EETs mediate cardioprotection of salvianolic acids through MAPK signaling pathway

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Abstract Salvianolic acids, including salvianolic acid A (SAA) and salvianolic acid B (SAB), are the main water-soluble bioactive compounds isolated from the Chinese medicinal herb Danshen and have been shown to exert in vitro and in vivo cardiovascular protection. Recent studies suggest that epoxyeicosatrienoic acids (EETs), the primary cytochrome P450 2J (CYP2J) epoxygenase metabolites of arachidonic acid, are involved in the progression of ischemic injury in diverse organs. Here, we investigated the relation between the protective effects of salvianolic acids and EETs/sEH as well as MAPK signaling pathway. In the present study, the rat acute myocardial infarction (AMI) model was established by the left anterior descending coronary artery occlusion. Our results showed that salvianolic acids significantly reduced ST-segment elevation and serum levels of CK-MB, LDH, and ALT in AMI rats, and significantly attenuated the caspase 3 expression and reduced the ratio of Bax/Bcl-2. ELISA measurement showed that salvianolic acids significantly increased the 14,15-EET levels in blood and heart, and attenuated hydrolase activity of sEH in heart of AMI rat. Western blotting analysis suggested that salvianolic acids significantly attenuated the phosphorylation of JNK and p38, and increased phosphorylation of ERK in heart. In conclusion, these results indicate that EETs/sEH and MAPK signaling pathways are important processes in cardioprotection of salvianolic acids.

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1. Introduction

Danshen, the dried root of *Salvia miltiorrhiza*, is used in Chinese medicine to treat vascular disease. According to Chinese medicine theory, Danshen promotes blood flow and resolves blood stasis. Therefore, it is widely prescribed to patients with angina pectoris, acute myocardial infarction (AMI), hyperlipidemia, and stroke. Recent studies show that there are more than 18 components in Danshen. They can be classified as water-soluble (hydrophilic) phenolic compounds and nonpolar (lipid-soluble) diterpenoidal compounds. Salvianolic acids are the main water-soluble compounds in Danshen. Among salvianolic acids, salvianolic acid A (SAA) and salvianolic acid B (SAB) are the most abundant components. The structures of salvianolic acids are shown in Fig. 1. Although the therapeutic potential of salvianolic acids on hepatic protection, neural protection, and cancer treatment has been proposed in recent years, the greatest clinical impact of salvianolic acids is cardiovascular protection.

In the past few years, mechanisms of how salvianolic acids exert the cardiovascular protective effects have been investigated. Many studies on endothelial cells, vascular smooth muscle cells and cardiomyocytes suggest salvianolic acids may exert cardiovascular protection via the multiple mechanisms in terms of reactive oxygen species (ROS) scavenging ability, leukocyte–endothelial adhesion regulation, inflammation inhibition and immune-modulation.

Epoxyeicosatrienoic acids (EETs), the primary cytochrome P450 2J (CYP2J) epoxygenase metabolites of arachidonic acid, possess potent and diverse biological effects within the vasculature, i.e., vasodilatory, anti-inflammatory, anti-apoptotic, and mitogenic effects. Recent studies suggest that active EETs are involved in the progression of ischemic injury in diverse organs. Some studies show that the anti-apoptotic effects of EETs were significantly attenuated by inhibition of MAPK signaling pathway. It indicates that MAPK signaling pathway plays an important role in beneficial biological effects of EETs. Soluble epoxide hydrolase (sEH) is considered as the primary enzyme responsible for degradation of EETs to dihydroxyeicosatrienoic acids (DHETs) which are less potent. Many studies indicate pharmacological inhibition of sEH protects rats against ischemic damage.

Based on the perceived beneficial effects of EETs, there was little information about the relation between cardioprotection of salvianolic acids and EETs/sEH. Here, we investigate the protective effects of salvianolic acids involving EETs/sEH and explore the role of MAPK signaling pathway using the rat acute myocardial infarction model.

2. Materials and methods

2.1. Chemicals and reagents

SAA and SAB (purity > 99%) were supplied by the Department of Phytochemistry, Institute of Materia Medica (Beijing, China). When used, they were freshly prepared in physiological saline to reach appropriate concentrations. Antibodies of p38, phospho-p38 and β-actin were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). Antibodies of caspase 3, MAPK/JNK, phospho-MAPK/JNK, ERK, and phospho-ERK were obtained from Cell Signaling Technology, Inc. (MA, USA). All other chemicals and reagents were of analytical grade.

2.2. Animals

Male Sprague-Dawley rats (220–250 g) were provided by Vital River Laboratory Animal Center (Beijing, China). The protocol was approved by the institutional animal care and use committee and the local experimental ethics committee. All rats were kept on a 12-h light/12-h dark regime, with free access to food and water. Room temperature (25 ± 1 °C) and humidity (55 ± 10%) were controlled.

2.3. Experimental procedure and treatment

AMI was induced by permanent ligation of the left anterior descending coronary artery in rats, as described previously. Briefly, rats were anaesthetized with urethane (1.0 g/kg, ip) and ventilated with a volume-regulated respirator. After left thoracotomy, the heart was exposed, and the left anterior descending coronary artery was ligated approximately 2 mm from its origin between the pulmonary artery conus and the left atrium with a 5-0 prolene suture. The successful coronary occlusion was verified by typical changes in Lead II ECG. Five minutes after ligation, the rats were randomly divided into five groups of 10 animals each: sham group, AMI group, 20 mg/kg SAB group (SAB 20), 1 and 3 mg/kg SAA groups (SAA 1 and SAA 3). All drugs were administered via tail vein injection 5 min after coronary occlusion. Sham group received the same volume of saline.

2.4. Electrocardiogram measurements

A bipolar lead with electrodes was placed on the right arm and left leg. Lead II ECG monitoring was undertaken throughout the experimental duration, and ST-segment (J point) elevation
in the experimental animals 30 min after coronary occlusion was considered.

2.5. Measurements of cardiac marker enzyme activity

After 6 h of coronary ligation, the rats were re-anaesthetized and 3 mL blood was drawn from the abdominal aorta. After centrifuging at 1000 × g, the serum was collected. The activities of serum creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) were determined spectrophotometrically using diagnostic kits according to the manufacturer’s instructions (Jiancheng, Nanjing, China).

2.6. Measurement of 14,15-EET by ELISA

The 14,15-EET levels in the blood and heart were measured with a commercial ELISA kit. Briefly, blood or heart homogenate was collected in TPP (triphenylphosphine) with a final concentration of 0.1 mM. After acidification with acetic acid to a pH of approximately 3.0–4.0, the samples are extracted three times with ethyl acetate. The collected organic phases were dried up residue was dissolved in 20 μL of ethanol, and then 20 μL of acetic acid was added to make the pH approximately 3.0–4.0, reacting for 12 h at 45 °C. In the acidic conditions, EET is hydrolyzed to DHET. After the reaction, add 1.5 volumes of water to the sample and extract three times with equal volume of ethyl acetate. After three times of extraction, all the organic phase (ethyl acetate) were pooled together and evaporated under nitrogen. The above dried up residue was dissolved in 20 μL of ethanol for ELISA assay of 14,15-DHET according to the manufacturer’s instructions. At the same time, measure the 14,15-DHET level without hydrolysis of 14,15-EET in the same sample.

2.7. Assay for hydrolase activity of sEH

Hydrolase activity of sEH in heart was measured by incubating with 14,15-EET and assaying for 14,15-DHET. Briefly, heart was homogenized in 4 volumes of ice-cold buffer (20 mM Tris–HCl, 0.32 M sucrose, 1 mM EDTA), centrifuged at 10000 × g for 10 min, and supernatant was further centrifuged at 10000 × g for 20 min. Hydrolytic enzymatic reactions were initiated by adding 1 μM 14,15-EET and incubated in shaking water bath at 37 °C for 1 h. Then the 14,15-DHET level was measured by ELISA according to the manufacturer’s instructions.

2.8. Western blotting

Heart was homogenized for 30 min at 4 °C in 4 volumes of RIPA lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton x-100, 1% sodium deoxycholate, 0.1% SDS, and 1 mM PMSF). After centrifuging at 12000 × g at 4 °C for 30 min and the protein concentration of the supernatant was quantified using BioRad De Protein Assay kit. Equal amounts of protein samples (n=3 in each group) were separated by SDS-PAGE and transferred to PVDF membranes. After being blocked with Tris buffered saline, 0.1% Tween 20 with 5% (w/v) non-fat milk, membranes loaded with protein of interest were incubated with primary antibodies at 4 °C overnight. HRP-conjugated secondary antibody (goat anti-rabbit, 1:1000 dilution, Rockland, PA, USA) was used to identify primary antibodies. Blots were developed with SuperSignalWest Pico Chemiluminescent Substrate (Pierce, IL, USA) and visualized using a ChemiDoc XRS system (BioRad, PA, USA). The intensity of immunoreactive bands was quantified using Quantity One Software (BioRad, PA, USA).

2.9. Statistical analysis

Data are presented as mean±SD. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s test. All calculations were performed with SPSS version 13.0 (SPSS Inc., IL, USA). Differences were considered significant if P<0.05.

3. Results

3.1. Effects of salvianolic acids on electrocardiographic parameters and levels of CK-MB, ALT and LDH

Fig. 2A demonstrated that the rats in AMI group showed a marked elevation in ST-segments comparing to rats in sham group. Treatment with SAB (20 mg/kg) and SAA (1 and 3 mg/kg, respectively) significantly attenuated the elevation in ST-segments at 240 min post infarction compared with the AMI group (P<0.05 and P<0.01).

Fig. 2B–D showed that AMI induced increase in serum cardiac marker enzyme activity in the rats. Treatment with SAB (20 mg/kg) and SAA (1 and 3 mg/kg, respectively) significantly attenuated the increase in CK-MB, LDH and ALT compared with the AMI group (P<0.01).

3.2. Effects of salvianolic acids on expression of caspase 3, Bcl-2 and Bax in myocardial tissues

AMI resulted in a significant reduction in Bcl-2 expression and a significant increase in Bax and caspase 3 expression. Treatment with SAB (20 mg/kg) and SAA (1 and 3 mg/kg, respectively) significantly attenuated the caspase 3 expression and reduced the ratio of Bax/Bcl-2 (P<0.05 and P<0.01) (Fig. 3).

3.3. Effects of salvianolic acids on the level of 14,15-EET in blood and heart

As shown in Fig. 4A and B, the 14,15-EET levels in blood and heart in AMI rats were not different compared to sham rats. Treatment with SAB (20 mg/kg) and SAA (1 and 3 mg/kg, respectively) significantly increased the 14,15-EET levels in blood and heart (P<0.05 and P<0.01).

3.4. Effects of salvianolic acids on sEH activity in heart

As shown in Fig. 4C, there was no significant difference in heart sEH activity between sham and AMI rats as indicated by the corresponding 14,15-DHET content. Treatment with SAB (20 mg/kg) and SAA (1 and 3 mg/kg, respectively) significantly attenuated the sEH activity in heart (P<0.01).
Figure 2  Salvianolic acids reduced ST-segment elevation and levels of CK-MB, ALT and LDH in serum after AMI. (A) ST-segment elevation in lead II ECG was calculated at the time point of 30 min after AMI. (B), (C) and (D) Levels of CK-MB, ALT and LDH in serum were assayed using commercial diagnostic kits. Data are presented as mean ± SD \((n=10)\). **\(P<0.01\) vs. sham group; *\(P<0.05\), *\(P<0.01\) vs. AMI group.

Figure 3  Salvianolic acids altered levels of caspase-3, Bcl-2 and Bax after AMI. (A) Representative immunoblotting (upper) of caspase-3 and \(\beta\)-actin at 1:500 dilution of antibodies and densitometric analysis (bottom). (B) Representative immunoblotting (upper) of Bcl-2 and Bax at 1:500 dilution of antibodies and densitometric analysis (bottom). Data are presented as mean ± SD. The experiments were repeated for 3 times. **\(P<0.01\) vs. sham group; *\(P<0.05\), *\(P<0.01\) vs. AMI group.
3.5. Effects of salvianolic acids on MAPK signaling pathway

As shown in Fig. 5, there were significant differences in phosphorylation level of MAPK signaling pathway between sham and AMI rats, as indicated by the ratio of phospho-/total protein. Treatment with SAB (20 mg/kg) and SAA (1 and 3 mg/kg, respectively) significantly attenuated the phosphorylation of JNK and p38, and increased phosphorylation of ERK in heart ($P \leq 0.01$).

4. Discussion

Salvianolic acids, which contain polyphenolic structure, are potent antioxidants. Salvianolic acids reduce intracellular and intravascular oxidative stress, which protects endothelial cells, arterial smooth muscle cells, cardiomyocyte, and LDL from free radical damage and peroxidation. ROS scavenging ability has been regarded as the major mechanism of cardiovascular protection. Recent researches focus on elucidating the other potential pathways.

In the present study, we tested the hypothesis that EETs/sEH and MAPK signaling pathways would play a vital role in cardioprotection of salvianolic acids. Our results showed that SAA and SAB, two representative ingredients of salvianolic acids, provided potent protective effects on the rats with acute myocardial infarction, as indicated by a significant reduction in ST-segment elevation and serum levels of cardiac ischemic markers such as CK-MB, LDH and ALT after permanent LAD occlusion. Acute myocardial infarction resulted in significant apoptosis of cardiomyocyte. This process characterizes with reduction in Bcl-2 expression and increase in Bax and caspase 3 expression. Salvianolic acids significantly attenuated the caspase 3 expression and reduced the ratio of Bax/Bcl-2.

Like many eicosanoids, EETs have multiple biological functions, including reduction of blood pressure, inflammation, ischemic injury and atherosclerosis in multiple species. In vitro and in vivo studies verify that exogenous EETs have potent protective effects on myocardial infarction17–19. Our results showed that AMI did not significantly alter the 14,15-DHET and 14,15-EET levels in blood and heart compared to sham rats, similar to previous findings. Maybe it is attributed to the reason that AMI did not directly affect heart sEH activity and there are other pathways than sEH pathway in the metabolism of EETs. The results demonstrated that salvianolic acids significantly increased the 14,15-EET levels in blood and heart of rat subjected to AMI. EETs are regulated through conversion to less active corresponding diols by sEH. Inhibition of the sEH stabilizes EETs, thus, enhancing the beneficial effects of EETs. Our previous study showed that SAA could inhibit recombinant human sEH activity in a concentration-dependent manner (IC$_{50}$=1.62 µM) using a

![Figure 4](image-url)  
**Figure 4** Salvianolic acids increased the 14,15-EET levels in blood and heart and attenuated heart sEH activity in AMI rats. (A) and (B) The 14,15-EET levels in blood and heart. (C) The activity of sEH in heart as indicated by the corresponding 14,15-DHET content. Data are presented as mean±SD ($n=10$). *$P<0.05$, **$P<0.01$ vs. AMI group.
fluorescent assay in vitro"). The results of this study also showed that salvianolic acids significantly attenuated heart sEH activity of AMI rats as indicated by the corresponding 14,15-DHET content.

MAPKs play an important role in myocardial ischemia/reperfusion injury. Recent studies suggest that the beneficial biological effects of EETs require MAPK signaling pathway. Our results showed that salvianolic acids significantly attenuated the phosphorylation of JNK and p38, and increased phosphorylation of ERK in heart. These effects may be derived from the direct action of salvianolic acids, also being considered as the sequence of increasing EETs induced by salvianolic acids. It will be confirmed in further studies.

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References


