Hypoxia-Dependent Regulation of Nonphagocytic NADPH Oxidase Subunit NOX4 in the Pulmonary Vasculature

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Abstract—Nonphagocytic NADPH oxidases have recently been suggested to play a major role in the regulation of physiological and pathophysiological processes, in particular, hypertrophy, remodeling, and angiogenesis in the systemic circulation. Moreover, NADPH oxidases have been suggested to serve as oxygen sensors in the lung. Chronic hypoxia induces vascular remodeling with medial hypertrophy leading to the development of pulmonary hypertension. We screened lung tissue for the expression of NADPH oxidase subunits. NOX1, NOXA1, NOXO1, p22phox, p47phox, p40phox, p67phox, NOX2, and NOX4 were present in mouse lung tissue. Comparing mice maintained for 21 days under hypoxic (10% O2) or normoxic (21% O2) conditions, an upregulation exclusively of NOX4 mRNA was observed under hypoxia in homogenized lung tissue, concomitant with increased levels in microdissected pulmonary arterial vessels. In situ hybridization and immunohistological staining for NOX4 in mouse lungs revealed a localization of NOX4 mRNA and protein predominantly in the media of small pulmonary arteries, with increased labeling intensities after chronic exposure to hypoxia. In isolated pulmonary arterial smooth muscle cells (PASMCs), NOX4 was localized primarily to the perinuclear space and its expression levels were increased after exposure to hypoxia. Treatment of PASMCs with siRNA directed against NOX4 decreased NOX4 mRNA levels and reduced PASMC proliferation as well as generation of reactive oxygen species. In lungs from patients with idiopathic pulmonary arterial hypertension (IPAH), expression levels of NOX4, which was localized in the vessel media, were 2.5-fold upregulated. These results support an important role for NOX4 in the vascular remodeling associated with development of pulmonary hypertension. (Circ Res. 2007;101:258-267.)

Key Words: hypoxia ■ hypoxic pulmonary vasoconstriction ■ NADPH oxidase ■ pulmonary hypertension ■ vascular smooth muscle cell proliferation

The NADPH oxidases are superoxide-generating enzymes that release superoxide by electron transfer from NADPH to oxygen. The classical leukocyte NADPH oxidase plays an important role in host defense against bacterial and fungal pathogens.1,2 This phagocytic type of NADPH oxidase consists of 2 membrane-bound subunits, gp91phox and p22phox which form the flavocytochrome b55 complex, together with the cytosolic subunits p47phox, p40phox, and p67phox. Superoxide production by this complex is induced by assembly of the cytosolic and membrane-bound subunits. Such an assembly can be induced by the phosphorylation of p47phox.3 Rac GTPases are also involved in this activation process. Recently, several additional isoforms of the membrane-bound subunit gp91phox have been described. The first described homolog of gp91phox, called mox1 (later NOX1), is primarily expressed in the colon and is suggested to be involved in mitogenic activity.4 Additional homologs, including NOX3, NOX4 (Renox), NOX5, Duox1, and Duox2, were subsequently described.5–8 According to this new nomenclature, gp91phox is synonymous with NOX2. It was suggested that each of these homologs can replace gp91phox in the NADPH oxidase complex, and it has been demonstrated that these nonphagocytic NADPH oxidases release lower amounts of superoxide.9,10 However, very recently 2 new isoforms of the cytosolic subunits p47phox and p67phox have been identified. These new subunits, NOXO1 and NOX1A, have been demonstrated to interact with NOX1 to generate significant amounts of superoxide without being activated by protein kinase C–dependent phosphorylation.11,12 Isoforms of gp91phox have been identified in different organs and cell...
types, including the colon, kidney, uterus, testis, liver, vascular smooth muscle cells, fibroblasts, endothelial cells, pancreatic islets, and lymphocytes. The NOX2 homologs have been suggested to be associated with the development of atherosclerosis, systemic hypertension, diabetic vascular disease, and diseases of the brain. The 2 vascular isoforms, NOX1 and NOX4, are thought to play a role in vascular pathology. However, with respect to the lung, relatively few reports of expression and regulation of the recently-identified new vascular NADPH oxidase subunits exist. Hoidal and coworkers have demonstrated that NOX4 is the predominant homolog in human airway and pulmonary artery smooth muscle cells. In addition Hohler et al identified a low output NADPH oxidase in pulmonary artery endothelial cells. Against this background, we screened the lung for expression of the new NADPH oxidase isoforms. We sought to assess the regulation of the various isoforms, including the classical phagocytic NADPH oxidase subunits by hypoxia because (1) NADPH oxidases recently have been proposed as possible pulmonary oxygen sensors for the acute response to lung alveolar hypoxia (hypoxic pulmonary vasoconstriction), (2) reactive oxygen species are thought to play a role in the vascular remodeling that occurs during chronic alveolar hypoxia, and (3) the phagocytic

Figure 1. RT-PCR screening for NADPH oxidase subunits in various mouse organs, and real time PCR quantification of NADPH oxidase subunits in homogenized lungs after 3 days and 3 weeks of hypoxia. a, Analysis by RT-RCR of RNA extracts from homogenized tissue. An ethidium bromide-stained gel is illustrated. b and c, Real-time PCR quantification of NADPH oxidase subunits in the lung homogenate after 3 days (duplicate measurements from 3 independent lungs, b) or 3 weeks (duplicate measurements from 5 independent lungs, c) of hypoxia. *Significant difference compared with normoxic controls; boxes, percentiles 25 and 75; black bar, median; whiskers, percentiles 0 and 100; O = value is more than 1.5 lengths of a box away from the edge of a box.
NADPH oxidase subunit NOX2 has recently been suggested to play an important role in hypoxia-induced pulmonary hypertension. The hypoxia-induced vascular remodeling process is characterized by hypertrophy and de novo muscularization of the vessel media, leading to a decrease in vascular luminal area, increased vascular resistance, and thus development of pulmonary hypertension and right ventricular hypertrophy. In essence, we found that NOX4 is the only subunit prominently upregulated in pulmonary arterial vessels and in smooth muscle cells during chronic hypoxia, both at the transcriptional and protein level. Cell culture experiments demonstrated a proproliferative activity of NOX4 during hypoxia, because targeted knock-down of NOX4 with siRNA suppressed pulmonary arterial smooth muscle cell (PASMC) proliferation. Most interestingly, NOX4 expression was upregulated in the vessel media of lungs from patients with idiopathic pulmonary hypertension (IPAH), in comparison to lungs from healthy donors, suggesting an important role of this NADPH oxidase subunit in human IPAH.

**Materials and Methods**

**Chronic Hypoxia Exposure**

All animal experiments were approved by local authorities. Mice (C57BL/6N) of either sex (Charles River Laboratories, Sulzfeld, Germany; 20 to 22 g) were exposed to normobaric hypoxia [inspiratory O₂ fraction (FiO₂) 0.10] in a ventilated chamber for up to 3 weeks as described previously.

**Mouse Lung Preparation for Laser Assisted Microdissection and Right Heart Hypertrophy Assessment**

Mouse lungs were prepared as described previously. For details see the supplemental materials (available online at http://circres.ahajournals.org).

**Laser-Assisted Microdissection**

Laser-microdissection was performed as described previously.

**RNA-Extraction and RT-PCR**

The RNA was extracted from cells using guanidine thiocyanate-acid phenol (RNAzol B, WAK-Chemie, Germany) or with spin-columns.
Real-Time PCR

Relative quantification of the NADPH oxidase subunits was done using ABI prism 7700 detection system (Applied Biosystem, Weiterstadt, Germany). For details please refer to the supplemental materials.

In Situ Hybridization

For a detailed description of the in situ hybridization protocol please refer to the supplemental materials.

Immunohistochemistry for Mouse Lung Sections

Immunohistochemistry was performed as described previously.26 For details see the supplemental materials.

Immunohistochemistry for NOX4 and NOX2 in Human Lung Sections

Lung tissue samples from healthy individuals and from patients with IPAH were formalin-fixed, paraffin-embedded, and cut into 3-μm sections. The immunostaining of the human lung sections was performed with a custom-made rabbit anti-human NOX4 polyclonal antibody29 or rabbit anti-human NOX2 polyclonal antibody (Upstate, Germany) as previously described.26,28

Western Blot of NOX4 in Frozen Human Lung Tissue

For the detection of NOX4 by Western blot, a custom-made polyclonal anti-NOX4 antibody in rabbits was used.28 For details see the online supplemental materials.

Cell Culture

Smooth muscle cells from human and murine pulmonary arteries were isolated and cultured as described previously.29,30 For the investigation of the effect of hypoxia on NOX4 mRNA levels, cells were either exposed to 1% O2 (hypoxia) or to 21% O2 (normoxia).

Immunocytochemistry of Murine PASMCs

Isolated PASMCs were cultured on chamber slides, treated as indicated, fixed in ice cold acetone and methanol (1:1), and blocked with 3% (m/v) BSA in PBS for 1 hour, followed by overnight incubation with an anti-NOX4 antibody (1:25) diluted in 3% (m/v) BSA in PBS.28 Indirect immunofluorescence was obtained by incubation with a Cy3-conjugated anti-goat antibody (Dako, Denmark) diluted 1:100 in PBS for 90 minutes. Nuclear counterstaining was performed with Hoechst-33258 (1:10 000 dilution in PBS; Invitrogen, Karlsruhe, Germany) for 10 minutes.

RNA Interference and Proliferation Assay

A detailed description of the siRNA transfection and the proliferation assay is available in the supplemental materials.

Statistics

Values are given as mean±SEM if not indicated differently. For statistical analysis a Student t test was used for comparison of 2 groups. For more than 2 groups, ANOVA with LSD posthoc test was performed. A probability value of less then 0.05 was considered significant. Empirical assessment of NOX4 immunoreactive vessels was performed in blinded fashion. Two conditions were evaluated for assessment of NOX4-immunoreactive vessels: first, the number of NOX4-immunoreactive vessels different between the groups, and second, the mean diameter of NOX4-immunoreactive vessels different between the groups. Statistical analysis was performed by a nonparametric variance analysis (Kruskal-Wallis test). If the probability value in that test was <0.05, a comparison of the groups between each other was performed using a Mann-Whitney test, where P<0.05 was regarded as significant. Comparison of groups was stopped after P>0.05 to prevent α-inflation.

Results

To investigate the role of NADPH oxidases in the development of hypoxia-induced pulmonary hypertension, expression of the NADPH-oxidase subunits NOX1, NOX2, NOX4, p22phox, p40phox, p47phox, p67phox, as well as NOXO1 and NOXA1, was assessed by RT-PCR in different mouse tissues. As evident from Figure 1a, all subunits were detected in the colon, heart, lung, and pulmonary arteries. In pulmonary arteries NOX2 and NOX4 appeared to be more prominently expressed, as compared with the other subunits investigated (Figure 1a). Focusing on the hypoxic regulation of NADPH oxidation and redox balance, the expression of NOX4 in the mouse lung was assessed by Western blot (Figure 3B).
oxidase subunits in mouse lungs, NOX4 was the only subunit significantly upregulated in homogenized tissue over the time-course of exposure to chronic hypoxia (Figure 1b and 1c). In contrast NOX2, NOXA1, and p67phox were not significantly regulated and NOX1 as well as the other cytosolic NADPH oxidases exhibited an overall downregulation after 21 days of chronic hypoxia (Figure 1b and 1c). Considering that NOX4 was prominently upregulated and NOX2 was previously suggested to play an important role in hypoxia-induced pulmonary hypertension,24 we next investigated the expression of NOX4 and NOX2 in microdissected small pulmonary arterial vessels (~100 μm diameter), the major site of pulmonary vascular remodeling in chronic hypoxia by real-time PCR (Figure 2a through 2c). Comparing these vessels from animals exposed to normoxic (21% O₂) and chronic hypoxic (10% O₂) conditions for up to 3, 7, and 21 days, it was observed that NOX4 mRNA
expression was upregulated in the pulmonary arteries over the course of exposure to hypoxia, with the highest elevation after 3 weeks (Figure 2a). In contrast to NOX4, no regulation of NOX2 was observed (Figure 2b). Under normoxic conditions NOX2 mRNA levels were not different from those of NOX4 (Figure 2c). The hypoxic upregulation of NOX4 paralleled the development of pulmonary hypertension in mice induced by chronic hypoxia. The ratio of the right to the left ventricular mass was 0.27±0.01 in mice maintained under normoxic conditions, and increased to 0.28±0.03, 0.31±0.02, and 0.37±0.01 (n=5 each) after 3, 7, and 21 days of hypoxia, respectively (Figure 2d). In situ hybridization demonstrated NOX4 mRNA expression in different cell types with prominent presence in the vessel media, as confirmed by its colocalization with α-smooth muscle actin (Figure 3a through 3f). Nonvascular area that stained positive for NOX4 mRNA (Figure 3) comprises bronchial smooth muscle cells and may include alveolar type II cells. In this regard we detected NOX4 transcripts in isolated type II cells from the mouse (supplemental Figure I). Our observations that NOX4 mRNA was the predominant NOX mRNA present in the vessel media was also confirmed on the protein level (Figure 4). NOX4 immunoreactivity was observed in a subset of cells of the medial wall of the pulmonary artery, as well as in some smaller pulmonary arteries (Figure 4a through 4e). After exposure to chronic hypoxia, the number of NOX4-positive vessels was significantly increased after 3 days of exposure to hypoxia (Figure 4f). The number of small NOX4 immunopositive vessels was also significantly increased after 7 and 21 days of hypoxia (Figure 4g), indicating that the newly-formed smaller vessels were also NOX4-immunoreactive. At the sub-cellular level, NOX4 protein exhibited a predominantly perinuclear localization in mouse PASMCs with increased intensity after 48 hours of hypoxic incubation (Figure 5).

Histological staining of human lung sections from healthy donors and from patients with idiopathic pulmonary arterial hypertension (IPAH) confirmed NOX4 expression in the vessel media of the pulmonary arteries (Figure 6a through 6d). In contrast to NOX4 we found that NOX2 was primarily expressed in the endothelial layer of the human pulmonary arteries (Figure 6e and 6f). Western blot analysis revealed a significant (P<0.001) 2.5-fold higher NOX4 protein level in lungs from IPAH patients compared with healthy donor lungs (Figure 7a, full blot and specificity of the NOX4 antibody see supplemental Figure IIa). In addition, NOX4 transcripts quantified by real-time PCR were increased in human donor PASMCs from passage 3 exposed to hypoxia for 24 hour, compared with normoxic controls (Figure 7b). To confirm a functional role for NOX4 in cell proliferation, we demonstrated that siRNA directed against human NOX4 significantly reduced the NOX4 mRNA level (Figure 8a and suppressed the proliferation of human passage 3 PASMCs (Figure 8b) correlating with a decrease of reactive oxygen species (ROS) generation (Figure 8c). Reduced proliferation of human PASMCs after siNOX4 treatment was additionally confirmed by cell counting (supplemental Figure III). As previously reported for systemic and PASMCs the NOX4 levels decreased with higher passages (supplemental Figure IVa). However, siRNA against NOX4 decreased cell proliferation of both passage 3 and passage 5 cells with higher efficacy in passage 5 cells (see supplemental Figure IVb).

Discussion

No extensive analysis of the expression of the NADPH-oxidase subunits, particularly nonphagocytic NADPH oxidase subunits, in the lung and their regulation in hypoxia has been performed to date. Thus, in screening for the expression of the NADPH oxidase subunits NOX1, NOX2, NOX4, p22phox, p40phox, p47phox, p67phox, NOXA1, and NOXO1 in different mouse organs, it was observed that all of these subunits could be detected by RT-PCR in lung tissue with a similar signal intensity compared with the colon and heart.
In 2 recent investigations, elevated expression of NOX1 and NOX4 was demonstrated for the colon and kidney by Northern blotting. In a previous study, we identified NOX1 in homogenized rabbit lungs and in PASMCs. Recently, NOX4 was suggested to be the predominant NOX2 homolog in human airway PASMCs, and Liu and colleagues provided evidence that the phagocytic NADPH oxidase subunit NOX2 plays an important role in the development of hypoxia-induced pulmonary hypertension.

Investigations into the recently identified new isoforms of phagocytic NADPH oxidase subunits in the lung is of interest, because NADPH oxidases have been proposed as possible pulmonary oxygen sensors. Suliman and co-workers supported a possible role for NOX4 in the context of oxygen sensing in the mouse kidney, demonstrating induced expression of the renal-specific NADPH oxidase (NOX4) under hypoxic conditions.

With respect to the lung vasculature, oxygen sensing is important under circumstances of acute hypoxia (lasting seconds to minutes) as well as of chronic hypoxia (lasting days to months and years). Acute alveolar hypoxia induces constriction of pulmonary arterial vessels, which is an essential mechanism to adapt perfusion to ventilation, and thus to optimize pulmonary gas exchange. Recently we have demonstrated that a nonphagocytic NADPH oxidase may play an important role in the acute hypoxic response of the pulmonary arteries in the lung.

In contrast, chronic alveolar hypoxia induces remodeling of the pulmonary vasculature, characterized by hypertrophy of the vessel media, and thus a narrowing of the vascular lumen. This leads to an increased pulmonary vascular resistance, pulmonary hypertension, and ultimately resulting in right heart failure. For both acute and chronic alveolar hypoxia, a possible role for reactive oxygen species has been
widely discussed\textsuperscript{19,22,35,36} and the phagocytic NADPH oxidase NOX2 has been demonstrated in a knockout mouse model to be of major importance for the development of hypoxia-induced pulmonary hypertension. With respect to an additional role for nonphagocytic NADPH oxidase subunits in the pathophysiology of hypoxia-induced pulmonary hypertension, the present study focused on NOX4, because: (1) NOX4 is the only nonphagocytic NADPH oxidase subunit prominently expressed in pulmonary arteries, (2) NOX4 was the only NADPH oxidase subunit upregulated in chronic hypoxia in homogenized mouse lung tissue (Figure 1b and 1c), (3) NOX4 acts as an oxygen sensor to regulate TASK-1 activity in HEK 293 cells, and (4) it was recently suggested that this subunit may contribute to pathophysiological changes in the systemic vasculature and in the pulmonary arteries.\textsuperscript{1,10,20,37} Moreover, we compared the regulation of NOX4 to that of NOX2 considering the recent findings by Liu et al.\textsuperscript{24} As remodeling of small pulmonary arteries is thought to be the major cause of the increase in vascular resistance occurring during chronic hypoxia, we focused on the hypoxia-dependent regulation of NOX4 and NOX2 mRNA in these vessels of the murine pulmonary vasculature. Our analysis revealed that NOX4, in contrast to NOX2, is elevated in the pulmonary vasculature by chronic hypoxia: upregulation of NOX4 but not of NOX2 occurred in the pulmonary arteries within 21 days of exposure to hypoxia, as demonstrated by quantitative PCR of microdissected vessels. Moreover, in situ hybridization revealed that NOX4 transcripts were localized to the pulmonary artery smooth muscle layer. The hypoxia-dependent increase in NOX4 expression levels in the pulmonary vasculature correlated well with the development of pulmonary hypertension,\textsuperscript{38} and was corroborated further at the protein level: (1) NOX4-immunoreactivity was detected in the pulmonary vasculature...

\begin{figure}[h]
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\caption{Detection of NOX4 by Western blot in human donor and IPAH lungs and hypoxia-induced upregulation of NOX4 in isolated PASMCs. a, Western blots of human IPAH lungs (n=4) compared with healthy donor lungs (n=6) revealed a 2.5-fold upregulation of NOX4 expression in human IPAH lungs (specific band at 64 kDa). The NOX4 was normalized to β-actin. The full blot as well as the specificity of the antibody is shown in supplemental Figure Ila. *Significant difference as compared with donor lungs. b, Isolated human PASMCs were maintained under hypoxic (1% O\textsubscript{2}) or normoxic (21% O\textsubscript{2}) conditions for 24 hours. The NOX4 mRNA levels were quantified by real-time PCR and standardized to β-2-microglobulin mRNA levels. A significant increase in NOX4 mRNA was observed after 24 hours of hypoxic vs normoxic treatment (*). Data are derived from duplicate measurement of n=3 independent cell preparations.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure8}
\caption{Suppression of human PASMC proliferation and reactive oxygen species generation by siRNA directed against NOX4. a, Quantification of NOX4 in siNOX4-transfected human PASMCs showing significant downregulation of NOX4 as compared with scrambled control. b, The normoxic proliferation of PASMCs was investigated using 3H-thymidine. c, Reactive oxygen species quantification by dihydroethidium fluorescence in scrambled and NOX4 siRNA transfected human PASMCs. *Significant differences between NOX4 siRNA and scrambled siRNA experiments. Data are derived from duplicate cell isolations of n=5 independent lungs. cpm indicates counts per minute.}
\end{figure}
by immunostaining, and (2) the percentage of NOX4 immunoreactive vessels was strongly increased by chronic alveolar hypoxia with an increase in the number of NOX4-positive small vessels. The upregulation at the protein level (Figure 4f) preceded the regulation on the mRNA level (Figure 2a). This suggests that NOX4 can be regulated on both the mRNA and the protein level. It was also evident from immunohistochemical data that the upregulation of NOX4 occurs in the vessel media, further supporting the idea that NOX4 contributes to the pathophysiological process of hypoxia-induced vascular remodeling in the lung, which is triggered by ROS-dependent smooth muscle cell proliferation. In line with this observation and the recent finding of Sturrock et al, the silencing of NOX4 by siRNA reduced human PASM C proliferation as well as ROS generation. A possible role for NOX4 in the pathogenesis of pulmonary hypertension in general was supported by the fact that NOX4 is upregulated in the vessel media of lung sections from patients with IPAH, compared with healthy donor lungs. The perinuclear localization of NOX4 in the PASMCs supports the notion of the presence of the protein in the endoplasmic reticulum (ER), as recently demonstrated in microvascular endothelial cells by Petry and coworkers. The presence of NOX4 in the ER further suggests an important role of NOX4 in maintaining the redox potential and Ca\(^{2+}\)-homeostasis in PASMCs.

The findings of Liu et al that NOX2 is essential for development of hypoxia-induced pulmonary hypertension, together with the fact that we as well as Liu et al were unable to detect regulation of NOX2 in pulmonary arteries by hypoxia, are suggestive that NOX2 and NOX4 play a differential role in the development of hypoxia-induced pulmonary hypertension. Hypothetically, endothelial ROS generation by NOX2 may stimulate NOX4 upregulation in the vessel media, which would be important for hypoxia-dependent PASMC proliferation. In line with this argumentation is the detection primarily of NOX2 in pulmonary vascular endothelial cells in our study, as well as 2 recent reports demonstrating a ROS-dependent upregulation of NOX4 in cardiac cells.

The fact that NOX4 is upregulated in the vessel media in both hypoxia-induced pulmonary hypertension and in human IPAH may be explained by distinct or common regulators of NOX4. With regard to the latter it has been shown that TGF-\(\beta\) can upregulate NOX4 in human PASMCs, that hypoxia can increase TGF-\(\beta\) in PASMCs, and that interference with TGF-\(\beta\) blocks hypoxia-induced vascular remodeling. Interestingly, it has been shown that TGF-\(\beta\) can vice versa be regulated by ROS. Thus, hypoxia-induced and human IPAH may share some common pathophysiological mechanisms with regard to NOX4.

In conclusion, we demonstrate in the present study that all major subunits of the phagocytic as well as nonphagocytic NADPH-oxidase subunits are expressed in the lung. Furthermore, NOX4 was found to be upregulated in the pulmonary vasculature, both in chronic hypoxic pulmonary hypertension as well as in human IPAH. The correlation of NOX4 expression with the development of pulmonary hypertension suggests a contribution of NOX4 to the development of this disease. With respect to the upregulation of NOX4 in IPAH patients, a functional interference with NOX4 may offer a new therapeutic approach for the treatment of this disease.

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Disclosures
None.

References
of the differentiated vascular smooth muscle cell phenotype. Arterioscler


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EXPANDED MATERIALS AND METHODS

Mouse lung preparation for laser assisted microdissection and right heart hypertrophy assessment.

After 3, 7 and 21 days of hypoxia, the animals were euthanized by intraperitoneal injection of a lethal ketamine and xylazine dose, and the lungs were flushed with Krebs Henseleit buffer (125.0 mM NaCl, 4.3 mM KCl, 1.1 mM KH$_2$PO$_4$, 2.4 mM CaCl$_2$, 1.3 mM MgCl$_2$ and 13.32 mM glucose) through a catheter in the pulmonary artery at a pressure of 20 cm H$_2$O at room temperature. The buffer was pre-equilibrated with a gas mixture of 3%O$_2$, 5.3%CO$_2$, balanced N$_2$. NaHCO$_3$ was adjusted to result in a pH of 7.4. During perfusion of the lungs, the buffer was allowed to drain freely from a catheter in the left ventricle. Once the effluent was clear of blood, lungs were dissected from the thoracic cavity and immediately frozen in liquid nitrogen for mRNA analysis.

For immunohistochemistry, in situ hybridization and laser assisted microdissection, 800 µl pre-warmed TissueTek® (Sakura Finetek, Zoeterwoude, The Netherlands) was instilled into the airways via a tracheal cannula. After ligation of the trachea, the lungs were excised and immediately snap frozen in melting isopentane. Preparation of the hypoxic animals was performed continuously in a hypoxic environment (10% O$_2$). Lungs from normoxic animals (control) were prepared accordingly under normoxic conditions. The right ventricular wall (RV) was separated from the left ventricle plus septum (LV+S) to calculate the ratio of RV/(LV+S) of the dehydrated heart tissue. For analysis of colon, heart, and pulmonary arteries, these tissues and organs were removed immediately after the lung had been flushed.

RT-PCR

For reverse transcription (RT) of extracted RNA, 1 µg of total RNA was denatured at 65 °C for 5 min. After cooling on ice, the following components were added to the samples: 5 µl of 5× RT buffer, 2 µl of 10 mM deoxynucleotide mixture, 1 µl of random-hexamer primer, 0.5 µl of 0.1 M dithiothreitol, 1 µl Moloney murine leukemia virus reverse transcriptase (MMLV) (Gibco-BRL, Karlsruhe, Germany) and volume for adjustment to 20 µl. After 10 min of incubation at room temperature, and
60 min at 39 °C, reverse transcriptase was inactivated by heating the mixture to 95 °C. For PCR reactions, 4 µl of 10 µM forward primer, 4 µl of 10 µM reverse primer, 10 µl of 10× PCR buffer, 61 µl of water and 0.5 µl of hot-start-Taq-polymerase (Qiagen, Hilden, Germany) were added to 20 µl of the cDNA. The thermal cycler profile consisted of an initial incubation at 95°C for 15 min, followed by 40 cycles of 95 °C 30 s, 62 °C, 30 s and 72 °C, 60 s, and a final extension at 72 °C for 10 min. For the sequences of primers used in RT PCR see Online Table I.

Real-time PCR

All primers used in the real time PCR experiments were intron-spanning. The Ct values of NOX4 were normalized to the endogenous control B2M (β2-microglobulin) in both mouse and human samples (1). For cDNA synthesis reagents and incubation time were applied as described before (2). The fold change $2^{ΔΔCt}$ was calculated as described previously (3). For the sequences primers used in real time PCR please see Online Table II.

Riboprobes for in situ hybridization

Single-stranded digoxigenin (DIG)-labeled riboprobes for non-isotopic in situ hybridization were generated by the in vitro transcription method. The template for the generation of single-stranded RNA probes was amplified by nested PCR. Briefly, 1 µg of the purified PCR-amplified template harbouring T3 and T7 RNA polymerase promoter sequences was mixed with 2 µl digoxigenin-11-uridine triphosphate (Roche, Mannheim, Germany), 4 µl of 5× transcription buffer (Promega, Mannheim, Germany), 1 µl of RNasin (Peqlab, Erlangen, Germany) and 2 µl of T3 or T7 Phage polymerase (Promega, Mannheim, Germany) in a total reaction volume of 20 µl. The reaction mixture was incubated at 37 °C for 2 h. The RNA probes were purified with a Qiagen PCR purification kit (Qiagen, Hilden, Germany).
Combined approach of non isotopic in situ hybridization and immunofluorescence on mouse lung sections

The non-isotopic in situ hybridization (NISH) was performed on 8 µm thick TissueTek®-embedded mouse lung cryostat sections. The sections were heated at 55 °C for 15 min followed by transferring the sections in to 2× SSC buffer for 30 min at 70 °C, diethylpyrocarbonate-treated water for 1 min, Proteinase K (5 µg/ml) for 10 min at room temperature, 0.2% (m/v) glycine in PBS solution (for the inactivation of Proteinase K) for 30 s, PBS for 30 s, freshly prepared cold 4% (m/v) paraformaldehyde for 20 min, PBS for 5 min, 0.1 M acetylated triethanolamine (0.5 ml of acetic anhydride/200 ml of triethanolamine) on a shaking platform for 10 min, PBS for 3 min, followed by dehydration of slides by passing through 70% (v/v), 80% (v/v) and 90% (v) ethanol (each for 2 min). The slides were then pre-hybridized with 2× Prehyb solution (1 M NaCl, 0.02 M Tris (pH 7.5), 2× Denhardt’s reagent, 2 mM EDTA, 10 mg/ml salmon sperm DNA, 0.2 mg/ml yeast tRNA) at 55 °C for 2-3 h in a humidified chamber, followed by hybridization with a denatured antisense NOX4 probe in 2× hybridization solution (1 M NaCl, 0.02 M Tris (pH7.5), 2× Denhardt’s reagent, 2 mM EDTA, 2 g dextran sulphate, 0.2 mg/ml yeast tRNA) at 55 °C for overnight. The following day, slides were washed from low to very highly stringent conditions as follows: on shaking platform 2× SSC for 1 h at room temperature, 0.1× SSC at 60 °C and finally to pre-heated 0.1x SSC (at 60 °C), followed by incubation at room temperature. The sections were treated with blocking buffer (2% Blocking reagent (Roche, Mannheim, Germany), 0.1% (m/v) BSA, 0.1 M Tris (pH 7.5), 5 M NaCl) for 30 min at room temperature, followed by incubation with a peroxidase-labeled anti-DIG antibody (Roche, Mannheim, Germany) in 1:20 dilution for 2 h at room temperature. After antibody incubation, sections were washed in TBT buffer (50 mM, 1 M Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Triton X-100; 3 × 15 min). The fluorescent substrate Alexa fluor 488 tyramide (Molecular Probes, Invitrogen, Karlsruhe, Germany) at a dilution of 1:60 in amplification buffer was applied to the sections for 2 h. Subsequently, sections were washed (3 × 20 min) in PBT buffer (PBS, 0.1% (v/v) Tween 20) and incubated with a mouse monoclonal Cy3-labeled α-smooth muscle actin antibody (Sigma, Hamburg, Germany) at 1:500 in PBS, for 1 h. The sections were washed (3 × 3 min) in PBS and subsequently
incubated with Hoechst-33258 (1:10.000 in PBS; Invitrogen, Karlsruhe, Germany) for 10 min, washed and mounted in carbonate-buffered glycerol (pH-8.6).

**Type II cell isolation**

Mouse alveolar type II cells were isolated as described previously (4).

**Immunohistochemistry for mouse lung sections**

Cryosections (10 µm) were fixed in ice-cold acetone for 10 min and unspecific binding of primary antibodies was blocked by incubation with 50% (v/v) heat-inactivated normal swine serum in phosphate buffered saline (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate and 150 mM NaCl, pH 7.4 (PBS)) with double the salt concentration (PBS+S) for 1 h. Incubation was performed overnight with an affinity-purified rabbit polyclonal anti-NOX4 antibody diluted 1:200, together with a mouse monoclonal FITC-conjugated α-smooth muscle actin antibody (clone 1A4, 1:500; Sigma, Deisenhofen, Germany) in PBS+S (5). After washing in PBS, the sections were incubated with a Cy3-conjugated donkey anti-rabbit antibody (Chemicon, Hofheim, Germany) diluted 1:2000 in PBS+S for 1 h, and after a final wash step, the sections were mounted with carbonate-buffered glycerol, pH 8.6. To test specificity, the NOX4 antibody was pre-incubated for 1 h with the corresponding peptide antigen (20 µg/ml). All labeling reported was specific as judged from its absence in sections incubated with pre-adsorbed antibody. Immunoreactivity was evaluated using an Axioplan 2 imaging epifluorescence microscope (Zeiss, Göttingen, Germany) equipped with a Axiocam digital camera, Axiovision software (Zeiss, Göttingen, Germany), and with appropriate filter sets for Cy3 and FITC or with a TCS-SP2 AOBS confocal laser scanning microscope (Leica, Heidelberg, Germany).

**Quantitative analysis of mouse lung sections**

Examination of sections was performed blinded. For every animal, 50 α-smooth muscle actin-immunoreactive vessels were evaluated for anti-NOX4-immunoreactive cells in the vessel wall, and
the vessel diameter was measured using the Axiovision software. To restrict analysis to smaller blood vessels, vessels with a diameter larger than 60 µm were not examined.

Western blot of NOX4 in frozen human lung tissue

Protein extracts were prepared from frozen lung tissues from healthy human donor lungs and lungs from patients with IPAH in RIPA buffer containing 1 mM sodium vanadate, Protease-Inhibitor Mix complete (Roche, Mannheim, Germany) and 0.1 mM PMSF. Equivalent amounts of protein were resolved on 10% SDS polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corporation, Dreieich, Germany) by semi-dry electro-blotting. Nonspecific antibody binding was blocked by incubation in 6% (m/v) non-fat dry milk powder in T-TBS (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) at room temperature for 1 h. Incubation with a 1:5000 diluted anti-NOX4 primary antibody was performed at 4 °C overnight. After washing the membranes in T-TBS buffer, specific immunoreactive signals were detected by enhanced chemiluminescence (ECL, Amersham, Freiburg, Germany) using a secondary antibody coupled to horseradish-peroxidase.

RNA interference and proliferation assay

Human pulmonary arterial smooth muscle cells (10,000 cells per well) from passage 3 were cultured in 48-well tissue culture plates, and used for the RNA interference and proliferation assays. Transfection of NOX4 siRNA was performed in low-serum and antibiotic-free medium (1% (v/v) FCS in DMEM). The medium was changed at least 4 h before transfection. Approximately 100 nM of NOX4 siRNA (5'-GGUACAGCUGGAUGUUGAC-3') (Eurogentec, Seraing, Belgium) was transfected using 1 µl X-tremeGENE siRNA Transfection Reagent (Roche, Mannheim, Germany) per cm² of the well. Both siRNA and transfection reagent were diluted in OPTI-MEM medium (Gibco, Karlsruhe, Germany). For controls, a FITC-labelled, scrambled siRNA (Invitrogen, Karlsruhe, Germany) was employed. After five hours of transfection, the medium was changed to low-serum medium containing antibiotics (1% (v/v) FCS, 1% (m/v) penicillin and streptomycin in DMEM and incubated overnight for cellular synchronisation. The following day, the cells were stimulated with
smooth muscle cell medium (Promocell, Heidelberg, Germany) supplemented with medium containing 5% (v/v) FCS and antibiotics for 20 h, followed by ³H-thymidine (Amersham, Munich, Germany) for 4 h. Subsequently, cells were washed 3× with ice-cold PBS and lysed with NaOH on a shaker for 4 h. The radioactivity in the lysate was measured by liquid scintillation counting (Rotiszint® eco plus, Roth, Germany) with a Packard liquid scintillation counter. For determination of cell numbers cells/ml were counted after synchronization for 72 h.

**ROS measurement and quantification**

ROS in human PASMC were measured using superoxide sensitive dye DHE (Dihydroethidium, Invitrogen). Briefly, the cells were grown on chamber slides, transfected with scrambled and NOX4 siRNA and incubated in normoxic and hypoxic chambers for 24 h. After 24 h the cells were incubated with 5 µM of DHE for 15 min in normoxic and hypoxic chamber. Subsequently the cells were washed in PBS, fixed in acetone and methanol mixture (1:1) for 10 min and stained with nuclear stain Hoechst-33258. The cells were visualized under a fluorescent microscope (excitation: 514 nm; emission: 560 nm). A total of eight images was captured from each group in a blinded fashion and were quantified using Image J software (U.S. National Institutes of Health, Bethesda, MD).

**Online Table I: Nucleotide sequences of primers used for Reverse Transcriptase-Polymerase Chain Reaction (RT PCR)**

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<th>Species</th>
<th>Primer</th>
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### Online Supplement

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**Online Table II: Nucleotide sequences of primers used for quantitative real time PCR**

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SUPPLEMENTARY FIGURES

Supplementary figure 1
RT-PCR of isolated alveolar type II cells.
RT-PCR revealed the presence of NOX4 mRNA transcripts in mouse alveolar type II cells.

Supplementary figure 2
Specificity of the NOX4 antibody.
a) Full Western-blot for NOX4 in human donor and IPAH lungs
Western-blot of human IPAH lungs (n=4) compared to healthy donor lungs (n=6) revealed a 2.5-fold up-regulation of NOX4 expression in human IPAH lungs (specific band at 64 kDa). NOX4 was normalized to β-actin.
b) Down-regulation of NOX4 protein by siRNA directed against NOX4 as recognized by the NOX4 antibody. PASMC were transfected with siRNA directed against NOX4 or with scrambled siRNA 65 h prior to western-blotting. β-actin was used as loading control.

Supplementary figure 3
Suppression of human pulmonary arterial smooth muscle cell proliferation by siRNA directed against NOX4.
Passage 3 isolated human pulmonary arterial smooth muscle cells (PASMC) were either transfected with 100 nM NOX4 siRNA or scrambled siRNA as control. The normoxic proliferation of PASMC was investigated by changes in cell numbers. Data are derived from duplicate cell isolations from n=3 independent lungs.

Supplementary figure 4
a) Comparison of NOX4 mRNA transcript levels between different passages in human PASMC.
NOX4 mRNA transcript levels were quantified by real-time PCR for cell passage 3, 4 and 5. Data are from duplicate measurements of at least 3 different cell isolations. ($\Delta Ct = Ct_{B2M} - Ct_{NOX4}$).
b) Cell proliferation and its suppression by siRNA directed against NOX4 in human PASMC from passage 5. Isolated human human pulmonary arterial smooth muscle cells (PASMC) were either transfected with 100 nM NOX4 siRNA or scrambled siRNA as control. Data are from n=3-5 cell isolations.

REFERENCES


Supplementary Figure 2a
Supplementary Figure 2b
Supplementary Figure 3

Cell numbers after proliferation (%)

- Scrambled siRNA
- NOX4 siRNA
Supplementary Figure 4

a) 

ΔCt

<table>
<thead>
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<th>Passage 3</th>
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b) 

Thymidine incorporation [cpm]

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Passage 5