Reactive Oxygen Species Activate the HIF-1α Promoter Via a Functional NFκB Site

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Objective—Reactive oxygen species have been implicated as signaling molecules modulating the activity of redox-sensitive transcription factors such as nuclear factor kappa B (NF-κB). Recently, the transcription factor hypoxia-inducible factor-1 (HIF-1), known to mediate gene expression by hypoxia, has been found to be also activated by nonhypoxic factors in a redox-sensitive manner. We therefore aimed to elucidate the link between these 2 important redox-sensitive transcription factors.

Methods and Results—In pulmonary artery smooth muscle cells, reactive oxygen species generated either by exogenous H_2O_2 or by a NOX4-containing NADPH oxidase stimulated by thrombin activated or induced NF-κB and HIF-1α. The reactive oxygen species-mediated HIF-1α induction occurred on the transcriptional level and was dependent on NF-κB. Transfection experiments with wild-type or mutant HIF-1α promoter constructs revealed the presence of a yet unidentified NF-κB binding element. Gel shift analyses and chromatin immunoprecipitation verified binding of NF-κB to this site. Furthermore, reactive oxygen species enhanced expression of plasminogen activator inhibitor-1, which was prevented by dominant-negative IκB or mutation of the HIF-1 binding site within the plasminogen activator inhibitor-1 promoter.

Conclusion—These findings show for the first time to our knowledge that reactive oxygen species directly link HIF-1α and NF-κB, implicating an important pathophysiological role of this novel pathway in disorders associated with elevated levels of reactive oxygen species. (***Arterioscler Thromb Vasc Biol.* 2007;27:755-761.)**

Key Words: hypoxia-inducible factor ■ NADPH oxidase ■ nuclear factor kappa B ■ reactive oxygen species ■ thrombin

Oxidative stress has been implicated to play an important role in the pathophysiology of many cardiovascular diseases including systemic and pulmonary hypertension and atherosclerosis as well as in tumor progression and vascularization.[1–3]

Moderate levels of reactive oxygen species (ROS), especially superoxide anions and hydrogen peroxide, have been shown to activate signaling cascades mediating the responses to vasoactive peptides, growth factors, cytokines, hormones, and coagulation factors, as well as to physical and chemical stress. ROS participate in the regulation of vascular proliferation, migration, apoptosis, modification of the extracellular matrix, and procoagulant activity.[4–9] Moreover, ROS can activate angiogenesis,[10] a process known to be primarily mediated by vascular endothelial growth factor under hypoxia. Under hypoxia, vascular endothelial growth factor expression is induced by the transcription factor hypoxia-inducible factor-1 (HIF-1).[11] Aside from vascular endothelial growth factor, HIF-1 regulates >100 genes encoding for metabolic enzymes, growth factors, and factors contributing to modulation of extracellular matrix and thrombosis such as plasminogen activator inhibitor-1 (PAI-1).[12–15]

HIF-1 is composed of an inducible α-subunit (HIF-1α) and a constitutive β-subunit (also termed ARNT).[12] HIF-1α contains an oxygen-dependent degradation domain that, when hydroxylated by specific prolyl hydroxylases, binds the von Hippel Lindau protein, leading to HIF-1α ubiquitinylation and degradation by the 26S proteasome. At low oxygen levels, the prolyl hydroxylases lose their activity, which prevents von Hippel Lindau protein binding.[13,16] This results in HIF-1α stabilization, nuclear translocation, dimerization with ARNT, recruitment of coactivators, and binding to hypoxia-response elements in the promoter of target genes.

Recently, HIF-1α has also been shown to be upregulated under normoxia in response to growth factors, thrombin, lipopolysaccharide, angiotensin II, cytokines, or insulin.[17–22] Interestingly, many of these factors also stimulate the generation of ROS, whereas inhibition of ROS formation decreased

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HIF-1α protein levels by thrombin, PDGF, transforming growth factor-β, angiotensin II, cytokines, or $CoCl_2$,[18,20,21,23] suggesting that ROS play an important role in the regulation of HIF-1. However, although a number of details of how hypoxia stabilizes HIF-1α have been unraveled, the mechanisms by which ROS induce HIF-1α have not been fully elucidated. We therefore aimed to investigate how ROS either applied exogenously as mild oxidative stress mimicked by low levels of H_2O_2, by overexpression of the NADPH oxidase subunit NOX4 or endogenously, induced by the NADPH oxidase activator thrombin, would regulate HIF-1α in pulmonary artery smooth muscle cells (PASMCs). We identified a novel pathway in which ROS upregulate HIF-1α transcription by activating NFκB, thus linking these important pathways in a common mechanism induced by ROS.

Materials and Methods

Reagents

All chemicals were of analytical grade and purchased from Sigma if not otherwise stated.

Cell Culture

Human PASMCs were from Cambrex and cultured in the medium provided. PASMCs (passages 3 to 11) were serum-deprived for 24 hours before stimulation with thrombin (3 U/mL) or H_2O_2 (50 μmol/L). Cell viability was not affected by this treatment. For reporter gene assays, human embryonic kidney cells (HEK293) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Plasmids and Luciferase Assays

Expression vectors for the NFκB subunits p50 and p65, dominant-negative IκBα, dominant-negative IκKα, p22phox, and NOX4, as well as the HIF-1α promoter luciferase constructs HIF1α-538 and HIF1α-106, the PAI-1 promoter constructs PAI-796 and PAI-796m, and the luciferase construct NFκB-Luc have been described.[24–27] The construct HIF1α-538m containing a mutated NFκB site at −197 bp was generated with the QuickChange mutagenesis kit (Promega) and confirmed by sequencing. Short hairpin RNA encoding for 19mer siRNA against HIF-1α (1272 to 1290 nt) (5′-GCA ACG ACA CAG AAA CTG A-3′), NOX4[28] and for control siRNA (siCtr)[28] were created using the siSTRIKE U6 Hairpin Cloning System (Promega). Transfection of PASMCs and HEK293 cells was performed as described.[24,27] After 24 hours, transfected cells were serum-starved for 16 hours and then stimulated. Transfection efficiency was 60% to 70%. A Renilla luciferase expression vector (pRLSV40) (Promega) was co-transfected in reporter gene assays to adjust for variations in transfection efficiencies.

Measurement of ROS Production

ROS generation was measured using the fluoroprobe 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA; Molecular Probes) as described.[25]

Immunofluorescence

Immunofluorescence using a p50 antibody (Santa Cruz) and a Alexa 488-coupled secondary antibody (Mobitech) was performed as described.[18]

Northern Blot Analysis

Total RNA was isolated as described[18] and analyzed by Northern blot. Hybridization was performed using digoxigenin-labeled antisense RNA probes for HIF-1α or NOX4 overnight at 65°C.

Western Blot Analysis

Western blot analysis was performed as described previously[18] using antibodies against HIF-1α (Transduction), PAI-1 (American Diagnostica), β-Actin (Dako), and goat anti-mouse or anti-rabbit immunoglobulin G (Calbiochem). The enhanced chemiluminescence Western blotting system (Amersham) was used for detection.

Electrophoretic Mobility Shift Assay

Nuclear extraction was performed as described.[18,27] The oligonucleotides contained the sequence 5′-ggg gtg ggg act tgc cgc ctg cg-3′ from the HIF-1α promoter. For competition assays, unlabeled oligonucleotides were added 15 minutes before adding the labeled probe at 25-fold molar concentration. Supershift analysis was performed by addition of a p65 antibody (Santa Cruz).

Chromatin Immunoprecipitation

Proteins were cross-linked with formaldehyde. After adding 0.125 mol/L glycine, cell lysis buffer (5 mmol/L PIPES, 1 mol/L KCl, 10% NP40) and nuclear lysis buffer (5 mmol/L PIPES, 85 nmol/L KCl, 0.5% NP40) supplemented with protease inhibitors were added, followed by sonication and centrifugation. The supernatant was precleared with Sepharose beads (Amersham Biosciences), incubated with p50 or p65 antibodies (Santa Cruz) overnight, and then with beads for 2 hours. The complexes were eluted with buffer (100 mmol/L $NaHCO_3$, 1% SDS) and incubated with RNAse A. DNA was purified using QIAquick polymerase chain reaction purification kit (Qiagen). Polymerase chain reaction was performed with primers for the HIF-1α promoter (forward: 5′-gaa cag aga gcc cag cag ag-3′; reverse: 5′-tgt gca ctg agg agc tga gg-3′) flanking the NFκB binding site (−197/188 bp) at 55°C for 35 cycles or primers for the PAI-1 promoter (forward: 5′-gct ctt tcc tgg agg tgg tc-3′; reverse: 5′-ggg cac aga gag agt ctg ga-3′).

Statistical Analysis

Values presented are means±SEM. Results were compared by ANOVA for repeated measurements followed by Student-Newman-Keuls t test. $P \leq 0.05$ was considered statistically significant.

Results

HIF-1α mRNA and Protein Are Increased by ROS Via a Transcriptional Mechanism

Stimulation of PASMCs with increasing concentrations of H_2O_2 for 2 hours enhanced not only HIF-1α protein but also HIF-1α mRNA levels, with a maximal response at 50 μmol/L (Figure 1A). Treatment with 50 μmol/L H_2O_2 for increasing time periods transiently elevated HIF-1α mRNA and protein with maximal levels at 1 and 2 hours, respectively (Figure 1B). We have previously shown that thrombin activates ROS production.[23,24] Indeed, stimulation with thrombin resulted in a time-dependent increase in ROS levels, peaking at 4 hours (Figure 2A). Interestingly, thrombin concomitantly enhanced HIF-1α mRNA and protein levels, peaking at 2 and 4 hours, respectively (Figure 2B). Treatment with the antioxidant N-acetylcysteine inhibited thrombin-stimulated HIF-1α mRNA and protein expression by 85±8% and 94±4%, respectively (Figure 2C). Similarly, siRNA targeting the NADPH oxidase subunit NOX4, which is known to contribute to ROS production in PASMCs,[25] reduced NOX4 mRNA levels by 80±7% and decreased HIF-1α levels to a similar degree (Figure 2D), further suggesting the involvement of ROS. Consistently, similar to H_2O_2 or thrombin, overexpression of NOX4 enhanced HIF-1α mRNA and protein levels (Figure 2D), indicating that a NOX4-containing NADPH oxidase contributes to thrombin-induced HIF-1α regulation.

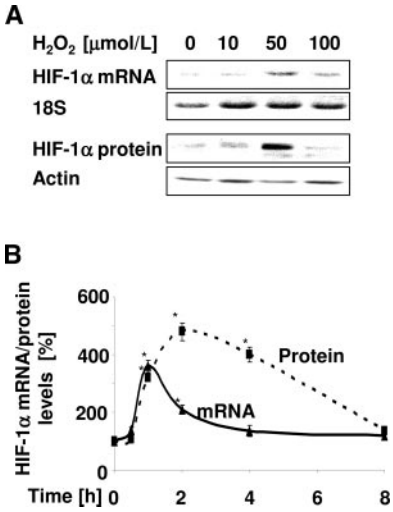

Figure 1. ROS induce HIF-1α mRNA and protein levels. PASMCs were (A) treated with different concentrations of H_2O_2 for 2 hours or (B) stimulated with H_2O_2 (50 μmol/L) for increasing time periods. HIF-1α mRNA or protein levels were assessed by Northern and Western blots. Blots for 18S RNA or actin protein are shown as loading controls. Levels without H_2O_2 (0) were set to 100%. Data are shown as relative increase to control (n=3; *P<0.05 vs. 0).

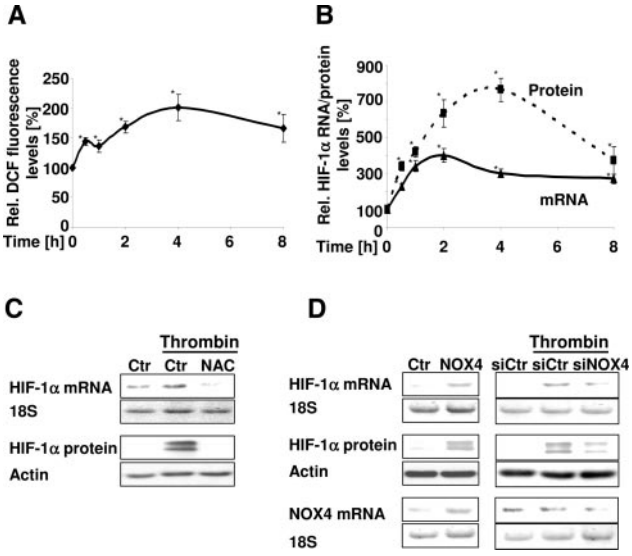

Figure 2. Thrombin induces HIF-1α mRNA and protein levels involving ROS and NOX4. PASMCs were stimulated with thrombin (3 U/mL) for increasing time periods. A, ROS levels were determined by measuring DCF fluorescence. ROS levels without thrombin (0) were set to 100%. Data are shown as relative increase to control (n=3; *P<0.05 vs. 0). B, HIF-1α mRNA or protein levels were assessed by Northern and Western blots. HIF-1α mRNA or protein levels without thrombin (0) were set to 100%. Data are shown as relative increase to control (n=3; *P<0.05 vs. 0). C, PASMCs were pretreated with N-acetylcysteine (NAC) (5 mmol/L) for 30 minutes or (D) transfected with control vector (Ctr) or vectors encoding for NOX4 or siRNA against NOX4 (siNOX4) or for an unrelated control siRNA (siCtr) and stimulated with thrombin (3 U/mL) for 2 hours. HIF-1α mRNA and protein levels were measured by Northern and Western blot analyses. The efficiency of siNOX4 was assessed by Northern blot. Representative blots from 3 to 5 experiments are shown.

Comparable results were obtained by overexpression of the NADPH oxidase subunit p22phox (data not shown). Because upregulation of HIF-1α mRNA by H_2O_2, NOX4, and thrombin suggested the involvement of a transcriptional mechanism, PASMCs were pretreated with the transcription inhibitor actinomycin D (5 μmol/L). This treatment diminished the induction of HIF-1α mRNA and protein by H_2O_2 by 85±8% and 92±7% or by thrombin by 94±5% and 82±9%, respectively (supplemental Figure I, available online at http://atvb.ahajournals.org).

NFκB Is Activated by H_2O_2 and Thrombin and Increases HIF-1α Expression

The transcription factor NFκB has been suggested to contribute to the transcriptional response to ROS.[29] Indeed, H_2O_2 or thrombin promoted nuclear translocation of NFκB p50 in PASMCs (supplemental Figure II) and increased NFκB activity in HEK293 cells transfected with a NFκB-dependent luciferase construct (NFκB-luc) (Figure 3A). Cotransfection of dominant-negative IκBα (IκBdn) abolished NFκB activation by these stimuli.

To determine the involvement of the NFκB pathway in the regulation of HIF-1α, PASMCs were transfected with vectors

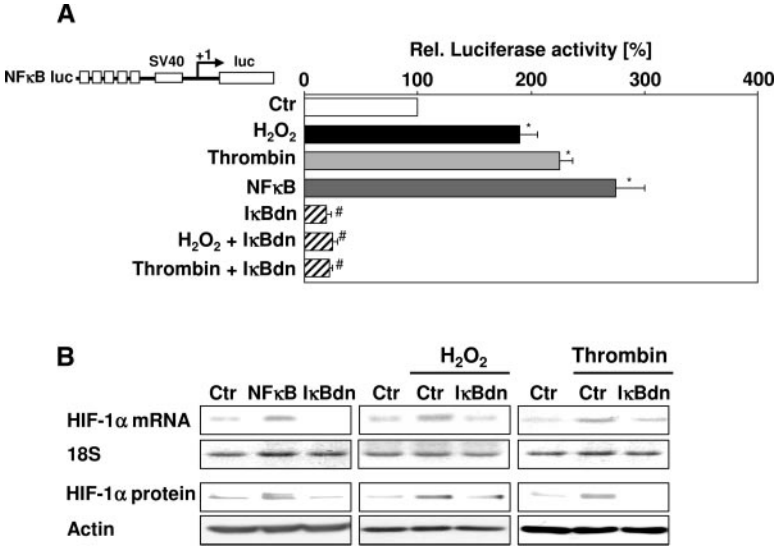

Figure 3. NFκB activation by H_2O_2 and thrombin mediates HIF-1α expression. A, HEK293 cells were cotransfected with vectors encoding p50 and p65 (NFκB), dominant-negative IκBα (IκBdn), or with control vector and a luciferase construct containing 5 NFκB elements (NFκB-luc) and stimulated with H_2O_2 (50 μmol/L) or thrombin (3 U/mL). Luciferase activity measured in untreated controls (Ctr) was set equal to 100%. Data shown represent % induction of luciferase activity (n=3; *P<0.05 vs. unstimulated cells transfected with control vector). B, PASMCs were transfected with expression vectors encoding p50 and p65 (NFκB) or dominant-negative IκBα (IκBdn) and stimulated with H_2O_2 or thrombin for 2 hours. Northern and Western blot analyses for HIF-1α were performed. Representative blots from 3 to 5 experiments are shown.

encoding the NFκB subunits p50 and p65 (NFκB) or IκBdn. NFκB, but not IκBdn, enhanced HIF-1α mRNA and protein levels. However, IκBdn diminished HIF-1α mRNA and protein induction by H_2O_2 by 82±9% and 79±8%, respectively, and inhibited HIF-1α mRNA and protein levels in the presence of thrombin by 89±8% and 95±6%, respectively (Figure 3B).

The HIF-1α Promoter Contains a Functional NFκB Binding Site

Next, we performed reporter gene assays using a construct containing the HIF-1α promoter (−538 to +284) fused to the luciferase gene (HIF1α-538). Stimulation with H_2O_2, thrombin, or overexpression of NOX4 or p22phox enhanced HIF-1α promoter activity (Figure 4A). A similar response was observed when the NFκB subunits p50 and p65 were co-expressed. However, H_2O_2-stimulated or thrombin-stimulated HIF-1α promoter activity was decreased in the presence of dominant-negative IκBα or IκKα. In contrast, luciferase activity of the construct containing only 106 bp of the HIF-1α promoter (HIF1α-106) could not be enhanced by thrombin, H_2O_2, or NFκB (Figure 4A). Analysis of the HIF-1α promoter revealed a putative NFκB site at −197/−188 bp. To determine whether this site is functional, site-directed mutagenesis was performed on the HIF1α-538 construct. The reporter gene assays showed that the mutated HIF-1α promoter failed to respond to H_2O_2, thrombin, or overexpression of NFκB, IκBdn, IκKdn, NOX4, or p22phox (Figure 4A). These data indicate that the site −197/−188 was responsible for transcriptional activation of HIF-1α by NFκB.

Consistently, nuclear extracts from PASMCs stimulated with H_2O_2 or thrombin showed enhanced binding to an oligonucleotide containing this NFκB site as demonstrated by electrophoretic mobility shift assays (supplemental Figure III). This binding was diminished by a 25-fold excess of unlabeled oligonucleotides. Addition of an antibody against p65 prevented binding of this complex.

Binding of p50 and p65 to the HIF-1α promoter was also detected by chromatin immunoprecipitation (Figure 4B). In contrast, as expected, chromatin immunoprecipitation did not reveal binding of p50 to the PAI-1 promoter (Figure 4B). These data clearly show that p50 and p65 specifically bind to the HIF-1α promoter element −197/−188.

NFκB Modulates the Expression of the HIF-1 Target Gene PAI-1

PAI-1 has been described to be regulated by HIF-1 in response to hypoxia.[15] Similarly, thrombin enhanced luciferase activity mediated by the human PAI-1 promoter (PAI-796), whereas depletion of HIF-1α by siRNA prevented this response (supplemental Figure IV). To determine whether induction of HIF-1α via NFκB would play a role in PAI-1 regulation, NFκB was co-expressed with PAI-796, which contains a hypoxia response element, but no NFκB binding sites. Reporter assays revealed that NFκB increased luciferase activity similar to H_2O_2 or thrombin (Figure 5A), whereas IκBdn diminished H_2O_2-stimulated and thrombin-stimulated PAI-1 promoter activity. In contrast, mutation of the hypoxia-response elements blunted luciferase activity induced by NFκB, H_2O_2 or thrombin (Figure 5A). Consistently, overexpression of NFκB increased PAI-1 secretion from PASMCs, whereas IκBdn decreased PAI-1 secretion by H_2O_2 or thrombin (Figure 5B).

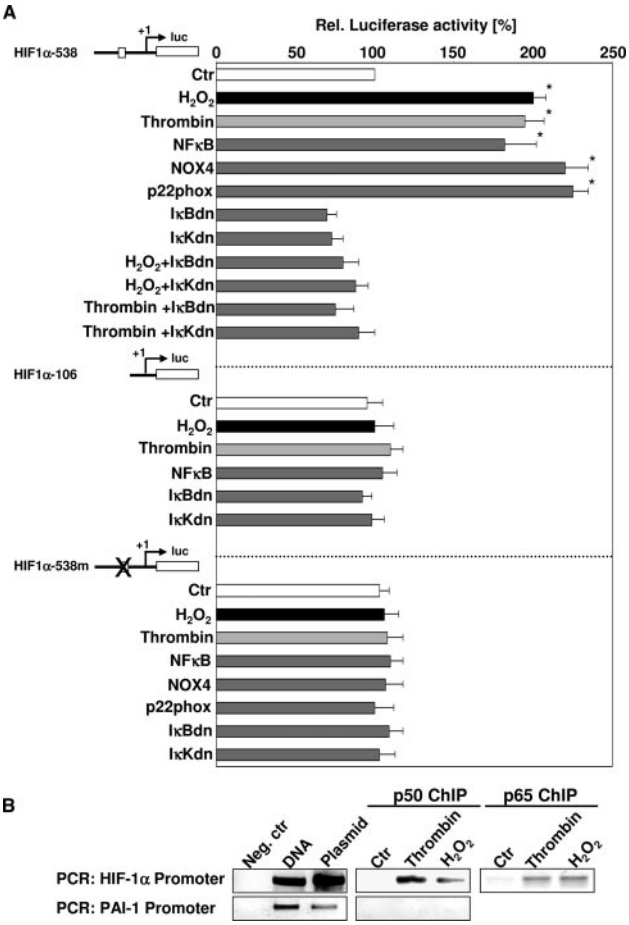

Figure 4. NFκB regulates HIF-1α promoter activity. A, HEK293 cells were cotransfected with the HIF-1α promoter constructs HIF1α-538, HIF1α-106, or with HIF-1α-538m containing a mutated NFκB binding site and control vector (Ctr) or vectors encoding for the NADPH oxidase subunits NOX4, p22phox, the NFκB subunits p50 and p65 (NFκB), dominant-negative IκBα (IκBdn), or dominant-negative IκKα (IκKdn). Cells were either left untreated (Ctr) or stimulated with H_2O_2 (50 μmol/L) or thrombin (3 U/mL) for 2 hours. HIF1α-538 luciferase activity under control conditions (Ctr) was set equal to 100%. Data represent % induction of luciferase activity (n=3; *$P<0.05$ vs. untreated control). B, Chromatin immunoprecipitation was performed using p50 and p65 antibodies. Polymerase chain reaction was performed on a sequence of the HIF-1α promoter including the NFκB binding site at −197/−188 bp. As negative control a polymerase chain reaction was performed with the p50 immunoprecipitate and primers for the PAI-1 promoter.

Discussion

In this study we demonstrated that ROS generated by direct application of H_2O_2, overexpression of the NADPH oxidase subunit NOX4, or by stimulation of the NADPH oxidase with thrombin induced HIF-1α transcription via binding of NFκB to a novel site in the HIF-1α promoter. Together with the observation that this pathway regulates the expression of PAI-1, these findings suggest an important role of this novel mechanism in various disorders associated with high levels of ROS and thrombotic activity.

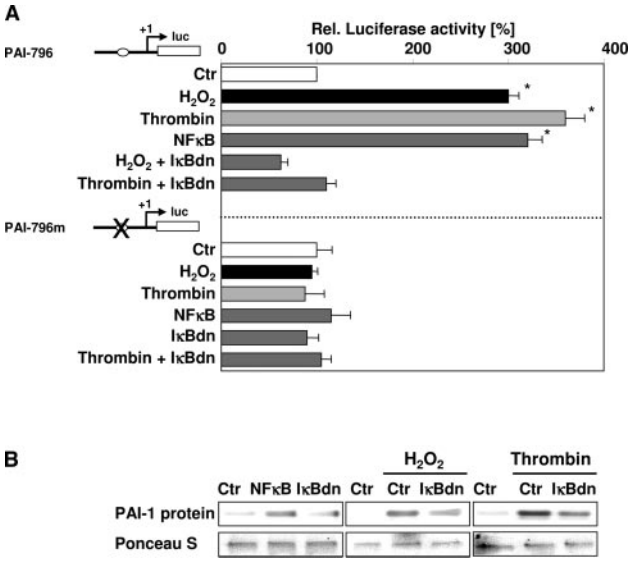

Figure 5. NFκB mediates PAI-1 expression by H_2O_2 and thrombin. A, HEK293 cells were cotransfected with PAI-796 or a respective construct containing a mutated HIF-1 binding site (PAI-796m) and vectors encoding NFκB p50 and p65 (NFκB), dominant-negative IκBα (IκBdn), dominant-negative IκKα (IκKdn), or with control vector (Ctr). Cells were stimulated with H_2O_2 (50 μmol/L) or thrombin (3 U/mL) for 2 hours. PAI-796 luciferase activity determined under control conditions (Ctr) was set equal to 100%. Data represent % induction of luciferase activity (n=3; *$P<0.05$ vs. untreated control). B, Pulmonary artery smooth muscle cells were cotransfected with vectors encoding p50 and p65 (NFκB) or dominant-negative IκBα (IκBdn) and stimulated with H_2O_2 (50 μmol/L) or thrombin (3 U/mL) for 8 hours. Western blot analyses were performed from supernatants using a PAI-1 antibody. Ponceau S staining is provided as loading control. Representative blots from 3 to 5 experiments are shown.

Nonhypoxic Stimulation of HIF-1α: Role of ROS

HIF-1 has been initially described to specifically mediate the response to hypoxia because of its instability under normoxia. Despite these findings, HIF-1α protein has been shown to be upregulated under normoxia in response to growth factors, hormones, coagulation factors, cytokines, and vasoactive peptides.[17,18,20,21,30,31] In this study we demonstrated that low levels of H_2O_2 as well as activation of ROS production by thrombin increase HIF-1α mRNA and protein levels. Interestingly, stimulation with thrombin rapidly increased ROS levels within 30 minutes of exposure followed by a second more prominent peak at 4 hours of stimulation. Concomitantly, HIF-1α protein was rapidly increased, followed by a sustained increase in HIF-1α mRNA levels peaking at 2 hours, which was then followed by a substantial increase in HIF-1α protein peaking at 4 hours. Although actinomycin D prevented thrombin and H_2O_2-induced HIF-1α mRNA and protein upregulation clearly pointing to a transcriptional mechanism, it cannot be excluded that the initial rapid increase in ROS and HIF-1α protein levels by thrombin may also involve HIF-1α stabilization, possibly by affecting the activity of prolyl hydroxylases. Furthermore, the decrease in HIF-1α protein levels after prolonged exposure to thrombin despite a sustained elevation of HIF-1α mRNA and ROS levels may be the result of a compensatory mechanism, such as upregulation of prolyl hydroxylases. Indeed, prolyl hydroxylases have been shown to be HIF target genes.[32] However, further experiments are required to dissect the exact contribution of transcriptional versus other mechanisms regulating HIF-1α levels in response to thrombin and ROS at different time points.

The importance of ROS derived from a NOX4-containing NADPH oxidase in response to thrombin was supported by our findings that antioxidants and depletion of NOX4 decreased HIF-1α upregulation by thrombin, whereas overexpression of NOX4 increased HIF-1α mRNA and protein. Similar results have been obtained with the NADPH oxidase subunit p22phox (data not shown) consistent with our previous observation that p22phox antisense oligonucleotides or antioxidants prevented HIF-1α protein upregulation by thrombin, transforming growth factor-β1, or PDGF in vascular smooth muscle cells.[18] Importantly, however, an effect of thrombin or ROS on HIF-1α mRNA levels has not been reported previously.

A role for NADPH oxidase-derived ROS in the regulation of HIF-1α was further suggested by an in vivo study in mice in which overexpression of p22phox increased ROS and HIF-1α protein levels in smooth muscle cells.[33] Consistently, overexpression of CuZnSOD, which promotes the formation of H_2O_2 from superoxide anions, enhanced HIF-1α protein levels in PASMCs, whereas catalase, which promotes the degradation of H_2O_2, prevented thrombin-induced and $CoCl_2$-induced HIF-1α protein,[23] further confirming that enhanced H_2O_2 levels are important in upregulating HIF-1α under normoxia in smooth muscle cells.

In contrast to these findings obtained under normoxic conditions (21% oxygen) in PASMCs, the role of ROS in the regulation of HIF-1α under hypoxia appears to be complex, and controversial data exist showing that ROS can promote or prevent the induction of HIF-1α under different oxygen concentrations in different cell types.[34] Although the reasons for the conflicting data are not clear to date, and await further experimental clarification, the cellular redox steady-state and microenvironmental conditions together with cell-type specific properties appear to be crucial. This is supported by findings showing that in HepG2 hepatoma cells cultivated under 16% or 8% oxygen, expression of active Rac, known to activate NADPH oxidases under normoxic conditions, decreased HIF-1α protein levels.[35] However, this response was not observed or even reversed when these cells were cultivated under 21% oxygen (data not shown) consistent with our study in which expression of NOX4 enhanced HIF-1α levels in PASMCs cultivated under 21% oxygen. These findings suggest that ROS are important signals in fine-tuning HIF-1α levels even in a narrow range of different microenvironmental conditions. They further implicate that there are no "absolute" ROS levels that will have a specific effect on HIF-1α, but that a shift toward a pro-oxidant or anti-oxidant state relative to a cell's redox steady-state will affect HIF-1α levels.

NFκB Is a Transcriptional Regulator of HIF-1α

Our data show for the first time that enhanced levels of ROS by H_2O_2, NOX4, or thrombin induce HIF-1α transcription because these stimuli increased HIF-1α promoter activity,

whereas actinomycin D almost completely prevented thrombin-induced and H_2O_2-induced HIF-1α mRNA and protein levels.

Although enhanced HIF-1α mRNA levels have also been described by angiotensin II, lipopolysaccharide, IL-1, or HGF,[19,31,36–38] only limited data exist with regard to the underlying mechanisms in particular with regard to the involvement of transcriptional regulation. Importantly, the involvement of ROS in transcriptional regulation of HIF-1α has not yet been addressed.

In this study we show that enhanced HIF-1α transcription by ROS was mediated by NFκB. Indeed, overexpression of NFκB increased HIF-1α mRNA levels and stimulated HIF-1α promoter activity similar to thrombin or H_2O_2, whereas dominant-negative IκBα and IκKα, which inhibit activation of NFκB, decreased H_2O_2-dependent and thrombin-dependent induction of HIF-1α mRNA and promoter activity. We further identified a NFκB binding site in the HIF-1α promoter at $-197/-188$ bp. Mutation of this site abolished stimulation of HIF-1α promoter activity by NFκB, H_2O_2, NOX4, and thrombin, confirming that ROS transcriptionally upregulate HIF-1α by activation of NFκB. Consistently, electrophoretic mobility shift assays and chromatin immunoprecipitation demonstrated that the NFκB subunits p50 and p65 were able to bind to the HIF-1α promoter at this specific site.

NFκB has been previously shown to contribute to the regulation of HIF-1α protein, but not to HIF-1α mRNA, by tumor necrosis factor-α or colchicine in fibroblasts, and to HIF-1α translation by HGF in HepG2 cells.[37,39–41] Thus, the transcriptional regulation of HIF-1α by ROS and NFκB may act in concert with translation and stabilization of HIF-1α and appears to be cell type and/or stimulus specific. A recent study showed upregulation of HIF-1α mRNA by lipopolysaccharide, which was prevented by the nonspecific NFκB inhibitor bortezomib in monocytes.[38] Interestingly, in this study it was suggested that this response was mediated by binding of NFκB to a site within the HIF-1α promoter although this site has not been verified by mutagenesis. In contrast to the NFκB site we identified in the HIF-1α promoter at $-197/188$ bp by site-directed mutagenesis, gel shift analysis and chromatin immunoprecipitation, the NFκB site suggested in this earlier study lies at position $+149$ bp, well within the HIF-1α 5′-untranslated region. Thus, its contribution to transcriptional activity is unclear. In contrast, our data demonstrated that the truncated (HIF1α-106) and the mutated (HIF1α-538m) HIF-1α promoter constructs, both of which exclude the NFκB site at $-197/-188$ bp but include the full untranslated region and the intact NFκB consensus sequence at $+149$ bp, were not modulated by H_2O_2, thrombin, or the NFκB subunits. This confirms that a NFκB binding element at $-197/-188$ bp upstream of the transcription initiation site of the HIF-1α promoter is functional and is responsible for the transcriptional activation of HIF-1α by H_2O_2, thrombin, and NADPH oxidases.

The importance of NFκB as a regulatory factor of HIF-1 in response to exogenous or endogenous ROS was further highlighted by our findings that H_2O_2, thrombin, and NFκB increased promoter activity and expression of the HIF-1 target gene PAI-1. This response was dependent on HIF-1, because the PAI-1 promoter construct that does not contain a NFκB site did not respond to H_2O_2, thrombin, or NFκB when the HIF-1–binding hypoxia response element in the PAI-1 promoter was mutated or when HIF-1α was downregulated. PAI-1, known as an inhibitor of fibrinolysis, also plays an important role in matrix remodeling and angiogenesis and has been implicated in the pathogenesis of many cardiovascular disorders associated with enhanced levels of ROS and thrombin including systemic and pulmonary hypertension and atherosclerosis.[42] Interestingly, these disorders have also been associated with increased activity of NFκB, suggesting that this pathway can upregulate HIF-1α even under normoxia in the presence of ROS or on activation of the coagulation cascade.

In summary, this study shows that elevated levels of ROS can activate NFκB involving IκKα and IκBα. This results in enhanced transcription of HIF-1α via a specific element in the HIF-1α promoter leading to increased HIF-1α mRNA and protein levels, which mediate the upregulation of PAI-1 and possibly other HIF-1 target genes. Because enhanced ROS, thrombotic activity, and matrix remodeling have been implicated in cardiovascular disorders including systemic and pulmonary hypertension and atherosclerosis, as well as in tumor progression, the findings that HIF-1α is a target gene of NFκB under normoxia indicate a new role for HIF-1 in the pathogenesis of these diseases independent of oxygen availability and suggest the requirement of new therapeutic strategies for these disorders.

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Disclosure

C.M. is a senior research associate of FNRS (Belgium).

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Arteriosclerosis, Thrombosis, and Vascular Biology

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Reactive Oxygen Species Activate the HIF-1α Promoter Via a Functional NFκB Site

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Figure I

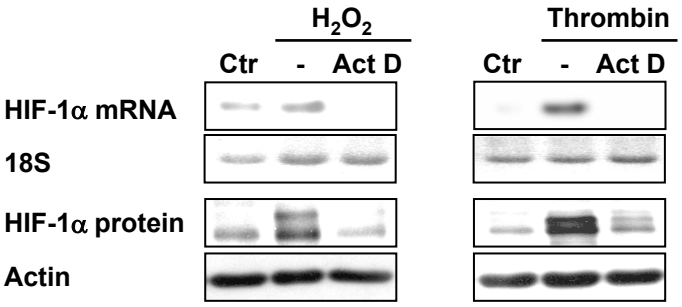

H_2O_2
Thrombin
Ctr
-
Act D
Ctr
-
Act D
HIF-1α mRNA
18S
HIF-1α protein
Actin

Figure II

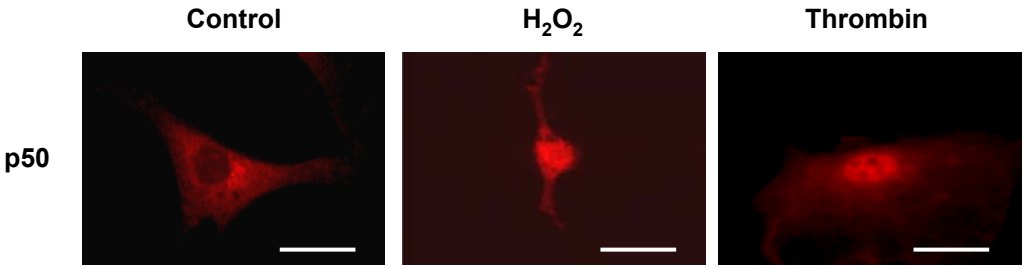


Figure III

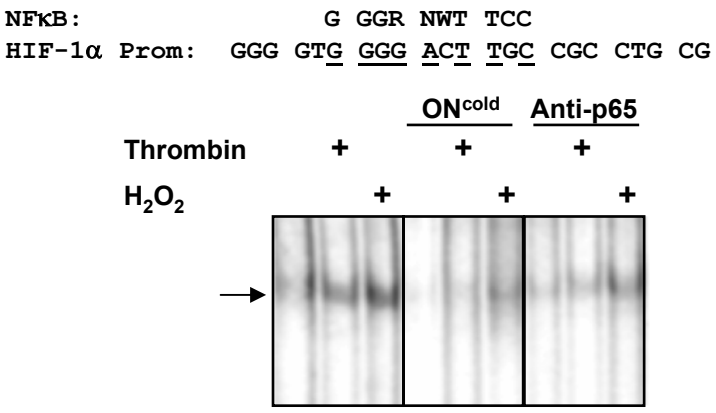
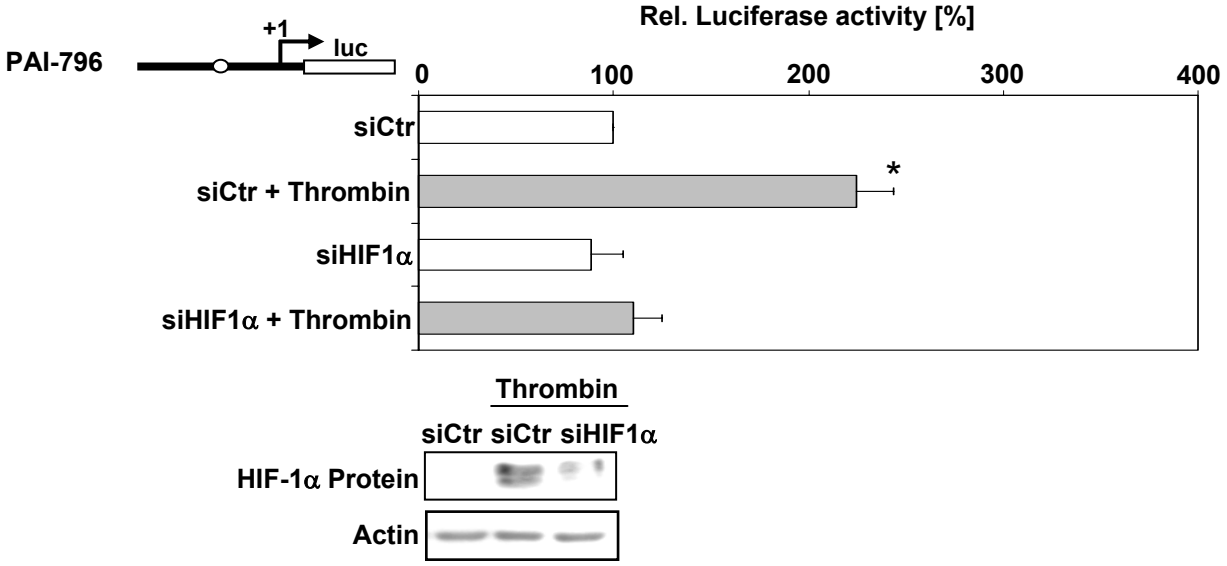

NFκB: G GGR NWT TCC
HIF-1α Prom: GGG GTG GGG ACT TGC CGC CTG CG
ONcold
Anti-p65
Thrombin
H2O2

Figure IV


Rel. Luciferase activity [%]
+1
luc
PAI-796
0
100
200
300
400
siCtr
siCtr + Thrombin
siHIF1α
siHIF1α + Thrombin
*
Thrombin
siCtr siCtr siHIF1α
HIF-1α Protein
Actin

Legends

Figure I. Induction of HIF-1α mRNA by H_2O_2 and thrombin is transcriptionally regulated. Pulmonary artery smooth muscle cells were pretreated with actinomycin D (ActD, 5 μmol/L) for 30 minutes before stimulation with thrombin (3 U/mL) or H_2O_2 (50 μmol/L) for 2 hours and Northern and Western blot analyses for HIF-1α were performed. Representative blots from 3-5 experiments are shown.

Figure II. H_2O_2 and thrombin induce NFκB translocation. Pulmonary artery smooth muscle cells (PASMC) were stimulated with H_2O_2 (50 μmol/L) or thrombin (3 U/mL) for 2 hours. Immunofluorescence was performed with an antibody against the NFκB subunit p50. The bars indicate 20 μm.

Figure III. NFκB binds to the HIF-1α promoter. Electrophoretic mobility shift assays were performed using oligonucleotides corresponding to the HIF-1α promoter sequence containing a putative NFκB binding site at -197/-188 bp (HIF-1α Prom). Competition assays were performed by adding 25-fold molar concentration of unlabelled oligonucleotides (ON cold). For supershift assay, anti-p65 was added.

Figure IV. PAI-1 expression is dependent on HIF-1α. HEK293 cells were cotransfected with a luciferase reporter containing the human plasminogen activator inhibitor-1 promoter (PAI-796) and a vector encoding a random siRNA sequence (siCtr) or siRNA targeting HIF-1α (siHIF1α) and stimulated with thrombin (3 U/mL) for 2 hours (n=3; *$p<0.05$ versus unstimulated control). The efficiency of the HIF-1α siRNA was assessed by Western blot.