Interferon-Gamma Induces Prolyl Hydroxylase (PHD)3 Through a STAT1-Dependent Mechanism in Human Endothelial Cells

Scott A. Gerber, Bogdan Yatsula, Cheryl L. Maier, Timothy J. Sadler, Laurence W. Whittaker, Jordan S. Pober

Objective—We previously reported that interferons (IFNs) regulate transcription of HIF-1α in human endothelial cells (ECs), linking immunity and hypoxia. Prolyl hydroxylases (PHDs) regulate expression of HIF-1α in response to hypoxia. We examined whether IFNs affect PHD expression and whether PHDs regulate the EC response to IFNs.

Methods and Results—Human cell cultures were treated with various cytokines, and PHD expression was examined using qRT-PCR and immunoblotting. IFNγ and, to a lesser extent, IFNα significantly induced PHD3, but not PHD1 or 2, mRNA, and protein expression selectively in ECs directly via a JAK/STAT1 pathway as demonstrated by pharmacological inhibition, siRNA knockdown, and chromatin immunoprecipitation. Inhibition of PHD activity with dimethylallyl glycine or desferroxamine reduced IFNg-dependent responses in these same cells.

Conclusions—IFNγ induces PHD3 through a JAK/STAT1-dependent mechanism in human ECs. Induction is independent of HIF-1α and may contribute to expression of IFNγ-dependent genes. (Arterioscler Thromb Vasc Biol. 2009;29:1363-1369.)

Key Words: IFNγ ■ signaling ■ endothelial cells ■ hypoxia ■ PHD3 ■ HIF-1α

Hypoxia activates transcription of genes necessary for adaptation to low oxygen.1,2 The best described response system uses hypoxia-inducible factors (HIFs) composed of the constitutively expressed subunit HIF-1β bound to labile subunits HIF-1α or HIF-2α, forming HIF-1 or HIF-2, respectively.3 HIF expression is regulated by a family of prolyl hydroxylases, PHD1, PHD2, and PHD3, that sense oxygen tension through its binding to an associated iron atom.4,5 When the PHD iron is occupied by O2, these enzymes catalyze a reaction in which one oxygen atom reacts with 2-oxoglutarate to form succinate and CO2 while the other is transferred to a proline residue in a protein substrate, such as HIF-1α, to form a hydroxyproline side chain. Hydroxylation of proline in HIF-1α recruits the von Hippel-Lindau (pVHL) complex, targeting HIF-1α for ubiquitination and proteasomal degradation.6-9 Molecular oxygen is normally rate limiting, and hypoxia causes HIF-1α protein stabilization and accumulation by inhibiting PHD-mediated proline hydroxylation. As its levels increase, HIF-1α enters the nucleus and dimerizes with HIF-1β to form active HIF-1, initiating transcription of genes that aid in adaptation to hypoxic conditions including enzymes that favor anaerobic glycolysis and factors that stimulate both angiogenesis and erythropoiesis.5,9 PHD3 transcription is induced by HIF-1 under hypoxic conditions through a functional hypoxic response element (HRE) located in the first intron of PHD3.10 HIF-1α-dependent induction of PHD3 most likely serves as a negative feedback mechanism during hypoxia, and may promote the rapid degradation of HIF-1α or HIF-2α on reoxygenation.6,11,12 Although most closely identified with the hypoxic response, PHDs may also regulate other molecular systems. PHD1 negatively regulates the NF-κB pathway by repressing the activity of the positive regulator, IKKβ, through hydroxylation in an oxygen-sensitive reaction.13 In rodents, PHD3 is required for normal neurological development through induction of neuron apoptosis.14,15 Loss of PHD3 impairs development of sympathetic neurons and may lead to the formation of pheochromocytomas.16 PHD3 interacts with activating transcription factor-4 (ATF-4) and negatively regulates the stability of this stress-induced protein in an oxygen-dependent manner.17 Finally, PHD3 has been shown to induce subcellular aggregation of proteasomal components that are similar to aggresome-like structures.18 These protein aggregates may activate apoptosis in certain cell types in an oxygen-dependent manner that requires PHD3 hydroxylase activity. Inhibition of PHD3 activity by hypoxia or pharmacological inhibitors, such as dimethylallyl glycine (DMOG), prevents PHD3-induced protein aggregation and subsequent apoptosis.

The interaction of the immune system and elements of the HIF system have recently been observed in several cell types.19,20 Cytokines that activate NF-κB may increase HIF-1α by driving increased transcription.21 We have re-
ently reported that IFNα, a type I IFN, may also increase HIF-1α in human endothelial cells (ECs) by activating its transcription through a Janus activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway involving the transcription factor interferon–stimulated gene factor (ISGF)3 (a complex consisting of STAT1, STAT2 and interferon response factor [IRF]9), and that this mechanism may contribute to the antiproliferative effects of this cytokine.22 Although IFNα primarily mediates antiviral effects, IFNγ, also known as type II IFN, primarily exerts immunomodulatory effects.23 In ECs, IFNγ enhances the expression of surface adhesion molecules and chemokines that promote effector memory T cell activation and trafficking.24 IFNγ also enhances antigen presentation to effector memory T cells by ECs through the upregulation of MHC Class I and II molecules as well as certain costimulators that selectively act on memory cells.25–28 IFNγ only minimally increases HIF-1α in ECs, mostly after prolonged treatment of 24 hours or more, and exerts its largest effects by potentiating the response to IFNα through induction of IRF9.23

ECs, like other cells, respond to IFNγ through the type II IFN receptor.29 On ligand binding, JAK2 is activated by autophosphorylation and, in turn, transphosphorylates and activates JAK1. Activated JAKs phosphorylate tyrosine residues in the receptor that promote binding and subsequent JAK-mediated tyrosine phosphorylation of STAT1. Phosphorylated STAT1 dissociates from the receptor, forming homodimers that translocate to the nucleus and bind Gamma interferon-activated sequence (GAS) elements to initiate transcription of IFN-stimulated genes (ISGs).29 Although IFNα acts through a different receptor, uses Tyk2 instead of JAK2, and principally activates ISGF3, it also may activate STAT1 homodimers, especially in ECs which display only limited expression of the SHP-2 tyrosine phosphatase that normally limits STAT1 signaling.30 Here we report that IFNγ (and to a lesser extent IFNα) induces the transcription of PHD3 in human ECs. This response depends on STAT1 and is independent of IRF9. Interestingly, IFNγ-mediated induction of PHD3 is selectively observed in ECs, is independent of HIF-1, and may contribute to the immunomodulatory actions of IFNγ on this cell type.

Methods

Cell Culture

Human cells were isolated from discarded anonymized tissues or from anonymized adult volunteer blood donors according to protocols approved by the Yale Human Investigation Committee. Human umbilical vein endothelial cells (HUVECs), human dermal microvascular endothelial cells, human aortic smooth muscle cells, human umbilical artery smooth muscle cells, human placental pericytes, and peripheral blood CD4+ T cells were isolated and cultured as previously described.22

Cytokines, Reagents, and Antibodies

Sources of cytokines, reagents and antibodies used in this study are listed in the supplemental Methods (available online at http://atvb.ahajournals.org).

Real-Time Quantitative RT-PCR

RNA was isolated from cells and mRNA was measured by real-time quantitative (qRT-PCR) of 5 μL of cDNA template using primers listed in supplemental Table I as described in supplemental methods.

siRNA Delivery to Cells

All siRNAs were from Qiagen and transduced via electroporation or Oligofectamine (Invitrogen) as described in supplemental methods.

Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation (ChIP) analysis was performed on IFNγ- or vehicle control-treated HUVECs as described in supplemental methods.

Induction of Hypoxia

HUVECs were plated on fibronectin (10 μg/mL)-coated glass plates and exposed to hypoxic conditions (<0.5% O2) in a ProOx nitrogen-induced hypoxia system (BioSpherix, Red Field, NY) for 6 hours before isolation of protein.

Immunoblotting

Protein lysates were subjected to SDS-PAGE and immunoblotted as previously described22 and presented in supplemental methods.

Statistical Analysis

Data are presented as mean±SE from a minimum of 3 replicates. Statistical analysis was performed using ANOVA for single and repeated measures with the Bonferroni or Dunnett posthoc test for comparisons of groups greater than two. Paired t tests were used when appropriate.

Results

IFNγ Induces PHD3 in ECs

We recently reported that IFNα can induce transcription of HIF-1α in cultured HUVECs.22 In the present study, we initially examined whether inflammatory cytokines known to act on ECs could alter expression of HIF-regulatory PHD molecules in HUVECs. Cells were treated with IL-1α (2 ng/mL), TNF (10 ng/mL), IFNα (100 ng/mL), or IFNγ (50 ng/mL) for 1.5 hours, and mRNA levels of PHD1, PHD2, and PHD3 were determined by qRT-PCR. These concentrations are optimal to induce new cytokine-dependent gene expression in HUVECs (Figure 1A). PHD3 mRNA was the least abundant prolyl hydroxylase at basal conditions (data not shown). None of these treatments altered the expression of PHD1 or PHD2 after 1.5 or 8 hours of cytokine treatment (Figure 1A and data not shown). IFNγ and, to a lesser extent, IFNα consistently induced PHD3 mRNA at 1.5 hours. Examination of mRNA levels at 8 hours showed continued IFNγ responses, but the IFNα effect was not sustained (data not shown). Interestingly, at the 1.5-hour time point IFNα and IFNγ stimulated the expression of PHD3 mRNA to a higher level than DFO, a potent inducer of PHD3 mediated via HIF-1 (Figure 1A), although DFO induced higher levels of PHD3 mRNA than IFNγ treatment at 8 hours (Figure 1A). We focused our subsequent studies on the IFNγ response.

We next examined whether IFNγ could induce PHD3 mRNA expression in other primary human cell types. All cells tested were responsive to IFNγ as indicated by IRF1 mRNA induction (Figure 1B). Although the basal levels of PHD3 mRNA differed among cell types, IFNγ treatment for 1.5 hours (Figure 1B) stimulated the expression of PHD3 mRNA to a small extent in human aortic smooth muscle cells, and much more so in human dermal microvascular endothelial cells and HUVECs. IFNγ treatment failed to induce PHD3 mRNA in human peripheral blood CD4+ T cells, human placental-derived pericytes, and human umbilical
artery smooth muscle cells. Additionally, IFNγ failed to significantly induce PHD1 and PHD2 mRNA in all cell types tested (data not shown). Thus the induction of PHD3 by IFNγ appears selective for ECs.

IFNγ induced PHD3 mRNA in a dose- (Figure 2A) and time- (Figure 2B) dependent manner. IFNγ stimulated a rapid increase in PHD3 mRNA levels (between 1 and 2 hours) that slowly decreased over time. Although PHD3 transcripts may exist in alternatively spliced forms,31,32 the primers used selectively identify the full-length active transcript, and this was confirmed by sequencing the PCR product amplified from IFNγ-treated HUVECs. We also detected an increase in the levels of a PHD3 protein of the expected size for the product of the full-length transcript by immunoblotting as early as 2 hours of IFNγ treatment, and these levels remained constant throughout the time course (Figure 2c). Protein isolated from HUVECs exposed to 6 hours of hypoxia served as a positive control for PHD3 induction. The effects of IFNγ on protein levels appeared smaller than that observed in response to hypoxia but were highly reproducible.

**IFNγ-Induced PHD3 Is an Immediate Early Gene**

We next investigated the mechanism by which IFNγ induces the expression of PHD3 in ECs. Pretreatment of HUVECs with the transcription inhibitor DRB (50 μmol/L) blocked the induction of PHD3 mRNA (Figure 3A) and protein (Figure 3B) after IFNγ treatment, indicating a requirement for transcription. Additionally, the stability of PHD3 mRNA was examined and found to be unchanged in cells treated with vehicle control or IFNγ for 2 hours followed by the addition of DRB (data not shown). Treatment with the protein synthesis inhibitor CHX (10 μg/mL) did not alter the PHD3 mRNA levels after IFNγ stimulation (Figure 3B). These data suggest that the induction of PHD3 mRNA by IFNγ is dependent on transcription but does not require new protein synthesis, fitting the definition of an immediate early response gene.

**IFNγ-Expressed PHD3 Is Dependent on JAK/STAT Signaling and STAT1**

IFNγ transcribes immediate early genes predominately through a JAK/STAT1 signaling pathway. To determine the role of JAK/STAT signaling on PHD3 induction, cells were pretreated with different doses of JAK inhibitor 1, a pharmacological inhibitor of JAK enzymes, followed by the addition of IFNγ or vehicle control. As a positive control, 2 known immediate early IFNγ-induced genes, IP-10 and IRF1, were suppressed after treatment with this inhibitor (data not shown). Figure 4A illustrates that JAK inhibitor 1 reduced IFNγ-induced PHD3 mRNA expression in a dose-dependent manner supporting a role for JAK signaling on PHD3 induction.

The transcription factor involved in immediate early responses to IFNγ signaling is typically a STAT1 homodimer. We examined whether IFNγ-induced PHD3 was dependent on STAT1. siRNA against STAT1 was effective in reducing...
STAT1 protein as assayed by immunoblotting (Figure 4B) and inhibited the expression of IRF1 mRNA (data not shown), as well as induction of PHD3 after IFNγ stimulation (Figure 4C).

To determine whether STAT1 directly regulates the transcription of PHD3, we identified 4 putative STAT1-binding sites within the 9.0-kb 5’ promoter region of the PHD3 (Figure 4D, top) and then examined whether STAT1 was bound to these sequences in HUVECs treated with IFNγ or vehicle control using ChIP. Figure 4D shows that the DNA sequences 760 and 4678 (relative to the transcriptional start site) bound STAT1 after IFNγ treatment but not in untreated cells. Putative STAT1 sites at positions −8076 and −5840 failed to demonstrate STAT1 binding after either IFNγ or vehicle control treatment.

Type I IFNs (IFNα/β) signal predominately through the ISGF3 complex that comprises IRF9, STAT1, and STAT2 and binds to interferon-stimulated response elements (ISREs). Our laboratory has previously reported that IFNγ can transiently induce the formation of STAT1 homodimers in HUVECs but not HeLa cells, likely because of lesser expression of SHP-2 in ECs. To evaluate whether the weak and transient response of PHD3 in HUVECs in response to IFNα (Figure 1A) is mediated by a STAT1 homodimer, we used siRNA against IRF9 or STAT1 to inhibit the formation of ISGF3 and evaluated PHD3 mRNA levels after IFNγ or IFNα treatment. siRNA knockdown of IRF9 resulted in a 75% reduction of IRF9 mRNA (supplemental Figure Ia) and inhibited the induction of an ISGF3-dependent gene, viperin (supplemental Figure Ib), but had no effect on the STAT1 homodimer–driven gene, IRF1 (supplemental Figure Ic) or the induction of PHD3 in either IFNγ- or IFNα-treated ECs (supplemental Figure Id). Knockdown of STAT1 abrogated the induction of PHD3 in IFNα-treated ECs (data not shown). These data indicate that both IFNγ and IFNα can induce PHD3 via a STAT1 homodimer-dependent mechanism.

IFNγ-Induced PHD3 Does Not Require HIF-1 nor Affects its Stability During Normoxia and Hypoxia

PHD3 transcription may be induced by HIF-1, serving as a negative regulator of HIF-1α during periods of hypoxia. We therefore explored the possibility that HIF-1 was a transcription factor that could contribute to the induction of PHD3 by IFNγ. Our previous data had demonstrated that IFNγ failed to induce HIF-1α at early time points, but was effective in increasing HIF-1α protein levels at later periods. Because PHD3 is strongly induced as early as 1.5 hours
after IFN treatment, it seemed unlikely that HIF-1α plays a role in the IFNγ response. Nevertheless, a siRNA approach was used to determine whether knockdown of HIF-1α protein altered the ability of IFNγ to induce PHD3. siRNA knockdown of HIF-1α was consistently more than 90% effective (Figure 5A), and suppressed hypoxia-induced HIF-1α protein (Figure 5B) resulting in the abrogation of PHD3 protein induction during periods of hypoxia (Figure 5C). However, HIF-1α knockdown had no effect on IFNγ-mediated induction of PHD3 (Figure 5D). Similarly siRNA knockdown of HIF-1β strongly inhibited induction of HIF-1–dependent genes under hypoxic conditions, but had no effect on IFNγ-mediated induction of PHD3 during normoxia (supplemental Figure II).

PHD3 may promote the rapid degradation of hypoxia-induced HIF-1α during reoxygenation. We therefore tested whether IFNγ treatment during hypoxia would result in an increase of PHD3 when compared to hypoxia alone and whether such an increase of PHD3 would accelerate HIF-1α protein decay during reoxygenation. ECs were pretreated with IFNγ or vehicle control then subjected to hypoxia/reoxygenation. IFNγ treatment+hypoxia did significantly increase the levels of PHD3 when compared to vehicle control+hypoxia (supplemental Figure IIa), but did not alter the rate of HIF-1α protein decay during reoxygenation (supplemental Figure IIb). Additionally, IFNγ-induced PHD3 does not alter the basal level of HIF-1α protein during normoxia at early time points after IFNγ stimulation (supple-

Figure 4. IFNγ-induced PHD3 is dependent on JAK signaling and STAT1. A, Cells treated with JAK inhibitor 1 or DMSO before addition of 50 ng/mL IFNγ or vehicle (0 and 333 μmol/L; n=3). B and C, HUVECs were transfected with STAT1 or control siRNA. B, Immunoblot of STAT1 or β-actin. C, PHD3 mRNA levels in cells treated with IFNγ for 1.5 hours (n=3). D, ChIP analysis of 4 putative STAT1 binding sites upstream of the TSS as determined by qRTPCR (n=2). A representative agarose gel illustrating the resultant amplicons is provided. H2O was added in place of DNA to serve as a negative control. P<0.05; ANOVA/Bonferroni post test.

Figure 5. IFNγ induces PHD3 independently of HIF-1α. A and B, Protein levels of HIF-1α after HIF-1α or control siRNA-treated cells were stimulated with 50 ng/mL IFNγ (A) for 1.5 hours or hypoxia (0.5% O2) (B) for 5 hours (n=3). C, siRNA-treated cells were exposed to hypoxia or normoxia and PHD3 protein examined by immunoblot (n=2). D, siRNA-treated cells were stimulated with 50 ng/mL IFNγ or vehicle for 1.5 hours, and PHD3 mRNA was analyzed (n=3). P<0.05; ANOVA/Bonferroni post test.
DMOG, an Inhibitor of PHD Activity, Suppresses the Induction of IFNγ-Dependent Genes

Because PHD3 levels increase rapidly after IFNγ treatment, we examined whether IFNγ-induced PHD3 may affect IFNγ signaling and subsequent induction of IFNγ-dependent genes. ECs were treated with 1 mmol/L DMOG, a 2-oxoglutarate analog inhibitor that inhibits PHD activity, for 2 hours followed by stimulation with IFNγ or vehicle control for 2 hours, and mRNA levels of 6 known IFNγ-stimulated genes were determined (treatment with DMOG for periods over 12 hours was toxic for HUVECs, but cells appeared viable under conditions used in this study). The Table demonstrates that DMOG treatment significantly suppressed all of the IFNγ-inducible genes examined after IFNγ stimulation. DMOG had minimal effects on the basal levels of these genes with only IRF1 and TAP1 demonstrating small but statistically significant decreases. HUVECs treated with 250 μmol/L DFO, an iron chelator which also inactivates PHDs, for 4 hours followed by IFNγ treatment or vehicle control for 2 hours, yielded similar results as DMOG treatment (supplemental Table II). Our results suggest that PHD hydroxylase activity, likely from IFNγ-induced PHD3, contributes to optimal stimulation of IFNγ-dependent genes and thus to the immunomodulatory effect of this cytokine on ECs.

Discussion

Our data demonstrate that IFNγ can induce PHD3 and this effect is at least partly selective for human ECs. PHD3 behaves as an immediate early response gene, and IFNγ-induced transcription is activated by JAK/STAT1 signaling and not HIF. In an attempt to determine the function of this induction, 2 separate PHD inhibitors, DMOG and DFO, were both shown to suppress the induction of IFNγ-dependent genes in ECs consistent with the conclusion that PHD3 contributes to IFNγ signaling. In addition to the pharmacological inhibitors of PHDs, we treated ECs with IFNγ or vehicle control and subjected them to hypoxia (0.5% O2), which also inactivates PHD activity, in an attempt to assess what role this may have on IFNγ gene transcription. Hypoxic treatment of ECs did inhibit the expression of IFNγ-dependent genes, but it also inhibited gene expression in response to other cytokines that do not induce PHDs (S.G., unpublished observations, 2008). We have placed more emphasis on DMOG and DFO effects, which are more likely linked to PHD inhibition, as opposed to the global inhibitory effects we have observed with hypoxia.

While DMOG inhibits the hydroxylase activity of all PHDs, it could have PHD-independent effects. To confirm whether the suppression of IFNγ signaling was a result of inhibiting IFNγ-induced PHD3, we attempted to knock down PHD3 using siRNA. Unfortunately, we were unable to significantly reduce the levels of PHD3 mRNA and protein in HUVECs even though 5 separate previously validated siRNA sequences and 2 different means of siRNA delivery were attempted (S.G., unpublished observations, 2008). The reason for this technical failure is unclear. It is also unclear how prolyl hydroxylation may effect IFNγ-dependent gene transcription. PHD3 can be localized to both cytoplasm and nucleus and has been shown to bind and form complexes with other proteins6,34 and thereby stabilize (or destabilize) factors, such as coactivators, that may enhance IFNγ gene transcription. A related aspargynyl hydroxylase, factor inhibiting HIF-1 (FIH), functions to modulate recruitment of coactivators p300 and CBP to HIF-1.6 Further experiments are warranted to decipher the exact role PHD3 plays in IFNγ signaling.

Two splice variants exist for PHD3, one of which lacks the majority of exon 1 (PHD3Δ1), and the other is missing exon 4 (PHD3Δ4). PHD3Δ1 has been shown to lack hydroxylase activity, whereas PHD3Δ4 retains activity.31,32 PHD3Δ4 has only been described in malignant tissues. The PHD3Δ1 variant can be found in all tissues where wild-type PHD3 is also located. We were able to isolate both full-length PHD3 and PHD3Δ1 from HUVECs treated with IFNγ using DNA primers designed to detect all variants (S.G., unpublished observations, 2008). DNA sequencing confirmed these results. Interestingly, hypoxia also induced both forms of PHD3 in ECs. We do not know what role, if any, PHD3Δ1 plays during hypoxia or IFNγ stimulation.

In summary, we report here a further example of an interaction between immune and hypoxic responses, namely that IFNγ is capable of inducing transcription of PHD3 in human ECs. A physiological role for this phenomenon is suggested by the ability of PHD inhibitors to blunt the response to IFNγ.

Table. DMOG Treatment Inhibits IFNγ-Inducible Genes

<table>
<thead>
<tr>
<th>CXCL11</th>
<th>IDO</th>
<th>IP-10</th>
<th>IRF1</th>
<th>MIG</th>
<th>TAP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>DMOG</td>
<td>PBS</td>
<td>DMOG</td>
<td>PBS</td>
</tr>
<tr>
<td>Fold increase</td>
<td>1</td>
<td>0.78</td>
<td>1</td>
<td>0.58</td>
<td>1</td>
</tr>
<tr>
<td>% Control</td>
<td>78±12</td>
<td>58±13</td>
<td>109±32</td>
<td>49±4</td>
<td>108±46</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Fold increase</td>
<td>240</td>
<td>100</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>% Control</td>
<td>41±2</td>
<td>32±3</td>
<td>39±13</td>
<td>78±4</td>
<td>36±5</td>
</tr>
</tbody>
</table>

HUVECs were treated with PBS or DMOG (1 mmol/L) for 2 hours followed by vehicle or IFNγ for 2 hours. IFNγ-inducible genes were assessed by qRT-PCR and expressed as mean fold increase, or % of control (PBS) ± SE. n=3. ns indicates not significant. *P<0.05; t test.
Acknowledgments

We thank Dr Deepak Rao for providing T cells, and Dr George Tellides (New Haven, Conn., USA) for providing aortic SMCs. We thank Louise Benson, Gwen Davis-Arrington, and Lisa Gras for help with endothelial cell isolation and culture.

Sources of Funding

This work was supported by the National Institutes of Health (RO1-HL62188 [to J.P.], T32-AR7107 [to S.G.], and T32-A1071001930 [to S.G.], and an Anna Fuller Fund Fellowship [to S.G.]). T.J.S. and L.W.W. were supported by student fellowships from Trinity Hall, Cambridge University.

Disclosures

None.

References

Interferon-Gamma Induces Prolyl Hydroxylase (PHD)3 Through a STAT1-Dependent Mechanism in Human Endothelial Cells

Scott A. Gerber, Bogdan Yatsula, Cheryl L. Maier, Timothy J. Sadler, Laurence W. Whittaker and Jordan S. Pober

Arterioscler Thromb Vasc Biol. 2009;29:1363-1369; originally published online July 2, 2009; doi: 10.1161/ATVBAHA.109.192542

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/9/1363

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/08/21/ATVBAHA.109.192542.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
**Supplemental Materials and Methods:**

*Cytokines, reagents, and antibodies:*

Recombinant human IL-1α was from PeproTech (Rocky Hill, NJ). TNF and IFNα were from R&D systems (Minneapolis, MN). Recombinant human IFNγ was from Invitrogen Life Technologies (Carlsbad, CA). JAK inhibitor 1 was from Calbiochem (San Diego, CA). Dimethyloxallyl glycine (DMOG) was from Cayman Chemicals (Ann Arbor, MI). Cycloheximide (CHX), desferoxamine (DFO), 5,6-dichloro-1-β-D ribofuranosylbenzimidazole (DRB), and mouse anti-human β-actin were from Sigma-Aldrich (St. Louis, MO). Rabbit anti-human PHD3 (NB-100-303) was from Novus Biologicals (Littleton, CO). Rabbit anti-human STAT1 (sc-346) was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human STAT1 (#9172) was from Cell Signaling (Danvers, MA). Normal rabbit IgG was from BD Biosciences. Protein A agarose beads were from Millipore (Billerica, MA). HIF-1α was quantified using a Human/Mouse Total HIF-1α ELISA kit (R&D systems) as previously described 22.

*mRNA isolation and real-time quantitative RT-PCR:*

Cells were treated with the specified concentration of cytokine or vehicle control and either mRNA or total protein was isolated at the indicated times. Where needed,
pharmacologic inhibitors were added to the cultures at the indicated concentrations and minutes prior to treatment with cytokine. Total RNA was isolated using an RNeasy minikit (Qiagen, Valencia, CA) according to manufacture’s instructions and RNA quantified by a spectrophotometer. Equal amounts of input RNA was used to generate first strand cDNA using TaqMan reverse transcriptase reagents (Applied Biosystems, Foster City, CA) according to manufacturer’s protocol. Real-time quantitative (qRT-PCR) was performed on 5ul of cDNA template using nested primers designed to span introns and SYBR Green (Bio-Rad, Hercules, CA) master mix equaling 25ul. Samples were run on an Icycler IQ5 real-time detection system (Bio-Rad). All data were normalized using GAPDH as reference values and expressed as relative fold increases over vehicle control. Primer sequences used in this study are listed in Supplemental Table I.

**siRNA delivery to cells:**

All siRNAs were purchased from Qiagen. siRNA against control, HIF-1α, or IRF9 was delivered to cells via electroporation. Briefly, 1x10^6 cells were exposed to one round of electroporation using a HUVEC Nucleofactor kit (Amaxa, Gaithersburg, MD) with either 3 ug of siRNA of a non-binding control sequence or siRNA against HIF-1α or IRF9 as previously described 22. Cells were assayed for knockdown or used experimentally 15-24 hours later. siRNA against STAT1 and HIF-1β were delivered to cells using Oligofectamine (Invitrogen). Briefly, 1x10^5 cells were plated in 12 well plates (BD Biosciences, San Jose, CA) 24 hours before 2-3 rounds of oligofectamine transfection at either a final concentration of 20 nM in OptiMem for HIF-1β (target sequence:
AACCCGAGACTTGCCATAAA, catalog no. SI00304234 & target sequence: CCCAGCCAATATACAACTGTA, catalog no. SI00304227) or 80nM for STAT1 (validated target sequence proprietary; catalog no. SI02662884 & SI02662324). Cells were examined for knockdown or used experimentally 24 hours following the last round of transfection. Knockdown specificity for the desired protein and off-target effects were examined by testing 2-3 different siRNA sequences against the protein of interest, with similar outcomes resulting each time.

Chromatin immunoprecipitation analysis:
ChIP analyses were performed on IFNγ-treated and control-treated HUVECs (3 x10⁶). Following 1.5 h of treatment, the cells were crosslinked with 1% formaldehyde at room temperature for 10 min. Cells were then lysed with RIPA buffer on ice for 15 min. Chromatin was sheared by sonication (<700 bp) followed by centrifugation for 10 min. Supernatants were pre-cleared with protein A agarose beads for 1 h at 4 °C. Immunoprecipitation was performed on 500 ug of total protein with anti-STAT1 antibody (Cell Signaling, #9172) or control normal rabbit IgG at 4 °C overnight, and immune complexes were collected with protein A agarose beads. The samples were washed four times in RIPA buffer. DNA was eluted, purified, and subjected to quantitative PCR using primer pairs (Supplemental Table 1) specific to PHD3 promoter regions containing the putative STAT1-binding sites. PCR reaction was carried out as follows: denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 20 s. Resultant amplicons were run on an agarose gel, purified, and sequenced to confirm specificity.
Protein Isolation & Immunoblotting:

To isolate protein, semiconfluent cultures in tissue culture-treated polystyrene plates (BD Biosciences) were placed on ice, media removed, and washed with 5 mls of cold HBSS (Invitrogen). Lysis buffer (50 mM Tris (pH 7.4), 300 mM NaCl, 10% glycerol, 3 mM EDTA, 1 mM MgCl₂, 20 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100, 25 ug/ml leupeptin, 25 ug/ml pepstatin, and 3 ug/ml aprotinin) was added directly to plates and lysates collected using a cell scraper (BD Biosciences). Samples were vortexed and centrifuged at 10000 x g for 10 minutes at 4 degree C to remove debris. Total protein was quantified by a Bio-Rad protein assay kit. Twenty to 30 ug of total protein was loaded per lane, subjected to SDS-PAGE, and immunoblotted as previously described 22. Densitometry was performed using a Memorex 6142u scanner and analyzed by Image J software.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANGPTL4</td>
<td>TCTCCGTACCCTTCTCCACT</td>
<td>AGTACTGGGCCGTTGAGGTTG</td>
</tr>
<tr>
<td>CXCL11</td>
<td>CGCGATATGTAAGGAGTAGAAGGCT</td>
<td>AGCTTTATGGCAGAGAATTTC</td>
</tr>
<tr>
<td>E-selectin</td>
<td>AAATTGTGAAGCTGTACAGCC</td>
<td>CTGATAGAGCAGGAAGATGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAAGGTGAAGGTCGAGGTC</td>
<td>GAAGATGGTGATGGAGATTTC</td>
</tr>
<tr>
<td>IDO</td>
<td>GGACAATCAGTAAGAGATACCA</td>
<td>GGCAGATGTTTAGCAATGAA</td>
</tr>
<tr>
<td>IP-10</td>
<td>TGAATCAAAAAGTGGCTTCTTCTG</td>
<td>GTACAGCGTACAGTTCTAGA</td>
</tr>
<tr>
<td>IRF1</td>
<td>GATGATCTTCCAGATCCCAT</td>
<td>TCTTTCACCTCCTCAGATAC</td>
</tr>
<tr>
<td>IRF9</td>
<td>GATACTAAAGACCATTGTTC</td>
<td>CCCTCTTTATACCCCTCTAA</td>
</tr>
<tr>
<td>MIG</td>
<td>TCCACCTCAATCCTTGAAGA</td>
<td>TGCTGAATCTGGGTGAGCA</td>
</tr>
<tr>
<td>PHD1</td>
<td>ACGGGCTCGGGGTACGTAAG</td>
<td>CCCAGTTCTGATTCAGGTAATAGATAACA</td>
</tr>
<tr>
<td>PHD2</td>
<td>GACCTGATACGCCACTGTACG</td>
<td>CCCGGATAACCAAGACCAACCAT</td>
</tr>
<tr>
<td>PHD3</td>
<td>ATACTACGCAAGAGGAGGT</td>
<td>TCGACATCAAGTGACCA AGA</td>
</tr>
<tr>
<td>TAP1</td>
<td>CAAGAGCCACAGGTTATTTG</td>
<td>ACTGCAGCACGCTGTGATTTTC</td>
</tr>
<tr>
<td>Viperin</td>
<td>CTTTGTCGTGCCCCCTTGAGGAA</td>
<td>CTCTCAGGATACGGCTCTCAA</td>
</tr>
<tr>
<td>-760 (PHD3</td>
<td>CAAAGTCTTTCTCAGTGTTGT</td>
<td>TTCTGTAACAGTGACGGA</td>
</tr>
<tr>
<td>promoter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4678 (PHD3</td>
<td>CTTCCCATATTCTCTGTGG</td>
<td>CTCTCAAAGTGCGTGGGATTA</td>
</tr>
<tr>
<td>promoter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5840 (PHD3</td>
<td>TCGGCTTAATGAGAGCTGAG</td>
<td>CAGCTCTAAAAGGATGGCAA</td>
</tr>
<tr>
<td>promoter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-8076 (PHD3</td>
<td>CAGGTTTACCCCTGCTATT</td>
<td>CATCAGAAGTCTGACTCCAT</td>
</tr>
<tr>
<td>promoter)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Supplemental Table I** Primer pairs for qRT-PCR.
<table>
<thead>
<tr>
<th></th>
<th>CXCL11</th>
<th>IDO</th>
<th>IP-10</th>
<th>IRF1</th>
<th>MIG</th>
<th>TAP1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fold Increase</strong></td>
<td>Veh</td>
<td>DFO</td>
<td>Veh</td>
<td>DFO</td>
<td>Veh</td>
<td>DFO</td>
</tr>
<tr>
<td>Veh</td>
<td>1</td>
<td>.94</td>
<td>1</td>
<td>.85</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>IFNγ</td>
<td>402</td>
<td>188</td>
<td>579</td>
<td>166</td>
<td>404</td>
<td>188</td>
</tr>
<tr>
<td>% Control</td>
<td>-</td>
<td>95+/21ns</td>
<td>-</td>
<td>85+/17ns</td>
<td>-</td>
<td>552+/302ns</td>
</tr>
<tr>
<td>% Control</td>
<td>-</td>
<td>46+/6*</td>
<td>-</td>
<td>28+/2*</td>
<td>-</td>
<td>43+/12*</td>
</tr>
</tbody>
</table>

Supplemental Table II. DFO treatment inhibits IFNγ-inducible genes. HUVECs were treated with vehicle control or DFO (250uM) for 4 hours followed by vehicle control or IFNγ for an additional 2 hours. IFNγ-inducible genes were assessed by qRT-PCR and expressed as mean fold increase, or % of control +/- SE, where control is PBS treated cells. n=3. ns = not significant; * = p < 0.05. t-test.
**Supplemental Figure I: IFNs do not require IFR9 to induce PHD3.** HUVECs were electroporated with siRNA against IRF9 or a control sequence and stimulated with cytokines 15-20 hours later. Cells were treated with vehicle control, 50 ng/ml IFNγ, or 100 ng/ml IFNα for two hours and mRNA was analyzed by qRT-PCR for IRF9 (A), viperin (B), IRF1 (C), and PHD3 (D). Data pooled from three independent experiments.

**Supplemental Figure II: IFNγ induces PHD3 independently of HIF-1β.** HIF-1β mRNA expression in HUVECs treated with control or HIF-1β siRNA. siRNA-treated cells were treated with DFO for 6 hrs and angiopoietin-like 4 (ANGPTL4) mRNA examined (B), or 50 ng/ml IFNγ for 1.5 hours and PHD3 mRNA examined by qRT-PCR (C). (Data pooled from three independent experiments).

**Supplemental Figure III: IFNγ-induced PHD3 does not alter levels of HIF-1α protein during normoxia or hypoxia.** HUVECs were treated with 50 ng/ml IFNγ or vehicle then subjected to normoxia or hypoxia for 4 hours and PHD3 mRNA levels (A) were examined before hypoxic cells were allowed to reoxygenate (Data pooled form three independent experiments). (B) At the indicated timepoints following reoxygenation, protein lysates were collected and examined for HIF-1α protein (Data pooled from two independent experiments). The half-lives of HIF-1α protein under each condition are indicated on the graph. (C) HIF-1α protein levels following IFNγ treatment during normoxia.
Supplemental Figure I

A

% of Control (IFR9 mRNA)

B

Viperin mRNA (Fold Increase)

C

IRF1 mRNA (Fold Increase)

D

PHD3 mRNA (Fold Increase)

siCon  siIRF9

siCon  siIRF9

siCon  siIRF9

Veh

IFNγ

IFNα

Veh

IFNγ

IFNα

Veh

IFNγ

IFNα

Veh

IFNγ

IFNα

Veh

IFNγ

IFNα

Veh

IFNγ

IFNα

siCon

siIRF9

siCon

siIRF9

siCon

siIRF9
Supplemental Figure III:

A) PHD3 mRNA (Fold increase over Veh Control)

B) HIF-1α Protein (pg/ml) over time

C) HIF-1α Protein (Fold increase over Veh Control) over time