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Prolyl hydroxylase-1 regulates hepatocyte apoptosis in an NF-κB-dependent manner.

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Running title: PHD1 regulates apoptosis via NF-kappaB.

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Abstract

Hepatocyte death is an important contributing factor in a number of diseases of the liver. PHD1 confers hypoxic sensitivity upon transcription factors including the hypoxia inducible factor (HIF) and nuclear factor-kappaB (NF-κB). Reduced PHD1 activity is linked to decreased apoptosis. Here, we investigated the underlying mechanism(s) in hepatocytes. Basal NF-κB activity was elevated in PHD1−/− hepatocytes compared to wild type controls. ChIP-seq analysis confirmed enhanced binding of NF-κB to chromatin in regions proximal to the promoters of genes involved in the regulation of apoptosis. Inhibition of NF-κB (but not knock-out of HIF-1 or HIF-2) reversed the anti-apoptotic effects of pharmacologic hydroxylase inhibition. We hypothesize that PHD1 inhibition leads to altered expression of NF-κB-dependent genes resulting in reduced apoptosis. This study provides new information relating to the possible mechanism of therapeutic action of hydroxylase inhibitors that has been reported in pre-clinical models of intestinal and hepatic disease.

Keywords: prolyl hydroxylase, NF-κB, apoptosis, hypoxia.
Introduction

The chemical reduction of molecular oxygen is central to eukaryotic metabolism. Hypoxia therefore represents a significant threat to bioenergetic homeostasis and cell survival. Eukaryotic cells evolved an adaptive transcriptional response to hypoxia which is mediated primarily by the hypoxia inducible factor (HIF) [1]. The enzymes responsible for conferring hypoxic sensitivity upon HIF are termed prolyl hydroxylases (PHDs) [1].

A second key hypoxia-sensitive transcription factor is NF-kappaB (NF-κB). In unstimulated cells, NF-κB is retained in the cytoplasm by IkappaB (IκB) proteins [2]. Upon stimulation of cells by pro-inflammatory signals, IκB is phosphorylated by IκB kinase (IKK) targeting it for proteasomal degradation. Liberated NF-κB dimers then translocate to the nucleus and regulate transcription of a range of genes including a number associated with the regulation of apoptosis [3]. Hypoxia regulates NF-κB activity in a hydroxylase-dependent manner although this is a complex relationship. In the basal state, hydroxylase inhibition induces a low level activation of NF-κB, whereas in cells stimulated with Interleukin-1beta (IL-1β), hydroxylase inhibition suppresses NF-κB [4, 5].

Pharmacologic hydroxylase inhibition is protective in a number of models of intestinal inflammation [6-13]. Based on experiments utilizing isoform-specific PHD knock-out mice, it was shown that knock-out of PHD1 (but not PHD2 or PHD3) is responsible for protection against the development of colitis through inhibition of apoptosis in epithelial cells and consequent improvement in intestinal barrier function [14]. Hepatocytes also express functional PHD1 and PHD1 knock-out mice are protected against liver ischemia/reperfusion injury in a manner which is associated with reduced hepatocyte apoptosis [15]. However, the molecular mechanisms linking PHD-1 activity and apoptosis remain unclear and are the topic of this study.
Materials and Methods

Reagents. All chemicals were obtained from Sigma Aldrich except DMOG (Cayman Chemicals). FLAG-EGLN2 was from William Kaelin (Harvard Medical School). FLAG-IKKβ was from Warner Greene (Addgene plasmid #23298) [16]. HIF HepG2 cells were from Bernhard Brüne (University of Frankfurt).

Cell culture. Primary hepatocytes, derived from wild type or PHD1−/− Swiss/129 mice, Caco-2, HepG2 and HEK293 cells were cultured using standard conditions.

Site directed mutagenesis. The P191A-IKKβ mutant was generated using QuikChange Lightning mutagenesis kit (Agilent Technologies). Forward and reverse primers for IKKβ were 5’-CCCTGC AGTACCTGGCCGCAGAGCTACTGGAGC-3’ and 5’-GCTCCAGTAGCTCTGCGGCCAGGTACTGCAGGG-3’, respectively.

Transient transfections. HEK293 cells were transfected using 1 μg DNA in 1 μL/mL Lipofectamine 2000 (Life Technologies). HepG2 cells were transfected with siRNA (ON-Target Plus #L-004277-00-0010 and #D-001810-01-05, Dharmacon) using 5 μL/mL Trans-IT X2 (Mirus Bio LLC, Medison, WI, USA) in culture medium.

Immunohistochemistry. 4 μM sections of tissue were cut from paraffin blocks, deparaffinised and rehydrated using standard protocols. Sections were treated with proteinase K (20 μg/mL in 10mmol/L Tris-HCl, pH 7.5) for 15 minutes at RT before quenched with 3% hydrogen peroxide solution for 10 minutes, blocked using 5% normal goat serum in PBS containing 0.1% Triton X-100 (PBST) for 1 hour at RT and incubated with a primary anti-PHD1
antibody (Epitomics, Burlingame, CA) in 2% goat serum in PBST at 4 °C overnight. Slides were stained with a biotinylated goat anti-rabbit antibody (Vector Laboratories, Peterborough, UK) and developed using 3,3’-diaminobenzidine. Images were acquired using an Aperio Scanscope XT digital slide scanner.

**In vitro transcription/translation.** A rabbit reticulocyte-based transcription/translation system was used as per manufacturer’s instructions (Promega) using cDNA of FLAG-tagged PHD-1 cloned into pcDNA3 vector or the empty vector alone as template as indicated.

**Mass spectrometry.** N-terminally biotinylated synthetic peptides of GSLATSFVGTLQYLAPELLEQQKY (GL Biochem, Shanghai) were incubated with the complete reaction mixture from the *in vitro* transcription/translation assay as described previously [17]. Peptides were then isolated using monovalent streptavidin resin (Amersham), eluted, desalted and subjected to reverse-phase liquid chromatography coupled to mass spectrometry (LC-MS/MS) as described in [5]. The IKKβ peptide containing the Proline 191 residue was quantified by comparing the XIC of the threefold charged modified peptide and unmodified peptides using a window of 0.02 Da. Normalization of hydroxylated peptide was performed by dividing the intensity of the modified peptide by the matching unmodified peptide.

**In vitro decarboxylation assay.** The assay was performed as described previously [18].

**Chromatin immunoprecipitation sequencing (ChIP-Seq).** Libraries were prepared from immunoprecipitated chromatin and sequence analysis was performed on an Illumina HiSeq 2000 (GATC-Biotech AG, Konstanz, Germany). Sequences were mapped to NCBI mouse
genome (mm9). Reads were aligned with BWA aligner [19]. For peak calling, FindPeaks version 4.0 was used with standard parameters for control/case with a p-value cut-off of 0.01 [20]. The overlaps of the peaks were analysed using the R/bioconductor package ChIPpeakAnno [21, 22]. When computing the overlaps between different subunits, the maximum gap allowed between the peaks was 100 base pairs. For the same subunit no gap was permitted. Raw data can be sourced using the following URLs for (1) p50 binding in PHD1-/− cells, (2) p50 binding in WT cells, (3) p65 binding in PHD1-/− cells and (4) p65 binding in WT cells respectively:

1) http://titin.ucd.ie/~tom/susan/p50.allsample.bed
2) http://titin.ucd.ie/~tom/susan/p50.allcontrol.bed
3) http://titin.ucd.ie/~tom/susan/p65.allsample.bed
4) http://titin.ucd.ie/~tom/susan/p65.allcontrol.bed

Corresponding peak height information can be found at:

1) http://titin.ucd.ie/~tom/susan/p50.allcontrol.peaks
2) http://titin.ucd.ie/~tom/susan/p50.allsample.peaks
3) http://titin.ucd.ie/~tom/susan/p65.allcontrol.peaks
4) http://titin.ucd.ie/~tom/susan/p65.allsample.peaks

**Real-time quantitative PCR.** Total RNA was isolated, analysed and transcribed into cDNA as described in [23]. PCR reaction was performed using the apoptosis specific human TaqMan Array Microfluidic Card #4378716 and ABI Prism 7900HT Sequence Detection System according to manufacturer instructions (Applied Biosystems). Expression levels were calculated by the delta delta Ct (ΔΔCt) method using β-Actin as selected endogenous control.
**Immunobloting.** Analyses were carried out as detailed previously [24]. Primary antibodies used are summarized in Table 1.

**NF-κB luciferase reporter assay.** Luciferase reporter assays were carried out as described previously [4, 5].

**Flow Cytometry.** HepG2 cells were incubated with YO-PRO-1® and propidium iodide (Membrane Permeability/Dead Cell Apoptosis Kit, Invitrogen (Thermo Fisher Scientific, Waltham, MA USA) and subjected to flow cytometric analysis using the BD Accuri C6 flow cytometer (BD Bioscience, Oxford, UK) and CFlow software (version 1.0.264.21). 15000 cells were analyzed per sample and a minimum of three replicates per experiments were performed.

**Statistical Analysis** Data are presented as mean±SEM for n≥3 independent experiments. Statistical significance was evaluated by using one-way ANOVA or Student’s t test for unpaired or paired data, respectively. Asterisks correspond to P≤0.05.
Results

NF-κB activity is elevated in PHD1⁺/- hepatocytes

Immunohistochemical analysis of liver sections from wild type (WT) and PHD1⁺/- mice revealed nuclear enrichment of phosphorylated p50 in tissue from PHD1⁺/- mice compared to WT suggesting that PHD1 depletion leads to the activation of NF-κB in hepatocytes (Figure 1A). A potential target for PHD-dependent hydroxylation in the NF-κB pathway is IKKβ as it contains an LxxLAP hydroxylation motif similar to those found in HIFα subunits between amino acids 186 and 191 [4, 18]. This suggests that proline 191 (Pro191) could be a target for functional hydroxylation [4]. Rabbit reticulocyte-translated PHD1 was incubated with a synthetic peptide containing the putative hydroxylation motif of IKKβ. Mass spectrometric analysis revealed increased levels of proline hydroxylated peptide in the samples incubated with PHD1-reticulocyte lysates compared to those incubated with control lysates (Figure 1B). While peptides incubated in control lysates were predominantly in the non-hydroxylated state (Figure 1C), peptides incubated with lysates containing PHD1 showed two fractions of comparable abundance (Figure 1D). One of these fractions was identified as peptide hydroxylated on the proline residue corresponding to Pro191 of IKKβ. Mass spectrometric analysis also revealed that incubation with reticulocyte lysate resulted in the oxidation of the N-terminal affinity label biotin. In the presence of PHD1 we additionally detected the hydroxylation of Pro191. (Figure 1B,D).

These in vitro data support the possibility that Pro191 of IKKβ is a target for hydroxylation by PHD1. It is noteworthy that Pro191 hydroxylation was detected in samples incubated in the absence of PHD1 (Figure 1B) suggesting either that this site is prone to non-enzymatic oxidation or modified by other hydroxylases in the rabbit reticulocyte lysate.

To investigate whether PHD1 alone is sufficient to catalyze IKKβ hydroxylation, we next incubated IKKβ peptides with purified PHD1 in a cell-free reaction mixture and performed a
decarboxylation assay. Purified full length HIF-1α was used as a positive control substrate for PHD1. In contrast to the experiment in which peptides were incubated with whole reticulocyte lysates, peptide hydroxylation was not consistently detected in the lysate-free hydroxylation assay (Figure 1F). This may reflect the requirement for additional co-factors (present in the reticulocyte lysate) which are required for the PHD1-mediated IKKβ hydroxylation but not for HIF-1α hydroxylation. While identifying such factor(s) is beyond the scope of the current manuscript, future studies will be directed towards investigating this important question.

We next investigated a possible functional role for Pro191 in NF-κB signaling. We generated a P191A IKKβ mutant and compared its NF-κB activating potential to WT IKKβ. Overexpression of WT IKKβ reduced basal levels of IκBα and increased p65 phosphorylation on serine 536 indicating enhanced NF-κB signaling (Figure 2A) [25]. In cells stimulated with TNFα, IκBα degradation and Ser536 phosphorylation of p65 were increased further when WT IKKβ is overexpressed (Figure 2A and 2B). In contrast, the P191A IKKβ failed to exert similar effects on NF-κB pathway (Figure 2A and 2B). Overexpression of WT IKKβ also resulted in robustly elevated basal NF-κB activity (as measured by luciferase reporter assay) compared to mock-transfected cells and this activation was refractive to further stimulation by TNFα while the P191A mutant failed to increase basal NF-κB activity and rendered cells unresponsive to TNFα stimulation (Figure 2C). These data support the hypothesis that proline 191 of IKKβ is important for the catalytic activity of the kinase. Thus, its PHD1-mediated hydroxylation may affect its critical role in the signal transduction of the canonical NF-κB pathway.

**PHD1 knockout changes the DNA binding pattern of NF-κB in primary hepatocytes**
We next employed chromatin immunoprecipitation combined with DNA sequencing (ChIP-seq) to determine the distribution of binding sites of the p50 and p65 subunits of NF-κB throughout the genome. We found elevated numbers of binding sites for both p50 and p65 in chromatin from primary hepatocytes derived from PHD1−/− mice compared to WT controls (Figure 3A). We identified 3445 and 6945 unique binding sites for p50 and p65, respectively, in PHD1−/− cells compared with 61 and 584 unique binding sites for p50 and p65, respectively, in WT cells. The average relative distances of these binding sites from the nearest transcription factor start site was not significantly different between the wild type and the PHD1−/− (Figure 3B). Further analysis identified differential NF-κB binding to chromatin close to a cohort of genes known to be involved in the regulation of apoptosis (Figure 3C-H). In some cases, e.g. TRAIL, different peak heights were observed between WT and PHD1−/− cells, indicative of the differential degree of binding. In other cases (e.g. CASPASE-3, CASPASE-6, BIRC2, BIRC4 and FADD) unique binding sites were identified in PHD1−/− cells. It is noteworthy, that unique binding sites for p50 were frequently found in genes considered to be pro-apoptotic whereas p65 was more often enriched proximal to anti-apoptotic genes (Figure 3C-H).

In order to take an alternative approach to investigating the role of PHD1 in regulating apoptotic gene expression, we next carried out a quantitative PCR-based screening array of a panel of apoptosis-related genes. Using this approach, we identified a cohort of pro-apoptotic genes that are downregulated in HepG2 cells in which PHD1 was knocked down using siRNA compared to mock transfected controls (Table 1). These included critical elements of the intrinsic apoptotic pathway e.g. APAF1, DIABLO or PUMA. Interestingly, a number of caspase encoding genes were also found to be down-regulated as well as elements of the NF-κB pathway itself including the IKKγ and ε subunits, the β and ζ isoforms of the IκB and the TNF-related apoptosis inducing ligand (TRAIL) receptor 1 and 2. These data indicate the
genome-wide relocation of p50 and p65 in response to inhibition of PHD1 results in
differential expression of genes involved in the regulation of apoptosis. Overall, these
findings suggest that gene expression is biased toward an anti-apoptotic / pro-survival state
upon the inhibition of PHD1 in hepatocytes.

**Pharmacological PHD inhibition promotes hepatocyte survival.**
To test whether hydroxylase inhibition protects hepatocytes by reducing the rate of apoptosis
in an NF-κB-dependent manner, we exposed HepG2 cells to TRAIL in the presence or
absence of the hydroxylase inhibitor DMOG and Bay-117082 which inhibits TNFα-induced
NF-κB activity in HepG2 cells (Figure 4A) [26]. TRAIL profoundly increased apoptosis in a
manner that was partially reversed by DMOG treatment. The protective effects of DMOG
were reversed by Bay-117082 indicating that NF-κB signaling mediates the anti-apoptotic
effect of hydroxylase inhibitors in hepatocytes (Figure 4B and 4C). To investigate whether
there was also a role for HIF1 or HIF2, we investigated whether the anti-apoptotic effect of
DMOG is lost in HIF^-/- cells. RNA interference-mediated knock-down of either HIF-1 or HIF-
2α (Figure 4D) had no significant impact on the anti-apoptotic effects of DMOG (Figure 4E
and 4F). These data indicate that the anti-apoptotic effects of the hydroxylase inhibition are
independent of the HIF pathway and due instead to the activation of NF-κB.
Discussion

Recent work has shown that protein hydroxylation is not restricted to HIFα subunits and that components of other pathways such as NF-κB may also be functional targets for hydroxylases [4, 5, 27-29]. Consistent with these studies, we identified a global increase in NF-κB binding to chromatin in PHD1−/− hepatocytes. This may explain the observed anti-apoptotic effects of hydroxylase inhibition.

Protective effects of PHD1 knockout, due to decreased apoptosis, have been reported in models of colitis and liver ischemia/reperfusion injury [14, 15]. Furthermore, PHD1−/− mice display decreased cardiomyocyte apoptosis and are protected against ischemia reperfusion injury [15, 28, 30]. However, the molecular mechanisms underpinning the link between hydroxylase inhibition and apoptosis is unclear.

In this manuscript, we propose a model whereby PHD1 promotes apoptosis by suppressing NF-κB activity. PHD1 inhibition prevents apoptosis by increasing the activity of IKKβ leading to phosphorylation and degradation of IκB with a resultant increase in NF-κB which upregulates anti-apoptotic genes. Although our data indicate that the IKKβ may be a bona fide target for PHD1, whether this occurs in vivo and is regulated by hypoxia remains to be determined [4, 18].

The dominant-negative behaviour of the P191A mutant supports the concept that Pro191 is important for the catalytic activity of IKKβ. It was previously demonstrated that the intact C-terminal IKK complex-related phosphor-acceptor site of p105 is a prerequisite for the TNFα-responsive degradation of p105 and the consequent formation of the p50-containing trans-activator NF-κB complexes [31]. Since Pro191 is located in the close proximity of the kinase regulatory loop of IKKβ, PHD1-mediated hydroxylation of Pro191 on IKKβ may determine the composition of NF-κB dimers generated and, consequently, the functional outcome of the activation of the pathway. This raises the question of whether hydroxylation of Pro191
represents a molecular switch directing altered NF-κB-dependent biological responses upon hypoxia.

Although these mechanistic questions need further investigation, we hypothesize that the PHD1-mediated regulation of the NF-κB pathway promotes anti-apoptotic processes that underpins part of the protective effects of hydroxylase inhibition in a range of inflammatory and ischemic diseases. In intestinal epithelial cells, this may result in an enhancement of the barrier function reflected by protection against the initiation and development of colitis while in hepatocytes it may contribute to the observed resistance to various pro-apoptotic stimuli. Our current data provide additional mechanistic insight into the potential therapeutic usefulness of hydroxylase inhibitors.
Acknowledgements

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Disclosures:

The authors have no disclosures to declare.
References


the PHD1 prolyl hydroxylase protects livers of mice against ischemia/reperfusion injury, Gastroenterology, 138 (2010) 1143-1154 e1141-1142.


Figure legends

**Figure 1. Hydroxylase inhibition activates NF-κB.** (A) Immunohistological staining of phospho-p50 in liver sections from wild type (WT) and PHD1−/− mice. Representative images from 4 animals. (B-E) Mass spectrometric analysis reflecting hydroxylation of a synthetic peptide corresponding to the putative hydroxylation site of IKKβ following exposure to either control or PHD1-translated rabbit reticulocyte lysates. (F) Cell-free decarboxylation assay using synthetic IKKβ peptides or full length HIF-1α and purified PHD1.

**Figure 2. Pro191 mutation diminishes IKKβ-dependent NF-κB activity.** (A) Cells were transfected with empty vector, WT IKKβ or P191A IKKβ, stimulated with TNFα (0-30 mins) and markers of NF-κB activity were assessed by western blot. Representative blots of 3 independent experiments are shown. (B) Normalized densitometry results of quantitative p65 phosphorylation. (C) Normalized basal and TNFα-induced NF-κB-luciferase reporter activity in cells transfected with empty vector, WT IKKβ or P191A IKKβ (n=3).

**Figure 3. PHD1 removal alters the DNA-binding pattern of NF-κB in hepatic tissues.** (A) A Venn diagrams displaying the numbers of unique and shared p50 (upper panel) and p65 (lower panel) binding sites in hepatocytes derived from wild type and PHD1−/− mice. (B) Diagrammatic representation of distances of p50 and p65 binding sites from nearest known transcriptional start site (TSS) in hepatocytes derived from wild type (WT) and PHD1−/− mice. (C-H) The number of binding sites (represented by the individual bars in the histograms) and the peak height data (represented by the height of the bars) for p50 (left hand side) and p65 (right hand side) were compared between wild type (white bars) and PHD1−/− (black bars) hepatocytes for apoptosis-related genes as indicated in the figure.
Figure 4. Inhibition of PHD1 promotes hepatocyte survival. (A) NF-κB luciferase reporter activity in hepatocytes treated with TNFα (1 ng/ml) +/- Bay 11-7082 (10µM). Assessment of live (B) and apoptotic (C) cells treated with TRAIL, DMOG and Bay117802. (D) Knock-down of HIF1α and HIF2α protein in HepG2 cells. A representative immunoblot of n=3 independent experiments is shown. (E-F) HepG2 cells lacking either HIF1α or HIF2α were exposed to TRAIL (100ng/ml) and cycloheximide (CHX, 1µg/ml) for 3 hours following DMOG pre-treatment (1mM, 24h). The percentage of live (E) and apoptotic (F) cells were assessed by flow cytometry. Data represent mean ± SEM of 3 (A) or 4 (B and C) independent experiments (* p< 0.05, **p< 0.01, ***p> 0.0001, two way ANOVA, Bonferroni post-test for A, one way ANOVA, Tukey post-test for B, C, D and E).

Table 1. Summary of details of antibodies used in the study.

Table 2.
Summary of down-regulated apoptotic genes in PHD1−/− HepG2 cells (shown as mean fold change compared to cells transfected with non-targeting siRNA (n=4 independent experiments).
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B

Fold change of untreated pcDNA3-transfected sample

C

Normalized RLU
Highlights

- Genetic ablation of PHD1 upregulates NF-kappaB (NF-κB) in hepatocytes
- PHD1 blockade-mediated activation of NF-κB leads to differential DNA-binding of p50 and p65 and result in differential regulation of apoptotic genes
- We identified proline 191 in the beta subunit of the I-kappaB kinase as a target for PHD1-mediated hydroxylation.
- Blockade of prolyl-4-hydroxylases has been found cytoprotective in liver cells