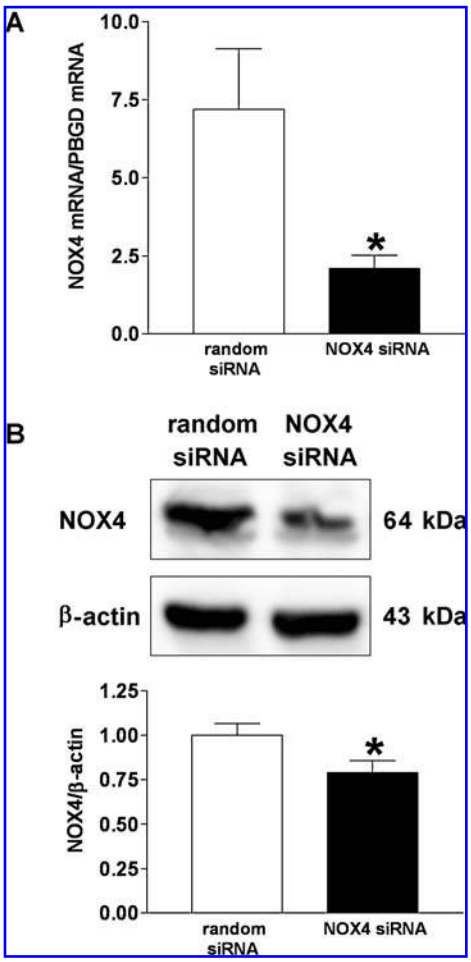

FIG. 4. NOX4 inhibition by NOX4 siRNA in PAFB. (**A**) NOX4 mRNA analyses by real-time RT-PCR from RNA extracts after transfection of PAFB with NOX4 siRNA or random siRNA ($n = 5$). (**B**) *Top*: Western blot analysis of NOX4 and β-actin from protein extracts of PAFB treated in the same way as in (A). *Bottom*: Densitometric analysis of NOX4 normalized to β-actin ($n = 6$). *Significant downregulation of NOX4 as compared with random siRNA ($p < 0.05$).

broblasts (PAFB). We focused on NOX4 regulation and analyzed cellular responses such as ROS generation, proliferation, and apoptosis. We found that (a) NOX4 is the predominant NADPH oxidase in PAFB when compared to NOX1 and (b) NOX4 is upregulated under hypoxic conditions. Employing specific inhibition of NOX4 by siRNA, we concluded that (c) NOX4 strongly contributes to basal ROS levels under normoxic conditions and to an increase of ROS levels after long-term hypoxic conditions (24 h). Furthermore, we observed that (d) the proliferation depends on NOX4 expression and (e) the apoptosis is suppressed by NOX4 expression.

Regulation of NOX4 and ROS generation

The regulation of NOX4 appears to differ from that of NOX2 (gp91phox) and NOX1. Indeed, NOX2 is regulated by assembly with different cytosolic subunits (16, 48). The assembly is required for an active NADPH oxidase complex, which is triggered by phosphorylation of the p47phox subunit and by the GTP/GDP binding protein Rac (9, 20). For the assembly of NOX1, NOXO1, and NOXA1, which represent homologs of p47phox and p67phox, appear to be critical for enzymatic activity (3). The regulation of NOX4 is less clear. Recent data from recombinant NOX4 expression suggest that NOX4 enzymatic activity depends on the membrane-associated p22phox subunit, whereas cytosolic subunits, phosphorylation, or interaction with Rac are apparently not required for its activation (1, 24, 31). However, this depends upon the cell type and experimental design. For example, in endothelial cells, activation of NOX4 by phorbol 12-myristate 13-acetate (PMA) has been observed (27). A recent study with controlled recombinant expression of NOX4 demonstrated a strong correlation between NOX4 mRNA level and ROS generation (42). Thus, in this experimental setting, the activity of NOX4 was demonstrated to depend directly on its gene expression levels. A further study (41) demonstrated that siRNA against NOX4 reduced basal ROS level in rat smooth muscle cells by ~20%. This value is in accordance with the measurements of our study. Interestingly, we report in the present study that NOX4 gene expression is induced under hypoxic conditions, and this is related to an increase of ROS. This observation is important in the context of altered ROS generation under hypoxic conditions. Recent data employing different approaches for the measurement of ROS suggest an increase in ROS under hypoxic conditions (7, 18, 25, 28, 30). One source of ROS under these conditions appears to be the mitochondria. Electron leakage at complex II and complex III of the respiratory chain is crucial for ROS generation (7, 18, 34). In particular, one study observed an upregulation of ROS levels after 1 h of hypoxia as measured by a redox sensitive FRET protein sensor. Inhibition of the Rieske iron–sulfur protein by siRNA supports the importance of complex III of the mitochondrial electron transport chain (18). This mitochondrial-dependent induction of ROS generation under hypoxic conditions occurs in the early phase. During sustained hypoxia, the mitochondrial respiration becomes optimized. Then, a switch in cytochrome c oxidase subunit expression and the induction of pyruvate dehydrogenase kinase result in reduced mitochondrial ROS production (12, 26, 36). Interestingly, we observed a hypoxic upregulation of ROS generation after 4 h, a time period after which NOX4 mRNA and NOX4 protein was not significantly upregulated (Fig. 1B and C). Thus, at earlier time points (up to 4 h) mitochondrial sources may be relevant for the ROS increase, whereas the upregulation of NOX4 (after 24 h) as observed in this study likely causes the long-lasting ROS increase. Also, other studies provide evidence that NADPH oxidases contribute to changes in ROS levels under hypoxic conditions (14, 28). However, NADPH oxidase-derived ROS have been suggested either to be decreased or increased under hypoxic conditions (2, 13, 14, 33, 35, 52). A decreased ROS release has been attributed to the lowered concentration of oxygen as a substrate. On the other hand, the K_m values of NOX are rather low, allowing ROS synthesis even under hypoxic conditions (13). Accordingly, different studies support that NADPH oxidases are involved in an increase in ROS generation under hypoxic conditions, because of an upregulation of this enzyme (14, 33). In the present study, a role of increased NOX4 gene expression could be related to an increase in ROS generation under hypoxic conditions, since the inhibition of NOX4 by siRNA prevented the significant hypoxia-induced ROS increase after

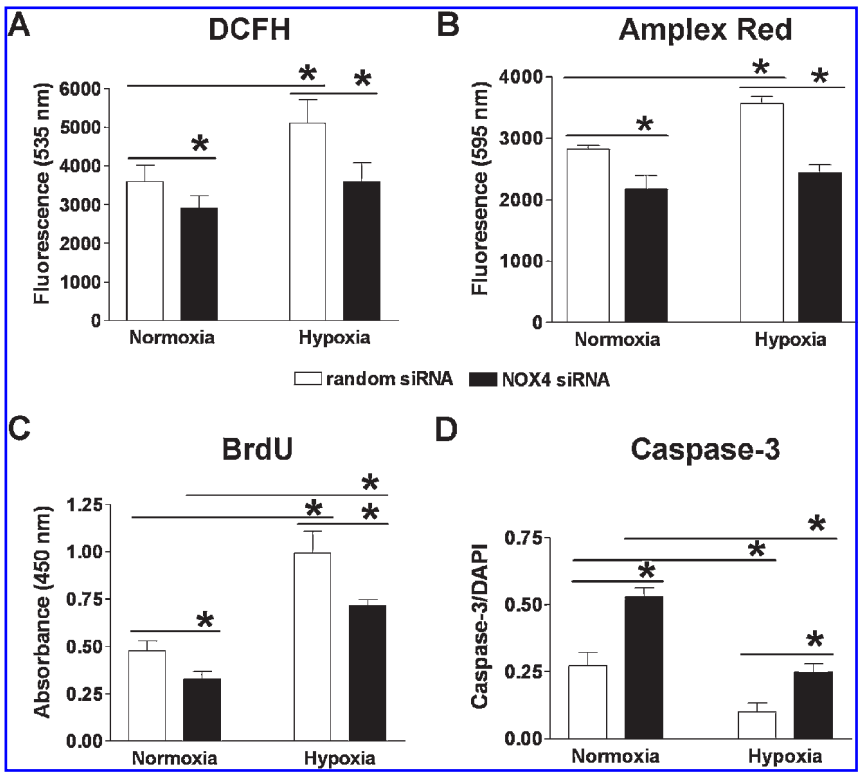

FIG. 5. Effect of inhibition of NOX4 by NOX4 siRNA on ROS generation, proliferation, and apoptosis in PAFB. Intracellular reactive oxygen species (ROS) generation was measured by the (**A**) dichlorofluorescein (DCFH) or (**B**) Amplex Red techniques after normoxic or hypoxic incubation (24 h) ($n = 3$). (**C**) Analysis of proliferation by BrdU incorporation after incubation under normoxic or hypoxic conditions (24 h). Data are derived from duplicate measurements of three independent experiments. (**D**) Analysis of apoptosis by caspase-3 staining after normoxic or hypoxic incubation (24 h). Data are derived from triplicate measurements of three independent experiments. *Significant differences compared with random siRNA treatment ($p < 0.05$).

24 h. However, siNOX4 treatment resulted in equal effects on proliferation and apoptosis under normoxic and hypoxic conditions and could not block the hypoxia-induced alterations of proliferation and apoptosis, suggesting that other components may contribute to hypoxia-mediated responses such as proliferation and apoptosis.

Also, a recent study observed upregulation of NOX4 in smooth muscle cells from pulmonary artery under hypoxic conditions. In this study, predominant staining of NOX4 in the medial layer of pulmonary artery, as demonstrated with histological techniques (*in situ* hybridization and immunocytochemistry for NOX4) was observed (33).

A further study compared ROS generation in smooth muscle cells (SMC) from the coronary and pulmonary artery (54). Interestingly, ROS generation was increased under long-term hypoxia (48 h) only in SMC from the pulmonary artery. The authors suggested either NOX1- or NOX4-containing NADPH oxidases as the likely source of these ROS. The regulation of these NADPH oxidases may differ at the expression level or subunit assembly level. However, to date, the regulation of NOX4 gene expression, and the relevant transcription factors have not been described. It is also not known if NOX4 is under the direct regulation of hypoxia-inducible factors, since promoter studies of NOX4 are lacking. An alternative explanation for the cell-specific regulation of NADPH oxidases is the availability of NADPH. Apart from the availability of oxygen as regulator of NADPH oxidase activity, the availability of cytosolic NADPH is also crucial for NADPH oxidase activity. One study reported elevated ROS generation in bovine pulmonary artery segments when compared to coronary artery segments (17). This was attributed to higher NADPH levels in the pulmonary artery segments. The elevated NADPH levels appeared to be caused by increased expression of glucose-6-phosphate dehydrogenase in pulmonary artery segments, compared to coronary artery segments. Interestingly, glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway which generates NADPH.

The precise cellular localization of NOX4 has not been defined. However, there is evidence that NOX4 is localized within intracellular membranes producing intracellular ROS (13, 42, 55). Hence, we performed the ROS measurements in cell lysates. Also, we employed exogenously added catalase to support the relevance of ROS in the cellular responses of PAFB. Exogenously added native catalase was shown to increase cellular catalase activity in endothelial cells with impact on oxidant stress tolerance (5). We observed downregulation of ROS by catalase in the cell lysates, resulting in inhibition of proliferation and increase of apoptosis. However, we cannot exclude that the addition of catalase also exerts its effects by scavenging of extracellular ROS.

Proliferation

The increased proliferation in PAFB under hypoxic conditions is a typical response of these cells to hypoxia, and differs from that of other cells in this respect (40, 45). The concomitant increase in ROS appeared to be related to the increased proliferation. Several studies have demonstrated that increased ROS production affects proliferation. It is less clear if the NOX-derived ROS is relevant for proliferation. Both NOX1 and NOX4 induced proliferation of smooth muscle cells (32, 33, 37, 46). In particular, NOX4 is known to be induced by TGF-β, and has been related to the TGF-β-dependent proliferation of SMC in the pulmonary artery. Furthermore, NOX2, NOX4, and p22phox are important for pro-

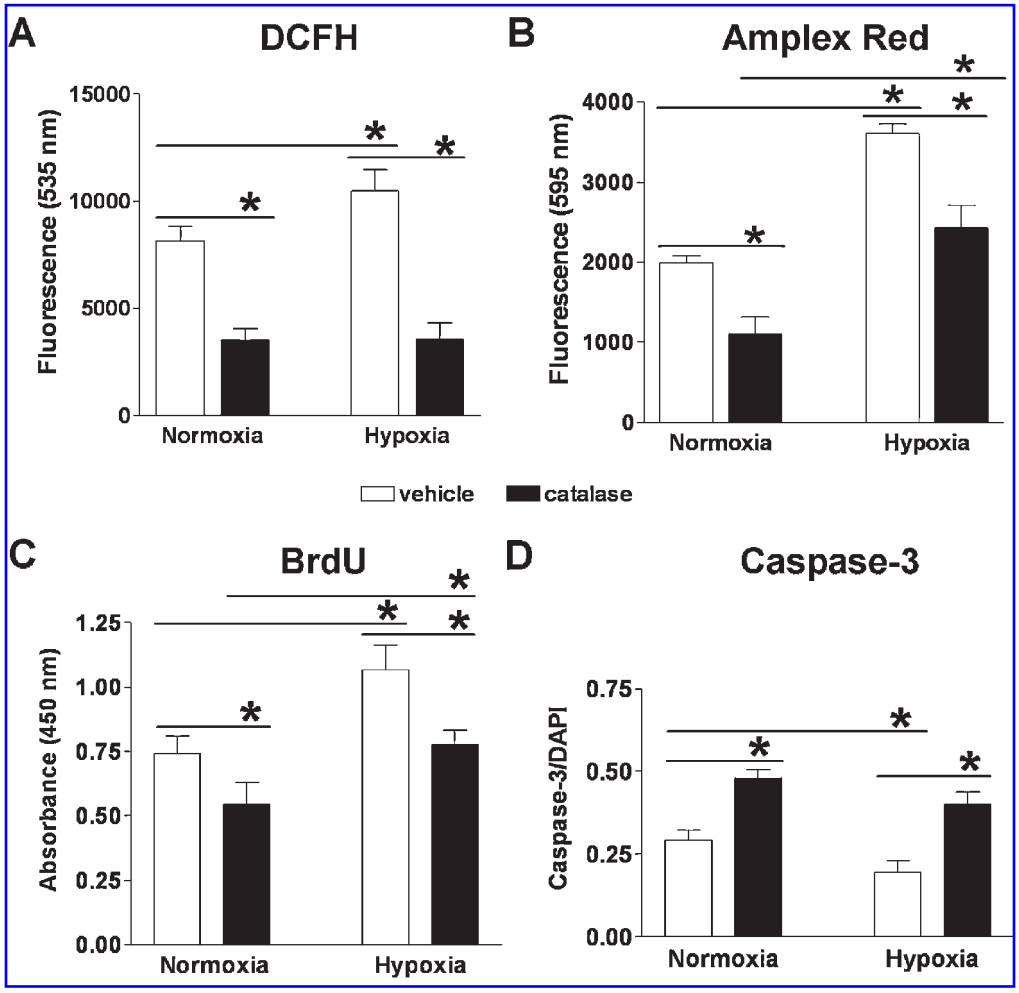

FIG. 6. Effect of catalase treatment on ROS levels, proliferation, and apoptosis in PAFB. Native catalase (2 units/μl) was added to the medium and cells were washed with medium before ROS was measured in the cell lysates. ROS was measured by the (**A**) dichlorofluorescein (DCFH) or (**B**) Amplex Red techniques after normoxic or hypoxic incubation (24 h) ($n = 5$). (**C**) Analysis of proliferation by BrdU incorporation after incubation under normoxic or hypoxic conditions (24 h). Data are derived from duplicate measurement of four independent experiments. (**D**) Analysis of apoptosis by caspase-3 staining after normoxic or hypoxic incubation (24 h). Data are derived from triplicate measurements of three independent experiments. *Significant difference ($p < 0.05$).

liferation of endothelial cells (4, 38). In tumor cells, NOX1 is relevant for the transformation of tumor cells (47). Interestingly, in our study, treatment of cells by NOX4 siRNA as well as the breakdown of hydrogen peroxide by catalase reduced proliferation and increased apoptosis in PAFB, supporting pro-proliferative role of ROS in this experimental setting.

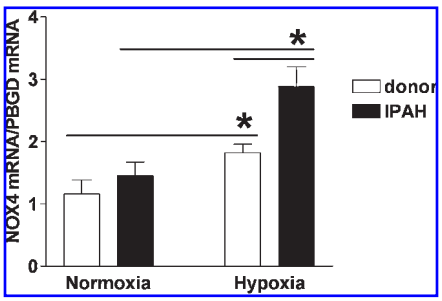

FIG. 7. NOX4 in PAFB from donors and IPAH patients. NOX4 mRNA analysis by real-time RT-PCR of RNA extracts from PAFB of healthy donors or IPAH patients. *Significant difference compared with healthy donors ($n = 3$, $p < 0.05$).

Apoptosis

In our study, we observed that hypoxia inhibited the apoptosis rate of PAFB, and treatment of cells with NOX4 siRNA significantly increased apoptosis both under normoxic and hypoxic conditions. In one study performed on fetal PASMC, endothelin caused mitogenic effects by ROS generation, and ROS scavenging in this process blocked proliferation by induction of apoptosis (51). Similarly, in the context of cancer biology, NOX-derived ROS were also shown to be anti-apoptotic, where DPI and NOX4 siRNA were employed for the inhibition of ROS production in pancreatic cancer cell lines (50). These observations are in line with our results, where an inhibition of NOX4-dependent ROS generation results in decreased proliferation and enhanced apoptosis. In contrary, it has also been described that NOX-derived ROS can trig-

ger apoptosis indirectly through toxic effects exerted on DNA, lipids and proteins, or through the MAP kinase pathway, or by inhibition of tyrosine phosphatases (21).

Expression of NOX4 in IPAH

We observed a significant increased expression of NOX4 mRNA under hypoxic conditions in PAFB isolated from IPAH lungs, when compared with PAFB isolated from healthy donor lungs. It is noteworthy that these experiments were performed on cells in the third passage, since progressive downregulation of NOX4 expression has been correlated with increasing passage number (46). A recent study analyzed NOX4 expression in healthy and IPAH lungs in lung homogenates. Expression of NOX4 protein in IPAH lungs was significantly increased compared with healthy donor lungs. This study demonstrated NOX4 immunostaining predominantly in the medial layer of pulmonary arteries (33). Taken together, NOX4 upregulation addresses a possible role of NOX4 in the pathogenesis of pulmonary hypertension.

Acknowledgments

We thank Christiane Hild and Sabine Graef–Hoechst for excellent technical assistance. We thank Dr. Rory Morty for proofreading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft SFB547-B9.

Abbreviations

BrdU, bromodeoxyuridine; C_T, threshold cycle; DAPI, 4′,6-diamidino-2-phenylindol; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetra acetate; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IPAH, idiopathic pulmonary arterial hypertension; MAPK, mitogen-activated protein kinase; PAFB, pulmonary artery adventitial fibroblasts; PDK1, pyruvate dehydrogenase kinase 1; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SMC, smooth muscle cells; siRNA, small interfering RNA.

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Date of first submission to ARS Central, January 29, 2008; date of final revised submission, May 13, 2008; date of acceptance, May 14, 2008.

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