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Original Contribution

UPREGULATION OF NAD(P)H OXIDASE 1 IN HYPOXIA ACTIVATES HYPOXIA-INDUCIBLE FACTOR 1 VIA INCREASE IN REACTIVE OXYGEN SPECIES

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Abstract—Hypoxia sensing and related signaling events, including activation of hypoxia-inducible factor 1 (HIF-1), represent key features in cell physiology and lung function. Using cultured A549 cells, we investigated the role of NAD(P)H oxidase 1 (Nox1), suggested to be a subunit of a low-output NAD(P)H oxidase complex, in hypoxia signaling. Nox1 expression was detected on both the mRNA and protein levels. Upregulation of Nox1 mRNA and protein occurred during hypoxia, accompanied by enhanced reactive oxygen species (ROS) generation. A549 cells, which were transfected with a Nox1 expression vector, revealed an increase in ROS generation accompanied by activation of HIF-1-dependent target gene expression (heme oxygenase 1 mRNA, hypoxia-responsive-element reporter gene activity). In A549 cells stably overexpressing Nox1, accumulation of HIF-1α in normoxia and an additional increase in hypoxia were noted. Interference with ROS metabolism by the flavoprotein inhibitor diphenylene iodonium (DPI) and catalase inhibited HIF-1 induction. This suggests that H_2O_2 links Nox1 and HIF-1 activation. We conclude that hypoxic upregulation of Nox1 and subsequently augmented ROS generation may activate HIF-1-dependent pathways.

Keywords—Catalase, Hypoxia-inducible factor 1, NAD(P)H oxidase 1, Oxygen sensing, Reactive oxygen species, Free radicals

INTRODUCTION

Oxygen supply and sensing play a fundamental role in lung pathology. In acute alveolar hypoxia, lung resistance vessels constrict to match lung perfusion with ventilation [1]. In chronic alveolar hypoxia, vascular remodeling of pulmonary vessels occurs, with prominent smooth muscle and adventitial fibroblast proliferation, leading to pulmonary hypertension and cor pulmonale [2,3]. The underlying O_2-sensing mechanisms for the short- and long-term response to hypoxia remain under investigation. Distinct mechanisms, including mitochondrial and NAD(P)H oxidase-dependent reactive oxygen species (ROS) formation, have been suggested [4]. Hypoxia-inducible factor 1 (HIF-1) has been shown to be involved in pulmonary responses to hypoxia [5–7], and specifically transfers oxygen sensing to the level of gene expression [8–10].

HIF-1 ubiquitously adapts homeostatic responses to hypoxia by regulating the expression of approximately 50 identified target genes encoding growth factors and receptors, as well as vascular, glycolytic, and cell cycle regulatory active proteins [11–14]. These genes include vascular endothelial growth factor, endothelin 1, phosphoglycerate kinase, and hemeoxygenase 1, which are activated by binding of HIF-1 to the hypoxia responsive element (HRE) of regulatory DNA gene regions [15]. HIF-1 is a heterodimer of HIF-1α and HIF-1β. HIF-1α is

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continuously degraded during normoxia via the ubiquitin proteasomal pathway, whereas HIF-1β levels are constant. Degradation of HIF-1α involves binding of Von Hippel–Lindau (VHL) protein to the oxygen-dependent degradation domain (ODDD), which targets HIF-1α for ubiquitination [16]. This interaction requires an oxygen-dependent enzymatic hydroxylation of a proline residue in the ODDD domain, and does not occur in hypoxia [17–19]. In addition to this well-explored basic mechanism of oxygen-dependent HIF-1 regulation by protein stabilization, the loss of hydroxylation of an asparagine residue in the transactivation domain during conditions of hypoxia has been proven to be crucial for HIF-1 transactivation [20].

Various oxygen sensing systems and transduction pathways upstream of, or unrelated to, the hydroxylation events are suggested to be linked to HIF-1 activation [21–23]. ROS, including superoxide anion and H_2O_2 of different cellular origins, are thought to be involved in oxygen sensing [24–27]. Physiological studies using intact lungs favored the role of NAD(P)H oxidase in oxygen-dependent events, with ROS formation increasing, rather than decreasing, in hypoxia [6,7,28,29]. Whereas it seems unlikely that the leukocyte-type NAD(P)H oxidase contributes to the regulation of HIF-1 [30], recently described low-output isoforms of NAD(P)H oxidases, in particular Nox1 [31–33], may provide such a function. In the present study, we tested this hypothesis by analyzing and manipulating Nox1 expression in a lung epithelial cell line, focusing on the impact of these manipulations on ROS formation and HIF-1 activation. We provide evidence that Nox1 is upregulated during hypoxia, and that this event is linked with enhanced HIF-dependent gene regulation via increased ROS formation. These findings suggest a major role of Nox1 in oxygen-dependent transcriptional regulation, which may also be important in nonpulmonary hypoxia-driven cellular events.

MATERIALS AND METHODS

Cell culture and hypoxic incubation

Culturing of the human pulmonary epithelial cell line A549 was performed according to the protocol given by the American Type Culture Collection. Hypoxic incubation was done in custom made humidified chambers at a level of 1% O_2. Normoxic cells were exposed to 21% O_2. Experiments were carried out under normobaric conditions at 37°C.

Western blot analysis

Western blot analysis of HIF-1α was performed using a previously described polyclonal HIF-1α antibody [34]. In brief, normoxic or hypoxic cells were scraped from dishes, and cellular protein extracts were prepared by homogenization in an ice-cold buffer (8 M urea, 10% glycerol, 1% sodium dodecyl sulfate, 5 mM dithiothreitol, 10 mM Tris–HCl pH 6.8) and a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). Twenty micrograms of protein was run per lane on a sodium dodecyl sulfate–polyacrylamide gel. For Nox1 and β-actin Western blot, cells were scraped from the dishes and lysed using a detergent buffer containing a protease inhibitor cocktail (Sigma–Aldrich). After centrifugation of cell debris, protein concentrations were determined and 20 μg protein per lane was run for Nox1 and 5 μg protein was run for β-actin on a 6% SDS–PAGE. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore) by electroblotting. Protein transfer was confirmed by Ponceau S staining. After the membrane was blocked in buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.25 % gelatin, and 0.05% Triton X-100 (TBST)) for 2 h at room temperature, Nox1 antibody was added at a dilution of 1:5000 and incubated overnight at 4°C. The polyclonal anti-Nox1 antibody was raised in rabbit using a synthetic peptide from the Nox1 C terminus as described [35]. After the membrane was washed in TBST buffer, specific immunoreactive signals were detected by chemiluminescence (ECL, Amersham, Buckinghamshire, England) by using a second antibody coupled with horseradish peroxidase.

Measurement of ROS

Intracellular ROS generation was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA Sigma–Aldrich) as described previously [24,36]. ROS in the cells cause oxidation of DCFH-DA to the fluorescent 2′,7′-dichlorofluorescein (DCF). In brief, confluent cells plated on six-well cell culture plates (Corning Inc., Corning, NY, USA) were washed and incubated with DCFH-DA (30 μM) in serum free culture medium under normoxic (21% O_2, 5% CO_2, balanced N_2) or hypoxic (1% O_2, 5% CO_2, balanced N_2) conditions for 2 h. The DCFH-DA-containing medium was removed; cells were washed twice with serum-free medium, lysed in liquid nitrogen in the presence of serum-free medium, and centrifuged (1200*g*, 5 min, 4°C). The fluorescence of the supernatant was measured at 530 nm with an excitation wavelength of 485 nm using a spectrofluorometer (FL-600, BIO-TEK Instruments, Inc., Winooski, VT, USA). Values are given as fluorescence at 530 nm from wells containing cells subtracted by background (wells without cells).

RNA extraction and real-time RT-PCR

RNA was extracted from cells using guanidine thiocyanate–acid phenol (RNAzol B, WAK-Chemie, Ger-

many). Two micrograms of RNA per sample was copied to cDNA using reverse transcriptase (MMLV-RT; Invitrogen, Carlsbad, CA, USA) in a standard protocol with $p(dT)_{15}$ primers. For the negative control, MMLV-RT was omitted. Real-time PCR was performed using the ABI Prism 7700 Detection System (Applied Biosystem, Foster City, CA, USA) with SYBR-Green as fluorescent dye, enabling real-time detection of PCR products according to the manufacturer's protocol. The cDNA was submitted to real-time PCR using the primer pairs as given below. Cycling conditions were 95°C for 15 min, followed by 50 cycles of 94°C for 10s, 55°C for 30s, 72°C for 30s. For quantification, the target gene was normalized to the internal standard gene porphobilinogen deaminase (PBGD) using the following equation: $T_0/R_0 = K(1+E)^{CT_R - CT_T}$, where T_0 = initial number of target gene mRNA copies, R_0 = initial number of standard gene mRNA copies, E = efficiency of amplification, CT_T = threshold cycle of target gene, CT_R = threshold cycle of standard gene, and K = constant. The efficiencies were shown to be in the range $0.90 < E < 1.0$. The following primer sets (+, forward; −, reverse) derived from the Genbank sequences were used: (Accession No.: human Nox1 NM_007052) Nox1+: 5′-CTT GCC TCC ATT CTC TCC AG-3′, Nox1−: 5′-CAC TCC AGT GAG ACC AGC AA-3′; PBGD (Accession No.: NM_000190) PBGD+: 5′-GCA CGA TCC CGA GAC TCT GC-3′ PBGD−: 5′-CCT TCC AGG TGC CTC AGG A-3′; heme oxygenase 1 (HO-1) (Accession No.: NM_002133) HO-1+: 5′-ATG ACA CCA AGG ACC AGA GC-3′, HO-1−: 5′-AGA CAG-CTG CCA CAT TAG GG-3′.

Construction of plasmid vectors for Nox1 and HIF-1α

For isolation of full-length cDNA fragments of HIF-1α and Nox1, we employed a nested RT-PCR from

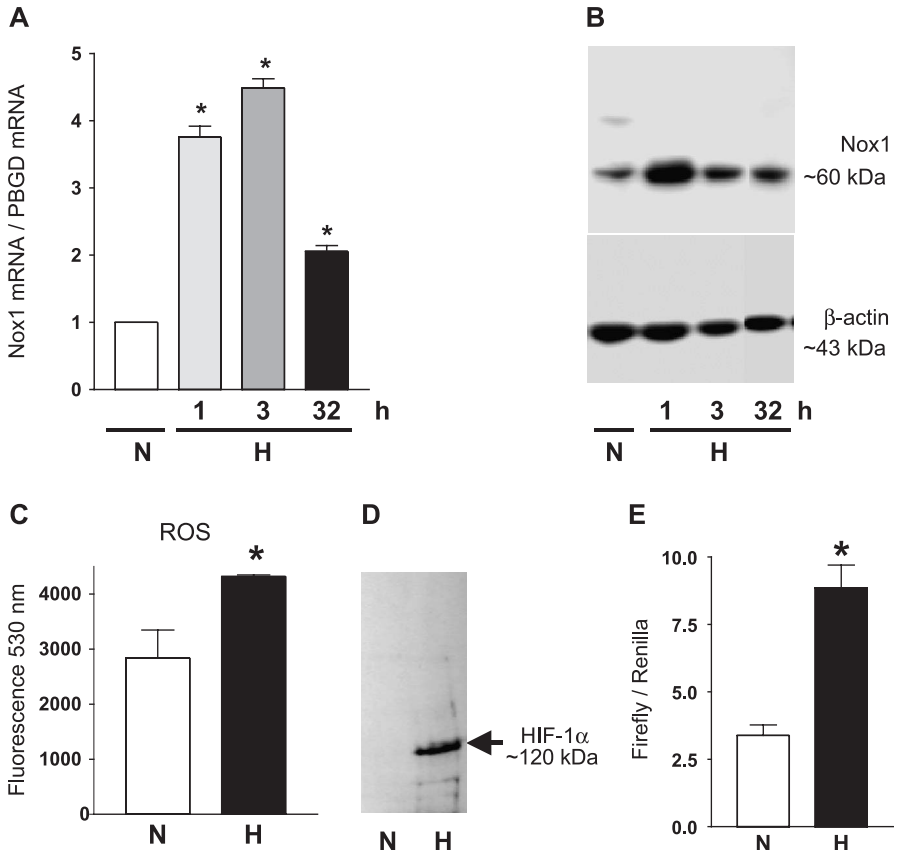

Fig. 1. Nox1 expression, reactive oxygen species (ROS) formation, and HIF-1α activation in A549 cells: (A) Nox1 mRNA analysis by real-time RT-PCR of RNA extracts from human pulmonary epithelial cell line A549 cultured under normoxic (N) or hypoxic (H) conditions. *Significant differences compared with N ($n = 3$, SEM, $p < .001$, ANOVA). (B) Western blot analysis of Nox1 and β-actin. Cellular protein extracts incubated under conditions of normoxia or hypoxia for up to 32 h were analyzed. Induction of Nox1 in hypoxia is observed. (C) Intracellular ROS generation measured by the DCF assay in A549 cells after 2 h of incubation under normoxic or hypoxic conditions. *Significant difference compared with N ($n = 3$, SEM, $p < .01$, paired t test). (D) HIF-1α Western blot analysis after 24 h of normoxia or hypoxia. (E) Hypoxia-responsive-element (HRE) reporter gene assay after 24 h of normoxic or hypoxic incubation. *Significant difference compared with N ($n = 4$, SEM, $p < .05$, paired t test).

RNA extracts of human lung cells. In all constructs, we inserted the described Kozak sequence (GCCGCCAC-CATGG) for maximal translation efficacy [37]. The following primer sets were designed: HIF-1α (Accession No.: - NM_001530) for first PCR, HIF1+: 5′-TGA AGA CAT CGC GGG GAC CG-3′ and HIF1−: 5′-TAA CCA AGT TTG TGC AGT ATT GTA GCC AGG-3′; and for second PCR: HIF-1-nested-Kozak+: 5′-GCC GCC ACC ATG GAG GGC GCC GGC GGC-3′ and HIF-1-nested-5′-GAC TGC TTT AGG TAG TGA GCC ACC AGT GTC C-3′; Nox1 (Accession: No. NM_007052) for first PCR, Nox1+: 5′-GAA TCT TCC CTG TTG CCT AG-3′ and Nox1−: 5′-GAG GCA CAT TCT TAT GCT AAA-G 3′; and for second PCR: Nox1-nested-Kozak+: 5′-GCC GCC ACC ATG GGA AAC TGG GTG GTT AAC CAC-3′ and Nox1-nested-5′-GCA ATC AAA AAA AGA ATA CCA GGG AG-3′. These fragments were cloned into the pGEMT-Easy Vector (Promega, Madison WI, USA) and inserts were verified by DNA sequencing (ABI Prism 310, Applied Biosystems). Sequencing of Nox1 cDNA revealed a variant with an amino acid exchange from proline to serine at position 276. This sequence was deposited to EMBL under Accession No. AJ438989. Nox1 and HIF-1 fragments were then subcloned into the pcDNA3.1+ vector (Invitrogen) Carlsbad, CA, USA) by *Not*I restriction digestion. Orientations of the inserts were verified and constructs with both sense and antisense orientation were selected.

Construction of bicistronic plasmid vectors

For isolation of full-length cDNA fragments of catalase, we performed RT-PCR using RNA extracts from A549 cells using the following primer sets designed from sequence Accession No. NM_001752: Cat-Kozak+: 5′-GCC GCC ACC ATG GCT GAC AGC CGG GAT CC-3′; Cat-5′-GCT TCG CTG CAC AGG TGC-3′. The fragment was cloned into the pGEMT-Easy Vector (Promega) and insert was verified by DNA sequencing (ABI Prism 310, Applied Biosystems). This fragment was then subcloned into the pcDNA3.1+ vector by *Not*I restriction digestion. Nox1 and HIF-1α fragments were also subcloned into the pMG Vector (Invivogen, San Diego, CA, USA) multiple cloning site 1 by *Xba*I and *Nhe*I restriction digestion. Constructs with sense orientation were selected. These were named pMG−Nox1 and pMG−HIF-1α, respectively. HIF-1α from pcDNA3.1+ construct was further subcloned into pMG−Nox1 in multiple cloning site 2 by *Xba*I and *Nhe*I restriction digestion. New construct was named pMG−Nox1−HIF-1α. Catalase was also further subcloned into pMG−Nox1 by *Xba*I and *Nhe*I restriction digestion in multiple cloning site 2. The constructs were named pMG−Nox1−Cat.

Reporter gene assay for HIF-α— dependent gene transcription

For studying HIF-1-dependent gene regulation a dual reporter gene assay was performed. One vector carrying a firefly luciferase gene, controlled by a three-tandem repeat of the HRE coupled to a thymidine kinase (TK) promoter, was cotransfected with a second reporter

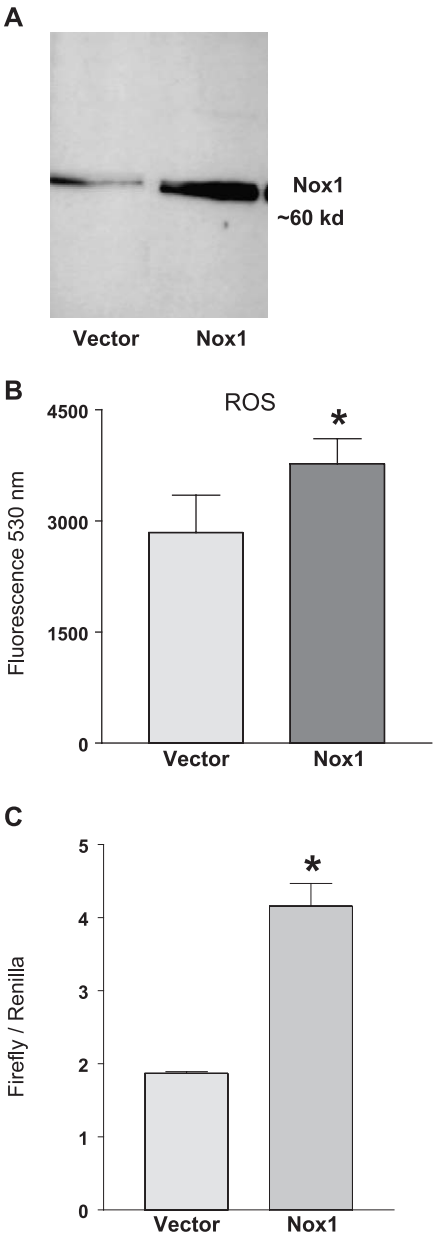

Fig. 2. Characterization of A549 cells transiently transfected with Nox1 expression vector (Nox1) or empty vector (Vector) 24 h after transfection: (A) Nox1 Western blot analysis. (B) Measurement of reactive oxygen species (ROS) with dichlorofluorescein. *Significant difference compared with vector ($n = 3$, SEM, $p < .05$, paired t test). (C) Hypoxia-responsive element reporter gene assay. *Significant difference compared with vector ($n = 4$, SEM, $p < .01$, paired t test).

vector carrying a renilla luciferase gene controlled by the TK promoter without HRE. The values represent the ratio of chemiluminescence measured for firefly and renilla, respectively, thus giving the HRE-specific luciferase activity. Hypoxic responsive promoter constructs were prepared by inserting the consensus HRE element of the erythropoietin gene promoter (HIF-HRE: 5′-GCCCTACGTGCTGTCTCA-3′) as three-tandem repeat 5′ upstream of the herpes simplex thymidine kinase promoter (TK) into the pGL3 plasmid (Promega) containing the firefly luciferase. The insert was verified by sequencing. Either the pcDNA–Nox1 sense, pcDNA–Nox1 antisense, pcDNA–HIF-1α sense, pcDNA–HIF-1α antisense, or pcDNA3.1+ plasmid constructs were cotransfected with the HRE-TK and pRL-TK plasmid in a molar ratio of 5:5:1 into A549 cells by incubation with a mixture of cationic lipids and plasmid DNA for 6 h according to the manufacturer's protocol (Lipofectamin 2000, Invitrogen) in a 48-well plate. Analogous cotransfection experiments were performed using the bicistronic constructs. Either empty vector pMG, pMG–Nox1, pMG–HIF-1α, pMG–Nox1–Cat, or

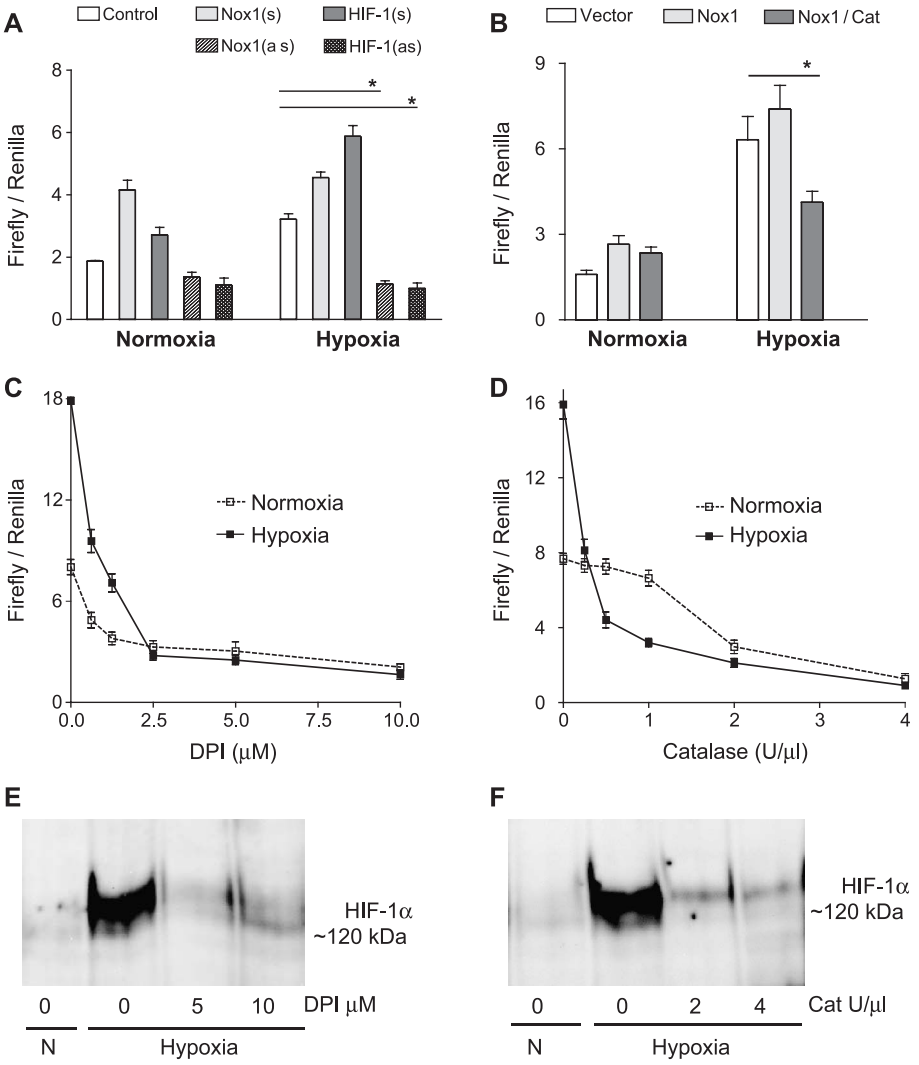

Fig. 3. Hypoxia-responsive-element reporter gene assay in A549 cells treated with Nox1 and ROS interfering compounds. Luciferase activity was measured after 20 h of normoxic or hypoxic incubation: (A) Cells were transfected with Nox1 sense (Nox1(s)), Nox1 antisense (Nox1(as)), HIF-1α sense (HIF-1(s)), or HIF-1α antisense (HIF-1(as)) expression vector. *Significant difference of Nox1(as) compared with empty vector ($n = 4$, SEM, $p < .05$, paired t test). (B) Cells were transfected by a bicistronic vector containing two expression cassettes. Either these carried no insert (Vector), or Nox1 insert (Nox1), or both a Nox1 insert and catalase insert (Nox1/Cat). *Significant difference of Nox1/Cat compared with Nox1 ($n = 4$, SEM, $p < .01$, paired t test). (C) Cells were incubated with the flavoprotein inhibitor diphenylene iodonium (DPI). (D) Cells were incubated with catalase (Cat) ($n = 4$, SEM). (E) HIF-1α Western blot analysis of hypoxically (24 h) incubated cells treated with DPI. Data from normoxically incubated cells (N) are used as the control. (F) HIF-1α Western blot analysis of hypoxically (duration 24 h) incubated cells treated with catalase. Data from normoxically incubated cells (N) are used as the control.

pMG–Nox1–HIF-1α were cotransfected with HRE-TK and pRL-TK. Transfected cells were then incubated for 6 h in FCS-containing medium, after which medium was replaced by serum-free medium, and culture under normoxic or hypoxic conditions was conducted for 20 h. The cells were lysed in 4°C using passive lysis buffer (Promega), and firefly and renilla luciferases activities were measured with a bioluminometer (TECAN, Crailsheim, Germany).

Generation of cells stably overexpressing Nox1

Culture of the human pulmonary epithelial cell line A549 was performed according to the protocol given by the American Type Culture Collection. A549 cells were transfected with either the pMG–Nox1 construct described above or empty pMG vector by incubating cells with a mixture of cationic lipids and plasmid DNA for 6 h according to the manufacturer's protocol (Lipofectamin 2000, Invitrogen) in 100-mm plates. Twenty-four hours posttransfection, cells were treated with medium containing 800 μg/ml hygromycin B (Roche Diagnostics, Indianapolis, IN, USA). The medium containing hygromycin was replaced every 48 h for 2 weeks. The concentration of hygromycin was reduced to 500 μg/ml after 2 weeks for 2 more weeks and finally cells were maintained in 300 μg/ml hygromycin continuously. The cells were characterized by an increase in Nox1 mRNA by real-time PCR and an increase in ROS generation by the measurement described above.

Chemicals

Catalase and diphenylene iodonium (DPI) used in some experiments were from Sigma–Aldrich.

RESULTS

Effect of hypoxia on human lung A549 cell line

Real-time RT-PCR of A549 cells revealed endogenous Nox1 mRNA expression, which was markedly upregulated during hypoxia (Fig. 1A). Concomitantly, we observed transient hypoxic Nox1 protein induction, as shown by Nox1 Western blot (Fig. 1B). Measuring ROS generation by dichlorofluorescein, we found an increase in ROS in hypoxia. (Fig. 1C). Analysis of HIF-1α Western blot and HIF-1α target gene expression by hypoxia-responsive-element reporter gene assay revealed the typical hypoxic induction of HIF-1 (Figs. 1D, 1E).

Effect of transient overexpression of Nox1 on HIF-1α—dependent gene transcription

Considering the effect of hypoxia on Nox1, ROS, and HIF-1, we aimed to investigate the possibility of a causal link between these components. To analyze the role of Nox1 in ROS generation and its possible influence on HIF-1α-dependent target gene expression, transfection experiments were undertaken. A Nox1 cDNA expression vector was employed and Nox1 transfection was controlled by Nox1 Western blot analysis (Fig. 2A). In Nox1-overexpressing cells, an increase in ROS generation was observed (Fig. 2B). In parallel, Nox1-transfected cells were analyzed by HRE reporter gene assay. Cotransfection of a mixture of Nox1 plasmid and HRE reporter plasmid was performed to ensure that luciferase activity derived from HRE reporter plasmid was measured with high probability in cells overexpressing Nox1. Thus, HRE activation is measured in transfected cells only (~12% fraction), and not from nontransfected cells. These experiments revealed a significant induction of hypoxia-dependent reporter gene activation in the case of Nox1 transfection, when compared with the empty vector control (Fig. 2C).

Effect of inhibition of Nox1 on HIF-1 induction

For analysis of the signaling cascade between hypoxia, Nox1, and HIF-1 regulation, we employed various strategies for Nox1 inhibition or interference with ROS and combined these with a HRE reporter gene assay. Transfection of a Nox1 antisense expression vector diminished the induction of hypoxia-dependent reporter gene activation (Fig. 3A). In these experiments we used

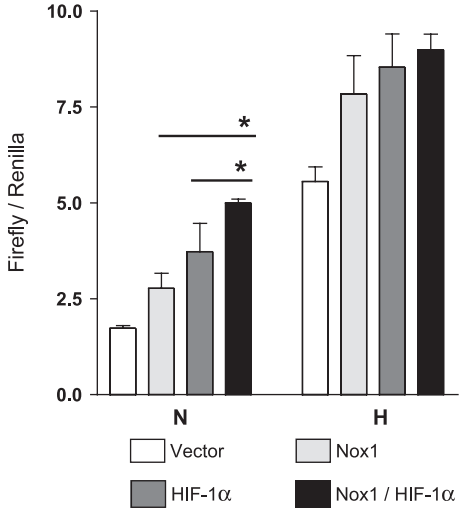

Fig. 4. Hypoxia-responsive-element reporter gene assay in A549 cells transfected with a bicistronic vector containing two expression cassettes. These either carried no insert (vector), a Nox1 insert (Nox1), a HIF-1α insert (HIF-1α), or both a Nox1 insert and a HIF-1α insert (Nox1/HIF-1α). Luciferase activities were measured under both normoxic (N) and hypoxic (H) conditions. *Significant difference of Nox1/HIF-1α compared with Nox1 or compared with HIF-1α ($n = 4$, SEM, $p < .01$, paired t test).

HIF-1α sense and antisense expression plasmids as a control revealing an increase or decrease in HRE activation as anticipated. Employing the flavoprotein inhibitor DPI to target Nox1, we observed a dose-dependent decrease in reporter gene activation (Fig. 3C). Similarly, addition of exogenous catalase, which cleaves H_2O_2, inhibited HRE activation (Fig. 3D), suggesting that H_2O_2 may be relevant for HIF-1α induction. The effects of DPI and catalase treatment on HIF-1α protein levels were also analyzed by Western blot. Both compounds prevented hypoxia-induced HIF-1α protein accumulation (Figs. 3E, 3F). To further validate the finding that Nox1 derived ROS trigger HIF induction, we employed coexpression of catalase and Nox1. Interestingly, the inductive effect of hypoxia and Nox1 on HIF-1 was strongly suppressed when coexpressing catalase in a bicistronic expression vector having two expression cassettes (Fig. 3B). On the other hand, coexpression of Nox1 and HIF-1α had an additive effect on HIF-1α activation in this vector system (Fig. 4).

Analysis of HIF-1, Nox1, and ROS in cells stably overexpressing Nox1

Next, we analyzed selected cells stably overexpressing Nox1. These cells have the advantage over transient transfected cells that the majority of the cell population is overexpressing Nox1. This allows different approaches for analysis such as direct measurement of HIF-1α protein and HIF-1α-dependent target gene expression analysis. Cells stably overexpressing Nox1 showed 6-fold higher expression of Nox1 mRNA levels when compared with the empty vector control in normoxia. Hypoxic induction of Nox1 mRNA was approximately 4-fold for control cells and 1.2-fold for Nox1 transfected cells (Fig. 5A). We found increased ROS generation in Nox1 stably transfected cells when compared with empty vector control (Fig. 5B). Interestingly, in Nox1-trans-

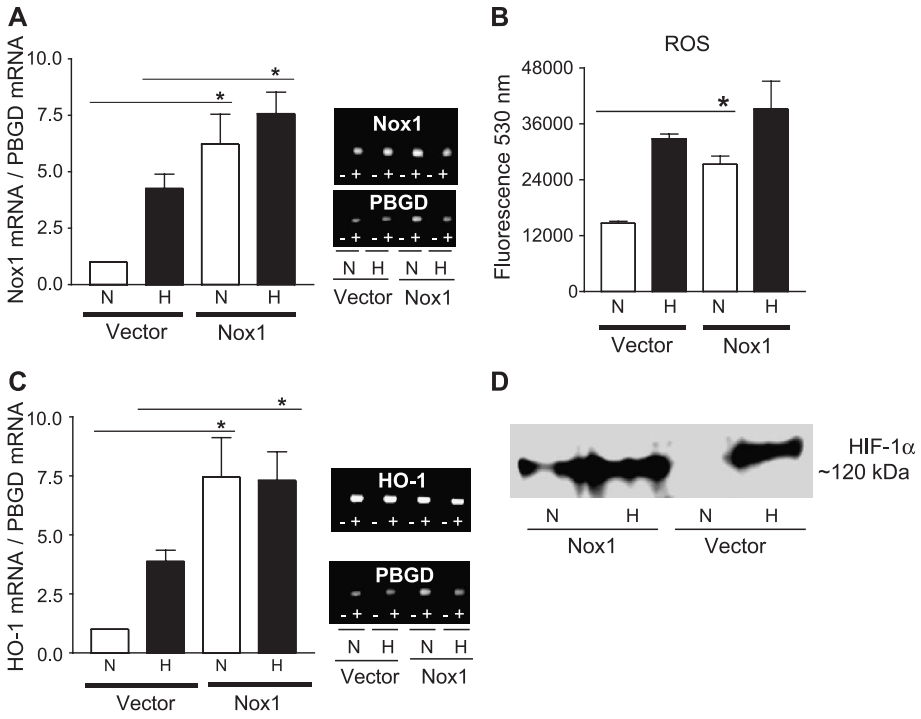

Fig. 5. Characterization of A549 cells stably transfected with Nox1. Nox1-expressing cells were selected by hygromycin resistance coupled to the Nox1 cDNA insert by internal ribosomal entry site, ensuring common expression of Nox1 and hygromycin. (A) Nox1 mRNA quantification by real-time RT-PCR using porphibilinogen deaminase (PBGD) gene as housekeeping reference gene in empty vector control cells (Vector) and Nox1-overexpressing cells (Nox1) after normoxic or hypoxic incubation for 24 h. *Significant difference compared with vector (n = 3, SEM, $p < 0.05$, paired t test, performed on C_T differences). The Insets: Ethidium bromide-stained agarose gel electrophoresis of endpoint RT-PCR products, for validation of fragment length and minus (−) and plus (+) RT control, respectively. (B) ROS measurements (dichlorofluorescein) in Nox1-overexpressing cells (Nox1) and empty vector control cells (Vector). *Significant difference compared with vector (n = 4, SEM, $p < .01$, paired t test). (C) Measurement of HIF-1-dependent target gene heme oxygenase 1 expression by real-time RT-PCR using PBGD gene as a reference gene in empty vector control cells (Vector) and Nox1-overexpressing cells (Nox1) after both normoxic and hypoxic incubation for 24 h. *Significant difference compared with vector (n = 3, SEM, $p < .05$, paired t test). Insets: Ethidium bromide-stained agarose gel electrophoresis of endpoint RT-PCR products, for validation of fragment length and minus (−) and plus (+) RT control, respectively. (D) HIF-1α Western blot analysis in cells overexpressing Nox1 (Nox1) and empty vector control cells (Vector).

fected cells, the increased levels of HIF-1α protein, typically observed only in hypoxia, were observed in normoxia (Fig. 5D). Additionally, we measured heme oxygenase 1 mRNA, an endogenously expressed HIF-dependent target gene. As for HIF-1α, we found strongly activated heme oxygenase 1 expression in Nox1-overexpressing cells, even during normoxia, when compared with empty vector control (Fig. 5C).

DISCUSSION

Regulation of the transcription factor HIF-1α has been suggested to be linked to ROS generation. It is not known whether generation of ROS in hypoxia is mediated by mitochondria, by NAD(P)H oxidases, or by both. In addition, there are conflicting data in relation to the increase or decrease in ROS during hypoxia. The present studies addressed these issues by transfecting lung cells with different molecular tools.

The studies were performed using the human pulmonary epithelial cell line A549. The first important finding was the detection of Nox1 mRNA and protein expression in these cells. When addressing the regulation of Nox1, we found a strong increase in Nox1 mRNA in hypoxia peaking at 3 h, which was accompanied by upregulation of Nox1 protein. Hypoxic induction of Nox1 may explain the enhanced ROS generation encountered in these cells under hypoxia. The regulation of superoxide generation by Nox1 in terms of the oxygen concentration is currently not known; however, it is conceivable that increased expression of Nox1 causes enhanced ROS formation.

The second important finding is that enhanced Nox1 expression is linked to an accumulation of HIF-1α and stimulation of HIF-dependent target gene transcription. Various approaches using transient and stably transfected Nox1 cells support this link: A specific HRE-dependent reporter gene assay showed an increase in activation on transient transfection of Nox1, which was further enhanced on subsequent exposure to hypoxia. The same features were noted for cells transfected with HIF-1α, which was anticipated and served as a positive control. Interestingly, combined transfection of HIF-1α and Nox1 produced a further increase in HRE-dependent luciferase activation surpassing each single transfection. In a further approach, using cells stably overexpressing Nox1, we demonstrated that Nox1 causes HIF-1α protein accumulation, even under normoxic conditions. Control cells stably transfected with empty vector displayed no HIF-1α in normoxia, while the typical hypoxic HIF-1α induction was retained. Moreover, we investigated these cells for endogenous expression of heme oxygenase 1, a well-studied HIF-1-dependent target gene [38]. Nox1-transfected cells showed a strong induction of heme oxygenase 1 in accordance with the increased HIF-1α expression in these cells.

The third major finding is the link between Nox1-derived ROS and HIF-1 activation. Using DCF fluorescence, a well-established technique for cell culture experiments [24], we found enhanced ROS generation during hypoxia. More importantly, we noticed an enhancement of ROS generation after Nox1 transfection. This finding is concordant with the previous observation of increased H_2O_2 levels in cells stably transfected with Nox1 [39], but is in contrast with investigations in HEPG2 cells, where ROS generation decreased under hypoxic conditions [40]. Irrespective of the underlying molecular mechanisms, the present observations, in accordance with indirect evidence from inhibitor studies in intact organs [4,27,41–43], strongly support the notion that Nox1-related ROS generation may increase during hypoxia. Nox1-dependent superoxide anion production may thus represent an alternative nonmitochondrial source for hypoxia-induced upregulation of ROS [27,44]. Interestingly, we were able to find the expression of mRNAs for Nox1 organizer and Nox1 activator in A549 cells (data not shown). These proteins represent homologs of the classic $p67^{phox}$ and $p47^{phox}$ subunits, and are important for ROS generation involving Nox1 [45]. When using the NAD(P)H oxidase inhibitor DPI (Fig. 3E), we found a decrease in HRE-dependent gene activation accompanied by reduced HIF-1α protein levels. However, due to its lack of specificity [46], this inhibitor may also interfere with the mitochondrial respiratory chain [24,26,27]. For this reason, we used transfection of a plasmid expressing Nox1 antisense mRNA as a tool for inhibition of Nox1. This resulted in a decrease in HRE-dependent transcriptional activation, again supporting a role for Nox1 in HIF-1 regulation.

As an additional tool to interfere with ROS generation, we transiently transfected cells with catalase or treated them with exogenously added catalase, to cleave H_2O_2. Catalase attenuated Nox1-induced upregulation of HIF-1-dependent gene transcription as measured by reporter gene assay. Also, a decrease in the HIF-1α protein level was noted in cells treated with exogenous catalase. This finding is in contrast with investigations in HEPG2 cells stably overexpressing catalase that demonstrated unchanged levels of HIF-1α during hypoxia [47]. Cell type differences or long-term adaptive processes in these cells may explain their dissimilar hypoxic response when compared with our study.

Our data suggest a sequence of NAD(P)H-dependent superoxide formation, conversion to H_2O_2, and subsequent HIF-1 activation. This activation may be mediated on the translation level, HIF-1α stabilization level, and/or transactivation level. A crucial role of H_2O_2 in HIF-1 activation was previously suggested by Chandel et al. for

Hep3B cells [24], with evidence for a mitochondrial source of ROS in these cells. In that study, the increase in HIF-1-dependent transcriptional activity was reproduced by exogenous H_2O_2. Three recent studies investigated the link between HIF-1 activation, induction of NAD(P)H oxidase complexes, and ROS generation. The first study documented activation of HIF-1 by thrombin, mediated via activation of a $p22^{phox}$-containing NAD(P)H oxidase complex and increased ROS production in endothelial cells [48]. The second study demonstrated that angiotensin induces HIF-1α via ROS-dependent activation of PI3 kinase, leading to increased HIF-1α translation [49]. The third study revealed involvement of the small GTPase rac1 in the activation of HIF-1α [50]. Interestingly, rac1 is involved in assembly of the classic NAD(P)H oxidase complex containing $gp91^{phox}$ [51] and may play a similar role for an unidentified hypothetical Nox1-containing NAD(P)H oxidase complex. Our investigation, together with these studies, favors the overall concept that NAD(P)H oxidase activities are linked to HIF-1-dependent gene regulation via ROS signaling.

The mechanism of action of ROS on HIF-1α activation may involve cell specific pathways, such as ROS-dependent regulation of prolyl or asparagyl hydroxylases. These enzymes depend on the amount of oxygen, ascorbate, 2-oxoglutarate, and Fe^{2+}. Fe^{2+} concentration can be affected by the redox status, which is influenced by ROS generation. Moreover, ROS may regulate the complex assembly of HIF-1 with CBP/P300, which also depends on the redox status [52]. This may support the finding that overexpression of catalase also interferes with HRE-dependent gene regulation, after overexpression of HIF-1α (data not shown).

In conclusion, we report that Nox1 expression is increased during hypoxia. Furthermore, enhanced Nox1 activity, as mimicked by overexpression, is linked to HIF-1α accumulation and subsequent HRE-dependent gene expression. Conversely, inhibition of Nox1 suppresses HIF-1-dependent gene transcription. Our data suggest that hypoxia-induced Nox1 upregulation is an important signaling mechanism upstream of HIF-dependent gene regulation and involves ROS as an intermediate signal.

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