Dexmedetomidine pretreatment inhibits cerebral ischemia/reperfusion-induced neuroinflammation via activation of AMPK

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Abstract. Focal ischemia/reperfusion (I/R) injury induced cerebral inflammation, aggravates brain damage. The aim of the present study was to investigate the protective mechanisms of dexmedetomidine (DEX) on I/R brain injury in rats. Sprague-Dawley rats were divided to seven experimental groups (18 rats/group): Sham surgery; middle cerebral artery occlusion (MCAO) surgery (90 min); DEX10 [10 μg/kg intraperitoneal (i.p.) injection 30 min prior to MCAO]; DEX50 (50 μg/kg i.p. 30 min prior to MCAO); DEX100 (100 μg/kg i.p. 30 min prior to MCAO); DEX50+Yohimbine [YOH; 5 mg/kg 10 min prior to DEX (50 μg/kg i.p.) administration and MCAO] and YOH (5 mg/kg 40 min prior to MCAO). At 24 h post-MCAO surgery, neurological deficit was examined by staining damaged brain tissues with 2,3,5-triphenyltetrazolium chloride. Neuronal apoptosis in the cerebral cortex was histologically assessed by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling staining, and the expression levels of phosphory-lated (p)-AMP-activated protein kinase (AMPK; Thr172) was detected by western blotting. In addition, the expression levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β were assessed by ELISA. At days 1, 2 and 5 following I/R, motor functions were assessed by an observer blinded to the study. The brain infarct size, neurological deficit scores, number of apoptotic neurons, expression levels of pro-inflammatory cytokines TNF-α and IL-1β were increased following MCAO, whereas the motor function scores were reduced. Pretreatment with DEX prior to MCAO can reverse the effects induced by I/R.

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Compared with rats in the Sham group, the expression levels of p-AMPK were mildly increased in the MCAO group and highly increased in the three DEX-treatment groups. Pretreatment with YOH reversed the above effects of DEX and produced a similar level of cerebral I/R injury. The results demonstrated that precondition with DEX exhibited anti-inflammatory effects on brain ischemic injury mediated by AMPK signal pathway.

Introduction

Stroke is a heterogeneous disease and is the leading cause of mortality in the world. For patients with ischaemic stroke, anticoagulant therapy has been the mainstay of treatment to prevent recurrent ischaemic stroke and venous thromboembolism. Specifically, anticoagulant intervention with oral vitamin K antagonists, including warfarin, is used to prevent recurrent stroke. However, this drug is substantially underused owing to concerns over the risk of bleeding (1). Among the different types of stroke, ischemia stroke is the main type, which makes up 60-80% of all stroke evens. Neuronal infarct and behavioral dysfunction caused by ischemia induced brain injury are common in patients with stroke. Tissue plasminogen activator (tPA) is the only approved pharmacological treatment for ischemic stroke (2) In cases that result in a negative outcome with tPA (3), the identification of a neuroprotective therapy is required. Inflammation may serve an important role in regulating tissue damage and dysfunction (4). Previous reports indicated that dysfunctional energy metabolism may induce inflammation in cerebral ischemia injury (5). Therefore, pharmacological treatment with an anti-inflammatory drug may have obvious effects in treating stroke.

AMP-activated protein kinase (AMPK) responds to changes in the AMP:ATP ratio and is considered an index of cellular energy levels (6), and serves as a sensor of energy balance and is activated in response to low energy supply (7). AMPK expression is abundant in the brain, and cerebral AMPK is rapidly activated in response to cerebral ischemia (8). The effects of AMPK on the nervous system under pathological conditions are being examined (9). Based on its sensitivity to AMP, AMPK may be activated by nutrient deprivation-induced metabolic stresses, such as hypoxia or glucose deprivation (10); ischemia may induce phosphorylation of AMPK at Thr172 (11). Furthermore, previous studies have

indicated that activation of AMPK protected against global cerebral ischemia and focal ischemia (8,12,13).

Dexmedetomidine (DEX), an α2 adrenergic receptor (AR) agonist, has been reported to have protective effects against I/R injury in different tissues (14,15), particularly in the brain (16). DEX has also been reported to serve an anti-inflammatory role in ischemia injury in rats (17-21). However, the protective effects and mechanisms of DEX on brain ischemic injury have not been fully examined. For example, it is unknown if the anti-inflammatory effects of DEX may be associated with AMPK pathway. Therefore, the present study aimed to investigate the effects and mechanism of DEX on cerebral ischemia-induced inflammation.

Materials and methods

Materials. DEX was purchased from Jiangsu Singch Pharm. Co., Ltd. (Jiangsu, China) and the α2-AR antagonist yohimbine (YOH) was purchased from Tocris Bioscience (Bristol, UK) 2,3,5-Triphenyltetrazolium chloride (TTC) and pentobarbital sodium were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Primary antibodies against AMPK and ELISA kits were purchased from R&D Systems Europe, Ltd., (Abingdon, UK). Rabbit anti-goat, goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from R&D Systems Europe, Ltd.

Animals. A total of 126 male Sprague-Dawley (SD) rats (8 weeks, 220-250 g) were purchased from the Experimental Animal Center of Shanghai Jiaotong University (Shanghai, China) and housed in a controlled environment with a 12-h light/dark cycle, 60±5% humidity and 22±2°C with access to water and food *ad libitum*. All procedures were conducted in accordance with The National Institute of Health Guide for the Care and Use of Laboratory Animals and this study was approved by the Renji Hospital Laboratory Animal Ethics Committee (reference no. 20170723-006).

As illustrated in Fig. 1, SD rats were divided to seven experimental groups (18 rats/group): Sham surgery [treated with intraperitoneal (i.p.) saline]; middle cerebral artery occlusion (MCAO) surgery (90 min); DEX10 [10 μg/kg i.p. injection 30 min prior to MCAO]; DEX50 (50 μg/kg i.p. 30 min prior to MCAO); DEX100 (100 μg/kg i.p. 30 min prior to MCAO); DEX50+Yohimbine [YOH; 5 mg/kg 10 min prior to DEX (50 μg/kg i.p.) administration and MCAO] and YOH (5 mg/kg 40 min prior to MCAO).

Establishment of middle cerebral artery occlusion (MCAO) model rats. The MCAO model was established as previously described (22). Briefly, rats were anesthetized with an i.p injection of 3% pentobarbital sodium (50 mg/kg; Sigma-Aldrich; Merck KGaA), and the middle cerebral artery (MCA) was occluded by threading a monofilament sterile nylon suture with a heat-rounded tip through the internal carotid artery, which was advanced until it blocked the origin of the MCA. At 90 min following ischemia induction, reperfusion was initiated by withdrawal of the monofilament. In the Sham surgery group, the MCA was separated only and MCAO was not performed. During all surgical procedures rats were maintained at 37°C using a heating blanket and a heat lamp.

Drug treatments. DEX was dissolved in normal saline and administered by *i.p* injection at three single doses (10, 50 100 μg/kg; 0.25 ml administration) 30 min prior to MCAO surgery. YOH (5 mg/kg; 0.25 ml) was administered by *i.p* 10 min prior to the dexmedetomidine (50 μg/kg). The concentrations of DEX and YOH were selected according to the previous reports (19,23-26). The vehicle control was normal saline, which was administrated by *i.p* injection in 0.25 ml 30 min prior to ischemia induction (Fig. 1). SD rats were divided to seven experimental groups (18 rats/group): Sham surgery; middle cerebral artery occlusion (MCAO) surgery (90 min); DEX10 [10 μg/kg i.p injection 30 min prior to MCAO]; DEX50 (50 μg/kg i.p 30 min prior to MCAO); DEX100 (100 μg/kg i.p 30 min prior to MCAO); DEX50+Yohimbine [YOH; 5 mg/kg 10 min prior to DEX (50 μg/kg i.p) administration and MCAO] and YOH (5 mg/kg 40 min prior to MCAO).

Evaluation of neurological deficit. Neurological deficit scores were evaluated as reported previously (26): 0, no motor deficits (normal); 1, forelimb weakness and torso turning to the ipsilateral side when held by tail (slight); 2, circling to the contralateral side, with normal posture at rest (moderate); 3, unable to bear weight on the affected side at rest (severe); 4, no spontaneous locomotor activity or barrel rolling (serious).

Evaluation of infarct volume. Infarct volume was evaluated by TTC staining at 24 h following I/R. A total of 6 rats/group were euthanized and the brains were quickly removed. The brains were sliced into five coronal sections (2 mm) and stained with 1% solution of TTC at 37°C for 20 min, followed by fixation in 4% paraformaldehyde at room temperature for 30 min. Images of TTC-stained sections were captured by light microscopy (x1; DSC-HX9V, SONY Corporation, Tokyo, Japan) and the digital images were analyzed using Image-Pro Plus v6.0 (Media Cybernetics, Inc., Rockville, MD, USA) image analysis software. Lesion volumes were calculated by multiplying the area by the thickness of slices. The percentage of hemisphere lesion volume (%HLV) was calculated by the following formula (27): %HLV = {[total infarct volume-(the volume of intact ipsilateral hemisphere-the volume of intact contralateral hemisphere)]/contralateral hemisphere volume} x100.

Detection of tumor necrosis factor (TNF)-α and interleukin (IL)-6 expression in cortex. At 24 h post-I/R, 6 rats/group were sacrificed and the brain cortex tissue were obtained from the infarcted cerebral hemisphere and homogenized at 4°C in lysis buffer for ELISA. The production levels of TNF-α and IL-1β were measured in brain tissue homogenates using specific ELISA kits (TNF-α: cat. no. MTA00B; IL-1β: cat. no. MLB00C; R&D Systems Europe, Ltd.), according to the manufacturer's protocol; results were expressed as pg/ml.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) staining. At 24 h following I/R, 6 rats/group were euthanized, rats were anesthetized with 4% chloral hydrate and intracardially perfused first with 250 ml saline and then fixed with 250 ml 4% paraformaldehyde (at 4°C for 30 min). Then the fixed brains were processed by embedding in paraffin blocks, and the brains (2-μm) were sectioned. Apoptotic cells in the cortex were determined by the terminal
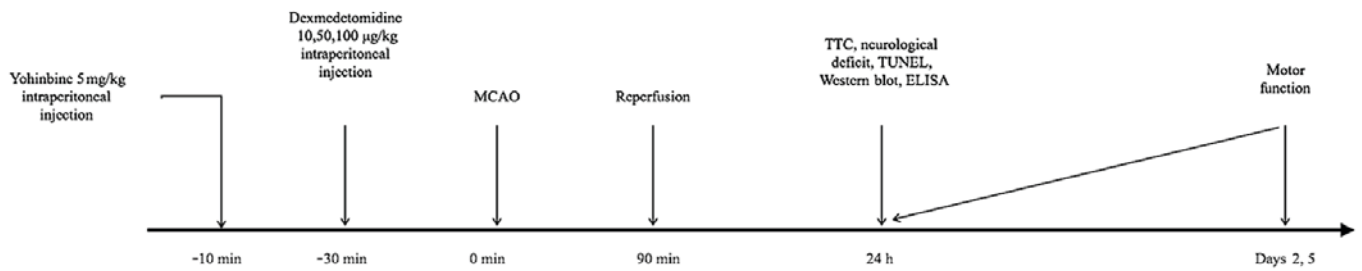
Figure 1. Protocol flowchart for investigating the effects of Dexmedetomidine pretreatment on MCAO-induced injury. MCAO for 90 min was used to establish focal ischemia followed by reperfusion as detailed in Methods. MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling.

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. TUNEL staining was performed 6 times according to the manufacturer's protocol (R&D Germany); TUNEL-positive cells emitted a green fluorescent color and were quantified using fluorescence microscopy (Olympus BX53; Olympus corporation) at magnification, x40, and 5 fields for each section were examined from the ischemic cortex. The average percent of TUNEL-positive cells out of the total number of cells were determined. Nuclei were stained with DAPI.

Western blot analysis. At 24 h post-I/R, the cortex of 6 rats/group were obtained and homogenized. Total protein extracted from cortex homogenates with a ProteinExt™ Mammalian Total Protein Extraction kit (TransGenBiotech, Beijing, China) was used to analyze protein expression by western blotting. Following that, protein concentrations were measured using a BCA Protein Quantification kit (Yeasen, Shanghai, China) with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein samples (40 μg/lane) were separated by 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were placed in QuickBlock Blocking Buffer for Western Blot (Beyotime Institute of Biotechnology, Beijing, China) for 30 min to block non-specific binding sites at 4˚C prior to incubating with mouse primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) against AMPKα (1:1,000; cat. no. 2532), phosphorylated (p)-AMPK (1:1,000; cat. no. 4186) and β-actin (1:2,000; Abcam; cat. no. ab8226) at 4˚C overnight. The membranes were subsequently washed with TBST (0.05% Tween-20), incubated with the appropriate secondary antibodies (horseradish peroxidase-conjugated anti-mouse IgG secondary antibody; R&D Systems Europe, Ltd.; cat. no. HAF007, 1:2,000) at room temperature for 2 h and washed with TBST. This experiment was repeated 6 times. The protein bands were detected using a Bio-Rad Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified using the Quantity One software package (version 2, Bio-Rad Laboratories, Inc.). The expression of p-AMPK was normalized to β-actin.

Motor function tests. A total of 6 rats/group were subjected to neuromotor tests (screen clinging, horizontal bar and prehensile traction) at days 1, 2 and 5 post-I/R, as previously described (28), with a score of 9 being the best possible score. The rats were placed on a screen (29x30 cm), which was rotated in the vertical plane. The time that the rat was able to

hold onto the vertical screen was recorded for a maximum of 15 seconds (screen clinging). In addition, the rats were placed at the center of a horizontal wooden rod (diameter, 2.5 cm) and the time that the rat was able to remain balanced on the rod was recorded for a maximum of 30 sec (horizontal bar). Finally, the time that the animal was able to cling to a horizontal rope was recorded for a maximum of 5 sec (prehensile traction). The tests were performed by a researcher who was blinded to animal group assignments.

Statistical analyses. Data were analyzed by one-way analysis of variance followed by Tukey's post hoc test except total motor scores using GraphPad Prism 5.0 (Graphpad, Inc., La Jolla, CA, USA). Neuromotor scores using the Tukey's Multiple Comparison Test data were expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. Each experiment was repeated six times.

Results

DEX suppresses I/R-induced neuronal injury and apoptosis in the cortex of rats. Compared with rats in the MCAO group, the neurological scores and infarct volumes were significantly decreased in rats in each of the three DEX pre-treatment groups (P<0.01; Fig. 2A and B). No significant differences were identified for neurological scores and infarct volume in the YOH group compared with the MCAO group. Compared with the DEX10 group, the neurological scores and infarct volumes were significantly decreased in DEX50 and DEX100 groups (P<0.05); However, no significant differences were identified between the DEX50 and the DEX100 group (P>0.05). The neurological scores and infarct volumes were significantly increased in rats in the DEX50+YOH group (P<0.001).

Compared with the Sham group, the percent of TUNEL-positive cells and infarct size in the cortex of ischemic hemisphere in MCAO group increased (P<0.001; Fig. 2C and D). Compared with the MCAO group, the percent of TUNEL-positive cells was significantly decreased in the DEX treatment groups (P<0.05 or P<0.01); however, no significant difference in apoptotic cells was identified in the YOH group compared with the MCAO group. Compared with the DEX10 group, the percent of TUNEL-positive cells was significantly decreased in the DEX50 and DEX100 groups (Fig. 2D; P<0.01). Compared with the DEX50 group, there was a significant increase in apoptotic cells in the DEX50+YOH group (P<0.01).
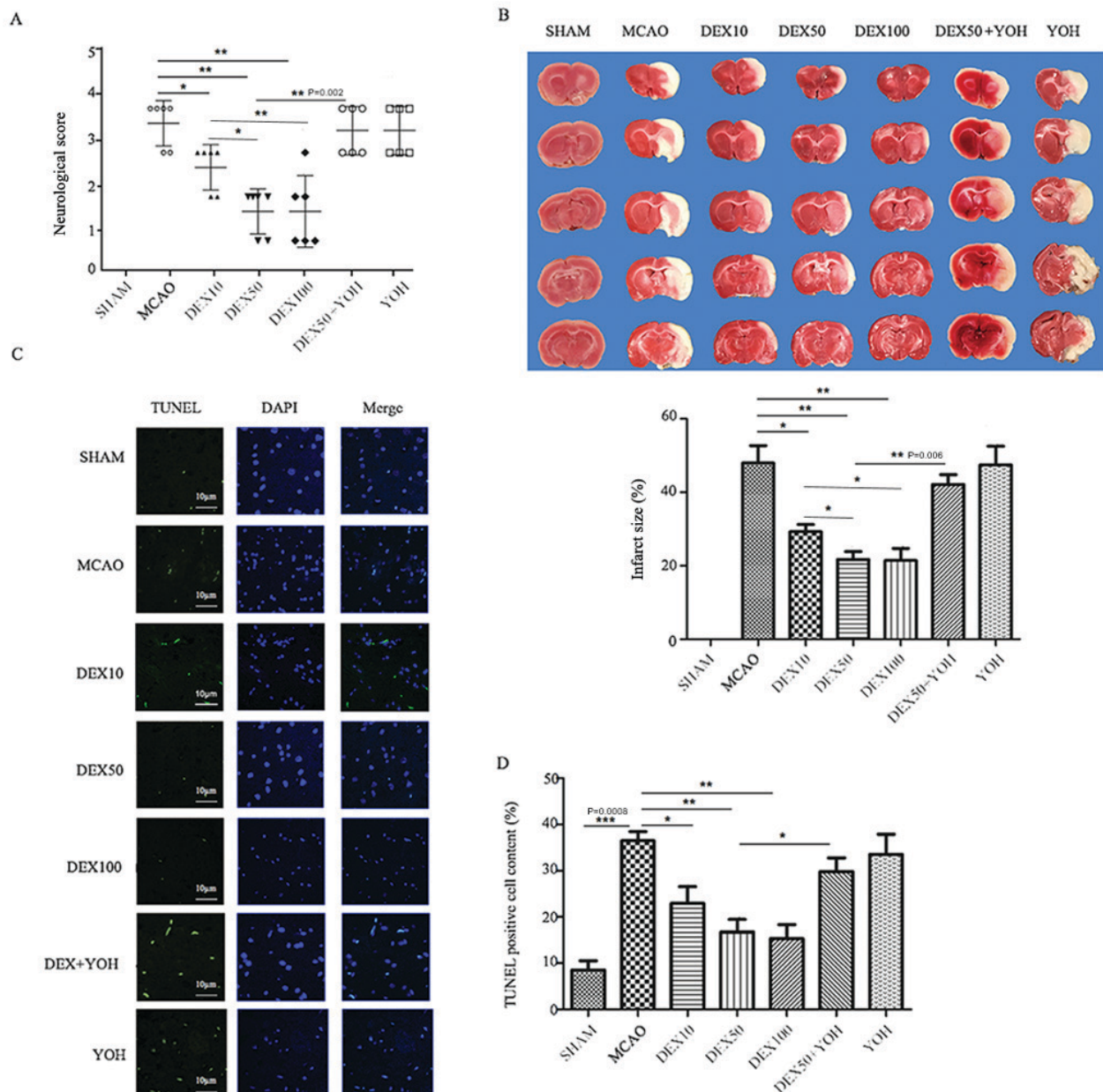
Figure 2. DEX suppresses MACO-induced neuronal death and apoptosis in rats. (A) Neurological score. Score of Sham group was 0. (B) TTC staining of brain tissue was used to determine ischemia-induced infarct size in the cortexes in rats in the various treatment groups. Infarct size, the Sham group was scored 0. (C) Apoptotic neurons were detected using TUNEL staining (green); nuclei were stained with DAPI (blue); scale bar, 10 µm. (D) The percent of TUNEL-positive cells was determined for each experimental group. Data are presented as the mean ± standard deviation; *P<0.05, **P<0.01 and ***P<0.001. MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling. DEX10/50/100, dexmedetomidine pre-treatment (10/50/100 µg/kg); YOH, yohimbine.

DEX alleviates I/R-induced inflammation in rats via activation of AMPK. The production levels of TNF-α and IL-1β in the brain tissues were examined to elucidate the effects of DEX on MCAO-induced inflammation (Fig. 3A and B, respectively). Compared with the Sham group, the levels of TNF-α and IL-1β were significantly increased in the MCAO group (P<0.001). Compared with the MCAO group, the levels of TNF-α and IL-1β were significantly decreased in the three DEX treatment groups (P<0.01). YOH had no observable effects on the production of TNF-α and IL-1β in the cortex compared with the MCAO group. Compared to DEX50 group, the levels of TNF-α and IL-1β were higher in the DEX50+YOH-treatment group (P<0.01).

The expression levels of p-AMPK were also examined in the ischemic brain cortex. The levels of p-AMPK were elevated in ischemic cortex (Fig. 3C and D). Compared with the MCAO group, pretreatment with DEX (10, 50 and 100 µg/kg) resulted in a significant increase in the expression levels of p-AMPK (P<0.01). p-AMPK expression levels were increased in DEX50 and DEX100 groups compared with the DEX10 group (P<0.05). Compared with the DEX50 group, p-AMPK expression was significantly lower in the DEX50+YOH group (P<0.01). Furthermore, the ratio of p-AMPK/AMPK as illustrated in Fig. 4.

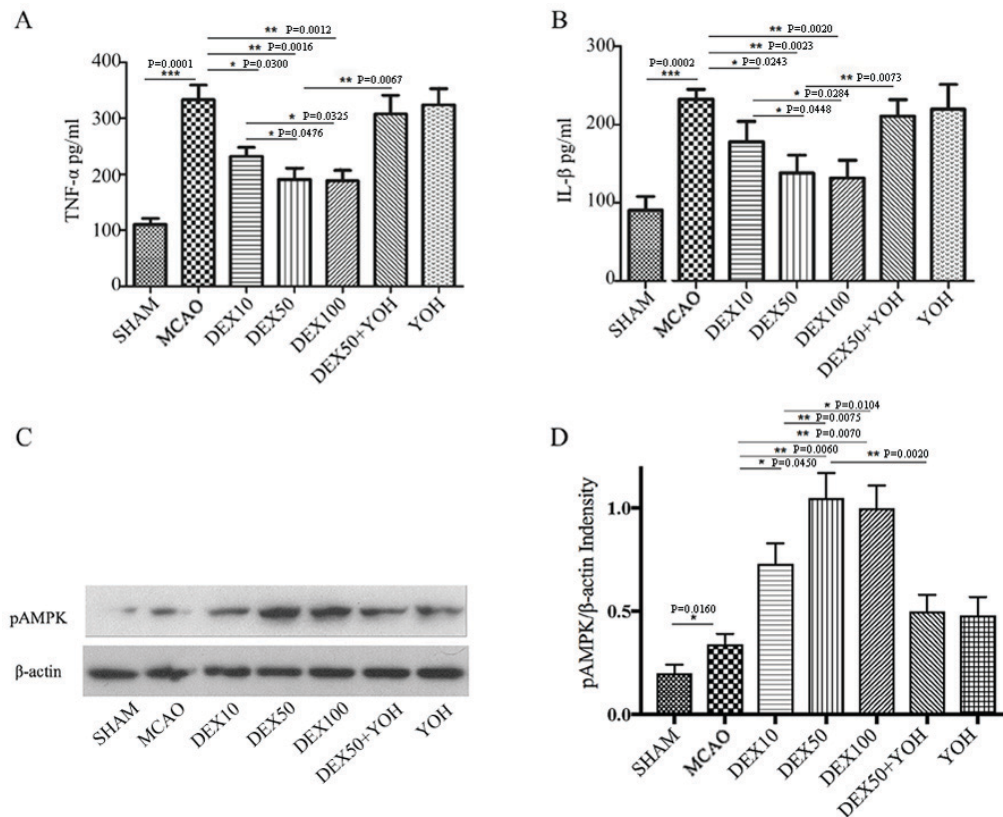*DEX improves motor functions following forebrain ischemia.* Focal cerebral ischemia resulted in reduced motor

Figure 3. DEX alleviates ischemia-induced brain inflammation in MCAO rats by activating AMPK. (A and B) Effects of DEX pre-treatment on the expression levels of (A) TNFα and (B) IL-1β in brain tissues from rats in each of the various treatment groups at 24 h following focal ischemia induction. (C and D) p-AMPK expression in the cortex was (C) detected by western blotting and (D) relative expression levels were determined by densitometric analysis. Data are presented as the mean ± standard deviation; *P<0.05, **P<0.01 and ***P<0.001. AMPK, AMP-activated protein kinase; DEX10/50/100, dexmedetomidine pre-treatment (10/50/100 µg/kg); IL-1β, interleukin 1β; MCAO, middle cerebral artery occlusion; p, phosphorylated; TNF-α, tumor necrosis factor α; YOH, yohimbine.
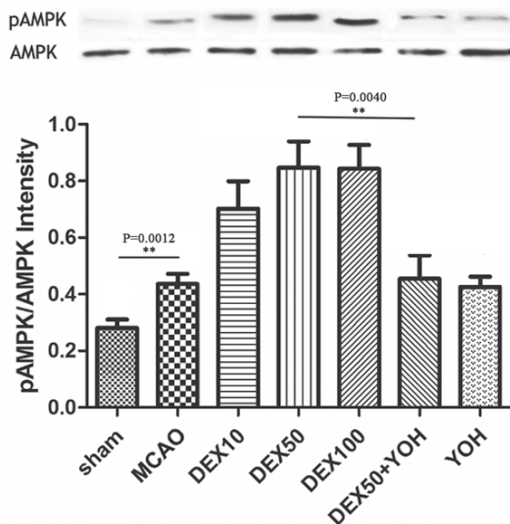

Figure 4. DEX increases the ratio of p-AMPK/AMPK in MCAO rats. The expression of p-AMPK and AMPK in the cortex was detected by western blotting and relative expression levels of p-AMPK/AMPK were determined by densitometric analysis. Data are presented as the mean ± standard deviation. **P<0.01. MCAO, middle cerebral artery occlusion; DEX10/50/100, dexmedetomidine pre-treatment (10/50/100 µg/kg); p, phosphorylated; YOH, yohimbine.

function scores in MCAO rats compared with Sham group rats at day 1 following I/R (Fig. 5A), and the motor functions remained low at days 2 and 5 (Fig. 5B and C, respectively).

The decline of motor function was partially improved by pre-treatment with DEX, and the higher doses (50 and 100 µg/kg) improved the motor function scores more significantly compared with the lower dose (10 µg/kg; P<0.05). No significant differences in motor functions were identified in rats in the YOH groups compared with the MCAO group; However, compared with the DEX50 group, pretreatment of YOH resulted in a significant reduction in the motor score (P<0.01).

Discussion

Results from the present study demonstrated that different doses of DEX (10, 50 100 µg/kg), an α2-AR agonist, were able to effectively reduce the cortex area of brain injury, cell apoptosis and inflammation; DEX pre-treatment also significantly improved motor function scores. The protective effects of DEX on I/R-induced cerebral brain cell injury may function through the AMPK pathway. In addition, the α2-AR antagonist YOH was revealed to inhibit the effects of DEX.

DEX functions include antianxiety, analgesia and restraining the sympathetic nerve activity (29). Previous clinical and animal studies have demonstrated that DEX attenuated cerebral injury and decreased the occurrence of disordered cerebral function during the perioperative period (30,31); *in vivo* experiments demonstrated that DEX decreased cerebral infarction area and improved the function of
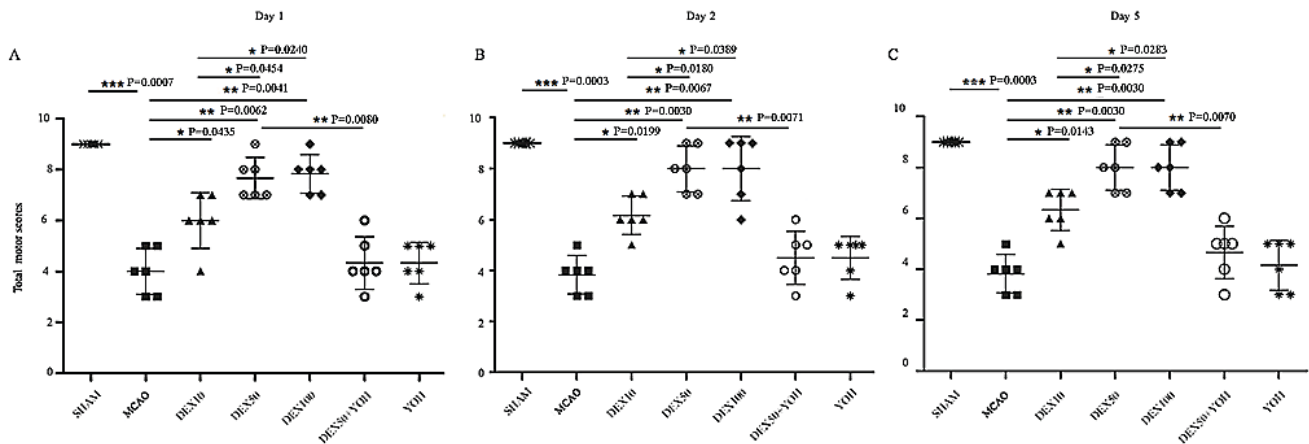
Figure 5. DEX improves motor function of MCAO rats. (A-C) The motor function of rats in each of the various treatment groups was examined at (A) day 1, (B) day 2 and (C) day 3 following MCAO surgery. Horizontal bars represent the median motor score; *P<0.05, **P<0.01 and ***P<0.001. DEX10/50/100, dexmedetomidine pre-treatment (10/50/100 μg/kg); IL-1β, interleukin 1β; MCAO, middle cerebral artery occlusion; p, phosphorylated; TNF-α, tumor necrosis factor α; YOH, yohimbine.

motor (32,33). In order to investigate further the effects of DEX pretreatment on MCAO and the related molecular mechanism. In the present study, DEX was administered 30 min prior to ischemia induction, and the results demonstrated that DEX pre-treatment effectively alleviate MCAO-induced cerebral injury, reduce necrotic areas (TTC staining area) of cerebral ischemia, improve the neurological score and motor score. Post-I/R analysis of cortex cell apoptosis demonstrated that DEX pre-treatment was able to reduce brain cell apoptosis. The effects of DEX on both neurological and motor revealed that the different concentrations DEX protected the brain from ischemic injury, and the efficacy of high concentrations if DEX (50 and 100 μg/kg) were more efficient compared with the lower concentration of DEX (10 μg/kg).

The present study confirmed that inflammation may be one of the important factors that induced I/R injury (34,35). I/R-induced inflammation has been explored previously as a treatment target. DEX has been widely used as a sedative drug (36), many previous studies also demonstrated the DEX inhibited infection or non-infection induced by inflammation *in vivo* or *in vitro* (37-39). Particularly, DEX was reported to serve an anti-inflammatory role in ischemic injury in intestines, lungs and kidneys (20,40,41). The present study explored the effects of DEX on inflammation following cerebral ischemia in rats, and the results demonstrated that DEX pre-treatment decreased the levels of TNF-α and IL-1β in MCAO rats, which was similar to previous studies (16,19,42). In addition, the effects of different concentrations of DEX on inflammation demonstrated that the anti-inflammatory effects are increased with high concentrations of DEX compared with the lower concentration, as indicated by the reduced levels of TNF-α and IL-1β expression in DEX50 and DEX100 groups compared with the DEX10 group. The present study explored the effects of DEX on MCAO and focused on the changes in expression of inflammation-related factors in brain tissue in MCAO rats. In the future, the changes of inflammation factor expressions will be examined in plasma.

Although DEX was able to reduce I/R-induced inflammation, the related mechanisms were not clear. Abnormal cellular energy metabolism has been reported as an important factor for inflammation reactions (43). AMPK is considered a key enzyme in the process of energy metabolism and the AMPK pathway is the energy sensitive protein kinase (44). These factors serve a key role in inflammatory disease development. Activated AMPK has been reported to inhibit the inflammation reaction (45,46). The present study demonstrated that DEX affected the expression of P-AMPK in the cortex of MCAO rats in a dose-dependent manner, as illustrated in Fig. 3C and D. A previous study reported that increased cAMP inhibits AMPK through the activated protein kinase A (PKA) pathway. The PKA inhibitor H89 was previously demonstrated to increase the activity of AMPK (47). Other studies reported that DEX suppresses the expression of cAMP via α2-AR and subsequently affects the generation of PKA (48,49). Therefore, it was speculated that DEX may activate the AMPK pathway by inhibiting PKA and attenuate the levels of inflammatory factors, thus inhibiting the inflammation reaction following MCAO. Local cerebral I/R injury reduced the function of cerebral pallium, which is the center of motor functions, and the changes of motor functions were examined for 5 days post-MCAO surgery; the motor function score was reduced at days 1, 2 and 5 in untreated rats. However, motor function scores were significantly improved in rats that received DEX pre-treatment. DEX exerts neuroprotective effects via α2-AR, and this effect was demonstrated to be reversed by the α2-AR antagonist YOH.

There are some limitations in the present study. First, the dose of DEX should be studied further, including the post-ischemia and intravenous dose. Furthermore, the effects of DEX post-treatment on MCAO-induced injury and the associated molecular mechanism require more investigation. Second, the activation mechanism of DEX on AMPK is unclear or whether the upstream and downstream signal pathways of AMPK were involved in regulating MCAO-induced injury

In conclusion, DEX was demonstrated to inhibit inflammation, alleviate cerebral injury and improve the motor function score. DEX may exert protective effects by activating AMPK and inhibiting the generation of inflammatory factors. Future studies will continue to explore the mechanism of DEX on activating AMPK.

Acknowledgements

Availability of data and materials

All data generated or materials during this study are included in this published article.

Authors' contributions

ZH and ZW participated in research design; WZ, HD and ZW conducted experiments; WZ and XM performed data analysis; and WZ and ZW wrote or contributed to the writing of the manuscript.

Ethics approval and consent to participate

Ethics approval was obtained from the Renji Hospital Laboratory Animal Ethics Committee (reference no. 20170723-006).

Patient consent for publication

Not applicable.

Competing interests

Not applicable.

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