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ORIGINAL ARTICLE

Hyperbaric Oxygen Alleviates the Inflammatory Response Induced by LPS Through Inhibition of NF-κB/MAPKs-CCL2/CXCL1 Signaling Pathway in Cultured Astrocytes

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Abstract—The purpose of this study was to investigate the inhibition neuroinflammation mechanisms of hyperbaric oxygen therapy (HBOT). Primary astrocytes were incubated with lipopolysaccharide (LPS) after which they underwent HBOT and separate administration of inflammatory cytokine inhibitors. The respective expression of inflammatory factors was then detected. Results showed that LPS significantly induced increases in the expression levels of chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine C-C motif ligand 2 (CCL2), phospho-nuclear factor-kappa B (p-NF-κB), phospho-c-Jun N-terminal kinase (p-JNK), phospho-extracellular signal-regulated kinase (p-ERK), and phospho-p38 (p-p38) in cultured astrocytes and peaked at 3 h. HBOT downregulated the expression of some inflammation mediators including CXCL1 and CCL2. Furthermore, HBOT inhibited the expression of some up-stream regulators of inflammation mediators including p-NF-κB, p-JNK, p-p38 (at 3 and 6 h), and p-ERK (3 h). Inhibitors of NF-κB, ERK, and JNK (BAY117082, PD98059, and SP600125) significantly suppressed the expression of CXCL1 and CCL2 that were induced by LPS for 3 h. However, the p38 inhibitor, SB203580, had no obvious effect on expression levels of CXCL1 and CCL2. In conclusion, we found that HBOT inhibits neuroinflammation *via* regulation of the LPS-induced NF-κB/mitogen-activated protein kinases (MAPKs, JNK, and ERK) - CCL2/CXCL1 signaling pathways.

KEY WORDS: hyperbaric oxygen; astrocytes; CCL2; CXCL1; LPS.

INTRODUCTION

Neuroinflammation is prominent in the short- and long-term responses of neuronal injuries that occur after traumatic brain injury (TBI). Neuroinflammation involves the activation of astrocytes and microglia followed by the active release of inflammatory mediators such as chemokines and cytokines by activated astrocytes [1, 2]. The chemokine C-C motif ligand 2 (CCL2, also known as monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-X-C motif) ligand 1 (CXCL1) are key mediators of inflammation following TBI [3–5]. Consistent with the above, our previous study showed that astrocytes of injured brains activated significantly in TBI rats [6]. We also found that CCL2 protein was mainly co-localized with the astroglial marker glial fibrillary acidic protein (GFAP) and that the expression of

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CCL2 was significantly increased in the injured cortex of TBI rats [7].

Hyperbaric oxygen therapy (HBOT) is widely used in TBI and has a neuroprotective effect by inhibiting the inflammatory response to TBI. Current reports have demonstrated that the neuroprotective effect of HBOT is partly realized by inhibiting or alleviating the inflammatory response with changes in expression levels of inflammatory factors such as interleukin (IL)-6, IL-1, IL-10, IL-1β, IL-18, CXCL1, CCL2, and tumor necrosis factor-α (TNF-α) in TBI animal studies [8–12]. Our preliminary studies demonstrated that the number of astrocytes in the ipsilateral hippocampal region and cortex in TBI rats was significantly reduced after 2 weeks of HBOT [6].

Based on above studies, HBOT reduced the activation of astrocytes and inhibited inflammation responses, but the mechanisms have not been fully clarified. In this study, the main aim was to investigate the effects of HBOT on lipopolysaccharide (LPS)-activated inflammatory mediator production (CXCL1, CCL2) in primary astrocytes from cerebral cortices of neonatal rats. In addition, we also studied whether HBOT has any effect on mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB), as well as explore possible mechanisms of inhibition of inflammation by HBOT.

MATERIALS AND METHODS

Primary Astrocytes Cultures

Primary astrocyte cultures were prepared from cerebral cortices of neonatal rats [13, 14]. Neonatal rats were provided by the Experimental Animal Center of Nantong University, China (Nantong, China). Bilateral cerebral cortices were isolated and transferred to ice-cold D-Hank's buffer and the meninges were carefully removed. The tissues were then dissociated and filtered through nylon mesh with a pore size of 100 μm and collected by centrifugation at ~3000×*g* for 5 min. The cell pellets were dispersed with a pipette and resuspended in a medium containing 10% fetal bovine serum (FBS) in low glucose Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA). After trituration, the cells were filtered through a 10-μm screen and then seeded into 6-well plates at a density of 2.5×10^5 cells/cm^2 and cultured for approximately 10 days. The medium was replaced every 3 days with 10% FBS. Dibutyryl cAMP (d-cAMP, 0.15 mM, Sigma, CA, USA) was added to induce morphological and functional differentiation when the cells were grown to 90% confluence.

The cells were used 3 days later. Opti-MEM (Gibco, Grand Island, NY, USA) was replaced and then the cells were incubated with lipopolysaccharide (LPS, 1 μg/mL, Santa Cruz, CA, USA) for different time periods from 1 to 12 h depending on the following experiments.

HBOT Intervention

When the astrocytes were ready, they were randomly divided into 4 groups: (1) control group; (2) control + HBO group; (3) LPS group; and (4) LPS + HBO group. The cells of the control + HBO group and LPS + HBO group at 2 or 5 h were transferred individually to HBO chamber [15]. The oxygen pressure was first increased over 10 min to 2 atm absolute (ATA), held at 2 ATA for 60 min (O_2 concentration above 98%), and then decreased over 10 min to atmospheric pressure for an 80-min HBO exposure. After the treatments, the cells were collected for ELISA or western blotting.

Drugs Administration

Inhibitors of NF-κB, ERK, JNK, and p38 were purchased from Calbiochem (Merck, Darmstadt, Germany). Before 30-min LPS induction, cells were pre-treated with specific inhibitors, including NF-κB inhibitor (BAY117082), JNK inhibitor (PD98059), ERK inhibitor (SP600125), and p38 inhibitor (SB203580) using two different doses: 20 and 50 μmol/L. Cultured cells were then collected for further experiments.

ELISA

Rat CCL2 ELISA kit was purchased from R&D systems (MN, USA). Rat CXCL1 ELISA kit was purchased from Lianke-Bio systems (Hangzhou, China). For *in vitro* experiments, cultured cells were collected after treatment or stimulation. Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL, USA). For each reaction in a 96-well plate, 100 μg of proteins or 100 μL of culture medium were used, and ELISA was performed according to manufacturer's protocol. The standard curve was included in each experiment.

Western Blotting

Protein samples were prepared in the same way as for ELISA analysis, and 30 μg of proteins were loaded for each lane and separated on SDS-PAGE gel (10%, Beyotime, Shanghai, China). After the transfer, the blots

were incubated overnight at 4 °C with polyclonal antibody against p-NF-κB (anti-rabbit, 1:1000, Cell signaling, Boston, USA), p-JNK (1:500, anti-rabbit, Cell signaling, Boston, USA), p-ERK (rabbit, 1:1000, Cell signaling, Boston, USA), and p-p38 (anti-rabbit, 1:1000, Cell signaling, Boston, USA). For loading control, the blots were probed with GAPDH antibody (1:10000, anti-mouse, Millipore, Billerica, MA, USA). These blots were further incubated with IRDye® 800CW (1:10000, Goat anti-Rabbit IgG, LICOR, Lincoln, NE, USA; 1:10000, Goat anti-Mouse IgG, LICOR, Lincoln, NE, USA) for 2 h. Blots were exposed with CLx Infrared Imaging System (LICOR, Lincoln, NE, USA). The intensity of the selected bands was analyzed using Image J software (NIH, Bethesda, MD, USA).

Quantification and Statistical Analysis

All data were expressed as mean ± SEM. For the quantification of Western blot, the density of specific bands for p-NF-κB, p-JNK, p-ERK, p-p38, and GAPDH were measured with imaging J software. The differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* multiple comparison tests. Differences between two groups were compared using Student's *t* test. The criterion for statistical significance was $P < 0.05$.

RESULTS

LPS Induces Increase of CXCL1 and CCL2 Expression in Cultured Astrocytes

The change in levels of expression of CXCL1 and CCL2 in primary astrocytes by LPS induction were detected by ELISA at 1, 3, 6, and 12 h. As shown in Fig. 1, the expression levels of CXCL1 and CCL2 in primary astrocytes were higher at 1 h compared to the control group. The peak expression levels for both transcripts were reached at 3 and 6 h of coculture with LPS and gradually decreased thereafter.

LPS Induces Upregulation of p-NF-κB, p-JNK, p-ERK, and p-p38 Expression in Cultured Astrocytes

LPS-induced NF-κB, JNK, ERK, and p38 activation in primary astrocytes was detected by western blotting at 1, 3, and 6 h. As shown in Fig. 2, the expression levels of p-NF-κB, p-JNK, p-ERK, and p-p38 in primary astrocytes were higher at 1 h compared with the control group. The peak expression levels of p-NF-κB, p-JNK, p-ERK, and p-p38 were all reached at 3 h of coculture with LPS and gradually decreased thereafter.

HBOT Suppresses LPS-Induced Expressions of CXCL1 and CCL2

To investigate the anti-inflammatory effects of HBOT on neuroinflammation, in LPS-induced primary astrocytes, ELISA was used to explore the changes of CXCL1 and CCL2 expression. As shown in Fig. 3a, b, the results showed that the exposure of primary astrocytes to LPS increased the production of CXCL1 and CCL2. Compared with the LPS group, CXCL1 and CCL2 expression in the HBO + LPS group was significantly reduced with HBO therapy 1 h after LPS stimulation at 3 and 6 h. These results demonstrate HBOT can downregulate the expression of CXCL1 and CCL2.

HBOT Reduces LPS-Induced Activation of p-NF-κB, p-JNK, p-ERK, and p-p38

To investigate the anti-inflammatory mechanism of HBOT, LPS-induced NF-κB, JNK, ERK, and p38 activation was detected in this study. As shown in Fig. 4a, a

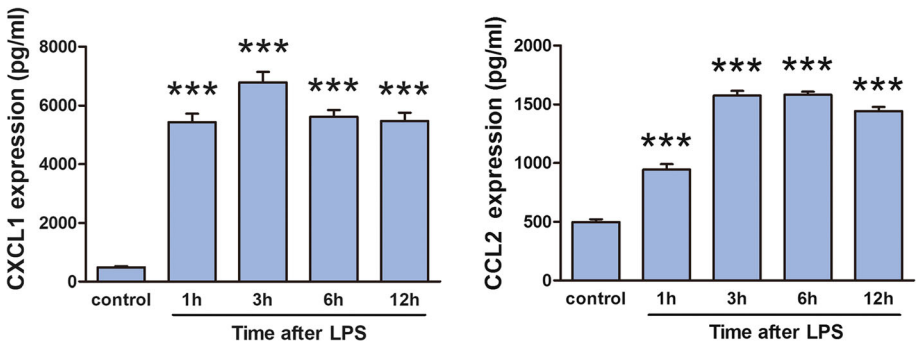

Fig. 1. LPS (1 μg/mL) induced high expressions of CXCL1 and CCL2 in cultured primary rat astrocytes. The values presented are the means ± SEM. ***$P < 0.001$ *vs.* control group.

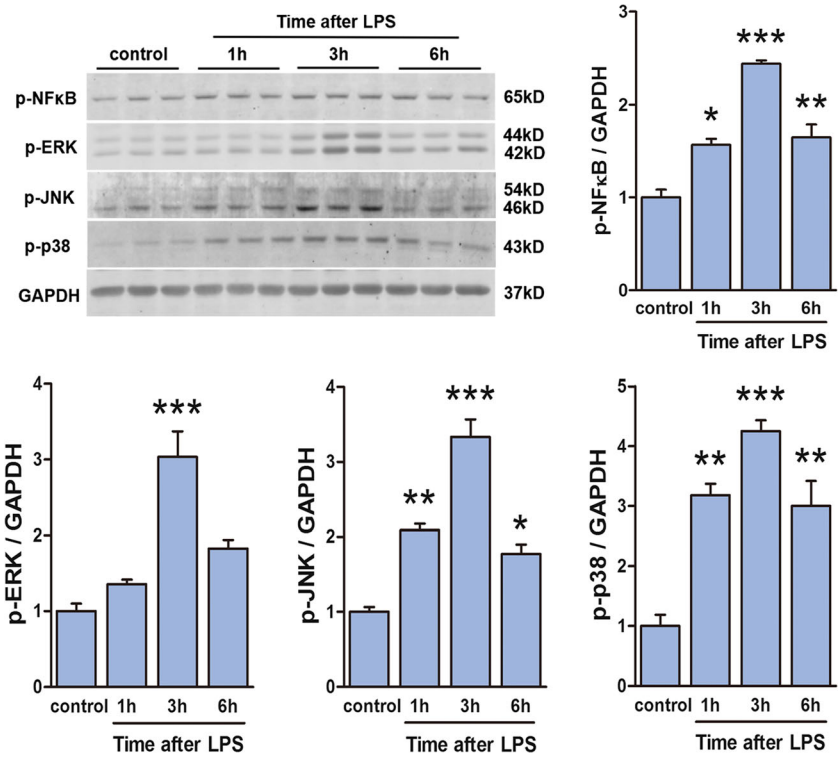

Fig. 2. LPS (1 μg/mL) induced NF-κB, JNK, ERK, and p38 activation in cultured primary rat astrocytes. The values presented are the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group.

significant increase in expression of p-NF-κB, p-JNK, p-ERK, and p-p38 resulted from LPS stimulation for 3 h in primary astrocytes was suppressed by HBO therapy for 1 h. As shown in Fig. 4b, HBO therapy on primary astrocytes for 1 h after coculture for 5 h with LPS resulted in a significant reduction in expression of p-NF-κB, p-JNK, and p-p38, although HBOT had no significant effect on p-ERK expression. From the above results, we concluded that HBOT may inhibit the expression of some up-stream regulators of mediators of inflammation. This needs to be examined more carefully.

Inhibitors NF-κB, JNK, and ERK Decrease CXCL1 and CCL2 Expressions

Based on the above results, we speculated that HBOT suppressed expression of CXCL1 and CCL2 by inhibiting the production of up-stream regulators of CXCL1 and CCL2. To verify this, we explored the changes in levels of expression of CXCL1 and CCL2 after pre-treated with inhibitors of NF-κB, ERK, JNK, or p38 at different doses (20 and 50 μM). As shown in Fig. 5a, b, inhibitors of NF-κB, ERK, and JNK (BAY117082, PD98059, and SP600125) significantly suppressed expression of CXCL1 and CCL2, which had been induced by LPS for 3 h. However, p38 inhibitor SB203580 (20 and 50 μM) had no obvious effect on expression of CXCL1 and CCL2. From the above results, we concluded that HBOT suppressed expression of CXCL1 and CCL2, by inhibiting the production of some up-stream regulators of CXCL1 and CCL2, including NF-κB, ERK, and JNK.

DISCUSSION

Astrocyte secreted chemokines CCL2 and CXCL1 are important mediators of inflammation that recruit inflammatory cells to sites of tissue injury in the central nervous system [7, 16–19]. CXCL1 secreted by astrocytes, and endothelial CXCR2 play essential roles in cerebral endothelial activation and subsequent leukocyte recruitment during neuroinflammation after intracerebroventricular injection of LPS [19]. LPS molecules are essential outer membrane components of most Gram-negative bacteria, which is composed of lipid, O-antigen, and the core oligosaccharide [20]. Astrocytes play a key role in the maintenance of neuronal functions in the central nervous

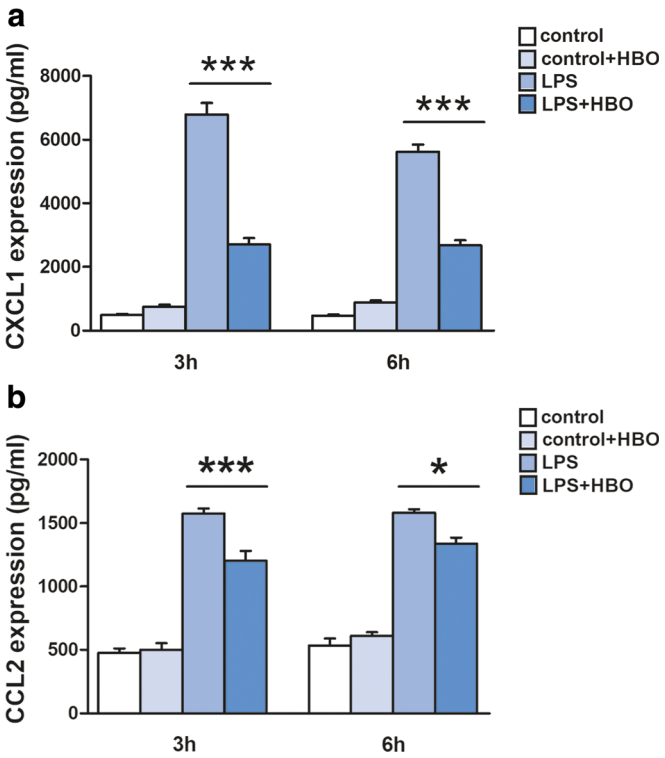

Fig. 3. a HBOT inhibits CXCL1 expression in activated astrocytes. The values presented are the means ± SEM of four independent experiments. *$P < 0.05$, ***$P < 0.001$ *vs.* LPS group. b. HBOT inhibits CCL2 expression activated astrocytes. The values presented are the means ± SEM of four independent experiments. *$P < 0.05$, ***$P < 0.001$ *vs.* LPS group.

system by producing various cytokines and chemokines. LPS-induced astrocytes release inflammatory molecules such as TNF-α, IL-10, CCL2 (MCP-1), NF-κB, ERK, JNK, and p38 in animal experiments [21–23]. In cultured human astrocytes, some expressed chemokines include CXCL1 and CCL2. The NF-kB signaling pathway differentially regulates gene expression of chemokines that play both neuroprotective, and neurotoxic roles in neuroinflammation in human astrocytes under physiological and inflammatory conditions [24].

In this study, LPS induced significant upregulation of CXCL1, CCL2, p-NF-κB, p-JNK, p-ERK, and p-p38 expression in cultured primary astrocytes. Inhibitors of NF-κB, ERK, and JNK significantly suppressed expression of CXCL1 and CCL2 that was induced by 3-h LPS treatment. However, p38 inhibitor had no obvious effect on expressions of CXCL1 and CCL2. The results confirmed the role of NF-κB, ERK, and JNK signaling pathways by using specific inhibitors, which mediated suppression of LPS-induced CXCL1 and CCL2 production.

TBI secondary injury involves a cascade of molecular mechanisms that are activated over hours to days and include the critical mechanism of neuroinflammation [25, 26]. TBI significantly up-regulated MAPKs (ERK, JNK, and p38) and NF-κB signaling, in injured brain regions of a rodent model of TBI, which also involved a proinflammatory response [27–29]. HBOT is defined as the inhalation of pure oxygen in a hyperbaric chamber that is pressurized higher than 1 absolute atmosphere and proposed widely as an effective treatment for TBI. HBOT reduces proinflammatory factor generation and promotes anti-inflammatory factor production, thereby reducing inflammatory responses in brain tissue and assisting in reducing TBI secondary injury [8, 30, 31]. Our study demonstrated HBOT alleviated the expression of proinflammatory mediators CXCL1 and CCL2, in LPS induced in primary astrocytes. HBOT has recognized therapeutic effect through inhibition of the Toll-like receptor 4 (TLR4)/NF-kB and vascular endothelial growth factor (VEGF)/ERK signaling pathways after TBI in rats [10, 32]. We furthermore found that HBOT downregulated the expressions of up-stream regulators of inflammatory mediators including p-NF-κB, p-JNK, p-p38, and p-ERK. Taken together, these results suggested that HBOT inhibited LPS-induced CXCL1 and CCL2 by inhibiting NF-κB, JNK, and ERK signaling pathways in cultured primary rat astrocytes.

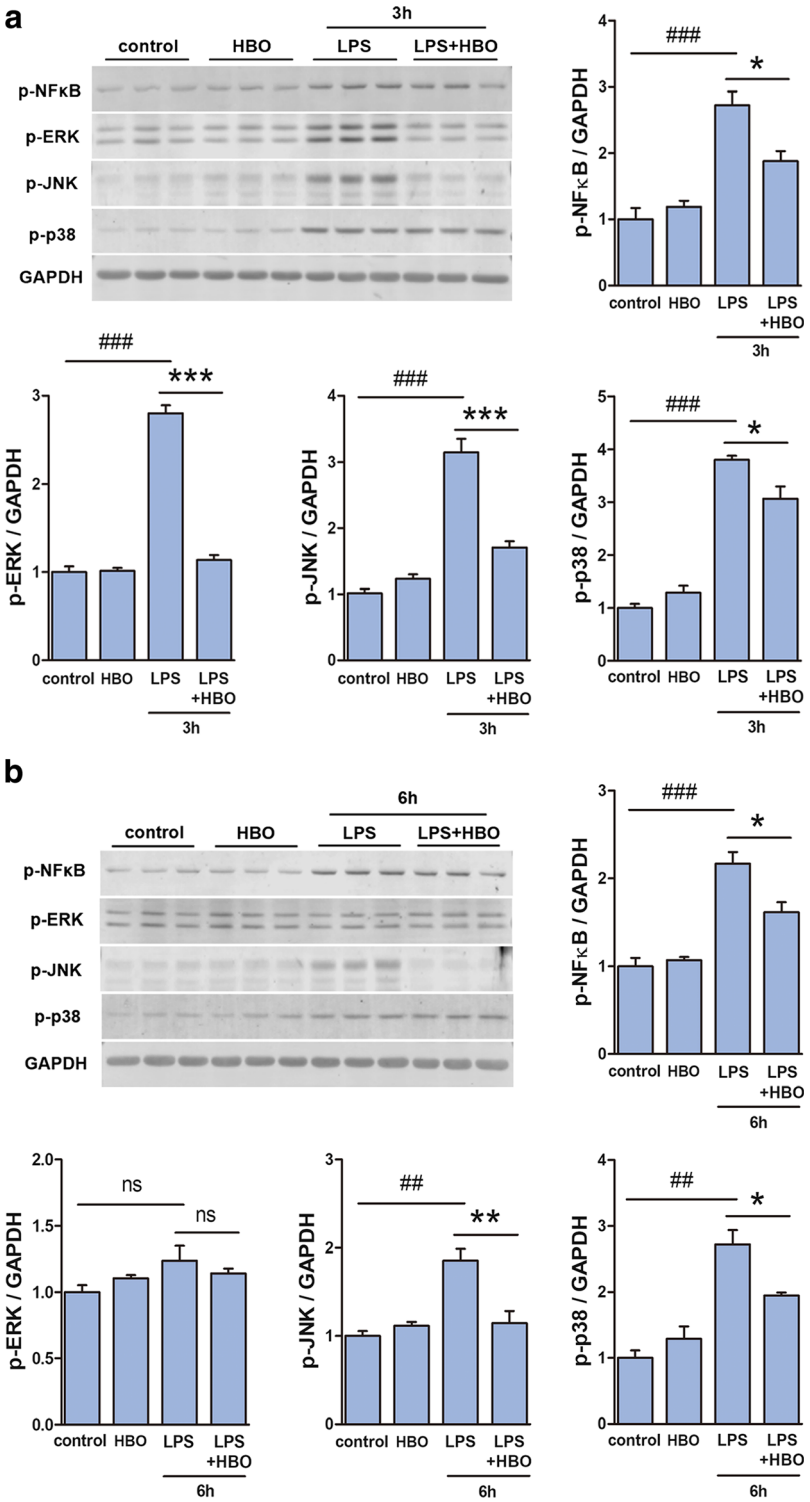

Fig. 4. a HBOT inhibits p-NF-κB, p-JNK, p-ERK, and p-p38 expressions after astrocyte activation by LPS-induced 3 h. The values presented are the means ± SEM of four independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* LPS group. $^{###}P < 0.001$ *vs.* control group. b HBOT inhibits p-NF-κB, p-JNK, and p-p38 expressions after astrocyte activation by LPS-induced 6 h. The values presented are the means ± SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* LPS group. $^{##}P < 0.01$, $^{###}P < 0.001$ *vs.* control group.

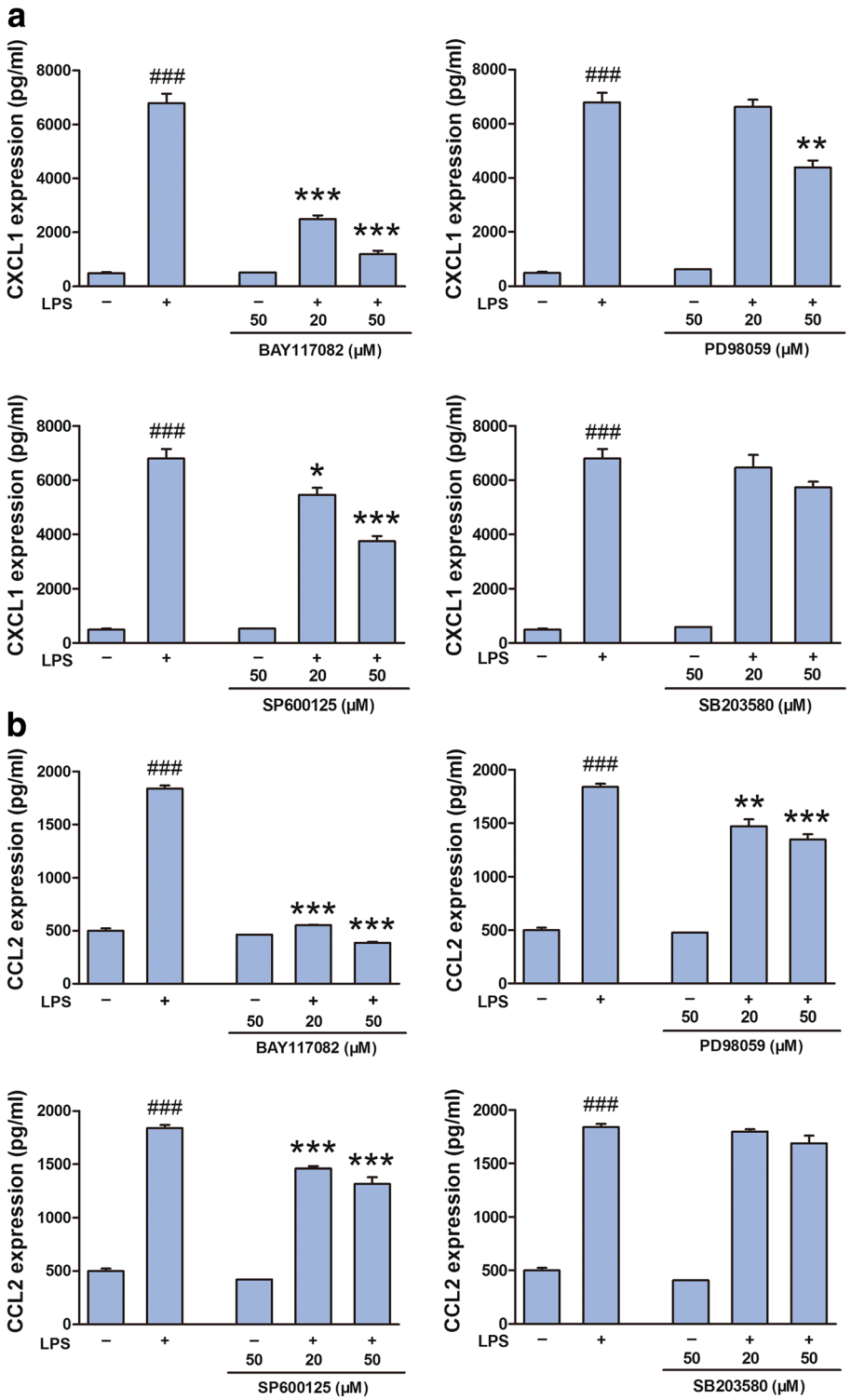

Fig. 5. a Inhibitors p-NF-κB (BAY117082, 20 and 50 μM), p-JNK (PD98059, 50 μM), and p-ERK (SP600125, 20 and 50 μM) reduce CXCL1 expression in activated astrocytes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* LPS group. $^{\#\#\#}P < 0.001$ *vs.* control group. b Inhibitors of p-NF-κB (BAY117082, 20 and 50 μM), p-JNK (PD98059, 20 and 50 μM), and p-ERK (SP600125, 20 and 50 μM) reduce CCL2 expression in activated astrocytes. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* LPS group. $^{\#\#\#}P < 0.001$ *vs.* control group.

In conclusion, our results showed that HBOT inhibited neuroinflammation *via* inhibiting the LPS-induced NF-κB/MAPKs (JNK and ERK)-CCL2/CXCL1 signaling pathways. This provides the theoretical basis for clinical treatment with HBOT.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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