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The involvement of p38MAPK in the rat model of lower-extremity arterial ischemia-reperfusion injury

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Abstract. – OBJECTIVE: This study aims to investigate the regulatory role of p38 mitogen-activated protein kinase (p38MAPK) in rats with lower-extremity arterial ischemia-reperfusion injury.

MATERIALS AND METHODS: A total of 60 Sprague-Dawley (SD) rats were randomly divided into four groups: control group (Group A), lower-extremity arterial ischemia-reperfusion group (Group B), lower-extremity arterial ischemic postconditioning group (Group C), and lower-extremity arterial ischemic postconditioning + SB203580 group (Group D, 5 μmol/L SB203580, the inhibitor of MAPK pathway, was injected after lower-extremity arterial ischemic postconditioning). The lower-extremity arterial vessels were collected after 24 h. The apoptosis in the lower-extremity arterial vessel in each group was detected via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. The expression of phosphorylated (p)-p38MAPK was measured via Western blotting, and the level of p-activating transcription factor-2 (ATF-2) was detected via immunohistochemical method.

RESULTS: The positive rate of apoptotic cells (%) in Group B was significantly increased compared to that in Group A ($p<0.05$). However, the positive rate was statistically decreased by postcondition in Group C, the rate was further reduced after injection of SB203580 in Group D compared to Group B ($p<0.05$). Compared with that in Group C, the expressions of p-p38MAPK and *p*-ATF-2 in Group D were significantly downregulated after injection of SB203580 ($p<0.05$).

CONCLUSIONS: Lower-extremity arterial ischemia-reperfusion postconditioning can significantly reduce the apoptosis level in vascular tissues, decrease the expressions of p-p38MAPK and downstream factor ATF-2, and alleviate the damage in lower-extremity arterial vessels. The inhibition of MAPK pathway further restricted the apoptosis and contributed to a promoting role in the recovery of lower-extremity arterial ischemia-reperfusion injury.

Key Words
Lower-extremity arterial ischemia-reperfusion, p38MAPK, ATF-2..

Introduction

Lower-extremity arterial ischemia-reperfusion injury refers to the changes in physiological and biochemical functional status of tissues and cells in the body due to the recovery of blood supply after vascular obstruction, which commonly occurs after vascular surgery[1,2]. The concept of ischemia-reperfusion was first proposed in the 1960s, and increasing studies have focused on the research of ischemia-reperfusion since then. Lower-extremity arteriosclerosis occlusion, replantation of severed limbs, etc., lead to lower-extremity ischemia, and the extremity injury is aggravated sometimes after recovery of blood supply, resulting in unnecessary damage to distant organs in the body, multiple organ dysfunction syndrome (MODS), or even death in severe cases[3-5]. Mitogen-activated protein kinase (MAPK) is a kind of protein kinase with dual-phosphorylation capacity[6], among which, p38MAPK plays an important role in the inflammatory response, apoptosis, and immune regulation of the body[7]. Previous evidence indicated that the limb ischemic postconditioning counteracted the injury through activation of MAPK pathway, especially extracellular signal-regulated kinase 1/2 (ERK1/2) pathway[8]. However, the exact role of p38MAPK pathway in the lower-extremity arterial ischemia-reperfusion injury remains elusive. This study thus aims to determine the effects of postconditioning and the role of p38MAPK pathway on apoptosis in artery vessels during the ischemia-reperfusion injury.

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Materials and Methods

Experimental Animals

A total of 60 Sprague-Dawley (SD) rats weighing (190±10) g aged 8-10 weeks old were provided by Chengdu Dashuo Laboratory Animal Co., Ltd., and randomly divided into four groups: control group (Group A), lower-extremity arterial ischemia-reperfusion group (Group B), lower-extremity arterial ischemic postconditioning group (Group C), and lower-extremity arterial ischemic postconditioning + SB203580 group (Group D), with 15 SD rats in each group. All procedures were approved by the Animal Ethics Committee of our hospital.

Reagents and Instruments

Reagents: rabbit anti-mouse β-actin, p-p38 and p-activating transcription factor-2 (ATF-2) polyclonal antibodies were from Wuhan Servicebio Technology Co., Ltd. (Wuhan, Hubei, China). Dimethyl sulfoxide was bought from Shanghai Haoyang Biotechnology Co., Ltd. (Shanghai, China). 5 μmol/L SB203580, the inhibitor of MAPK pathway was purchased from Nanjing Signalway Antibody Biotechnology Co., Ltd. (Nanjing, Jiangsu, China). Bicinchoninic acid (BCA) protein concentration assay kits was obtained from Shanghai Biotech well Co., Ltd. (Shanghai, China), Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reagent was provided by Shanghai Rongbio Technology Co., Ltd. (Shanghai, China). Ultra-sensitive enhanced chemiluminescence (ECL) kits was collected from Beijing Chreagen Biotechnology Co., Ltd. (Beijing, China), and diaminobenzidine (DAB) developing kits was produced by Shanghai Kanglang Biological Technology Co., Ltd. (Shanghai, China)

Instruments: UV-2410 ultraviolet spectrophotometer was made by Hangzhou Noted Scientific Equipment Co., Ltd. (Hangzhou, Zhejiang, China). Optical microscope was from Olympus (Shinjuku, Tokyo, Japan). The low-temperature high-speed centrifuge was from Eppendorf tube (Hamburg, Germany). Full-automatic gel imaging analyzer was provided by Thmorgan Biological Technology Co., Ltd. (Beijing, China), and CLARIOstar full-featured multi-functional microplate reader was produced by Hong Kong BioGene Technology Ltd. (Hongkong).

Lower-Extremity Arterial Ischemia-Reperfusion Modeling

The lower-extremity arterial ischemia-reperfusion model of rat was established according to the method previously reported[9]. Rats were anesthetized via intraperitoneal injection of chloral hydrate (0.004 ml/g). The left and right thighs were wound using rubber rings and fixed above the hip joint to block the normal blood flow. After the rubber ring was cut off after 3 h, followed by recovery of blood perfusion for 2 h, rats were in a constant-temperature environment at about 25°C during the modeling process.

Immunohistochemistry

After the experimental animals were anesthetized, perivascular connective tissues were collected, washed with normal saline, fixed via paraformaldehyde, embedded in paraffin and sliced. The sections were dewaxed, hydrated, washed with phosphate-buffered saline (PBS) and treated in blocking solution for 20 min to reduce the non-specific background staining caused by endogenous peroxidase. It was then incubated with 10% serum at room temperature for 10 min. The primary antibody solution was added at 4°C for overnight incubation. Sections were washed with PBS, and added with secondary antibody solution for 30 min incubation at room temperature. Then sections were washed again with PBS, and incubated with streptavidin-peroxidase solution for 0.5 h at room temperature, followed by washing via PBS, color development via DAB, rinsing via distilled water, re-staining, sealing and observation under a light microscope.

Detection of Apoptosis Via TUNEL Assay

Tissue sampling, fixation and dewaxing processes were the same as those in the immunohistochemical method. Tissues were washed 3 times with normal saline. After tissues were dried with absorbent paper, TUNEL mixed reaction solution was added for incubation in a dark wet box for 1 h. After being washed, tissues were added with converter-POD and incubated in the same environment for 0.5 h, followed by washing 3 times, hematoxylin staining and color development via DAB. After sealing, cells were observed and counted under a microscope. Brown yellow nuclei indicated the TUNEL-positive cells, while blue nuclei indicated the TUNEL-negative cells. The average number of positive cells in 5 non-repetitive fields of view was counted as the number of positive cells in each section.

Positive rate of apoptotic cells = number of positive cells/(number of positive cells + number of negative cells) × 100%.

Detection of Protein Expression Via Western blotting

The lower-extremity arteries of rats in each group were collected and ground with liquid nitrogen. The total protein was extracted according to the instructions of tissue protein extraction kit. The concentration of protein extracted was determined via BCA protein assay, and the protein was stored at -70°C for standby application. The gels were prepared for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and the size of protein was roughly determined according to the marker bands. Then the protein was transferred onto a membrane for 35 min, blocked by using 5% skim milk powder at 37°C for 90 min, and incubated with primary antibody at 4°C overnight. Tris-Buffered Saline with Tween-20 (TBST-20) was added to wash the membrane on a shaker 3 times (15 min/time). After the protein was incubated with secondary antibody at 37°C for 1 h, the membrane was washed again with TBST on the shaker for 3 times (15 min/time). Then, the ultra-sensitive ECL developing solution was added in a dark room for exposure, development and fixation. Finally, the image was scanned by ChemiDocTMMP imaging system and analyzed using Image J professional image analysis software, and the absorbance value was recorded with β-actin as an internal reference.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Armonk, NY, USA) was used for statistical analysis. Measurement data were presented as mean ± standard deviation ($\bar{x}\pm s$), and one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used for the intergroup comparison. $\alpha=0.05$ was used as the test statistical criterion.

Results

Apoptosis

Results of TUNEL staining showed that the positive rate of apoptotic cells in lower-extremity arterial vessels in rats in Group A was (1.15±0.58), which was significantly lower than those in Group B, C and D ($p<0.05$). Lower-extremity arterial ischemia-reperfusion caused the marked increase of apoptotic cells in Group B while the postconditioning significantly reduced the apoptosis of tissues ($p<0.05$). The inhibition of MAPK pathway by SB203580 even restricted the apoptotic effect compared with that in Group C ($p<0.05$) (Table I and Figure 1).

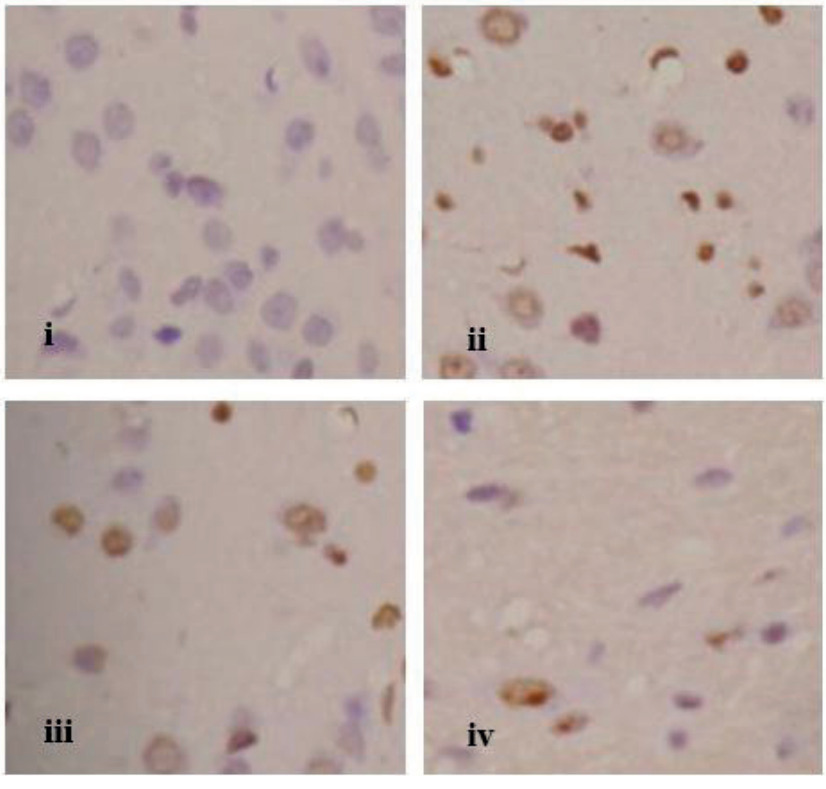

Figure 1. Apoptosis in lower-extremity arterial vessels of rats in each group (TUNEL, 400×).
i: Group A, ii: Group B, iii: Group C, iv: Group D.

Table I. Apoptosis in rats in each group ($\bar{x}$±s).

Group	n	Positive rate of apoptotic cells (%)
Group A	15	1.15±0.58
Group B	15	61.39±9.94*
Group C	15	46.56±9.02*#
Group D	15	30.17±3.59*#+

Note: *p<0.05 vs. Group A. #p<0.05 vs. Group B. +p<0.05 vs. Group C.

Detection of p-p38MAPK Expression Via Western Blotting

The expression level of p-p38MAPK in Group B (0.295±0.038) was significantly higher than that in Group A (0.133±0.017), indicating that the lower-extremity arterial ischemia-reperfusion injury led to the activation of MAPK pathway (p<0.05). Of note, the treatment of postcondition significantly downregulated the level of p-p38MAPK protein compared to that in Group B while this inhibitory effect was enhanced by using SB203580 (p<0.05) (Table II and Figure 2).

Immunohistochemical Detection of p-ATF-2 Expression

Results of immunohistochemical assay showed brown yellow p-ATF-2-positive cells under the light microscope. Compared with that in Group A, the expression of p-ATF-2 in Group B was significantly increased (p<0.05), which was mainly located in the nucleus. However, the expressions of p-ATF-2-positive cells in Group C and D were significantly lower than that in Group B (p<0.05), and the expression of p-ATF-2-positive cells in D was significantly reduced compared to that in Group C (p<0.05) (Table III and Figure 3).

Table II. Detection of p-p38MAPK expression via Western blotting ($\bar{x}$±s).

Group	n	p-p38MAPK protein expression level
Group A	15	0.133±0.017
Group B	15	0.295±0.038*
Group C	15	0.236±0.015#
Group D	15	0.099±0.013#+

Note: *p<0.05 vs. Group A. #p<0.05 vs. Group B. +p<0.05 vs. Group C.

Discussion

MAPK represents a kind of serine/threonine protein kinase, and exerts important signal transduction during cell activities. It mainly includes three pathways currently: ERK, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38MAPK, which facilitate the transmission from extracellular signals into nucleus, and regulate the gene transcription process. Among them, ERK pathway is closely related to the intracellular signal that transmits the polypeptide mitogen stimulus[10], and JNK/SAPK and p38MAPK mediate the apoptosis, inflammatory response, etc., induced by cytokines and stress stimulation[11,12]. It has been demonstrated that p38MAPK-specific inhibitors can effectively delay the process of lipopolysaccharide-induced apoptosis[13], suggesting that p38MAPK may be involved and play a role in the process of apoptosis. In this study, we found that the vascular apoptosis of rats in Group B was significantly increased compared with that in control group. Nevertheless, the number of apoptotic cells was significantly decreased after treatment of p38MAPK inhibitor SB203580 (p<0.05), indicating that p38MAPK is involved in the process of lower-extremity arterial ischemia-reperfusion injury, which is consistent with previous finding[14].

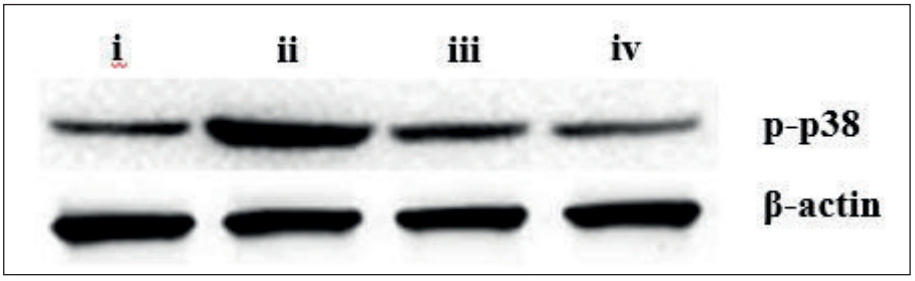

Figure 2. Detection of p-p38MAPK expression via Western blotting.
i: Group A, ii: Group B, iii: Group C, iv: Group D.

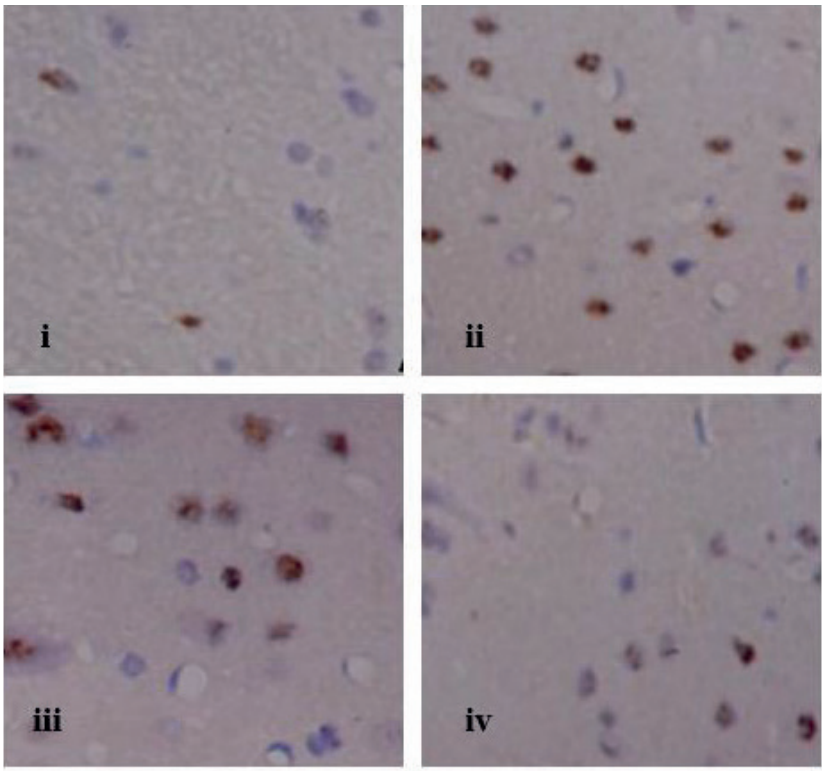

Figure 3. Immunohistochemical observation of *p*-ATF-2 in lower-extremity arterial vessels of rats in each group (SP method, 400×).
i: Group A, ii: Group B, iii: Group C, iv: Group D.

A previous study[15] reported that the repeated ischemia/reperfusion treatment for body's organs or tissues after ischemia can significantly improve the tolerance of myocardial tissues to ischemic injury, indicating that ischemic postconditioning of limbs can alleviate the myocardial ischemic injury. In our study, postconditioning markedly alleviated the damage of arterial ischemia-reperfusion, which was in agreement with previous study. It was shown that p38MAPK mediated the apoptosis by activating its downstream substrate[16]. ATF-2, one of the downstream substrates of p38MAPK, presents as one of the essential components for activation of protein transcription complexes[17]. P-ATF-2 binds to cyclic adenosine monophosphate response element (CRE) to form the homodimeric structure, which can activate the transcription-associated genes and exert functions[18]. Accumulative studies have revealed that ATF-2 mediates the response to body stimulation mainly through JNK and p38MAPK, and a variety of external stimuli can activate the above two signaling pathways, thereby upregulating the ATF-2 activity. After ATF-2 is activated, it eventually causes the damage to the function or structure of organs or tissues during the apoptosis process[19,20]. A recent evidence[21] unraveled the pathway of PERK relieved the apoptosis during ischemia/reperfusion injury. In our data, we found that after the treatment of inhibitor SB203580, the expression level of p38MAPK was decreased, and the downstream substrate ATF-2 was also reduced, which promoted the inhibitory effect of lower-extremity arterial ischemia-reperfusion postconditioning on apoptosis in vascular tissues.

Table III. Immunohistochemical detection of *p*-ATF-2 expression ($\bar{x}$±s).

Group	n	Number of p-ATF-2 -positive cells
Group A	15	18.37±6.35
Group B	15	93.29±13.04*
Group C	15	71.24±11.93$^{\#}$
Group D	15	38.72±5.26$^{\#+}$

Note: *p<0.05 vs. Group A. $^{\#}p$<0.05 vs. Group B. ^{+}p<0.05 vs. Group C.

Conclusions

We demonstrated that lower-extremity arterial ischemia-reperfusion postconditioning can significantly reduce the apoptosis in vascular tissues, with the decrease of p-p38MAPK and down-

stream factor ATF-2 levels. The inhibitory role of postconditioning was enhanced by suppression of MAPK pathway, which provides academic support for the future therapy of lower-extremity arterial ischemia-reperfusion injury.

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Conflict of Interests:

The Authors declare that they have no conflict of interests.

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