Cyclooxygenase-2 contributes to the hypoxia-induced aggravation of the neuroinflammation response stimulated by lipopolysaccharide in microglia

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Abstract. Hypoxia and neuroinflammation are key risk factors involved in various pathophysiological neural disorders. Hypoxia can aggravate neuroinflammation *in vitro* and *in vivo*; however, the underlying mechanisms remain unknown. In the present study, hypoxia [either 3 or 1% oxygen (O_2)] increased lipopolysaccharide (LPS)-induced expression of the IL-6, IL-1β and TNF-α proinflammatory cytokines in BV2 cells. At the molecular level, both hypoxia and FG-4592, an hypoxia inducible factor 1 pathway activator, effectively induced cyclooxygenase-2 (COX-2) expression. The COX-2 inhibitor celecoxib significantly reduced the expression of cytokines induced by LPS under hypoxic conditions. Additionally, the administration of celecoxib inhibited the activation of microglia as well as cytokine expression in mice administered with hypoxia exposure and LPS injection. The present data demonstrated that COX-2 is involved in the hypoxia-induced aggravation of neuroinflammation stimulated by LPS.

Introduction

Neuroinflammation is involved in the pathophysiology of several neurological disorders, including stroke, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, epilepsy, traumatic brain injury and depression (1-3). During hypoxic-ischemic brain injuries, neuroinflammation can occur just minutes after damage (4,5). A number of studies report that hypoxia can aggravate the development of neuroinflammation. Song *et al* revealed that pretreatment with lipopolysaccharide (LPS) followed by hypoxia exposure markedly increases TNF-α and IL-1β levels compared with treatment of LPS alone in the plasma and brain cortex of rats (6). Another study previously reported that, compared with LPS alone, pretreatment with LPS followed by hypobaric hypoxia increases the development of neuroinflammation in a mouse model (7). These results indicate that hypoxia can aggravate LPS-induced neuroinflammation. However, the underlying mechanisms remain unclear.

Under hypoxic conditions, the activation of the hypoxia inducible factor (HIF) pathway is considered to be involved in the inflammatory process (8,9). The relationship between HIF-1 target genes that are activated by hypoxia and neuroinflammation has attracted considerable attention from researchers. Some studies suggest that hypoxia activates cyclooxygenase-2 (COX-2) expression in a HIF-1α-dependent manner, as a functional hypoxia response element has been identified in the COX-2 promoter sequence (10-12). Moreover, COX-2 is a key mediator of the inflammatory process (13). In response to growth factors, cytokines and pro-inflammatory molecules, COX-2 is rapidly expressed at mRNA levels and has emerged as the isoform (COX-1 is considered to be constitutively expressed) responsible for prostanoid production in acute and chronic inflammatory conditions. The selective inhibition of COX-2 may relieve the symptoms of inflammatory diseases (14). COX-2 inhibition has been reported to suppress the upregulation of IL-6, both at the mRNA and protein expression levels in BV2 cells. At the same time, the upregulation of IL-1β, TNF-α and monocyte chemoattractant protein-1 is also blocked (15). These results confirmed that inhibition of COX-2 could suppress neuroinflammation response. Whether inducible COX-2 plays an important role in the hypoxia-induced aggravation of neuroinflammation needs to be determined.

In the present study, the role of COX-2 in hypoxia-induced aggravation of neuroinflammation was investigated using both *in vitro* (microglial BV2 cells) and *in vivo* (C57BL/6 mice) models of neuroinflammation induced by LPS under hypoxic conditions. In addition, the current study demonstrated that celecoxib, a COX-2 inhibitor, attenuated neuroinflammation both *in vivo* and *in vitro*. The present results suggested COX-2

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is a promising therapeutic target in future strategies for the treatment of neuroinflammatory diseases.

Materials and methods

Cell culture. Mouse microglial BV2 cells (Cell Resource Center of the Chinese Academy of Medical Sciences) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Beijing Aoqing Biotechnology Co., Ltd.) and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2 and the medium was changed every 2 days. Cells were split with 0.125% trypsin when they reached 80% confluence and the passages 2-10 were used for carrying out experiments. For LPS treatment, the cells were treated with 100 ng/ml LPS (cat. no. L2630; Sigma-Aldrich; Merck KGaA) for the indicated times. To perform the hypoxia exposure experiments, BV2 cells seeded in 60-mm dishes ($8x10^5$ cells/dish) were put in the cell culture chamber with 1 or 3% oxygen (O_2) at 37˚C for the indicated times. For the FG-4592 (Selleck Chemicals) treatment, cells were treated with 10 μM FG-4592 for 6, 12 and 24 h at 37˚C. For the celecoxib (Selleck Chemicals) treatment, cells were pretreated with 10 and 20 μM celecoxib for 1 h at 37˚C.

Animal treatment. C57BL/6 mice (8-week-old; male; 18±2 g) were purchased from the Laboratory Animal Center of Vital River Experimental Animal Company. Mice were housed under a 12-h light/dark cycle at 22±2˚C with humidity 50±5% and with free access to standard rodent chow and water. The mice were maintained under specific pathogen-free conditions. All animal experimental procedures fully complied with the related laboratory animal regulations. A total of 16 mice were equally divided into four groups: i) Normoxia; ii) celecoxib; iii) LPS + hypoxia; and iv) celecoxib/LPS + hypoxia). The mice were intraperitoneally injected with celecoxib (20 mg/kg). After 30 min, mice were intraperitoneally injected with LPS (0.5 mg/kg). Subsequently, the mice were placed for 6 h in a hypobaric hypoxia chamber mimicking 6,000 m of altitude. After the treatment, the mice were anesthetized with sodium pentobarbital (50 mg/kg) via intraperitoneal injection, followed by cardiac perfusion with prechilled saline solution (0.9%) for 2 min. Thereafter, tissue samples were collected.

Western blotting. BV2 cells were collected and homogenized using RIPA lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 1.0 mM EDTA at a pH of 7.4) supplemented with 50X Protease Inhibitor Cocktail; Roche Diagnostics) on ice. The protein concentration was determined using the BCA assay (Applygen Technologies, Inc.). The total protein (20 μg/per lane) from each sample was separated using 8% SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes. After 1 h of blocking with 5% non-fat milk at room temperature, the membranes were incubated with primary antibodies against COX-2 (1:1,000; cat. no. A3560; ABclonal Biotech Co., Ltd.), HIF-1α (1:1,000; cat. no. 36169; Cell Signaling Technology, Inc.) and β-actin (1:5,000; cat. no. A2228; Sigma-Aldrich; Merck KGaA) overnight at 4˚C. After washing three times, the membranes were incubated with anti-mouse/rabbit IgG HRP-conjugated secondary antibodies (1:2,000; cat. no. 7076 and 7074, respectively; Cell Signaling Technology, Inc.) for 2 h at room temperature. Protein bands were visualized using an ECL detection kit (Bio-Rad Laboratories, Inc.). Quantification of the band intensities was performed using ImageJ Software (version 1.8.0; National Institutes of Health) and normalized to the band intensities of β-actin.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA in BV2 cells or hippocampus tissue was isolated using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Reverse transcription was performed using a HiScript III All-in-one RT SuperMix kit (Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. qPCR was subsequently performed using the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) and a CFX96 Real-Time PCR Detection System (Bio-Rad). The thermal cycling conditions include an initial denaturation step at 95˚C for 10 min, followed by 40 cycles at 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. The mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene β-actin (16). The primer sequences used for qPCR are presented in Table I.

Immunofluorescence staining. After the hypoxia treatment, the mice were collected from the hypoxia chamber, which was previously brought back to the local altitude. The mice were immediately anesthetized with sodium pentobarbital (50 mg/kg) via intraperitoneal injection and perfused with prechilled saline (0.9%) to remove circulating blood cells. The brain was fixed in 4% paraformaldehyde overnight. Each brain was dehydrated with 15 and 30% sucrose solutions, and then frozen sectioned (-20˚C) at a thickness of 40 μm. Thereafter, sections were permeabilized with 5% Triton X-100 at room temperature and blocked with 5% BSA for 30 min at room temperature. The sections were incubated with a specific primary antibody against ionized calcium-binding adapter molecule 1 (IBA1; 1:1,500; cat. no. 019-19741; Wako Chemicals USA, Inc.) at 4˚C overnight and then incubated with Alexa Fluor 594-conjugated donkey anti-goat secondary antibodies (1:1,000; cat. no. A32758; Thermo Fisher Scientific, Inc.) for 60 min at room temperature. The nuclei were counterstained with DAPI-containing mounting medium (ZSGB-BIO; OriGene Technologies, Inc.) for 15 min at room temperature. Images were captured using a scanning confocal microscope (Nikon Corporation).

Statistical analysis. Data were analyzed using GraphPad Prism version 7.0 (GraphPad Software, Inc.). Data are presented as the arithmetic mean ± standard error of the mean and the experiments were performed three times. Statistically significant differences between groups were determined using one-way ANOVA followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Hypoxia increases the expression of cytokines stimulated by LPS in BV2 cells. The impact of different hypoxic conditions on neuroinflammatory response stimulated by LPS in BV2 cells was determined by measuring the mRNA levels of IL-6, TNF-α and IL-1β via RT-qPCR analysis. The LPS treatment

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Genes	Forward (5'-3')	Reverse (5'-3')
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL-1β	TTCAGGCAGGCAGTATCACTC	GAAGGTCCACGGGAAAGACAC
IL-6	AGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Cyclooxygenase-2	AGGTCATTGGTGGAGAGGTG	CCTGCTTGAGTATGTCGCAC
β-actin	ACTGTCGAGTCGCGTCCA	GTCATCCATGGCGAACTGGT

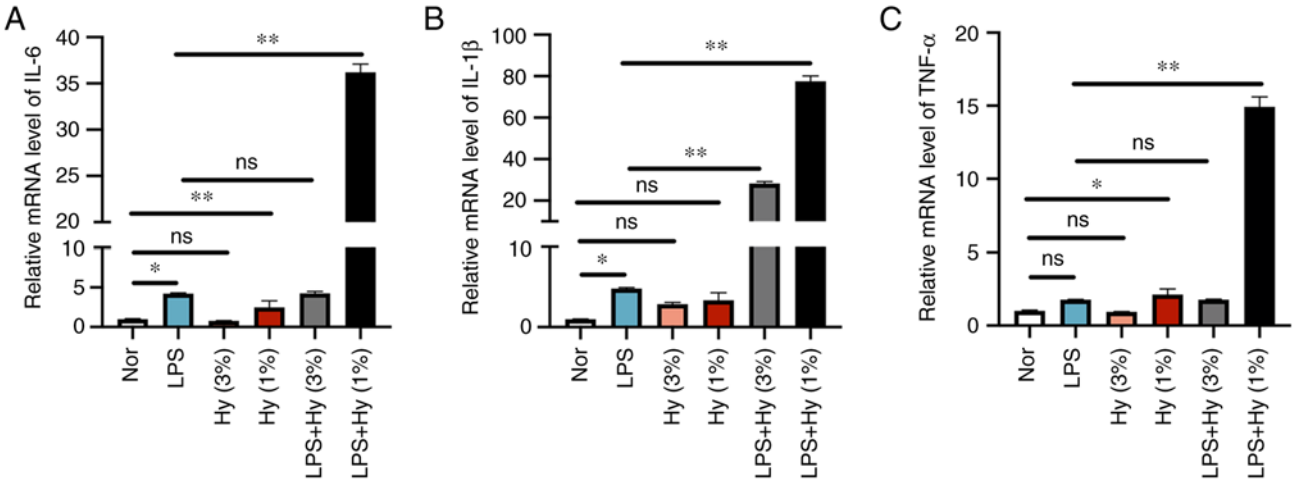

Figure 1. Hypoxia aggravates the LPS-induced neuroinflammatory response in BV2 cells. (A-C) BV2 cells were treated with 1% or 3% O_2 hypoxia and/or LPS (100 ng/ml) for 24 h. Total RNA was isolated and used to perform reverse transcription-quantitative PCR assays to measure the mRNA levels of (A) IL-6, (B) IL-1β and (C) TNF-α. The results are expressed as the mean ± SEM (n=3). *P<0.05 and **P<0.01. LPS, lipopolysaccharide; SEM, standard error of the mean; Nor, Normoxia; Hy, hypoxia; ns, not significant.

alone induced a significant increase in mRNA levels of the three cytokines IL-1β, TNF-α and IL-6 (Fig. 1A-C). When the cells were treated with LPS combined with hypoxia with 3% O_2, the mRNA levels of IL-1β were increased compared with those in the LPS treatment group. However, when the cells were treated with LPS combined with hypoxia with 1% O_2, the mRNA levels of IL-6, TNF-α and IL-1β were all increased compared with those in the LPS treatment group (Fig. 1A-C). Hypoxia with 1% O_2 without LSP treatment also induced a marked increase in the mRNA levels of these three cytokines, which was related to heavier hypoxia exposure. These results might indicate that hypoxia aggravated the inflammatory response in BV2 cells. Since hypoxia with 1% O_2 aggravated LPS-induced inflammation response more evidently than hypoxia with 3% O_2, 1% O_2 was selected as the representative hypoxia treatment condition for the subsequent experiments.

Hypoxia exposure increases the expression of COX-2 at both the mRNA and protein levels in BV2 cells. The activation of HIF-1 signaling pathway was first detected because it is a well-known marker responding to hypoxia exposure in microglia. Western blotting demonstrated that the protein levels of HIF-1α increased significantly following hypoxia exposure (Fig. 2A and B). To determine the effect of hypoxia on COX-2 expression, the mRNA levels of COX-2 were measured via RT-qPCR. Exposure to hypoxia for 6, 12 and 24 h significantly induced the upregulation of COX-2 by 4.1, 5.2 and 19.7-fold, respectively, compared with that in the normoxia control group (Fig. 2C). Moreover, western blotting demonstrated that exposure to hypoxia (1% O_2) for 6, 12 and 24 h increased the protein levels of COX-2, while there were no significant differences in the groups that were treated with LPS alone for 6, 12 and 24 h. Notably, the combination of hypoxia and LPS significantly increased the expression of COX-2 compared with that in the hypoxia group (Fig. 2D and E). These results might indicate that hypoxia and LPS treatment had a synergic effect on the expression of COX-2 protein.

HIF-1 activator FG-4592 induces the expression of COX-2. To further confirm the inducible effect of hypoxia on the expression of COX-2 in microglia, FG-4592, a prolyl hydroxylase (PHD) inhibitor, was applied. FG-4592 can stabilize HIF-1 and promote the expression of its target genes. Western blotting (Fig. 3A and B) demonstrated that FG-4592 significantly increased the protein levels of HIF-1α at 6, 12 and 24 h compared with that at 0 h (Fig. 3B). Moreover, the protein levels of COX-2 were also significantly increased at 12 and 24 h compared with that at 0 h (Fig. 3C), indicating that hypoxia induced the COX-2 expression via the HIF-1 pathway in BV2 cells.

Celecoxib inhibits the inflammatory response in BV2 cells. To determine whether COX-2 was involved in neuroinflammation

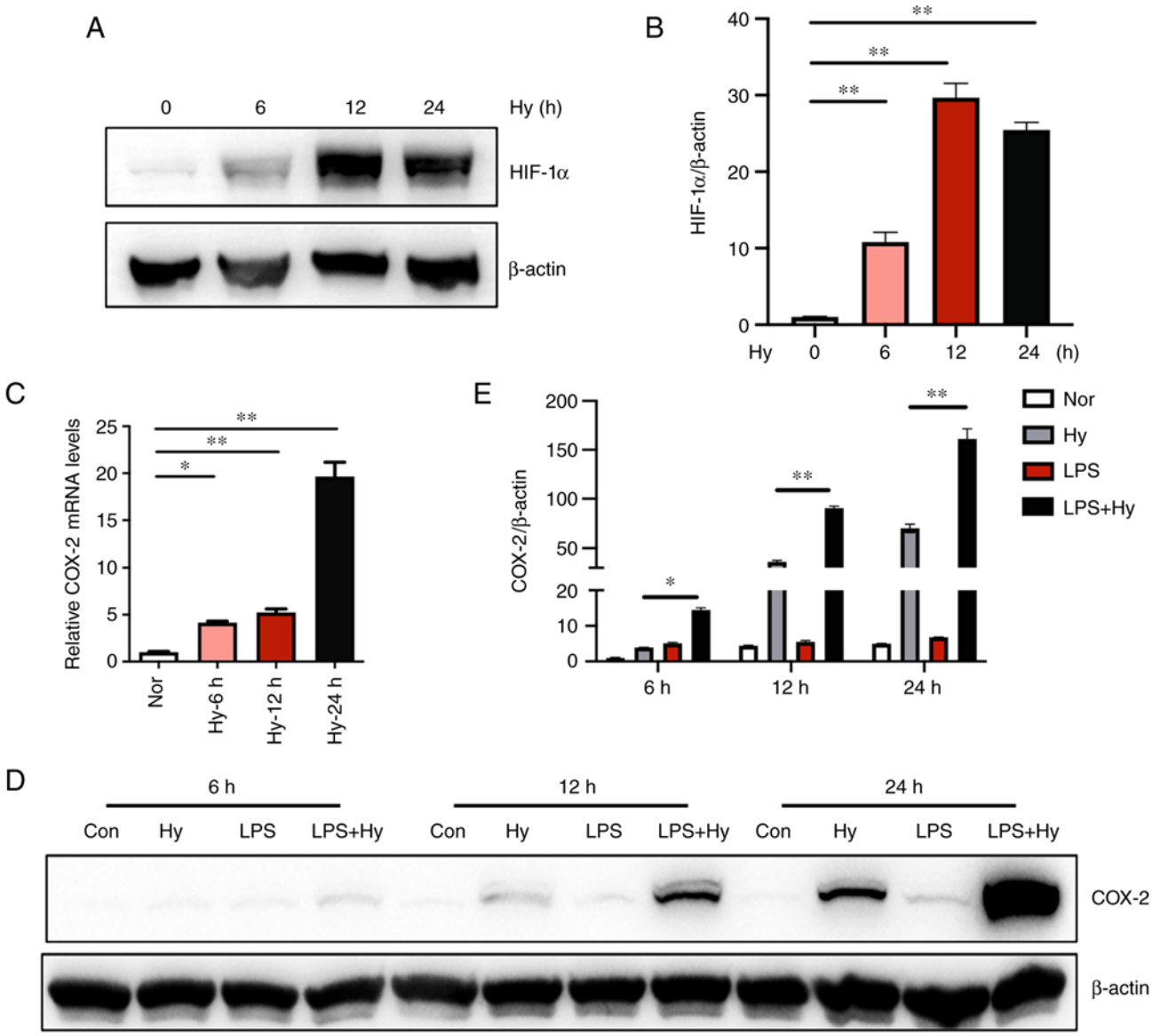

Figure 2. Hypoxia increases COX-2 expression at both the mRNA and protein levels in BV2 cells. (A and B) BV2 cells were exposed to 1% O_2 hypoxia for 6, 12 and 24 h. (A) Protein levels of HIF-1α were measured by western blotting and β-actin served as the internal control. (B) Quantification of the protein band densities of HIF-1α are presented as a bar graph. (C) BV2 cells were exposed to 1% O_2 hypoxia for 6, 12 and 24 h. Total RNA was isolated and used to perform reverse transcription-quantitative PCR assays to measure the mRNA levels of COX-2. (D) BV2 cells were treated with hypoxia (1% O_2) and/or LPS (100 ng/ml) for 6, 12 and 24 h. The protein levels of COX-2 were measured by western blotting and β-actin served as the internal control. (E) Quantification of the protein band densities of COX-2. The results are expressed as the mean ± SEM (n=3). *P<0.05 and **P<0.01. SEM, standard error of the mean; COX-2, cyclooxygenase-2; HIF-1α, hypoxia inducible factor 1α; Nor, normoxia; Hy, hypoxia; LPS, lipopolysaccharide.

under hypoxic conditions, the effect on inflammation of the COX-2 selective inhibitor celecoxib was investigated in BV2 cells. RT-qPCR demonstrated that exposure to hypoxia (1% O_2) combined with LPS (100 ng/ml) for 12 and 24 h significantly increased the expression of proinflammatory cytokines, such as IL-6, TNF-α and IL-1β. By contrast, pretreatment with celecoxib dose-dependently inhibited the increased mRNA levels of these cytokines caused by the combination of LPS treatment and hypoxia (Fig. 4A-C). These results might indicate that COX-2 was involved in the neuroinflammatory response under hypoxic conditions in BV2 cells.

Celecoxib suppresses microglial activation and decreases the mRNA levels of proinflammatory cytokines in mice. The effect of celecoxib on inflammation in mice was investigated. To evaluate neuroinflammation, immunofluorescence staining for IBA1 was performed to measure microglial activation. The proportion of IBA1-positive microglia was significantly increased by the combination of LPS treatment and hypoxia compared with the normoxic group, but this effect was significantly inhibited by celecoxib treatment (Fig. 5A and B). In addition, the mRNA levels of the three proinflammatory cytokines in the hippocampal region of the brain were measured. The RT-qPCR results indicated that celecoxib treatment significantly attenuated the significantly increased mRNA levels of TNF-α caused by the combination of LPS treatment and hypoxia (Fig. 5C-E). The mRNA levels of IL-6 and IL-1β were also decreased, but without statistical significance. These results indicated that blocking COX-2 inhibited the neuroinflammatory response under hypoxic conditions in a mouse model.

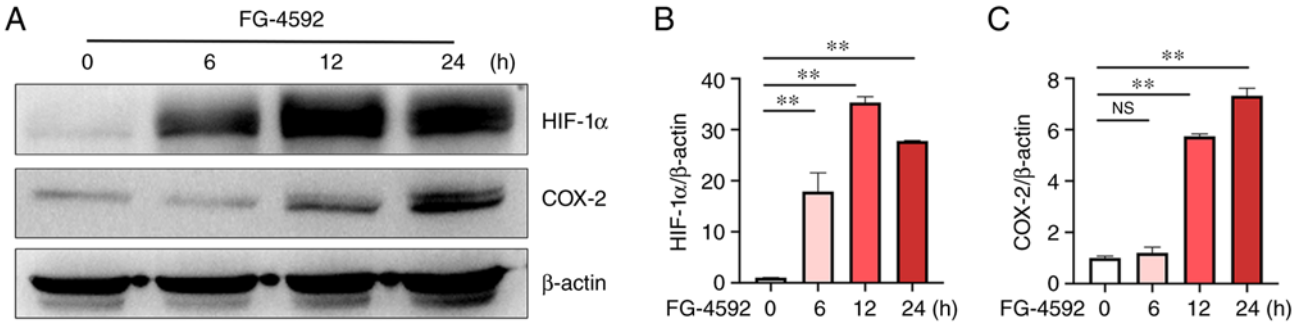

Figure 3. FG-4592 induces COX-2 expression. (A) BV2 cells were treated with FG-4592 (10 μM) for 6, 12 and 24 h. The protein levels of HIF-1α, COX-2 and β-actin in BV-2 cells in different groups were determined by western blotting. Quantification of the protein band densities of (B) HIF-1α and (C) COX-2. The results are expressed as the mean ± standard error of the mean (n=3). **P<0.01. COX-2, cyclooxygenase-2; HIF-1α, hypoxia inducible factor 1α; ns, not significant.

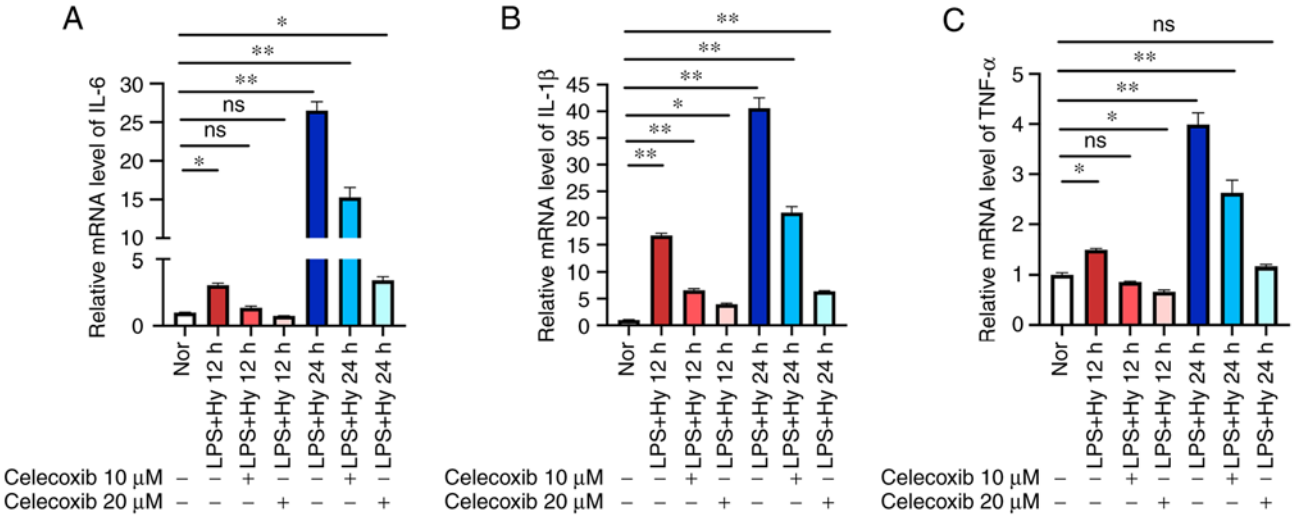

Figure 4. Celecoxib inhibits the neuroinflammatory response induced by the combination treatment of LPS and hypoxia in BV2 cells. (A-C) BV2 cells were pretreated with different doses of celecoxib (10 and 20 μM) for 1 h and then treated with 100 ng/mg LPS combined with 1% O_2 hypoxia for 12 and 24 h. Total RNA was isolated and used to perform RT-qPCR assays to measure the mRNA levels of (A) IL-6, (B) IL-1β and (C) TNF-α. The results are expressed as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01. LPS, lipopolysaccharide; Hy, hypoxia; Cel, celecoxib; ns, not significant.

Discussion

Microglia, resident mononuclear macrophage-like cells in the CNS, play an important role in cerebral inflammation (17). Microglia activation can be triggered in various injury processes, such as hypoxia, ischemia and immune responses (18). Hypoxia-induced neuroinflammation is the key pathological mechanism involved in acute mountain sickness (due to high altitude) or other various neural diseases, such as Alzheimer's disease, multiple sclerosis and traumatic brain injury. In addition, the peripheral immune system contributes to this process (19). Hypoxia has a direct role in microglial activation. It has been reported that hypoxia triggers the M1 phenotype in BV2 cells through the activation of STAT1 signaling (20). Zhang *et al* also revealed that acute hypoxia induces an imbalanced M1/M2 activation of microglia through the NF-κB signaling pathway (21). Furthermore, even preexposure to hypoxia for 3-6 days can lead to persistent and aberrant inflammatory responses (22). The present study revealed that compared with 3% O_2, 1% O_2 increased the expression of proinflammatory cytokines, including IL-6, TNF-α and IL-β. In addition, hypoxia markedly increased the neuroinflammatory response to LPS stimulation. Hypoxia and inflammation are two major pathogenic mechanisms of brain injury (23,24). Prompted by this phenomenon, the current study attempted to explore the underlying mechanisms of inflammation response under hypoxia conditions.

Numerous observational and experimental studies have indicated that there are several mechanisms involved in neuroinflammation under hypoxia (25,26). A recent study demonstrated that autophagy participates in neuroinflammation induced by hypoxia (26). In addition, it has been reported that membrane receptors are involved in neuroinflammation (27). Moreover, crosstalk between the NF-κB and HIF-1 signaling pathways may play important roles in this process (28). MicroRNAs also are involved in hypoxia-induced neuroinflammation (29). For example, induction of miR-3473b, which likely targets suppressor of cytokine signaling 3, contributes to stroke pathogenesis by enhancing poststroke neuroinflammation injury (30). Therefore, based on these different pathophysiological models and cell types and several treatment parameters, increasing numbers of signaling mediators have been revealed.

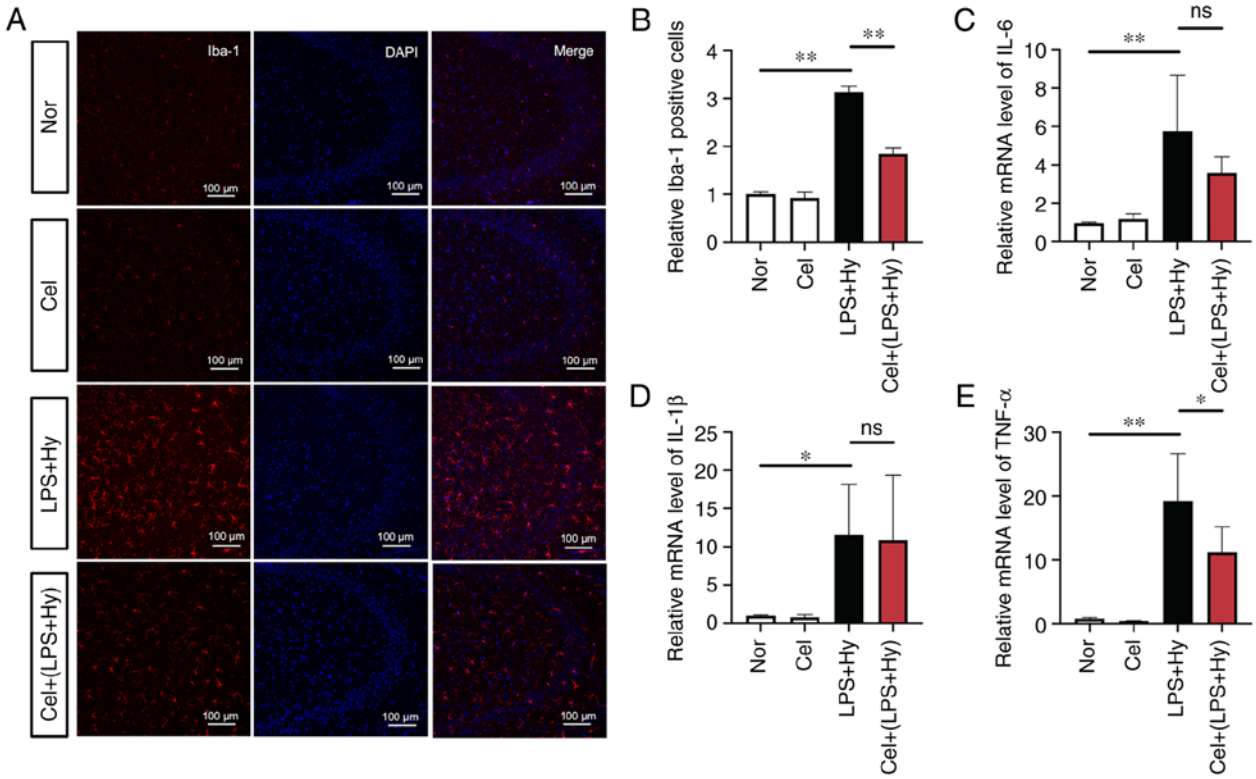

Figure 5. Celecoxib inhibits microglial activation and decreases the mRNA levels of proinflammatory cytokines in the mouse model. (A) Mice were pretreated with celecoxib (20 mg/kg) and then treated with lipopolysaccharide injection (0.5 mg/kg) and hypoxia exposure (mimicking 6,000 m of high altitude) for 24 h. The brain slices were subjected to immunofluorescence staining. Representative images of ionized calcium-binding adapter molecule 1 staining in the hippocampus. The nucleus was stained with DAPI. Scale bar, 100 μm. (B) Statistical analysis of microglial activation, as presented in A. The mRNA levels of (C) IL-6, (D) IL-1β and (E) TNF-α were measured via reverse transcription-quantitative PCR assay. The results are expressed as the mean ± SEM (n=4). *P<0.05 and **P<0.01. SEM, standard error of the mean; IBA1, ionized calcium-binding adapter molecule 1; LPS, lipopolysaccharide; Nor, normoxia; Hy, hypoxia; Cel, celecoxib; ns, not significant.

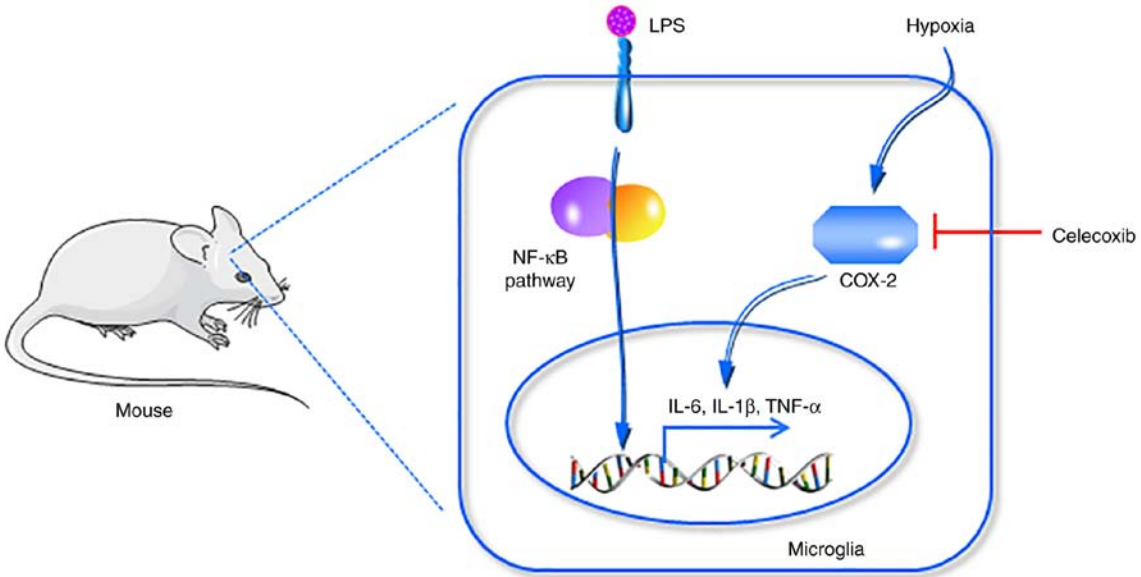

Figure 6. Schematic representation depicting the role of COX-2 in mediating hypoxia-induced aggravation of neuroinflammation stimulated by LPS. Hypoxia aggravated LPS-induced neuroinflammation responding *in vitro* and *in vivo*. Under hypoxic conditions, the expression of COX-2 was induced in microglia. Celecoxib, a specific inhibitor of COX-2, inhibited neuroinflammation response under hypoxia conditions. LPS, lipopolysaccharide; COX-2, cyclooxygenase-2.

COX-2 and prostaglandin E2 are well-known *in vitro* and *in vivo* inflammatory inducers. Previous studies have indicated that the expression of COX-2 is induced by the hypoxic microenvironment in various systems, such as tumors and epithelial cells (11,31). COX-2 is a direct HIF-1 target gene and acts as a mediator of both inflammation

and angiogenesis (32,33). However, the contribution of COX-2 to pro-inflammatory responses under hypoxic conditions remains unclear. The present study revealed that LPS produced no significant effect on COX-2 expression, but hypoxia induced COX-2 expression. This was further confirmed by treating cells with FG-4592, a well-known PHD inhibitor, which resulted in the activation of the HIF-1 signaling pathway. In addition, the increase in COX-2 expression observed upon treatment with both hypoxia and LPS was concomitant with the cytokine burst, indicating that COX-2 probably mediated hypoxia-induced aggravation of LPS-induced neuroinflammation. Consistently, celecoxib effectively inhibited the expression of cytokines induced by the combination treatment of hypoxia and LPS and a similar result was observed in the mouse neuroinflammation model. These results demonstrated that COX-2 served as an important mediator of hypoxia-induced aggravation of LPS-induced neuroinflammation.

COX-1 is constitutively expressed in a wide range of tissues, while COX-2 is an inducible enzyme that produces prostaglandins in inflammatory settings (34). Chauhan *et al* (35) revealed that COX-1 and COX-2 isoforms contribute to downstream proinflammatory responses in a high-altitude hypoxia exposure rat model (35). Based on a hypoxic postnatal rat model, Li *et al* (36) also demonstrated that prostaglandin E2 regulates inflammatory mediators in activated microglia via the prostaglandin E2 receptor-cAMP signaling pathway under hypoxic conditions. COX-2 is an intracellular, inducible protein that positively regulates cytokine signaling in numerous cell types (37). The present report focused on microglial cells, which are the main components of the innate immune system in the CNS (38). In BV2 cells, pretreatment with celecoxib decreased the expression of cytokines. Additionally, the administration of celecoxib also inhibited microglial activation in a mouse model injected with LPS treatment. The neuroprotective roles of celecoxib in other neural diseases, such as neurodegeneration caused by exposure to high altitude hypoxia (35), Alzheimer's disease (39), neonatal brain injury (40), autoimmune encephalomyelitis (41) and ischemic injury (42), should be considered. The present results indicated the potential significance of targeting COX-2 in the treatment of neuroinflammatory diseases, even under hypoxic conditions.

In summary, the current study revealed that COX-2, a downstream mediator of HIF-1, contributed to neuroinflammation response to hypoxic conditions. Blocking COX-2 function with celecoxib effectively inhibited neuroinflammation *in vivo* and *in vitro* (Fig. 6). The present results demonstrated that COX-2 is an important mediator of hypoxia-induced aggravation of LPS-induced neuroinflammation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MZ conceived and designed the experiments. YY, YG, XC, JG and ZS performed experiments. YY and YG analyzed and interpreted the data. MZ wrote and revised the manuscript. YY, YG and MZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Institutional Animal Care and Use Committee of the Beijing Institute of Basic Medical Sciences (approval no. IACUC-DWZX-2021-648).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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