Original Article
Role of ornithine decarboxylase/polyamine pathway in focal cerebral ischemia-reperfusion injury and its mechanism in rats

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Abstract: Objective: To observe the role of ornithine decarboxylase (ODC)/polyamine pathway in focal cerebral ischemia-reperfusion injury and to explore its mechanism in rats. Methods: This study was randomly divided into 3 groups including sham-operation (sham) group, ischemia-reperfusion (I/R) group and α-difluoromethylornithine (DFMO) group (each group with 80 rats). In DFMO group, 300 mg/kg of DFMO was injected by tail vein 24 h before reperfusion. According to different time points (3 h, 12 h, 24 h, 48 h and 72 h) after reperfusion, each group was divided into 5 subgroups (each subgroup with 16 rats). Results: In I/R group, apoptosis began increasing 3 h after reperfusion, reached a peak 24 h after perfusion and began decreasing 48 h after perfusion. Compared with sham group, apoptosis significantly increased in I/R and DFMO groups (P<0.05). However, apoptosis was significantly lower in DFMO group than in I/R group at each time point (P<0.05). In I/R group, CHOP expression began increasing 3 h after reperfusion, reached a peak 24 h after perfusion and began decreasing 48 h after perfusion. CHOP expression was significantly lower in DFMO group than in I/R group at each time point (P<0.05). The level of polyamines was significantly higher in I/R and DFMO groups than in sham group, and in I/R group than in DFMO group 12 h, 24 h and 48 h, respectively (P<0.05). Conclusion: Down-regulation of ODC/polyamine pathway may inhibit CHOP-mediated apoptosis caused by endoplasmic reticulum stress and plays a protective role in cerebral I/R injury.

Keywords: Cerebral ischemia-reperfusion injury, endoplasmic reticulum stress, ornithine decarboxylase/polyamine pathway

Introduction
Cerebral ischemia-reperfusion (I/R) injury is common in thrombolytic therapy, craniocerebral trauma and operation. In most cases, injured structure may be restored after reperfusion of ischemic brain tissue. However, sometimes, rather than relieving cerebral tissue injury, reperfusion aggravates nerve cell damage. In cerebral I/R injury, elevation of reactive oxygen species, overload of Ca2+, enhancement of excitatory amino acid toxicity, changes in cell membrane permeability, leukocyte aggregation and reduction of ATP all may induce endoplasmic reticulum stress (ERS). Durative and severe ERS can activate CHOP and/or caspase-12 to induce apoptotic pathways, leading to neuron death [1]. CHOP/GADD153 is one of the classic markers of ERS [2]. In cerebral I/R injury, ERS usually occurs and CHOP expression increases, which activate a series of apoptotic pathways and induces nerve cell apoptosis [3, 4]. It has been reported that in ischemic environment, polyamines and their metabolites can affect neurons [5]. Ornithine decarboxylase is a key enzyme for polyamine synthesis and its specific inhibitor, α-difluoromethylornithine (DFMO), can inhibit polyamine synthesis. In this study, we used DFMO to inhibit polyamine synthesis, and then observed the effect of down-regulation of ODC/polyamine pathway on cerebral I/R injury and explored its possible mechanism.

Materials and methods
All study methods were approved by ethics committee of the First Affiliated Hospital, Liaoning Medical University.
Cerebral ischemia-reperfusion injury

Animals and grouping

Two hundred and forty SD rats weighing between 180 g and 240 g were provided by the Experimental Animal Center, Liaoning Medical University (Jinzhou, China). DFMO (70052-12-9) and dansyl chloride were purchased from Sigma (Silicon Valley, USA). TUNEL kit was purchased from Promega (Madison, State of Wisconsin, USA). Rabbit anti rat GADD153/CHOP kit was purchased from Boosen biological engineering company (Beijing, China). Hydral was purchased from Chemical Reagent Factory (Shanghai, China). According to random digits table, 240 rats were divided into sham-operation (sham) group, ischemia-reperfusion (I/R) group and α-difluoromethylornithine (DFMO) group (each group with 80 rats). According to different time points (3 h, 12 h, 24 h, 48 h and 72 h) after reperfusion, each group was divided into 5 subgroups (each subgroup with 16 rats). In each subgroup, samples of the right cerebral hemisphere between optic chiasma and stalk hypophysial from 8 rats were used for TUNEL and immunohistochemistry, and from other 8 rats were used for high performance liquid chromatography (HPLC) and Western-blot.

Modeling

Rat models of the right middle cerebral artery occlusion (MCAO) were prepared with thread occlusion method [6, 7]. The blood supply was restored 1.5 h after ischemia. In sham group, the thread was not inserted into the right middle cerebral artery, but other procedures were the same as that in rat MCAO models. Successful models were that rats exhibited adduction and inflection of the left forelimb in tail suspension, and left tumble or counterclockwise circling in crawl. In DFMO group, 300 mg/kg of DFMO was injected by tail vein 24 h before reperfusion.

Nerve cell apoptosis detected by TUNEL method

After brain tissue from the right cerebral hemisphere between optic chiasma and stalk hypophysial was subjected to paraffin embedding and slicing, nerve cell apoptosis was detected according to the instructions of TUNEL kit. Nuclei of apoptotic cells were brown. Eight sections were randomly selected from each rat, and 8 high-power fields (×400) were selected in each section to count the number of apoptotic cells.

CHOP-positive cells detected by immunohistochemistry

Rats were fixed after they were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 ml/kg). A cut was made on the neck to expose the right common carotid artery followed by perfusion of 50 ml physiological saline and 4% paraformaldehyde at 4°C, respectively. The brain was taken after decapitation. The brain tissue from the right cerebral hemisphere between optic chiasma and pituitary stalk was fixed in 4% paraformaldehyde for 24 h, and then were subjected to gradient dehydration, transparency and paraffin embedding. Samples were sectioned at a thickness of 5 μm for future use. SABC method was performed according to the instructions of immunohistochemistry kit. In negative control group, PBS was used instead of primary antibody. One section was taken from each rat, and then 8 high-power fields (×400) in hippocampal CA1 region were selected to count the number of positive cells. CHOP protein expression was calculated by the following formula: CHOP protein expression = number of positive cells/total number of cells ×100.

CHOP protein expression detected by Western-blot

Rat brain was taken after rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 ml/kg). Brain tissue was made into pieces which were placed in lysate followed by centrifuged at 12000 r at 5°C for one hour. Supernatant fluid was taken to determined protein concentration. Samples were placed in boiling water for 5 min, underwent electrophoresis, and then were transferred on nitrocellulose filter. The membrane was washed with TTBS balanced solution containing 5% milk powder, and then primary antibody (goat anti rabbit 1:500) was added at 4°C overnight. After washing the membrane, horseradish peroxidase link-coupled secondary antibody (rabbit anti rat 1:400) was added at room temperature for one hour. After washing the membrane, film exposure was performed by enhanced chemiluminescence followed by analysis of CHOP protein. The relative expression of sample was
Figure 1. Nerve cell apoptosis detected by TUNEL. A. In sham group 24 h after reperfusion ×400; B. In I/R group 24 h after reperfusion ×400; C. In DFMO group 24 h after reperfusion ×400; D. Statistical analysis of nerve cell apoptosis in each group at different time points. Notes: sham group: sham-operation group; I/R group: ischemia-reperfusion group; DFMO group: α-difluoromethylornithine group. *indicates \( P < 0.05 \) as compared to sham group at the same time point. *, * and * indicate \( P < 0.05 \) or \( P < 0.01 \) as compared to I/R group at the same time point.
obtained by the ratio of optical density of sample to β-actin.

**Levels of polyamines determined by HPLC**

Levels of polyamines in brain tissue were determined according to the method reported by Kabra et al. [8]. Chromatographic conditions were as follows: mobile phase consisting of methanol and double distilled water, gradient elution, column temperature at 40°C, 1 ml/min of flow rate, fluorescence detection including 330 nm of excitation wave length and 510 nm of emission wave length.

![Figure 2. CHOP-positive cells in each group. A. In sham group 24 h after reperfusion ×400; B. In I/R group 24 h after reperfusion ×400; C. In DFMO group 24 h after reperfusion ×400; D. Statistical analysis of CHOP-positive cells in each group at different time points. Notes: sham group: sham-operation group; I/R group: ischemia-reperfusion group; DFMO group: α-difluoromethylornithine group. * indicates *P* < 0.05 as compared to sham group at the same time point. " indicates *P* < 0.05 as compared to I/R group at the same time point.
Cerebral ischemia-reperfusion injury

Statistical analysis

Data were expressed as mean ± standard deviation. One-factor analysis of variance was performed using SPSS17.0 software. Statistical significance was established at $P<0.05$.

Results

Nerve cell apoptosis

In sham group, less apoptosis was found at each time point. In I/R group, apoptosis began increasing 3 h after reperfusion, reached a peak 24 h after perfusion and began decreasing 48 h after perfusion. Compared with sham group, apoptosis significantly increased in I/R and DFMO groups ($P<0.05$). However, apoptosis was significantly lower in DFMO group than in I/R group at each time point ($P<0.05$ or $P<0.01$) (Figure 1).

CHOP protein expression in hippocampal CA1 region detected by immunohistochemistry

In CHOP-positive cells, cytoplasm was brown and nucleus was occasionally light brown. Most CHOP-positive cells had nuclear shrinkage. In sham group, there were a few of CHOP-positive cells, and there was not significant difference in CHOP-positive cells between each time points ($P>0.05$). In I/R group, CHOP-positive cells began increasing 3 h after reperfusion, reached a peak 24 h after perfusion and began decreasing 48 h after perfusion. CHOP-positive cells in DFMO group were significantly decreased as compared to I/R group at each time point ($P<0.05$) (Figure 2).

Level of CHOP protein determined by Western-blot

Western-blot showed no CHOP protein expression in sham group. In I/R group, CHOP protein
expression began increasing 3 h after reperfusion, reached a peak 24 h after perfusion and began decreasing 48 h after perfusion. CHOP protein expression was significantly higher in I/R and DFMO groups than in sham group at each time point \((P<0.05)\). CHOP protein expression in DFMO group was significantly decreased as compared to I/R group at each time point \((P<0.05)\) (Figure 3).

**Level of polyamines in each group at different time points**

The peak of polyamines including putrescine, spermine and spermidine occurred 24 h after reperfusion in I/R and DFMO groups. The level of polyamines was significantly higher in I/R and DFMO groups than in sham group 12 h, 24 h and 48 h after reperfusion, respectively \((P<0.05)\). Compared to I/R group, the level of polyamines significantly decreased in DFMO group 12 h, 24 h and 48 h after reperfusion, respectively \((P<0.05)\) (Figure 4).

**Discussion**

Cerebral I/R injury can induce ERS which leads to unfolded protein response (UPR). In mammals, UPR regulates transcription and translation through IRE1-XBP1, ATF-6 and PERK pathways. ERS inhibits protein translation mostly through PERK protein activation and elf2 protein kinase phosphorylation, which leads to accumulation of peroxides in cells and promotes apoptosis \([9, 10]\). CHOP is a classic marker of ERS and CHOP-induced apoptotic pathway is one of the ERS-apoptotic signal pathways.

Polymamines include putrescine, spermidine and spermine. Ornithine decarboxylase (ODC) is a key enzyme in polyamine synthesis and DFMO, an inhibitor of ODC, may reduce the production of polymamines. Polymamines have toxic effect on neurons in cerebral I/R because ① they can interfere with permeability of blood brain barrier, promote vasogenic edema and increase cellular sensitivity to N-methyl-D-aspartic acid receptor (NMDA)-mediated signal response, leading to neuronal damage \([11, 12]\); ② they can inhibit the activity of nitric oxide synthase which is a protective agent in ischemic condition \([13]\). Furthermore, in cerebral I/R, elevation of activity and content of ODC also aggravate neuronal damage \([14]\).

In this study, we detected apoptosis in rats at different time points after cerebral I/R, and found that apoptotic cells occurred 3 h after reperfusion and reached a peak 24 h after reperfusion in hippocampal CA1 region. In I/R group, most apoptotic cells were located in ischemic area and were brown. Apoptotic cells significantly increased in I/R group as compared to sham group; but they significantly decreased after DFMO treatment. This suggests that DFMO has inhibitory effect on cerebral I/R-induced apoptosis, but the mechanism has not been completely clear. Therefore, we observed CHOP expression to explore the mechanism that DFMO inhibits apoptosis.

In this study, we also found that CHOP-positive cells began increasing 3 h after reperfusion, reached a peak 24 h after reperfusion and began decreasing 48 h after reperfusion. CHOP-positive cells were significantly higher in I/R group than in sham and DFMO groups. At the same time, CHOP protein expression detected by Western-blot was consistent with CHOP-positive cells determined by immunohistochemistry. This demonstrated that ERS occurrence and CHOP elevation after cerebral I/R trigger CHOP-induced apoptotic pathway \([15]\).

In this study, compared to sham group, level of polymamines and apoptotic cells were significantly increased in I/R group, demonstrating that polymamines have toxic effect on neurons in cerebral I/R. DFMO, a specific inhibitor of ODC, significantly decreased level of polymamines and apoptotic cells, suggesting that down-regulation of polyamine pathway has a protective role in cerebral I/R injury. This conclusion is consistent with the report \([16]\). At the same time, we also found that DFMO also decreased CHOP protein expression and CHOP-positive cells, suggesting that ODC/polyamine pathway is related to CHOP-induced apoptotic pathway in cerebral I/R injury. It has been reported that over-expression of ODC can inhibit CHOP expression through depression of PERK phosphorylation and caspase-4 activation \([17]\).

In summary, down-regulation of ODC/polyamine pathway plays a protective role in cerebral I/R injury through inhibiting CHOP-induced apoptotic pathway. This study provides a new idea for the association between ODC/polyamine pathway and ERS in cerebral I/R injury.
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Disclosure of conflict of interest
None.

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