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Hypoxia Up-Regulates Hypoxia-Inducible Factor-1α Transcription by Involving Phosphatidylinositol 3-Kinase and Nuclear Factor κB in Pulmonary Artery Smooth Muscle Cells

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The oxygen sensitive α-subunit of the hypoxia-inducible factor-1 (HIF-1) is a major trigger of the cellular response to hypoxia. Although the posttranslational regulation of HIF-1α by hypoxia is well known, its transcriptional regulation by hypoxia is still under debate. We, therefore, investigated the regulation of HIF-1α mRNA in response to hypoxia in pulmonary artery smooth muscle cells. Hypoxia rapidly enhanced HIF-1α mRNA levels and HIF-1α promoter activity. Furthermore, inhibition of the phosphatidylinositol 3-kinase (PI3K)/AKT but not extracellular signal-regulated kinase 1/2 pathway blocked the hypoxia-dependent induction of HIF-1α mRNA and HIF-1α promoter activity, suggesting involvement of a PI3K/AKT-regulated transcription factor. Interestingly, hypoxia also induced nuclear factor-κB (NFκB) nuclear translocation and activity. In line, expression of the NFκB subunits p50 and p65 enhanced HIF-1α mRNA levels, whereas blocking of NFκB by an inhibitor of nuclear factor-κB attenuated HIF-1α mRNA induction by hypoxia. Reporter gene assays revealed the presence of an NFκB site within the HIF-1α promoter, and mutation of this site abolished induction by hypoxia. In line, gel shift analysis and chromatin immunoprecipitation confirmed binding of p50 and p65 NFκB subunits to the HIF-1α promoter under hypoxia. Together, these findings provide a novel mechanism in which hypoxia induces HIF-1α mRNA expression via the PI3K/AKT pathway and activation of NFκB.

INTRODUCTION

An adequate supply of oxygen is mandatory for the life of aerobic organisms. Thus, a decrease in O_2 availability needs to initiate processes enabling adaptation to these changed conditions. A crucial regulator of these adaptational responses is the transcription factor hypoxia-inducible factor-1 (HIF-1) (Wenger *et al*., 2005; Semenza, 2006). To date, HIF-1 has been shown to regulate >100 genes, products of which control angiogenesis, oxygen transport, glucose metabolism, vascular tone, and cell proliferation and survival (Wenger *et al*., 2005; Semenza, 2006). A pathophysiological role for HIF-1 has been established for ischemic diseases and cancer and for chronic hypoxic pulmonary hypertension (Semenza, 2005; Brahimi-Horn and Pouyssegur, 2007).

Pulmonary hypertension is a progressive disease that is characterized by elevated vascular resistance and vascular remodeling (Semenza, 2005; Stenmark *et al*., 2006). Indeed, chronic hypoxia induces many changes within the lung vasculature that correlate with an increase in pulmonary artery pressure and dramatic structural and biochemical changes in both large and small vessels. These changes have been associated with the activation of HIF-1 (Semenza, 2005; Stenmark *et al*., 2006) because *Hif-1α*$^{+/-}$ mice showed delayed development of pulmonary hypertension and pulmonary vascular remodeling in response to hypoxia (Yu *et al*., 1999).

HIF-1 is a heterodimer composed of an inducible α-subunit that confers the sensitivity to oxygen and a constitutively expressed β-subunit, arylhydrocarbon receptor nuclear translocator (Kallio *et al*., 1999; Wenger *et al*., 2005; Semenza, 2006). The predominant mode of HIF-1α regulation occurs at the level of protein stability. This is brought about by the oxygen-dependent degradation domain termed ODD. This domain contains proline residues (Pro402 and Pro564) that under normoxic conditions are targets of O_2-dependent prolyl hydroxylases (PHDs) (Schofield and Ratcliffe, 2004). Once the prolines within the ODD are hydroxylated, they are recognized by the von Hippel-Lindau tumor suppressor protein, which initiates the ubiquitinylation process and degradation by the 26S proteasome system. Under hypoxic conditions, PHD activity is inhibited, thereby allowing stabilization and accumulation of the HIF-1α protein.

In addition to the regulation of HIF-1α by protein stabilization, several in vivo studies showed increased levels of HIF-1α mRNA when mice, rats, and ferrets were exposed to hypoxia (Wiener *et al*., 1996; Semenza *et al*., 1997; Yu *et al*., 1998; Chen *et al*., 2006). Enhanced HIF-1α mRNA levels could also be observed in lungs, further supporting the importance of this regulation for the development of pul-

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monary vascular remodeling (Palmer *et al*., 1998). However, the mechanisms underlying up-regulation of HIF-1α mRNA in pulmonary cells has not been investigated so far.

Therefore, we sought to determine the mechanism by which hypoxia is able to affect HIF-1α mRNA levels in pulmonary artery smooth muscle cells (PASMC), the main cell type responsible for vascular remodeling in pulmonary hypertension. We found that hypoxia initiates a rapid but transient increase of HIF-1α mRNA. This response involves activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and of nuclear factor-κB (NFκB), which in turn binds and activates the HIF-1α promoter. These results provide a new mechanism that explains the observed increases of HIF-1α mRNA levels in tissues exposed to hypoxia.

MATERIALS AND METHODS

Reagents

Kinase inhibitors were from Calbiochem (San Diego, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Animals

Mice (C57BL/6J) were placed for 2 h in a custom-made normobaric chamber and maintained at 10% O_2. Mice were killed by cervical dislocation, and lungs were dissected for RNA extraction. Control mice breathing room air were killed in the same manner. For ex vivo experiments, lungs were dissected and washed two times in DMEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Lung tissue samples were exposed to hypoxia (1% O_2) for 2 h in DMEM before RNA extraction. Animal use and experiments were reviewed and approved by the Animal Care Committee of the local institutions.

Cell Culture

Human PASMC were from Lonza (Verviers, Belgium) and cultured in the medium provided as recommended. PASMC (passages 3–11) were serum deprived for 24 h before exposure to hypoxia (1% oxygen) for the indicated times in a hypoxia workstation (InVivo400; Biotrace, Bothell, WA). When inhibitors were used, cells were pretreated for 1 h before exposure to hypoxia. Because PASMC do not efficiently express luciferase constructs, A7r5 rat smooth muscle cells (rSMC) were used for reporter gene assays, and they were grown in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin as described previously (BelAiba *et al*., 2004).

Plasmids

Expression vectors for the NFκB subunits p50 and p65 and dominant-negative inhibitor of nuclear factor-κB (IκBα), as well as the HIF-1α promoter luciferase constructs pHIF1α-538 and pHIF1α-106 and the luciferase construct NFκB-Luc have been described previously (Minet *et al*., 1999b; Djordjevic *et al*., 2004). The constructs pHIF1α-538m(−197) containing a mutated NFκB site at −197 base pairs (Bonello *et al*., 2007), and HIF1α-538m(+149), containing a mutated NFκB site at +149, were generated with the QuikChange mutagenesis kit (Promega, Madison, WI) and confirmed by sequencing. Transfection of PASMC or rSMC and luciferase assays were performed as described previously (BelAiba *et al*., 2004).

Immunofluorescence

Immunofluorescence was performed as described previously (Görlach *et al*., 2001). PASMC were grown on glass coverslips to ~80% confluence, serum starved, and exposed to hypoxia. Cells were then fixed with ice-cold methanol/acetone and incubated with antibodies against p50 or p65 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Secondary antibodies used were coupled to Alexa Fluor 594 (MoBiTech, Göttingen, Germany).

Northern Blot Analysis

Total RNA was isolated as described previously (Djordjevic *et al*., 2004). Ten micrograms of RNA was subjected to Northern blot analysis, and hybridizations were performed with a digoxin-labeled HIF-1α cDNA fragment (Kietzmann *et al*., 2001) .

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as described previously (Djordjevic *et al*., 2004). PCR was performed from first-strand cDNA synthesized from 1 μg of total RNA by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR was performed using the following primers to detect 1) mouse HIF-1α: 5′-GTCGGACAGCCTCACCAAACAG-3′ as forward primer and 5′-TAGGTAGTGAGCCACCAGTGTCC-3′ as reverse primer (542 base pairs); and 2) 18S: 5′-CGGCTACCACATCCAAGGAA-3′ as forward primer and 5′-GCTGGAATTACCGCGGCT-3′ as reverse primer (186 base pairs). The PCR for HIF-1α was performed in 32 cycles with the following cycle profile: 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The PCR for 18S was set up for 25 cycles with the following cycle profile: 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min. The PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized using GelDoc software (Bio-Rad, Hercules, CA).

Western Blot Analysis

Western blot analysis was performed as described previously (BelAiba *et al*., 2004). Fifty micrograms of protein was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and blotted onto nitrocellulose membranes. The monoclonal antibody against HIF-1α was from BD Biosciences (San Jose, CA), and it was used in a 1:1000 dilution. The antibodies against phosphorylated AKT (phospho-Ser473 and phospho-Thr308) and against phospho-Thr202/phospho-Tyr204-extracellular signal-regulated kinase (ERK) were from BD Biosciences, and they were used in a 1:1000 dilution. The antibody against smooth muscle cell actin (1:1000 dilution) was from Santa Cruz Biotechnology. Goat anti-mouse or goat anti-rabbit immunoglobulin (Calbiochem) was used as secondary antibody. The enhanced chemiluminescence Western blotting system was then used for detection.

Chromatin Immunoprecipitation

Confluent cells were grown on 15-cm dishes, serum starved for 24 h, and exposed to hypoxia for 1 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 either with the p50 or the p65 antibody (Santa Cruz Biotechnology) overnight at 4°C. PCR was performed with primers for the HIF-1α promoter (forward, 5′-GAACAGAGAGCCCAGCAGAG-3′; and reverse, 5′-TGTGCACTGAGGAGCTGAGG-3′) flanking the NFκB binding site (−197/188 base pairs) at 55°C for 35 cycles or primers for the PAI-1 promoter (forward, 5′-GCTCTTTCCTGGAGGTGGTC-3′; and reverse, 5′-GGGCACAGAGAGAGTCTGGA-3′).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extraction was performed as described previously (Dimova and Kietzmann, 2006). Nuclear extracts were hybridized with ^{32}P-labeled oligonucleotides containing the sequence ggg gtg ggg act tgc cgc ctg cg from the HIF-1α promoter or a mutated sequence ggg gtg aat tct tgc cgc ctg cg in binding buffer (50 mM Tris, 750 mM KCl, 2.5 mM EDTA, 0.5% Triton-X 100, 62.5% glycerol, and 1 mM dithiothreitol) for 45 min at 4°C. For competition assays, unlabeled oligonucleotides were added to the mixture 15 min before adding the labeled probe at 25× molar concentration.

Statistical Analysis

Values are presented as means ± SD. Results were compared by Student–Newman–Keuls *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

Hypoxia Increases HIF-1α mRNA and Protein Levels

First, we investigated whether exposure to hypoxia would increase HIF-1α mRNA levels. Indeed, when mice were exposed to hypoxia (10% O_2) for 2 h, HIF-1α mRNA levels were elevated in lung tissues (Figure 1A). Similarly, ex vivo exposure of lung tissue samples to hypoxia (1% O_2) resulted in increased HIF-1α mRNA levels compared with lung tissue exposed to normoxia (Figure 1A). Next, PASMC were exposed for different times to hypoxia (1% O_2). HIF-1α mRNA levels were increased in response to hypoxia within 0.5 h, peaking at 1 h after stimulation and returning to basal levels after 4 h of stimulation (Figure 1B). As expected, HIF-1α protein levels were rapidly increased in response to hypoxia after 0.5 h, and they remained elevated for up to 8 h (Figure 1B). Pretreatment of PASMC for 1 h with the transcription inhibitor actinomycin D abolished the increase in HIF-1α mRNA levels by hypoxia, indicating a transcriptional mechanism (Figure 1C). To further confirm that HIF-1α mRNA levels are regulated at a transcriptional level, cells were transfected with pHIF1α-538 in which 538 base pairs of the HIF-1α promoter were cloned in front of the luciferase gene (Minet *et al*., 1999a). Exposure of the transfected cells to hypoxia for 2 h resulted in a significant increase

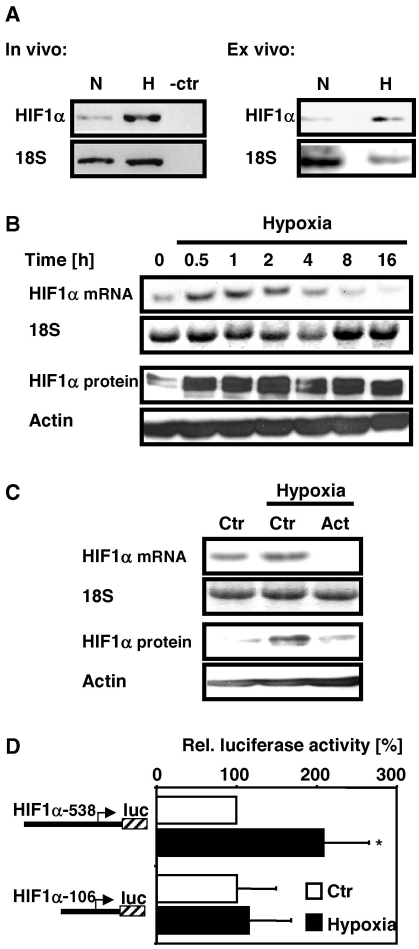

Figure 1. Hypoxia increases HIF-1α mRNA and protein levels. (A) Left, Mice were exposed for 2 h to 10% O_2 (H) or room air (N). Lungs were dissected and HIF-1α mRNA levels were determined by RT-PCR by using specific HIF-1α primers. 18S RNA levels were determined by RT-PCR and used as control. Accuracy of RT-PCR was tested by omitting cDNA (−Ctr). Right, lungs were dissected and exposed ex vivo to 21% O_2 (N) or 1% O_2 (H) for 2 h. HIF-1α mRNA levels were determined by Northern blot by using an HIF-1α RNA probe. 18S RNA levels used as loading control. Representative experiments are shown. (B) PASMC were incubated for different times under hypoxia (1% oxygen). HIF-1α mRNA or protein levels were determined by Northern or Western blots by using a HIF-1α RNA probe or a HIF-1α antibody. 18S or actin were used as loading controls. Representative blots of three experiments are shown. (C) PASMC were pretreated with actinomycin D (Act; 5 μM) for 1 h and exposed to hypoxia for 1 h. HIF-1α mRNA and protein levels were determined by Northern blot and Western blot, respectively. Blots are representative of three experiments. (D) rSMC were transfected with luciferase constructs where part of the HIF-1α promoter was cloned in front of the luciferase gene (pHIF1α-538 and pHIF1α-106) and exposed to hypoxia for 2 h. Luciferase activity was measured (n = 3; *p < 0.05 vs. Ctr).

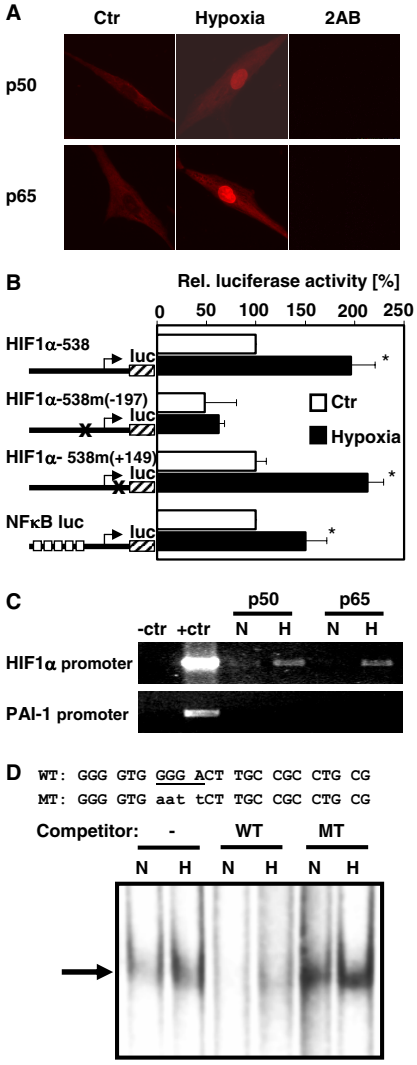

Figure 2. Hypoxia activates HIF-1α transcription via a NFκB site located at −197/−188 base pairs within the HIF-1α promoter. (A) PASMC were incubated for 30 min under hypoxia (1% oxygen). The subcellular localization of the NFκB subunits p50 and p65 was investigated by immunofluorescence by using specific antibodies directed against each subunit. As negative control, the first antibody was omitted (2AB). Pictures are representative of three experiments. (B) rSMC were transfected with luciferase constructs containing the wild-type HIF-1α promoter (pHIF1α-538) or the HIF-1α promoter mutated at the NFκB site at −197 [pHIF1α-538m(−197)] or the putative NFκB site at +149 [pHIF1α-538m(+149)]. In addition, cells were transfected with luciferase constructs driven by five NFκB sites. Transfected cells were exposed to hypoxia for 2 h before luciferase activity was measured (n = 3; *p < 0.05 vs. Ctr). (C) Cells were incubated for 1 h under hypoxia (1% oxygen) and chromatin immunoprecipitation was performed using antibodies against p50 and p65. PCR was performed using specific oligonucleotides for the HIF-1α promoter or for the PAI-1 promoter as negative control. (D) Cells were exposed for 1 h to hypoxia, and EMSAs were performed using labeled oligonucleotides containing the putative NFκB binding site located at −197/−188 base pairs of the HIF-1α promoter. Competition assays were performed by adding a 25-fold molar excess of unlabeled wild-type (WT) or mutated oligonucleotides (MT). Gels are representative of three experiments.

in luciferase activity (Figure 1D). Interestingly, hypoxia did not enhance luciferase activity when a deletion construct (pHIF1α-106) was transfected (Figure 1D), suggesting that transcription factors binding to the deleted sequence were responsible for HIF-1α transcription in response to hypoxia.

Hypoxia Increases HIF-1α Transcription via the Activation of NFκB

Because we have previously identified a binding site for NFκB within the HIF-1α promoter at −197/−188 base pairs (Bonello *et al.*, 2007), we tested whether this site could be involved in the regulation of HIF-1α transcription by hypoxia in PASMC. First, we examined whether hypoxia is able

to activate NFκB. Exposure of PASMC to 1% O_2 for 30 min induced nuclear translocation of the NFκB subunits p50 and p65, as demonstrated by immunofluorescence (Figure 2A). In addition, we transfected a luciferase construct with five NFκB binding sites in front of the simian virus 40 promoter and the luciferase gene (NFκB-Luc). We found that hypoxia resulted in an increase in NFκB-dependent luciferase activity, confirming that hypoxia induces the activation of NFκB (Figure 2B). We then used in addition to the wild-type HIF-1α promoter construct (pHIF1α-538), a construct mutated at the NFκB site in the HIF-1α promoter [pHIF1α-538m(−197)] (Bonello *et al*., 2007) and another construct mutated at a putative NFκB site located in the HIF-1α 5′-untranslated region (UTR) [pHIF1α-538m(+149)] (Frede *et al*., 2006). Mutation of the NFκB site at −197 resulted in a decrease in basal luciferase activity under normoxia, whereas the hypoxia-dependent induction was abolished (Figure 2B). By contrast, mutation of the putative binding site in the 5′-UTR did not affect basal or hypoxia-stimulated luciferase activity (Figure 2B).

To confirm NFκB binding to the HIF-1α promoter, chromatin immunoprecipitation assays were performed using antibodies against p50 and p65 in cells exposed to hypoxia for 1 h. PCR performed with specific primers for the HIF-1α promoter revealed that binding of p50 and p65 was enhanced after exposure to hypoxia. By contrast, NFκB did not bind to the PAI-1 promoter, confirming the specificity of the NFκB binding to the HIF-1α promoter (Figure 2C). In addition, electrophoretic mobility shift assays by using nuclear extracts from PASMC exposed to hypoxia showed enhanced protein binding to an oligonucleotide sequence homologous to the NFκB binding site at −197/188 of the HIF-1α promoter (Figure 2D). The binding activity was reduced by addition of excess unlabeled wild-type oligonucleotides, but not by adding unlabeled mutated oligonucleotides (Figure 2D).

The importance of NFκB activation for HIF-1α mRNA up-regulation under hypoxia was further determined by transfecting PASMC with expression vectors encoding either p50 and p65 or a dominant-negative mutant of IκB (IκBdn) known to inhibit NFκB, and exposing the transfected cells to hypoxia for 1 h. Northern blot analysis revealed that overexpression of NFκB subunits resulted in an increase in HIF-1α mRNA levels under normoxic conditions and further enhanced the hypoxic up-regulation. Inhibition of the NFκB pathway by IκBdn abolished the hypoxia-dependent induction of HIF-1α mRNA, although it also slightly decreased HIF-1α mRNA levels under normoxia (Figure 3A). Similar responses were observed at the protein level. Furthermore, expression of NFκB enhanced HIF-1α promoter activity under normoxic conditions, whereas inhibition of NFκB prevented HIF-1α promoter activation by hypoxia (Figure 3B).

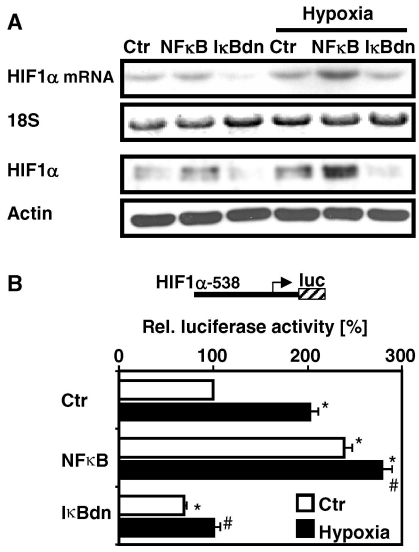

Figure 3. NFκB increases HIF-1α mRNA levels and promoter activity. (A) PASMC were cotransfected with vectors encoding for p50 and p65 (NFκB), IκBdn, or control (Ctr) vector. Cells were incubated for 1 h under hypoxia (1% oxygen). HIF-1α mRNA and protein levels were investigated by Northern and Western blot, respectively. 18S and actin were used as loading controls. Blots are representative of three experiments. (B) rSMC were cotransfected with luciferase constructs driven by the HIF-1α promoter (pHIF1α-538) and with vectors encoding for p50 and p65, IκBdn, or Ctr. Cells were stimulated with hypoxia for 2 h and luciferase activity was measured (n = 3; *p < 0.05 vs. Ctr; #p <0.05 vs. hypoxia-stimulated Ctr).

Hypoxia-induced NFκB Activation Involves the PI3K/AKT Pathway

Several kinase pathways, including PI3K, AKT, and ERK1/2, have been described to be activated by hypoxia and to regulate HIF-1 (Berra *et al*., 2000; Kietzmann *et al*., 2003; Mottet *et al*., 2003a,b). Likewise these kinase pathways also seemed to be effective in the regulation of NFκB (Sizemore *et al*., 1999; Madrid *et al*., 2000). However, their combined impact on HIF-1α transcription is not understood. Therefore, we tested whether hypoxia involves these kinases to trigger the NFκB-dependent HIF-1α transcription. We found that hypoxia induced a rapid but transient increase in the phosphorylation of AKT, which was prevented by the PI3K inhibitors LY294002 (10 μM) and wortmannin (20 nM) (Figure 4A). Hypoxia also enhanced the phosphorylation of ERK1/2, which was inhibited by PD98059 (25 μM) but not by PI3K inhibitors (Figure 4A). Pretreatment with LY294002 or wortmannin reduced hypoxia-induced HIF-1α mRNA, whereas PD98059 was not effective (Figure 4B). In line, hypoxia-stimulated pHIF1α-538–dependent luciferase activity was decreased by PI3K/AKT inhibitors, but it was not altered by PD98059 (Figure 4C). Furthermore, the hypoxia-dependent induction of NFκB-dependent luciferase activity was decreased by LY294002 and wortmannin but not by PD98059, confirming the involvement of PI3K/AKT in NFκB-mediated HIF-1α expression in response to hypoxia (Figure 4C).

DISCUSSION

The present study describes for the first time the central role of NFκB in the induction of HIF-1α mRNA by hypoxia. We found that short-term hypoxia increased HIF-1α levels not only by protein stabilization but also by de novo transcription of the HIF-1α gene. This latter mechanism involved the activation of the PI3 kinase pathway, the activation of NFκB, and its binding to a specific element in the HIF-1α promoter.

HIF-1α mRNA Levels Are Increased in Response to Hypoxia

In this study, we demonstrated that in lungs, and more specifically in PASMC, short-term hypoxia resulted not only in HIF-1α protein accumulation but also in a transient increase in HIF-1α mRNA levels with a maximum at 1 h returning to basal levels within 4 h. This was the result of a transcriptional mechanism, because hypoxia increased HIF-1α promoter activity and actinomycin D treatment pre-

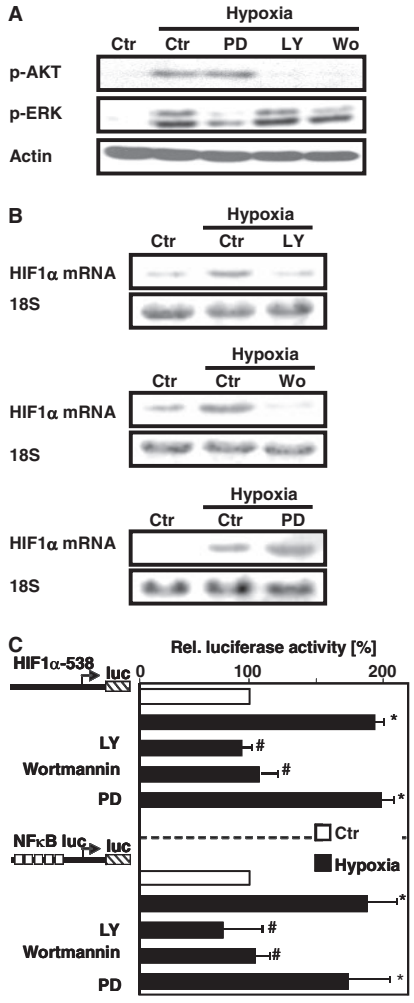

Figure 4. Hypoxia activates HIF-1α transcription via PI3K/AKT and NFκB. (A) PASMC were pretreated for 1 h with LY294002 (LY; 10 μM), wortmannin (20 nM), or PD98059 (PD; 25 μM) and exposed to hypoxia for 15 min. Phosphorylation levels of AKT and ERK1/2 were determined by Western blot. Actin was used as loading control. Blots are representative of three experiments. (B) PASMC were pretreated for 1 h with 10 μM LY, 20 nM wortmannin (Wo) or 25 μM PD98059 (PD) and exposed to hypoxia for 1 h. HIF-1α mRNA levels were measured by Northern blot analysis. Blots are representative of three experiments. (C) rSMC were transfected with luciferase constructs driven by the HIF-1α promoter (pHIF1α-538) or by five NFκB sites. Cells were pretreated for 1 h with 10 μM LY, 20 nM wortmannin, or 25 μM PD and exposed to hypoxia for 2 h before luciferase activity was measured (n = 3; *p < 0.05 vs. Ctr; #p < 0.05 vs. hypoxia-stimulated Ctr).

vented HIF-1α mRNA and protein up-regulation by hypoxia. In contrast, a variety of studies in cultivated cells did not detect up-regulation of HIF-1α mRNA levels under hypoxia. The reasons for obtaining these different results on HIF-1α mRNA regulation under hypoxia are not clear. Given the transient nature of this response, inappropriate exposure times may have prevented the detection of HIF-1α mRNA up-regulation. Interestingly, it was proposed that sequences within the HIF-1α promoter exist that may prevent transcription of the HIF-1α gene in a cell type-specific way (Minet *et al*., 1999a). This may provide another explanation for the controversial data found in the literature.

Importantly, our data are supported by initial studies in Hep3B cells showing that HIF-1α mRNA transiently increased in response to hypoxia with a first peak at 1–2 h and a subsequent decline to basal levels within 8 h (Wang *et al*., 1995) and that actinomycin D prevented HIF-1 DNA binding under hypoxia (Wang and Semenza, 1993). Subsequently, a rapid but transient increase in HIF-1α mRNA levels was also observed in Caki-1 cells exposed to 1% O_2 (Turcotte *et al*., 2003). These in vitro studies were accompanied by a number of in vivo studies demonstrating a rapid increase in HIF-1α mRNA levels upon hypoxia (7% O_2) within 1 h in lung, brain, and kidney of rat or mouse (Wiener *et al*., 1996; Semenza *et al*., 1997), similar to results observed in this study. In ferret lungs ventilated with 0% O_2 HIF-1α mRNA levels were maximally increased at 2 h (Yu *et al*., 1998). A delayed upregulation of HIF-1α mRNA was observed in pulmonary artery endothelial cells upon exposure to hypoxia for 48 h and in rat lungs after 3 wk of chronic hypoxia (Palmer *et al*., 1998), suggesting that in addition to the rapid transcriptional upregulation of HIF-1α by hypoxia described in this study, further mechanisms may contribute to the regulation of HIF-1α mRNA levels under hypoxia.

The importance of HIF-1α regulation at the mRNA level is further supported by findings showing that angiotensin-II, lipopolysaccharide, interleukin-1, hepatocyte growth factor, thrombin, or H_2O_2 also enhance HIF-1α mRNA levels in different cell types (Thornton *et al*., 2000; Page *et al*., 2002; Blouin *et al*., 2004; Tacchini *et al*., 2004; Frede *et al*., 2006; Bonello *et al*., 2007). Our study now shows that such a mechanism also takes place under hypoxic conditions.

NFκB Is Activated by Hypoxia and Mediates the Induction of HIF-1α mRNA

Our results indicate that the NFκB pathway is activated by hypoxia and that it plays a critical role in regulating HIF-1α mRNA levels in response to hypoxia. Although NFκB has been long considered to be activated by oxidative stress, in contrast to HIF-1 being activated by low O_2, recent studies provided evidence that both transcription factors are responsive to both conditions (Koong *et al*., 1994; Görlach *et al*., 2001; BelAiba *et al*., 2004; Zampetaki *et al*., 2004; Ryan *et al*., 2005; Cummins *et al*., 2006; Bonello *et al*., 2007). In line, we could demonstrate by using immunofluorescence and reporter gene assays that hypoxia activates NFκB within 30 min. This response was prevented by the PI3K inhibitors LY294002 and wortmannin. Similarly, inhibition of PI3K or expression of dominant-negative AKT prevented NFκB-driven luciferase activity in response to hypoxia (Figueroa *et al*., 2002).

Our data provide evidence that NFκB regulates HIF-1α via a transcriptional mechanism by binding to the HIF-1α promoter in response to hypoxia, because p50 and p65 directly bound to the HIF-1α promoter under hypoxia as shown by chromatin immunoprecipitation and EMSA. Furthermore, overexpression of p50 and p65 increased HIF-1α mRNA levels and HIF-1α promoter activity, whereas expression of dominant-negative IκB abolished these responses to hypoxia. Importantly, mutation of a NFκB site in the HIF-1α promoter at −197/188 base pairs prevented hypoxic induction of HIF-1α promoter activity. This indicates that NFκB binding to the HIF-1α promoter is a prerequisite for transcriptional activation of HIF-1α by hypoxia. This mechanism seems to be conserved, because our data show an induction of HIF-1α mRNA by hypoxia in lungs of mice and in human PASMC. In addition, sequence alignments confirmed that similar NFκB binding sites are present in the rat and mouse HIF-1α promoter where they can be found at positions −354 and −92, respectively. Interestingly, expression of mutant IκB also decreased HIF-1α mRNA levels

under normoxic conditions, suggesting a role of the NFκB pathway in maintaining basal HIF-1α mRNA levels. Indeed, HIF-1α mRNA has been shown to be expressed under normoxic conditions, and it has been suggested that cRel may play an important role in the constitutive expression of HIF-1α, explaining the decreased levels of HIF-1α mRNA upon blocking of the NFκB pathway (Minet *et al*., 1999a).

Previously, NFκB has been suggested to contribute to the up-regulation of HIF-1α by LPS (Frede *et al*., 2006) by binding of NFκB to a putative consensus site in the HIF-1α 5′-UTR ,which would suggest the involvement of NFκB in HIF-1α translation. However, our data showing that mutation of this NFκB site in the HIF-1α 5′-UTR did not affect reporter gene activity under normoxia or hypoxia, together with earlier data that HIF-1α translation is preserved under hypoxia (Görlach *et al*., 2000; Lang *et al*., 2002), further confirm that a transcriptional rather than a translational mechanism is induced by NFκB to regulate HIF-1α mRNA levels under hypoxic conditions.

The PI3K/AKT Pathway Regulates HIF-1α mRNA Transcription via NFκB

Our study showed that hypoxia activates AKT in a PI3K-dependent manner and that this pathway is responsible for activation of NFκB by hypoxia and subsequent induction of HIF-1α mRNA. In contrast, activation of ERK1/2 did not affect HIF-1α mRNA levels. In line, several studies have identified the PI3K/AKT pathway as an important element in hypoxic induction of HIF-1α protein and activity (Kietzmann *et al*., 2003; Mottet *et al*., 2003a; Schnitzer *et al*., 2005; Lee *et al*., 2006). In addition, the PI3K pathway is also involved in the activation of NFκB (Sizemore *et al*., 1999; Madrid *et al*., 2000), and in fact, inhibitors of PI3K but not of ERK1/2 prevented activation of NFκB by hypoxia, indicating that PI3K/AKT activates HIF-1α indirectly by inducing NFκB. In contrast, ERK1/2 has been previously reported to regulate HIF-1α (Minet *et al*., 2000; Mottet *et al*., 2003b; Kaluz *et al*., 2006; Lee *et al*., 2006), possibly by phosphorylation of HIF-1α (Berra *et al*., 2000; Mylonis *et al*., 2006). Thus, ERK1/2 may act on the level of HIF-1α protein stability and/or transactivation, but it does not seem to be involved in transcriptional activation of HIF-1α by NFκB.

Together, this study provides evidence that hypoxia leads to activation of NFκB involving a PI3K-dependent pathway. NFκB binds to the HIF-1α promoter, resulting in a rapid and transient increase in HIF-1α mRNA and protein levels in PASMC. Because reduced oxygen availability together with a predisposition to inflammatory responses are found in a variety of diseases, including pulmonary vascular remodeling in pulmonary hypertension or tumor progression, our findings indicate a direct link between HIF-1 and NFκB in the pathogenesis of these diseases. Together with the finding that members of the NFκB pathway may be HIF target genes (Walmsley *et al*., 2005), new therapeutic strategies may have to be designed to inhibit this putative vicious cycle.

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