The NADPH Oxidase Subunit NOX4 Is a New Target Gene of the Hypoxia-inducible Factor-1

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NADPH oxidases are important sources of reactive oxygen species (ROS), possibly contributing to various disorders associated with enhanced proliferation. NOX4 appears to be involved in vascular signaling and may contribute to the response to hypoxia. However, the exact mechanisms controlling NOX4 levels under hypoxia are not resolved. We found that hypoxia rapidly enhanced NOX4 mRNA and protein levels in pulmonary artery smooth-muscle cells (PASMCs) as well as in pulmonary vessels from mice exposed to hypoxia. This response was dependent on the hypoxia-inducible transcription factor HIF-1α because overexpression of HIF-1α increased NOX4 expression, whereas HIF-1α depletion prevented this response. Mutation of a putative hypoxia-responsive element in the NOX4 promoter abolished hypoxic and HIF-1α–induced activation of the NOX4 promoter. Chromatin immunoprecipitation confirmed HIF-1α binding to the NOX4 gene. Induction of NOX4 by HIF-1α contributed to maintain ROS levels after hypoxia and hypoxia-induced proliferation of PASMCs. These findings show that NOX4 is a new target gene of HIF-1α involved in the response to hypoxia. Together with our previous findings that NOX4 mediates HIF-1α induction under normoxia, these data suggest an important role of the signaling axis between NOX4 and HIF-1α in various cardiovascular disorders under hypoxic and also nonhypoxic conditions.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion radicals (O₂⁻) and hydrogen peroxide (H₂O₂) have been for a long time recognized as unwanted byproducts of oxidative ATP generation in the mitochondria. More recently, ROS have also been shown to act as important signaling molecules in a variety of physiological and pathophysiological conditions (Wolin, 1996; Finkel, 1999; Griendling et al., 2000). Thereby, the family of NADPH oxidases represents the only known enzyme system whose primary biological function is to produce ROS (Babior, 1999). This group of multicomponent enzymes generates superoxide anion radicals in a regulated manner by allowing the transfer of electrons from NADPH via a catalytic core protein (NOX) containing flavin and heme moieties to molecular oxygen. The NOX family comprises five isoforms that display distinct patterns of tissue specificity (NOX1 to NOX5; Cheng et al., 2001; Bedard and Krause, 2007; BelAlba et al., 2007). Initially, a NOX2-containing NADPH oxidase has been described to be part of the innate immune response in phagocytes by generating superoxide anion radicals in the respiratory burst (Babior et al., 2002). Stimulation of ROS production by this enzyme requires a complex signaling mechanism involving phosphorylation and translocation of cytoplasmic proteins such as p47phox and p67phox as well as the GTPase Rac (Babior et al., 2002). Compared with NOX2, the other family members, which are often coexpressed in the same cells, differ in their subunit requirements and subcellular localization (Lambeth et al., 2007; Ushio-Fukai, 2009). NADPH oxidases gained increasing interest and importance as sources of ROS in the vasculature (Griendling et al., 2000). Vascular smooth-muscle cells express NOX1-, NOX4- and NOX5-containing enzymes, whereas endothelial cells express in addition the NOX2 protein (Gorlach et al., 2000; Cheng et al., 2001; Bedard and Krause, 2007; BelAlba et al., 2007). Various humoral factors have been shown to activate NADPH oxidases within minutes due time involving a complex cascade of signaling events including protein phosphorylation and translocation of cytosolic subunits (Brown and Griendling, 2009). In contrast, NOX4 has been suggested to act constitutively and not to require cytosolic regulators for its function (Martyn et al., 2006). Thus, changes in NOX4-dependent oxidase activity appear to be directly related to NOX4 abundance. NOX4 has been reported to be the predominant homolog in human airways and pulmonary artery smooth-muscle cells (PASMCs; Djordjevic et al., 2005a; Sturrock et al., 2006; Mittal et al., 2007). PASMCs have been suggested to be particularly sensitive to oxygen availability and to be responsible for acute hypoxic vasoconstriction and the development of pulmonary hypertension due to chronic hypoxia (Weir et al., 2005; Aaronson et al., 2006; Stenmark et al., 2006; Gupte and Wolin, 2008). Interestingly, enhanced NOX4 levels have been identified in PASMC exposed to hypoxia (Mittal et al., 2007; Ismail et al., 2009), although the underlying mechanisms have not been clarified.

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Hypoxia-inducible transcription factors (HIFs) have been recognized as master regulators of oxygen-regulated gene expression (Ratcliffe et al., 1998; Semenza, 2000). HIF-1 is the most abundant family member. It is a heterodimer composed of an inducible α-subunit (HIF-1α) and a constitutive β-subunit and has been implicated in physiological and pathophysiological responses toward hypoxia (Wenger et al., 2005), but has also been shown to be up-regulated under nonhypoxic conditions (Richard et al., 2000; Gorlach et al., 2001; Gorlach and Kietzmann, 2007). In this regard, we could demonstrate that NOX4 is involved in the up-regulation of HIF-1α (Bonello et al., 2007). On the other hand, a link between HIF-1α and NOX4 levels has not been established.

In this study we cloned the human NOX4 promoter and evaluated the role of HIF-1α in the regulation of NOX4 expression under hypoxic conditions.

MATERIALS AND METHODS

Materials

All reagents were from Sigma (Taufkirchen, Germany) unless otherwise stated.

Cell Culture

Human PASMCS were obtained from Cambrex (Verviers, Belgium), cultured in the medium provided as recommended, and used from passages 3–11. Human embryonic kidney cells (HEK293, ATCC CRL-1573, Manassas, VA) and human epithelial bronchial cells (HepG2, ATCC HB-8065) were grown in DMEM (GIBCO, Darmstadt, Germany) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Plasmids and Transfections

A 2355-base pair fragment from the NOX4 gene (gene Id: 58057) upstream from the transcriptional start site was amplified with primers containing restriction sites (forward: 5'-GTA AAG CAA CAT AAA CAG ATC TCA GTCC CCC TC-3' and reverse: 5'-ACG GCC ATG GCC GCG CCG C-3') and digested with KpnI to reveal a 968-base pair fragment containing 730 base pairs of the NOX4 promoter and 238 base pairs of the first exon. This fragment was inserted into pGL3basic (Promega, Mannheim, Germany) to reveal pGLNOX4-730. Site-directed mutagenesis was performed at a putative hypoxia-responsive element (HRE) at −391 to −387 base pairs upstream of the transcription start site by inserting a G→T base exchange at −387 base pairs using the QuickChange mutagenesis kit (Promega) and the following primers: forward: 5'-GAG GAA GGG TGG GAG AAA CGT TAA CTA GCA CAC-3' and reverse: 5'-TCT GCA TGC CTT CAC AA-3'. Site-directed mutagenesis was performed at a putative hypoxia-responsive element (HRE) at −391 to −387 base pairs upstream of the transcription start site by inserting a G→T base exchange at −387 base pairs using the QuickChange mutagenesis kit (Promega) and the following primers: forward: 5'-GTA AAG CAA CAT AAA CAG ATC TCA GTCC CCC TC-3' and reverse: 5'-ACG GCC ATG GCC GCG CCG C-3'.

Cloning of the NOX4 promoter

The vectors encoding short hairpin RNA (shRNA) against HIF-1α, HIF-1β, and human hepatoblastoma cells (HepG2, ATCC HB-8065) were grown in T75 flasks to confluency and exposed to 1% oxygen. Cells were fixed with formaldehyde, lysed, and human hepatoblastoma cells (HepG2, ATCC HB-8065) were grown in T75 flasks to confluency and exposed to 1% oxygen. Cells were fixed with formaldehyde, lysed, and cell pellets were harvested. Total RNA was isolated and treated with RNase free DNase. Blots were scanned and analyzed using GelDoc software (BioRad, Munich, Germany).

RNA Extraction and RT-PCR

RNA was extracted from human PASMCS or mouse tissue or from cells using RNaseasy Mini or Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1 μg RNA using reverse transcriptase (Invitrogen, Karlsruhe, Germany).

RT-PCR primers were as follows: mouse NOX4: forward, 5'-TGT TGC ATG TTC CAT GTG TT-3' and reverse, 5'-TAC TGG CCA TTC CTG CTT T-3'; mouse 18S: forward, 5'-GGC GCT GCC TGG TGA ACT C-3' and reverse, 5'-GTC GCT TCC GTG AAT TTT GC-3'; mouse human NOX4: forward, 5'-GAA GTA TGC CAT GTA ATC TCA-3' and reverse, 5'-GCC TCT ACA AAA ACC CCA AA-3'; mouse human 18S: forward, 5'-CCA AGG CAG TAT GAG AGC G-3' and reverse, 5'-GAC TTG GTT GGC GCT ACT GC-3'; mouse and human actin: forward, 5'-GTT GGT TTT CGG AAC TGA GG-3' and reverse, 5'-GCC GCC TTA GCC TCC TC-3'; mouse α-actin gene (gene ID: 60) not containing a putative HRE (5'-ACGTG-3') and reverse, 5'-ACGTG-3').

Western Blot Analysis

Confluent HepG2 cells were grown in T75 flasks to confluence and exposed to hypoxia (1% oxygen) for 3 h. Cells were fixed with formaldehyde, lysed, and cell pellets were harvested. Total RNA was isolated and treated with RNase free DNase. Blots were scanned and analyzed using GelDoc software (BioRad, Munich, Germany).

Phenol Red-free DMEM

Phenol Red-free DMEM (Corbett). As negative control to analyze unspecific binding and precipitation, a Rotor-Gene 6000 (Corbett). As negative control to analyze unspecific binding and precipitation, a Rotor-Gene 6000 (Corbett).

Chromatin Immunoprecipitation

Confluent HepG2 cells were grown in T75 flasks to confluence and exposed to hypoxia (1% oxygen) for 3 h. Cells were fixed with formaldehyde, lysed, and cell pellets were harvested. Total RNA was isolated and treated with RNase free DNase. Blots were scanned and analyzed using GelDoc software (BioRad, Munich, Germany).

Measurement of ROS Production

ROS generation was detected using the fluoroprobe dihydroethidium (DHE; Invitrogen) as described previously (Bonello et al., 2007). As background control, chromatin immunoprecipitation (Chip) without antibody was performed. Statistical analysis was performed using a Student’s t-test of the input. Hif-1α binding to chromatin was revealed after background subtraction as relative amount of the input used.

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KCl, 25 mM NaHCO₃, 1.03 mM KH₂PO₄, 5.6 mM d-glucose, 20 mM Na-HEPES, 2.5 mM CaCl₂, 1.2 mM MgSO₄ supplemented with 25 μM desferoxamine and 5 μM o,o-dimethyl-thio-phosphoryl-chloride (DETC). Before measurements, the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen, Elzach, Germany) was added to a final concentration of 100 μM. Cell suspension was scanned 20 times at 37°C for a total of 10 min in an EPR spectrometer with temperature control (e-scan, Noxygen) with the following parameters: microwave power = 23.89 mW; center field = 3459–3466 G; modulation frequency = 86 kHz; scan time 10.49 s per scan; and modulation amplitude = 2.93 G. Superoxide generation rate was calculated using linear regression and normalized to the protein content.

**Proliferation and Migration Assays**

DNA synthesis was assessed by 5-bromo-2-deoxyuridine (BrdU) labeling (Roche) as described previously (Djordjevic et al., 2005b) after exposure to hypoxia for 4 h. Similarly, total cell numbers were evaluated after stimulation by hypoxia for 4 h by cell counting in a standard hemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany). Cell migration was measured using a 35-mm gridded μ-dish (Ibidi, Martinsried, Germany). Transfected cells were grown to confluency, wounded with a 10-μl tip, and exposed to hypoxia for 4 h. Phase-contrast images were captured at 0 h (control) and 24 h using an inverted microscope. The number of migrated cells was counted. Relative number of migrated cells per 250-μm² scratch area was calculated and related to control or transfected cells.

**Animal Experimentation**

C5BL/6 female mice (3 wk old, 20–25 g; Charles River, Sulzfeld, Germany) were exposed to normobaric hypoxia (10% O₂) in a ventilated chamber (Ing. Humbs, Valley, Germany) for 1 d. Age-matched control animals breathed room air. The mice were killed, and lungs were dissected and either snap-frozen in liquid nitrogen or inflated and Formalin-fixed. All animal procedures were approved by the local legislation on protection of animals (Regierung von Oberbayern, Munich, Germany).

**Immunohistochemistry**

Immunohistochemistry was performed using the LSAB2 HRP Systems (Dako, Hamburg, Germany) with antibodies against smooth muscle α-actin (Dako) or with the antibody against NOX4. The antibodies were diluted at 1:500 in 5% BSA. Hemalum was used for counterstaining.

**Statistical Analysis**

Values are presented as means ± SD. Results were compared by ANOVA for repeated measurements followed by Student-Newman-Keuls t test. p < 0.05 was considered statistically significant.

**RESULTS**

**Hypoxia Increases the Expression of the NADPH Oxidase Subunit NOX4**

To investigate whether hypoxia regulates NOX4 expression, PASMCs were exposed to 1% oxygen for increasing time periods. NOX4 mRNA levels were rapidly elevated 1–2 h after stimulation as was analyzed by Northern blot (Figure 1A) and real-time PCR (Figure 1B). Interestingly, the induction of NOX4 mRNA by hypoxia showed a similar kinetics than that of PAI-1 mRNA (Figure 1B), which is known to be regulated by hypoxia through the hypoxia-inducible transcription factors of the HIF family (Kietzmann et al., 1999).
In line, NOX4 protein levels were also elevated by hypoxia peaking at 4–8 h (Figure 1C). As expected, HIF-1α protein levels were also induced by hypoxia, but peaked before NOX4 at 1–4 h of hypoxia (Figure 1C). Similar responses were observed in HEK293 cells and human microvascular endothelial cells (data not shown). To verify the specificity of the NOX4 antibody used, a plasmid coding for a GFP–NOX4 fusion protein was coexpressed together with a vector encoding an shRNA against NOX4. Western blot analyses were performed with antibodies against GFP and NOX4 (Figure 1D). In both cases, more than 50% reduction of protein levels was observed. In addition, two different shRNAs against NOX4 were expressed, and PASMcs were exposed to 4 h of hypoxia (Figure 1E). Again, in both cases, NOX4 protein levels were significantly reduced, further indicating that the antibody used detected NOX4 protein, and that expression of shRNA against NOX4 effectively decreased endogenous NOX4 protein levels.

To determine, whether hypoxia up-regulates NOX4 also in vivo, lung tissue samples were obtained from mice exposed to 10% oxygen for 1 d. Compared with lung tissue from normoxic mice, NOX4 mRNA levels were up-regulated in samples from hypoxic mice as was determined by semiquantitative RT-PCR and real-time PCR (Figure 2, A and B). In line, NOX4 protein levels were increased in these samples to a similar extent than HIF-1α protein levels (Figure 2C).

In addition, immunohistochemistry was performed from lung tissue sections from normoxic and hypoxic mice (Figure 2D). As expected, the levels of HIF-1α were markedly increased in hypoxic pulmonary vessels. Interestingly, NOX4 protein levels were also strongly enhanced after 1 d of hypoxia, whereby NOX4 was primarily localized in the α-actin–positive media but also in the endothelial layer of small pulmonary arteries.

### HIF-1 Regulates NOX4 Expression

To further evaluate the mechanisms of up-regulation of NOX4 by hypoxia, PASMcs were treated with actinomycin D before exposure to hypoxia. Compared with untreated cells, the induction of NOX4 mRNA and protein by hypoxia was decreased (Figure 3, A and B), suggesting that a transcriptional mechanism contributes to this response. As expected, induction of HIF-1α protein levels by hypoxia was only slightly affected by actinomycin D treatment (Figure 3B).

We then cloned the human NOX4 promoter in front of the luciferase gene and evaluated NOX4 promoter activity under hypoxia using a luciferase reporter system. Compared with normoxia, exposure to hypoxia increased NOX4 promoter activity (Figure 3C), further indicating that hypoxia induces NOX4 transcription.

To further investigate the molecular mechanisms underlying NOX4 promoter activation by hypoxia, bioinformatic analysis of the NOX4 promoter was performed (MathInspector, Genomatix, Munich, Germany). Interestingly, a putative HRE was found at base pairs −387 to −391. This sequence contained the core consensus sequence “ACGTG” known to be indispensable for HIF binding (Wenger et al., 2005) and was also identical in the flanking sequences to HREs of known HIF target genes (Wenger et al., 2005). In addition, this putative NOX4 HRE was not only in its sequence, but also in its relative position in the NOX4 promoter similar to several known HREs in HIF target genes.

We therefore investigated, whether HIF-1α would contribute to hypoxic induction of NOX4. To this end, PASMcs were transfected with shRNAs against HIF-1α, which effectively blocked hypoxic induction of HIF-1α protein (Figure 4A). In fact, Northern blot and Western blot analyses showed that, compared with control cells, the induction of NOX4 mRNA and protein by hypoxia was diminished in HIF-1α–deficient PASMcs (Figure 4, A and B). In addition, HIF-1α overexpression enhanced NOX4 mRNA and protein levels (Figure 4, A and B). Again, NOX4 mRNA levels were similar to PAI-1 mRNA levels in HIF-1α–overexpressing cells as was determined by real-time PCR (Figure 4C). In line, HIF-1α overexpression increased NOX4 promoter activity (Figure 3B). Importantly, neither hypoxia nor HIF-1α overexpression were able to stimulate luciferase activity of a construct where the putative HRE in the NOX4 promoter was mutated (Figure 3B).

To further evaluate whether HIF-1α can directly bind to the NOX4 promoter, we performed ChIP using an antibody against HIF-1α and analyzed the precipitates by real-time PCR. Compared with control conditions, hypoxia enhanced binding of HIF-1α to the NOX4 promoter, and this effect was similarly observed for the PAI-1 promoter (Figure 4D), further indicating that NOX4 is directly activated by HIF-1 under hypoxia.
Because NOX4-dependent NADPH oxidases have been shown to generate ROS, we hypothesized that enhanced levels of HIF-1α should be able to increase ROS levels via NOX4. We therefore overexpressed HIF-1α in PASMCs and found significantly elevated ROS levels that were similar to those in NOX4-overexpressing PASMCs (Figure 5A). However, when NOX4 was depleted from HIF-1α-overexpressing cells, the HIF-1α-induced increase in ROS levels was abolished, indicating that induction of NOX4 by HIF-1α elevates ROS levels in PASMCs (Figure 5A). Because hypoxia increased the levels of NOX4, we next tested the contribution of NOX4 to ROS generation after hypoxia. Interestingly, when ROS levels were determined by DHE fluorescence after exposure of PASMCs to 30 min of hypoxia, a condition, where NOX4 levels were not elevated, yet, ROS levels were decreased compared with normoxic cells (Figure 5B). However, overexpression of NOX4 could restore ROS levels after short-term hypoxia to normoxic values, suggesting that NOX4 could be involved in ROS generation after hypoxia. In fact, when ROS levels were determined in PASMCs after removal from hypoxic incubation for 4 h, a condition where NOX4 levels were increased, ROS levels not only returned to baseline, but were even
higher than in the normoxic control cells (Figure 5B). However, when NOX4 or HIF-1α were depleted by shRNAs, ROS levels measured after exposure to 4 h of hypoxia were significantly diminished as determined by DHE fluorescence (Figure 5C) as well as by EPR using CMH as a spin trap (Figure 5C), suggesting that NOX4 induction by HIF-1α during hypoxic incubation was sufficient to allow restoration of ROS levels after prolonged hypoxia.

HIF-1α-dependent NOX4 Induction Promotes Proliferation and Migration by Hypoxia Involving ROS

To further test the functional relevance of HIF-1α–dependent NOX4 induction for the proliferative capacity of PASMCs, BrdU incorporation or cell numbers were determined in HIF-1α–overexpressing cells. Compared with control cells, proliferative activity was significantly enhanced (Figure 6A and B). However, depletion of NOX4 by shRNA from HIF-1α–overexpressing cells not only reduced the levels of NOX4 (Figure 6C), but also the proliferative activity under these conditions (Figure 6A and B). Western blot analysis was performed with antibodies against HIF-1α, NOX4, or ARNT. Actin served as loading control.

Figure 5. HIF-1α and NOX4 modulate ROS levels under normoxia and hypoxia. (A) Pulmonary artery smooth-muscle cells (PASMCs) were transfected with plasmids coding for NOX4 or for HIF-1α and were cotransfected with siRNA against NOX4 (siN4I) or with control shRNA (siCtr). ROS levels were evaluated by DHE fluorescence. ROS levels of control vectors were set to 100% (n = 3; *p < 0.05 vs. control, #p < 0.05 vs. HIF-1α). (B) PASMCs were transfected with a plasmid coding for NOX4 and were exposed to hypoxia for 30 min (0.5 h hypoxia), or with plasmids coding for siRNA against NOX4 (siN4I, siN4II), HIF-1α (siH1I, siH1II) or with control siRNA (siCtr) and exposed to hypoxia for 4 h. ROS levels were evaluated by DHE fluorescence thereafter. ROS levels of control vectors under normoxic conditions were set to 100% (n = 3; *p < 0.05 vs. normoxic control, #p < 0.05 vs. hypoxic control). (C) PASMCs were transfected with plasmids coding for either siRNA against NOX4 (siN4I), HIF-1α (siH1I), or control siRNA (siCtr). Cells were exposed to hypoxia for 4 h, and ROS levels were measured by EPR using the spin-trap CMH. ROS levels of control hypoxic cells (siCtr) were set to 100% (n = 3; *p < 0.05 vs. hypoxic control).

Figure 6. NOX4 mediates proliferative activity by HIF-1α and hypoxia. Pulmonary artery smooth-muscle cells (PASMCs) were transfected with vectors encoding different shRNAs against NOX4 (siN4I, siN4II) or control shRNA (siCtr) and were either cotransfected with a plasmid coding for HIF-1α or were exposed to hypoxia for 4 h. Proliferative activity was determined by (A) BrdU incorporation or (B) determination of cell numbers using a hemocytometer. Data are shown as relative change to normoxic control (100%; n = 3, *p < 0.05 vs. control; #p < 0.05 vs. HIF-1α–transfected or hypoxia-stimulated control). (C) Western blot analysis was performed with antibodies against HIF-1α, NOX4, or ARNT. Actin served as loading control.
ROS levels by NOX4 or HIF-1α
following hypoxia, and prevented induction of ROS levels by NOX4 or HIF-1α (Figure 8, A and B).

Interestingly, treatment with vitamin C also blocked the proliferative activities of PASMCs exposed to hypoxic conditions to a similar extent than those of NOX4 or HIF-1α-overexpressing PASMCs. This indicates that HIF-1α-dependent up-regulation of NOX4 under hypoxic conditions mediates the increased generation of ROS that is observed after prolonged exposure to hypoxia and that this pathway also contributes to the enhanced proliferation of PASMCs in response to hypoxia.

**DISCUSSION**

The results of this study demonstrate that the NADPH oxidase subunit NOX4 is a new target gene of HIF-1 under hypoxia and may contribute to the regulation of ROS levels and PASMC proliferation after hypoxic exposure because 1) hypoxia induced NOX4 mRNA and protein levels in vitro and in vivo, and this response was abrogated by HIF-1α depletion; 2) HIF-1α bound to the NOX4 promoter at a HRE, thereby enhancing NOX4 promoter activity; 3) HIF-1α-dependent up-regulation of NOX4 contributed to the restoration of ROS levels after prolonged hypoxia; and 4) induction of NOX4 by HIF-1α promoted proliferative activity of PASMCs in response to hypoxia.

NADPH oxidases have been identified as important regulators of ROS in vascular cells and also in other cell types, and the NADPH oxidase subunit NOX4 has been associated with systemic and pulmonary smooth-muscle function (Brown and Griendling, 2009). In this study, we showed that the NADPH oxidase subunit NOX4 is up-regulated by hypoxia at the mRNA and protein levels in several cell types including PASMCs and HEK293 cells, but also in human microvascular endothelial cells, as well as in human A549 lung carcinoma cells (data not shown) within 4 h of exposure, indicating that NOX4 expression is sensitive to oxygen availability independently of the cell type. In support, NOX4 mRNA levels have been reported to be up-regulated by hypoxia for 24 h in PASMCs (Mittal et al., 2007; Ismail et al., 2009). Furthermore, NOX4 mRNA and protein levels were enhanced in lung tissue from mice exposed to hypoxia for only 1 d. Interestingly, NOX4 protein was mainly found in the smooth muscle and endothelial cell layers as was confirmed by immunohistochemistry, further indicating that NOX4 may be part of an early vascular response to hypoxia in vitro and in vivo. These findings complement earlier reports demonstrating NOX4 expression in the media of remodeled pulmonary vessels in mice exposed to hypoxia for 3 wk (Mittal et al., 2007). In contrast to our findings of an immediate effect of hypoxia on NOX4 expression, however, the effect of chronic hypoxia on NOX4 expression may not be a direct one, but may be due to secondary effects mediated by growth factors, vasoactive peptides, or cytokines induced by chronic hypoxia as has been suggested earlier (Ismail et al., 2009). In fact, NOX4 has been shown to be induced, for example, by urotensin-II (Djordjevic et al., 2005a), transforming growth factor-β1 (TGFβ1; Cucurano et al., 2005; Sturrock et al., 2006), angiotensin-II (Wingerl et al., 2001; Gorin et al., 2003), and TNF-α (Moe et al., 2006). All these factors have been associated with pulmonary vascular remodeling, and several of them, including urotensin-II, TGFβ1, and angiotensin-II, have been shown to be inducible by hypoxia (Falanga et al., 1991; Zhang et al., 2002; Lam and Leung, 2003).

In contrast, our findings that NOX4 is rapidly up-regulated by hypoxia in vitro and in vivo clearly point to a direct regulatory mechanism responsible for these effects. In fact, we could show that NOX4 promoter activity was enhanced...
under hypoxic conditions, and also our findings that actinomycin D prevented up-regulation of NOX4 by hypoxia pointed to a transcriptional mechanism underlying NOX4 regulation by hypoxia. Promoter analysis indeed indicated the presence of an HRE in the proximal NOX4 promoter. This sequence contained the essential core sequence for HIF binding and was also very similar to other HIF target genes in the flanking sequences (Wenger et al., 2005), and mutation of this HRE abolished NOX4 promoter activation by hypoxia. Furthermore, HIF-1α directly bound to the NOX4 promoter, as was determined by ChIP, and consequently depletion of HIF-1α diminished hypoxia-induced NOX4 expression. Thus, our data showed for the first time that NOX4 is a target gene of HIF-1α.

Interestingly, depletion of HIF-2α also decreased NOX4 protein levels (data not shown), suggesting that NOX4 may be, in addition to HIF-1, also regulated by HIF-2, although further studies that are beyond the scope of this manuscript are required to delineate the specific role of HIF-2 versus HIF-1 in the regulation of NOX4. In this regard, however, it is of note that NOX4 itself cannot only up-regulate HIF-1α in PASMCs (Bonello et al., 2007), but also HIF-2α in several cell lines including von Hippel-Lindau protein (VHL)-deficient RCC4 cells (Block et al., 2007) and PASMCs (Diebold et al., 2010b). Moreover, NOX4 itself can also be up-regulated by ROS (Djordjevic et al., 2005a).

Because ROS are important regulators of vascular tone and function (Rhoades et al., 1990; Demiryurek and Wads-worth, 1999; Cai and Harrison, 2000; Lee and Griendling, 2008) and increased expression of NADPH oxidase subunits correlated with enhanced vascular superoxide production in various cardiovascular diseases (Brennan et al., 2003; Selemidis et al., 2008; Lassegue and Griendling, 2010), we tested whether the induction of NOX4 by HIF-1α has functional relevance for ROS generation in PASMCs. In fact, overexpression of HIF-1α increased ROS levels, and this effect was blunted by down-regulation of NOX4 or treatment with the antioxidant vitamin C, emphasizing that NOX4 acts as a downstream target of HIF-1α and contributes to enhanced ROS levels under normoxic conditions.

Furthermore, we could show that NOX4 is also responsible for the adaptation of ROS levels after hypoxia because depletion of NOX4 prevented the restoration of ROS generation after 4 h of hypoxia, and a similar response was observed when HIF-1α was depleted or when cells were treated with vitamin C. On the other hand, the decreased ROS levels observed after brief hypoxia, when adaptive NOX4 expression was not yet completed, were restored when NOX4 was overexpressed, mimicking the situation after longer hypoxia when NOX4 levels were up-regulated. These findings indicate that acute hypoxia diminishes ROS levels, and this response is even maintained after short-term reoxygenation (as in our experimental set-up). However, after prolonged hypoxia, adaptive processes take place, among them the HIF-1α-dependent up-regulation of NOX4, which then allows restoration and even elevated ROS production immediately after reoxygenation. These findings are in line with previous reports demonstrating either enhanced or decreased levels of ROS under hypoxic conditions determined by various protocols (Kietzmann and Gorlach, 2005; Cash et al., 2007; Prabhakar et al., 2007; Wolin et al., 2007; Archer et al., 2008; Waypa and Schumacker, 2008). In fact, our study may explain some of the controversial findings indicating either high or low levels of ROS after hypoxia. Accordingly, hypoxia decreases ROS levels, and ROS levels remain low even during short-term reoxygenation as long as no adaptive responses take place. However, when exposure to hypoxia is sufficiently long to induce adaptive responses, such as the up-regulation of NOX4 by HIF-1α, ROS generation can be restored to basal normoxic levels or even above as soon as oxygen is available again.

Figure 8. ROS mediate proliferation by hypoxia, NOX4, and HIF-1α. (A and B) Pulmonary artery smooth-muscle cells (PASMCs) were treated with vitamin C (VitC, 100 µM) for 60 min before exposure to hypoxia for 4 h. (A) ROS levels were evaluated by DHE fluorescence. (B) Proliferative activity was determined by BrdU incorporation. Untreated cells (Ctr) were set to 100% (n = 3; *p < 0.05 vs. control, #p < 0.05 vs. hypoxic control). (C and D) PASMCs were transfected with plasmids encoding for HIF-1α or NOX4 or control plasmid (Ctr) and treated with VitC for 60 min. (C) ROS levels were evaluated by DHE fluorescence. (D) Proliferative activity was determined by BrdU incorporation. Untreated cells (Ctr) were set to 100% (n = 3; *p < 0.05 vs. control).
A functional relevance of NOX4 up-regulation under hypoxic conditions was further shown by our findings that hypoxia induces proliferation as well as migration of PASMCs in a NOX4-dependent manner. Furthermore, mimicking the hypoxic situation by overexpressing HIF-1α enhanced proliferation and migration of PASMCs, and also here depletion of NOX4 diminished the proliferative and migratory responses. Thus, these findings clearly show that activation of HIF-1 by hypoxia results in up-regulation of NOX4, which contributes to adaptation of ROS levels and proliferation and migration of PASMCs. In support, NOX4 has been described to contribute to PASMC proliferation also in response to chronic hypoxia, although, in contrast to our findings, this delayed effect seemed to be indirectly mediated by TGFβ1 (Ismail et al., 2009).

Furthermore, our findings may provide a molecular link for previous reports describing enhanced HIF-α levels as well as NOX4 levels in pulmonary hypertension induced by chronic hypoxia (Yu et al., 1998, 1999; Brusselmann et al., 2003; Mittal et al., 2007), although our findings clearly demonstrate that up-regulation of NOX4 by HIF-1α takes place already after 4 h of hypoxia.

Our novel findings that NOX4 is a target gene of HIF-1, and possibly also of HIF-2, together with previous data that NOX4 regulates HIF-1α and HIF-2α levels (Bonello et al., 2007; Block et al., 2007; Diebold et al., 2010b) suggest a positive-feedback loop whereby NOX4 would induce HIF-α proteins and vice versa. In fact, we could previously show that HIF-1α and HIF-2α as well as NOX4 contribute to proliferative activity of PASMCs in response to thrombin or urotensin-II (Djordjevic et al., 2005a; Diebold et al., 2008, 2010b). We now demonstrate that HIF-1α and NOX4 also contribute to the proliferative and migratory response of PASMCs toward hypoxia and that this response is dependent on ROS.

Thus, our findings that NOX4 is a novel target gene of HIF-1 and is up-regulated by hypoxia together with previous findings that NOX4 is redox-sensitive and is contributing to the up-regulation of HIF-α proteins under normoxic conditions implicate an important role of the HIF-NOX4 axis in various disorders associated with ROS generation and proliferation under hypoxia as well as under normoxia.

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REFERENCES

Aaronson, P. I., Robertson, T. P., Knock, G. A., Becker, S., Levis, T. H., Snetskoy, V., and Ward, J. P. (2006). Hypoxic pulmonary vasoconstriction: mechanisms and controversies. J. Physiol. 570, 53–58.

Archer, S. L., Gomberg-Maitland, M., Maitland, M. L., Rich, S., Garcia, J. G., and Weir, E. K. (2008). Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1alpha-Kv1.5 O2-sensing pathway, at the intersection of pulmonary hypertension and cancer. Am. J. Physiol. Heart Circ. Physiol. 294, H570–H578.

Babior, B. M. (1999). NADPH oxidase: an update. Blood 93, 1464–1476.

Babior, B. M., Lambeth, J. D., and Nauseef, W. (2002). The neutrophil NADPH oxidase. Arch. Biochem. Biophys. 397, 342–344.

Bedard, K., and Krause, K. H. (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. 87, 325–313.

BelAiba, R. S., Djordjevic, T., Bonello, S., Fligel, D., Hess, J., Kietzmann, T., and Gorlach, A. (2004). Redox-sensitive regulation of the HIF pathway under non-hypoxic conditions in pulmonary artery smooth muscle cells. Biol. Chem. 385, 249–257.

BelAiba, R. S., Djordjevic, T., Petry, A., Diemer, K., Bonello, S., Banfi, B., Hess, J., Porgrebniak, A., Bickel, C., and Gorlach, A. (2007). NOX5 variants are functionally active in endothelial cells. Free Radic. Biol. Med. 42, 446–459.

Block, K., Gorin, Y., Hoover, P., Williams, P., Chelmicki, T., Clark, R. A., Yoneda, T., Abboud, H. E. (2007). NAD(P)H oxidases regulate HIF-2alpha protein expression. J. Biol. Chem. 282, 8019–8026.

Bonello, S., Zahringer, C., BelAiba, R. S., Djordjevic, T., Hess, J., Michiels, C., Kietzmann, T., and Gorlach, A. (2007). Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site. Arterioscler. Thromb. Vasc. Biol. 27, 755–761.

Brennan, L. A., Steinhorn, R. H., Wedgwood, S., Mata-Greenwood, E., Roark, E. A., Russell, J. A., and Black, S. M. (2003). Increased superoxide generation is associated with pulmonary hypertension in fetal lambs: a role for NADPH oxidase. Circ. Res. 92, 683–691.

Brown, D. L., and Griendling, K. K. (2009). NADPH oxidases in signal transduction. Free Radic. Biol. Med. 47, 1239–1253.

Brusselmann, K., Compernolle, V., Tjwa, M., Wiesener, M. S., Maxwell, P. H., Collen, D., Carmeliet, P. (2003). Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. J. Clin. Invest. 111, 1519–1527.

Cai, H., and Harrison, D. G. (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidative stress. Circ. Res. 87, 840–844.

Cash, T. P., Pan, Y., and Simon, M. C. (2007). Reactive oxygen species and cellular oxygen sensing. Free Radic. Biol. Med. 43, 1219–1225.

Cheng, G., Cao, Z., Xu, X., van Meir, E. G., and Lambeth, J. D. (2001). Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. Gene 259, 131–140.

Cucoranu, I., Clempps, R., Dakalova, A., Pehlan, P. J., Aryian, S., Dakalov, S., and Sorescu, D. (2005). NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. Circ. Res. 97, 900–907.

Demireyurek, A. T., and Wadsworth, R. M. (1999). Superoxide in the pulmonary circulation. Pharmacol. Ther. 84, 355–365.

Diebold, I., Djordjevic, T., Hess, J., and Gorlach, A. (2008). Rac-1 promotes pulmonary artery smooth muscle cell proliferation by upregulation of plasminogen activator inhibitor-1, role of NFkappaB-dependent hypoxia-inducible factor-1alpha transcription. Thromb. Haemost. 100, 1021–1028.

Diebold, I., et al. (2010a). Reciprocal regulation of Rac1 and PAK-1 by HIF-1alpha: a positive-feedback loop promoting pulmonary vascular remodeling. Antioxid. Redox. Signal. (in press).

Diebold, I., Fligel, D., Becht, S., Belaiba, R. S., Bonello, S., Hess, J., Kietzmann, T., and Gorlach, A. (2010b). The hypoxia-inducible factor-2alpha is stabilized by oxidative stress involving NOX4. Antioxid. Redox. Signal. (in press).

Djordjevic, I., BelAiba, R. S., Bonello, S., Petschlicher, J., Hess, J., and Gorlach, A. (2005a). Human urotensin II is a novel activator of NADPH oxidase in human pulmonary artery smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 25, 519–525.

Djordjevic, T., Porgrebniak, A., BelAiba, R. S., Bonello, S., Wotzlav, C., Acker, H., Hess, J., and Gorlach, A. (2005b). The expression of the NADPH oxidase subunit p22phox is regulated by a redox-sensitive pathway in endothelial cells. Free Radic. Biol. Med. 38, 616–630.

Falanga, V., Qian, S. W., Danielpour, D., Katz, M. H., Roberts, A. B., and Sporn, M. B. (1991). Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. J. Invest. Dermatol. 97, 634–637.

Finkel, T. (1999). Signal transduction by reactive oxygen species in non-phagocytic cells. J. Leukoc. Biol. 65, 337–340.

Gorin, Y., Ricono, J. M., Kim, N. H., Bhandari, B., Choudhury, G. G., and Abboud, H. E. (2003). Nox4 mediates angiotensin II-induced activation of Akt/protein kinase B in mesangial cells. Am. J. Physiol. Renal Physiol. 285, F219–F229.

Gorlach, A., Brandes, R. P., Nguyen, K., Amid, M., Dehghani, F., and Busse, R. (2000). A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. Circ. Res. 87, 26–32.

Gorlach, A., Diebold, I., Schini-Kerth, V. B., Bercher-Pfannschmidt, U., Roth, U., Brandes, R. P., Kietzmann, T., and Busse, R. (2001). Thrombin activates the hypoxiainducible factor-1 signaling pathway in vascular smooth muscle cells: Role of the p22(phox)-containing NADPH oxidase. Circ. Res. 89, 47–54.
Gorlach, A. (2003). Reactive oxygen species modulate HIF-1 mediated PAI-1 expression: involvement of the GTPase Rac1. Thromb. Haemost. 89, 926–935.

Gorlach, A., and Kietzmann, T. (2007). Superoxide and derived reactive oxygen species in the regulation of hypoxia-inducible factors. Methods Enzymol. 435, 421–446.

Griendling, K. K., Sorescu, D., and Ushio-Fukai, M. (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. Circ. Res. 86, 494–501.

Gupte, S. A., and Wolin, M. S. (2008). Oxidant and redox signaling in vascular oxygen sensing: implications for systemic and pulmonary hypertension. Antioxid. Redox Signal. 10, 1137–1152.

Ismail, S., Sturrock, A., Wu, P., Cahill, B., Norman, K., Huecksteat, T., Sanders, K., Kennedy, T., and Hoidal, J. (2009). NOX4 mediates hypoxia-induced proliferation of human pulmonary artery smooth muscle cells: the role of autocrine prostaglandins. Cardiovasc. Res. 82, 296–307.

Kietzmann, T., Roth U., and Jungermann, K. (1999). Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia-inducible factor-1 in rat hepatocytes. Blood 94, 4177–4185.

Kietzmann, T., and Gorlach, A. (2005). Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. Semin. Cell Dev. Biol. 16, 474–486.

Klein, A., Flugel, D., and Kietzmann, T. (2008). Transcriptional regulation of serine/threonine kinase-15 (STK15) expression by hypoxia and HIF-1. Mol. Biol. Cell 19, 3667–3675.

Lam, S. Y., and Leung, P. S. (2003). Chronic hypoxia activates a local angiotensin-generating system in rat carotid body. Mol. Cell. Endocrinol. 203, 147–153.

Lambeth, J. D., Kawahara, T., and Diebold, B. (2007). Regulation of Nox and Duox enzymatic activity and expression. Free Radic. Biol. Med. 43, 319–331.

Lassegue, B., and Griendling, K.K. (2010). NADPH oxidases: functions and pathologies in the vasculature. Arterioscler. Thromb. Vasc Biol. 30, 653–661.

Lee, M. Y., and Griendling, K. K. (2008). Redox signaling, vascular function, and hypertension. Antioxid. Redox Signal. 10, 1034–1059.

Martyn, K. D., Frederick, L. M., von Loehneysen, K., Dinauer, M. C., and Knuts, L. G. (2006). Functional analysis of Nox reveals unique characteristics compared to other NADPH oxidases. Cell Signal. 18, 69–82.

Mittal, M., et al. (2007). Hypoxia-dependent regulation of nonphagocytic NADPH oxidase subunit NOX4 in the pulmonary vasculature. Circ. Res. 101, 258–267.

Moe, K. T., Aulia, S., Jiang, F., Chua, Y. L., Koh, T. H., Wong, M. C., and Dusting, G. J. (2006). Differential upregulation of Nox homologues of NADPH oxidase by tumor necrosis factor-alpha in human aortic smooth muscle and embryonic kidney cells. J. Cell. Mol. Med. 10, 231–239.

Petry, A., Djordjevic, T., Weitnauer, M., Kietzmann, T., Hess, J., and Gorlach, A. (2006). NOX2 and NOX4 mediate proliferative response in endothelial cells. Antioxid. Redox Signal. 8, 1473–1484.

Prabhakar, N. R., Kumar, G. K., Nanduri, J., and Semenza, G. L. (2007). ROS signaling in systemic and cellular responses to chronic intermittent hypoxia. Antioxid. Redox Signal. 9, 1397–1403.

Ratcliffe, P. J., O’Rourke, J. F., Maxwell, P. H., and Pugh, C. W. (1998). Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J. Exp. Biol. 201, 1153–1162.

Rhoades, R. A., Packer, C. S., Roepke, D. A., Jin, N., and Meiss, R. A. (1990). Reactive oxygen species alter contractile properties of pulmonary arterial smooth muscle. Can. J. Physiol. Pharmacol. 68, 1581–1589.

Richard, D. E., Berra, E., and Pouyssegur, J. (2000). Nonhypoxic pathway mediates the induction of hypoxia-inducible factor alpha in vascular smooth muscle cells. J. Biol. Chem. 275, 26765–26771.

Sakamichi, S., Sobey, C. G., Wingler, K., Schmidt, H.H., and Drummond, G.R. (2008). NADPH oxidases in the vasculature: molecular features, roles in disease and pharmacological inhibition. Pharmacol. Ther. 120, 254–291.

Semenza, G. L. (2000). HIF-1, mediator of physiological and pathophysiological responses to hypoxia. J. Appl. Physiol. 89, 1474–1480.

Stenmark, K. R., Fagan, K. A., and Frid, M. G. (2006). Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. Circ. Res. 99, 675–691.

Sturrock, A., et al. (2006). Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. Am. J. Physiol. Lung Cell Mol. Physiol. 290, L661–L673.

Ushio-Fukai, M. (2009). Compartimentalization of redox signaling through NADPH oxidase-derived ROS. Antioxid. Redox Signal. 11, 1289–1299.

Waypa, G. B., and Schwemmer, P. T. (2008). Oxygen sensing in hypoxic pulmonary vasoconstriction: using new tools to answer an age-old question. Exp. Physiol. 93, 133–138.

Weir, E. K., Lopez-Barneo, J., Buckler, K. J., and Archer, S. L. (2005). Acute oxygen-sensing mechanisms. N. Engl. J. Med. 353, 2042–2055.

Wenger, R. H., Stiehl, D. P., and Camenisch, G. (2005). Integration of oxygen signaling at the consensus HRE. Sci STKE 2005, re12.

Wingerl, K., Wunsch, S., Kreutz, R., Rothermund, L., Paul, M., and Schmidt, H. H. (2001). Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system in vitro and in vivo. Free Radic. Biol. Med. 31, 1456–1464.

Wolin, M. S. (1996). Reactive oxygen species and vascular signal transduction mechanisms. Microcirculation 3, 1–17.

Wolin, M. S., Ahmad, M., Gao, Q., and Gupte, S. A. (2007). Cytosolic NAD(P)H regulation of redox signaling and vascular oxygen sensing. Antioxid. Redox Signal. 9, 671–678.

Yu, A. Y., Frid, M. G., Shimoda, L.A., Wiener, C. M., Stenmark, K., and Semenza, G. L. (1998). Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am. J. Physiol. 275, L818–L826.

Yu, A. Y., et al. (1999). Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor alpha. J. Clin. Invest. 103, 691–696.

Zhang, Y., Li, J., Cao, J., Chen, J., Yang, J., Zhang, Z., Du, J., and Tang, C. (2002). Effect of chronic hypoxia on contents of urotensin II and its functional receptors in rat myocardium. Heart Vessels 16, 64–68.