Inhibition of Prolyl Hydroxylase Domain-Containing Protein Suppressed Lipopolysaccharide-Induced TNF-α Expression

Kotaro Takeda, Toshihiro Ichiki, Eriko Narabayashi, Keita Inanaga, Ryohei Miyazaki, Toru Hashimoto, Hirohide Matsuura, Jiro Ikeda, Toshio Miyata, Kenji Sunagawa

Objective—Prolyl hydroxylase domain-containing proteins (PHDs) play pivotal roles in oxygen-sensing system through the regulation of α-subunit of hypoxia-inducible factor (HIF), a key transcription factor governing a large set of gene expression to adapt hypoxia. Although tissue hypoxia plays an essential role in maintaining inflammation, the role of PHDs in the inflammatory responses has not been clearly determined. Here, we investigated the role of PHDs in lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNF-α) induction in macrophages.

Methods and Results—Northern blot analysis and ELISA revealed that LPS-induced TNF-α upregulation was strongly suppressed by PHD inhibitors, dimethylxallyl glycine (DMOG), and TM6008 in RAW264.7 macrophages. DMOG suppressed LPS-induced TNF-α upregulation in HIF-1α-depleted cells and HIF-1α overexpression failed to suppress the induction of TNF-α. DMOG rather suppressed LPS-induced NF-κB transcriptional activity. Downregulation of Phd1 or Phd2 mRNA by RNA interference partially attenuated LPS-induced TNF-α induction. DMOG also inhibited LPS-induced TNF-α production in peritoneal macrophages as well as human macrophages.

Conclusions—PHD inhibition by DMOG or RNA interference inhibited LPS-induced TNF-α upregulation in macrophages possibly through NF-κB inhibition, which is independent of HIF-1α accumulation. This study suggests that PHDs are positive regulators of LPS-induced inflammatory process, and therefore inhibition of PHD may be a novel strategy for the treatment of inflammatory diseases. (Arterioscler Thromb Vasc Biol. 2009;29:2132-2137.)

Key Words: tumor necrosis factor -alpha ■ prolyl hydroxylase domain-containing protein ■ hypoxia-inducible factor ■ inflammation ■ hypoxia

Inflammation is a fundamental process for the protection of our body against outside pathogen. Tissues with inflammation are characterized by several features including the accumulation of inflammatory cells such as macrophages, lymphocytes, and neutrophils, limited blood supply attributable to impaired local microcirculation, and abnormal angiogenesis. Inflammatory cells are metabolically active and consume a large amount of oxygen and nutrient. These cells are, therefore, eventually exposed to hypoxic and nutrient-deprived condition. Thus, the inflammatory cells need to adapt these hypoxic conditions to perpetuate inflammatory reaction.

The reduced oxygen concentration is directly sensed by an innate oxygen-sensing system. The hypoxia-inducible factor (HIF) is a key transcription factor that mediates cellular adaptive responses to hypoxia. HIF is a heterodimer consisting of an oxygen-labile α-subunit and a stable β-subunit. The stability of the α-subunit of HIF-1 and HIF-2 (HIF-1α and HIF-2α) is regulated through the hydroxylation at the 4-position of specific proline residues in HIF-1α and HIF-2α by prolyl hydroxylase domain-containing proteins (PHDs).

Because PHD activity depends on the availability of molecular oxygen, PHDs are able to serve as a sensor for oxygen concentration. Under normal oxygen concentration, HIF-α is well hydroxylated by PHDs and tagged by von Hippel-Lindau (VHL) E3 ubiquitin ligase complex to be targeted for proteosomal degradation. When oxygen concentration is reduced, the activity of PHDs is decreased. This results in the accumulation of HIF in the nucleus, followed by upregulation of a series of genes suited for hypoxic condition. Because hypoxia is closely associated with an inflammatory reaction, it is reasonable that HIF is essential to maintain inflammatory processes. By switching energy production from oxidative phosphorylation to an anaerobic metabolism, macrophages generate ATP and thereby preserve its bacterial ability in the hypoxic tissues.

HIF-1α-deficient myeloid cells showed impaired inflammatory responses attributable to inefficient energy production. In contrast to HIF, the role of PHD in the inflammation is somewhat controversial. A specific knockdown of Phd gene led to the activation of NF-κB and hence upregulation of proinflamma-
tory molecules in HeLa cells. On the other hand, chemical PHD inhibitors attenuated inflammatory responses in several models including colitis and myocardial inflammation after an ischemic insult. Thus, in the present study, we focused on the question whether PHD inhibition suppresses or activates inflammatory responses in macrophages. We demonstrated that the PHD inhibition by pharmacological inhibitors or RNA interference suppressed lipopolysaccharide (LPS)-elicited induction of tumor necrosis factor α (TNF-α), a pivotal proinflammatory cytokine. However, interestingly the suppression was mediated not by a HIF-α accumulation but by suppression of NF-κB transcriptional activity. Our data suggest that suppression of PHD may be a novel antiinflammatory mechanism.

Methods
To clarify the role of PHD inhibition on inflammatory response, murine macrophage cell line, RAW264.7 cells were stimulated with LPS in the presence or absence of PHD inhibitor. The effect of LPS on mouse peritoneal macrophage, and human monocyte cell line, THP-1 was also examined. PHD isoforms were selectively knocked down by stable transfection of small hairpin RNA expression vector. Expression of TNF-α and other inflammatory cytokines were examined by quantitative reverse-transcription PCR (qPCR) or Northern blot analysis. Promoter activity was examined by luciferase assay. Nuclear translocation of NF-κB was examined by electrophoretic mobility shift assay and ELISA-based TransAM NF-κB p65 Transcription Factor Assay Kits. Cell viability was measured by flow cytometry after propidium iodide staining.

Detailed information of materials and methods used in this article is available in the online Data Supplement (please see http://atvb.ahajournals.org).

Results
DMOG Suppressed LPS-Induced TNF-α Upregulation in Macrophages
To assess the effect of the PHD inhibition on inflammatory response, RAW264.7 macrophages were pretreated with a vehicle DMSO or DMOG (1 mmol/L) for 1 hour before 100 ng/mL of LPS stimulation. Real-time qPCR and Northern blot analysis revealed that DMOG time- and dose-dependently inhibited LPS-induced Tnf-α mRNA upregulation (Figure 1A and 1B). TNF-α secretion in the supernatant during 24 hours of LPS treatment was also suppressed by DMOG (Figure 1C).

A luciferase gene regulated by murine Tnf-α gene promoter was introduced into the RAW264.7 cells, and luciferase activity was measured. A LPS treatment (100 ng/mL, 4 hours) significantly increased Tnf-α promoter activity and DMOG significantly suppressed the upregulation (Figure 1D). In contrast, DMOG did not affect Tnf-α mRNA stability (data not shown). We tested another novel PHD inhibitor, TM6008. Pretreatment with TM6008 (100 μmol/L) for 1 hour significantly suppressed TNF-α secretion in the supernatant after 24 hours of LPS treatment (supplemental Figure II). In addition to TNF-α, DMOG suppressed LPS-induced TNF-α converting enzyme (Tace) expression (supplemental Figure III).

Phd Knockdown Strongly Attenuated the LPS-Induced Cytokine Production
To examine whether the suppressive effect of DMOG is indeed mediated by the PHD inhibition, Phd gene expression was knocked down by shRNA introduction. Because there are at least three PHD isoforms (PHD1, PHD2, and PHD3) in mice, we determined the expression of Phd isoforms in RAW264.7 macrophages. Real-time qPCR analyses revealed that Phd3 gene was expressed at very low level in RAW264.7 cells (Figure 2A). We, therefore, downregulated Phd1 and Phd2 expression by shRNA. Phd1 and Phd2 shRNA efficiently decreased Phd1 and Phd2 mRNA expression by 91±1% and 67±2%, respectively (Figure 2B). Although Phd2 shRNA did not affect Phd1 mRNA expression, Phd1 shRNA increased Phd2 mRNA expression by 1.4-fold (Figure 2B). Then, these Phd1- or Phd2-depleted cells were stimulated with 100 ng/mL of LPS. LPS-induced Tnf-α mRNA upregulation and TNF-α secretion were significantly inhibited in both Phd1- and Phd2-depleted cells (Figure 2C and 2D and Figure IV). However, Phd1 depletion showed stronger suppression of Tnf-α expression than Phd2 depletion.

Activation of HIF Pathway by DMOG or Phd2 Knockdown but not by Phd1 Knockdown
To confirm whether the DMOG inhibition of PHD activates the HIF pathway in RAW264.7 macrophages, the levels of 2 main HIF-α isoforms (HIF-1α and HIF-2α) were determined
by Western blot analyses. Whereas HIF-1α was dramatically accumulated by DMOG treatment, HIF-2α protein remained undetectable (Figure 3A). Western blot for HIF-2α was validated by clear detection of HIF-2α expression in placenta lysate as a positive control. A HRE-driven luciferase expression vector21 was transiently introduced and a luciferase activity was measured. DMOG treatment for 24 hours strongly increased the HRE-dependent transcriptional activity (Figure 3B).

We also determined the levels of HIF-α in Phd1- or Phd2-depleted cells. Introduction of Phd2 shRNA, but not Phd1 shRNA, induced HIF-1α accumulation, whereas HIF-2α was not induced by either Phd1 or Phd2 shRNA (Figure 3C). HRE-dependent transcriptional activity was only increased in Phd2-depleted cells (Figure 3D).

A HIF-1α Overexpression Failed to Suppress the LPS-Induced Tnf-α Promoter Activation

To test whether accumulated HIF-1α by DMOG is responsible for the suppression of LPS-induced TNF-α induction, we determined the effect of overexpression of CA-HIF-1α.22 The expression of CA-HIF-1α strongly increased HRE-dependent transcriptional activity (Figure 3E). However, Tnf-α gene transcriptional activity was not suppressed in CA-HIF-1α-expressing cells after 6 hours or 24 hours of LPS stimulation (Figure 3F).

DMOG Suppressed LPS-Induced Tnf-α Upregulation in Hif-1α-Depleted Cells

We next examined whether DMOG would be able to suppress the LPS-induced Tnf-α upregulation in the absence of HIF-1α. shRNA specific for Hif-1α gene strongly decreased the HIF-1α mRNA level and the DMOG-induced HIF-1α accumulation (Figure 4A and 4B). Then, Hif-1α-depleted cells were pretreated with DMOG for 1 hour and stimulated with 100 ng/mL of LPS for 4 hours. Consistent with a previous report,12 the induction of Tnf-α mRNA was significantly
DMOG Treatment Suppressed LPS-Induced NF-κB Transcriptional Activation

Because both activation of NF-κB and mitogen-activated protein kinases (MAP kinases) is responsible for the LPS-induced TNF-α induction, the authors examined whether DMOG would suppress an activation of MAP kinases such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). Phosphorylation of these kinases, a surrogate marker of kinase activation, was strongly induced by 100 ng/mL of LPS but the activation was not reduced by DMOG pretreatment (Figure 5A). The expressions of c-Jun, p38, JNK, and ERK were suppressed by DMOG, as determined by electrophoretic mobility shift assay and ELISA-based DNA-binding assay by using nuclear protein extract after LPS stimulation, respectively. However, translocation of NF-κB to the nucleus and binding capacity to NF-κB consensus site were not decreased by DMOG pretreatment (supplemental Figure VIA through VIC).

DMOG Suppressed LPS-Induced TNFα Upregulation in Resident Peritoneal Macrophages and Human THP-1

Finally, to generalize the effect of DMOG on LPS-induced TNF-α upregulation, the authors analyzed the effect of DMOG on 2 different types of macrophages. One is murine peritoneal macrophages from normal mice, and the other is human monocyte cell line THP-1. Consistent with the results of RAW264.7 macrophages, DMOG pretreatment significantly suppressed LPS-induced TNF-α secretion in peritoneal macrophages (Figure 6A). DMOG also time- and dose-dependently suppressed LPS-induced TNF-α mRNA upregulation in differentiated THP-1 macrophages (Figure 6B and 6C).

Discussion

In this article, we demonstrated that PHD inhibition by DMOG significantly suppressed LPS-induced expression of several proinflammatory genes encoding not only TNF-α but IL-6, IL-1β, iNOS, and antiinflammatory gene IL-10 in macrophages. Although DMOG treatment apparently raised HIF-1α level, the increased HIF-1α was not responsible for the suppression. And PHD1 among three PHD isoforms may be mainly responsible for the suppressive effect of DMOG on LPS function. These data indicated that PHD inhibition decreased cellular sensitivity to inflammatory stimuli and may have a therapeutic implication.
How does PHD inhibition suppress LPS-induced TNF-α upregulation? Because PHD is a negative regulator for HIF-1α or HIF-2α expression, one would expect that increased HIF-α might be responsible for the suppression. HIF-2α was undetectable in RAW264.7 macrophages, excluding the possible involvement of HIF-2α. In contrast, DMOG strongly induced HIF-1α accumulation and activated HRE-dependent transcription. However, DMOG suppressed LPS-induced Tnf-α upregulation even in Hif-1α–depleted cells. Moreover, Phd1 knockdown significantly inhibited LPS-elicited TNF-α upregulation but did not increase HIF-1α levels. In addition, forced expression of stable form of HIF-1α (CA-HIF-1α) failed to inhibit TNF-α promoter activity. These evidences consistently indicate that DMOG-mediated suppression of the LPS effect does not depend on HIF-1α as well as HIF-2α.

The mechanism by which PHD inhibition attenuated LPS-induced TNF-α production is not clear at this point, but several possibilities may be considered. First of all, because NF-κB activation is an essential step for the induction of cytokines such as TNF-α, IL-6, IL-1β, iNOS, and antiinflammatory IL-10, DMOG-induced NF-κB suppression may be a potential mechanism. We observed that DMOG did not suppress nuclear translocation or binding capacity to NF-κB consensus site but reduced NF-κB–dependent transcriptional activity. The mechanism by which DMOG suppressed NF-κB activation remains elusive. However, recent studies suggest that phosphorylation of NF-κB on Ser536 phosphorylation is essential for the NF-κB transcriptional activation. Thus, DMOG may affect the phosphorylation of NF-κB to suppress LPS-induced transcriptional activation.

In contrast to our data, a previous report suggest that DMOG may enhance inflammatory reaction. Cummins et al described that PHD-induced hydroxylation of IkB kinase-β (IKKβ), an activator of NF-κB pathway, attenuated its kinase activity. DMOG activates NF-κB pathway and induces proinflammatory cyclooxygenase 2 expression in HeLa cells. The reason for the discrepancy between their study and ours is not immediately clear, but it may be possible that PHD inhibition causes different effects on different cell type, which is most likely reflecting differential expression pattern of PHD isoforms. Alternatively, DMOG may increase the basal expression of proinflammatory genes while decreasing the induction of these genes on inflammatory stimuli. Therefore, further study is needed to clarify the effect of PHD inhibition in several different experimental conditions of inflammation.

Other possible mechanisms for DMOG-elicited suppression of LPS-induced TNF-α upregulation may be the suppression of oxidative phosphorylation and global energy consumption. DMOG treatment significantly inhibits electron transport chain activity during mitochondrial respiration, leading to the reduced ATP production in cardiomyocytes. DMOG also inhibits intracellular ATP consumption, an example of which is the reduction of contraction in cardiomyocytes. Therefore, DMOG may suppress energy metabolism, leading to attenuation of inflammatory responses in macrophages.

Our isoform-specific knockdown experiments indicated that PHD1 was mainly responsible for LPS-induced TNF-α upregulation. In IKKβ hydroxylation, PHD1 is also mainly responsible. PHD1 knockout mice caused reduced ATP production and consumption in skeletal muscle. These data indicate that although PHD2 is generally important for HIF regulation, PHD1 might have a distinct pathway rather than HIF to regulate various biological activities. Thus, further study is needed to identify a target molecule of hydroxylation by PHD1 to clarify the role of PHD1 in LPS-induced inflammation. In this study, we did not analyze the role of PHD3, because PHD3 was not expressed in RAW264.7 cells. However, PHD3 has a potential to compensate the role of PHD1 in other cells that express PHD3. Thus, we cannot exclude the possible involvement of PHD3 in other inflammatory models in which PHD3 is present substantially.

In general, hypoxia is considered to induce or augment inflammatory responses. For instance, hypoxia augments LPS-induced TNF-α and iNOS expression in several cell lines including RAW264.7 macrophages and murine dendritic cells. Thus, it may be counterintuitive that PHD inhibition suppresses LPS-induced TNF-α expression, because both hypoxia and PHD inhibition induce HIF-α accumulation and upregulation of HIF target gene expression. However, the biological effects caused by hypoxia or PHD inhibition are not necessarily the same or even opposite. One example is a production of reactive oxygen species (ROS); hypoxia increases ROS production, whereas PHD inhibition decreases ROS. If hypoxia-induced ROS production potentiates inflammation, reduced ROS production by PHD inhibition may attenuate inflammation. Thus, it is possible that hypoxia and PHD inhibition induce opposite biological responses in some cases. Our study indicates that PHD inhibition suppresses LPS-induced TNF-α upregulation, which is usually augmented by hypoxic exposure.

Taken together, we provided the first-line evidence that PHD inhibition suppressed LPS-induced proinflammatory TNF-α production independently of HIF-1α in macrophages. TNF-α is involved in various pathological conditions, including sepsis, autoimmune disorders, atherosclerosis, and obesity-associated insulin resistance. Antagonizing TNF-α has been shown to be protective for several inflammatory diseases. Therefore, PHD inhibition might be a novel strategy for the treatment of inflammatory diseases.

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Disclosures
None.
References

30. Takeda et al Suppression of TNF-alpha by PHD Inhibition 2137
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Supplemental Figure I

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\[ Tnf-\alpha \]

\[ rRNA \]

B

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\[ Tnf-\alpha \text{mRNA} / rRNA \]

\[ (\text{folds}) \]
Supplemental Figure II

The graph shows the levels of TNF-α in the tissue samples. The control group (C) has the lowest levels, followed by the LPS group, which has significantly higher levels (indicated by **). Treatment with LPS + TM6008 resulted in levels similar to the control group. The error bars represent the standard deviation.
Supplemental Figure IV

The figure shows a Western blot analysis of Tnf-α and rRNA expression under different conditions: Control shRNA, Phd1 shRNA, and Phd2 shRNA with and without LPS stimulation. The blot indicates a significant increase in Tnf-α expression in the Phd2 shRNA group compared to the control and Phd1 shRNA groups. The bar graph on the right confirms these findings with quantification of Tnf-α mRNA levels relative to rRNA, showing a significant difference (N.S. and **).
Supplemental Figure V

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![Graph showing TNF-α mRNA levels](attachment:image.png)

- **Control**
- **LPS**
- **LPS + DMOG**
Supplemental Figure IX

**LPS**

**LPS + DMOG**

Vegf / Hprt mRNA (folds)

C  1  2  4  6 (h)