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Original article

Salvianolic acid a attenuates limb ischemia/reperfusion injury in skeletal muscle of rats

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ABSTRACT

Ischemia and reperfusion(I/R) injury can cause complications in applying blood flow treatment for atherosclerosis occlusion syndrome. Platelet activation and inflammatory reaction play a role in the procession of I/R injury. This study was designed to investigate the effects of Salvianolic Acid A(SAA) on limb I/R injury via inhibition of platelet activation and inflammatory reaction. Rats were divided into sham, I/R, I/R + SAA-Low (5 mg/kg) and I/R + SAA-high (10 mg/kg) groups with a procession of 6 h for ischemia and 24 h for reperfusion in the femoral artery of the right hind limb, with the exception of the sham group. SAA was injected into the right jugular vein before reperfusion. Reperfusion recovery was monitored by Laser Doppler. HE staining, electron microscopy examination and MDA were used to evaluate the I/R injury. ELISA, Western Blot and RT-PCR were used to measure the levels of P-selectin, IL-8(KC), ICAM-1, TNF-α, IL-1β, CK and NF-κB in plasma or tissues. Pretreatment with SAA attenuated skeletal muscle edema and mitochondria changes, and decreased the levels of MDA and CK. Meanwhile, there was significant reduction of P-selectin, KC, ICAM-1, TNF-α, IL-1β and NF-κB with treatment of SAA. Pretreatment with SAA may attenuate the I/R injury in the skeletal muscle tissues of rats via inhibition of platelet activation and inflammatory reaction.

1. Introduction

Atherosclerosis occlusion syndrome has become a serious concern, affecting human living quality and safety [1]. Some drugs have been used to recover blood flow artificially, but clinical results are not satisfactory [2]. Ischemia-reperfusion (I/R) injury is the most common complication in applying blood flow treatments. High incidence rates and poor prognoses indicate the seriousness of I/R injury [3]. I/R can affect the recovery of limb function in skeletal muscle cells resulting in increased risk of physical disability. Severe limb I/R injuries cause injuries to other organs, which may result in systemic inflammatory response syndrome (SIRS) [4], and even multiple organ dysfunction syndrome (MODS), named reperfusion syndrome [5]. In addition, the pathogenesis of I/R injury is complex and can include cellular energy metabolic disorder, calcium overload, oxygen free radical damage, platelet activation and leukocyte activation, inflammatory reaction and endothelial cell apoptosis and necrosis, and so on. It has been proven that platelet activation and the particles released from platelets play an important role in I/R injury [6,7]. A large number of oxygen free radicals results from the process of I/R injury that induce platelet activation, leading to microthrombosis and platelet aggregation, destruction and consumption. Activated platelets release dense granules, alpha granules, and lysosomal inflammatory mediators, resulting in inflammation and tissue injury.

The traditional Chinese medicine Salvia miltiorrhiza is widely used in the treatment of coronary artery disease and cerebrovascular diseases [8,9]. Salvianolic acid A (SAA) is one of the major active components of *S. miltiorrhiza*, extracted from *S. miltiorrhiza* Bunge [10]. SAA has a lot of pharmacological activities, such as anti-inflammation [11], anti-platelet and anti-thrombosis properties, improvement of microcirculation [12], antioxidant activity [13], and so on. Our previous study found that SAA inhibited platelet aggregation induced by adenosine diphosphate (ADP), thrombin, collagen and U46619 *in vitro* [14]. In this study, we investigate the effect of SAA on platelet activation and inflammation during skeletal muscle I/R injury.

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2. Materials and methods

2.1. Animals and ethics statement

In this study, male Sprague-Dawley (SD) rats, weighing between 200 and 250 g, aged 6–8 weeks, were obtained from Laboratory Animal Center of Zhejiang province (certificate no. SCXL (Zhe) 2008 – 0033). Animals were acclimated for at least one week at room temperature (18–25 °C), 55 ± 5% humidity and a 12 h light/dark cycle. The animals were given free access to a standard diet and tap water. All experimental procedures were approved by the Ethics Review of Animal Use Application of Fifth Affiliated Hospital of Wenzhou Medical University (Zhejiang, China) and in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. Preparation of SAA

In this study,SAA was purchased from Plant Bio-Engineering Co, Ltd (Purity 98%,Xi'an, China).And it was dissolved in 5% glucose liquid with 10% final concentration.

2.3. Treatment protocol in vivo

Sixty rats were randomly divided into four groups. After giving anesthetic with 3% pentobarbital solution, the femoral artery of the right hind limb was exposed, and a line was used to block blood flow. All animals were treated with the protocol of 6 h of ischemia and 24 h of reperfusion, with the exception of sham group. sham group (n = 15): The femoral artery was exposed without any additional surgery for 6 h and injected with 5% glucose solution from the right jugular vein before reperfusion. I/R group (n = 15): The femoral artery was blocked for 6 h and injected with 5% glucose solution from the right jugular vein before reperfusion. I/R + SAA-Low group (n = 15, 5 mg/kg): The femoral artery was blocked for 6 h and injected with 5% glucose solution + SAA (5 mg/kg) from the right jugular vein before reperfusion. I/R + SAA-High group (n = 15, 10 mg/kg): The femoral artery was blocked for 6 h and injected with 5% glucose solution + SAA (10 mg/kg) from the right jugular vein before reperfusion.

2.4. Femoral artery blood flow monitoring with laser doppler imaging

Under standardized conditions, Laser Doppler Imaging (LDI) (Perimed, Sweden) was used to measure the blood flow in the hind limb. Measurements were performed once on 24 h after operation. During the measurements, rats were placed on a heating pad (37 °C). The perfusion ratio was calculated by dividing the perfusion of the right hind limb (RL) by perfusion of the left hind limb (LL).

2.5. Enzyme-Linked immunosorbent assay (ELISA)

Quantikine ELISA kits were purchased from Abcam (England). After 24 h of reperfusion, blood samples were collected from the abdominal aorta with 3.8% sodium citrate as an anticoagulant. The samples were centrifuged at 3500*g* for 10 min to isolate plasma. The plasma levels of P-selectin, IL-8 (KC), CK, ICAM-1 and TNF-α were measured according to the manufacturer's instructions.

2.6. MDA measurements

Tissue samples were homogenized in PBS (pH = 7.4) to make a 10% homogenate. The homogenates were centrifuged at 5000 rpm for 10 min and the supernatants were collected. The levels of MDA were measured as previously described [15].

2.7. Histopathology

Gastrocnemius muscle tissues were fixed for 72 h, then dehydrated in graded alcohol, embedded in paraffin and cut into 3–5 μm-thin sections. The tissue sections were stained with hematoxylin and eosin (HE).

2.8. Electron microscopy examination

The gastrocnemius muscle tissue was sectioned into small pieces (approximately 1 × 1 × 3 mm^3), fixed with 2.5% glutaraldehyde, dehydrated with acetone, and embedded in epoxy resin. The fixed tissues were then cut into ultrathin sections with ultramicrotome and examined by electron microscopy (JEM1230, Japan) after staining with uranyl acetate and lead citrate.

2.9. Western blot assay

Frozen gastrocnemius muscle tissues were homogenized in RIPA pyrolysis liquid with protease- and phosphatase-inhibitor, and centrifuged at 12,000*g* for 15 min. Bicinchoninic acid (Thermo, USA) reagent was used to measure the supernatant protein content. Extracts containing 80 μg protein were separated by electrophoresis with 10% SDS-PAGE and transferred onto nitrocellulose membranes (Merck Millipore, Germany).The separated proteins were blocked with 5% skimmed milk at room temperature for one hour, and incubated over night at 4 °C with primary antibodies including IL-1β (diluted 1:1000;Abcam, England), TNF-α (diluted 1:1000; Abcam, England), NF-κB (diluted 1:1000; Abcam, England) and rabbit anti-GAPDH (diluted 1:1500; Cell Signaling Technology, USA). The nitrocellulose membranes were then incubated with HRP secondary antibodies (diluted 1:1000; Beyotime institute of technology, Shanghai, China) at room temperature for 1 h. The bands were detected using a Super Signal ECL kit (Merck Millipore, Germany) in a Western blot detection system (Bio-Rad, CA, USA) and quantified by density values relative to GAPDH.

2.10. Quantitative real-Time PCR assay

Total RNA was isolated from gastrocnemius muscle samples. Reverse transcription and quantitative real time PCR (RT-qPCR) was carried out using a SYBR Green PCR kit (Thermo, USA). Primers for genes, including ICAM-1 and GAPDH, were obtained from Shanghai Generay Biotech Corperation. The relative amount of each gene was normalized to the amount of GAPDH. The primer sequences used are shown in Table 1.

2.11. Statistical analysis

All data are given as means ± standard deviation (SD). The significance difference was analyzed by one-way ANOVA with SPSS19.0 statistical software followed by the least-significant-difference (LSD) test for multiple comparisons with a P value less than 0.05 regarded to be significant.

Table 1
List of gene-specific primer sequences.

Gene	Sequence
ICAM-1	Primer F 5′ CCCACCTACATACATTCCTACC 3′ Primer R 5′ TCTCCCAGGCATTCTCTTTG 3′
GAPDH	Primer F 5′ GTCGGTGTGAACGGATTTG 3′ Primer R 5′ TCCCATTCTCAGCCTTGAC 3′