Baiacalin Attenuates Acute Myocardial Infarction of Rats via Mediating the Mitogen-Activated Protein Kinase Pathway

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Received January 8, 2013; accepted March 18, 2013; advance publication released online March 30, 2013

Baiacalin is a bioactive ingredient from the herb and has possessed various pharmacological actions. The present study was performed to evaluate the cardioprotective potential of baiacalin against myocardial infarction and explore the potential mechanism. Baiacalin was intraperitoneally injected into the rats by the doses of 50, 100 and 200 mg/kg, respectively, once a day for 7 d and, 30 min after the last administration, the left coronary artery was ligated. Infarct size was measured to analyze the myocardial damage. Myocardial specific enzymes, including creatine kinase (CK), the MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin T (cTnT) were determined with the colorimetric method. Evidence for myocardial apoptosis was detected by caspase-3 activity measurement and Western blot analysis. We also examined the protein levels of three major subgroups of mitogen-activated protein kinases (MAPKs), namely, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 by immublotting. Our results indicated that baiacalin significantly reduced the infarct size and myocardial enzymes (CK, CK-MB, LDH and cTnT). Administration of baiacalin also suppressed the activity and protein expression of caspase-3. Moreover, the protein level of phosphorylated ERK (p-ERK) was found to be evidently augmented while the phosphorylated JNK (p-JNK) and phosphorylated p38 (p-p38) were strikingly diminished in infarcted rats with baiacalin treatment. These findings suggest that the baiacalin’s cardioprotection associates with mediation of MAPK cascades in acute myocardial infarction of rats.

Key words baiacalin; caspase-3; mitogen-activated protein kinase; myocardial infarction

Myocardial infarction is one of the most common ischemic heart diseases. It often occurs when the myocardial blood supply is interrupted suddenly or persistently, finally leading to the loss of cardiomyocytes. Although some western drugs such as angiotensin-converting enzyme inhibitors, calcium channel blockers, angiotensin II receptor antagonists had been proved to be effective on the treatment of myocardial ischemia, their usefulness has always been limited due to serious adverse effects such as cardiac depression or even proarrhythmic effects. It is very essential to explore new types of cardioprotective drugs in this area.

It has been shown the mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that connect cell-surface receptors to intracellular critical regulatory targets and conceived of as important therapeutic targets of baiacalin following cerebral ischemia. At least three distinct subgroups including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 are classified into the MAPK family in mammalian cells. There is emerging evidence that MAPKs have been shown to be expressed in myocardium and involved in the important pathophysiological process in the heart. It is commonly accepted that ischemic heart diseases including acute myocardial infarction could activate numerous cell signaling cascades, causing dramatic myocard cell necrosis and apoptosis. Activation of p38 MAPK was reported to initiate the signal for apoptosis of colon cancer cells. Besides, p38 activation has been implicated in modulating apoptosis in PC12 and rat cerebellar granule cells. A recent study also supported the fact that p38 was activated in neonatal rat cardiomyocytes subjected to ischemia and that inhibition of p38 evidently protected cardiac myocytes from apoptosis. In the meantime, Wang et al. found that specific activation of JNK pathway through transfecting cultured rat neonatal cardiomyocytes with MKK7, an upstream activator of JNK, led to induction of hypertrophic responses rather than apoptosis. As to the role of ERK signaling pathway in the heart, a recent investigation illustrated that ERK was activated transiently in cultured rat neonatal cardiac myocytes after exposure to hydrogen peroxide and inhibition of its activation would exacerbate cardiomyocytes apoptosis.

Baiacalin is a compound, belong to flavonoid. It is extracted from extracted from the plant Scutellaria baicalensis Georgi and has been demonstrated to possess a variety of pharmacological actions including anti-viral, anti-oxidative, anti-tumor, anti-thrombotic, anti-apoptotic properties and so on. A recent investigation has elucidated that baiacalin exerts protection against global ischemia/reperfusion injury in gerbils through anti-oxidative and anti-apoptotic pathways. Besides, Lin et al. also reported a remarkable attenuation of inflammatory responses in tumor necrosis factor (TNF-α) induced injury in cultured rat cardiomyocytes after baiacalin treatment.

The authors declare no conflict of interest.

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to further explore whether baicalin’s cardioprotection was associated with the MAPKs pathway.

MATERIALS AND METHODS

Ethics Statement Great efforts were made to diminish the number of animals used and animal suffering. All experimental procedures were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine.

Animals and Induction of Acute Myocardial Infarction Adult male Wistar rats (250–300g) were provided by Beijing Animal Center (Beijing, China) and allowed to acclimatize in the animal cage for 4d before use, with free access to water and food.

The model of acute myocardial infarction was prepared according to the previous description with minor modification. Briefly, animals were anesthetized intraperitoneally (i.p.) with sodium pentobarbitone (40 mg/kg). Under anesthesia, they were intubated and artificially ventilated with a respirator at a rate of 5 mL/min. The normal electrocardiogram (II) was recorded via a transducer attached to a multi-channel recorder (BL-420F, Cheng Du Tai Meng, China) after the electrodes were subcutaneously penetrated into four limbs. Then the left anterolateral thoracotomy was carried out in the third and fourth intercostals space and the pericardium was removed. A 5-0 silk suture 1–2 mm was used to encircle the left anterior descending coronary artery below the left atrial appendage. The rats underwent the occlusion of the left coronary artery. Sham-operated animals were treated identically except for the coronary artery ligation. Efforts were made to minimize suffering and reduce the number of animals used. Successful infarction was verified by regional cyanosis of myocardial surface and ST-segment elevation.

Group Design and Drug Administration Baicalin (Sigma, with a purity >95%) was dissolved in physiological saline. The rats were randomly divided into five groups as follows: (1) sham-operated group (n=8), which underwent identical surgical surgery except for the coronary artery ligation and injected with physiological saline (0.1 mL/100 g, i.p.); (2) vehicle group (n=8), which underwent the occlusion of the left coronary artery and injected with physiological saline (0.1 mL/100 g, i.p.); (3–5) baicalin groups (n=8), which were subjected to the occlusion of the left coronary artery and treated with baicalin 50, 100 and 200 mg/kg (i.p.), respectively. Physiological saline or baicalin was injected once a day for 7 consecutive days. The dosage and dosing frequency were determined according to the previous report. Thirty minutes after the last administration, rats were operated on by occlusion of coronary artery.

Infarct Size Measurement Six hours after the coronary artery was ligated, the hearts were quickly removed and kept at −20°C for 2h. Frozen ventricles were then sliced into 2 mm thick sections from the apex to the atrioventricular groove and incubated in 1% triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, U.S.A.) at 37°C for 30 min. The normal myocardium was stained brick red, but the infarcted areas remained unstained. The size of the infarcted area was evaluated by the volume and weight as a percentage of the left ventricle.

Serum Necroenzyme Determination The blood samples were collected 6h after the occlusion of the coronary artery, in order to determine myocardial specific enzymes, including creatine kinase (CK), MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin T (cTnT). The activities of CK, CK-MB and LDH were measured with the colorimetric method according to the manufacturer’s protocols (Nanjing Jiancheng Bioengineering Institute, China). Serum cTnT was measured by an immunoassay (Roche Diagnostics Elecsys 2010, Germany).

Caspase-3 Activity Assay Caspase-3 activity of hearts was determined by cleavage of chromogenic caspase substrates, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp-p-nitroanilide), a caspase-3 substrate. The amount of caspase-3 was measured with a spectrophotometer at the wavelength of 405 nm using a commercial kit (Beyotime Institute of Biotechnology, China). Heart protein samples were obtained as indicated in Western blot analysis. Approximately 50 µg protein was added to a reaction buffer containing Ac-DEVD-pNA (2 mM), incubated at 37°C for 4h, and the absorbance of yellow pNA was calculated by a spectrometer at the wavelength of 405 nm. The specific caspase-3 activity which was normalized for total protein in heart was then expressed as fold of the baseline caspase-3 activity of control group.

Western Blot Analysis Western blot analysis was carried out on the heart samples of each group. For short, samples were lysated in ice-cold radioimmunoprecipitation assay buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 5 mM ethylenediamine tetraacetic acid and protease inhibitor cocktail. After the centrifugation at 13200×g for 20 min at 4°C, the supernatant was collected and total protein level was determined by bicinchoninic acid method (Beyotime Institute of Biotechnology, China).

An equal amount of protein (60 µg) was separated on 8% or 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, MA, U.S.A.). The membranes were blocked in 5% fat-free milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 2h and then incubated respectively with the following primary antibodies: rabbit anti-caspase-3 (1:300; Santa Cruz, U.S.A.), mouse anti-phospho-ERK (Tyr204) (1:200, Santa Cruz, U.S.A.), mouse anti-phospho-JNK (Thr183/Tyr185) (1:200, Santa Cruz, U.S.A.), rabbit anti-phospho-p38 MAPK (Tyr182) (1:200, Santa Cruz, U.S.A.) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000, Kang Chen, China), overnight at 4°C. After the membranes were rinsed in TBS-T for three times, they were incubated with horseradish peroxidase-conjugated goat antirabbit antibody (1:5000, Santa Cruz, U.S.A.) or goat antimouse antibody (1:5000, Santa Cruz, U.S.A.) for 2h at room temperature at constant stirring. Immunodetection was conducted with an enhanced chemiluminescence (ECL) kit (Pierce, CA, U.S.A.). Protein bands were visualized by exposure to X-ray film. GAPDH was selected as an internal reference for relative quantification. The protein band intensities were quantified using the Quantity One software (BioRad, U.S.A.).

Statistics Values were expressed as mean±S.D. Statistical analysis was conducted using one-way ANOVA followed by Dunnett’s test for individual comparisons between group means. All statistical analyses were carried out by SPSS 13.0 software. A p<0.05 was deemed statistically significant.

RESULTS

Effects of Baicalin on Myocardial Infarct Size in a Rat
Model of Acute Myocardial Infarction  The chemical structure of baicalin was indicated in Fig. 1. The infarct size in the vehicle-treated myocardial infarction group was 37.63±1.68%.

After the administration of baicalin by the dose of 50, 100 and 200 mg/kg, the infarcted area was significantly decreased to 31.58±1.25% (p<0.01, n=8), 27.63±1.18% (p<0.01, n=8) and 25.36±1.59% (p<0.01, n=8), respectively, compared with the infarcted group, as displayed in Fig. 2.

Effects of Baicalin on Serum CK, CK-MB and LDH Activities together with cTnT Level in a Rat Model of Acute Myocardial Infarction  The serum CK activity was evident -

Fig. 2. Effects of Baicalin on Infarct Size of Hearts in a Rat Model of Acute Myocardial Infarction (Mean±S.D., n=8)

Table 1. Effects of Baicalin on the Activities of CK, CK-MB and LDH together with the cTnT Level in Serum of Control and Acute Myocardial Infarction-Induced Rats (Mean±S.D., n=8)

<table>
<thead>
<tr>
<th>Groups</th>
<th>CK (U/mL)</th>
<th>CK-MB (I/U/L)</th>
<th>LDH (U/L)</th>
<th>cTnT (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.26±0.03</td>
<td>80.52±7.46</td>
<td>1774.13±331.38</td>
<td>0.09±0.05</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.56±0.07**</td>
<td>187.75±9.44**</td>
<td>3600.75±413.26**</td>
<td>0.30±0.07**</td>
</tr>
<tr>
<td>B50</td>
<td>0.42±0.05**</td>
<td>113.00±8.75**</td>
<td>3080.25±319.96*</td>
<td>0.16±0.05a</td>
</tr>
<tr>
<td>B100</td>
<td>0.34±0.03**</td>
<td>95.72±11.49**</td>
<td>2603.75±364.55**</td>
<td>0.14±0.05a</td>
</tr>
<tr>
<td>B200</td>
<td>0.29±0.03**</td>
<td>84.85±8.22**</td>
<td>2343.63±374.82**</td>
<td>0.13±0.03a</td>
</tr>
</tbody>
</table>

**p<0.01 vs. sham-operated group, *p<0.05, #p<0.01 vs. vehicle-treated group. Sham, sham-operated; Vehicle, vehicle-treated; B50, baicalin (50 mg/kg)-treated; B100, baicalin (100 mg/kg)-treated; B200, baicalin (200 mg/kg)-treated groups.

Effects of Baicalin on the Caspase-3 Activity in a Rat Model of Acute Myocardial Infarction  In order to confirm that baicalin could attenuate the apoptotic damage induced by myocardial infarction, the activity of caspase-3, an executioner molecule in the generation of apoptosis, was determined with colorimetric method. As indicated in Fig. 3, caspase-3 activity in the vehicle group was markedly enhanced by 289.88% (p<0.01, n=8) compared with the sham group. In the baicalin treatment (50, 100, 200 mg/kg) groups, there was an evident decline in caspase-3 activity by 21.03% (p<0.01, n=8), 37.59% (p<0.01, n=8) and 48.28% (p<0.01, n=8), respectively, compared to that in the vehicle group.

Effects of Baicalin on the Caspase-3 Protein and MAPK Cascades in a Rat Model of Acute Myocardial Infarction  Western blot analysis was conducted to further evaluate the effect of baicalin on the caspase-3 protein in a rat model of myocardial infarction. Figure 4A illustrated that the Western blot with caspase-3 antibody exhibited the specific bands of 20 kDa. The protein expression of caspase-3 in vehicle-treated
myocardial infarction group was abundantly increased from 0.39 ± 0.06 to 1.28 ± 0.04 (p < 0.01, n = 8) compared with the sham control. Nevertheless, when treatment with baicalin (50, 100, 200 mg/kg), the caspase-3 protein level was remarkably reduced from 1.28 ± 0.04 to 0.74 ± 0.07 (p < 0.01, n = 8), 0.61 ± 0.05 (p < 0.01, n = 8) and 0.46 ± 0.06 (p < 0.01, n = 8), respectively, in comparison to the myocardial infarction-subjected group, as shown in Fig. 4E. Thus, these results by Western blot assay further confirmed that baicalin treatment diminished the caspase-3 protein level in a rat model of myocardial infarction, which was in accordance with our result of caspase-3 activity determination.

The activation of MAPK proteins (ERK, JNK and p38) in rats hearts was also examined in the present work (Figs. 4B–D). The quantitative analysis revealed that an evident reduction in the level of phosphorylated ERK (p-ERK), the active form of ERK, was detected in the hearts of myocardial infarction-induced rats from 0.93 ± 0.07 to 0.57 ± 0.09 (p < 0.01, n = 8) than that in the control group, as displayed in Fig. 4F. However, administration of baicalin (50, 100, 200 mg/kg) to the infarcted rats dramatically increased the expression level of p-ERK protein from 0.57 ± 0.09 to 0.69 ± 0.10 (p < 0.05, n = 8), 0.72 ± 0.06 (p < 0.01, n = 8) and 0.86 ± 0.05 (p < 0.01, n = 8), respectively, compared to the vehicle-treated group. In contrast, the expression levels of phosphorylated JNK (p-JNK) protein was found to be elevated in myocardial infarction group from 1.64 ± 0.08 to 2.15 ± 0.14 (p < 0.01, n = 8) than sham control. Interestingly, when treatment with baicalin by different doses (50, 100, 200 mg/kg), the p-JNK protein level was obviously reduced from 2.15 ± 0.14 to 1.96 ± 0.16 (p < 0.05, n = 8), 1.73 ± 0.08 (p < 0.01, n = 8) and 1.79 ± 0.10 (p < 0.01, n = 8), respectively, in comparison to the vehicle-treated group, as shown in Fig. 4G. Besides, following one-way ANOVA analyses, an evident elevation in the level of phosphorylated p38 (p-p38) was also detected in the hearts of infarction-subjected rats from 0.29 ± 0.06 to 1.04 ± 0.07 (p < 0.01, n = 8), as illustrated in Fig. 4H. In the baicalin treatment (50, 100, 200 mg/kg) groups, there was a significant decline in the p-p38 protein from 1.04 ± 0.07 to 0.88 ± 0.06 (p < 0.01, n = 8), 0.85 ± 0.06 (p < 0.01, n = 8) and 0.43 ± 0.04 (p < 0.01, n = 8), respectively, compared to that in the vehicle group.

Fig. 4. Effects of Baicalin on the Caspase-3 Protein and MAPK Cascades in a Rat Model of Acute Myocardial Infarction (Mean ± S.D., n = 8)

(A)–(D) Displayed the representative images of immunoblots with antibodies against caspase-3, phospho-ERK (p-ERK); phosphor-JNK (p-JNK) and phosphor-p38 MAPK (p-p38), respectively, in rats hearts from different groups. caspase-3: 20 kDa; p-ERK: 44 kDa and 42 kDa; p-JNK: 54 kDa and 46 kDa; p-p38: 38 kDa; GAPDH: 36 kDa; (E)–(H) were the quantitative analysis of the protein levels of caspase-3, p-ERK, p-JNK, and p-p38, respectively, in rats hearts from different groups. The data were normalized to the loading control GAPDH. **p < 0.01 vs. sham-operated group, *p < 0.05, ##p < 0.01 vs. vehicle-treated group. Sham, sham-operated; Vehicle, vehicle-treated; B50, baicalin (50 mg/kg)-treated; B100, baicalin (100 mg/kg)-treated; B200, baicalin (200 mg/kg)-treated groups.
DISCUSSION

Flavonoids are naturally occurring polyphenolic compounds possessing multiple biological actions such as anti-oxidative, anti-tumor and anti-apoptotic effects. There is cumulative evidence supporting that flavonoids reduce the risk of coronary artery disease. Baicalin is a major flavonoid isolated from the plant *Scutellaria baicalensis GeorGi*. A previous report confirmed that baicalin could have a protective effect on heart injury in rats with severe acute pancreatitis. Baicalin has also shown potent protection against ischemia/reperfusion injury in cultured chick cardiomyocytes. In the cultured rat cardiomyocytes exposed to hypoxia/reoxygenation, baicalin treatment effectively inhibited the nuclear translocation of NF-xB and exerted cardioprotective efficacy. The present investigation illustrated for the first time that baicalin could possess the cardioprotective action in a rat model of acute myocardial infarction and its cardioprotection might be associated with the modulation of MAPK cascades.

Infarct size and cardiac marker enzymes (CK, CK-MB, LDH and cTnT) are conceived of as important indices for evaluating the cardiac damage in the generation of ischemic heart diseases. Numerous reports have revealed that infarct size and the activities of CK, CK-MB and are strikingly increased during isoproterenol-induced acute myocardial infarction. The Serum cTnT is a very sensitive and specific indicator in detecting myocardial infarction. It is a contractile protein that is rarely found in serum but markedly released when myocardial necrosis occurs. Consistently, our present study illustrated that the infarction size and the activities of CK, CK-MB, LDH and cTnT were evidently increased. Furthermore, they were all remarkably decreased after treating with baicalin to acute myocardial infarction-induced rats, suggesting the cardioprotective effect of baicalin.

It has been well recognized that myocardial infarction, induced by severe impairment of myocardial blood supply, often leads to marked cell apoptosis. Caspases are a family of cystein-dependent proteases which play a pivotal role in the initiation and execution of cellular apoptosis. In response to the apoptotic stimuli, caspases are specifically activated. Caspase-3, the major form of caspase, is served as an “apoptotic executor” that cleaves substrates, such as ploy(ADP-ribose) polymerase, finally leading to DNA fragmentation and cell loss. A remarkable elevation of caspase-3 was previously found in isoproterenol-induced acute myocardial infarction in Wistar rats. Our current study reported the consistent alteration of caspase-3 in infarcted hearts and meanwhile baicalin treatment obviously decreased caspase-3 expressions as well as its activity in the rats undergoing acute myocardial infarction. It is plausible that enhanced therapeutic effect by baicalin might be associated with its better regulation of caspase-3, finally exerting anti-apoptotic action in infarcted rats.

Cardiac myocytes apoptosis triggers a multiple intracellular signaling processes. It has been shown that alteration of MAPKs signaling cascades plays a pivotal role in the modulation of myocardial apoptosis. ERK was discovered as the first member of MAPK family and phosphorylation of Thr and Tyr residues is essential for activation of ERKs signaling. It is view that inhibition of ERK pathway enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exacerbates myocardial injury in isolated rat heart following ischemia/reperfusion. The sustained activation of ERK pathway during ischemia could mediate adaptative cytoprotection. Results from our current work showed that the p-ERK was strikingly reduced in rat model of acute myocardial infarction which was in line with the (activated form of ERK) the previous study. And, what’s more important, there was an evident increase of the phosphorylated level of ERK after treatment with baicalin. This implies that the activation of ERK cascade might be considered as a critical signaling pathway for baicalin’s cardioprotection in rats with acute myocardial infarction. In the meantime, the MAPKs such as JNK and p38 exert a detrimental effect when activated at the time of myocardial reperfusion through a variety of mechanisms including the proapoptotic mechanism, increasing the expression of adhesion molecules, enhancing the production of cytokines and reducing the components of the prosurvival pathway. Hreniuk et al. found that inhibition of JNK46 obviously suppressed reoxygenation-induced apoptosis in rat cardiac myocytes. Conversely, the activation of JNK via transfection of cultured rat cardiomyocytes with MKK7, an upstream activator of JNK, was reported to induce myocardial hypertrophy but not apoptosis. As to p38 MAPK, its activation has been involved in the modulation of cellular apoptosis in several cell types. In cultured neonatal rat cardiomyocytes subjected to ischemia, overexpression of activated MAPK kinase 3b, which phosphorylated and activated p38 MAPK, led to cell apoptosis and this phenomenon was enhanced by co-expression of p38α. Additionally, Barancik et al. reported that inhibition of cardiac p38 MAPK pathway by SB203580, a specific inhibitor, protected against ischemic cell death in pig myocardium. In agreement with part of the previous result, our findings illustrated that acute myocardial infarction resulted in the elevated protein level of p-JNK (the activated form of JNK) and p-p38 (the activated form of p38). Furthermore, administration of baicalin significantly diminished the expression of p-JNK and p-p38 in rats after acute myocardial infarction. Considering this, together with our results, the activation of ERK and the suppression of JNK as well as p38 signaling pathways is regarded as one of the important cardioprotective mechanisms of baicalin against myocardial infarction-induced cardiac damage in rats.

CONCLUSION

In summary, our findings demonstrated that baicalin effectively protected heart from acute myocardial infarction impairment by reducing the infarction size, decreasing the activities of myocardial specific enzymes including CK, CK-MB, LDH and cTnT as well as suppressing the activity and protein expression of caspase-3 in the rat in vivo. The cardioprotective effect might be associated with the activation of prosurvival kinase including ERK and suppression of apoptotic kinases such as JNK and p38. Our results support the fact that baicalin could be used as a promising cardioprotective agent for the treatment of acute myocardial infarction. This study also hints that MAPK cascades could be ischemic a potential therapeutic target for ischemic heart disease in future.

REFERENCES


