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Puerarin alleviates aggravated sympathoexcitatory response induced by myocardial ischemia via regulating $P2X_3$ receptor in rat superior cervical ganglia

Shuangmei Liu [1], Shicheng Yu [1], Changshui Xu [1], Lichao Peng [1], Hong Xu [1], Chunping Zhang, Guilin Li, Yun Gao, Bo Fan, Qicheng Zhu, Chaoran Zheng, Bing Wu, Miaomiao Song, Qin Wu, Shangdong Liang *

Department of Physiology, Medical School of Nanchang University, Nanchang 330006, PR China

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ABSTRACT

Myocardial ischemia elicits a sympathoexcitatory response characterized by increase in blood pressure and sympathetic nerve activity. Puerarin, a major active ingredient extracted from the traditional Chinese plant medicine Ge-gen, has been widely used in treatment of myocardial and cerebral ischemia. However, little is known about the mechanism. Our study was aimed to explore the effect of puerarin on sympathoexcitatory response induced by myocardial ischemic injury and possible relationship with $P2X_3$ receptor. Our results showed that puerarin alleviated systolic blood pressure and heart rate, and decreased the up-regulated of $P2X_3$ mRNA and protein in SCG of myocardial ischemic rats. The amplitude of ATP-activated currents of SCG neurons was much larger in myocardial ischemic group than that in control group. Puerarin reduced ATP-activated currents in myocardial ischemic group and control group, and the inhibiting effects of puerarin in myocardial ischemic group were stronger than those in control group. Puerarin also significantly inhibited ATP-activated currents in HEK293 cells transfected with $P2X_3$ receptor. These results suggest that puerarin can depress up-sympathoexcitatory response induced by myocardial ischemia via acting on $P2X_3$ receptor in rat SCG to protect myocardium.

1. Introduction

There exist cardiac sympathetic postganglionic neurons in superior cervical ganglia (SCG) (Pather et al., 2003). The sympathetic ganglia not only relay the sympathetic pre-ganglionic signals, but also play an integrative role in the regulation of the autonomic function (Boehm and Kubista, 2002). Therefore, the autonomic nervous system can be viewed as the neuronal circuits that receive sensory visceral information and take control of efferent output to cardiovascular target (Armour, 2008; Hoover et al., 2008; Pan and Chen, 2002; Zipes, 2008). Clinical studies revealed that removal of SCG and other sympathetic ganglia disappear 50–60% of angina symptoms in the patients of coronary heart disease (Pan and Chen, 2002). It indicates that the cardiac sympathetic nerves are involved in the signal transmission of myocardial ischemic injury. Myocardial ischemia of early stage often induces the increment of sympathetic activity (Armour, 2008, 1999; Li et al., 2010, 2011). The sympathoexcitatory response causes an increase of heart rate and blood pressure, aggravating myocardial ischemic injury (Li et al., 2010, 2011; Liu et al., 2013; Tu et al., 2013).

$P2X_3$ receptor participated in the transmission of nociceptive signal (Burnstock, 2006, 2007; Wirkner et al., 2007; Zhang et al., 2007, 2008; Wang et al., 2009). ATP is released by superior cervical ganglia (Erlinge and Burnstock, 2008; Li et al., 2010, 2011; Vizi et al., 1997). Previous experiments in our laboratory showed $P2X_3$ and $P2X_{2/3}$ receptors activated by ATP in SCG were involved in sympathoexcitatory response induced by myocardial ischemia (Li et al., 2010, 2011; Zhang et al., 2007, 2008; Shao et al., 2007; Liang et al., 2010). Puerarin, a major active ingredient extracted from the traditional Chinese medicine Ge-gen (Radix Puerariae, RP), has been widely used in treatment of myocardial and cerebral ischemia in clinical setting in China (Gao et al., 2007; Tan and Wu, 2007; Xu et al., 2005) The studies in our laboratory showed that

Abbreviations: DAB, diamino-benzidine; DEPC, diethyl pyrocarbonate; ECG, electrocardiogram; HR, heart rate; MI, myocardial ischemic; Pue, puerarin; SBP, systolic blood pressure; SCG, superior cervical ganglia; TH, tyrosine hydroxylase.

* Corresponding author. Address: Department of Physiology, Medical School of Nanchang University, Nanchang, Jiangxi 330006, PR China. Tel./fax: +86 791 86360552.

E-mail address: liangsd@hotmail.com (S. Liang).

[1] Joint first authors.

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puerarin decreased the sensitization of $P2X_3$ receptor involved in hyperalgesia (Xu et al., 2009). In order to illuminate the mechanism of puerarin in treatment of myocardial ischemia, the present works examined the effects of puerarin on the sympathoexcitatory response induced by myocardial ischemia and explored the relationship with $P2X_3$ receptor in rat SCG.

2. Materials and methods

2.1. Animals and myocardial ischemic models

Sprague–Dawley (SD) rats (180–220 g) of either sex were provided by Laboratory Animal Center of Medical College of Nanchang University. Use of the animals was reviewed and approved by the Animal Care and Use Committee of Medical College of Nanchang University. Rats were randomly divided into control (con) group (rats were subcutaneously injected with normal saline (2 ml/d) for 14 days), puerarin (Pue) group (rats were intraperitoneally injected with puerarin (100 mg/kg/day, for 14 days), myocardial ischemic (MI) group (rats were subcutaneously injected with isoproterenol (Shanghai Harvest Pharmaceutical Co. Ltd., 5 mg/kg/d) for 14 days to induce myocardial ischemic model (Wan et al., 2010), and MI + Pue group (myocardial ischemia rats were intraperitoneally injected with puerarin (Kangenbei Pharmaceutical Limited Corporation, China)). Electrocardiogram (ECG) of the four group rats were measured before and after injection of drugs.

2.2. Measurement of blood pressure, heart rate

Systolic blood pressure (SBP) and heart rate (HR) were measured by non-invasive blood pressure determinator. Tail cuff of 1.5 cm in diameter and 3.2 cm in length was used. Systolic pulsation was measured by an electrosphygmograph coupler (ZH-HX-Z, MD3000, Anhui). The pressure and pulsation were transduced by a pneumatic transducer and recorded on a physiograph. The rats were habituated to the entire test procedure before the experiments began, and all measurements were made by the same person and in a quiet room. Systolic blood pressure was averaged from four to eight measurements for each rat.

2.3. Hematoxylin–Eosin (H–E) stain of myocardial tissue

Myocardial tissues were washed by phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (PFA) for 24 h and then dehydrated with 20% sucrose for overnight at 4 °C. Using a cryostat, myocardial tissues were cut into 10 μm thick. The sections were stained with hematoxylin for 30 s, washed with tap water, stained with eosin for 35 s, and decolorizated in 95% ethanol.

2.4. Double-labeled immunofluorescence of $P2X_3$ and TH

Myocardial tissues and SCG were fixed in 4% PFA for 24 h at room temperature, and then transferred to 20% sucrose for dehydration at 4 °C for overnight. 10 μm sections were cut at a cryostat and stored at −20 °C. After washed with PBS for three times, the sections were pre-incubated with 10% normal donkey serum (NDS; Jackson ImmunoResearch Inc., West Grove, PA, USA) for 40 min in a moisture chamber at 37 °C, and then incubated with rabbit anti-$P2X_3$ (1:500; Chemicon International, Inc., USA) and mouse anti-TH (tyrosine hydroxylase) (1:500; Chemicon International, Inc., USA) diluted in PBS for overnight at 4 °C. After 3 rinses in PBS, the sections were incubated with fluorescent donkey anti-mouse TH CyT^{M3} and donkey anti-rabbit AMCA secondary antibody (1:200 dilution of both secondary antibody; Jackson Immunoresearch, PA, USA) at 37 °C for 40 min. The sections were washed another three times in PBS, mounted with glycerin and examined with fluorescence microscopy. Image-Pro Plus 6.0 image analysis software (Media Cybernetics Inc.) was used to quantify the co-expression of $P2X_3$ and TH. To specify the immunoreactivity of $P2X_3$ and TH, normal donkey serum and PBS worked as negative control instead of primary antibodies.

2.5. Immunohistochemistry and in situ hybridization (ISH)

SCG were fixed with 4% PFA for 24 h and dehydrated with 20% sucrose for 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) for 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 diaminobenzidine (DAB) (Beijing Zhongshan Biotech. CO.) substrate, then dehydrated and mounted with neutral gum. After mounting, the immunohistochemistry photos were photographed using a microscope and Image-Pro Plus 6.0 software in the computer. The software is also an image analysis software used for quantification in the immunohistochemical study. The immunohistochemical signal of positive expression in the picture was assessed by estimating the area of the objects and the medium pixel intensity per object, as the integrated optical density (IOD) (Stîngă et al., 2011). The IOD value represents the level of $P2X_3$ receptor protein expression in the picture. In order to eliminate the effect of photo shooting conditions, all the photos for analysis are acquired under the same shooting condition. We can get a set of data from different photos of the same group. So we calculate the mean and standard deviation of the four groups. And then compare the IOD values between different groups by using one way analysis of variance.

Diethyl pyrocarbonate (DEPC) water was used for all solutions and appliances necessary for ISH. The preparation of section was same with immunohistochemistry section. Sections were treated with 0.5% H_2O_2, followed by digestion with pepsin at 37 °C for 1–2 min, terminated with 0.5 mol/L PBS and washed with it for 15 min. Then the sections were incubated in prehybridization for 2 h at 37 °C and in hybridization overnight at 37 °C. The in situ hybridization kit for $P2X_3$ receptor (Wuhan Boster Co.) was used. The sections were washed with gradient SSC (2 × SSC 17.6 g sodium chloride, 8.8 g sodium citrate in 1000 ml distilled water) thoroughly, 2 × SSC for 10 min, 0.5 × SSC for 15 min and 0.2 × SSC for 15 min to remove the background signals and followed by treatment biotinylated digoxim antibody at 37 °C for 2 h. After strongly washed with PBS, the sections were incubated with SABC-POD for 30 min and with biotinylated peroxidase (Beijing Zhongshan Biotech. CO.) for 30 min at 37 °C. The color was developed in DAB substrate, then dehydrated and mounted with neutral gum. Image-Pro Plus 6.0 image analysis software was used to analyze the intergrated optical density (IOD) of $P2X_3$ mRNA. The analysis method is the same as in the immunohistochemistry experiment.

2.6. Western blotting

SCG were homogenized by mechanical disruption in lysis buffer (50 mM TrisCl, pH8.0, 150 mM NaCl, 0.1% dodecyl sodium sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin) and incubated on ice for 40 min. Homogenate was then pelleted at 6000 g for 10 min