AQP4-knockout alleviates the lipopolysaccharide-induced inflammatory response in astrocytes via SPHK1/MAPK/AKT signaling

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Abstract. To date, aquaporin-4 (AQP4) has been considered as a critical contributor to neuroinflammation, but little is known about the underlying mechanism. Previous studies have shown that a critical enzyme involved in the sphingomyelin cycle, sphingosine kinase 1 (SPHK1), is implicated in inflammatory processes and contributes to chronic neuroinflammation. The present study investigated the role of AQP4 in proinflammatory cytokine release from astrocytes, with an emphasis on the SPHK1/mitogen-activated protein kinase (MAPK)/protein kinase B (AKT) pathway. Using primary cultures isolated from AQP4$^{+/+}$ and AQP4$^{-/-}$ embryos, the production of tumor necrosis factor-α (TNF-α)/interleukin-6 (IL-6) from astrocytes challenged by lipopolysaccharide (LPS) was compared. The results showed increased secretion of TNF-α/IL-6 in the two groups following LPS treatment, but a significantly lower level was observed in the AQP4$^{-/-}$ group compared with that in the AQP4$^{+/+}$ group. Although upregulation of SPHK1 was detected in the two genotypes, only a mild increase in SPHK1 was found in the AQP4$^{-/-}$ genotype. The phosphorylation of MAPK/AKT was also confirmed to be attenuated in the AQP4$^{-/-}$ group, suggesting decreased MAPK/AKT signaling over time in AQP4$^{-/-}$ astrocytes. Overall, the study findings demonstrated that AQP4 deficiency alleviates proinflammatory cytokine release from astrocytes, in association with the SPHK1/MAPK/AKT pathway. This data improves our understanding of AQP4 in neuroinflammatory events, highlighting a novel profile of SPHK1 as a potential target for the treatment of CNS inflammation.

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Introduction

As pivotal events involved in central nervous system (CNS) disorders, neuroimmune variations are rather pivotal to the deterioration of neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (1,2). Neuroinflammatory responses are well-known to be associated with the progression of these diseases (3), and proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), have been widely identified as convincing hallmarks of inflammation (4,5). Astrocytes, a major component of the neural network, are increasingly being recognized for their importance in neuroimmunity, including responsiveness to IL-1 (6), removal of old cells and secretion of TNF-α/IL-6 (7,8). Impaired astrocytic function is regarded as a critical issue implicated in neuroinflammation (9); however, the underlying molecular mechanism remains obscure.

Aquaporin-4 (AQP4), the predominant isoform of AQPs in the adult brain, is primarily expressed on astrocytic foot processes throughout the CNS (10,11). It has been well documented that AQP4 is intimately involved in the modulation of astrocyte function (12), but the majority of knowledge on the transporter is limited to water balance (13), glial scar formation (14) and neuroexcitation (15). Little focus has been placed on the area of neuroinflammation until recently (9). As found by Manley *et al* (16), AQP4 deletion attenuates

inflammation-related brain edema, providing a novel perspective on AQP4 in the management of neuroinflammation. Furthermore, several studies provided direct evidence to show the contribution of AQP4 to neuroinflammatory responses, although AQP4 was shown to serve distinct roles in different stages of neuroinflammation (17,18). In addition, multiple studies provided evidence that the involvement of AQP4 in the neuroinflammatory cascade may be dependent on the mitogen-activated protein kinase (MAPK) pathway (17), but its precise control of intracellular signals are not well understood.

The role of sphingolipids in neuroinflammation has been well established. Sphingosine kinase 1 (SHPK1) and sphingosine-1-phosphate receptor 1 (S1P1) are predominantly distributed in astrocytes (18). Abnormal sphingolipid metabolism is observed during neuroinflammation progression. As previous studies have demonstrated, SPHK modulates the function of neurocytes by regulating sphingolipid metabolism. The SPHK inhibitor was shown to prevent glutamate uptake in astrocytes (19), SPHK1 activation was demonstrated to correlate with the histological grade of astrocytomas (20) and genetic deletion of SPHK1 led to the inhibition of glial cell proliferation (21). More recently, a patient suffering from neuromyelitis optica spectrum disorder developed a fulminant course of multiple white-matter lesions following a treatment with S1P mimics, which may be connected with the presence of AQP4 antibodies (22). Although preliminary evidence from clinical data has implied a link between AQP4 and sphingolipids, little is known about the role of AQP4 in sphingolipid metabolism.

The present study aimed to assess the role of AQP4 in cytokine release, with an emphasis on the SPHK1/MAPK/protein kinase B (AKT) pathway. Thus, AQP4 knockout (KO) mice was used to elucidate the potential effect of AQP4 on nureoinflammation by assessing the levels of inflammation associate kinases in LPS treated primary astrocyte cultures isolated from mouse embryos.

Materials and methods

AQP4-knockout mice. AQP4-deficient mice were generated as previously described (11) (generation and phenotype of a transgenic knockout mouse lacking the mercurial-insensitive water channel AQP4). CD-1 mice were kept under environmentally controlled conditions (3-day-old mouse embryos, ambient temperature, 22˚C; humidity, 40%) on a 12-h light/dark cycle with food and water *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee, Nanjing Medical University (Nanjing, China). All efforts were made to minimize animal suffering and to reduce the number of animals used for the experiments.

Primary cultures of astrocytes and treatments. Primary astrocytic cultures were prepared from cerebral cortices of 20 $AQP4^{+/+}$ and 20 $AQP4^{-/-}$ 3-day-old mouse embryos. Following mechanical dissociation, pooled dissected cortices were digested with 0.25% trypase (Amresco, Inc., Solon, OH, USA) at 37˚C for 10 min and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (200 U/ml) and streptomycin (200 μg/ml; Gibco; Thermo Fisher Scientific, Inc.). Following centrifugation at 400 x g for 5 min at room temperature, the cell pellets were resuspended and plated on a polylysine-treated (Sigma-Aldrich; Merck KGaA, Darmstadt, Germant) flask. The cultures were maintained at 37˚C in a humidified incubator with 5% CO_2. Half of the medium was replaced with fresh substratum 24 h after plating and then changed every 2-3 days. Astrocytes were treated with serum-free DMEM for 24 h and then exposed to LPS (100 ng/ml; Sigma-Aldrich; Merck KGaA).

Cell viability assay. Cell viability was measured by the Cell Counting Kit-8 (CCK-8) assay. Astrocytes were collected from flasks and plated in 96-well plates at a density of $5x10^3$ cells/well in growth medium and allowed to adhere for 48 h prior to the growth media being replaced with serum-free DMEM. Cells were treated with 100 ng/ml LPS, and CCK8 (Obio Technology, Shanghai, China) was applied after 1, 3, 6 and 24 h. The absorbance was measured at a wavelength of 490 nm.

Measurement of cytokines. The quantitative determination of mouse TNF-α and IL-6 in cell culture supernatants was assessed by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) (cat. nos. MTA00B and M6000B; R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's protocols. The total protein concentrations of the viable cells were determined using the Bradford reagent. Total amounts of the TNF-α and IL-6 in media were normalized to the total protein amounts of the viable cell pellets and expressed as pg/mg proteins.

Western blot analysis. Astrocytes were lysed by RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, Indianapolis, IN, USA). Lysates were centrifuged (400 x g for 10 min at 4˚C) and the protein concentration in the extracts was determined by the Bradford assay. Samples in the extracts were denatured with a SDS sample buffer. A total of 200 μg of protein was loaded into each well and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gels were transferred to a polyvinylidene difluoride membrane (Pierce; Thermo Fisher Scientific, Inc.) and immunoblotted, followed by blocking of the membranes with 5% skimmed milk dissolved in TBST [(pH 7.5), 10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20] at room temperature for 1 h. Subsequent to being washed three times with TBST buffer, the membranes were incubated with the following primary antibodies at 4˚C over one night: Phosphorylated (phospho)-Akt (Ser473) (1:1,000, cat. no. 4060), phospho-p44/42 MAPK [p-extracellular signal-regulated protein kinases 1 and 2 (ERK1/2)] (Thr202/Tyr204) (1:1000, cat. no. 9106), phospho-p38 MAPK (Thr180/Tyr182) (1:1,000, cat. no. 4511), AKT (1:1,000, cat. no. 9272), p44/42 MAPK (ERK1/2) (1:1,000, cat. no. 4695) and p38 MAPK (1:1,000, cat. no. 9212; all Cell Signaling Technology, Inc., Danvers, MA, USA), and SPHK1 antibody (1:1,000, cat. no. ab71700; Abcam, Cambridge, MA, USA), which were visualized by reaction with horseradish peroxidase-linked secondary antibodies for 1 h at room temperature (cat. nos. 29-0382-77 and 29-0382-78;

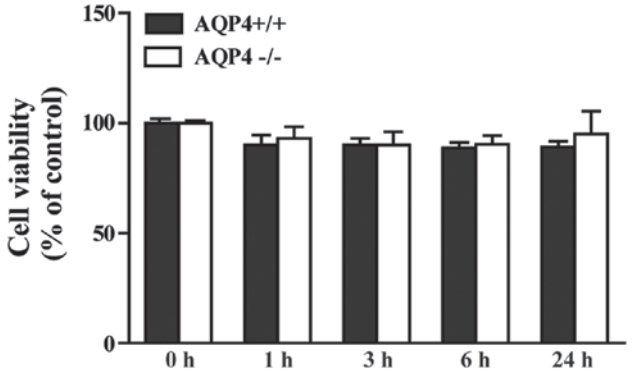

Figure 1. LPS has no toxic effect on astrocyte viability in 24 h. No significant change in the viability of the cells was observed after 0, 1, 3, 6 and 24 h of treatment with LPS (100 ng/ml). Values are presented as the mean ± standard deviation of 3 independent experiments. LPS, lipopolysaccharide.

GE Healthcare, Chicago, IL, USA) and an enhanced chemiluminescence (ECL; cat. no. 32106; Thermo Fisher Scientific, Inc.) or ECL-plus detection system (Quantity One Quantitation software, Bio-Rad Laboratories, Hercules, CA, USA). GAPDH (1:1,000, cat. no. D16H11; Cell Signaling Technology, Inc.) was used as an internal control.

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical analysis between $AQP4^{+/+}$ and $AQP4^{-/-}$ was performed with a two-tailed indirect Student's t-test using SPSS version 10.0 for Windows (SPSS, Inc., Chicago, IL, USA). Statistical analysis for multiple comparisons was performed by a one-way analysis of variance with Bonferroni's corrections. The level of statistical significance was defined as $P<0.05$.

Results

LPS has no significant effect on astrocyte viability. As shown in Fig. 1, LPS at 100 ng/ml exhibited no significant effect on astrocyte viability within 24 h in the $AQP4^{+/+}$ and $AQP4^{-/-}$ groups. Furthermore, no clear difference was observed between the two genotypes. The results of the CCK-8 assay indicate that the alterations in the cytokines and pathways found in the following experiments were not associated with LPS-induced cell damage.

AQP4-knockout reduces LPS-induced cytokine secretion in astrocyte cultures. To investigate the role of AQP4 in the inflammatory response of astrocytes, measurements of cytokine release were performed on differentiated primary astrocyte cultures from the brain cortex of neonatal $AQP4^{+/+}$ and $AQP4^{-/-}$ mice. As shown in Fig. 2, AQP4-knockout attenuated TNF-α and IL-6 concentrations in cell culture supernatant following 12 h of treatment with LPS. Compared with the $AQP4^{-/-}$ genotype, $AQP4^{+/+}$ showed only a slight elevation in IL-6 at 12 h after LPS administration (Fig. 2B), while the secretion of TNF-α in $AQP4^{+/+}$ astrocytes was robustly elevated at 24 h after LPS addition in astrocyte cultures (Fig. 2A). This supports the hypothesis that knockout of AQP4 alleviates LPS-induced cytokine secretion in astrocytes.

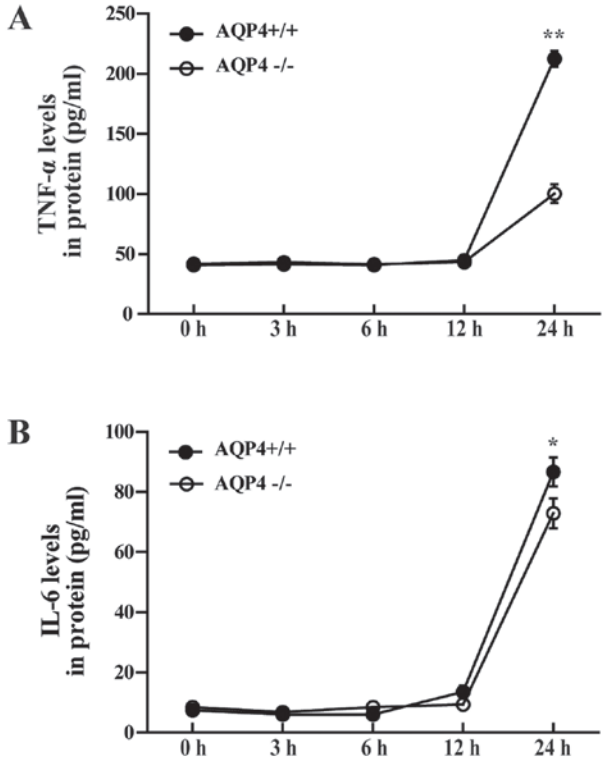

Figure 2. AQP4-knockout reduces LPS-induced cytokine secretion in astrocyte cultures. (A and B) IL-6 and TNF-α levels in the cell culture supernatant of mice increased significantly after 24 h of LPS treatment. $^{*}P<0.05$ and $^{**}P<0.01$ vs. $AQP4^{-/-}$. Values are presented as the mean ± standard deviation of 4 independent experiments. AQP4, aquaporin-4; LPS, lipopolysaccharide; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α.

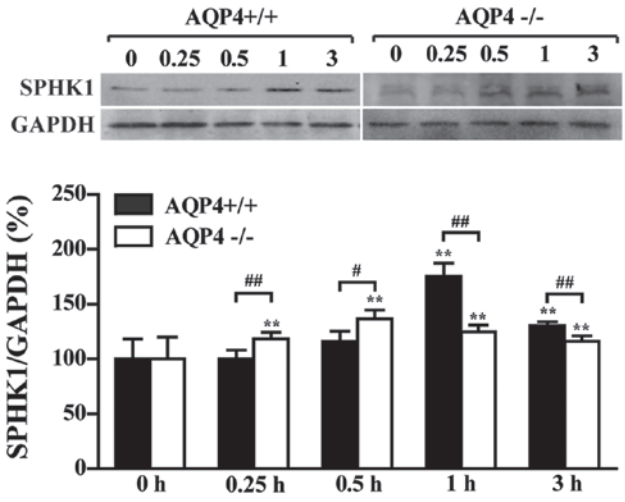

Figure 3. AQP4-knockout alleviates the increased levels of SPHK1 in astrocytes following treatment with LPS (100 ng/ml). The expression of SPHK1 in astrocytes was detected by western blot analysis following treatment with LPS at the time-points indicated. A representative autoradiogram and densitometric analysis of SPHK1 levels are shown. GAPDH served as a loading control and was used to normalize the western blot data. $^{**}P<0.01$ vs. 0 h; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. $AQP4^{+/+}$. Values are presented as the mean ± standard deviation of 4 independent experiments. AQP4, aquaporin-4; LPS, lipopolysaccharide; SPHK1, sphingosine kinase 1.

AQP4-knockout attenuates LPS-induced SPHK1 generation in astrocytes. Western blot analysis by semi-quantitative analysis showed that the expression of SPHK1 was induced by

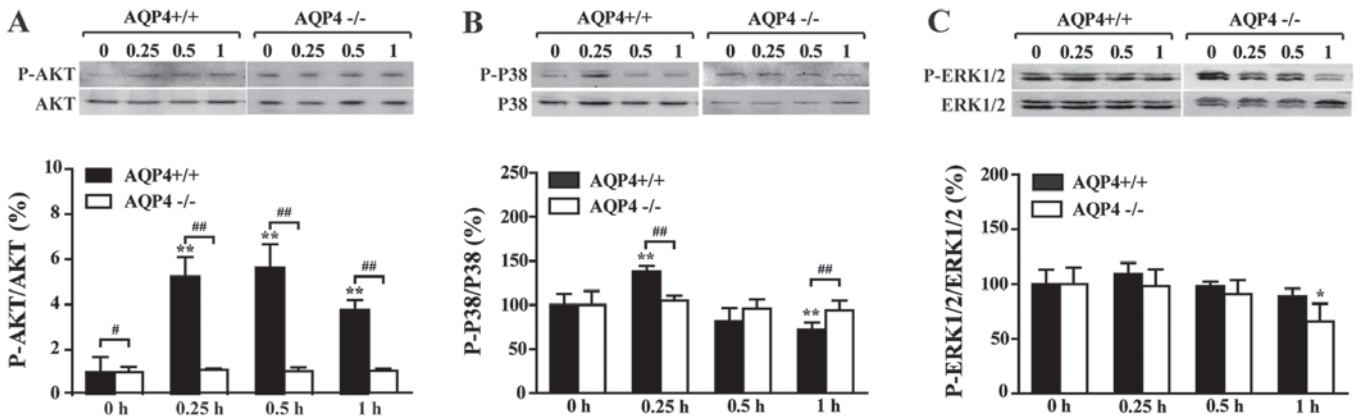

Figure 4. AQP4-knockout alleviates LPS-induced signal pathway activation in astrocytes. A representative autoradiogram and densitometric analysis of (A) p-AKT, (B) p-P38 and (C) p-ERK1/2 levels are shown. Values are presented as the mean ± standard deviation of 4 independent experiments. The protein concentrations in the extracts were determined by the Bradford assay. The lowest concentration was chosen as the standard concentration, and extracts from different experiment groups were diluted with ddH_2O to the standard concentration. Following the adjustment, same volumes of sample were equal to same loading quantities. Equal volumes of samples were loaded in the lane to ensure equal protein loading for the western blotting. *P<0.05 and **P<0.01 vs. 0 h; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. AQP4$^{+/+}$. AQP4, aquaporin-4; LPS, lipopolysaccharide; p-, phosphorylated; AKT, protein kinase B; ERK1/2, extracellular signal-regulated protein kinases 1 and 2.

LPS in AQP4$^{+/+}$ and AQP4$^{-/-}$ astrocytes, and peaked at 1 and 0.5 h, respectively (Fig. 3). However, the induction of SPHK1 was attenuated in the AQP4$^{-/-}$ group compared with that in the AQP4$^{+/+}$ group, demonstrating that AQP4 mediated the induction of SPHK1 expression by LPS in astrocytes.

AQP4-knockout alleviates LPS-induced AKT pathway activation in astrocytes. As shown in Fig. 4A, the phosphorylation of AKT was elevated by LPS after 0.25 h and peaked at 0.5 h in the AQP4$^{+/+}$ group, while in the AQP4$^{-/-}$ group, the phosphorylation of AKT was not affected following LPS administration within 1 h. It was demonstrated that AKT signaling in the AQP4$^{-/-}$ genotype is more stable than that in the AQP4$^{+/+}$ gentoype with regard to inflammatory stimulation.

AQP4-knockout alleviates LPS-induced MAPK pathway activation in astrocytes. Next, the phosphorylation ratio of p38 and ERK was determined in order to investigate the involvement of the MAPK pathway. As observed in Fig. 4B and C, the phosphorylation of p38 and ERK peaked at 0.25 h in the AQP4$^{+/+}$ group and no clear elevation was observed in the AQP4$^{-/-}$ group. A decline was actually found in the phosphorylation of ERK following the administration of LPS. Taken together, these results demonstrated that lack of AQP4 may interfere with the phosphorylation of ERK. Furthermore, a marked decline was found in the phosphorylation of p38 after 0.25 h, and the phosphorylation ratios at 0.5 and 1 h were lower than the basic level (0 h), which indicates an exhaustion of phosphorylation. The data suggests a decrease in MAPK signaling over time in AQP4$^{-/-}$ astrocytes.

Discussion

Neuroinflammatory responses have long been observed to be associated with neuroimmune changes outside and inside the brain. Apart from reactive peripheral immune cells that traverse the blood-brain barrier (23), glia cells in the CNS are also implicated in initiating an inflammatory cascade (24). Microglia are often assumed to serve the most vital role in brain inflammation (25). However, since astrocytes occupy nearly half of the total cells in the CNS (26) and exhibit important immune regulatory properties, the role of astrocytes in inflammation should not be neglected. AQP4 has been demonstrated to be dispersed in the cytoplasm of reactive astrocytes, particularly when inflammation occurs (27). This AQP holds the key to our understanding of brain inflammation. Astrocytes also express toll-like receptors (TLRs) that specifically recognize LPS, which may stimulate the release of proinflammatory cytokines and oxidative stress (28). It was previously found that TLR4 activation regulates AQP4 expression, and that IL-6 leads to an increase in AQP4 (29). In turn, AQP4 may modulate astrocyte-to-microglia communication during the development of inflammation (30). All these findings suggest an important role of AQP4 in neuroinflammation, but the underlying mechanism remains obscure.

As is known, it takes time to synthesize and transport cytokines, such as IL-6 and TNF-α, to the cell surface upon TLR4 activation. Previous studies have implied a different amplification loop formed in AQP4$^{-/-}$ and AQP4$^{+/+}$ genotypes once cytokines are secreted (31-33). The signaling between these two groups is distinct and complicated. The present study established a cell model to determine the role of AQP4 in the early phase of inflammation. From LPS administration to cytokine secretion, it was revealed that cytokines were not secreted during the first 12 h, yet intracellular pathways central to inflammation were activated. In addition, AQP4$^{-/-}$ astrocyte signaling (SPHK/MAPK/AKT) was decreased over time.

SPHKs serve an important role in sphingolipid metabolism and are heavily linked to inflammatory diseases (34). Compelling evidence from clinical trials has indicated a link between AQP4 and SPHK1, with an emphasis on their role in inflammation (25). Attention has become focused on SPHK1 in LPS-induced cytokine production (35), which could lead to a rapid increase in S1P, which has been hypothesized to be associated with the transactivation of different inflammatory pathways (36). The present study found that SPHK1 expression in AQP4$^{+/+}$ reached a peak at 1 h and then declined rapidly,

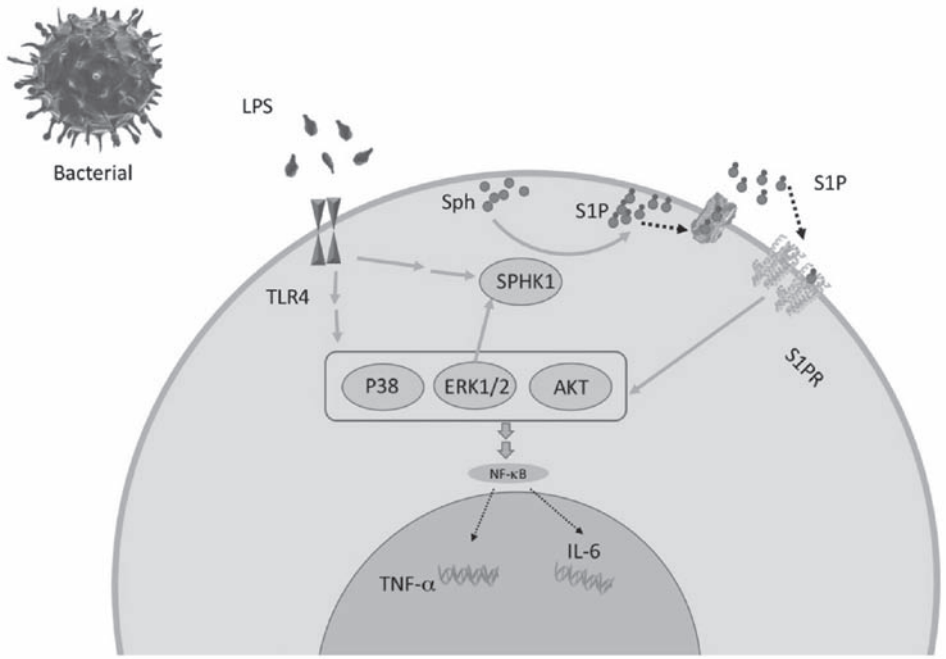

Figure 5. The potential mechanism by which LPS induces proinflammatory cytokine release from astrocytes via SPHK1/MAPK/AKT signaling. LPS derived from bacteria activate TLR4 and strengthen the SPHK1/S1PR/MAPK/SPHK1 loop through ERK1/2, which contributes to the expression of TNF-α and IL-6. AQP4-knockout alleviates LPS-induced cytokine secretion through inhibition of SPHK1/MAPK/AKT signaling. The present model enabled the examination of the interaction of signaling pathways involved in LPS-induced inflammation. It was found that LPS significantly increased TNF-α and IL-6 levels in the astrocyte culture supernatants. LPS derived from bacteria activate TLR4 and strengthen the SPHK1 and MAPK signals, which ultimately contribute to the expression of TNF-α and IL-6. As previously described, ERK1/2 was confirmed to activate SPHK1, and SPHK1 modulates the MAPK/AKT pathway through the S1P/S1PR signal in turn; an amplification loop is formed when TLR4 is activated. AQP4, aquaporin-4; LPS, lipopolysaccharide; SPHK1, sphingosine kinase 1; MAPK, mitogen-activated protein kinase; AKT, protein kinase B; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; S1P, sphingosine-1-phosphate.

while the increase in SPHK1 in the AQP4-knockout genotype was attenuated despite an earlier peak time (0.5 h). The level of SPHK1 in $AQP4^{-/-}$ astrocytes challenged by LPS was much lower than that in $AQP4^{+/+}$ astrocytes, implying that SPHK1 signaling may inhibited when AQP4 is deleted.

As previously reported, S1P is able to bind to S1PR and activate pathways such as those of MAPK, AKT and phospholipase C, which are generally considered as the triggers of inflammation and cell proliferation (37); their activation drive the transcription factor, nuclear factor (NF)-κB, to launch proinflammatory cytokine transcription. To date, in colon diseases, it has been found that upregulation of S1P promotes a persistent MAPK/NF-κB/IL-6/STAT3/S1PR amplification loop that is critical to chronic inflammation development (36). ERK1/2 has also been confirmed to mediate the activation of SPHK1 in turn (38). There is a potential clue among SPHK1/MAPK/AKT signaling involved in cytokine release from astrocytes (Fig. 5). We hypothesized that the attenuated increase of SPHK1 observed in the $AQP4^{-/-}$ genotype may result from the inhibited activation of MAPK. Attenuation of the phosphorylation of MAPK and AKT in LPS-induced $AQP4^{-/-}$ astrocytes was indeed found.

The highly conserved MAPK family transfers extracellular signals to nucleus in eukaryotic organisms (39,40). MAPK family is serine/threonine protein kinases belonging to p38 MAPKs, ERK1/2 and Jun amino-terminal kinases (JNKs) (41). LPS leads to the activation of p38 MAPKs and ERK, which contribute to the production of proinflammatory cytokines (42,43). The present study compared the level of MAPK phosphorylation in $AQP4^{+/+}$ and $AQP4^{-/-}$ astrocytes. The findings showed that the phosphorylation levels of p38 MAPKs and ERK peaked at 0.25 h in the $AQP4^{+/+}$ group, while the levels declined markedly in the $AQP4^{-/-}$ group. As previous studies implied, AQP4 overexpression may be associated with the activation of p38 MAPKs and ERK when inflammation occurs (20). The present study found a significant increase in MAPKs in the $AQP4^{+/+}$ group, but not in the $AQP4^{-/-}$ group, suggesting an AQP4-dependent mechanism underlying the LPS-initiated MAPK activation. Furthermore, AKT phosphorylation in $AQP4^{-/-}$ astrocytes remained at a very low level at 1 h compared with that in $AQP4^{+/+}$ astrocytes, also hinting at stable signaling of AKT in the absence of AQP4.

The role of AQP4 in inflammation has been investigated, but as the pathological conditions vary, as does the role of AQP4 in the inflammatory response. Previously, Li *et al* (31) revealed that AQP4 deficiency alleviated LPS-induced TNF-α production in astrocytes, which is consistent with the present findings. In an overhydration model, AQP4 deficiency presented the opposite effect (44,45), highlighting a distinct role of AQP4 when the type of stress changes. As previous studies reported, AQP4 deficiency alleviated LPS-induced TNF-α production, but augmented MPTP-induced TNF-α generation (46,47). This may be due to the distinct downstream signals. Further studies are required to investigate the signaling alterations under different pathological conditions. However, in the present study, it was indicated that AQP4-knockout could evoke inhibition of MAPKs and AKT, and attenuate the fluctuation of SPHK1, which ultimately contributed to the

secretion of cytokines. Further studies are required to confirm the SPHK1/MAPK/AKT loop.

In conclusion, the present study identified a specific role of AQP4 in regulating the initial astrocytic inflammatory response. It was found that astrocytes with AQP4 ablation are less responsive to inflammatory stress. This study indicates the importance of AQP4 in astrocyte activation and provides mechanistic insight into understanding the different kinase pathways involved in this response.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XZe and XZh conceived and designed the experiments; WD and JY performed the experiments; WD and GC analyzed the data; GH provided the AQP4 knockout mice; WD, JY and XZe wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Care and Use Committee, Nanjing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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