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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 (O_2^-) and hydrogen peroxide (H_2O_2) 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; BelAiba *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; BelAiba *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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Abbreviations used: ARNT, aryl hydrocarbon receptor nuclear translocator; DHE, dihydroethidium; EPR, electron paramagnetic resonance; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-responsive element; ROS, reactive oxygen species; PASMC, pulmonary artery smooth-muscle cell.

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 hepatoblastoma 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: 50507) upstream from the start codon was amplified by PCR using primers with appropriate restriction sites (forward: 5′-GTA AAG CAA CAT AAA CAG ATC TCA GTC CCC TC-3′; reverse: 5′-ACA GCC ATG GCG CCG GCC 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 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′; reverse: 5′-CAG CCT TTT GTG TGC TAG TTA ACG TTT CTC CCA CCC TTC CTC-3′ revealing pglNOX4-730m. All constructs were confirmed by DNA sequencing. The vectors encoding short hairpin RNA (shRNA) against HIF-1α, NOX4, or an unspecific random sequence (siCtr) were previously described (Bonello *et al.*, 2007). Additional vectors encoding for shRNA against HIF-1α (siH1II) with the sequence 5′-GCC ACC ACT GAT GAA TTA A-3′ or shRNA against NOX4 (siN4II) with the sequence 5′-GGG ACA AGA TTT GAA TAC A-3′ were generated using the pSTRIKE-U6 vector system (Promega). The plasmid encoding V5-tagged HIF-1α was kindly provided by Dr. T. Kietzmann (Kaiserslautern; Klein *et al.*, 2008). The plasmid encoding GFP-NOX4 was previously described (Petry *et al.*, 2006).

Transfections of PASMC were performed using FuGene reagent (Roche, Mannheim, Germany) as described (Diebold *et al.*, 2008). PASMCs were plated to a density of 70% and cultured for 24 h. Transfection efficiency was on average 40%. Because PASMCs do not express the luciferase gene well (BelAiba *et al.*, 2004), luciferase assays were performed in HEK293 cells transfected by calcium phosphate precipitation as described (Gorlach *et al.*, 2003). A Renilla luciferase expression vector (pRLSV40; Promega) was cotransfected in reporter gene assays to adjust for variations in transfection efficiencies.

Western Blot Analysis

Western blot analysis was performed as described (Diebold *et al.*, 2008). Briefly, 10 μg of isolated proteins was separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. To detect NOX4 or green fluorescent protein (GFP), membranes were blocked for 1 h in TBS (Tris-buffered saline) containing 5% nondry milk and were then incubated overnight at 4°C with a polyclonal antibody raised against a NOX4 peptide (NH3-CSYGTRFEY-NKESFS) diluted 1:500 in TBS with 5% milk or with an antibody against GFP (Roche) diluted 1:500 in TBS with 5% milk. Western blot analyses for HIF-1α or aryl hydrocarbon receptor nuclear translocator (ARNT) were performed with 50 μg of protein, separated by 8% SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked in TBS containing 5% milk and 0.3% Tween20 (TBS-T) and were incubated with antibodies against HIF-1α (Novus Biologicals, Littleton, CO) diluted at 1:500 in TBS-T or ARNT diluted at 1:1000 (Abcam, Cambridge, United Kingdom). After incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Calbiochem, Darmstadt, Germany) for 1 h, proteins were visualized by performing luminol enhanced chemiluminescence. Loading of equal amounts of proteins was confirmed by reprobing the membranes with an antibody against actin (Santa Cruz, Heidelberg, Germany). Blots were scanned and analyzed using GelDoc software (Bio-Rad, Munich, Germany).

RNA Extraction and RT-PCR

RNA was extracted from human PASMCs or mouse tissue or from cells using RNeasy Midi or Mini 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 TTT CAG GTG GT-3′; reverse, 5′-TAC TGG CCA GGT CTG CTT T-3′; mouse 18S: forward, 5′-GGC CTC ACT AAA CCA TCC AA-3′; reverse, 5′-GTT GGT TTT CGG AAC TGA GG-3′. For quantitative real-time PCR, the following primers were used: human NOX4: forward, 5′-CCG GCT GCA TCA GTC TTA ACC-3′ and reverse, 5′-TCG GCA CAG TAC AGG CAC AA-3′; human plasminogen activator inhibitor-1 (PAI-1): forward, 5′-CAC AAA TCA GAC GGC AGC ACT-3′ and reverse, 5′-CAT CGG GCG TGG TGA ACT C-3′; human β-actin: forward, 5′-CCAACCGCGAGAAGATGA-3′ and reverse, 5′-CCA GAG GCG TAC AGG GAT AG-3′; mouse NOX4: forward, 5′-GAA GAT TTG CCT GGA AGA ACC-3′ and reverse, 5′-AGG TTT GTT GCT CCT GAT GC-3′; and mouse 18S: forward, 5′-CCT GCG GCT TAA TTT GAC TC-3′ and reverse, 5′-AAC TAA GAA CGG CCA TGC AC-3′. Real-time PCR analysis was performed as described (Diebold *et al.*, 2010a) using the Perfecta SYBR Green FastMix (VWR, Darmstadt, Germany) in a Rotor-Gene 6000 (Corbett, Wasserburg, Germany). Quantification was performed using ΔCT calculation.

Northern Blot Analysis

Total RNA from PASMCs (10–15 μg) was separated on 1.3% agarose gels, transferred to nylon membranes, and cross-linked by UV irradiation. Northern hybridizations were carried out with digoxigenin-labeled antisense RNA probes for NOX4, which were transcribed from a NOX4 antisense construct using digoxigenin-labeled nucleotides and T7 polymerase (Roche) at 65°C for 16 h. Detection was performed after incubation with a digoxigenin antibody conjugated with alkaline phosphatase by using the chemiluminescent substrate CDPStar (Roche). Loading of equal amounts of RNA was confirmed by ethidium bromide staining of 18S.

Chromatin Immunoprecipitation

Confluent HepG2 cells were grown in T75 flasks to confluency and exposed to hypoxia (1% oxygen) for 3 h. Cells were fixed with formaldehyde, lysed, and sonicated to obtain DNA fragments in a size from 500 to 1000 base pairs. Chromatin was then precipitated with an antibody against HIF-1α (Novus) overnight at 4°C. Real-time PCR was performed with primers for the NOX4 promoter (forward, 5′-GAT AAA GAA ACT GGC GGC TG-3′ and reverse, 5′-GTA ACG AAA TTT GAG CCG GA-3′) flanking the potential HRE −391 to −387 bp and with primers for the PAI-1 promoter containing known HREs as positive control (forward, 5′-GCT CTT TCC TGG AGG TGG TC-3′ and reverse, 5′-GGG CAC AGA GAG AGT CTG GA-3′) using a Rotor-Gene 6000 (Corbett). As negative control to analyze unspecific binding and precipitation, real-time PCR using primers amplifying a region within the third intron of the β-actin gene (gene ID: 60) not containing a putative HRE (5′-ACGTG-3′) was performed (forward, 5′-AAC ACT GGC TCG TGT GAC AA-3′ and reverse, 5′-AAA GTG CAA AGA ACA CGG CT-3′). As background control, chromatin immunoprecipitation (ChIP) without antibody was performed. Statistical analysis was performed using a standard curve of the input. HIF-1α binding to chromatin was revealed after background subtraction as relative amount of the input used.

Measurement of ROS Production

ROS generation was detected using the fluoroprobe dihydroethidium (DHE; Invitrogen) as described previously (BelAiba *et al.*, 2007). PASMCs were incubated for the indicated time points at 1% oxygen. Cells were incubated in the dark with DHE (50 μM) for 5 min at 37°C. Thereafter, cells were quickly washed with HBSS to remove excess dye, and DHE fluorescence was monitored using 480-nm excitation and 620-nm emission wavelength in a microplate reader (Safire, Tecan, Crailsheim, Germany) for a total time of 3 min at normoxic conditions. DHE fluorescence was standardized to the number of viable cells using the AlamarBlue test according to the manufacturer's instructions (Biosource, Nivelles, Belgium) as described (Djordjevic *et al.*, 2005b). Additionally, electron paramagnetic resonance (EPR) for measurement of superoxide production was used. Cells were seeded in 10-cm dishes and exposed to hypoxia for 4 h. After washing with phosphate-buffered saline (PBS), cells were scraped in PBS, spun down (2000 rpm, 4°C, 5 min) and resuspended in 80 μl of Krebs HEPES buffer, pH 7.35 (99 mM NaCl, 4.69 mM

KCl, 25 mM $NaHCO_3$, 1.03 mM KH_2PO_4, 5.6 mM D-glucose, 20 mM Na-HEPES, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$) supplemented with 25 μM desferoxamine and 5 μM *o,o*-diethyl-thio-phosphoryl-chloride (DETC). Before measurement, 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-μm^2 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% O_2) 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).

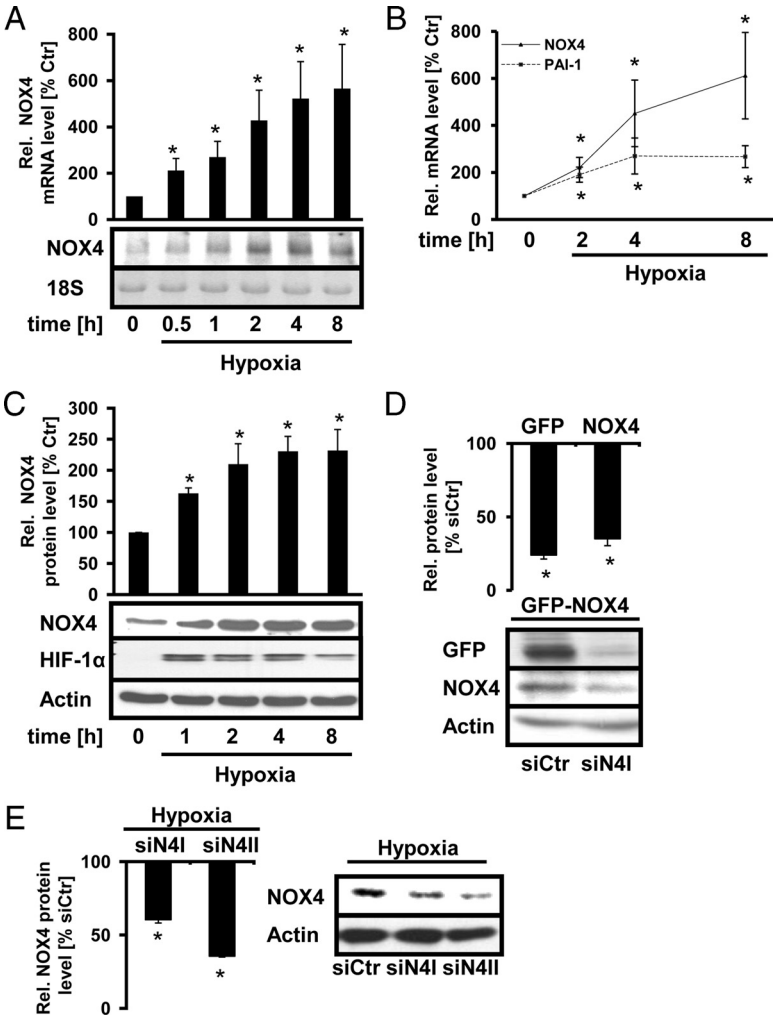

Figure 1. Hypoxia increases NOX4 expression in vitro. (A–C) Pulmonary artery smooth-muscle cells (PASMC) were stimulated for different time periods by hypoxia (1% O_2). (A) Northern blot analyses were performed using a specific probe for NOX4; 18S staining served as loading control. Data represent % change of NOX4 mRNA levels versus normoxic control (100%; n = 3, *$p < 0.05$ vs. control). (B) Real-time PCR was performed with primers amplifying cDNA fragments specific for NOX4, PAI-1, or actin. Quantification was performed by ΔCT calculation. NOX4 and PAI-1 mRNA levels were normalized to actin levels. Normoxic control (0) was set to 100% (n = 3; *$p < 0.05$ vs. control). (C) NOX4 and HIF-1α protein levels were determined by Western blot analyses. Actin levels served as loading control. Data represent % change of NOX4 protein levels versus normoxic control (100%; n = 5, *$p < 0.05$ vs. control). (D) HEK293 cells were cotransfected with a plasmid encoding GFP-NOX4 and plasmids encoding shRNA against NOX4 (siN4I) or control shRNA (siCtr). Western blot analyses were performed with antibodies against NOX4 and GFP. Staining with an antibody against actin served as loading control. GFP or NOX4 protein levels in siCtr-expressing cells were set to 100%. Data represent % decrease of GFP or NOX4 protein levels compared with control (n = 3; *$p < 0.05$ vs. control). (E) PASMCs were transfected with vectors encoding two different shRNAs against NOX4 (siN4I, siN4II) or control shRNA (siCtr) and exposed to hypoxia for 4 h. Western blot analyses were performed with an antibody against NOX4. Actin staining served as loading control. NOX4 protein levels in hypoxic siCtr-expressing cells were set to 100% (n = 3; *$p < 0.05$ vs. control).

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.

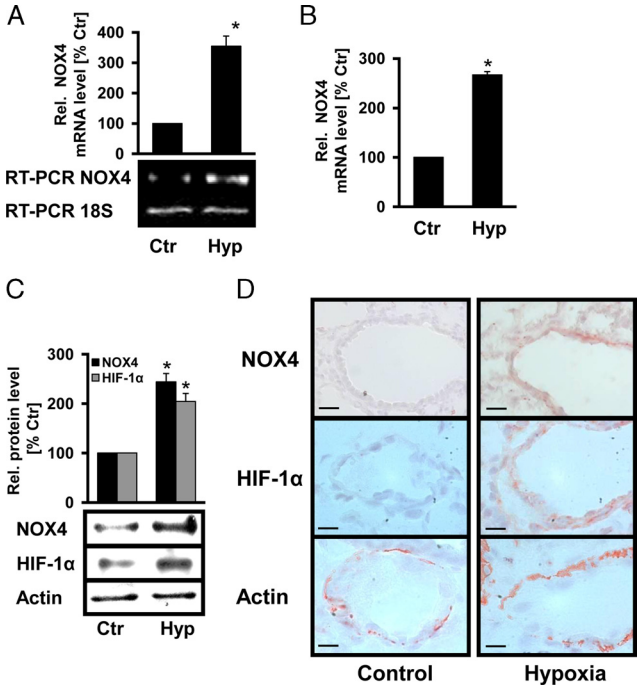

Figure 2. Hypoxia increases NOX4 expression in mouse lungs. Mice were exposed to normoxia (Ctr) or hypoxia (10% oxygen, Hyp) for 1 d, and lung tissue was obtained. (A and B) RNA was isolated from normoxic and hypoxic lung tissue. RT-PCR (A) or real-time PCR (B) were performed using specific primers for NOX4 or 18S. NOX4 mRNA levels under normoxia (Ctr) were set to 100%. Data represent % increase of NOX4 mRNA levels compared with control ($n = 3$; $^*p < 0.05$ vs. Ctr). (C) Protein was isolated and Western blot analysis was performed with antibodies against NOX4 or HIF-1α. Actin levels served as loading control. Data represent % change of protein levels versus normoxic control (100%; $n = 3$, $^*p < 0.05$ vs. control). (D) Immunohistochemistry was performed on lung tissue samples using antibodies against NOX4, HIF-1α, and α-actin (Actin). Images were taken with a 40x objective. Scale bars, 20 μm.

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 (MatInspector, 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.

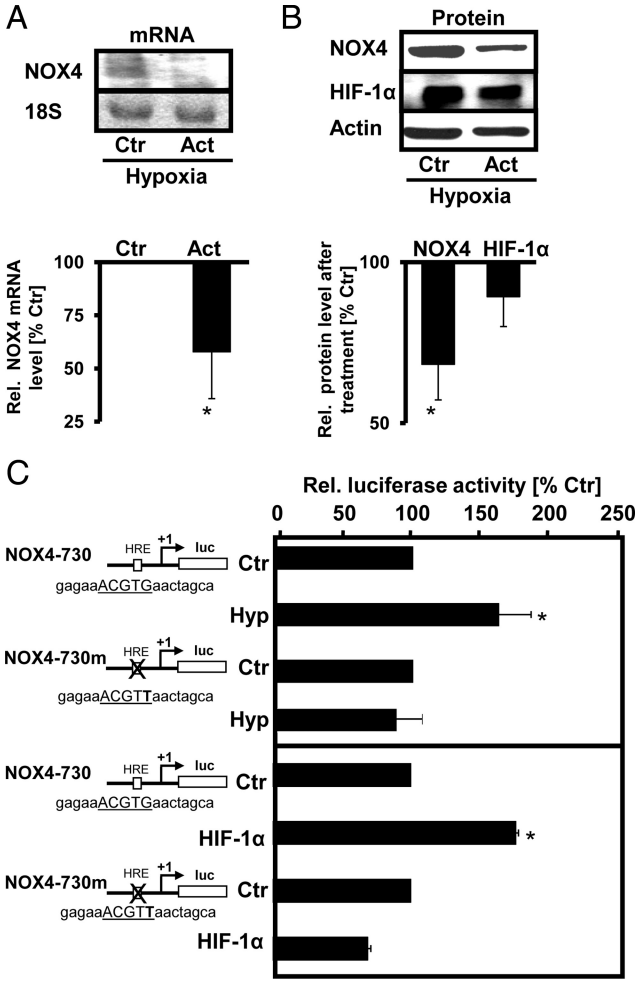

Figure 3. Hypoxia increases NOX4 transcription. (A and B) Pulmonary artery smooth-muscle cells (PASMCs) were treated with actinomycin D (Act, 5 μM) or DMSO (Ctr) for 1 h and exposed to hypoxia for 4 h. (A) Northern blot analyses were performed using a specific probe for NOX4, 18S staining served as loading control. NOX4 mRNA levels in DMSO-treated cells under hypoxia (Ctr) were set to 100%. Data represent % change of NOX4 mRNA levels versus hypoxic control (n = 3, *p < 0.05 vs. hypoxic control). (B) Western blot analyses were performed using antibodies against NOX4 or HIF-1α. Actin served as loading control. Protein levels in DMSO-treated cells under hypoxia (Ctr) were set to 100%. Data represent % change of protein levels versus hypoxic control (n = 3, *p < 0.05 vs. hypoxic control). (C) HEK293 cells were cotransfected with luciferase constructs containing either the wild-type NOX4 promoter (NOX4-730) or the NOX4 promoter mutated at the hypoxia-responsive element (HRE; NOX4-730m). Cells were exposed to hypoxia (Hyp) or were cotransfected with a plasmid coding for HIF-1α. Luciferase activities under the respective control conditions (Ctr) for each reporter plasmid were set equal to 100%. Data represent % induction of luciferase activity (n = 3; *p < 0.05 vs. control).

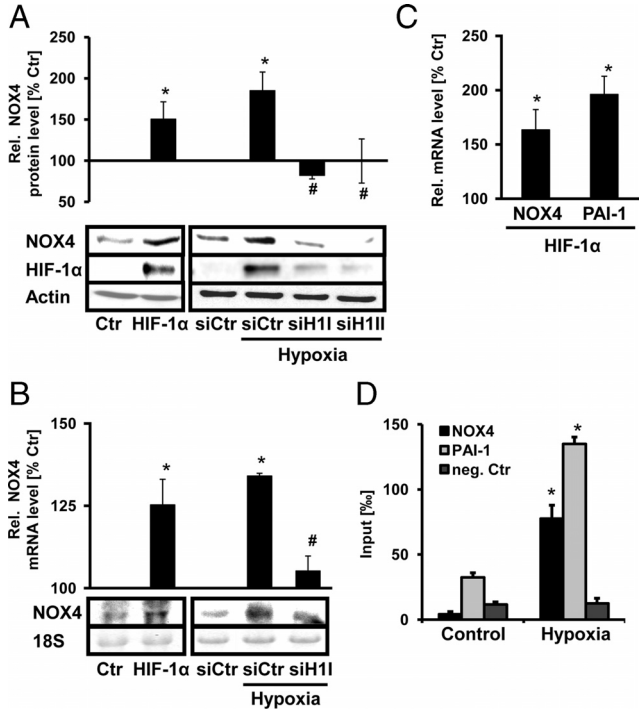

Figure 4. HIF-1α mediates NOX4 expression. (A–C) Pulmonary artery smooth-muscle cells (PASMCs) were transfected with vectors coding for HIF-1α or with vectors coding for shRNA against HIF-1α (siH1I, siH1II) or for control shRNA (siCtr) and exposed to hypoxia for 4 h. (A) Western blot analyses were performed using antibodies against NOX4 or HIF-1α. Actin was used as loading control. (B) Northern blots were performed using specific probes for NOX4 or 18S. Data represent % change of NOX4 protein (A) or NOX4 mRNA levels (B) versus the appropriate normoxic control set to 100% (n = 3, *p < 0.05 vs. control; #p < 0.05 vs. hypoxic control). (C) mRNA levels for NOX4 and PAI-1 were determined in HIF-1α–overexpressing cells by real-time PCR using primers specifically amplifying NOX4, PAI-1, or actin fragments. Quantification was performed by ΔCT calculation. NOX4 or PAI-1 mRNA levels were normalized to actin levels. Control cells (Ctr, siCtr) were set to 100%, and the relative change in HIF-1α–overexpressing cells is displayed (n = 3; *p < 0.05 vs. control). (D) HepG2 cells were exposed to hypoxia for 3 h. Chromatin immunoprecipitation (ChIP) was performed with an antibody against HIF-1α. Real-time PCR was performed on the precipitates using primers for the NOX4 promoter (black) or the PAI-1 promoter (gray) as positive control or for the third intron of β-actin lacking an HRE as negative control (dark gray, neg. Ctr). For background calculation, ChIP without antibody was performed. Quantification is shown in promille to chromatin input for all samples after background subtraction (n = 3, *p < 0.05 vs. control).

HIF-1α–dependent NOX4 Induction Contributes to ROS Generation after 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

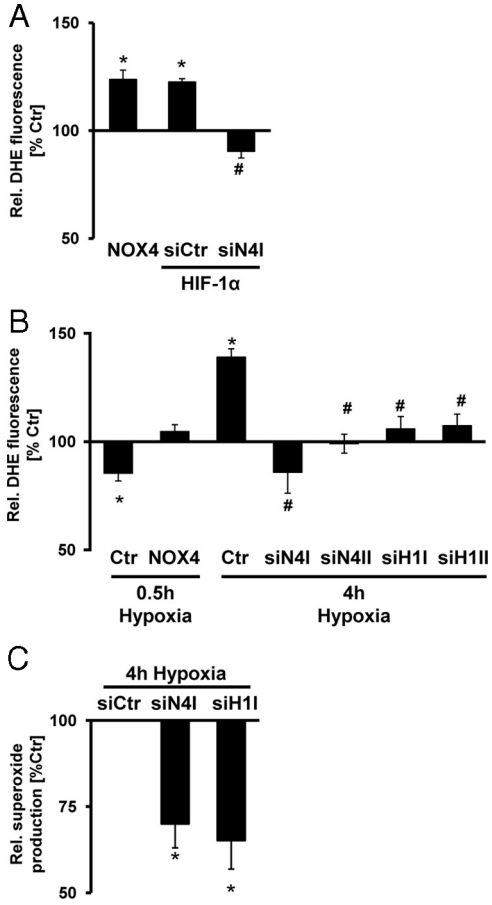

Figure 5. HIF-1α and NOX4 modulate ROS levels under normoxia and hypoxia. (A) Pulmonary artery smooth-muscle cells (PASMCs) were transfected with plasmids coding for NOX4 or for HIF-1α and were cotransfected with shRNA against NOX4 (siN4I) or with control shRNA (siCtr). ROS levels were evaluated by DHE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

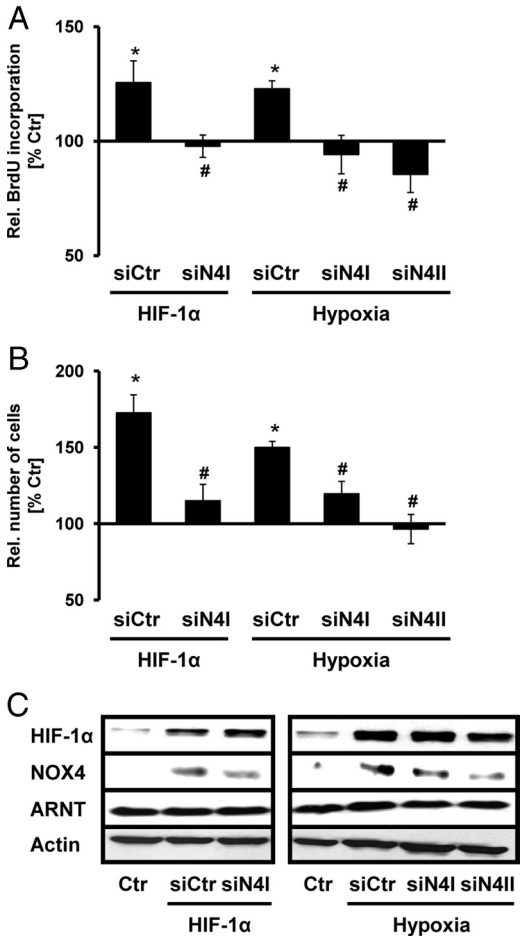

Figure 6. NOX4 mediates proliferative activity by HIF-1α and hypoxia. Pulmonary artery smooth-muscle cells (PASMCs) were transfected with vectors encoding different shRNAs against NOX4 (siN4I, siN4II) or control shRNA (siCtr) and were either cotransfected with a plasmid coding for HIF-1α or were exposed 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.

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 5B) 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 6, A 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 to control levels (Figure 6, A and B). We then evaluated the proliferative activity of PASMCs upon exposure to hypoxia for 4 h. Interestingly, PASMC proliferation was enhanced in hypoxic cells compared with normoxic cells to a similar degree as in HIF-1α–overexpressing cells (Figure 6, A and B). Expression of vectors encoding two different shRNAs against NOX4 decreased not only NOX4 induction by hypoxia (Figure 6C), but also the proliferative activity of PASMCs under these conditions (Figure 6, A and B), suggesting that HIF-1α–dependent NOX4 induction was important for the proliferative activity of PASMCs in response to hypoxia. Interestingly, these responses were not confined to

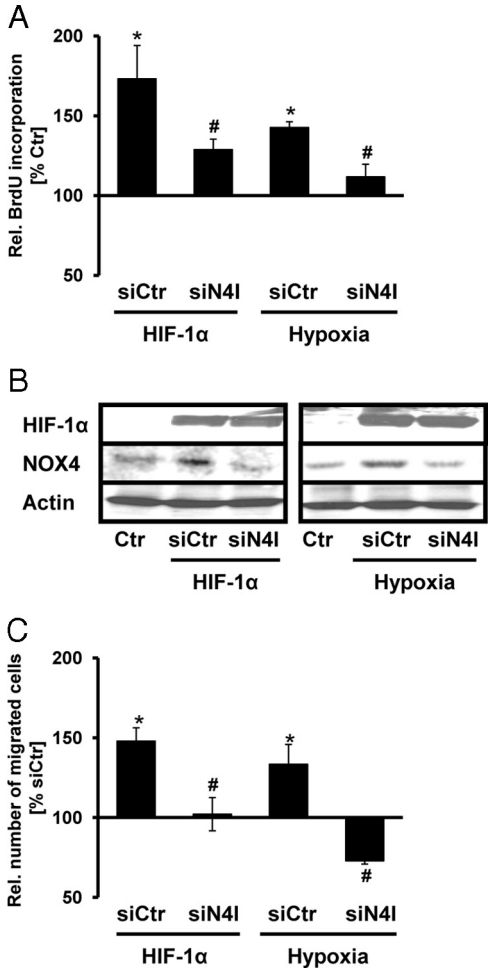

Figure 7. NOX4 mediates migration by HIF-1α and hypoxia. (A/B) HEK293 cells were transfected with vectors encoding shRNA against NOX4 (siN4I) or control shRNA (siCtr) and were cotransfected with an expression plasmid for HIF-1α or were exposed to hypoxia for 4 h. (A) Proliferative activity of HEK293 cells was determined by BrdU incorporation. 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). (B) Western blot analysis was performed with antibodies against HIF-1α or NOX4. Actin served as loading control. (C) Pulmonary artery smooth-muscle cells (PASMCs) were transfected with vectors encoding shRNA against NOX4 (siN4I) or control shRNA (siCtr) and were cotransfected with an expression plasmid for HIF-1α or were exposed to hypoxia for 4 h after wounding the cell layer with a 10-μl tip. The number of migrated cells into the wound was counted. Data are shown as relative change to control (n = 3; *$p < 0.05$ vs. control, #$p < 0.05$ vs. HIF-1α–transfected or hypoxia-stimulated control).

PASMCs, because similar results were obtained in HEK293 cells (Figure 7, A and B). In addition, hypoxia or HIF-1α overexpression enhanced migration of PASMCs, and these responses were also prevented by depletion of NOX4 (Figure 7C).

Finally, to analyze whether ROS mediate NOX4-dependent proliferation under conditions of elevated HIF-1α levels, PASMCs were pretreated with vitamin C which can act as an antioxidant. In fact, vitamin C decreased ROS production following 4 h of 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; Cucoranu *et al.*, 2005; Sturrock *et al.*, 2006), angiotensin-II (Wingler *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

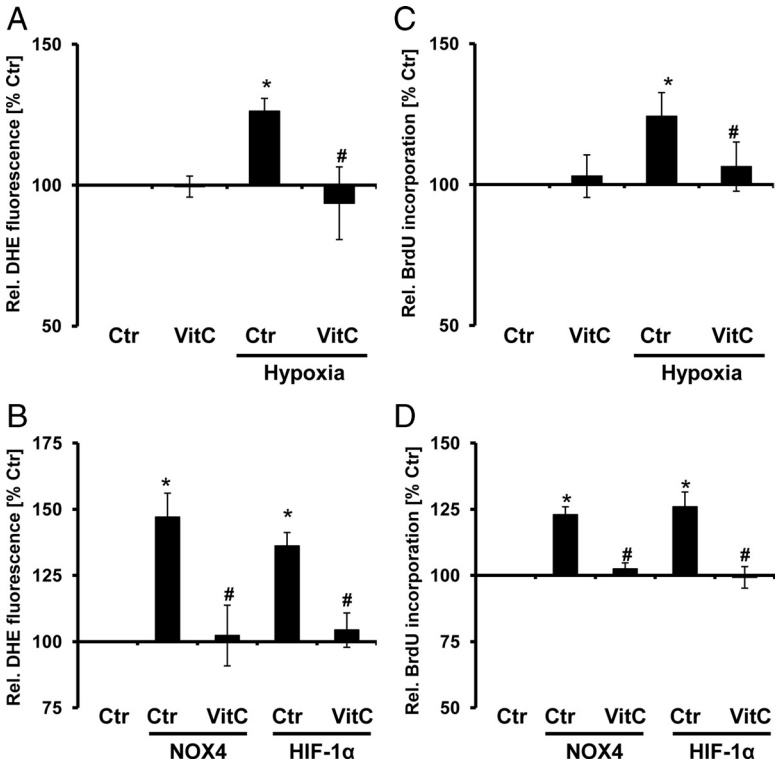

Figure 8. ROS mediate proliferation by hypoxia, NOX4, and HIF-1α. (A and B) Pulmonary artery smooth-muscle cells (PASMCs) were treated with vitamin C (VitC, 100 μM) for 60 min before exposure to hypoxia for 4 h. (A) ROS levels were evaluated by DHE fluorescence. (B) Proliferative activity was determined 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, $^{\#}p < 0.05$ vs. hypoxic control).

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 Wadsworth, 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.

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; Brusselmans *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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