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Prolyl hydroxylase-1 negatively regulates IκB kinase-β, giving insight into hypoxia-induced NFκB activity

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Edited by Laurie H. Glimcher, Harvard Medical School, Boston, MA, and approved October 4, 2006 (received for review March 22, 2006)

Hypoxia is a feature of the microenvironment of a growing tumor. The transcription factor NFκB is activated in hypoxia, an event that has significant implications for tumor progression. Here, we demonstrate that hypoxia activates NFκB through a pathway involving activation of IκB kinase-β (IKKβ) leading to phosphorylation-dependent degradation of IκBα and liberation of NFκB. Furthermore, through increasing the pool and/or activation potential of IKKβ, hypoxia amplifies cellular sensitivity to stimulation with TNFα. Within its activation loop, IKKβ contains an evolutionarily conserved LxxLAP consensus motif for hydroxylation by prolyl hydroxylases (PHDs). Mimicking hypoxia by treatment of cells with siRNA against PHD-1 or PHD-2 or the pan-prolyl hydroxylase inhibitor DMOG results in NFκB activation. Conversely, overexpression of PHD-1 decreases cytokine-stimulated NFκB reporter activity, further suggesting a repressive role for PHD-1 in controlling the activity of NFκB. Hypoxia increases both the expression and activity of IKKβ, and site-directed mutagenesis of the proline residue (P191A) of the putative IKKβ hydroxylation site results in a loss of hypoxic inducibility. Thus, we hypothesize that hypoxia releases repression of NFκB activity through decreased PHD-dependent hydroxylation of IKKβ, an event that may contribute to tumor development and progression through amplification of tumorigenic signaling pathways.

IKK

During cancer progression, a state of hypoxia occurs as the developing tumor outgrows the local blood supply, leading to a drop in pO_2 (1), a condition that should prove rate limiting for tumor growth. However, growth of the tumor is paradoxically facilitated through promotion of a pathway maladaptive for the host involving activation of the hypoxia-specific transcriptional regulator, hypoxia inducible factor-1 (HIF-1), which regulates the expression of genes promoting angiogenesis, vasodilatation, glycolysis, and erythropoesis (2). Furthermore, hypoxia also activates NFκB (3), a transcription factor important in the promotion and progression of tumor development and survival (4). The net result of HIF-1 and NFκB activation by hypoxia is increased tumor oxygenation and survival/growth, respectively. Thus, inhibition of these pathways represents a potentially important therapeutic window of opportunity in the treatment of cancer.

The mechanisms by which HIF-1 is activated in hypoxia are relatively well understood (5). HIF-1α is constitutively synthesized at a high level in normoxia, but its level is repressed by members of the 2-oxoglutarate-dependent dioxygenase superfamily, namely the prolyl hydroxylases (PHDs). Three PHD isoforms have been described to date (PHD-1, PHD-2, and PHD-3). Oxygen-dependent modification of specific proline residues within consensus LxxLAP motifs (P402 and P564) in HIF-1α by these enzymes, primarily the PHD-2 isoform, results in the targeting of HIF-1α for ubiquitination through an E3 ligase complex initiated by the binding of the Von Hipple Lindau protein (pVHL) and subsequent proteasomal degradation. A further hydroxylation of N803 in the transactivation domain of HIF-1α by Factor Inhibiting HIF (FIH), an asparagine hydroxylase, represents a second mechanism of oxygen-dependent repression through inhibition of transactivation (6). Similar mechanisms exist for HIF-2α (5). The hypoxic sensitivity of the HIF pathway is achieved by the absolute requirement of PHD enzymes for molecular oxygen as a cosubstrate. Inhibition of this pathway in hypoxia with the resultant stabilization and transactivation of HIF-α subunits represents a paradigm for oxygen sensing and hypoxia-responsive alterations in gene expression (7).

NFκB-like HIF-1 is a prosurvival transcription factor implicated in tumorigenesis through increasing the expression of genes that inhibit apoptosis and growth arrest in premalignant cells and promote tumor progression through production of cytokines (4). The mechanisms of NFκB activation have been best characterized for their role in inflammation in response to a host of proinflammatory ligands (e.g., TNFα, IL-1, and lipopolysaccharide). A complex sequence of events resulting from receptor occupation by these ligands triggers cascades involving a diverse array of adaptor molecules and enzymes that are ligand-specific (8). There is, however, a convergence point for these signals at the level of the IκB kinases (IKK α, β, and γ) that form the IKK complex (9). Although it is clear that hypoxia activates NFκB, the signaling pathways remain unclear. In the current study, we sought to interrogate the signaling pathways leading to NFκB activation in hypoxia. We investigated whether similar oxygen-sensing mechanisms that exist for HIF-1 may also exist for NFκB. Specifically, we investigated a role for the PHDs.

Results

Hypoxia Activates NFκB. Consistent with previous studies, hypoxia activated NFκB (10, 11). Hypoxia stimulated a 1.9 ± 0.1- and 5.9 ± 1.7-fold increase in basal NFκB-dependent transcriptional activity at 24 and 48 h, respectively, as measured by the NFκB-dependent luciferase reporter assay (Fig. 1*A*). Furthermore, this event is preceded by increased levels of nuclear NFκB (as demonstrated by p65 binding to immobilized oligonucleotides containing the con-

Author contributions: E.P.C., K.M.C., C.G., P.M., J.P., and C.T.T. designed research; E.P.C., E.B., K.M.C., A.G., K.T.F., F.S., and J.E.N. performed research; J.E.N. contributed new reagents/analytic tools; E.P.C., E.B., F.S., P.M., and J.P. analyzed data; and E.P.C. and C.T.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Freely available online through the PNAS open access option.

Abbreviations: HIF, hypoxia inducible factor; PHD, prolyl hydroxylase.

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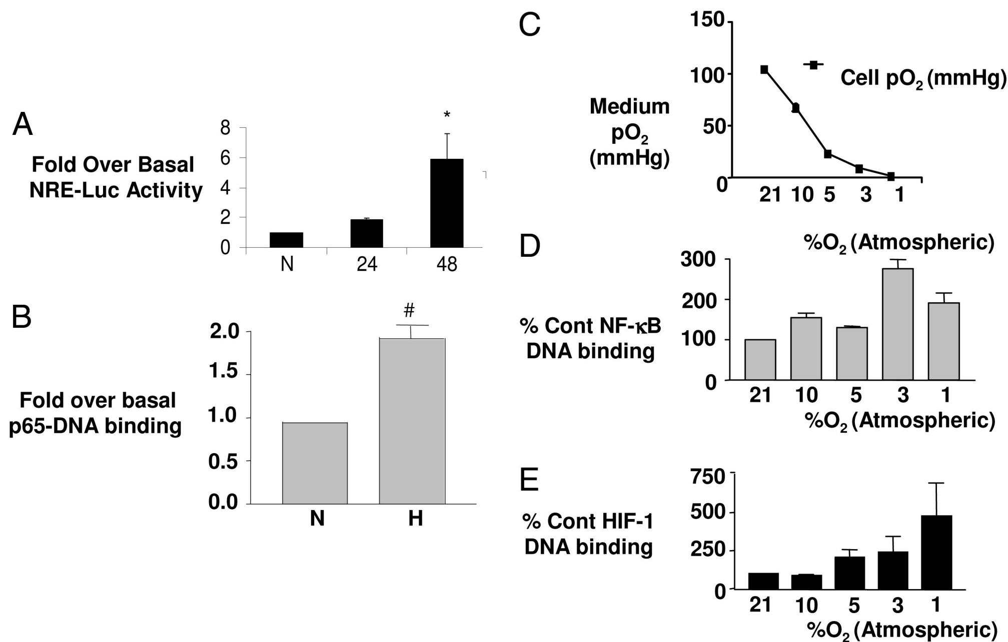

Fig. 1. Hypoxia activates NFκB. (*A*) HeLa cells transfected with an NFκB-luciferase reporter and exposed to 1% O_2 (0–48 h) demonstrate increased NFκB activity. (*B*) Nuclear extracts from HeLa cells exposed to preconditioned hypoxic medium (1% O_2; 4 h) demonstrate increased NFκB DNA binding. (*C*) HeLa cells exposed to graded hypoxia (21–1% O_2; 24 h) demonstrate decreased extracellular pO_2 values as measured by fluorescence quenching oxymetry and increased NFκB and HIF-1α DNA binding activity (*D* and *E*).

sensus NFκB response element) detectable 4 h after exposure to preconditioned hypoxic medium (1.8 ± 0.29-fold; $P < 0.05$; 1% O_2; Fig. 1*B*). Next, cells were exposed to graded hypoxia (21%, 10%, 5%, 3%, and 1% O_2) in separate ambient atmospheric hypoxia chambers. Using oxygen quenching oxymetry, we determined extracellular oxygen tensions at the level of the monolayer to be 105 ± 2.9, 67 ± 1.6, 23 ± 1.0, 9 ± 1.0, and 2 ± 0.2 mmHg (1 mmHg = 133 Pa), respectively (Fig. 1*C*). DNA binding activity of p65 and HIF-1 in nuclear fractions was measured in the same cell lysates and was related to oxymetry readings. Whereas the peak of p65 DNA binding in hypoxia occurred at a cell pO_2 of 9.2 ± 0.9 mmHg (Fig. 1*D*), peak HIF-1 DNA binding occurred at a relatively lower pO_2 value (1.7 ± 0.2 mmHg; Fig. 1*E*).

TNFα-Induced NFκB Nuclear Binding Is Enhanced in Hypoxia. To determine possible functional consequences for our observations, we examined whether hypoxia increased cellular sensitivity to a stimulus of the NFκB pathway, TNFα. HeLa cells were exposed to a preconditioned hypoxic medium (1% O_2 for 1 h) before treatment with low doses of TNFα (0.01–0.1 ng/ml for 1 h). Nuclear lysates were prepared, and DNA-binding assay was carried out. As predicted, TNFα-dependent activation of the NFκB pathway was significantly enhanced in cells in hypoxia ($P < 0.05$; Fig. 2).

Hypoxia Activates NFκB Through IKK. The IKK complex is the convergence point for many diverse NFκB-activating stimuli including TNFα, LPS, and IL-1 (8). We investigated whether hypoxia-dependent NFκB activation involves this pathway. Cells were exposed to a preconditioned hypoxic medium (1% O_2), and the phosphorylation status of IKKα/β was determined by Western blot analysis. Hypoxia results in a rapid phosphorylation of the IKKα/β complex detectable after 5 min, which is temporally upstream of IκBα phosphorylation on S32 and S36 (maximal after 15 min) and, in turn, is temporally upstream of IκBα degradation (Fig. 3*A*). The transient nature of IκBα phosphorylation is consistent with reports of the cyclical nature of NFκB activation in response to other stimuli (12). Critically, these measurements were made in the same cell lysates. Thus, hypoxia likely activates NFκB through IKK-dependent mechanisms. Abolition of HIF-1 expression in HeLa cells using siRNA did not alter hypoxia-induced IκBα phosphorylation (Fig. 3*B*), indicating that hypoxia-induced activation of the NFκB pathway is independent of HIF-1α.

PHD Inhibition Stimulates NFκB Activity. Prolyl hydroxylases are central to oxygen-sensing pathways leading to HIF-1α activation as a result of their absolute requirement upon molecular oxygen as a cofactor for enzymatic activity (2). The above data led us to the hypothesis that decreased PHD activity in hypoxia may also underlie NFκB activation. To further test this, we examined components of the NFκB pathway for the presence of the previously described, conserved LxxLAP motif for proline hydroxylation in HIF-1α (13). Whereas p65, p50, IκBα, and NEMO (IKKγ) are without this sequence, both IKKα and IKKβ contain the motif (Fig. 4*A*). This motif is evolutionarily conserved between humans, mice, and zebrafish. Interestingly, this motif is adjacent to the sites of phosphorylation of IKKβ (177/181) on the activation loop, making it likely accessible to modification by enzymes such as PHDs.

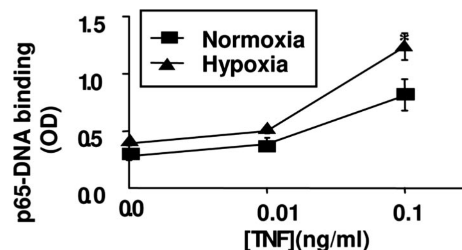

Fig. 2. NFκB DNA-binding assay in HeLa cells exposed to preconditioned hypoxic medium (1% O_2) for 1 h before TNFα treatment (0.01–0.1 ng/ml for an additional hour) demonstrates enhanced NFκB-nuclear binding when compared with normoxic controls.

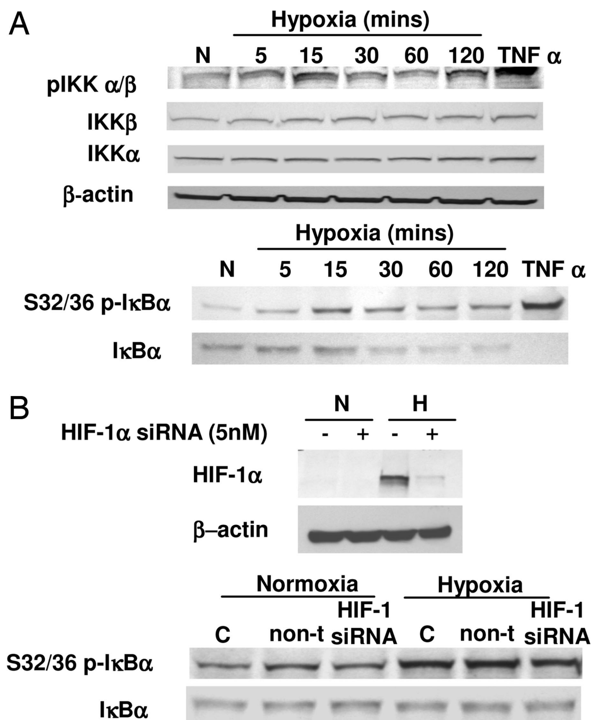

Fig. 3. Hypoxia activates NFκB through the IKK complex. (*A*) HeLa cells exposed to preconditioned normoxic (N; 21% O_2) or hypoxic (H; 1% O_2) media (5–120 min) demonstrate temporally sequential activation of IKKα/β, phosphorylation of IκBα, and degradation of IκBα. (*B*) HeLa cells transfected with siRNA against HIF-1α or nontarget siRNA exposed to hypoxia (1% O_2; 24 h) demonstrate effective HIF-1α knockdown. siRNA against HIF-1α does not prevent hypoxia-induced phosphorylation of IκBα.

To investigate the possibility that PHDs are playing a role in NFκB activation in hypoxia, we used PHD isoform-specific siRNA (14). Using this approach, we effectively knocked down each of the PHD-1, -2, and -3 isoforms by 73.3%, 80.3%, and 93.6%, respectively. Quantitation of PHD knockdown as measured by quantitative RT-PCR is demonstrated in Fig. 4*B*. Knockdown of each individual PHD isoform was without effect on expression levels of the other isoforms as measured by quantitative RT-PCR. HeLa cells transfected with an HIF-1-dependent luciferase reporter construct demonstrated sensitivity to PHD-2 knockdown as previously reported (ref. 13 and data not shown). Interestingly, cells transfected with an NFκB-dependent reporter construct demonstrated the greatest reporter activity in cells deficient in the PHD-1 isoform, indicating that the dominant isoform specificity for PHDs may differ for the HIF-1 and NFκB pathways (Fig. 4*C*). Conversely, artificial transient overexpression of PHD-1 in normoxia causes a decrease in TNFα-stimulated NFκB activity (Fig. 4*D*).

HeLa cells transfected with an NFκB-luciferase reporter construct were treated in normoxia with the pan-hydroxylase inhibitor DMOG (1 mM) for 24 h. DMOG treatment resulted in a 2.2 ± 0.4-fold increase in NFκB reporter activity compared with vehicle-treated control cells ($P < 0.05$, $n = 4$; Fig. 4*E*). To assess whether DMOG was acting proximally, at the level of initiation of the NFκB response, we exposed HeLa cells to 1 mM DMOG over a short time course (0–75 min) and demonstrated phosphorylation of S32/36 residues of IκBα, which was maximal after a 30-min exposure (Fig. 4*F*), thus indicating that PHD inhibition increases IKK activity. Collectively, these data indicate a repressive role for PHD-1 in NFκB signaling. Furthermore, PHD inhibition by DMOG (1 mM, 24 h) or PHD1 siRNA caused a significant increase in the expression of COX-2, an important NFκB-dependent inflammatory marker (Fig. 4 *G* and *H*).

Hypoxia Alters the Cellular Pool of IKKα and IKKβμ. HIF-1 stability is controlled by the PHD-dependent hydroxylation of specific residues in the CODD and NODD regions. The presence of an HIF-α LxxLAP consensus motif in IKKβ led us to examine the expression of these proteins under hypoxia. In line with previous studies (15), HeLa cells exposed to graded hypoxia for 24 h demonstrated increased IKKβ protein levels, whereas IKKγ/NEMO (which does not contain the hydroxylation motif) did not increase (Fig. 5*A*). We next investigated the impact of hypoxia on IKKβ expression in more physiologically relevant cell lines of immune and epithelial origin. Similar to HeLa cells, IKKβ levels were increased by graded hypoxia in THP-1 monocytic cells and in CaCo-2 intestinal epithelial cells in response to instantaneous hypoxia (1% O_2) (Fig. 5*B*).

Because IKKβ has been described as the primary positive regulator of NFκB activity in inflammatory processes and as the molecular link between inflammation and cancer (16–18), we focused our subsequent studies on this isoform. To assess the role of the proline residue within the LxxLAP motif of IKKβ (P191), we used a site-directed mutagenesis strategy to mutate P191 to an alanine residue. HeLa cells were transiently transfected with wild-type IKKβ or P191A IKKβ DNA. Forty-eight hours after transfection, cells were exposed to normoxia or preconditioned hypoxic medium (3% O_2 for 2 h; pO_2 = 19.9 mmHg). Cytosolic extracts were prepared, and IKKβ expression levels were compared by Western blot analysis. Cells transfected with the wild-type vector demonstrated elevated levels of IKKβ in hypoxia. Cells transfected with the P191A IKKβ vector demonstrated a loss of hypoxic inducibility (Fig. 5*C*). This is suggestive of a key role for P191 in the hypoxic regulation of IKKβ levels.

Interestingly, digital residue replacement modeling indicates that a modeled IKK peptide containing the LxxLAP motif has the characteristic boomerang structure of the HIF-1 CODD sequence, which is also subject to prolyl hydroxylation by PHDs (19). Furthermore, based on the crystal structure of the HIF-1 CODD peptide interaction with VHL (19), we were able to model an IKKβ-pVHL interaction without having to drastically modify the pVHL structure (data not shown).

Coimmunoprecipitation studies demonstrate an interaction between VHL and IKKβ in normoxic cells (Fig. 5*D*); however, this does not appear to result in ubiquitination or proteasomal degradation of IKKβ (data not shown). Furthermore, coimmunoprecipitation studies reveal the interaction between PHD1 and both IKK α and β isoforms. Although this indirectly indicates that IKK is hydroxylated, further critical experimentation using mass spectrometric analysis will be necessary to directly demonstrate hydroxylation. A distinct possibility is that inhibition of IKK activity by hydroxylation of P191 may be due to steric hindrance of the phosphorylation of the nearby residues S177 and S181 within the activation loop. Alternatively, binding of VHL to hydroxylated IKK may prevent activation through the blockage of the serine phosphorylation residues on the activation loop. Thus, we propose that hydroxylation represses IKKβ activity by altering protein expression or by preventing its activation through phosphorylation on S177/S181.

Discussion

PHD enzymes are critical in sensing and transducing the HIF-dependent transcriptional response to hypoxia, an event clearly demonstrating an absolute requirement for molecular oxygen as a cosubstrate (7, 20). Thus, PHDs are true oxygen/hypoxia sensors. However, the transcriptional response to hypoxia is not restricted to HIFs with NFκB also demonstrating hypoxic sensitivity and impacting the resultant hypoxic transcriptome (3). However, to date,

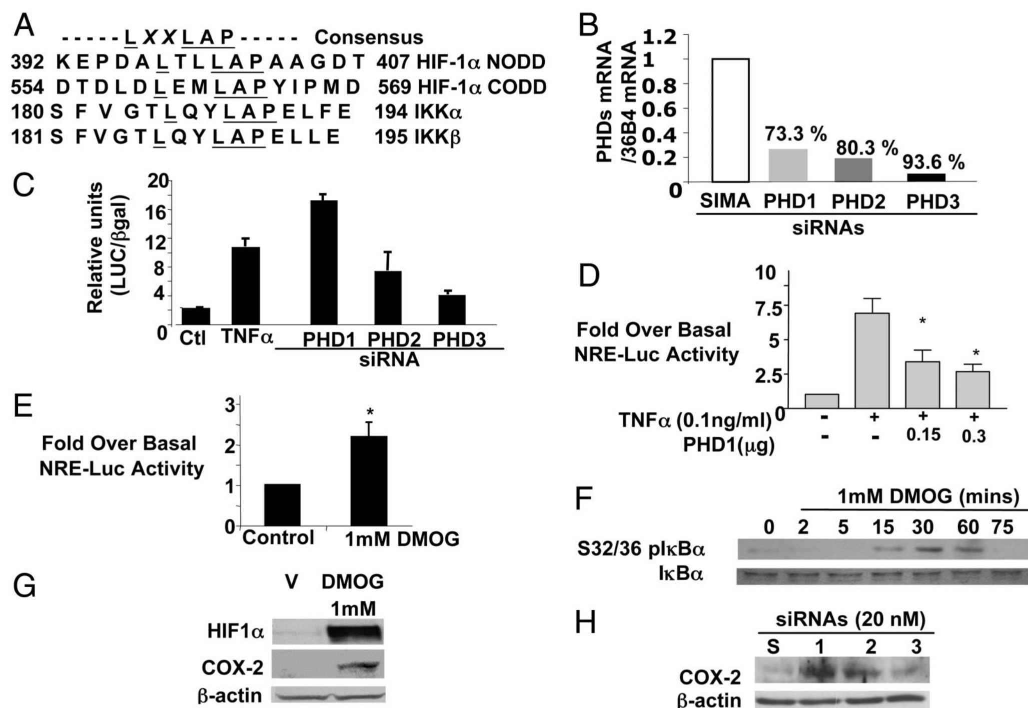

Fig. 4. PHDs suppress NFκB activity. (*A*) IKKα and IKKβ but not NEMO (not shown) contain conserved LxxLAP motifs. (*B*) HeLa cells transiently transfected with PHD siRNA demonstrate effective isoform-specific knockdown by quantitative RT-PCR. Data are shown from a representative experiment ($n = 3$ in total). (*C*) HeLa cells were cotransfected with isoform-specific PHD siRNAs (20 nM), the reporter vector (pNFκB-LUC), and 100 ng of a β-galactosidase construct. As a control for NFκB activation, cells were incubated with TNFα (10 ng/ml). Forty-eight hours after transfection, cells were lysed and luciferase and β-galactosidase activity were measured (37). Results are expressed as the fold induction over control. (*D*) HeLa cells were transiently cotransfected with pEGLN2-FLAG (PHD1; 0.15–0.3 μg) or empty pcDNA (0.15–0.3 μg) vector and pNFκB-LUC. After transfection, cells were treated with TNFα (0.1 ng/ml) for 24 h. Whole-cell lysates were prepared, and a luciferase assay was carried out. Results are protein-normalized RLU values expressed as fold over basal luciferase activity. (*E*) HeLa cells were transiently transfected with an NFκB-promoter reporter construct. After transfection, cells were exposed to DMSO-vehicle (control) or 1 mM DMOG and maintained at 21% O_2 for 24 h. Results shown are protein normalized RLU values. (*F*) Immunoblotting for anti-phospho-S32/36 IκBα and total IκBα was carried out on DMOG-treated HeLa cells (1 mM; 2–75 min). (*G*) HeLa cells were exposed to DMOG (1 mM, 24 h) or vehicle. Whole-cell extracts were immunoblotted for HIF-1α, Cox-2, or β-actin. (*H*) Three PHD isoforms were knocked down in HeLa cells as described above, and Cox-2 expression was determined by Western blot analysis.

our understanding of PHD involvement in hypoxic signaling has been restricted mainly to the HIF-1 pathway. Because of its role in tumor progression, NFκB has important pathophysiologic implications in cancer, a condition involving significant tissue hypoxia.

Hypoxia-activated NFκB is demonstrable by the nuclear accumulation of the p65 subunit as well as by reporter gene assay and correlates with decreased cellular pO_2. Furthermore, hypoxia-induced NFκB activity is preceded by temporally sequential IKK activation, IκB phosphorylation, and IκB degradation, indicating that hypoxia activates NFκB through increased IKK activity. The IKK-dependence of hypoxia-induced NFκB activity is consistent with most other stimuli of this pathway. Although previous studies have hypothesized roles for tyrosine phosphorylation (21), reactive oxygen species generation (22), and p42/44 or PI-3-kinase (23) in mediating NFκB responses in hypoxia, none of these theories clearly demonstrates a direct relationship between decreased molecular oxygen and NFκB activation such as through altered PHD activity. Our model proposes that while hypoxia activates NFκB it also increases the sensitivity of the NFκB pathway to activation through proinflammatory stimuli, such as cytokines. Thus, the importance of this pathway may be greatest where hypoxia occurs against the backdrop of increased inflammatory activity [such as in a growing tumor (24)] where amplification of a stimulated response may heavily affect tumor-promoting gene expression.

Alternative signaling targets for proline hydroxylation in the transcriptional response to hypoxia are attractive in developing the theory that hydroxylating enzymes are central to cellular oxygen sensing (7, 20). In the current study, we demonstrate that IKKβ, a critical regulator of NFκB activity, contains a conserved prolyl hydroxylation consensus site homologous with the two conserved sites within the oxygen-dependent degradation domain (ODD) of HIF-1 (LxxLAP). Like HIF-1α, the pool of IKKβ in cells is increased in hypoxia; however, unlike HIFα, the absolute expression of IKKs is not determined by the presence of oxygen because basal expression is clearly detectable. Thus, it appears that hypoxia is more a modulator of IKK expression and activation than an absolute determinant. This may be due to the presence of just a single LxxLAP motif in IKKβ rather than the two present in HIF-1α. A recent study has demonstrated the requirement of a leucine residue within 10 residues of the LxxLAP motif for hydroxylation of the consensus motif (25). Two leucine residues reside closely downstream of the LxxLAP motif in IKKβ. Importantly, the putative site of hydroxylation (P191) on IKKβ resides just 10 residues from the known phosphorylation site S181, which is required for IKKβ activation.

S181 is positioned in the vicinity of P191 in the well conserved protein kinase activation loop, and P191 itself is predicted to be positioned in the substrate binding cleft. Experimental studies (26) of a prototypic protein kinase (the cAMP-dependent protein kinase, PKA) show that the conformation and dynamics of the activation loop are known to be linked with the phosphorylation of S181. Thus, it is possible that hydroxylation of P191 alters the conformation of the activation loop, making phosphorylation difficult or ineffective (i.e., the kinase might be phosphorylated but inactive) and/or impairs the binding of the protein kinase substrate. This would cause a decrease in IKKβ (and subsequently NFκB) activity. Similarly, it is also conceivable that phosphorylation renders hydroxylation more difficult

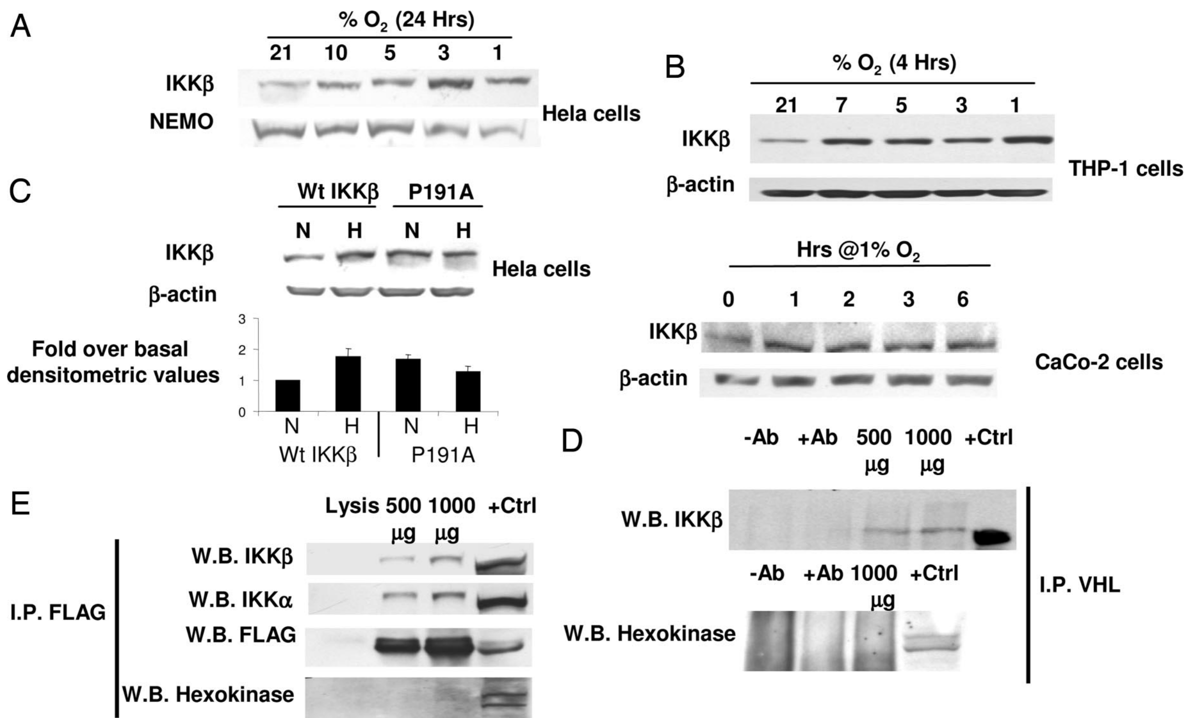

Fig. 5. The cellular pool of IKKβ is increased in hypoxia. HeLa (*A*) and THP-1 and CaCo-2 (*B*) cells were exposed to graded hypoxia (21–1% O_2) for 24 h or instantaneous hypoxia (1% O_2) for 0–6 h. Whole-cell extracts were immunoblotted for IKKβ, NEMO, and β-actin where indicated. (*C*) HeLa cells were transiently transfected with 1 μg of wild-type IKKβ or P191A mutant IKKβ. Forty-eight hours after transfection, the cells were maintained in normoxia (N; 21% O_2) or exposed to preconditioned hypoxic medium (H; 3% O_2) for 2 h. Cytosolic extracts were immunoblotted for IKKβ and β-actin. Alterations in protein expression were measured semiquantitatively by using densitometric analysis ($n = 4$). (*D*) pVHL was immunoprecipitated from whole HeLa cell extracts (500 or 1,000 μg of total protein), and immunoprecipitates were immunoblotted for IKKβ and hexokinase (similarly sized negative control). (*E*) NETN lysates from HeLa cells transiently overexpressing PHD-1-FLAG were immunoprecipitated by using a specific anti-FLAG resin. Immunoprecipitates were immunoblotted for IKKβ, IKKα, FLAG, and hexokinase.

because the hydroxylation site in active IKKβ could be masked by the substrate. Additionally, the activation loop would be less dynamic and hence less accessible to PHDs.

The role of IKKβ in oncogenesis and inflammation is well established, but there is emerging evidence that IKKα may have antiinflammatory functions (27, 28). We also observed an increase in IKKα in hypoxia (data not shown). The observation that both IKKα and IKKβ are hypoxia-sensitive, albeit differentially, suggests the possibility of a counterbalancing mechanism where a p65-mediated response could be resolved over a course of hypoxia.

Genetic or pharmacologic interference with PHD activity results in activation of NFκB-dependent signaling in normoxia. Mutation of the hydroxylated proline residues of HIF-1α results in a loss of hypoxic inducibility (29). Similarly, mutation of proline residue 191 in IKKβ, which resides within the consensus sequence, results in a similar effect. Although sensitive to knockdown of both PHD-1 and PHD-2, NFκB activation appears to be more sensitive to silencing of the PHD-1 isoform, suggesting somewhat differential regulation to HIF-1, which is predominantly regulated by PHD-2. These data suggest the intriguing possibility that hypoxia increases IKK stability through an inhibition of protein hydroxylation, resulting in evasion of degradation in a similar manner to HIF-1. Indeed, it is worth noting that a number of studies have demonstrated that the E3 pVHL suppresses NFκB activity in normoxia (30–32). However, although IKKβ interacts with VHL in normoxia, we found no evidence for its ubiquitination/degradation. Furthermore, the rapid kinetics of IKKβ activation in hypoxia suggests the likelihood that the primary repressive role of P191 hydroxylation would be through inhibition of phosphorylation of S177/S181. This may occur through VHL masking of the activation loop.

Thus, hypoxia increases the expression and activation of IKK, leading to increased sensitivity of cells to inflammatory stimuli such as cytokines. This has important implications in cancer where elevated levels of proinflammatory cytokines coexist with hypoxia in the microenvironment of the tumor (33). Furthermore, this is consistent with previous studies demonstrating synergy between hypoxia and inflammatory cytokines in the activation of cells (10, 34).

In summary, we propose that hypoxia, through inhibiting PHD activity, increases the activity of cellular IKKβ, an event that amplifies the cellular capacity for response to cytokines. Furthermore, we believe that such events may directly impact on tumor development through enhanced expression of genes that promote tumor development and growth.

Methods

Cell Culture and Hypoxia. HeLa, CaCo-2, and THP-1 cells were placed in one of four hypoxia chambers (Coy Laboratories, Grass Lake, MI; Ruskin Technologies, Leeds, U.K.) allowing the establishment of graded, humidified, ambient, atmospheric hypoxia of 21%, 10%, 5%, 3%, and 1% O_2 with 5% CO_2 and a balance of N_2 in all cases. Extracellular pO_2 measurements were made by using fluorescence quenching oxymetry (Oxylite-2000; Oxford Optronix, Oxford, U.K.). Hypoxia did not induce apoptosis or necrosis (data not shown). Temperature was maintained at 37°C. Where outlined, instantaneous hypoxia was achieved by exposure of cells in hypoxia chambers to preconditioned media.

Western Blot Analysis. Cytoplasmic or whole-cell lysates were separated by SDS/PAGE, transferred to nitrocellulose membranes, and immunoblotted as described (35). IKKα, IKKβ, and phospho-IKKα (S180)/IKKβ (S181) polyclonal antibodies (Cell Signaling), IKKγ/NEMO polyclonal antibody (Santa Cruz Biotechnology), IκBα polyclonal antibody (Upstate Biotechnology), FLAG poly-

clonal antibody (Sigma), Hexokinase polyclonal antibody (Biogenesis), Cox-2 polyclonal antibody (Cayman Chemical), phospho-IκBα monoclonal antibody (Cell Signaling), and HIF-1α monoclonal antibody (BD Transduction Laboratories) were used.

NFκB-DNA Binding Assays. Nuclear lysates for DNA-binding assays were prepared as per the manufacturer's instructions (TransAm kit; ActiveMotif). The nuclear extract was incubated with an immobilized oligonucleotide on a 96-well plate (containing a specific transcription factor binding site as indicated) and detected by the transcription factor ELISA.

Generation of IKKβ Mutant by Site-Directed Mutagenesis. The P191A IKKβ mutant was generated by using a site-directed mutagenesis kit described previously (36). Plasmid constructs were verified by using DNA sequencing. Primers designed for IKKβ were as follows: forward, 5′-CCCTGCAGTACCTGGCC*G*CAGAGCTACTGGAGC-3′; reverse, 5′-GCTCCAGTAGCTCTGCGGCCAGGTACTGCAGGG-3′.

Transient Transfection. Transient transfection of plasmid DNA was performed in HeLa cells (50–70% confluent) by using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. After transfection, cells were exposed overnight at 37°C (5% CO_2). Medium was replaced, and cells were exposed to experimental treatment as described.

Reporter Assay. Cells were transfected with an NFκB-luciferase construct (Stratagene *Cis*-Reporting Systems) with a synthetic promoter containing a concatomer of five NFκB response elements. Cell lysates were prepared by using a 1× luciferase lysis buffer (Promega). Substrate was added to the cell lysate, and luciferase activity was measured in a luminometer. Experiments were carried out in duplicate or triplicate, and luciferase values were normalized to cotransfected CMV *Renilla* luciferase, β-Gal, or protein control values.

RNA Interference by siRNA. HeLa cells were grown to 40–50% confluency and transfected with 5 nM specific siRNA against HIF-1α or control nontarget siRNA (Dharmacon) by using a Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions. Cells were maintained in antibiotic-free media for 48 h after transfection to achieve maximal knockdown of the target gene. RNA interference by siRNA against PHD isoforms was carried out as described (14) and quantified by quantitative RT-PCR (Applied Biosystems).

Coimmunoprecipitation Studies. Whole HeLa cell lysates were prepared in a lysis buffer as described above. Lysate was precleared with 50 μl of protein A/G PLUS agarose beads (Santa Cruz Biotechnology) and rotated for 1 h at 4°C. Five hundred micrograms and 1,000 μg of lysate were incubated with 2 μg of an anti-VHL antibody (BD Pharmingen) and brought to a final volume of 650 μl with lysis buffer. The lysis buffer (650 μl) ± anti-VHL antibody as well as the nonimmunoprecipitated whole-cell extracts were included as controls (+Ab, −Ab, and +Ctrl, respectively). Samples were rotated for 2 h at room temperature. Thirty microliters of protein A/G PLUS agarose beads was added to the samples and controls and rotated for 1 h at 4°C. Samples were centrifuged at 17,968 × *g* for 6 min at 4°C. Beads were washed three times in lysis buffer. The supernatant was discarded, and the pellet was resuspended in reducing sample buffer (65 μl) and boiled for 10 min before electrophoresis.

HeLa cells were transiently transfected with pEGLN2-FLAG. Whole-cell lysates were prepared in NETN buffer [20 mM Tris·HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, protease inhibitor mixture]. Five hundred micrograms and 1,000 μg of protein lysate were incubated with 30 μl of anti-FLAG agarose affinity gel (Sigma) and brought to a final volume of 500 μl with an NETN buffer. NETN buffer alone (Lysis) was incubated with the beads as a control, in addition to a nonimmunoprecipitated NETN lysate (+Ctrl). Samples were rotated overnight at 4°C. Samples were centrifuged at 17,968 × *g* for 6 min at 4°C. Beads were washed three times in NETN buffer. The supernatant was discarded, and the pellet was resuspended in reducing sample buffer (65 μl) and boiled for 10 min before electrophoresis.

Statistical Analysis. All data are presented as mean ± SEM for n independent experiments. Statistical significance was evaluated by using one-way ANOVA or Student's t test for unpaired or paired data where an asterisk corresponds to $P < 0.05$.

We thank Anne Marie Griffin for expert technical assistance. pEGLN2 plasmids were a kind gift from Dr. William Kaelin (Harvard Medical School, Boston, MA). This work was supported by grants from Science Foundation Ireland, the Health Research Board of Ireland, the Wellcome Trust, the Government of Ireland Programme for Research in Third Level Institutions, Centre National de la Recherche Scientifique, and La Ligue Nationale Contre le Cancer.

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