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Regulation of aquaporin 4 expression by lipoxin A4 in astrocytes stimulated by lipopolysaccharide

Jun Wu[a,*,1], Danhua Ding[a,1], Xinyu Wang[b,1], Qianqian Li[a], Yuying Sun[a], Lanjun Li[a], Yangyang Wang[a]

[a] Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China
[b] Department of Rheumatology, Peking University Third Hospital, Beijing 100191, China

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ABSTRACT

Aquaporin (AQP4) could be associated with inflammation, common in central nervous system diseases. We investigated the effect of lipoxin A4 (LXA4) on the activation of astrocytes, AQP4 expression, and inflammatory response induced by lipopolysaccharide (LPS). Astrocytes were cultured *in vitro* and changes in transcript and protein levels of AQP4, interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and cyclooxygenase-2 (COX-2), and protein levels of P38 and phospho-P38 were determined. The LPS group showed increased AQP4, IL-1β, TNF-α, and COX-2 levels, whereas they decreased in the LPS + LXA4 group, suggesting that LXA4 inhibits AQP4 expression. Furthermore, levels of phospho-P38 increased in the LPS group, but decreased in the LPS + LXA4 group. In conclusion, LXA4 alleviated the LPS-induced increase in AQP4 expression and inflammatory cytokine secretion by astrocytes, possibly by inhibiting P38 phosphorylation. For the first time, we found that LXA4 may inhibit the expression of inflammatory factors by regulating the expression of AQP4. AQP4 on astrocytes is likely to be the target of anti-inflammatory effect of LXA4.

1. Introduction

Inflammation is a common pathological phenomenon that affects various parts of the body. It plays an important role in the pathogenesis of various central nervous system diseases, such as stroke and nervous system degeneration [1–4]. As the most abundant cell type in the mammalian brain, astrocytes participate in central nervous system inflammation by synthesizing and secreting various neurotrophic factors and cytokines. Aquaporin 4 (AQP4) is expressed in astrocytic foot processes and primarily regulates the water balance inside and outside the brain cell membrane. Recently, it was reported that AQP4 could also be involved in the occurrence and development of nervous system inflammatory responses [5]. Lipoxins (LXs) are endogenous lipid mediators produced during the inflammatory response. They have strong anti-inflammatory effects and promote the timely elimination of inflammation. The anti-inflammatory and neuroprotective effects of LXs in nervous system inflammation have been reported [6–8], but the specific mechanism remains unclear. It is unclear whether AQP4 is the target of LXs. Therefore, we studied the effect of lipoxin A4 (LXA4) on lipopolysaccharide (LPS). We observed the expression of AQP4 and inflammatory mediators after activation of astrocytes by LPS. The effects of LXA4 on the expression of AQP4 in astrocytes induced by LPS and its antagonistic effect on inflammatory response were also observed. Further, we discuss a possible mechanism to provide theoretical basis for the treatment of inflammatory diseases of the nervous system using LXA4.

2. Materials and methods

2.1. In vitro culture and identification of astrocytes

The cortical tissue was taken from newborn BALB/c mice aged 0–1 day, and the cortex was cut into D-hanks solution to strip the meninges and blood vessels. The cortex was shred into pieces of approximately 1 mm^3. After trypsin digestion, the supernatant was centrifuged and neutralized in the medium, prior to another centrifugation and discarding the supernatant. DMEM/F12 medium containing 20% fetal bovine serum was added gently to blow the cells, which were filtered using 200 mesh cell filter, and the collected filtrate was transferred into 50 mL culture bottle for incubation at 37 °C, 5% CO_2. When the cells

* Corresponding author.
E-mail address: wjun365@163.com (J. Wu).
[1] These authors contributed to the work equally and should be regarded as co-first authors.

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reached approximately 80% fusion (10–12 days), the cultures were moved to 37 °C shaking table for 18 h, and the cells were digested with 0.25% trypsin to obtain purified astrocytes. Glial fibrillary acidic protein (GFAP) antibody was used for identification. Follow-up experiments were performed using cultured third-generation astrocytes.

2.2. Experimental groups

The different experimental groups were defined as control group, to which serum-free medium containing 0.005% ethanol was added; LXA4 group, to which serum-free medium containing 5 ng/mL LXA4 was added; LPS group, to which after pretreatment with serum-free medium containing 0.005% ethanol for 0.5 h, LPS was added to a final concentration of 5 mg/L; and LXA4 + LPS group, to which after pretreatment with serum-free medium containing 5 ng/mL LXA4 for 0.5 h, LPS was added to a final concentration of 5 mg/L. Each experiment at least 3 duplication.

2.3. Immunofluorescence staining of AQP4

When the cells were passaged to the third generation, cover slides placed in a 24-well plate were used for cell observing climbing. Each well was fixed with 200 μL of 4% paraformaldehyde for 30 min and cleaned with phosphate buffered saline (PBS) three times. Two hundred microliters of 1% Triton X-100 was added to each well for 10 min, washed three times with PBS and sealed with goat serum. Next, 120 μL GFAP antibody was added to each well, and the plate was placed in a wet box at 4 °C overnight. The following day, the primary antibody was removed and the plate was washed three times with PBS at 25 °C. FITC-labeled secondary antibody (120 μL) was added to each well, and incubated for 1 h at room temperature. After secondary antibody treatment, the plate was washed with PBS thrice in the dark. The cells were stained with 120 μL DAPI solution by incubating for 5 min at room temperature. The DAPI solution was aspirated, and the plate washed in the dark prior to mounting and photographing under an inverted fluorescence microscope.

2.4. Expression of AQP4 and inflammatory mediators

Quantitative real time – polymerase chain reaction (qRT-PCR) was performed to investigate the expression of AQP4, interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and cyclooxygenase-2 (COX-2). Total RNA was extracted from cells in the different experimental groups using TRIzol reagent, cDNA was reverse transcribed using HiScript Q RT SuperMix reverse transcription kit (Vazyme, Biotechnology, Nanjing), and the cDNA was used as template for PCR. Murine glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The primer sequences used for qRT-PCR are listed in Table 1. The PCR conditions were 95 °C for 5 min, and 40 cycles of 95 °C for 10 s, 60 °C for 30 s. The relative amount of initial cDNA in each sample was calculated by cell threshold cycle values and a standard curve.

2.5. Western blot analysis

The protein levels of AQP4, P38 and P-P38 proteins were determined by western blot analysis. The total protein of each sample was extracted using the RIPA method and protein concentration was determined with the BCA method. The proteins were separated on a 12% gel and transferred to PVDF membrane, which was blocked with 5% skim milk in PBST buffer at room temperature for 2 h. Primary antibody for AQP4 (1:200, SANTA CRUZ) was incubated overnight at 4 °C. After washing, the membrane was incubated in secondary antibody (1:10,000, (Proteintech Group, Wuhan) at 37 °C for 2 h. The membrane was washed and developed. Quantitative image analysis was performed on the Image J software.

2.6. Enzyme-linked immunosorbent assay (ELISA)

IL-1β, TNF-α and COX-2 protein levels in the cell culture supernatant were determined using the ELISA kit (ExCell Technology Company) according to manufacturer's instructions.

2.7. Statistical analysis

SPSS 21.0 statistical software was used to analyze the data, which is expressed as mean ± SD. One-way analysis of variance (ANOVA) was used for comparison between groups, and pairwise comparison between samples was tested using the Bonferroni method. The results were considered significant when $P < 0.05$.

3. Results

3.1. Primary astrocyte identification

Primary astrocytes were identified by immunofluorescence analysis. Fluorescence microscopy showed blue fluorescence of the nucleus after DAPI staining (Fig. 1A), and green fluorescence of the cell body after GFAP staining. Also, the cell body was stretched, which was consistent with typical astrocyte morphology (Fig. 1B). The cell purity was analyzed using image analysis software, and determined to be over 95%.

3.2. Effect of LXA4 on the expression of AQP4 induced by LPS in astrocytes

We first determined the effect of LPS treatment on astrocytes treated for 1 h. qRT-PCR results showed that AQP4 expression was significantly increased ($P < 0.001$) upon LPS treatment, compared to the control group. However, in the LXA4 group, AQP4 expression was significantly lower than that in the LPS group ($P < 0.001$) (Fig. 2).

Next, we determined the effect of treating astrocytes for 2 h with LPS. Western blot analysis showed that the protein level of AQP4 was significantly higher in the LPS group compared to the control group ($P < 0.001$). However, after treatment with LXA4, the protein level of AQP4 was lower than that of the LPS group ($P < 0.01$) (Fig. 3).

Immunofluorescence analysis of astrocytes treated with LPS for 2 h showed increased levels of AQP4 compared to the control. However, after treatment with LXA4, the protein level of AQP4 was lower than that in the LPS group (Fig. 4).

Table 1
Primer sequences for qRT-PCR.

Primer	Forward sequence	Reverse sequence
GAPDH	5′–GGGTGTGAACCACGAGAAAT–3′	5′–CCTTCCACAATGCCAAAGTT–3′
AQP4	5′–AATCCCGCTGTGACTGTAGC–3′	5′–GGGAGGTGTGACCAGGTAGA–3′
TNF-α	5′–CCGATGGGTTGTACCTTGTC–3′	5′–AGATAGCAAATCGGCTGACG–3′
IL1-β	5′–GCTGCTTCCAAACCTTTGAC–3′	5′–AGCTTCTCCACAGCCACAAT–3′
COX-2	5′–TCCTCACATCCCTGAGAACC–3′	5′–GTCGCACACTCTGTTGTGCT–3′

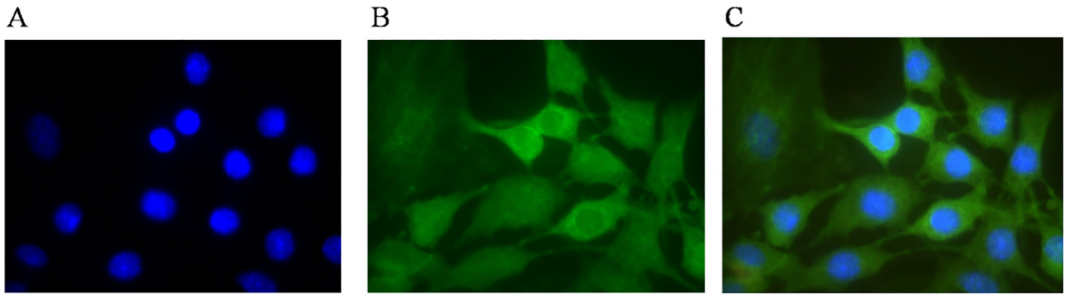

Fig. 1. Fluorescence microscopy of GFAP staining of mouse cortical astrocytes at a magnification of 40×. A The nucleus is blue after DAPI staining. B The cytoplasm of the cells is green after GFAP staining. C The morphology of cells after merging images in A and B.

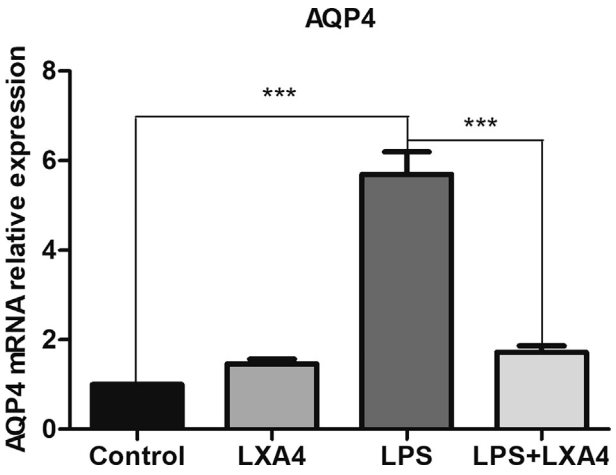

Fig. 2. qRT-PCR analysis of the expression level of AQP4. The expression of AQP4 in the LPS group was significantly higher than that in the control group ($P < 0.001$). The expression of AQP4 in the LPS + LXA4 group was significantly lower than LPS group ($P < 0.001$). RQ, mRNA relative quantification with the expression in control group set to 1.

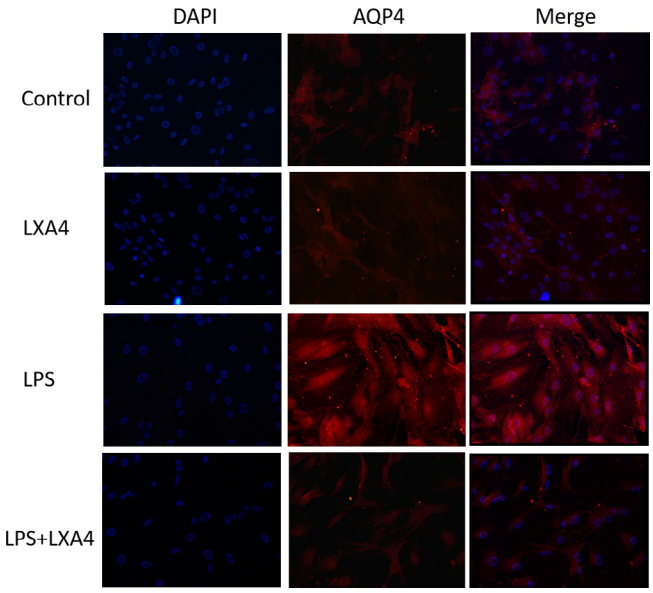

Fig. 4. Immunofluorescence detection of AQP4 protein level observed at a magnification of 400×. Higher levels of AQP4 protein was observed in the LPS group compared to the control and LPS + LXA4 groups.

3.3. Effect of LXA4 on the expression of inflammatory cytokines in astrocytes induced with LPS

qRT-PCR analysis of astrocytes treated with LPS for 2 h showed higher expression of IL-1β ($P < 0.001$), TNF-α ($P < 0.01$) and COX-2 ($P < 0.01$) compared to the control group. After treatment with LXA4, the expression of IL-1β, TNF-α and COX-2 was lower than that in the LPS group ($P < 0.05$) (Fig. 5).

When the astrocytes were treated with LPS for 6 h, ELISA showed that the protein levels of IL-1β, TNF-α and COX-2 were higher compared to the control group ($P < 0.001$). LXA4 treatment significantly decreased the levels of IL-1β ($P < 0.01$), TNF-α ($P < 0.001$) and COX-2 ($P < 0.01$) (Fig. 6).

3.4. Effect of LXA4 on the expression of phospho-P38 (P-P38) induced by LPS in astrocytes

We used western blot analysis to determine the level of P-P38 in astrocytes treated with LPS for 0.5 h. Significantly higher level of P-P38 was observed in the LPS group compared to the control group ($P < 0.001$). However, the P-P38 protein level was lower in the LPS + LXA4 group compared to the LPS group ($P < 0.001$) (Fig. 7).

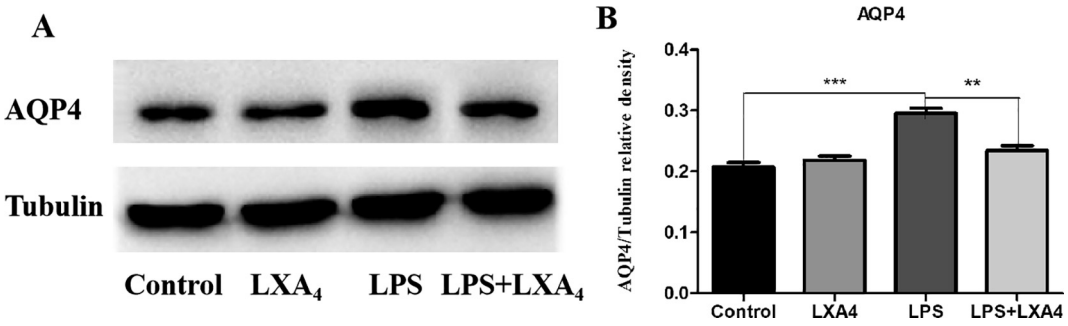

Fig. 3. Western blot detection of AQP4 protein showed significantly higher levels in the LPS group than in the control group ($P < 0.001$). LPS + LXA4 group showed significantly lower levels of AQP4 compared to the LPS group ($P < 0.01$).

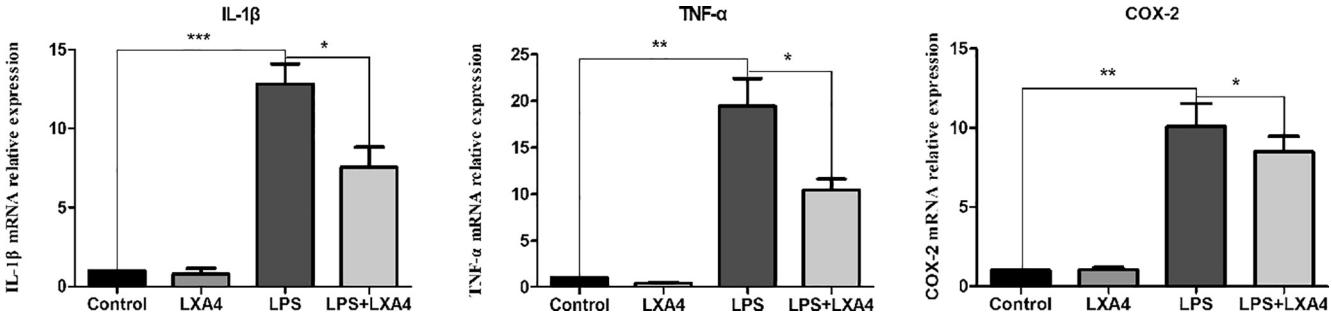

Fig. 5. qRT-PCR analysis of IL-1β, TNF-α, and COX-2. The transcript levels of IL-1β ($P < 0.001$), TNF-α ($P < 0.01$) and COX-2 ($P < 0.01$) in the LPS group was significantly higher than in the control and LPS + LXA4 ($P < 0.05$) groups. RQ, mRNA relative quantification with the expression in control group set to 1.

4. Discussion

Glial cells are widely distributed in the central nervous system. Astrocytes, the main type of glial cells, play an important role in the inflammatory response of the nervous system. Under physiological conditions, astrocytes play a role in maintaining glutamate homeostasis, water balance, ion homeostasis, blood-brain barrier integrity, regulation of cerebral blood flow and secretion of neuroprotective factors [9]. In stroke, Alzheimer's disease and other pathological conditions, astrocytes release potential virulence factors or participate in inflammatory processes, leading to further progression of the disease [10,11]. Among them, activated astrocytes secrete IL-1β, TNF-α and other inflammatory factors to induce neuronal death and aggravate nerve injury after stroke [11]. IL-1β activates signaling pathways, such as mitogen-activated protein kinase (MAPK) and aggravates the inflammatory response [12]. The combination of TNF-α and TNF receptor 1 triggers a signaling cascade resulting in cell death. In fact, the infarct area of TNF-α knockout mice is smaller, and the infarct volume increases with the injection of TNF-α [13]. TNF activates NF-κB, P38, and other signaling pathways, causing migration, apoptosis, and necrosis of cells, leading to inflammatory reactions [14]. Therefore, inhibiting or reducing the production of inflammatory factors is particularly important for reducing disease and improving prognosis.

AQPs are a group of membrane proteins that regulate the water balance inside and outside the cell and affect the transmembrane transport of water. Currently, 13 proteins AQP0-12 have been identified, which differ in size and permeability [15,16]. The main function of AQPs is to increase the permeability of the cell membrane to water and to regulate water transport inside and outside the cell. AQP4 is the most abundant aquaporin expressed in the central nervous system, mainly distributed in the astrocyte podocyte [17]. AQP4 is widely distributed in the intersection of brain tissue and blood and cerebrospinal fluid, which is the important position of water transport between cells, blood vessels and ventricles [5]. Recent studies have found that AQP4 also plays an important role in the inflammatory response of stroke, multiple sclerosis, Alzheimer's disease, and other pathological conditions. In a transient bilateral carotid occlusion model, AQP4 knockout mice had less blood-brain barrier damage, less inflammatory response, and fewer neuronal deaths than normal mice [18]. In a cerebral hemorrhage model, cerebrolysin was shown to inhibit the expression of AQP4 and the production of inflammatory factors such as TNF-α was reduced. This could be due to the decrease of AQP4 expression and the decrease of pro-inflammatory effect [19]. In addition, the application of apelin-13 [20] and autologous bone marrow monocytes [21] reduce the expression of AQP4 and production of inflammation-related substances such as matrix metalloproteinase after cerebral hemorrhage. In the mouse multiple sclerosis model, AQP4 gene knockout improved the symptoms of neurological deficit, tissue inflammation was mild, and the production of TNF-α and IL-6 was reduced [4]. Typical inflammatory features in patients with Alzheimer's disease include activation of astrocytes with increased AQP4 expression [22]. Knockout of the mouse AQP4 gene can attenuate LPS-induced inflammatory response and decrease the expression of IL-1β and TNF-α in astrocytes. The above studies collectively suggest that AQP4 could have an intrinsic pro-inflammatory effect [23]. AQP4 could be involved in an inflammatory process with important links and targets for regulation.

Lipoxin is a lipid mediator with strong anti-inflammatory effects. According to its molecular structure and conformation, lipoxins can be divided into four categories, LXA4, LXB4, and their stereoisomers 15-*epi*-LXA4 and 15-*epi*-LXB4 [24]. Lipoxins inhibit chemotaxis, exudation, and adhesion of granulocytes, enhances the phagocytic apoptosis of macrophages, promotes the adhesion and chemotaxis of monocytes, and downregulates the inflammatory response by downregulating the expression of pro-inflammatory genes, thus acting as an anti-inflammatory and pro-inflammatory mitigation [25]. Some studies have found that LXA4 can improve cerebral ischemia-reperfusion injury by inhibiting inflammatory response [26]. In the middle cerebral artery occlusion model, injection of LXA4 into the lateral ventricle can improve the degree of neurological deficit and reduce the volume of cerebral infarction [24]. In addition, LXA4 also inhibits the secretion of IL-1β and TNF-α in brain trauma models [27]. Although several studies have shown that lipoxin plays an anti-inflammatory and a neuroprotective role in the inflammatory process of nervous system diseases, its

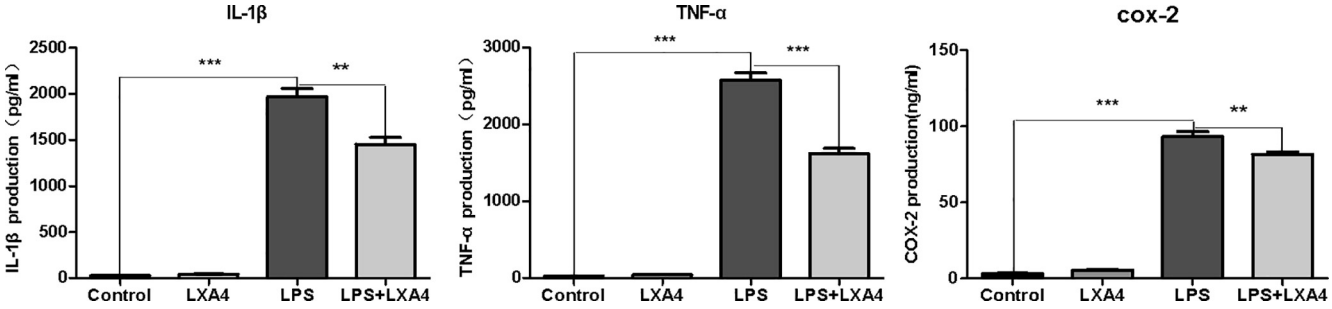

Fig. 6. Differences in protein levels of IL-1β, TNF-α and COX-2 measured in the supernatant using ELISA. The protein levels were higher in the LPS group compared to the control group ($P < 0.001$). LXA4 significantly decreased protein levels in the LPS group ($P < 0.01$).

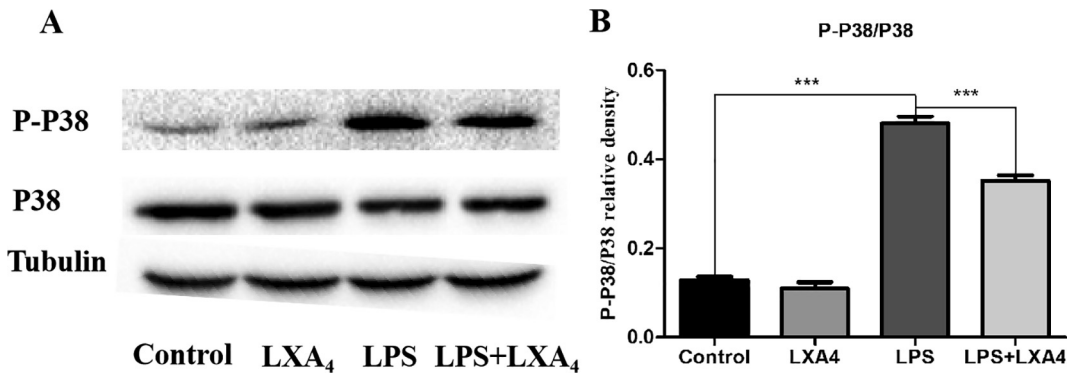

Fig. 7. Western blot analysis of P-P38 protein level, which was higher in the LPS group compared to control group ($P < 0.001$), and LPS + LXA4 group ($P < 0.001$).

specific mechanism of action is still controversial and has not yet been fully defined. It has not yet been reported whether AQP4 is a target of the anti-inflammatory effect of lipopolysaccharide; the purpose of this study was to test this connection.

We have shown here that LPS stimulation significantly increased the expression of AQP4 in astrocytes, as well as increasing the secretion of IL-1 β, TNF-α and COX-2. These findings suggested that AQP4 might have a pro-inflammatory effect. After pretreatment of astrocytes with LXA4, the expression of AQP4 in astrocytes was significantly down-regulated, and the LPS-induced inflammatory response of astrocytes was alleviated. Thus, LXA4 can down-regulate the expression of AQP4 in astrocytes and alleviate the corresponding inflammatory response. These findings suggest that AQP4 might be the target of LXA4 and that the latter likely attenuates the inflammatory response of astrocytes by regulating the expression of AQP4 during the treatment of inflammatory diseases of the central nervous system; this mechanism has not been reported by any of the previous studies on LXA4, and has been established for the first time through this study.

Here, we further explored the possible signaling pathway of LXA4 regulating AQP4 expression. The level of P-P38 protein was induced in astrocytes by LPS stimulation for 0.5 h, but LXA4 pretreatment reduced this increase in P-P38 protein level. The anti-inflammatory effect of LXA4 and the regulation of AQP4 could be achieved by regulating the P38 pathway. The MAPKs superfamily is an important signaling system that transmits extracellular stimuli to the nucleus to cause cellular responses. It mainly includes P38, extracellular signal-regulated kinase and c-Jun amino-terminal kinase [28]. The P38 signal transduction pathway could play a role in regulating inflammation and apoptosis in tissue damage of various nervous system diseases [29]. This study shows that LXA4 can regulate AQP4 through the P38 pathway and reduce the secretion of inflammatory factors such as IL-1β, TNF-α and COX-2, which further clarifies the anti-inflammatory mechanism of lipoxin and its subsequent clinical application.

In conclusion, this study found for the first time that LXA4 can inhibit the expression of inflammatory factors through the regulation of AQP4, suggesting that AQP4 may be a target of anti-inflammatory effect of LXA4. This provides new evidence for further understanding the anti-inflammatory mechanism of LXA4. There have been no similar reports before this study. At the same time, this study also provides a new basis for the treatment of inflammatory diseases of the central nervous system with LXA4. However, there are still some limitations of this study, which only involve the cellular level, and there are limitations in the depth of the study. Such as the research and verification of the AQP4 gene knockout cells and animal models, which will provide more powerful evidence, need to be further explored in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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