Original Article
Folic acid inhibits hypoxia-induced THP-1 cells inflammation by suppressing NF-κB signaling pathway

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Abstract: Lowering of homocysteine by folic acid is known to prevent cardiovascular events. However, the mechanism by which this impact related diseases is not clear. Therefore, we investigated whether folic acid has protective effects on the inflammatory response to hypoxia of human mononuclear cells in vitro, and the involved signaling pathways. The human monocyte cell line THP-1 cells were pretreated with folic acid for 30 min, and then incubated with 1% O_2 for 24 h to induce hypoxia injuries. The protein levels and mRNA expression of interleukin-1 beta (IL-1β) and interleukin-8 (IL-8) were respectively determined by ELISA and qRT-PCR. The expressions of hypoxia-inducible factor-1 alpha (HIF-1α), prolyl hydroxylase1 (PHD1), inhibitor of nuclear factor κB kinase beta (IKKβ), nuclear factor κB (NF-κB) were analyzed with western blotting and immunofluorescence. The results showed that hypoxia increased the expression of IL-1β and IL-8 accompanied by significant augmentation of HIF-1α, IKKβ and NF-κB expression as well as inhibition of PHD1. However, pretreatment with folic acid significantly reduced hypoxia-induced production of IL-1β and IL-8. Moreover, folic acid markedly attenuated hypoxia-induced upregulation of HIF-1α, IKKβ and NF-κB, and upregulated PHD1. In summary, folic acid inhibits the inflammatory response of THP-1 cells to hypoxia by inhibiting NF-κB pathways, which may represent one of the mechanisms by which folic acid exerts a protective effect in cardiovascular disorders.

Keywords: Folic acid, inflammation, hypoxia, nuclear factor-kappa B

Introduction

Folate, also named vitamin B9, is a water-soluble vitamin that can be found naturally in food or in its synthetic form, folic acid, in supplements and fortified foods. It is a necessary vitamin to support health and reduce disease risk [1]. Humans cannot synthesize folate and therefore must fulfill their requirements through diet. Inadequate folate intake may increase the risk of neural tube defects, megaloblastic anemia, cancer, cognitive dysfunction and hyperhomocysteinemia [1, 2].

Numerous studies have found that folic acid may prevent cardiovascular disease (CVD). Adequate levels of folic acid help reduce the risk of ischemic heart disease, deep vein thrombosis, and stroke [3], and the mortality of stroke [4]. Additionally, most studies have indicated that folic acid protects against arteriosclerotic vascular disease by lowering homocysteine [5], which is regarded as an independent risk factor for CVD [6]. However, how folic acid prevents CVD mechanistically is still unclear. It has been reported that folic acid improves vascular function by modulating endothelial nitric oxide synthase and the cofactor tetrahydrobiopterin, independent of homocysteine lowering. This modulation reduces vascular superoxide production and causes antioxidant effects [7-9]. It is worth noting that some researchers have found the anti-inflammatory effects of folic acid in vivo and in vitro, involving the reduction of several inflammatory cytokines and pro-inflammatory factors [10-12]. We have shown previously that folic acid significantly decreases lipopolysaccharide (LPS)-induced nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and interleukin-1 beta (IL-1β) production in RAW264.7 cells [13]. Moreover, folate deficiency can enhance the inflammatory response of macro-

phages [14]. In addition, our earlier studies have demonstrated that rats fed with deficient folate exhibited more severe inflammation of the vascular endothelium compared with those fed a methionine-rich diet [15]. It is clear that inflammation plays a central role in all phases of the atherosclerotic process and anti-inflammatory effect may yield novel therapeutic targets [16]. Therefore, folic acid may be protective against cardiovascular disease through its anti-inflammatory effect.

In early stages of atherogenesis, monocytes, an innate immune cell, are strongly linked to inflammation. Monocytes recruit and express pro-inflammatory cytokines in arterial wall [17]. Hypoxia has been proposed as an important underlying cause of the inflammatory response in atherosclerotic lesions [18]. Hypoxia not only activates the hypoxia-inducible factor (HIF) family by inhibiting prolyl hydroxylases (PHDs), but also inhibits the nuclear factor κB kinase (IKK) and nuclear factor κB (NF-κB). Activation of NF-κB can encode inflammatory mediator genes and increases secretion of inflammatory factors such as IL-1β, interleukin-8 (IL-8) [19, 20]. Moreover these inflammatory cells increase oxygen consumption. This creates a vicious circle of hypoxia, angiogenesis and inflammation which occurs deep in the plaque, enhancing plaque growth and increasing the risk of plaque rupture [21, 22]. However, little is known about the role of folic acid in the inflammation process in human mononuclear cells THP1 under hypoxia conditions.

In this study, we investigated the effects of folic acid on hypoxia-induced inflammatory responses, as measured by IL-1β and IL-8 production in THP-1 cells. We attempted to further identify the signaling pathway by measuring changes in the levels of HIF-1α, PHD1, IKKβ, and NF-κB. Our study may provide a possible therapeutic use of folic acid in vascular inflammatory disease.

Materials and methods

Materials

Folic acid and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). Materials for cell culture were purchased from GIBCO (NY, USA). Trizol reagent was obtained from Invitrogen (Carlsbad, USA). Antibodies against NF-κB p65 and IKKβ were purchased

from Cell Signaling Technology (Charlottesville, USA). The antibody of PHD1 and HIF-1α were purchased from Novus Biologicals (Littleton, USA) and BD Biosciences (New Jersey, USA) respectively. Antibody against β-actin was purchased from Boster (Wu Han, China). ELISA kits for IL-8 and IL-1β quantification were purchased from R&D Systems (Minneapolis, USA). Octyl-α-ketoglutarate was purchased from Cayman Chemical (Ann Arbor, USA). Fluorescent nuclei dye, DAPI, and donkey anti-rabbit IgG-FITC were purchased from Roche (Basel, Switzerland) and Santa Cruz Biotechnology (Dallas, USA), respectively. The Anoxomat instrument was purchased from Advanced Instruments, Inc. (Boston, USA) and was used to create hypoxic environments in jars.

Cell culture and viability assay

THP-1 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin at 37°C in a 5% CO_2 incubator. THP-1 cells were pretreated with folic acid at 0-40 µg/mL for 24 h, then moved to the respective incubators and incubated in either normoxia (21% O_2 and 5% CO_2) or hypoxia (1% O_2 and 5% CO_2) in a sealed jar that was pre-balanced for the desired O_2 concentration for 24 h.

A commercially available cell viability assay kit was used to evaluate the cytotoxic effects of folic acid, using MTT-based colorimetry. THP-1 cells ($2×10^5$ cells/well) were plated with a variety of concentrations of folic acid at 0-40 µg/mL in 96-well microtiter plates, and were then cultured for 24 h at 37°C in a 5% CO_2 incubator. At the termination of culture, 20 µL MTT (5 mg/mL) was added to each well, followed by incubation for 4 h at 37°C in a 5% CO_2 incubator. Then 100 µL mixed liquid containing 10% lauryl sodium sulfate, 5% isobutyl alcohol and 12 mM HCl was added to each well, followed by incubation for 24 h at 37°C in a 5% CO_2 incubator. Finally, 96-well microtiter plates were put on the shaker to shake for 10 min and the optical density values were determined using an EL×800 reader (Bio-Tek Instruments, Winooski, VT, USA) at 570 nm.

Measurement of cytokine levels

THP-1 was treated as previously described. The released IL-1β and IL-8 to the medium were determined using ELISA kits, according to the
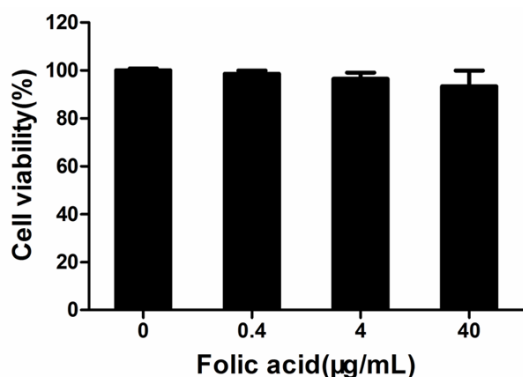
Figure 1. Effects of folic acid on THP-1 cell viability. The cytotoxic effect of folic acid in THP-1 cells was measured by MTT assay. Data shown are means ± SD representative of 5 independent experiments.

manufacturer's instructions. Optical density was measured at 450 nm, and the amount of cytokines was calculated from a standard curve prepared with the recombinant protein.

RNA preparation and qRT-PCR

THP-1 cells were prepared as previously described. Total RNA was extracted by using Trizol reagent. The reverse transcriptional reaction was performed with Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). The levels of IL-1β and IL-8 mRNA were measured by quantitative real-time polymerase chain reaction (qRT-PCR) using FastStart Universal SYBR Green Master (ROX) (Roche, Switzerland). The following primer pairs were used: β-actin forward 5'-CCTGGCACCCAGCAC-AA T-3' and reverse 5'-GGGCCGGACTCGTCAT-AC-3'. IL-1β forward 5'-TCTTCGACACATGGGAT-AACGA-3' and reverse 5'-TCCCGGA GCGTG-CAGTT-3'. IL8 forward 5'-CTCTGCACCCAGTTTT-CCTT-3' and reverse 5'-GTGCAGTTTTGCCAAG-GAGT-3'. The PCR conditions were as follows: 10 s at 95°C for denaturation, 30 s at 60°C for annealing with 40 cycles. The internal control was β-actin.

Western blot analysis

The protein levels of HIF-1α, PHD1, IKKβ and NF-κB p65 were analyzed by Western blot. Proteins (~30 μg) from whole cell lysates, cytosolic extracts, or nuclear extracts were separated by 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated subse-

quently with specific primary antibodies and secondary antibodies conjugated with horseradish peroxidase. The bands were visualized using the ECL system, and the band density was determined by Quantity One software (Bio-Rad, USA).

Immunofluorescence staining

THP-1 cells were fixed in PBS (pH 7.4) with 4% paraformaldehyde for 15 min. Next, fixed cells were permeabilized in PBS with 0.5% Triton X-100 for 10 min. After three washes, the cells were incubated with blocking solution (goat serum) for 15 min. Cells were then incubated with a rabbit anti-human NF-κB p65 antibody (dilution 1:200) overnight at 4°C. After washing with PBS, the cells were incubated for 1 h with Goat Anti-rabbit IgG-FITC antibody. After five washes, cells were stained with DAPI (1 μg/ml) for 15 min, followed by a wash with methyl alcohol. Confocal immunofluorescence analysis was performed using a Leica TCS SP5II microscope (Leica Microsystems, Germany) with a 40× oil-immersion objective lens.

Statistical analysis

Data are expressed as mean ± SD and analyzed using one way ANOVA followed by a Bonferroni post-hoc correction for multiple comparisons. All experiments were repeated at least three times. A *P*-value ≤ 0.05 was considered statistically significant. The SPSS 13.0 statistical software package was used in analysis.

Results

The cytotoxicity of folic acid in THP-1 cells

We evaluated cytotoxicity of folic acid at different concentrations (0-40 μg/mL) using MTT assay, to rule out the possibility that inflammatory cytokines result from the toxicity effect of folic acid. Folic acid-induced cytotoxicity was negligible in THP-1 cells at concentrations of 0.4-40 μg/mL (**Figure 1**).

Folic acid inhibits hypoxia-induced IL-1β and IL-8 mRNA expression and release in THP-1 cells

IL-1β and IL-8 release was examined after folic acid pretreatment and hypoxia exposure using an ELISA assay. IL-1β and IL-8 concentration in
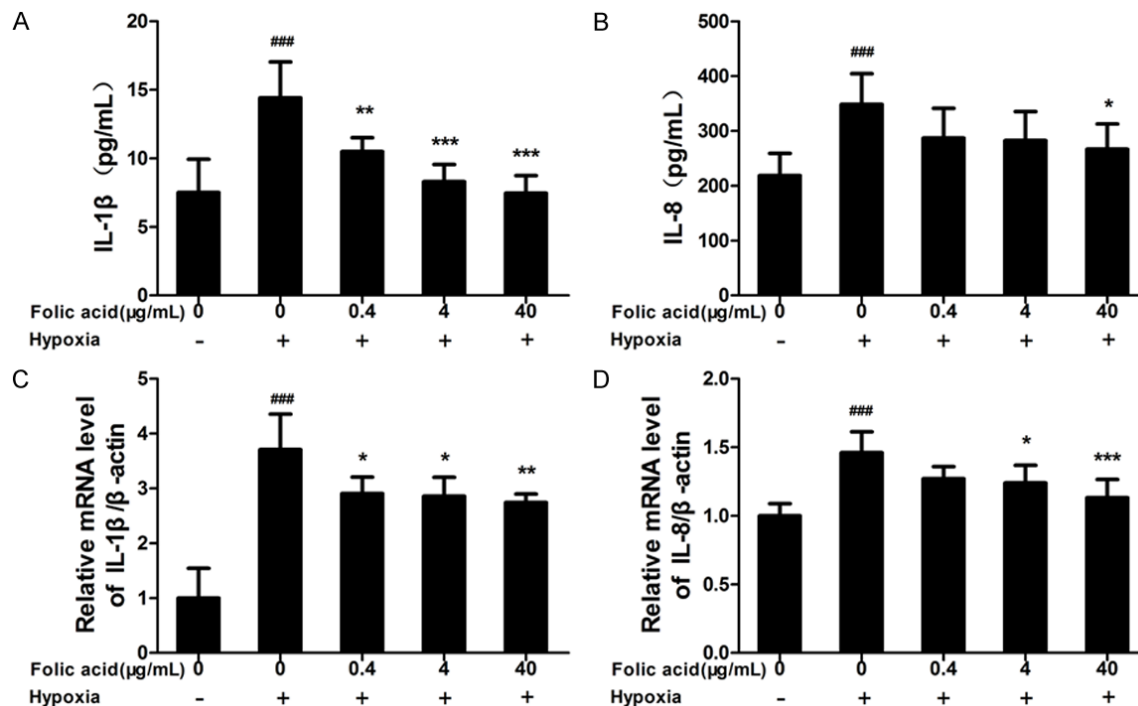
Figure 2. Effects of folic acid on hypoxia-induced IL-1β and IL-8 production and gene expression in THP-1 cells. THP-1 cells were pretreated with folic acid at 0.4-40 μg/mL for 30 min and then incubated under hypoxic conditions (1% O_2) for 24 h. The protein levels of IL-1β (A) and IL-8 (B) were measured by ELISA. The mRNA expression of IL-1β (C) and IL-8 (D) was analyzed by qRT-PCR. The results are representative of 5 independent experiments and expressed as mean ± SD. ###$P<0.001$ compared with control group. *$P<0.05$, **$P<0.01$ or ***$P<0.001$ compared with hypoxia alone.

the medium was significantly increased after hypoxic treatment for 24 h and was inhibited in a dose-dependent manner by treatment with folic acid (0.4-40 μg/mL) (**Figure 2A** and **2B**). QRT-PCR further confirmed that hypoxia caused marked increases in IL-1β and IL-8 mRNA expression, which were significantly attenuated by folic acid pretreatment (**Figure 2C** and **2D**).

Folic acid regulates PHD1, HIF-1α, IKKβ and NF-κB in THP-1 cells exposed to hypoxia

PHDs belong to a family of oxygen-sensing proteins. Under low oxygen conditions, the PHDs activity is inhibited, and the rate of HIF-1α hydroxylation is suppressed. This leads to HIF-1α accumulation in the cytoplasm, where it dimerizes with an HIF-1β and translocates to the nucleus. This complex then transcriptionally activates genes related to hypoxia adaptation and regulation of the inflammatory response [23]. PHD1, one of the PHD's subunits, also controls NF-κB activity through regulation of IKKβ. Once IKKβ is activated, it phosphorylates IκBα, leading to its degradation, and also

enhances NF-κB activity [19]. NF-κB is the master regulator of the inflammatory response. Its major subunit is NF-κB p65 [24].

As shown in **Figure 3A**, folic acid significantly inhibited hypoxia-induced activation of HIF-1α and increased PHD1 activities. Meanwhile, folic acid markedly suppressed hypoxia-induced activation of IKKβ and NF-κB p65 (**Figure 3B**). These data suggest that hypoxia-induced NF-κB signaling was significantly inhibited by folic acid in a dose-dependent manner.

Hypoxia induces IL-1β and IL-8 production by suppressing PHD1 and activating the NF-κB pathway in THP-1 cells

To further understand the mechanism underlying folic acid's effect on the NF-κB signaling, we used octyl-α-ketoglutarate (α-KG), an activator of PHD, to modulate the pathway. HIF-1α is activated and PHD1 is suppressed during the exposure to hypoxia (**Figure 4A**). Furthermore, PHD1-mediated repression of IKKβ was suppressed, possibly resulting in enhanced IKKβ
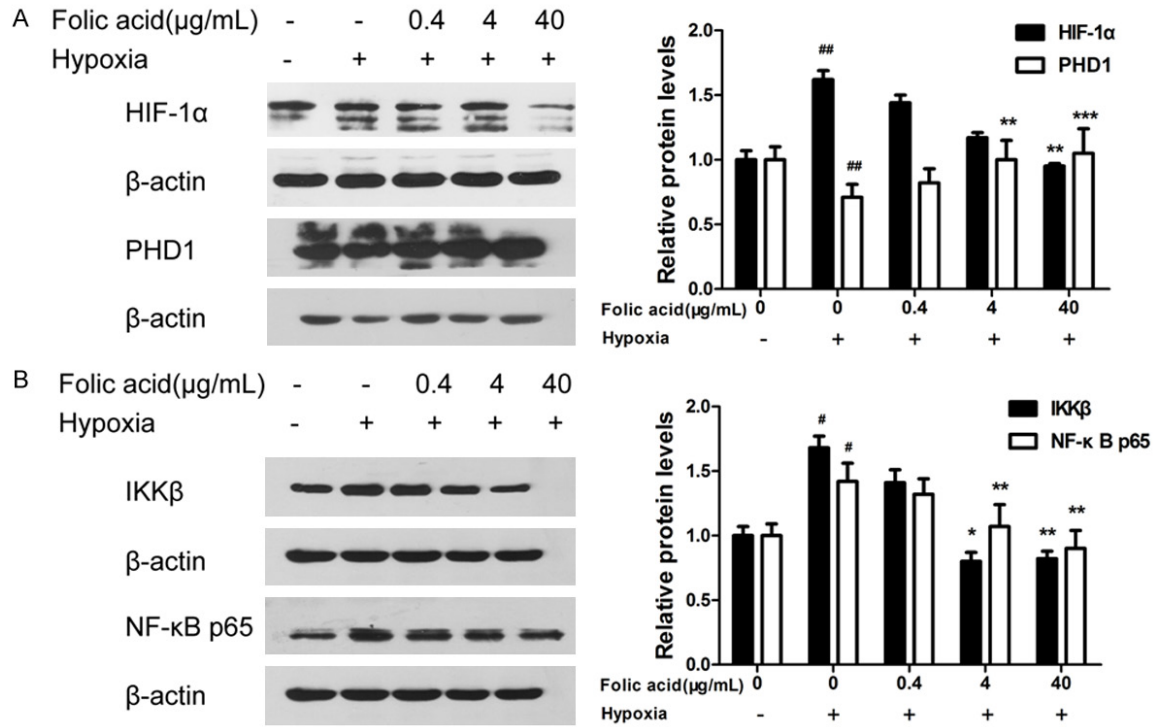
Figure 3. Folic acid inhibits hypoxia-induced activation of NF-κB pathway in THP-1 cells. THP-1 cells were pretreated with folic acid at 0.4-40 μg/mL for 30 min and then incubated under hypoxic conditions (1% O_2) for 24 h. PHD1, HIF-1α, IKKβ and NF-κB p65 protein levels were analyzed by Western blot. β-actin was used as a control. The results are representative of 3 independent experiments and expressed as mean ± SD. #$P<0.05$ or ##$P<0.01$ compared with control group. *$P<0.05$, **$P<0.01$ or ***$P<0.001$ compared with hypoxia alone.

activity and increased NF-κB p65 activity in the hypoxia conditions (**Figure 4B**). However, α-KG (1 mM) significantly inhibited hypoxia-induced activation of HIF-1α and increased PHD1 activities even under the hypoxia (**Figure 4A**). IKKβ and NF-κB p65 were significantly inhibited, possibly by activated PHD1 (**Figure 4B**).

We next determined the mechanistic roles of the inflammatory factors. IL-1β and IL-8 production was examined after the α-KG pretreatment and hypoxic exposure using an ELISA assay. IL-1β and IL-8 secretion was increased significantly after hypoxic treatment for 24 h, but had a marked suppression caused by α-KG (1 mM) treatment (**Figure 5A** and **5B**). The qRT-PCR further confirmed that hypoxia caused marked increases in IL-1β and IL-8 mRNA expression, which were significantly attenuated by α-KG pretreatment (**Figure 5C** and **5D**). The results above suggest that hypoxia induces IL-1β and IL-8 production via suppression of PHD1 and activation of the NF-κB pathway in THP-1 cells.

Folic acid and α-KG inhibit hypoxia-induced nuclear translocation of NF-κB p65 in THP-1 cells

To investigate the effects of hypoxia, folic acid and α-KG on the nuclear translocation of NF-κB, NF-κB p65 subunit was detected by immuno-fluorescence and imaged using confocal microscopy. As shown in **Figure 6**, the nuclear translocation of hypoxia-induced NF-κB p65 significantly increased, while NF-κB p65 was found exclusively in the cytoplasm in normoxia. Furthermore, the relative expression of NF-κB p65 was higher in hypoxia than in normoxia. In addition, when THP-1 cells were pretreated with folic acid (4 μg/mL) or α-KG (1 mM), nuclear translocation of NF-κB p65 was markedly reduced and the relative expression was decreased, in striking contrast to the effects of hypoxia alone. These results indicate that, under hypoxic conditions, folic acid has anti-inflammatory effects in THP1 cells via inactivation of the NF-κB pathway.
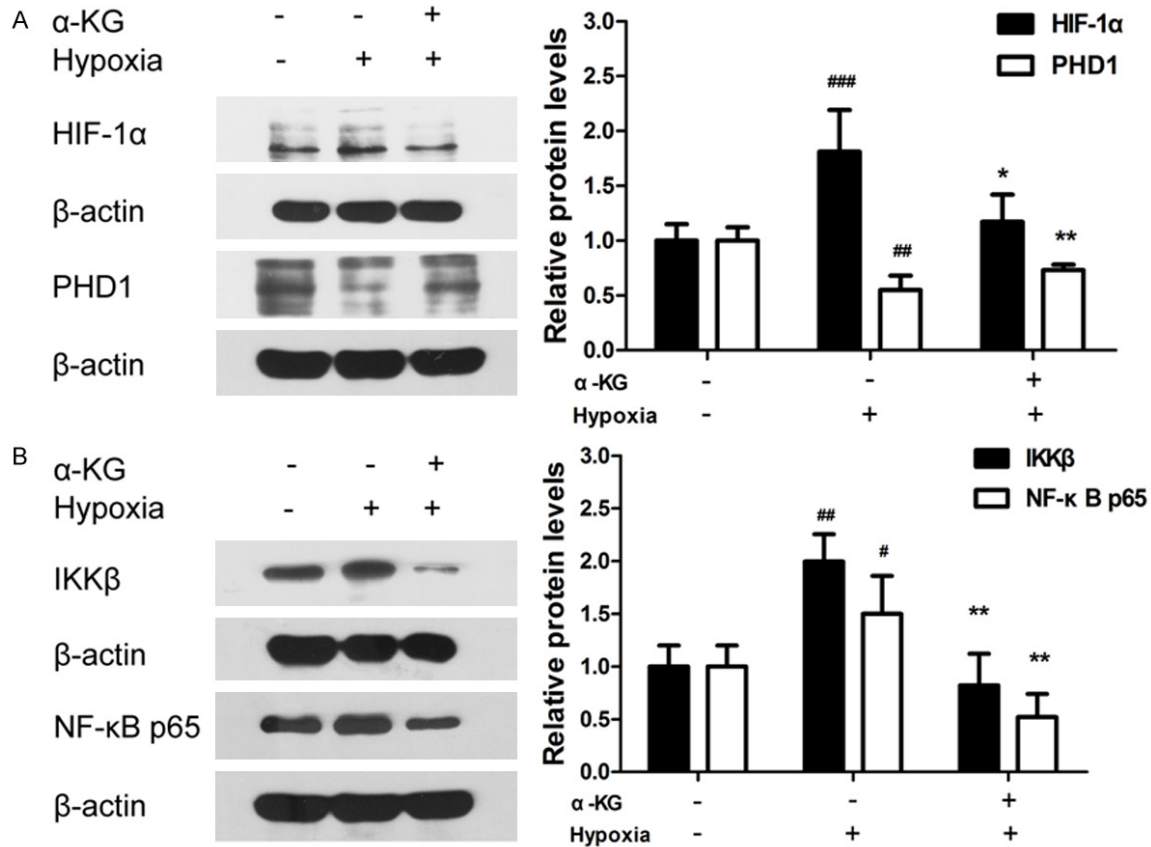
Figure 4. Effects of α-KG (PHD activator) on hypoxia-induced activation of NF-κB pathway in THP-1 cells. THP-1 cells were pretreated with α-KG at 1 mM for 30 min and then incubated under hypoxic conditions (1% O_2) for 24 h. PHD1, HIF-1α, IKKβ and NF-κB p65 protein levels were analyzed by Western blot. β-actin was used as a control. The results are representative of 3 independent experiments and expressed as mean ± SD. #$P<0.05$, ##$P<0.01$ or ###$P<0.001$ compared with control group. *$P<0.05$, **$P<0.01$ or ***$P<0.001$ compared with hypoxia alone.

Discussion

Hypoxia exists in vivo whenever oxygen demand exceeds oxygen supply. It is generally defined as levels between 0.5% and 3% oxygen by volume [25]. We chose to use a concentration of 1% O_2 because this level has been used in various cell culture models previously [26, 27]. Hypoxia is found to exist in atherosclerotic lesions, which causes an increase in lipid-loaded macrophages and local inflammation, two steps in the pathogenesis of human atherogenesis [18]. Monocytes and lymphocytes are recruited to the artery wall and express pro-inflammatory cytokines such as IL-1β and IL8. IL-1β is a highly inflammatory cytokine, particularly in humans, that promotes systemic and local inflammation, and causes acute and extensive damage in atherosclerotic lesions [28]. IL-8, a chemokine, is produced in several tissues upon inflammation, ischemia, trauma

etc., and has been found to be associated with atherosclerotic lesion formation. IL-8 may be involved in the recruitment of inflammatory cells into atherosclerotic plaques [29, 30]. This study demonstrates that in THP-1 cells hypoxia initiates an inflammatory response as measured by inflammatory markers. IL-1β and IL8 levels increase, both in levels of secreted protein and gene-expression. However, folic acid causes a pronounced effect, decreasing secretion and gene expression of IL-1β and IL-8.

NF-κB, a ubiquitous transcription factor, plays an important role in immune and inflammatory responses by regulating genes encoding pro-inflammatory cytokines (IL-1β), adhesion molecules, chemokines (IL-8), and enzymes [19]. The NF-κB canonical pathway is predominantly dependent on IKKβ. Once IKK is activated, the IKK complex phosphorylates IκB-α and dissociates from IκB-α, thereby freeing NF-κB to trans-
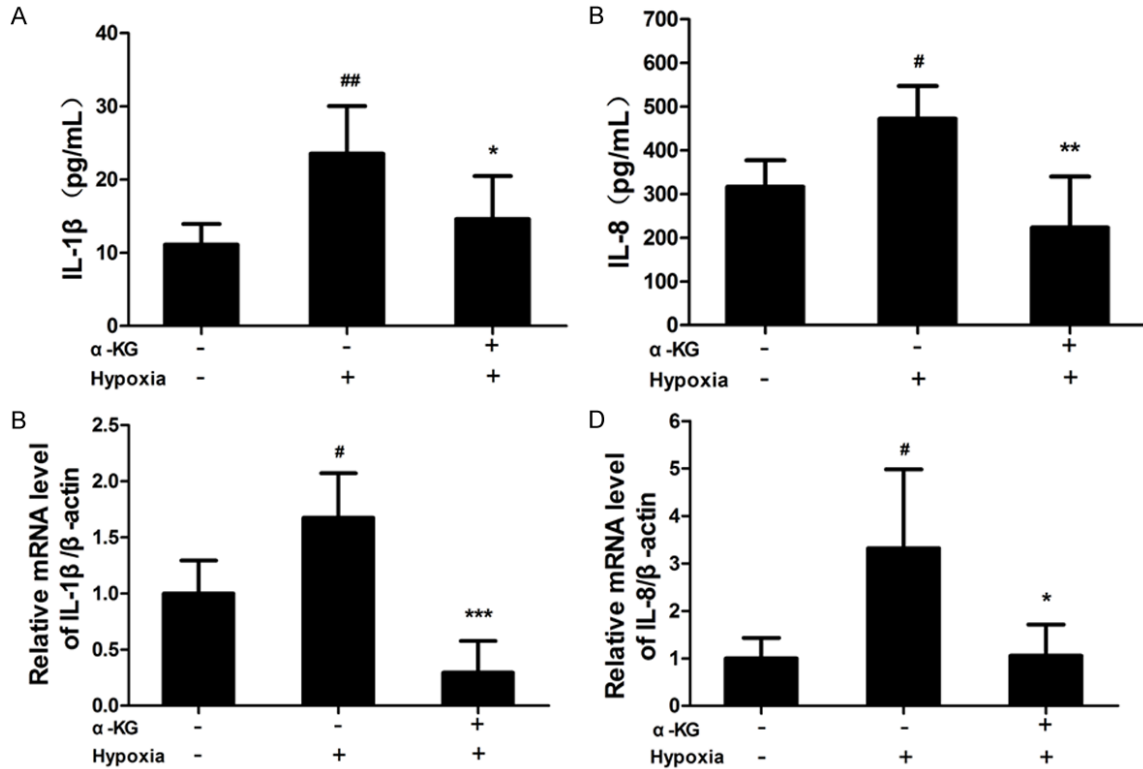
Figure 5. Effects of α-KG (PHD activator) on hypoxia-induced IL-1β and IL-8 production and gene expression in THP-1 cells. THP-1 cells were pretreated with α-KG at 1 mM for 30 min and then incubated under hypoxic conditions (1% O$_2$) for 24 h. The protein levels of IL-1β (A) and IL-8 (B) were measured by ELISA. mRNA expression of IL-1β (C) and IL-8 (D) was analyzed by qRT-PCR. The results are representative of 3 independent experiments and expressed as mean ± SD. #$P<0.05$ or ##$P<0.01$ compared with control group. *$P<0.05$, **$P<0.01$ or ***$P<0.001$ compared with hypoxia alone.

locate to the nucleus and activate transcription of target genes [31]. Hypoxia can activate NF-κB in an IKK-dependent manner. Some studies imply that IKKβ is a key physiological link between the hypoxic response and inflammation because IKKβ contains an evolutionarily conserved LxxLAP consensus motif for hydroxylation by PHDs. Moreover, silencing of PHD1 augments NF-κB activation more efficiently than silencing of PHD2 or PHD3 [26]. The present study demonstrates that hypoxia inhibits PHD1 and upregulates HIF-1α, IKKβ, and NF-κB p65 in THP-1 cells. When cells were pretreated with the α-KG, PHD1 was activated even though cells were under hypoxic conditions. Additionally, HIF-1α, IKKβ and NF-κB p65 were suppressed, and the inflammatory mediators (IL-1β and IL-8) were inhibited compared with the hypoxia only. These results suggest that hypoxia may act by inhibiting PHD1 activity, increasing the activity of cellular IKKβ/NF-κB pathway, and subsequently upregulating IL-1β

and IL-8 in THP-1 cells. We found that the effect of folic acid on this signaling pathway was similar to the effect of α-KG. This may imply that folic acid acts by activating PHD1, thereby inhibiting the IKKβ/NF-κB pathway to regulate the inflammatory response. Others have showed that folic acid may affect the NF-κB pathway as well [12, 13, 32]. However, further studies are needed to determine how folic acid affects this pathway. For the first time we have demonstrated the inhibitory effects and underlying mechanisms of folic acid on hypoxia-induced inflammation in THP-1 cells.

Folic acid is an important vitamin for humans. It is thought that folic acid prevents cardiovascular events by lowering homocysteine. However, there is still no consensus on the relation between folic acid and homocysteine. We hypothesized that folic acid alone might cause an anti-inflammatory effect. Previous evidence shows that folic acid can significantly attenuate
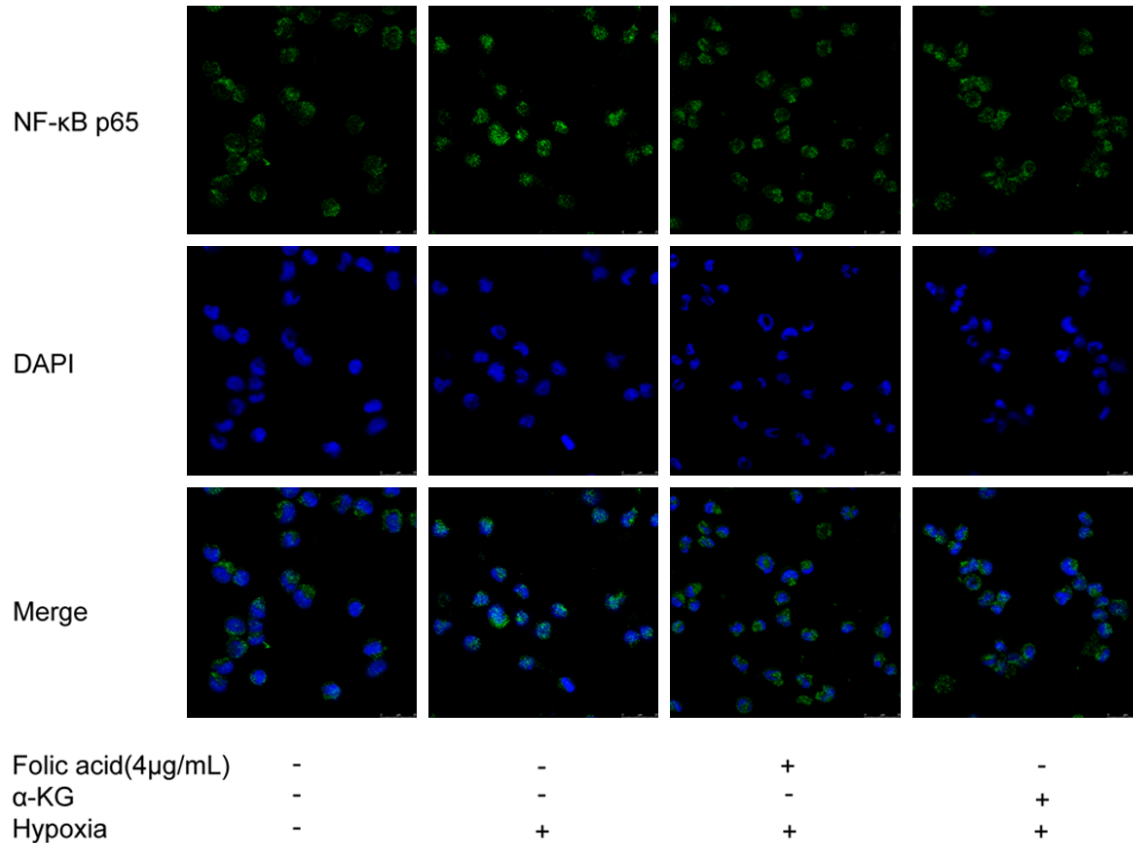
Figure 6. Effects of folic acid or α-KG on hypoxia-induced nuclear translocation of NF-κB p65. THP-1 cells were pretreated with folic acid (4 μg/mL) or α-KG (1 mM) for 30 min and then incubated under hypoxic conditions (1% O_2) for 24 h. The NF-κB p65 subunit was detected by immunofluorescence and imaged using confocal microscopy. FITC-labeled NF-κB p65 (green) and DAPI-labeled nuclei (blue) in the cells were visualized.

LPS-induced NO, TNF-α, and IL-1β production in RAW264.7 cells [13]. Maternal folic acid supplementation during pregnancy protects against LPS-induced preterm delivery, fetal death and intrauterine growth retardation through its anti-inflammatory effects [12]. Additionally, folic acid supplementation may markedly reduce the circulating level of IL-8 in healthy overweight subjects [11]. Folic acid has been found to possess anti-inflammatory effects, but its concentration differs in various studies. We and others have performed dose-response studies, showing that the most effective concentration of folic acid to reduce inflammatory mediators is about 40 μg/mL, while the least effective concentration is about 0.2 μg/mL [13, 32]. Our study demonstrates that folic acid pronouncedly inhibits hypoxia-induced IL-1β and IL-8 production, as measured by proteins levels and gene-expression in THP-1 cells. The most effective concentration of folic acid in suppressing IL-1β and IL-8 under hypoxia is 40 μg/ mL. The effect of folic acid may be attributed to

regulation of the PHD1, IKKβ, and NF-κB, where the NF-κB pathway may reverse hypoxia-induced IL-1β and IL-8 production.

In summary, this study reveals that hypoxia initiates an inflammatory response as measured by inflammatory markers (IL-1β and IL-8) in THP-1 cells, via inhibition of PHD1 and upregulation of HIF-1α, IKKβ, and NF-κB p65. In addition, folic acid has pronounced anti-inflammatory effects, inhibiting secretion and gene-expression of IL-1β and IL-8 via regulation of the NF-κB inflammatory signaling pathway. These findings may represent one of the mechanisms by which folic acid exerts a protective effect in atherogenesis and progression of vascular disease.

Acknowledgements

Disclosure of conflict of interest

None.

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