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Effect of lipoxin A_4 methyl ester from arachidonic acid on JAK2/STAT3 pathway after cerebral ischemia-reperfusion injury

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Abstract

It was determined if lipoxin A_4 methyl ester (LXA4 ME) affects JAK2/STAT3 pathway following transient focal cerebral ischemia-reperfusion injury (CIRI) in rats. Adult male SD rats were randomly assigned to sham-operated, CIRI group, DMSO + CIRI, AG490 + CIRI, and LXA_4 ME + CIRI. Neurologic deficit was evaluated in rats after 24 h reperfusion. IL-6 levels of brain tissue were determined 6 and 24 h post-reperfusion using an ELISA assay. STAT3 and SOCS3 mRNA levels were analyzed via real-time quantitative PCR. P-JAK2, p-STAT3, and SOCS3 were measured via Western blotting, and localized by fluorescent immunohitochemistry. Transient cerebral ischemia in rats caused a significantly elevated concentration of IL-6, levels of STAT3 and SOCS3 mRNA, phosphorylation of JAK2 and STAT3, and immunoreactive glia of ipsilateral cortex 24 h post-reperfusion versus sham rats. The AG490 intervention downregulated cerebral ischemia-induced JAK2 and STAT3 phosphorylation, and their immunoreactive glia located in the ischemic cortex. LXA_4 ME reduced the concentration of IL-6, increased SOCS3 expression, inhibited phosphorylation and immunoreactive glia of JAK2 and STAT3, as well as ameliorated neurologic dysfunction. In conclusions, LXA_4 ME inhibited JAK2/STAT3 activation, perhaps via upregulating SOCS3, as well as suppressed IL-6 expression to ameliorate cerebral ischemic injury.

Keywords: cerebral ischemia reperfusion; lipoxin A_4; interluekin-6; JAK/STAT; SOCS3.

Practical Application: We propose this mechanism by which LXA4 ME exerts an anti-inflammatory and neuroprotective effect.

1 Introduction

The inflammatory response is pivotal for cerebral ischemia-reperfusion (I/R) injury (CIRI), where pro- and anti-inflammatory signals are activated within ischemic brains (Jin et al., 2013). Indeed, large amounts of inflammatory cytokines are released, including pro- and anti-inflammatory cytokines (Ye et al., 2010), and function primarily via the Janus kinase (JAK) /signal transducers and activators of transcription (STAT) pathway (Huang et al., 2022; Yin et al., 2022), which exists as potential transcription factors and induce alterations in the pattern of cell death or survival-related gene transcription (Jin et al., 2013; Xin et al., 2020). Family JAK comprises JAK1-3 and TYK2, while family STAT consists of STAT1-4, STAT5a, STAT5b, and STAT6 (Xin et al., 2020).

A recent study showed the involvement of JAK/STAT pathway in central nervous system (CNS) growth and nerve cell proliferation, survival, and differentiation (Fan & Zhou, 2021a). The JAK/STAT pathway was likewise tightly involved in regulating the inflammatory response and apoptosis following brain injury (Nowery et al., 2021). CIRI triggers numerous inflammatory cytokines and growth factors released, ultimately activating JAK/STAT pathway (Jin et al., 2013; Ye et al., 2010). Additionally, a negative regulatory mechanism is also activated to control the duration and severity of the injury (Fan & Zhou, 2021a; Lasek-Bal et al., 2019). Family Proteins from the suppressors of cytokine signaling (SOCS) get involved in these processes by inhibiting of JAK/STAT activity and inhibiting pro-inflammatory cytokine signaling (Linossi et al., 2018). The SOCS family consists of CIS (CIS-1a, CIS-1b) and SOCS(SOCS1, SOCS2, SOCS3, SOCS4, etc.) (Linossi et al., 2018). Acting as one link in the negative regulatory loop of cytokine signal transduction, the functional studies of SOCS1 and SOCS3 are more common (Linossi et al., 2018). SOCS3 significantly inhibits the signal transduction of IL-6, IFN-γ, α, β, GH, and leptin, which may determine whether the outcome of brain ischemia-reperfusion injury is recovery or irreversible necrosis (Gao et al., 2017).

In the present study we determined whether lipoxin A_4 methyl ester (LXA_4 ME) influenced JAK2/STAT3 activation and SOCS3 expression in the acute inflammatory responses caused by CIRI in rats. Then, we attempted to demonstrate whether LXA_4 ME negatively regulated JAK2/STAT3 pathway closely implicated in the inflammatory cytokines via SOCS3, thereby exerting a neuroprotective effect.

2 Methods

2.1 Animal grouping

This research received authorization from the ethical review committee of our hospital, and was proceeded following the

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national guidelines of experimental animals. Sixty healthy male SD rats (225 ± 25 g) from the Experimental Animal Center of Tongji Medical College, were firstly kept fasting 12 h with the exception of water intake, and randomly grouped as sham operation, CIRI, DMSO (solvent for AG490) treatment, AG490 (specific inhibitor of JAK2) treatment, and LXA_4 ME treatment groups. For the treatment groups, the intracerebroventricular injection was performed using the following reagents: 3% DMSO (5 μL); AG490 (100 nM/5 μL dissolved in 3% DMSO just before use); and LXA4 ME 0.3 nM/5 μL (freshly prepared in sterile saline). For the sham and CIRI groups, the rats were given equal saline doses (solvent for LXA4 ME).

2.2 Construction of the CIRI model

As described in the literatures (Ye et al., 2010), a middle cerebral artery occlusion (MCAO) rat model was constructed with a thread occlusion technique. Thus, focal CIRI was induced. The rat was anesthetized, with neck incised medially for isolation of right external and internal carotid arteries. These arteries had the distal end ligated. The external carotid artery underwent severance with a little cut at its stump, through which a 4.0 nylon wire having spherical ends was plugged in. The wire was delivered to internal carotid artery about 1.8 ± 0.05 cm deep, thus occluding the blood supply to the middle cerebral artery. Then reperfusion was implemented with the wire withdrawn from the external carotid artery after 2 h of ischemia. In sham rats, the wire insertion depth was < 0.9 cm. During ischemia, tanal temperature was maintained at approximately 37 °C using a baking lamp. After reperfusion, rats were fed at 24 °C.

2.3 Neurologic deficit scoring

Neurologic deficit scoring was performed 24 h post-reperfusion according to the following numerical score criteria (Balkaya et al., 2013). A neurologic deficit score of 1-3 indicated that the MCAO model was successfully prepared. Rats scoring 0 and 4, as well as the dead rats, were eliminated. The MCAO model was further constructed in the remaining rats of the same batch to make up the amount.

2.4 Sample collection

Following 24 h reperfusion, 6 rats from each group were administered deep anesthesia before decapitation and brain harvesting. Tissues collected at 3 and 9 mm anterior to the frontal lobe were prepared as coronal sections. The brain tissue blocks were 6 mm thick. The median structures of both hemispheres were then excised from top-to-bottom along the sagittal suture of the brain mass, approximately 2 mm on either side. The right brain tissue block was used as the ischemic tissue and preserved at -70 °C prior to detection of various indicators.

2.5 ELISA detection of IL-6 content in brain tissues

The ischemic cerebral tissues were used for the detection of indicators. Precooled normal saline was added to the tissues at a specific weight-to-volume ratio to prepare a homogenate. Brain tissue homogenate was yielded using a 1-ml rotary homogenizer under 4 °C at 3500 rpm over 10 min, with supernatant gathered for subsequent detection. Then, an IL-1β ELISA kit (Neobioscience Technology Company, Shenzhen, China) was used to determine the IL-6 content in the supernatant. All procedures were undertaken following manufacturers' directions.

2.6 Real-time PCR assay

Total cellular RNA extracted through TRIzol (Invitrogen, USA), was reversely transcribed to cDNA with PrimeScriptTMRTase kits (Takara Bio, Japan).SYBR Green I dye was used for real-time PCR on the Bio-Rad iCycler 5 (Berkeley, California, USA). The molecular primers (Sangon, Shanghai, China) were listed below (gene name, forward, reverse): STAT3, 5'-AGGACATCAGTGGCAAGA-3', 5'-ATGGTAACTGGACGGCTA-3';SOCS3,5'-ACCCACAGCAAGTTTCCC-3', 5'-CACTGGATGCGTAGGTTCTT-3'; GAPDH, 5'-TATCGGACGCCTGGTTAC-3', 5'-CTGTGCCGTTGAACTTGC-3'. The relative mRNA expression was computed using the $2^{-\triangle\triangle Ct}$ approach using GAPDH as an internal reference.

2.7 Western blotting detection

The Western blot detection procedures were as follows. The ischemic cerebral cortex was retrieved after 24 h of reperfusion and immersed in cell lysis buffer. The proteins were extracted and detected utilizing the BCA method. After denaturation, 40 μg protein lysate was loaded onto SDS-PAGE (Amersham, Cytiva, England) using 8, 10%, and 12% gels, then electrophoretically transferred to PVDF membranes. The membrane was incubated in PBS with 5% bovine serum albumin (BSA) on ambient temperature for 1 h, and further subjected to the below antibodies against p-JAK2 (Cell Signal, Boston, USA) diluted in the freshly prepared TBS with 5% BSA (1: 500), p-STAT3 (1:800; Bioworld TechnologyInc, MN, USA), SOCS3 (1:500; Abcam, London,England), and β-actin (1:600; Sigma, MO, USA), succeeded by overnight incubation at 4 °C. Horseradish peroxidase-labeled secondary antibodies (Feiyi Technology, Wuhan, China) were added to label the membrane for 1 h. ECL reagent was added and the positive signals were detected. This was followed by exposure to X-rays and images were evaluated through Image J software. The targeted protein expression relative to β-actin was computed.

2.8 Double immunofluorescence staining for p-JAK2, p-STAT3, and SOCS3 proteins

Brain tissue blocks were fixed in pre-cooled 4% paraformaldehyde for 6 h as above. Then, 25% sucrose phosphate buffer was added to incubate the tissues at 4 °C overnight. When the tissue blocks sank, 20 μm thickness coronal sections were obtained utilizing a constant-temperature cryostat microtome, and placed upon superfrost slides with storage at -20 °C before use. The frozen sections were blown dry at room temperature using a fan, fixed in 4% paraformaldehyde for 30 min, then subjected to endogenous peroxidases blockage and cell permeabilization in 0.3% H_2O_2 methanol and 0.1% TritonX-100 (prepared in 0.01 M PBS) for 30 min, respectively. The slide was rinsed by PBS and incubated in 5% BSA (prepared in 0.01M PBS) at 37 °C for 60 min to block the binding to non-specific antigens. Excess BSA was removed by blotting

with an absorbent. The following primary antibodies were added successively into the slides of each group: anti-p-JAK2 (1:100); p-STAT3 (1:200); SOCS3 (1:200); and chicken anti-rat GFAP (1:100; Abcam, London,England) or mouse-derived OX42 (1:100; Abcam, London,England), succeeded by 60 min incubation at 37 °C and 48 h incubation at 4 °C. Then, secondary FITC or CY3-labeled goat anti-chicken or anti-rabbit IgG antibodies from Bioss (Beijing, China) were added successively with a 1:100 dilution ration, followed by 1 h incubation at 37 °C. Finally, the slides were sealed in glycerol:PBS (1:9) buffer. Photographs were taken with a confocal laser microscopy. Blank controls were set up for the above immunofluorescence staining assays with the addition of PBS instead of primary antibodies. Immunofluorescence staining procedures were performed in a dark room. Photography by a microscope was done immediately after slide mounting.

2.9 Statistical analysis

The data were indicated as mean ± standard deviation and analyzed through SPSS 13.0 software. One-way ANOVA and Dunnett's test were utilized for pairwise intergroup comparisons. $P < 0.05$ indicated a significant difference.

3 Result

3.1 LXA_4 ME improved neurologic deficits

Figure 1 revealed that the sham group had no neurologic deficits. The neurologic deficit scores of CIRI and DMSO groups were remarkably higher over AG490 and LXA_4 ME groups ($P < 0.05$).

3.2 Influence of LXA_4 ME on IL-6 content

As shown in Figure 2, each treatment group possessed an increased IL-6 content at 6 and 24 h, relative to sham rats ($P < 0.05$). After 6 h reperfusion, the IL-6 content declined in AG490 and LXA_4 ME groups in comparison to CIRI group ($P < 0.05$, both). The IL-6 content trend of each group 24 h post-reperfusion was the same as 6 h post-reperfusion, but its content was greater than the latter.

3.3 Effect of LXA_4ME on STAT3 and SOCS3 mRNAs levels

As shown in Figures 3 and 4, in comparison to sham rats, the CIRI and DMSO groups had overexpression of STAT3 and SOCS3 mRNAs ($P < 0.05$). In comparison to the CIRI rats, AG490 treated rats had downregulation of STAT3 mRNA ($P < 0.05$), while the SOCS3 mRNA level had no significant changes. STAT3 mRNA was downregulatedd ($P < 0.05$), but SOCS3 mRNA was upregulated ($P < 0.01$) in LXA_4 ME treated rats.

3.4 Influence of LXA_4 ME on protein levels of *p*-JAK2, *p*-STAT3, and SOCS3

As shown in Figure 5, specific bands were observed at the following molecular weights: 130 kD; 92 kD; 30 kD; and 43 kD. In the sham group, p-JAK2, p-STAT3, and SOCS3 proteins were nearly undetectable. The CIRI and DMSO groups showed

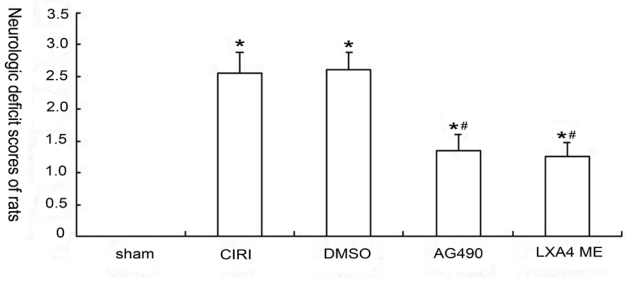

Figure 1. Neurologic deficit scores of rats in each group. Compared to the sham group, $^{*}P < 0.05$; compared to the CIRI group, $^{\#}P < 0.05$.

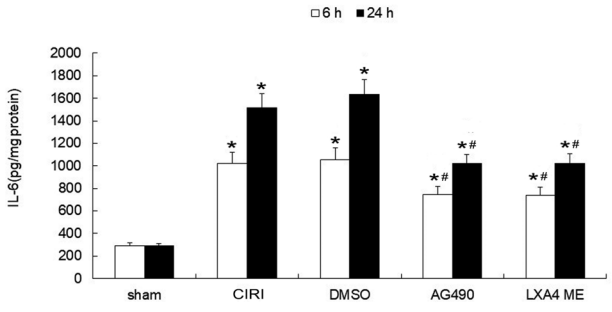

Figure 2. Changes in the IL-6 content in rats. Compared to the sham group, $^{*}P < 0.05$; compared to the CIRI group, $^{\#}P < 0.05$.

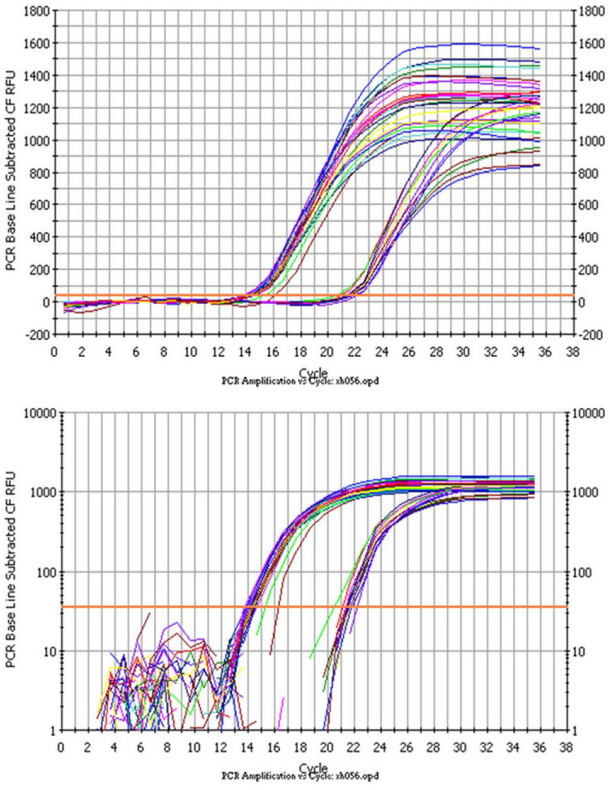

Figure 3. Amplification and logarithmic curves of STAT3 and SOCS3.

significantly upregulated p-JAK2 and p-STAT3 proteins following 24 h reperfusion ($P < 0.01$), and increased SOCS3 expression was also observed ($P < 0.05$), relative to the sham group. AG490 (a specific JAK2 inhibitor), and LXA4 ME significantly inhibited the phosphorylated JAK2 and STAT3 proteins, relative to the CIRI group ($P < 0.05$). In contrast, the SOCS3 protein expression was upregulated ($P < 0.01$).

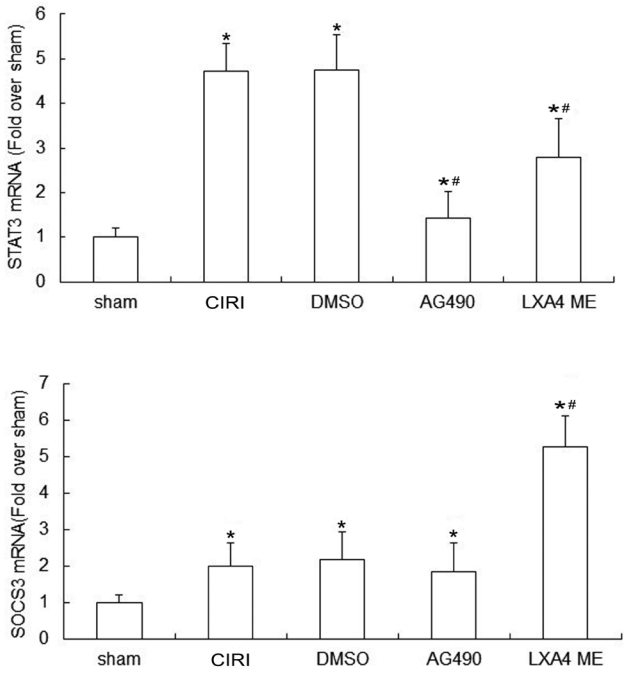

Figure 4. Changes in STAT3 and SOCS3 mRNAs expression. Compared to the sham group, $^{*}P < 0.05$; compared to the CIRI group, $^{\#}P < 0.01$.

3.5 Influence of LXA4 ME on protein levels of *p*-JAK2, *p*-STAT3, and SOCS3 in glial cells

As shown in Figures 6 and 7, p-JAK2 protein was more highly expressed in ischemic cortical cerebrum of rats following 24 h reperfusion, but was inhibited by AG490. LXA_4 ME also inhibited p-JAK2 protein expression. Double immunofluorescence staining revealed that p-JAK2 was mostly expressed in OX42-positive microglial cells. Only a small amount of p-JAK2 was expressed in GFAP-positive astrocytes. As shown in Figures 8 and 9, p-STAT3 protein was highly expressed in CIRI rats after 24 h reperfusion. In contrast, AG490 and LXA_4 ME inhibited p-STAT3 protein expression. Double immunofluorescence staining indicated that p-STAT3 was expressed in OX42-positive microglial cells and GFAP-positive astrocytes. As shown in Figures 10 and 11, SOCS3 protein was more highly expressed after 24 h of cerebral I/R. LXA_4 ME upregulated SOCS3expression dramatically. Double immunofluorescence staining indicated that SOCS3 was mostly expressed in OX42-positive microglial cells and GFAP-positive astrocytes.

4 Discussion

As an important intracellular signal transduction pathway, the JAK/STAT pathway regulates cellular activities of CNS development, and is closely related to the pathophysiologic processes of CNS diseases, including cerebral ischemia and brain tumors (Xin et al., 2020; Nowery et al., 2021). In normal situations, JAK2 and STAT3 are inactivated, and usually triggered by cytokines, growth factors, and oxidative stress following cerebral ischemia (Wu et al., 2018). Presenting in neuronal cytoplasm and a small number of glial cells, JAK-STAT signalling proteins are mainly distributed in the cortex and hippocampus (Wu et al., 2018). Cerebral ischemia injury models proved that STAT3 phosphorylation mainly occurs in the neurons in layers IV-V of the cortex, corpora striata, and hippocampal tissues (Sharma et al., 2011; Wang et al., 2022), along

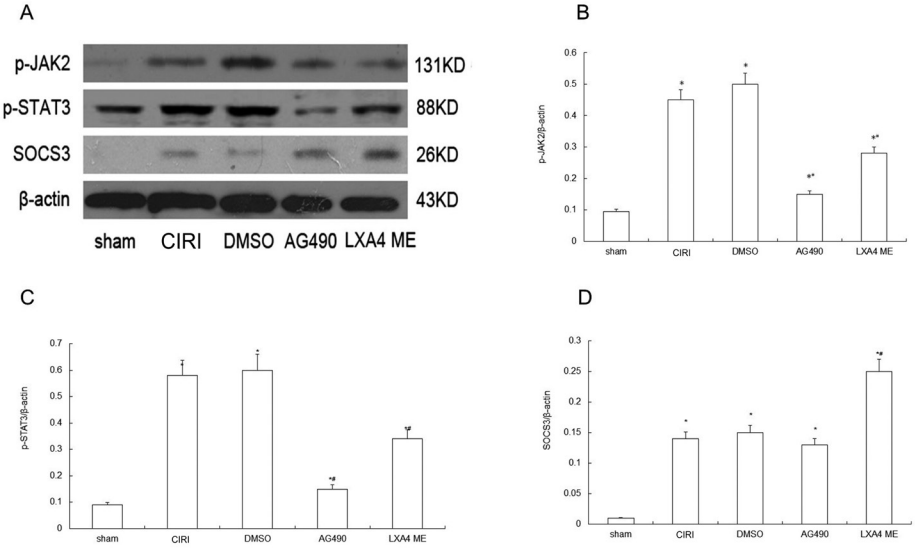

Figure 5. Changes in protein levels of p-JAK2 (B), p-STAT3 (C), and SOCS3 (D). Compared to the sham group, $^{*}P < 0.05$ or $P < 0.01$; compared to the CIRI group, $^{\#}P < 0.05$ or $P < 0.01$ (A).

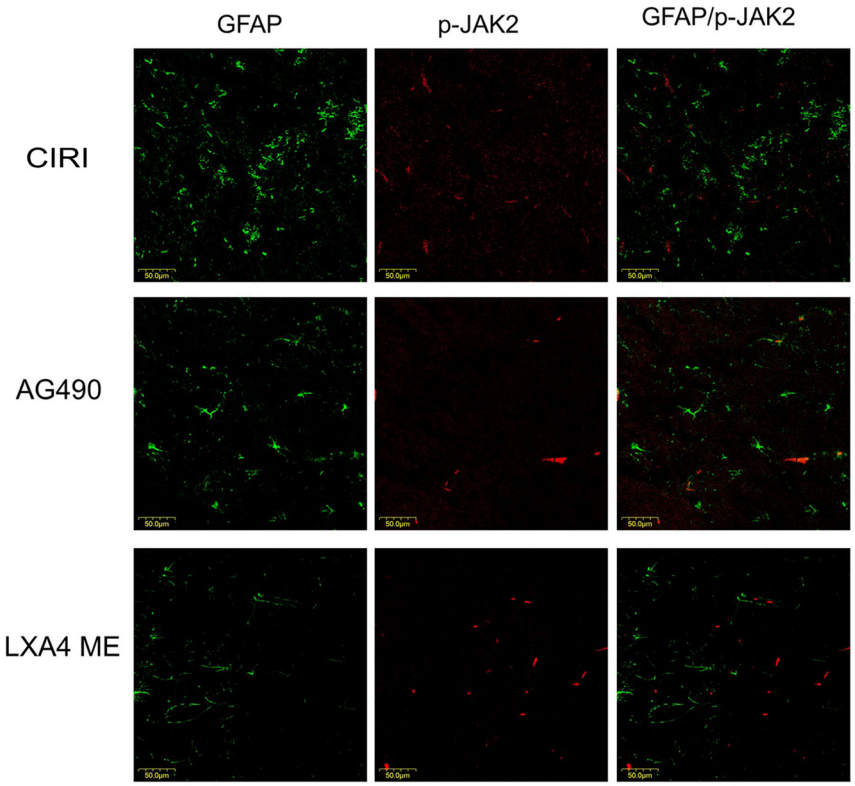

Figure 6. Changes of p-JAK2 protein expression in astrocytes of brain tissues.

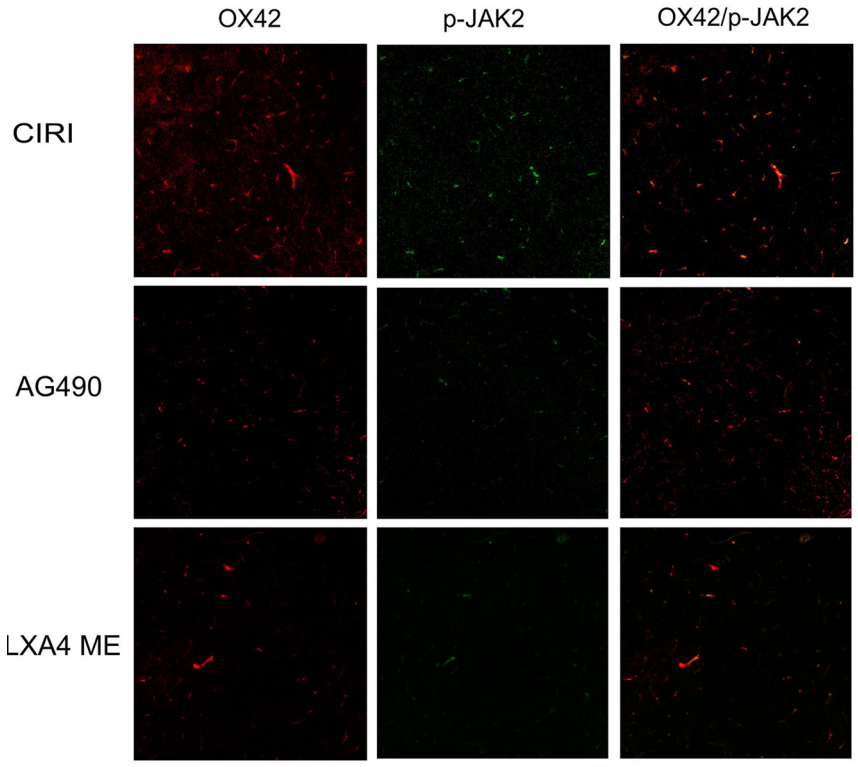

Figure 7. Changes of p-JAK2 protein expression in microglial cells of brain tissues.

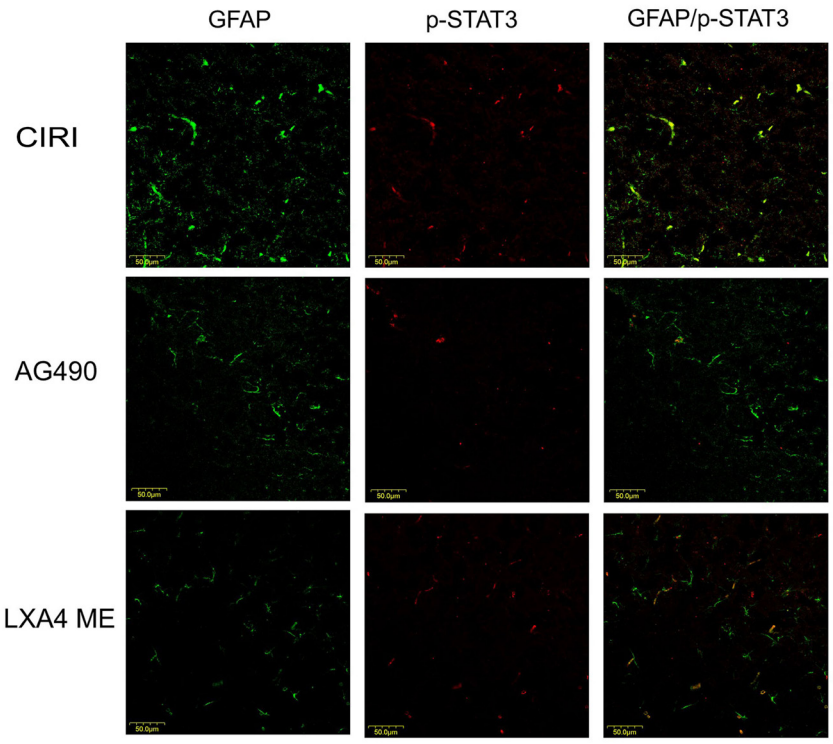

Figure 8. Changes of p-STAT3 protein expression in astrocytes of brain tissues.

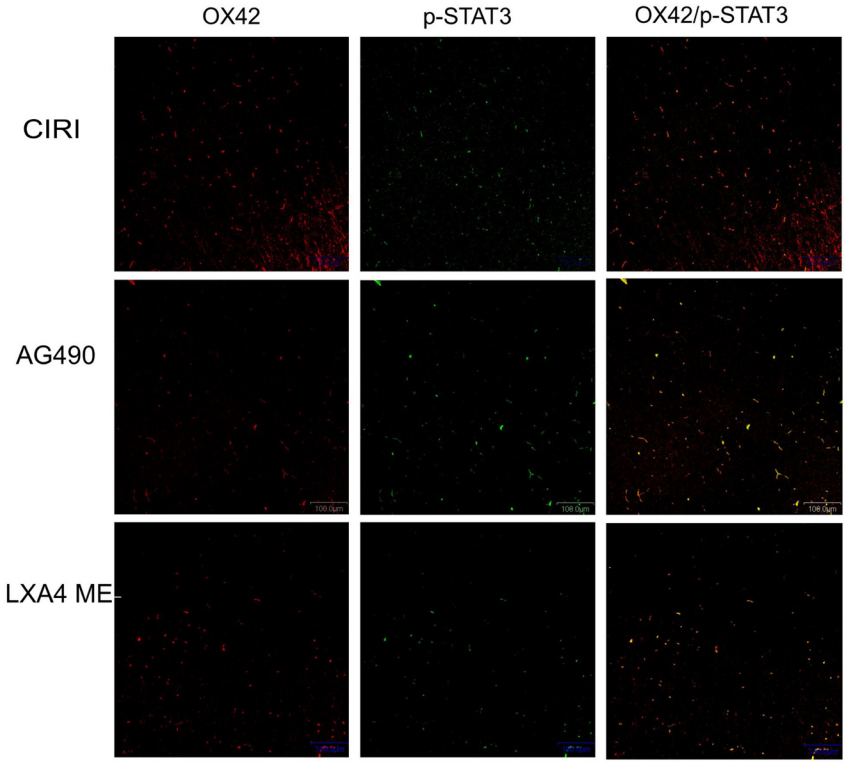

Figure 9. Changes of p-STAT3 protein expression in microglial cells of brain tissues.

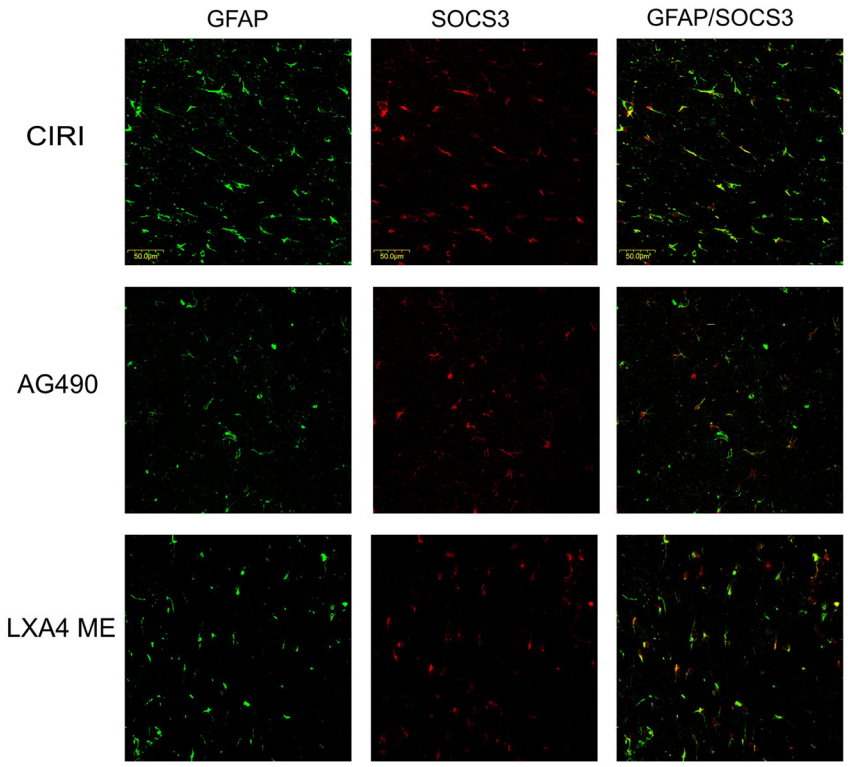

Figure 10. Changes of SOCS3 protein expression in astrocytes of brain tissues.

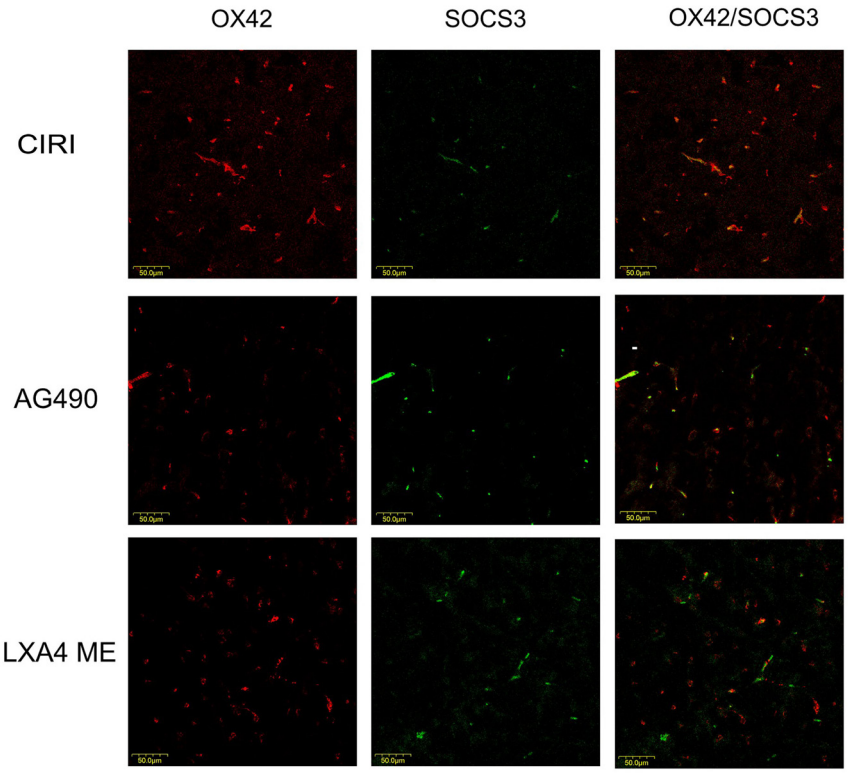

Figure 11. Changes of SOCS3 protein expression in microglial cells of brain tissues.

with astrocytes, microglial cells, oligodendrocytes, and vascular endothelial cells (Liang et al., 2016). These data (Wu et al., 2018; Sharma et al., 2011; Liang et al., 2016) revealed the effect of JAK2/STAT3 activation on regulating the physiologic status in cerebral injuries. We found that p-JAK2 protein was slightly expressed in brain tissues of sham rats. This result indicated that p-JAK2 protein was inactive in the brain tissues under a normal physiologic state, as previously reported (Wu et al., 2018). Following 24 h of CIRI, p-JAK2 and p-STAT3 were highly expressed within cytoplasm and nuclei of microglial cells, while p-STAT3 was upregulated in astrocytic cytoplasm. We also observed highly expressed p-JAK2 and p-STAT3 proteins 24 h after CIRI based on Western blotting, along with multiplied expression of STAT3 mRNA. The above findings suggested the CIRI-activated JAK2 and STAT3. The activated STAT3 translocated to the nuclei, bound to the target gene promoter, activated its expression, and caused a neurologic deficit in rats, which was in accordance with previous investigations (Wu et al., 2018; Sharma et al., 2011; Liang et al., 2016).

No consensus has been reached on how abnormal STAT3 activation induced by CIRI affects nerve cells. Satriotomo et al. (2006) reported a close connection between STAT3 phosphorylation and neuronal death in cerebral ischemia. Downregulation of JAK2/STAT3 activation improves nerve function and achieves neuroprotective effects in focal cerebral ischemia (Fan & Zhou, 2021b). Neuroprotective effect against CIRI can be activated by the JAK/STAT3 inhibitors like AG490 (Yang et al., 2017; Liang et al., 2016). Also, some drugs are demonstrated to provide protection on brain and improve neurological function through activating JAK2/STAT3 pathways (Feng et al., 2021; Hou et al., 2018). Moreover, a recent study showed that an antibody against IL-23 has protective properties against CIRI by JAK2/STAT3 (Lasek-Bal et al., 2019). More research is required to discuss actual function of STAT3 pathway in neuroprotection.

We also discussed the influence of AG490 on JAK2/STAT3 signal transduction. AG490, which is generally used as a selective inhibitor against JAK2, did not considerably affect the normal cell growth (Yang et al., 2017). Our experiments showed that early AG490 administration following focal cerebral ischemia in rats inhibited JAK2 phosphorylation and STAT3 mRNA and protein expression. Additionally, glial cells exhibited decreased phosphorated protein levels of JAK2 and STAT3, thus mitigating neurologic deficits in rats. The above results indicated that AG490 partially blocked or reduced intracellular signal transduction of inflammatory cytokines by inhibiting JAK2 phosphorylation and STAT3 tyrosine phosphorylation. Our results agreed with the results of international researchers (Yang et al., 2017; Fan & Zhou, 2021b; Liang et al., 2016).

The mechanisms activating and inhibiting inflammatory mediator production co-exist with the inflammatory response induced by CIRI (Jin et al., 2013; Ye et al., 2010). Lipoxin is an important endogenous mediator that promotes the resolution of inflammation in organisms, and exerts a negative regulatory effect on several types of inflammatory cells and inflammation-related genes (Freire & Van Dyke, 2013). Lipoxin functions as a "braking signal" in inflammation and its anti-inflammatory effect also involves several signal transduction pathways (Ye et al., 2010). LXA_4 ME downregulates the pro-inflammatory cytokines expression, while increases the anti-nflammatory cytokines expression in the ischemic brain (Ye et al., 2010). Lipoxin is activated by binding to lipoxin receptors or its G-protein coupled receptor as a complex, further triggering the Erk, p38, JNK, and PIPP pathways (Ge et al., 2020). Additionally, lipoxin might regulate the JAK/STAT pathway activation through influencing cytokines or SOCS family proteins (Gao et al., 2018; Machado et al., 2006). Lipoxin promotes SOCS2 expression, inhibits pro-inflammatory cytokine generation, and reduces neutrophil infiltration (Machado et al., 2006), which demonstrates SOCS2 to be an important intracellular mediator in the anti-inflammatory effect of lipoxin. Moreover, SOCS3 interacts with JAK2, inhibiting its phosphorylation and making JAK2 lose its kinase activity, which helps inhibitethe JAK/STAT pathway and suppress cytokine expression (Gao et al., 2018). These results show that Proteins of SOCS family may also be involved in regulating the synergistic or antagonist effects between the cytokines (Gao et al., 2018). The current study also illustrated that early LXA_4 ME treatment in CIRI rats markedly enhanced SOCS3 mRNA and protein expression within ischemic cortex. Oppositely, p-JAK2 and p-STAT3 protein expression declined within ischemic cortex and glial cells. LXA_4 ME might partially inhibit JAK2/STAT3 pathway activation via SOCS3, an intracellular negative regulator of JAK/STAT phosphorylation (Yu et al., 2017). Consequently, nerve injury following cerebral I/R is alleviated. This mechanism may explain the neuroprotective effect of LXA_4 ME.

IL-6, which is mainly found in neurons, astrocytes, microglial cells, mononuclear macrophages, and vascular endothelial cells, is an important inflammatory factor in CIRI (Lasek-Bal et al., 2019). High expression of IL-6 was found in saliva of ischemic stroke patients (Wang et al., 2014). It is thought that a high IL-6 level is positively correlated with the infarct area and the extent of neurologic deficit (Lasek-Bal et al., 2019). In our study IL-6 was upregulated 24 h post-reperfusion. A significant upregulation of IL-6 was shown, but SOCS3 expression did not increase dramatically, which suggests inadequate negative regulation. LXA_4 ME intervention decreased IL-6 expression, which might be related to SOCS3 upregulation or the inhibited NF-κB pathway activation. To conclude, LXA_4 ME partially inhibited IL-6-mediated activation of JAK/STAT pathway.

Our work preliminarily clarified the effects of lipoxin on astrocyte activation, proliferation and inflammatory response, as well as the regulatory effect of lipoxin on JAK2/ STAT3 pathway. The effects on cytokines, such as IL-6, that can induce the transcription factors (JAK and STAT) and on SOCS3, a negative regulator of the cytokine signal transduction, warrant further study. We showed that the LXA_4 ME had anti-inflammatory and neuroprotective effects via several signal transduction pathways in CIRI. The specific site of action for LXA_4 ME has not been identified.The repair mechanism of lipoxin on glial proliferation and nerve injury after ischemic brain injury needs to be further studied. In the future, we should extend investigations into the inflammatory response induced by ischemic cerebrovascular diseases to develop endogenous anti-inflammatory mediator analogs needed for the resolution of inflammation.

5 Conclusion

LXA_4 ME inhibited JAK2/STAT3 pathway activation following CIRI, probably via upregulating SOCS3 expression and inhibiting IL-6 overexpression. We propose this mechanism by which LXA_4 ME exerts an anti-inflammatory and neuroprotective effect.

Acknowledgements

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