

Neuroprotective effects of metformin on cerebral ischemia-reperfusion injury by regulating PI3K/Akt pathway

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Abstract

Metformin (Met) is a commonly used drug in the treatment of type 2 diabetes. Currently, it has been found that Met can effectively reduce the incidence of stroke and exert anti-inflammatory effects. However, its role in ischemia-reperfusion (I/R) induced nerve injury remains unclear. This study aims to investigate the neuroprotective effects of Met in I/R-induced neuron injury as well as the underlying mechanism. A middle cerebral artery occlusion (MCAO) model was established in SD rats, which were then treated with different doses of Met. Neurological deficits of rats were measured at different times post-surgery. TTC staining to observe the volume of cerebral infarction. HE staining was performed to observe pathological changes of brain tissues. Immunohistochemistry was performed to observe the expression of inflammatory factors in the cerebral tissues. qRT-PCR method was used to detect the relative expression of PI3K、Akt mRNA in cells after 24 h of drug action. Western blot method was used to detect the expression of PI3K、p-PI3K、Akt and p-Akt in hippocampus.

What's more, in vitro experiments were performed on BV2 microglia to verify

the role of Met against oxygen-glucose deprivation (OGD). As a result, Met dose-dependently attenuated neurological deficits and neuronal apoptosis. Besides, Met administration also significantly reduced BV2 cells apoptosis and inflammatory response. Mechanistically, Met inactivated PI3K/Akt pathway induced by I/R and OGD, while upregulated PI3K. In conclusion, Met protected rats from cerebral I/R injury via reducing neuronal apoptosis and microglial inflammation through PI3K/Akt pathway.

Keywords:Metformin;cerebral ischemia-reperfusion; inflammation; PI3K/Akt.

Introduction

Cerebrovascular diseases are the main cause of death among the elderly worldwide, among them, ischemic brain injury accounts for about 70% to 80% of all cerebrovascular patients, seriously threatening human's health (1). At present, intravenous injection of recombinant tissue plasminogen activator (r-TPA) and intra-arterial therapy have been widely used clinically and shown to reduce the risk of disability (2). However, the therapies may further aggravate neuronal death and neurological dysfunction at the same time (3). These side reactions involve a variety of pathophysiological mechanisms, among which inflammatory mechanisms have received increasing attention (4). Therefore, understanding the inflammatory mechanism after the cerebral ischemia-reperfusion injury is helpful to improve the treatment on cerebral I/R injury.

The results of epidemiological studies suggest that, Met as a first-line drug for the treatment of type II diabetes, Not only can play a hypoglycemic role, it also reduces the risk of stroke in diabetics (5,6). Importantly, Met plays an important role in various diseases through different mechanisms. For example, Tokuyama team research shows, after 2 hours of transient middle cerebral artery ischemia, three consecutive days of intraperitoneal administration, three times a day, it can effectively reduce the volume of cerebral infarction after cerebral ischemia (7). Mc Cullough team used a 60-minute transient middle cerebral artery occlusion model, intraperitoneal injection of Met was started 24 hours after ischemia, once a day, it was found that after 21 days

of injection, Met can reduce brain atrophy (8). Researchers believe that Met may inactivate PI3K, inhibition of inflammatory response after ischemia (9). Whether the mechanism of Met in I/R injury is related to PI3K and how it is related to PI3K regulation remains to be investigated. Therefore, in the past ten years, the research on whether Met can be used in therapeutic cerebral I/R injury has gradually become a hot spot.

The purpose of this study was to investigate the neuroprotective effects of Met on ischemic induced nerve injury. Rats suffered from cerebral I/R injury were treated with Met and we found that Met dose-dependently ameliorated neurological deficits of rats and neuronal damage in the brain lesions. Moreover, Met increased the expression of PI3K and Akt. Therefore, we speculated that Met could exert neuroprotective effect via modulating the PI3K/Akt signaling pathway.

Methods

Animals

All experiments were approved by the Animal Ethics Committee of School of Medicine, Yan'an University. Sixty male SD rats, aged 6-8 weeks, weighing 180-220g, were purchased from Animal Center of Air Force Military Medical University (Shanxi, China). The rats were housed in a temperature- and humidity-controlled animal dormitory, with light/dark cycles lasting 12 hours.

Establishment of experimental middle cerebral artery occlusion/reperfusion (MCAO/R) model in rats

A rat model of I/R injury was established using middle cerebral artery occlusion/reperfusion (MCAO/R) (10). The rats were anesthetized with 4% Sodium pentobarbital (350mg/kg) by intraperitoneal injection. Sterilize the neck with an ethanol cotton ball and open a 1 cm incision along the neck with surgical scissors. Blunt dissection was operated at the submandibular gland under a stereoscopic microscope, and the right common carotid artery, external carotid artery and internal

carotid artery were dissociated. The common carotid artery was fastened with a silk thread. We used a silk thread to tie a loose knot at the bifurcation of the external carotid artery and common carotid artery, ligated the distal end of the external carotid artery with a silk thread, and fused the external carotid artery with an electrocoagulation pen. Tighten the internal carotid artery with a silk thread, cut a small hole into the external carotid artery with micro ophthalmic surgical scissors, insert the threaded plug down through the small hole into the external carotid artery, loosen the knot on the internal carotid artery. Then we inserted the suture slowly into the internal carotid artery until resistance was felt (*Fig.1A-C*). Before the operation, the 60 rats were randomly divided into 5 groups (each group had 15 rats): sham group, MCAO/R group, MCAO/R+Met (3mg/kg,10mg/kg,30mg/kg). Rats were given Met (3,10,30mg/kg) by intraperitoneal injection one hour before operation and three times with 1 week of the operation. The sham and MCAO/R group were infused with same dose of solvent.

TTC staining

For the quantification of infarct volumes, the brains were removed after anesthesia. After being removed and frozen at -20 °C for 5 min, the brains were sliced into 5 slices (2mm thick coronal sections) and were stained with a 2% solution of TTC (Sigma Co., Ltd., St. Louis, MO) at 37 °C for 10 min in the dark. The slices were then fixed with 4% neutral paraformaldehyde for 24 h before photographed. Infarct areas were calculated by using Image J image analysis software (<http://rsb.info.nih.gov/ij/>), and were multiplied by slice thickness to give the infarct volume.

Water maze experiment

The Morris water maze experiment was used to observe the behavioral changes in animals. Specific steps refer to the method of Yao et al. (11). A circular stainless-steel pool with a diameter of 100cm and a height of 50cm was divided into 4 quadrants. A circular hidden platform with a diameter of 9 cm and a height of 27 cm was placed in the center of the target quadrant. The platform was hidden 1 cm below

the water. A camera was used to record the movement trajectory of rats above the pool, and the Ethovision XT monitoring system was used for analysis. The rats in different group were randomly placed into a quadrant (W, E, S, or N). The escape latency to the platform, the time spent in the targeting quadrant as well as the times crossing the platform were recorded, respectively and tested for three trials.

Modified neurological severity score

After 24 hours of I/R injury, modified neurological severity scores (mNSS) was used to evaluate the neurological functions of the rats. mNSS includes motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive), balance beam, and reflex tests. Professionally trained researchers who did not know the experimental groupings conducted the evaluation. The lowest total score was 0, indicating complete normal and no neurological deficits; the highest score was 18, which represents loss of consciousness or death in rats.

Cell culture and the establishment of OGD cell model

Microglial cell line BV2 was purchased from the Cell Center of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM (Thermo Fisher Scientific, MA, USA) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, MA, USA) and 1% penicillin/streptomycin (Invitrogen, CA, USA) at 37 °C and with 5% CO₂, 95% air. Cells were trypsinized with 0.25% trypsin (Thermo Fisher HyClone, Utah, USA) and sub-cultured during the logarithmic growth phase. To establish an OGD cell model, BV2 cells at logarithmic growth phase were cultured in D-Hank's medium and then incubated under hypoxic conditions (temperature 37°C, and atmosphere 95% N₂ and 5% CO₂). 12 hours of incubation, BV2 cells were then cultured in normal culture medium at 37 °C and with 5% CO₂, 95% air.

Flow cytometry

BV2 cells were seeded on 6-well plates for 24 hours. After being treated OGD and Met, the cells were harvested and suspended in PBS, and then treated for 30min with FITC-labeled Annexin V and PI (Nanjing KeyGen Biotech. Co. Ltd.) for 10min at room temperature in the dark. Next, the cells were incubated with binding buffer (400 μ L). Finally, the flow cytometry (EMD Millipore, Billerica, MA, USA) was used to test cell apoptosis.

Drug treatment

Met(Sigma-Aldrich, Shanghai, China) and EX 527 (Abcam, ab141506, Shanghai, China) were dissolved in 1% dimethyl sulfoxide (DMSO, Sigma-Aldrich) with 0.1M phosphate buffer solution (PBS). Rats were intraperitoneally given 3, 10, 30mg/kg Met or DMSO (0.1 M PBS + 1% DMSO). The total volume was 100 μ L. In the in vitro experiment, BV2 cells were treated with Met (3, 10, 30 μ g/ml) and/ or EX 527 (1 μ M) for 24 hours after undergoing OGD/R for 24 hours.

Immunohistochemistry

Immunohistochemistry was used to detect neuronal apoptosis and microglia activation. The fixed tissue was dehydrated, embedded, and sliced according to conventional methods, and baked at 60 °C for 1 hour. The sections were dewaxed in xylene I, xylene II, and xylene III for 5 min, respectively. After that, the sections were placed successively in anhydrous ethanol, 95% ethanol, and 75% ethanol for 5min, rinsed with tap water for 5min, sliced and placed in a citrate buffer for microwave repair for 10 min, cooled at room temperature, added with 0.3% hydrogen peroxide, and incubated in a wet box for 15 min in the dark. Next, the sections were incubated with the antibody anti-Caspase-3 (1:300, ab2302, Abcam) or anti-Iba1 (1:200, ab5076, Abcam) at 4 °C overnight. The working solution of HRP labeled secondary antibody was added at 37°C. Then, the sections were incubated with a peroxidase-conjugated polymer for 30min, and a DAB (Beyotime Institute of Biotechnology, Shanghai, China) system was used for detection. Finally, we observed and analyzed the average

gray level of positive expression sites using Image-proplus software (Media Cybernetics, USA).

Caspase-3 activity detection

Caspase-3 activity in the brain lesions was measured using Caspase3 activity assay kit (Cell Signaling Technology, Inc., Shanghai, China). Briefly, the tissues were homogenized in an appropriate lysis buffer at 4°C. Next, the lysate was centrifuged and the protein content of the supernatant was quantified. The Caspase3 viability was determined by a microplate reader (Bio-Rad) at 450 nm.

qRT-PCR

The total RNA of cells was routinely extracted using TRIzol reagent (Ambion, Life Technologies, USA). a cDNA reverse transcription kit was used to reverse transcription reaction according to the operation procedures. Next, the reversed cDNA was amplified using a quantitative PCR reaction system, which contained 500ng cDNA, 250nmol/L upstream and downstream primers, and 12.5μL of 2×SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan). The primers were as follows:

表 1. Statistics were performed using the $2^{-\Delta\Delta C_t}$ method. Each experiment was repeated three times.

Western blot

Total protein of the cells and tissues was extracted using RIPA lysate. Concentration of the protein measured by the BCA method. Then the total protein was separated by SDS-PAGE electrophoresis at a constant voltage of 100V for 60min. Next, the separated protein was electroporated into the PVDF membranes. After being blocked by 5% skimmed milk at room temperature for 2 hours, the membranes were incubated with primary antibodies PI3K (Abcam, ab110304, 1:1000), Akt (Abcam, ab16502, 1:1000), p-Akt (Abcam, ab86299, 1:1000), Caspase3 (Abcam, ab13847, 1:1000), Bcl2 (Abcam, ab32124, 1:1000), at 4 °C overnight. The next morning, the membranes were washed by TBST and then incubated with secondary antibodies for

1 hour at room temperature. Finally, the immunoblots were exposed by Chemistar™ High-sig ECL Western Blotting Substrate (Tanon Science & Technology Co., Ltd.). β -actin was used as the internal reference of the detected protein. Each experimental group was repeated 3 times.

CCK-8 assay

BV2 cells were seeded into 96-well plates with 100 μ l per well of complete medium. Cells were injured by 20 μ M Met, and treated with different concentrations of BAY 73-6691 (5, 10, 20 μ g/ml). CCK-8 (NeoBioscience, Shanghai, China) was utilized to evaluate cell viability according to the manufacturer's instruction. The absorbance was recorded at 450 nm using using a microplate reader (BioTek, USA).

Data statistics

SPSS23.0 statistical software (SPSS Inc.,Chicago,IL,USA) was used for analysis. The measurement data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). T-test was performed to compare the mean of the two sample groups. One-way analysis of variance was used to compare the mean of multiple samples. $P < 0.05$ was considered statistically significant.

Results

Met reduced infarct volumes in MCAO/R injury rats

Infarct volumes were obtained when ischemia was given for 2h and reperfusion for 24h. The white area represented infarcted tissues and red represented normal tissues. TTC staining results show the volume of cerebral infarctions significantly increased, compared with the sham group ($P < 0.001$); Met (3mg/kg, 10mg/kg, 30mg/kg) reduced the infarct volume compared with the MCAO/R group ($P < 0.001$, $P < 0.001$) (Fig. 2A, Fig. 2B). Moreover, the cerebral infarction volume of the Met30mg/kg group was significantly reduced, suggesting that the reduction of the cerebral infarction volume by Met is positively correlated with the measurement.

Neuroprotective effect of Met on MCAO/R injury rats.

In order to further verify the effect of Met on cerebral ischemia/reperfusion injury, we established a model of cerebral ischemia/reperfusion injury in rats, and treated the rats with Met. The neurological function defects of rats were assessed through water maze experiments, including spatial learning and memory, as well as motor function. The results showed that Met dose-dependently promoted the spatial learning and memory recovery of I/R rats (*Fig. 3A*). mNSS score display that Met attenuated neurological deficits of the I/R rats (*Fig. 3B*). Immunohistochemistry results show that Met significantly decreased the expression Caspase-3 apoptosis-related proteins(*Fig. 3C, D and E*). These results show that Met can reduce mNSS score by inhibiting the apoptosis of nerve cells, and realize the neuroprotection effect on I/R damaged rats.

Met inhibited I/R - mediated inflammatory response.

Met has certain neuroprotective effect on MCAO/R rats, to study the neuroprotective mechanism, we detected BV2 cells activation (labeled by CD11b) and the expression of inflammatory factors including IL-1, IL-6, IL-1 β and TNF- α . Immunohistochemistry results show that the number of CD11b BV2 cells as well as IL-1, IL-6, IL-1 β and TNF- α expression were all dramatically up-regulated in the brain lesions after I/R injury (*Fig. 4A, B, C, D, E and F*). Under the treatment of Met, CD11b positive cells were remarkably diminished and the expression of IL-1, IL-6, IL-1 β and TNF- α were also decreased (*Fig. 4A-D*). In order to further explore the anti-inflammatory mechanism of Met, we also studied the effect of Met on PI3K and Akt expression. Western blot results showed that Met attenuated the phosphorylated level of PI3K and Akt (p-PI3K, p-Akt) which was activated by I/R. Moreover, Met dose-dependently promoted the protein level of PI3K (*Fig. 4G*). Therefore, we speculated that Met can inhibit microglia induced inflammation following I/R injury, and probably through PI3K mediated Akt pathway.

Met inhibited the autophagy of BV2 cells induced by OGD.

The cerebral blood oxygen supply is insufficient, the region sensitive to ischemia and hypoxia will activate the autophagy pathway, and moderate autophagy can play a protective role in ischemia and hypoxia. However, the excessive occurrence of autophagy will cause neuronal damage. The results show that, Compared with the Control group, the relative expression of LC3 and Beclin-1 protein in the brain injury tissue of the OGD/R group was significantly increased, and the relative expression of p62 protein was significantly reduced ($P < 0.05$); Compared with OGD / R group, the relative expression of LC3 and Beclin-1 protein in met (5,10,20 $\mu\text{g} / \text{ml}$) group was significantly decreased, and the relative expression of p62 protein was significantly increased ($P < 0.05$), and positive correlation with dose (*Fig.5*).

Met inhibited the apoptosis of BV2 cells induced by OGD

In order to confirm the neuroprotective effects of Met, an in vitro model of I/R injury on BV2 cells was constructed by OGD. The viability of BV2 cells were detected by CCK8 assay, which showed that OGD induced obvious viability decrease, while Met treatment enhanced BV2 cells viability in a dose-dependent manner (compared with OGD group) (*Fig. 6A*). Moreover, the apoptosis of BV2 cells were also determine. The results of flow cytometry and western blot showed that compared with con group, the apoptotic rate and expression of Caspase-3 were markedly increased, and Bcl2 was downregulated under OGD treatment (*Fig. 6B 、 C and D*). However, Met significantly relieved the apoptosis of BV2 cells induced by OGD (*Fig. 6B 、 C and D*). Therefore, the above results indicated that Met exerted neuroprotective effects against OGD.

Met down-regulated OGD mediated microglial inflammation.

We treated BV2 microglia under OGD environment with Met , thus exploring the anti-inflammatory effects of Met. The results showed that OGD markedly promoted the expression of inflammatory cytokines, including IL-1 β and TNF- α (*Fig. 7A 、 B and C*). Next, the expression of PI3K and Akt was detected. We found that compared with the con group, the level of PI3K mRNA and protein were significantly

reduced after OGD treatment, while phosphorylated level of PI3K and Akt was increased (Fig.D、E and F). However, Met reversed OGD induced effects by promoting PI3K expression and inactivating Akt pathway. Consequently, the data verified that Met had anti-inflammatory effects by upregulating PI3K.

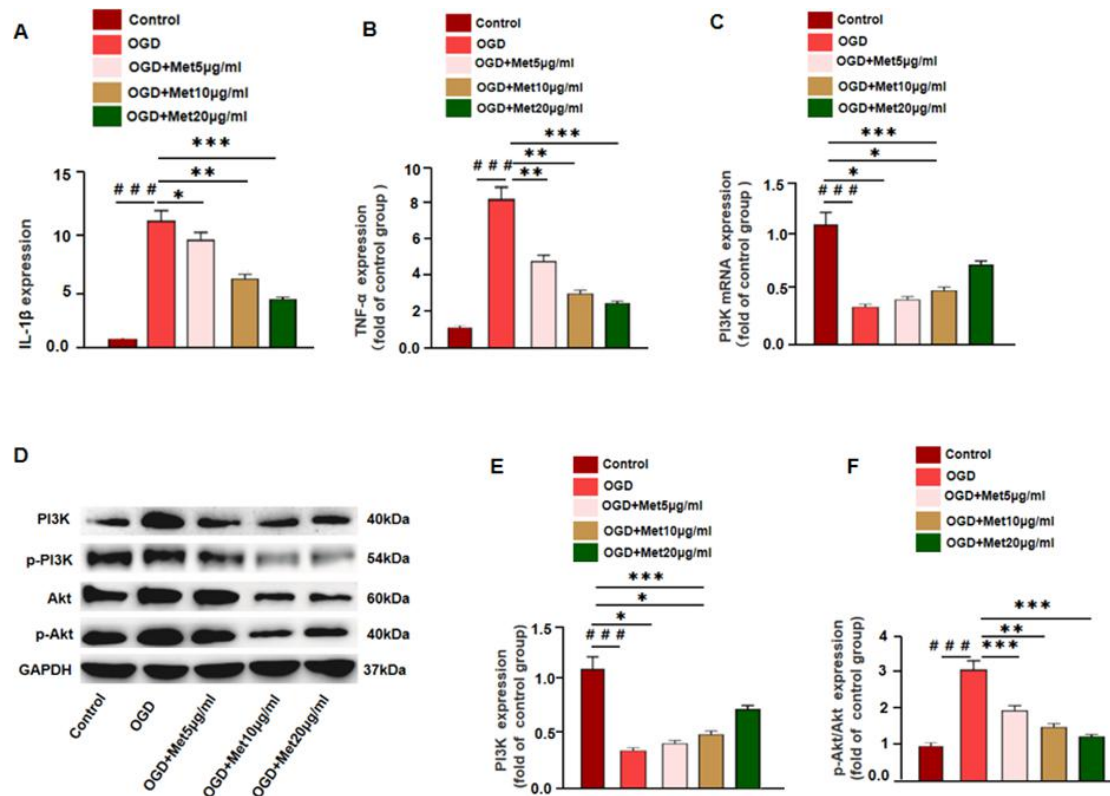


Figure7. Met down-regulated OGD mediated microglial inflammation. BV2 microglia was treated by OGD and/or Met (5,10,20 μ g/mL). A and B. The expression of inflammatory cytokines, including IL-1 β and TNF- α was tested by qRT-PCR. C. The expression of PI3K mRNA was detected by qRT-PCR. D. Western blot was used to detect the expression of PI3K、p-PI3K、Akt and p-Akt. vs. Control group: #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$; vs. OGD group: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

The anti-inflammatory effect of Met was further verified by inhibiting the expression of PI3K.

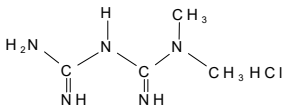
To further confirm the role of PI3K in Met mediated effects, we treated OGD activated microglia with PI3K inhibitor Calbiochem. Then the inflammatory response of microglia was detected. qRT-PCR results showed that compared with OGD+Met

group, the inflammatory cytokines (IL-1 β and TNF- α), phosphorylated Akt were all enhanced in the OGD+Met+Calbiochem group, while PI3K was downregulated (*Fig.7 A, B and C*). However, there were no significant differences between OGD group and OGD+Met+Calbiochem group in terms of the inflammatory cytokines and proteins (*Fig.7D, E and F*). Hence, the above results proved that Met exerted its effects dependently through PI3K.

Discussion

Stroke, the second-most cause of death worldwide, encompasses hemorrhagic stroke, but the majority of cases are caused by arterial occlusion causing ischemic injury (11). Stroke results in acute neuronal cell death and focal brain inflammation, which aggravates secondary brain injury by exacerbating blood-brain barrier damage, microvascular failure, brain edema, and oxidative stress (12). Thanks to technological innovations, the clinical management of ischemic stroke has greatly advanced, notably through the use of intravenous thrombolysis and endovascular thrombectomy, which reduces disability (13). However, identification of pathways and molecules that participate in cerebral ischemia could reveal novel approaches to improve the clinical outcome (14). In this regard, animal models mimicking human stroke such as the middle cerebral artery occlusion (MCAO) model enable the study on the pathogenesis of cerebral ischemia (15).

Metformin Hydrochloride is a biguanidine diabetes drug, Chemical name :1,1-methyl biguanidine hydrochloride, English name

(metformin, Met), Structure: . Cerebral I/R injury has been found to produce cytotoxicity as well as dysregulated neuroinflammation (16). In this study, we demonstrated that Met reduces neurological deficits and inflammatory responses in a MCAO rat model and the underlying mechanism of Met maybe through regulating the PI3K/Akt signaling pathway. Met as a classic hypoglycemic agent, current studies have found that it can reduce nerve damage caused by cerebral

ischemia and hypoxia. For example, Met inhibits H₂O₂-induced Bcl-2 down-regulation and Bax up-regulation in SH-SY5Y cells in a dose-dependent manner, thus increasing the Bcl-2/Bax ratio and exhibiting neuroprotective effects (17). In Alzheimer's disease (AD), Met inhibits A β aggregation and protects the neurons in AD (18). Besides, research also showed that Met plays an important role in anti-inflammation. For instance, it was found that in RAW 264.7 macrophages stimulated by lipopolysaccharide (LPS), Met exerts anti-inflammatory effects partly through PI3K/Akt signaling pathway (19). In acute lung injury (ALI), Met also reduces the number of inflammatory cells and expression of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 (20). Presently, it was found that Met not only dose-dependently promoted neurological rehabilitation of I/R rats, but also inhibited neuron apoptosis, microglia activation and the expression of TNF- α , IL-1 β and p-NF- κ B. These results indicated that Met has neuroprotective and anti-inflammatory effects in cerebral I/R injury.

A series of cascade reactions such as oxidative stress caused by cerebral ischemia and hypoxia can lead to over activation of autophagy and increase neuronal necrosis (21). PI3K / Akt pathway plays an important role in the regulation of autophagy. PI3K is an important signal transduction factor in cells, mainly composed of catalytic subunit P110 and regulatory subunit P85. Akt/PKB is a serine/threonine protein kinase, but also one of the important downstream target kinases in the process of PI3K signal transduction. PI3K is activated to produce PIP3, locating Akt to cell membranes, it binds to the PH region of Akt to activate Akt, this leads to a cascade of signal transduction pathways (22,23). Akt activated by PI3K can activate Akt phosphorylation, regulating autophagy-related protein Beclin1, LC3, P62 expression, participate in the regulation of autophagy. In this experiment, the results of TTC staining showed that, after 3, 10, 30 mg/kg Met intervention, in MCAO / R injury rats, the infarct volume and MNSs score decreased, the expression of IL-1, IL-6, IL-1 β , TNF- α , Beclin1, LC3 were significantly decreased, the expression of p62, p-PI3K and p-Akt were increased significantly, it was positively correlated with the

dose. These results suggest that Met can inhibit apoptosis and inflammation by regulating PI3K / Akt pathway, thereby alleviating MCAO / R injury.

In conclusion, our study revealed that Met alleviates brain injury caused by I/R through modulating inflammation, autophagy and apoptosis. The potential mechanism may be regulating PI3K/Akt pathway. Collectively, the above data indicates that Met has a potential role in the treatment of brain I/R injury.

Authors contribution

Conceived and designed the experiments: Cailian Ruan;

Performed the experiments: Hongtao Guo;

Statistical analysis: Zhiyong Liu, Jin Yi Yan, Xiao Ji Li;

Wrote the paper: Cailian Ruan, Hai Xia Lv.

All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics statement

Our study was approved by the Ethics Review Board of Yan'an University.

Data Availability Statement

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

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Figure Legends

Figure1. MCAO/R modeling process.A:Insert thread bolt, ligate common carotid artery.B.suture incision.C: MCAO/R pattern diagram.

Figure2.Met reduced infarct volumes in MCAO/R injury rats.A、B.The infarct volume was detected by TTC staining.A、B: TTC staining detection of cerebral infarction volume in rats.vs. Sham group: $^{\#}, P<0.05$, $^{##}, P<0.01$, $^{###}, P<0.001$;vs. MCAO/R group:*, $P<0.05$, **, $P<0.01$,***, $P<0.001$.

Figure3.Neuroprotective effect of Met on MCAO/R injury rats.A. Morris water maze experiment was assessed 14 days after brain injury and the representative images of Morris water maze in different groups were as shown. B. mNSS was used to evaluate the neurological damage of rats in each group. C. Caspase3 activity in the brain lesions were detected using a Caspase3 kit. D、E.Immunohistochemistry was conducted to detect apoptotic cells marked by Caspase3 and the number of Caspase3 positive cells was counted. vs. Sham group: $^{\#}, P<0.05$, $^{##}, P<0.01$, $^{###}, P<0.001$;vs. MCAO/R group:*, $P<0.05$, **, $P<0.01$,***, $P<0.001$.

Figure4.Neuroprotective effect of Met on MCAO/R injury rats.A.B.The infarct volume was detected by TTC staining.4A、B、C、D、E and F. Immunohistochemistry was used to examine microglia activation (marked by CD11b). G.Western blot and qRT-PCR was used to analyze the relative expression of IL-1、IL-6、IL-1 β 、TNF- α 、p-PI3K and p-Akt in each group. vs. Sham group: $^{\#}, P<0.05$, $^{##}, P<0.01$, $^{###}, P<0.001$;vs. MCAO/R group:*, $P<0.05$, **, $P<0.01$,***, $P<0.001$.

Figure5. Western blot detection of relative expression of autophagy-related proteins.

Figure6.Effect of Cell Experiment Met Anti-apoptotic Effect.BV2 cells were pre-treated by OGD for 24 hours, and then treated with different doses of Met (5,10,20 μ g/mL) for 24 hours. A. The viability of BV2 cells were detected by CCK8 assay. B and C. Flow cytometry was used to examine the apoptosis of BV2 cells. D.

Western blot was used to detect apoptotic proteins, including Caspase-3 and Bcl2. vs. Control group: [#], $P < 0.05$, ^{##}, $P < 0.01$, ^{###}, $P < 0.001$; vs. OGD group: ^{*}, $P < 0.05$, ^{**}, $P < 0.01$, ^{***}, $P < 0.001$.

Figure7. The anti-inflammatory effect of Met was further verified by inhibiting the expression of PI3K. BV2 microglia was treated by OGD, Met (5 μ g/ml) and/or Calbiochem (1 μ M). A and B. The expression of inflammatory cytokines, including IL-1 β and TNF- α was tested by qRT-PCR. C. The expression of PI3K mRNA was detected by qRT-PCR. D. Western blot was used to detect the expression of PI3K、p-PI3K、Akt and p-Akt. vs. Control group: [#], $P < 0.05$, ^{##}, $P < 0.01$, ^{###}, $P < 0.001$; vs. OGD group: ^{*}, $P < 0.05$, ^{**}, $P < 0.01$, ^{***}, $P < 0.001$.