Regulation of IL-1β–induced NF-κB by hydroxylases links key hypoxic and inflammatory signaling pathways

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Hypoxia is a prominent feature of chronically inflamed tissues. Oxygen-sensing hydroxylases control transcriptional adaptation to hypoxia through the regulation of hypoxia-inducible factor (HIF) and nuclear factor κB (NF-κB), both of which can regulate the inflammatory response. Furthermore, pharmacologic hydroxylase inhibitors reduce inflammation in multiple animal models. However, the underlying mechanism(s) linking hydroxylase activity to inflammatory signaling remains unclear. IL-1β, a major proinflammatory cytokine that regulates NF-κB, is associated with multiple inflammatory pathologies. We demonstrate that a combination of prolyl hydroxylase 1 and factor inhibiting HIF hydroxylase isoforms regulates IL-1β–induced NF-κB at the level of (or downstream of) the tumor necrosis factor receptor-associated factor 6 complex. Multiple proteins of the distal IL-1β–signaling pathway are subject to hydroxylation and form complexes with either prolyl hydroxylase 1 or factor inhibiting HIF. Thus, we hypothesize that hydroxylases regulate IL-1β signaling and subsequent inflammatory gene expression. Furthermore, hydroxylase inhibition represents a unique approach to the inhibition of IL-1β–dependent inflammatory signaling.

oxygen | inflammatory disease | OTUB1 | UBC13

Hypoxia occurs when the demand for oxygen necessary to satisfy metabolic requirements exceeds the vascular supply. Whereas it is well established that tissue hypoxia is a feature of a range of physiologic and pathophysiologic states, including fetal development, exercise, tumor growth, and ischemia, it has recently become appreciated that hypoxia is also a prominent feature in inflammatory pathologies, including rheumatoid arthritis and inflammatory bowel disease (IBD) (1, 2). Furthermore, hypoxia profoundly impacts upon important inflammatory processes, including the regulation of neutrophil survival, macrophage survival and differentiation, T-cell differentiation, and dendritic cell function (3).

A key mediator of the immunological and inflammatory sequelae of hypoxia is the hypoxia-inducible factor (HIF). HIF is suppressed in the presence of oxygen through the activity of a family of evolutionarily conserved hydroxylases, of which there are three prolyl hydroxylases (PHD1, PHD2, and PHD3) and a single asparaginyl hydroxylase termed factor inhibiting HIF (FIH). PHDs control the degradation of HIF through proline hydroxylation with a dominant role for PHD2, whereas FIH-dependent asparagine hydroxylation is involved in fine tuning HIF activity by regulating interactions with CREB-binding protein (CBP)/p300 (4). HIF has been identified as a key regulator of inflammation and immunity (5, 6), although whether its activation is ultimately pro- or anti-inflammatory in vivo is likely context specific. However, the net effect of pharmacologic activation of this pathway through inhibition of hydroxylases in vivo is anti-inflammatory. The complex role of HIF and inflammation and its potential as a therapeutic target have been recently reviewed (1–3, 7).

NF-κB, a key regulator of inflammation is another hypoxia-responsive transcription factor (8). The same hydroxylases that confer hypoxic sensitivity upon HIF have been reported to be responsible for the hypoxic sensitivity of NF-κB (9). Whereas PHDs have been implicated in the regulation of NF-κB, the functional site(s) of proline hydroxylation in the pathway has yet to be identified (9–11). Conversely, FIH-dependent asparagine hydroxylation sites on a number of key proteins in the NF-κB pathway have been identified; however, the functional impact of this remains unclear (12, 13). Little is known about whether hydroxylases regulate NF-κB in the stimulated state as occurs during active inflammation. However, the role of hydroxylases in inflammation is evidenced by the profoundly protective effect of pharmacologic hydroxylase inhibition in models of colitis, ischemia/reperfusion, infection, and sepsis (14, 15). The mechanism underpinning this anti-inflammatory effect of hydroxylase inhibition is the topic of the current study.

IL-1β is secreted from multiple cell types and is associated with a range of inflammatory, metabolic, and infectious diseases (16). Upon binding of IL-1β to its cognate receptor, a signaling cascade is initiated, which signals via tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and the IKK complex resulting in the activation of NF-κB, a master regulator of inflammatory gene expression (17, 18). IL-1β plays a key role in septic shock, rheumatoid arthritis, inflammatory bowel disease, and type II diabetes and is thus a major therapeutic target (16). Here, we investigated the regulation of IL-1β–induced NF-κB

Significance

Oxygen-sensing hydroxylases are a family of enzymes that control the cellular adaptive response to hypoxia. Hydroxylase inhibitors reduce inflammation in vivo; however, the anti-inflammatory mechanism of action remains unclear. IL-1β is a cytokine that potently promotes inflammation through activation of the transcription factor NF-κB. Here, we demonstrate that hydroxylase inhibition leads to a suppression of IL-1β–induced NF-κB activity and provide insight into the underlying mechanism involved. This work develops our understanding of how hydroxylase inhibition regulates IL-1β–induced inflammation and sheds light on our understanding of the association between hypoxic and inflammatory signaling pathways, underscoring the potential use of hydroxylase inhibitors for the treatment of inflammatory disease.

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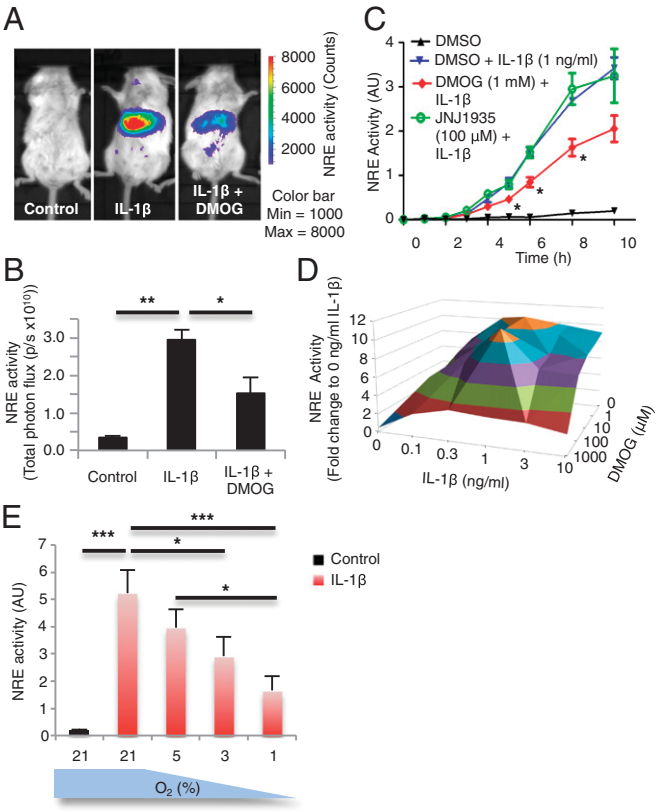

Fig. 1. Hydroxylase inhibition attenuates IL-1β–induced NF-κB activity in vivo and in vitro. (*A*) IL-1β was administered to NF-κB–luciferase reporter mice for 4 h with or without DMOG pretreatment (8 mg per mouse, 24 h) and luciferase activity was visualized by in vivo imaging. (*B*) In vivo NF-κB activity was quantified by measurement of photon release from NF-κB–luciferase reporter mice. (*C*) Measurement of IL-1β–induced NF-κB–dependent transcriptional activity by NF-κB *Gaussia* luciferase reporter assay in cells treated with DMOG or JNJ1935. (*D*) Matrix inhibition assay was used to measure the effect of DMOG on increasing concentrations of IL-1β–induced NF-κB–dependent transcriptional activity using a NF-κB *Gaussia* luciferase reporter assay. Samples were pretreated for 1 h with DMOG before IL-1β treatment. Samples were collected 10 h after the addition of IL-1β. (*E*) Measurement of IL-1β–induced NF-κB activity by NF-κB *Gaussia* luciferase reporter assay 8 h following stimulation in cells grown in graded hypoxic environments. Data are represented as mean + SEM. $n = 3$–6 throughout; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA followed by Tukey posttest.

activity by hydroxylases with an aim to identifying therapeutic targets in the control of IL-1β–induced inflammation.

Results

Hydroxylase Inhibition Attenuates IL-1β–Induced NF-κB Activity in Vitro and in Vivo. We have previously shown that hypoxia elevates basal NF-κB activity both in vitro and in vivo (19). In contrast to the regulation of basal NF-κB activity, in cells stimulated with IL-1β (but not TNFα), hypoxia inhibits stimulated NF-κB activity (Fig. S1 *A* and *B*). Because, hydroxylases are key oxygen-sensing enzymes in cells, we investigated the impact of hydroxylase inhibition on IL-1β–induced NF-κB activity in vivo. IL-1β treatment led to an increase in NF-κB activity in transgenic NF-κB–luciferase reporter mice in a manner that was significantly attenuated in animals that had been pretreated with the panhydroxylase inhibitor dimethyloxallyl glycine (DMOG) (Fig. 1 *A* and *B*). Ex vivo measurement of luciferase activity in tissues revealed that the majority of IL-1β–induced NF-κB activity was in the liver and that this was strongly attenuated in mice pretreated with DMOG (Fig. S1 *C* and *D*).

To gain mechanistic insight, we next investigated the effects of hydroxylase inhibition on IL-1β–induced NF-κB activity in cultured cells. HeLa cells were exposed to DMOG (which inhibits both PHDs and FIH) or the PHD-selective inhibitor JNJ-42041935 (JNJ1935) (Fig. S2) (20) before stimulation with IL-1β. Consistent with our in vivo experiments, pretreatment of HeLa cells with DMOG reduced IL-1β–induced NF-κB activity in a time- and dose-dependent manner and over a range of IL-1β concentrations (Fig. 1 *C* and *D*). However, JNJ1935 was without effect on IL-1β–induced NF-κB activity, leading us to hypothesize that inhibition of both prolyl and asparaginyl hydroxylation may be required. Supporting this hypothesis, graded hypoxia caused gradual reduction in IL-1β–induced NF-κB activity with maximal inhibition occurring at 1% O_2. This is consistent with PHD inhibition at higher oxygen levels and inhibition of both PHDs and FIH at lower oxygen levels (Fig. 1*E*) (21).

PHD1 and FIH Regulate IL-1β–Induced NF-κB Activity in a Combinatorial Manner. Four isoforms of HIF hydroxylases (PHD1, -2, -3, and FIH) have been described to confer hypoxic sensitivity on the HIF transcriptional pathway. We developed our investigation into the relative role of each of these isoforms alone and in combination in the hydroxylase-dependent regulation of IL-1β–induced NF-κB. siRNA-mediated RNA interference allowed us to specifically and potently inhibit expression of PHD1, PHD2, PHD3, or FIH either individually or in combination (Fig. S3 *A*–*F*). In a high-throughput screening assay, single knockdowns of FIH and PHD1 but not PHD2 or PHD3 reduced IL-1β–induced NF-κB activity (Fig. S3 *G*–*J*). Furthermore, in time course studies, both PHD1 and FIH knockdown significantly reduced IL-1β–induced NF-κB activity (Fig. S3 *K* and *L*). Notably however, the combinatorial knockdown of PHD1 and FIH was at least additive (Fig. 2*A*). Conversely, the overexpression of the combination of FIH and PHD1 (Fig. S4) significantly enhanced IL-1β–induced NF-κB activity (Fig. 2*B*). These data led us to hypothesize that it is a combination of inhibiting both PHD1 and FIH that is primarily responsible for the inhibitory effects of hydroxylase inhibitors on IL-1β–induced NF-κB activity.

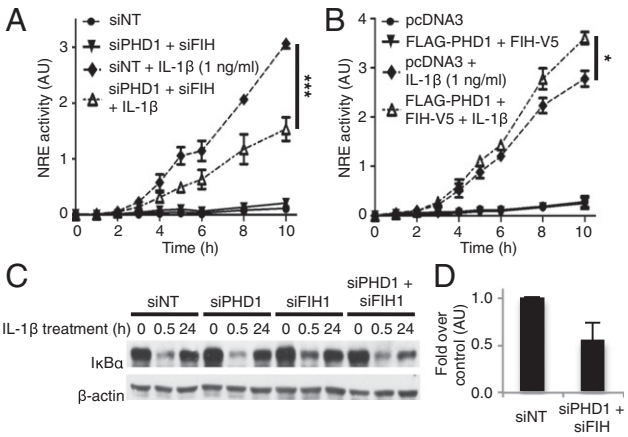

Fig. 2. IL-1β–induced NF-κB activity and endogenous gene expression is regulated in a combinatorial manner by PHD1 and FIH. NF-κB *Gaussia* luciferase reporter assay was used to determine the impact of (*A*) combinatorial knockdown and (*B*) combinatorial overexpression of PHD1 and FIH on IL-1β–induced NF-κB activation. (*C*) Western blot analysis of whole cell IL-1β–induced IκBα protein expression in cells where PHD1 and FIH have been knocked down alone and in combination. (*D*) Densitometric analysis of IκBα protein expression 24 h after IL-1β treatment with and without combinatorial knockdown of PHD1 and FIH. Data are represented as mean + SEM. AU, arbitrary unit; NS, not significant. $N = 4$ throughout; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for *A*; by one-way ANOVA for *B*.

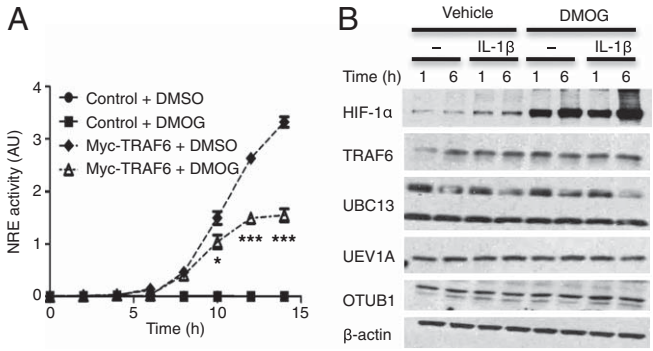

Fig. 3. Hydroxylase inhibition leads to a reduction of TRAF6-induced NF-κB activity. (*A*) NF-κB *Gaussia* luciferase reporter assay in HeLa cells demonstrates that NF-κB activity induced by overexpressing TRAF6 is inhibited by addition of DMOG 6 h after transfection with TRAF6 plasmid. (*B*) Expression levels of components of the TRAF6 complex in HeLa cells treated with DMOG and IL-1β. Data are represented as mean + SEM. AU, arbitrary unit. $N = 4$ throughout; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA followed by Tukey posttest.

Combinatorial PHD1 and FIH Knockdown Attenuates IL-1β–Induced NF-κB–Dependent Gene Expression. We next investigated the impact of combinatorial knockdown of PHD1 and FIH on the expression of inhibitor of NF-κB α (IκBα) (a known NF-κB target gene) in response to stimulation with IL-1β. Cells exposed to IL-1β demonstrated an acute and transient decrease in IκBα expression, which is associated with the activation of NF-κB, and which was followed by a rebound expression as a result of transcriptional up-regulation by NF-κB (22). Simultaneous knockdown of FIH and PHD1 reduced the recovery of IκBα, indicating that the combinatorial knockdown of PHD1 and FIH inhibits IL-1β–induced NF-κB–dependent gene expression (Fig. 2 *C* and *D*).

Hydroxylase Inhibition Reduces TRAF6-Dependent NF-κB Activity. We next investigated possible sites on the IL-1β pathway at which hydroxylases may regulate NF-κB. First, we investigated whether proteins upstream of the TRAF6 complex (Fig. S5*A*) could be targets for hydroxylation responsible for the regulation of IL-1β–induced NF-κB signaling. To do this, we bypassed the IL-1β receptor and associated proteins by activating NF-κB directly through overexpression of TRAF6 (23). DMOG strongly inhibited TRAF6-induced NF-κB activity (Fig. 3*A*), indicating that the functional hydroxylation occurs at the level of the TRAF6 complex or downstream of it. Importantly, whereas IL-1β and DMOG increased HIF expression as previously described (24), the abundance of components of the TRAF6 complex were not altered (Fig. 3*B*). Investigating the impact of DMOG on the formation of the functional E2 ubiquitin-conjugating enzyme of TRAF6, we found that the interaction between UBC13 and UEV1A (the two components of the E2 complex) overexpressed in HeLa cells was not affected by treatment with DMOG (Fig. S5*B*).

PHD1 and FIH Physically Interact with Proteins of the IL-1β Signaling Pathway. Having demonstrated that hydroxylase inhibitors regulate IL-1β–induced NF-κB signaling, we next investigated possible substrates for hydroxylation in the IL-1β pathway. To do this, we used an unbiased mass spectrometry-based approach to identify proteins that coimmunoprecipitate with individual hydroxylase isoforms. UEV1A and OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1), two proteins associated with the TRAF6 complex (18, 25), were found to be associated with PHD1 and FIH, respectively, but not with PHD2 or PHD3 (Tables 1 and 2 and Dataset S1). UBC13 is described to be a further, central component of the complex that interacts with both UEV1A and OTUB1 (26). To investigate this in our system, a pulldown of UBC13 was performed. Consistent with previous reports, we found that UBC13 interacted with both UEV1A and OTUB1 (Table 3). These data indicate that a complex containing UBC13, UEV1A, OTUB1, PHD1, and FIH exists in the IL-1β–signaling pathway.

Downstream of the TRAF6 complex, we found that IκBβ, an ankyrin-repeat domain (ARD)-containing protein, was associated with FIH. This is consistent with previous reports that ARD-containing proteins (including other IκB family members) are substrates for hydroxylation by FIH (12). We further found that interaction between FIH and OTUB1, IκBβ and previously identified substrates, was promoted in cells treated with DMOG (Fig. S6 and Dataset S1). As UEV1A is cytosolic and PHD1 has previously been described as predominantly nuclear (27), we investigated the cellular distribution of PHD1. We found that endogenous PHD1 is expressed in both nuclear and cytosolic compartments in the HeLa cell culture model used here (Fig. S3*M*). In summary, several proteins of the IL-1β–signaling pathway form complexes with either PHD1 or FIH.

Hydroxylation of Proteins in the IL-1β–Signaling Pathway. We next investigated whether proteins associated with IL-1β signaling were possible substrates for hydroxylation. For the unbiased identification of hydroxylated proteins of the IL-1β pathway, we searched a large-scale qualitative dataset (28) downloaded from the Trance repository against a human database including met, pro, trp, tyr, asp, asn, and his oxidations as variable modifications (Table S1 and Dataset S1) for proteins included in the IL-1β pathway (18). MS/MS analysis of this HeLa cell proteome demonstrated that UEV1A (shown to coimmunoprecipitate with PHD1) (Table 1) was hydroxylated on two adjacent proline residues (proline 153 and 154 in UEV1A isoform 1). These proline residues are conserved over five different UEV1A isoforms (Fig. 4 *A*, *D*, and *E* and Fig. S7). Furthermore, UBC13, the protein forming the functional E2-conjugating enzyme with UEV1A, was also shown to be hydroxylated on two different proline residues (Table S1), although no interaction with a PHD had been identified. OTUB1 (which was found to interact with FIH; Table 2) showed five hydroxylations on amino acid residues identified to be specifically targeted by FIH (asn, asp, and his) (Table S1) (29, 30). Additionally, we found evidence for prolyl hydroxylation of OTUB1 in this dataset, although, similar to UBC13, no direct interaction with a PHD was detected (Table S1). However, UBC13 interacts strongly with UEV1A (Table 3) (25) and UEV1A interacts with PHD1 (Table 1). Similarly, OTUB1 forms a complex with UBC13 and UEV1A (Table 3) (26, 31). The

Table 1. Coimmunoprecipitation of components of the IL-1β signaling pathway with PHD1, -2, and -3

Protein groups	Interacting proteins	Immunoprecipitation (IP) PHD1	PHD2	PHD3
Proteins of the HIF pathway	PHD1	+	+	+
	PHD2	+	+	+
	PHD3	–	+	+
	FIH	–	–	–
	HIF-1α	+	+	+
	ARNT	+	+	+
Other known interactors	HSP90	+	+	+
	FKBP38	–	+	+
Proteins of the IL-1β pathway	UBC13	–	–	–
	UEV1A	+	–	–
	OTUB1	–	–	–
	IκBβ	–	–	–

PHD1, -2, and -3 were immunoprecipitated and the coprecipitants were analyzed using mass spectrometry. Previously described hydroxylase targets/interactors served as controls demonstrating effective coimmunoprecipitation. Several different isoforms of HSP90 were found, which were combined in this table as "HSP90." For further information see Dataset S1.

Table 2. Coimmunoprecipitation of components of the IL-1β signaling pathway with FIH

Protein groups	Interacting proteins	IP FIH
Proteins of the HIF pathway	PHD1	–
	PHD2	+
	PHD3	–
	FIH	+
	HIF-1α	+
	ARNT	+
Other known interactors	Tankyrase-1	+
	Tankyrase-2	+
	Notch2	+
	RIPK4	+
Proteins of the IL-1β pathway	UBC13	–
	UEV1A	–
	OTUB1	+
	IκBβ	+

FIH was immunoprecipitated and the coprecipitants were analyzed using mass spectrometry. Previously described hydroxylase targets/interactors served as controls, demonstrating effective coimmunoprecipitation. For further information, see Dataset S1.

potential spatial proximity of both UBC13 as well as OTUB1 to PHD1 could therefore explain the observed prolyl hydroxylations. IκBβ, which, as well as OTUB1, specifically coimmunoprecipitated with FIH, was found to be hydroxylated on one aspartate residue (Table S1).

In addition to those listed above, a number of other proteins involved in the IL-1β pathway were also found to be hydroxylated (Table S1).

FLAG-UBC13 pulldown followed by mass-spectrometry–based analysis confirmed hydroxylations on proline 19 and 21 (Table S1). Furthermore, the analysis identified multiple sites for hydroxylation on UBC13, including proline 59 (Fig. 4*B*). MS/MS analysis of immunoprecipitated FLAG-HA-OTUB1 confirmed asparaginyl hydroxylation on N22 in isoform 1 (Otubain-1) (Fig. 4*C* and Table S1). In addition, we identified a hydroxylation of Y26, which is likely to be a nonenzymatic oxidation (Fig. 4*C*).

Overall, we have demonstrated that PHD1 and FIH play an important role in modulating IL-1β–induced NF-κB activity. A number of proteins in the IL-1β–signaling pathway were found to be associated with hydroxylases. Furthermore, peptides from these (and other) IL-1β–signaling proteins are found in the hydroxylated state. Importantly, although demonstration of association with hydroxylases and the detection of hydroxylated peptides indicate potential sites of action within this pathway, they do not definitively prove that enzymatic hydroxylation has taken place. Indeed, it is likely that nonenzymatic oxidation of proteins also occurs. Future studies will be aimed at deciphering spurious oxidations from enzymatic hydroxylations and identifying which hydroxylation(s) is functionally associated with altered IL-1β–induced NF-κB activity. Based on this data, we propose that hydroxylation is a key posttranslational modification in the IL-1β pathway. This may have important implications for the use of hydroxylase inhibitors in a number of inflammatory disorders.

Discussion

Hypoxia is a microenvironmental feature in chronically inflamed tissues due to increased metabolic activity and disrupted perfusion leading to increased oxygen demand and decreased oxygen supply at the inflamed site (1). Furthermore, hypoxia-sensitive pathways such as HIF and NF-κB, which are under the control of oxygen-sensing hydroxylases, drive proinflammatory responses in macrophages, T cells, dendritic cells, and neutrophils (1–3). Based on these observations, it would be predicted that pharmacologic hydroxylase inhibition in vivo would promote inflammation. However, a number of recent studies have somewhat paradoxically demonstrated a profoundly anti-inflammatory effect of hydroxylase inhibition in multiple models of acute and chronic inflammation (10, 11, 15, 32). In the current study, we investigated the underlying mechanism(s) of anti-inflammatory action of hydroxylase inhibition with an aim to develop our understanding of the role of hydroxylases in regulating inflammatory signaling pathways and the potential for hydroxylase inhibitors as anti-inflammatory therapeutics. In contrast to its effect on basal NF-κB activity, we found that hydroxylase inhibition strongly reduced IL-1β–induced NF-κB activity in a manner that was dependent upon the combinatorial blockade of both PHD1 and FIH. This is consistent with data demonstrating activation of basal NF-κB but inhibition of lipopolysaccharide (LPS)-induced NF-κB in models of sepsis (15). The regulation of a hypoxia-sensitive pathway by combinatorial activity of a prolyl hydroxylase together with FIH has been previously reported in HIF signaling where inhibition of PHD2 and FIH leads to optimal HIF-dependent transcriptional activity (4). We describe a comparable combinatorial role for a prolyl hydroxylase and an asparaginyl hydroxylase (in this case PHD1 and FIH) conferring optimal oxygen sensitivity upon the IL-1β–signaling pathway.

The mechanism underpinning the beneficial impact of hydroxylase inhibition on complex inflammatory pathways in vivo remains incompletely understood (11, 15, 32). A role for IL-1β in the pathogenesis of IBD has been implicated as it is increased in the diseased tissue and amplifies NF-κB activity, leading to an increase of the secretion of inflammatory mediators, the recruitment of inflammatory cells, and the secretion of enzymes such as matrix metalloproteinases (33, 34). Therefore, the down-regulation of IL-1β–induced NF-κB activity reported likely plays a role in the beneficial effects of hydroxylase inhibitors in models of IBD (14, 35).

Previous reports have demonstrated that basal and TNFα-induced NF-κB activity, which is TRAF6 independent, is increased with hydroxylase inhibition (11, 32). Furthermore, the noncanonical NF-κB–signaling pathway, which is also TRAF6 independent, is unaffected by hydroxylase inhibition (36). However, LPS-induced NF-κB activity, which is TRAF6 dependent, is down-regulated (15, 37). We found that similar to LPS, IL-1β–induced NF-κB activity is down-regulated by hydroxylase inhibition. Additionally, TRAF6-induced NF-κB activity was also down-regulated in the presence of DMOG. Therefore, it appears that the effects of hydroxylase inhibition on NF-κB signaling is dependent upon the stimulus used. Overall these data indicate that the effect of hydroxylase inhibition might be a general effect for pathways using TRAF6 as a major part of their signaling cascade.

Table 3. Coimmunoprecipitation of components of the IL-1β signaling pathway with UBC13

Protein groups	Interacting proteins	IP UBC13
Proteins of the HIF pathway	PHD1	–
	PHD2	–
	PHD3	–
	FIH	–
	HIF-1α	–
	ARNT	–
Other known interactors	STUB1	+
	UEV2	+
Proteins of the IL-1β pathway	UBC13	+
	UEV1A	+
	OTUB1	+
	IκBβ	–

UBC13 was immunoprecipitated and the coprecipitants were analyzed using mass spectrometry. Previously described interactors served as controls, demonstrating effective coimmunoprecipitation. For further information, see Dataset S1.

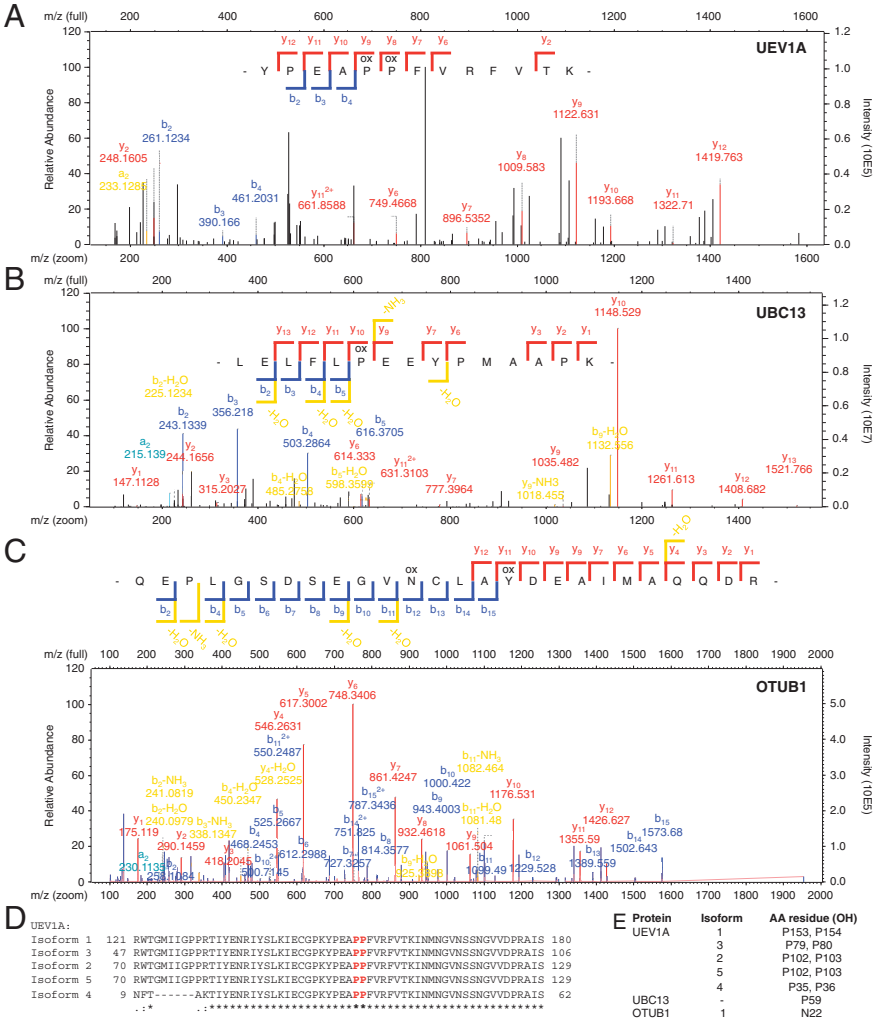

Fig. 4. Hydroxylation of UEV1A, UBC13, and OTUB1. Tandem mass spectrometric analysis shows hydroxylation of (*A*) UEV1A on two proline residues (data were obtained from searching a large-scale qualitative dataset downloaded from the Trance repository), (*B*) one proline residue in UBC13 in a FLAG-UBC13 immunoprecipitation, and (*C*) one asparaginyl hydroxylation in OTUB1 in a FLAG-HA-OTUB1 immunoprecipitation. (*D*) The hydroxylated proline residues of UEV1A identified in the large-scale qualitative dataset from the Trance repository are conserved over five isoforms (here shown as alignment using ClustalW2, www.ebi.ac.uk/Tools/msa/clustalw2). (*E*) Position of hydroxylated residues within the different proteins and their isoforms. AA residue (OH), hydroxylated amino acid residue.

We addressed the identification of putative functional hydroxylation sites in the IL-1β pathway, which may account for the effects of hydroxylases on IL-1β signaling. Using immunoprecipitation (IP) combined with mass spectrometric analysis, we identify UEV1A and OTUB1 as being associated with PHD1 and FIH, respectively, leading us to hypothesize that these may be sites of functional hydroxylation. Furthermore, we found that UEV1A and OTUB1 are also associated with UBC13, leading us to the hypothesis that PHD1, FIH, UEV1A, OTUB1, and UBC13 are part of one multiprotein complex in the IL-1β pathway. Investigating whether UEV1A is indeed a target for PHD1, we showed that hydroxylation occurred on two different proline residues with UBC13 and OTUB1 also being prolyl hydroxylated. Additionally, we also demonstrated that OTUB1 was hydroxylated on residues that are specific for FIH-dependent hydroxylation (N, D, and H) (29, 30). UEV1A and UBC13 together form the functional E2 ubiquitin-conjugating enzyme for TRAF6, whereas OTUB1 was reported to negatively regulate UEV1A-UBC13 function (25, 26). Furthermore, it has previously been reported that this complex signals through the TRAF6 ubiquitin ligase activity.

Downstream of the TRAF6 complex, we found IκBβ to interact with FIH and to be hydroxylated on an aspartate residue. Moreover, we showed that several proteins of the IL-1β–signaling pathway downstream of the TRAF6 complex are hydroxylated. Overall, these results demonstrate that hydroxylation occurs on multiple proteins in the IL-1β–signaling pathway and PHD1 and FIH are the main hydroxylase isoforms that regulate IL-1β–induced NF-κB activity.

The inhibition of IL-1β–induced NF-κB represents a mechanism by which hydroxylase inhibitors are anti-inflammatory in multiple models of IL-1β–driven inflammation. However, it is likely that other mechanisms also contribute to the protective effects of these drugs in vivo, such as expression of HIF-dependent epithelial barrier protective genes or hydroxylase-dependent regulation of intestinal epithelial cell death by apoptosis in inflammatory bowel disease (1). Further general mechanisms implicated in the anti-inflammatory activity of hydroxylase inhibitors, which may complement the inhibition of IL-1β signaling include the regulation of regulatory T-cell abundance (via FoxP3) and the promotion of adenosine signaling (38, 39).

In summary, pharmacologic hydroxylase inhibition represents a possible approach to anti-inflammatory therapy. In the current

paper, we provide evidence that a key aspect of the mechanism of anti-inflammatory action of hydroxylase inhibition is via suppression of IL-1β–induced NF-κB–dependent gene expression. Developing our understanding of the cross-talk, which exists between oxygen-sensing and inflammatory pathways will promote our understanding of how the microenvironment contributes to the development of inflammation and allow the development of unique approaches to its control.

Materials and Methods

Cell Culture and Plasmids. Unless otherwise indicated, HeLa cells were used for cell culture experiments under standard conditions.

The plasmids coding for FLAG-UBC13 and FLAG-HA-OTUB1 were obtained from Addgene [FLAG-UBC13: plasmid 12460 (40) and FLAG-HA-OTUB1: plasmid 22551 (41)].

Mass Spectrometric Analysis of Hydroxylated Proteins. We identified the UEV1A hydroxylation by searching a large-scale qualitative dataset (28) downloaded from the Trance repository against a human database including met, pro, trp, tyr, asp, asn, and his oxidations as possible modifications. These data originated from HeLa cells that were grown in normoxic conditions and lysed and proteins were digested with LysC, trypsin, or GluC. Higher-energy collisional dissociation (HCD) MS/MS Spectra were searched with the MaxQuant versions 1.2 and 1.3.

UBC13 and OTUB1 hydroxylations were detected in immunoprecipitated FLAG-UBC13 or FLAG-HA-OTUB1 samples using a Q-Exactive mass spectrometer (Thermo Scientific) and searching with the MaxQuant with met, pro, trp, tyr, asp, asn, and his oxidations as possible modifications. For the identification of isoform-specific hydroxylations, peptide sequences containing the hydroxylated amino acid were cross-checked with the Uniprot database (www.uniprot.org). All other Materials and Methods are described in *SI Materials and Methods*.

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