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EXPERIMENTAL STUDY

Total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) effect the expression of CaL-α1C and K_{ATP}-Kir6.1 mRNA of the myocardial cell membrane in myocardial ischemia-reperfusion arrhythmia rats

Yuxuan Ma, Yanli Wang, Yanyu Gao, Yin Fu, Ji Li

Yuxuan Ma, Periodical Office, Heilongjiang University of Chinese Medicine, Harbin 150040, China
Yanli Wang, Yanyu Gao, Yin Fu, College of Basic Medicine, Heilongjiang University of Chinese Medicine, Harbin 150040, China
Ji Li, Department of Prescription, College of Basic Medicine, Heilongjiang University of Chinese Medicine, Harbin 150040, China
Supported by National Natural Science Foundation of China (Proteomics research on the complexes of Ganshanbian (*Herba Hyperici Attenuati*) and Salvia miltiorrhiza anti-reperfusion arrhythmia based on the iTRAQ technology. No. 81173185); Key Project of Natural Science Foundation of Heilongjiang Province (Proteomics research on the complexes of Ganshanbian (*Herba Hyperici Attenuati*) and Salvia miltiorrhiza anti-reperfusion arrhythmia based on the iTRAQ technology. No. ZD201111); Heilongjiang Province Graduate Students Research Key Project (Proteomics research on the complexes of Ganshanbian (*Herba Hyperici Attenuati*) and Salvia miltiorrhiza anti-reperfusion arrhythmia based on the iTRAQ technology. No. YJSCX2012-334HLJ); Outstanding Talents Support Program of Heilongjiang University of Chinese Medicine (Proteomics research on the complexes of Ganshanbian (*Herba Hyperici Attenuati*) and Salvia miltiorrhiza anti-reperfusion arrhythmia based on the iTRAQ technology, 2012)
Correspondence to: Prof. Ji Li, Heilongjiang University of Chinese Medicine, Harbin 150040, China. wangyanli.300@163.com
Telephone: +86-451-82195331
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Abstract

OBJECTIVE: To observe the impact of total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) on the expression of vascular smooth muscle membrane L-type calcium channel alpha1 C subunit (CaL-α1C) and ATP-sensitive K^+ channel (K_{ATP})-Kir6.1 mRNA, and explore the mechanisms of the antiarrhythmic effect of Ganshanbian (*Herba Hyperici Attenuati*) total flavonoids.

METHODS: The treatment group was fed total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) for 7 days by gavage with 100 $mg \cdot kg^{-1} \cdot d^{-1}$. The blank control group and model control group were given the same amount of normal saline for 7 d. Arrhythmias were induced by performing a myocardial ischemia-reperfusion and electrocardiogram was observed. Reverse transcription-polymerase chain reaction was used to detect the expression of CaL-α 1C and K_{ATP}-Kir6.1 mRNA in the myocardial cell membrane of all groups of rats.

RESULTS: Total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) can delay the appearance of myocardial ischemia reperfusion arrhythmias, shorten the duration of myocardial ischemia reperfusion arrhythmias, reduce heart rate, reduce cell membrane expression of CaL-α1C mRNA and enhance the expression of K_{ATP}-Kir6.1 mRNA in myocardial ischemia-reperfusion arrhythmic rats.

CONCLUSION: Total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) can alleviate arrhythmias by affecting the expression of L-type calcium channels and ATP-sensitive K^+ channels.

Key words: Myocardial reperfusion injury; Hypericum; Flavonoids; CaL-α1C; K_{ATP} -Kir6.1

INTRODUCTION

Cardiac arrhythmias are common in the general population, especially among patients with heart disease. In extreme cases, patients are susceptible to sudden death. In China, many traditional medicines are used to treat arrhythmias including Ganshanbian (*Herba Hyperici Attenuati*), which is also known as metacentric grass. Ganshanbian (*Herba Hyperici Attenuati*) is a perennial herb and is mainly distributed in specific regions of China and other countries such as Korea, Japan, Mongolia, and the Russian Far East. Our previous studies have shown that the flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) have a potential ability to treat arrhythmias.[1-5] The purpose of this study is to observe the effect of total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) and the mechanisms involved in treating arrhythmic rats based on the expressions of vascular smooth muscle membrane L-type calcium channel alpha1 C subunit (CaL-α1C) and ATP-sensitive K^+ channel (K_{ATP})-Kir6.1 mRNA. This study will serve as a foundation for the broader clinical application of this drug.

MATERIALS AND METHODS

Equipment and reagents

Equipment and software used included: gradient PCR (Eppendorf Corporation, Hamburg, Germany), Medlab biological signal collecting and processing system (Nanjing Medease Science and Technology Co., Ltd., Nanjing, China), gel imaging system (Bio-Rad Company, Hercules, CA, USA), Integrated Broadband Access System (IBAS) 2.5 full automatic image analysis system (Kontron Company, Munich, Germany), 6010 UV-vis spectrophotometer (American Agilent Analytical Instrument Factory, Shanghai, China), DH-140 animal respirator (Taimeng Technology Co., Ltd., Chengdu, China), trace palm centrifuge (Sigma, Hamburg, Germany), - 80℃ refrigerator (Sanyo Company, Tokyo, Japan), desktop pH meter (Mettler Company, Zurich, Switzerland), and Primer Premier 5.0 (Primer Company, Ottawa, Canada).

Reagents and kits used included: TaKaRa PrimeScript® real-time polymerase chain reaction (RT-PCR) Kit [TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China], TaKaRa DNA 100 Marker [TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China], DNA Green (10000×, Tiandz Genetic Engineering Co., Ltd., Beijing, China), agarose (Bioscience Company, Barcelona, Spain), primer synthesis (Shanghai Generay Biotech Co., Ltd., Shanghai, China). All other experimental materials were from common commercial sources.

Animal groupings

Thirty-six adult Sprague-Dewey rats with a weight of (180±20) g (18 male rats and 18 female rats) were obtained from the Experimental Animal Center of Heilongjiang University of Chinese Medicine, which is accredited by the Institutional Animal Care and Use Committee. Living conditions of rats meet the standard. The experimenter does simulation exercises and reduce the death of animals, and at the end of the experiment, adopts euthanasia to the rats.

The rats were divided into three group randomly according random number table: a blank control group, a model control group, and a treatment group. Each group had six males and six females. The treatment group was fed total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) for 7 days by gavage with 100 $mg \cdot kg^{-1} \cdot d^{-1}$. The other two groups were given the same amount of normal saline for 7 days.

Model preparation

Animals were anesthetized by injecting of 25% urethane 5 mL/kg while fixed supinated. A normal electrocardiogram was recorded until a stable state that lasted for 15 min was observed. The median cervical skin was then cut and opened, and the trachea was exposed via blunt dissection. The center of the trachea was then cut with a T-type hole at the center, and a plastic pipe with a diameter of 2 mm was inserted. This pipe was used for artificial respiration by an animal respirator with a ventilation of 8-9 mL/100 g and a respiratory rate of 50-60 times/min. The ratio of inhalation to that of exhalation was 2∶1. The respirator was set to take a continuous positive pressure breathing of 4-5 kPa.

The skin of the left sternal border was longitudinally cut about 2 cm and opened. The subcutaneous tissues and muscles were separated layer by layer, and the chest was opened in the intercostal area where the beating of the heart was most obvious. The costa was dilated by eye speculum. The eye speculum was pressed down to access the heart. The left main coronary artery located between the left atrium and the right pulmonary conus was inserted with a 5/0 atraumatic suture needle through the myocardial surface. The needle was then twisted beneath the left anterior below the coronary artery and removed from the other side. A slipknot was made to ligate the left anterior below the coronary artery. The slipknot was then opened after 30 min. A dark color of the myocardial surface under the ligation point indicated ischemia, whereas a red color indicated ischemia-reperfusion.[6,7]

After ischemia-reperfusion, the rib, muscle and skin were sutured layer by layer. The breathing of the rats was ensured *via* the respirator but with decreased ventilation for 60 min. The blank control group had the same operation procedure except the ligation was not performed.

Throughout the surgical procedure, the authors had to abandon rats in which ligation or reperfusion were not successful, bled excessively or had a respiratory and cardiac arrest more than 30 s.

Detection of mRNA by RT-PCR

Primer design: primers were designed and amplified by

Primer Premier 5.0 software (Primer Company, Ottawa, Canada). The primer sequences were as shown in Table 1.

PCR reaction conditions

94℃ for 2 min, 94℃ for 30 seconds, 54℃ for 30 seconds, 72℃ for 2 min, 72℃ for 7 min, all of the above conditions turn 30 cycles.

Agarose gel electrophoresis

A 1% agarose gel as prepared with 0.5 g of agarose and 50 mL of 1× TAE (tris 242 g, $Na_2EDTA \cdot 2H_2O$ 37.2 g and glacial acetic acid 57.1 mL were dissolved and diluted by deionized water 1 L and diluted 50 times when it was used). The gel was melted in a microwave, cooled to 55℃, and 5 μL of DNA Green was added. The comb was removed after 30 min. The gum was placed in the electrophoresis buffer.

The sample was then mixed. About 10 μL of the sample plus 2 μL of 10× loading buffer was placed in a well. About 5 μL of DL2000 marker was used as the control sample. The samples were electrophoresed at 110 V for 30 min. Gene expression was calculated using the formula as follows: gene expression=(the measured intensity of the target gene×the area of the target gene)/(the intensity of the β-actin×the area of the β-actin).

Statistical analysis

Data analysis was performed using Microsoft Office Excel (Microsoft, Redmond, WA, USA). Mean ± standard deviation was used for data. A two-tailed unpaired *t*-test with equal variances was used in the estimation of statistical significance of the differences. $P<0.01$ and $P<0.05$ indicate statistical significance.

RESULTS

Myocardial ischemia -reperfusion arrhythmia

From the results of biological signal acquisition and processing, Total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) affect the appearance time, duration and heart rate of myocardial ischemia-reperfusion arrhythmia in rats significantly, as shown in Table 2.

Compared with the model control group, myocardial ischemia-reperfusion arrhythmias appeared later in the treatment group ($P=0.0008$, $P<0.01$) (Table 2). The duration of the myocardial ischemia-reperfusion arrhythmia in the treatment group was shorter ($P=0.0000$, $P<0.01$) than that in the model group. Compared with the model control group, the heart rate of the treatment group was slower ($P=0.0298$, $P<0.05$).

Expression of CaL-α1C mRNA in the cell membrane of myocardial ischemia-reperfusion arrhythmic rats

RT-PCR shows that, Total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) affect the expression of CaL-α1C mRNA in the cell membrane of myocardial ischemia-reperfusion arrhythmic rats significantly, as shown in Table 3.

The CaL-α1C mRNA level in the treatment group was significantly lower than that in the model control group ($P=0.0000$, $P<0.01$) (Table 3, Figure 1).

Expression of K_{ATP}-Kir6.1 mRNA in the cell membrane of myocardial ischemia-reperfusion arrhythmic rats

RT-PCR shows that, total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) affect the expression of K_{ATP}-Kir6.1 mRNA in the cell membrane of myocardial ischemia-reperfusion arrhythmic rats significantly, as shown in Table 4.

The K_{ATP}-Kir6.1 mRNA level in the treatment group was significantly higher than that in the model control group ($P=0.0000$, $P<0.01$) (Table 4 and Figure 2).

DISCUSSION

The L-type calcium channel is composed of α1c, α2δ, and β2 subunits. α1c has a pore through which Ca^{2+} ions enter the cell.[8,9] α1c is also the major subunit that determines the voltage of dependent Ca^{2+} channel and drug susceptibility. In this experiment, the high expression of α1c subunit mRNA indicates that the L-type calcium channel is highly open and results in more Ca^{2+} ions entering the cell. Studies have shown that the

Table 1 Primer sequences

Name of primer		Sequence of primer	Amplified product fragment length (bp)
β-actin	β-actin-EP	5'-AAATGCTGGTGACATCAAA-3'	546
	β-actin-RP	5'-AAGAAAGGGTGTAAAACGCA-3'	
CaL-α1C	CaL-α1C-EP	5'-AGAGCAAAAGCTCAAATTCACTG-3'	793
	CaL-α1C-RP	5'-ACTTTTAAAAATGCTTCCACGGT-3'	
K_{ATP}-Kir6.1	K_{ATP}-Kir6.1-EP	5'-TGATCATCTGCCATGTGATTGAT-3'	460
	K_{ATP}-Kir6.1-RP	5'-TTTCCTTCTGGAGTCATGAATTG-3'	

Notes: CaL-α1C: vascular smooth muscle membrane L-type calcium channel alpha1 C subunit; K_{ATP}: ATP-sensitive K^+ channel.

Table 2 Effect of total flavonoids from Hypericum attenuatum on myocardial ischemia-reperfusion arrhythmia ($\bar{x} \pm s$)

Group	*n*	Appearance time (s)	Duration (min)	Heart rate (beats/min)
Blank control	12	-	-	302.2±30.0[a]
Model control	11	180.4±48.6	15.6±3.2	345.9±30.6
Treatment	10	257.2±38.5[a]	8.0±2.2[a]	311.9±35.8[b]

Notes: treatment group were induced arrhythmias by preforming a myocardial ischemia-reperfusion and fed total flavonoids from Hypericum attenuatum for 7 days by gavage with 100 mg·kg^{-1}·d^{-1}; model control group rats were induced arrhythmias by preforming a myocardial ischemia-reperfusion and fed the same amount of normal saline; blank control group rats were not induced arrhythmias and fed the same amount of normal saline. Blank control group and treatment group were compared with model control group, [a]*P*<0.01, [b]*P*<0.05.

Table 3 Effect of total flavonoids from Hypericum attenuatum on the expression of CaL-α1C mRNA in the cell membrane of myocardial ischemia-reperfusion arrhythmic rats ($\bar{x} \pm s$)

Group	*n*	CaL-α1C/β-actin
Blank control	12	0.235±0.014[a]
Model control	11	0.808±0.027
Treatment	10	0.370±0.032[a]

Notes: treatment group rats were induced arrhythmias by preforming a myocardial ischemia-reperfusion and fed total flavonoids from Hypericum attenuatum for 7 days by gavage with 100 mg·kg^{-1}·d^{-1}; model control group rats were induced arrhythmias by preforming a myocardial ischemia-reperfusion and fed the same amount of normal saline; blank control group rats were not induced arrhythmias and fed the same amount of normal saline. CaL-α1C: vascular smooth muscle membrane L-type calcium channel alpha1 C subunit. Compared with model control group, [a]*P*<0.01.

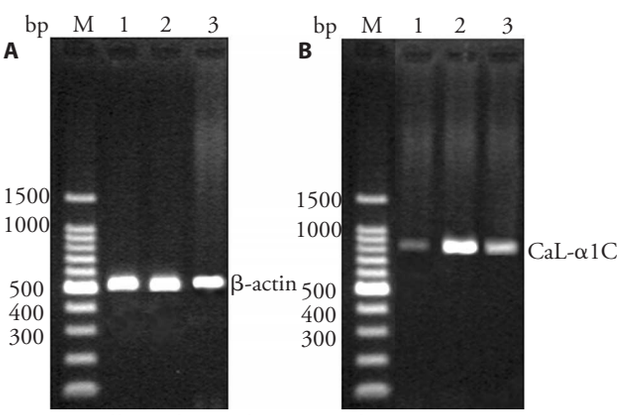

Figure 1 Effect of total flavonoids from Hypericum attenuatum on the expression of CaL-α1C mRNA in the cell membrane of myocardial ischemia-reperfusion arrhythmic rats
M: DNA Marker; 1: blank control group; 2: model control group; 3: treatment group. A: the intensity of the β-actin and the area of the β-actin; B: the intensity of CaL-α1C and the area of CaL-α1C. CaL-α1C: vascular smooth muscle membrane L-type calcium channel alpha1 C subunit.

increased expression of L-type Ca^{2+} channel or lengthening of opening time can prolong action potential duration. Therefore, the level of the plateau potential increases, the early stage of after-depolarization is induced, and the after-depolarization and other triggered arrhythmias are delayed.[10]

The K_{ATP} is a weak inward-rectifier potassium channel, which is regulated by ATP concentration in the cells. It is a transmembrane ion channel that is widely distributed in a variety of cells and organelles, coupling cell excitability and energy metabolism. K_{ATP} is an octamer composed of two-channel subunits.[11] One-channel subunits known as Kir6.x, which is an inward-rectifier subunit that includes Kir6.1 and Kir6.2. The other subunit is a regulatory subunit known as sulfonylurea receptor, or SUR subunit. Kir6.x forms the center pore of the K_{ATP} channel. Kir6.1 exists in the myocardial cell membrane and in the myocardial mitochondrial membrane.

Morrissey *et al*[12] found that Kir6.1 is expressed on the ventricular muscle cell membrane of mice and rats via

Table 4 Effect of total flavonoids from Hypericum attenuatum on the expression of K_{ATP}-K_{ir}6.1 mRNA in the cell membrane of myocardial ischemia-reperfusion arrhythmic rats ($\bar{x} \pm s$)

Group	*n*	Kir6.1/β-actin
Blank control	12	0.441±0.049[a]
Model control	11	0.625±0.044
Treatment	10	1.034±0.033[a]

Notes: treatment group rats were induced arrhythmias by preforming a myocardial ischemia-reperfusion and fed total flavonoids from Hypericum attenuatum for 7 days by gavage with 100 mg·kg^{-1}·d^{-1}; model control group rats were induced arrhythmias by preforming a myocardial ischemia-reperfusion and fed the same amount of normal saline; blank control group rats were not induced arrhythmias and fed the same amount of normal saline. Compared with model control group, [a]*P*<0.01.

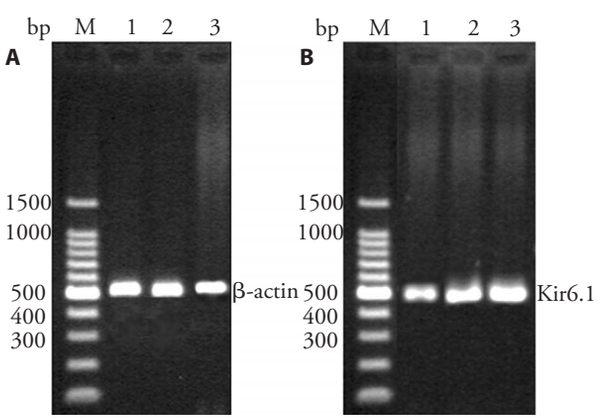

Figure 2 Effect of total flavonoids from Hypericum attenuatum on the expression of K_{ATP}-Kir6.1 mRNA in the cell membrane of myocardial ischemia-reperfusion arrhythmic rats
M: DNA Marker; 1: blank control group; 2: model control group; 3: treatment group. A: the intensity of the β-actin and the area of the β-actin; B: the intensity of Kir6.1 and the area of Kir6.1. K_{ATP}: ATP-sensitive K^+ channel.

immune localization. Ligations on the left coronary artery of rat hearts to mimic ischemia-reperfusion injury lead to significantly higher expression of Kir6.1 subunit mRNA and protein in the ischemic and non-ischemic compared with those in blank control groups. However, Kir6.2 mRNA does not significantly change.[13] Another *in vivo* study showed that Kir6.1 subunit mRNA expression in a hypoxia group was higher than that in a normal group, while the expression of Kir6.2 was lower.[14]

The results of the present study show that the total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) can reduce the cell membrane expression of CaL-α1C mRNA and enhance the expression of K_{ATP}-Kir6.1 mRNA in myocardial ischemia-reperfusion arrhythmic rats. Therefore, Ganshanbian (*Herba Hyperici Attenuati*) has direct or indirect regulatory functions on L-type Ca^{2+} and the K_{ATP} channels in myocardial cells. Total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) inhibit the inflow of Ca^{2+} ions and regulate the Ca^{2+} ion concentration in myocardial cells by inhibiting the duration of opening of L-type Ca^{2+} channels. Moreover, the total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) lower the prevalence of tachyarrhythmia by facilitating the opening of K_{ATP}.

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