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Study of baicalin on sympathoexcitation induced by myocardial ischemia via $P2X_3$ receptor in superior cervical ganglia

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ABSTRACT

After the myocardial ischemia, injured myocardial tissues released large quantity of ATP, which activated $P2X_3$ receptor in superior cervical ganglia and made the SCG postganglionic neurons excited. Excitatory of sympathetic postganglionic efferent neurons increased the blood pressure and heart rates, which aggravated the myocardial ischemic injury. Baicalin has anti-inflammatory and anti-oxidant properties. Our study showed that baicalin reduced the incremental concentration of serum CK-MB, cTn-T, epinephrine and ATP, decreased the up-regulated expression levels of $P2X_3$ mRNA and protein in SCG after MI, and then inhibited the sympathetic excitatory activity triggered by MI injury. These results indicated that baicalin acted on $P2X_3$ receptor was involved in the transmission of sympathetic excitation after the myocardial ischemic injury. Baicalin might decrease sympathetic activity via inhibiting $P2X_3$ receptor in rat SCG to protect the myocardium.

1. Introduction

ATP is involved in the neurotransmission and neuromodulation via acting on P2X receptors (Burnstock et al., 2011; Gourine et al., 2009; Ortega et al., 2009). P2X receptors are ligand-gated ion channel receptors, and its seven subunits have been identified (Burnstock, 2007a; Roberts et al., 2006). $P2X_3$ receptor is a member of P2X family which is expressed selectively in primary sensory nociceptive neurons (Abbracchio et al., 2006; Vizi et al., 1997; Vial et al., 1987).

After the myocardial ischemia, myocardial tissues and SCG sympathetic nerve endings released ATP through exocytosis or extravasation (Vizi et al., 1997; Vial et al., 1987; Li et al., 2010; Wang et al., 2008). ATP excited the cardiac afferent nerve endings and caused chest pain, thereby eliciting the sympathoexcitatory reflex. It was characterized with an increase of blood pressure and sympathetic nerve activity (Fu and Longhurst, 2010; Li et al., 2011; Liu et al., 2013a, 2013b; Tu et al., 2013). Clinical research found that removal of the cervical sympathetic ganglia, such as SCG or SG, causes the symptoms of angina pectoris disappear in 50–60% patients with coronary heart disease (Pather et al., 2003). It prompted that the sympathetic innervations and its activity in the heart tissues involved the myocardial ischemic injury reflex.

Cervical sympathetic ganglia were not only a simple relaying station for signals, but also an integration center for all kinds of neurotransmission (Armour, 2007; Armour, 2008; Zipes, 2008). Nociceptive signals of myocardial ischemia from sensory afferent nerves can strengthen the postganglionic sympathetic nerve activity. This will elevate blood pressure and heart rate, which will further exaggerate myocardial ischemic damage (Lobysheva et al., 2009; Li et al., 2011). Therefore, there were feedback loops in SCG between the cardiac sensory afferent nerves and the cardiac sympathetic postganglionic efferent neurons to modulate the postganglionic efferent effects (Armour, 2008; Hoover et al., 2008; Li et al., 2011; Liu et al., 2013a, 2013b; Pan and Chen, 2002; Tu et al., 2013; Zipes, 2008). Previous studies in our lab found that $P2X_3$ receptor in cervical sympathetic ganglia was involved in the transmission of myocardial ischemic nociceptive signals (Li et al., 2010, 2011; Liu

Abbreviations: ATP, adenosine 5′-triphosphate; ECG, electrocardiograph; ELISA, enzyme-linked immunosorbent assay; EPI, epinephrine; HE staining, hematoxylin and eosin staining; HR, heart rate; IOD, integrated optical density; MI, myocardial ischemia; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SBP, systolic blood pressure; SCG, superior cervical ganglia; SD, Sprague–Dawley; SDS, sodium dodecyl sulfate; SG, stellate ganglia

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et al., 2014a,b). After being treated with $P2X_3$ antagonist A-317491 in myocardial ischemic rats, the up-regulated systolic blood pressure and heart rate were decreased compare with those in the model rats (Li et al., 2010; Wang et al., 2008; Shao et al., 2007). It suggested that inhibition of $P2X_3$ receptor will contribute to alleviating myocardial ischemia injury, and further lessen the sympathoexcitatory reflex.

Creatine kinase isoenzyme MB (CK-MB) is one of the specific myocardium injury biomarkers, which are often elevated in myocardial infarction and other ischemic injuries. CK-MB was an isoenzyme approximately 15–30% of CK in the myocardium (Kemp et al., 2004). It has reported that serum activities of CK-MB were significantly increased in ischemia/reperfusion injury rats (Liu et al., 2013a, 2013b). Cardiac troponin T (cTn-T) is an ideal marker for the myocardial injury because there are marked changes in blood levels following small quantities of myocardial cellular injury, and these changes are easily measured and identified as abnormal. cTn-T remains elevated for 8 to 21 days during myocardial cellular injury (Rottbauer et al., 1996; Johnson et al., 1999). Thus, CK-MB and cTn-T are two major biochemical markers for the diagnosis of myocardial ischemic injury.

Baicalin ($C_{21}H_{18}O_{11}$) is a flavonoid compound purified from the dry roots of *Scutellaria baicalensis* — Georgi. It has been shown to have the pharmacological actions of anti-oxidation, anti-tumor, anti-inflammation and anti-proliferation (Gao et al., 2001; Shen et al., 2003; Dong et al., 2010). Previous studies have demonstrated that baicalin had the effects of cardiovascular protection and improving endothelial function (Woo et al., 2005; Chang et al., 2007). However, the mechanism of baicalin on cardiovascular protection is unclear. Little is known about the role of baicalin on the sympathoexcitatory reflex induced by myocardial ischemia via $P2X_3$ receptor in rat cervical sympathetic ganglia. This study was aimed to observe the change of blood pressure, heart rate after baicalin treatment in the myocardial ischemic rats, and the relationship between effects of baicalin on the sympathoexcitatory reflex and the up-regulated expression of $P2X_3$ receptor in the SCG of the myocardial ischemic rats.

2. Material and methods

2.1. Animals and groups

SD rats (200–250 g), male, were provided by Laboratory Animal Center of Medical School of Nanchang University. Use of the animals was reviewed and approved by the Animal Care and Use Committee of Medical School of Nanchang University. SD rats were randomly divided into 5 groups: normal control group, MI group, MI rats treated with baicalin group, baicalin control group and sham group. Rats were intraperitoneally injected with baicalin (40 mg/kg/day) for 14 days in MI plus baicalin group and baicalin control group. Rats of the normal control group were intraperitoneally injected with normal saline (10 ml/kg/day) for 14 days.

2.2. Myocardial ischemic injury rat model

Rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g) by intraperitoneal injection. The endotracheal intubation tube connected to a small animal ventilator was inserted into rat trachea to maintain animal breath. The ECG was recorded. The left anterior descending coronary artery was ligated with a fine line at the depth of 0.15–0.20 cm. The model of myocardial ischemia of rats was confirmed by abnormal Q wave and ST-segment displacement in the ECG recording.

2.3. Measurement of blood pressure and heart rate

Blood pressure and heart rate were measured by non-invasive blood pressure meter with an indirect tail-cuff method (Softron BP-98A, Softron Co., Tokyo, Japan) (Tu et al., 2013). At day 14 after surgery, blood samples from the vein blood of posterior orbital were collected via carotid arterial cannulation. Myocardial enzyme (CK-MB and cTn-T) in serum was measured by automatic electrochemiluminescence instrument (American Roche COBAS E411).

2.4. Hematoxylin and eosin staining

Ischemic injury changes of myocardial tissues were observed by HE staining. Myocardial tissues isolated from rats were washed by PBS, fixed with 4% PFA for 24 h and then dehydrated with 20% sucrose overnight at 4 °C. Using a cryostat, myocardial tissues were cut into 12 μm thick. The sections were stained with hematoxylin for 30 s, washed with tap water, stained with eosin for 35 s, and decolorized in 95% ethanol. The sections were stained with HE and then observed under a light microscope.

2.5. Immunohistochemistry

The SCG were fixed with 4% PFA for 24 h and dehydrated with 20% sucrose overnight at 4 °C. The ganglia were cut into 10 μm thick at a cryostat. The sections were incubated in 3% H_2O_2 for 10 min to block the endogenous peroxidase. Following incubation with normal goat serum for 10 min, the sections were incubated with rabbit anti-$P2X_3$ (1:2500 diluted in PBS; Chemicon International, Inc. USA) overnight at 4 °C. After 3 rinses in PBS, the sections were then incubated with biotinylated goat anti-rabbit secondary antibody (Beijing Zhongshan Biotech. CO.) for 1 h at room temperature and streptavidin-horseradish peroxidase (Beijing Zhongshan Biotech. CO.) for 30 min. The color was developed in DAB (Beijing Zhongshan Biotech. CO.) substrate, then dehydrated and mounted with neutral gum. Image-Pro Plus 6.0 image analysis software was used to analyze the integrated optical density (IOD) of $P2X_3$ receptor.

2.6. RNA preparation and reverse transcriptase (RT)-PCR

The rats were anesthetized by Nembutal (35 mg/kg i.p.). The SCG were isolated immediately and flushed with ice cold PBS. Total RNA samples were prepared from SCG in each group by using Trizol Total RNA Reagent (Beijing Tiangen Biotech CO.). Isolated RNA was resuspended in DEPC water and reverse transcribed to cDNA using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. PCR was performed on cDNA using primers listed below: 4 μl of cDNA, 6.5 μl DEPC water, 2 μl of 10 μmol/l primers (equal sense and antisense), and 12.5 μl 2× Easy Taq PCR SuperMix (Beijing Tiangen Biotech CO.) in 25 μl. PCR conditions included 3 min hot start at 94 °C, 30 s denaturation (94 °C), 30 s annealing (57 °C), and 30 s extension (72 °C) for 30 cycles, and 5 min final extension at 72 °C. PCR products (8 μl) were run on 1% agarose gels with EB using standard protocols. Gels were scanned with gel imaging system (Beijing Junyi CO.) and the intensity of PCR product bands was analyzed using

Table 1
Effects of baicalin on the blood pressure and heart rate in the MI rats.

Group	Number	Systolic blood pressure (mm Hg)	Diastolic blood pressure (mm Hg)	Heart rate (times/min)
Normal	5	69.13 ± 0.55	86.800 ± 8.248	366.533 ± 14.436
Ischemia	5	139.333 ± 7.218	98.467 ± 7.453	423.067 ± 18.266
Sham	5	116.500 ± 6.947	86.667 ± 10.210	372.200 ± 9.615
Bai + ischemia	5	123.333 ± 7.480	86.867 ± 12.374	390.267 ± 12.887
Baicalin	5	116.933 ± 4.383	86.125 ± 6.541	373.667 ± 14.435

Systolic blood pressure, diastolic blood pressure and heart rate in MI group rats were increased in comparison with those in normal control rats ($p < 0.05$). Systolic blood pressure, diastolic blood pressure and heart rate in MI rats treated with baicalin rats were decreased in comparison with those in MI group rats ($n = 5$, $p < 0.05$). No difference was found in the systolic blood pressure, diastolic blood pressure, and heart rate among the sham group, baicalin control group, and normal control group ($p > 0.05$).