Original Article
Prokineticin 2 preconditioning reduces arrhythmia induced by ischemia reperfusion in rats

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Abstract: Ischemia-reperfusion (I/R) arrhythmias are a common type of clinical arrhythmia. Prokineticin 2 (PK2) is a member of the pro-dynamic keratin family. The role and specific mechanisms of PK2 in arrhythmias remain unclear. Sprague-Dawley rats were randomly divided into a sham operation group, an ischemia/reperfusion arrhythmia model group (I/R), and a PK2 treatment group (PK2 group) followed by an analysis of the incidence of ventricular arrhythmia and the arrhythmia scores, the activity of superoxide dismutase (SOD), and the production of reactive oxygen species (ROS) by spectrophotometry, the secretion of neuropeptide SP by ELISA, caspase 3 activity, as well as the expressions of Bcl-2 and Bax by western blot. Compared with the sham group, the I/R group showed a significantly increased incidence of arrhythmia, increased ventricular arrhythmia (VA) scores, increased ventricular premature beats (VPB) and ventricular tachycardia (VT), decreased SOD activity, SP, and Bcl-2 expressions, increased ROS production, increased Bax expression as well as caspase 3 expression (P<0.05). After PK2 pretreatment, the incidence of arrhythmia, the VA scores, VPB, and VT were significantly decreased, but the SOD activity, SP expression, and Bcl-2 expression were increased with reduced ROS production, caspase 3 expression, and Bax expression. Compared with the I/R group, the differences were statistically significant (P<0.05). PK2 can inhibit myocardial cell apoptosis, increase SP substance expression, reduce the occurrence of ischemia-reperfusion arrhythmia, and then protect the myocardium by regulating the oxidation/antioxidant balance.

Keywords: Prokineticin 2, myocardial ischemia-reperfusion, arrhythmia, apoptosis

Introduction

Arrhythmia has the highest incidence of various cardiovascular diseases and can occur as one of the symptoms of various cardiovascular diseases, which can lead to sudden death, rapid onset, and a high degree of malignancy [1]. In China, due to the increased incidence of cardiovascular disease, the incidence of arrhythmia increases, so the mortality rate caused by ineffective prevention and control also increases [2, 3]. Arrhythmia can occur in the process of heartbeat frequency, rhythm and origin of heart rhythm, and impulse conduction, which can lead to an aggravation of the original heart disease and a subsequent progression to heart failure, and even death, which is a serious threat to human health [4, 5]. Myocardial ischemia-reperfusion (MIRI) can lead to severe myocardial ischemia, myocardial cell damage, myocardial remodeling, and a series of key points of myocardial pathological damage [6, 7]. MIRI arrhythmias are a common clinical type in many cardiovascular diseases that induce arrhythmias [8]. MIRI causes reperfusion arrhythmias (RA), which are a common type of clinical arrhythmia. The most common types of reperfusion arrhythmias include ventricular arrhythmias (VA), ventricular premature beats (VPB), ventricular tachycardia (VT), etc. [9]. Ischemia-reperfusion arrhythmia is one of the common causes of sudden death in patients with coronary heart disease [10]. At present, there are several drugs and methods for treating arrhythmia, but there is still a lack of curative drugs [11].

PK2 is a small molecular protein with a molecular weight of 8 kD first found in skin secretions and is a member of the pro-dynamic keratin family [12]. PK2 has a variety of biological functions, can promote the contraction of gastroin-
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testinal smooth muscle, and can also be expressed in the reproductive system, peripheral blood cells and central nervous system, which plays a role in feeding, hormone secretion, biological circadian rhythm, pain, immunity diseases, pregnancy, and other pathophysiological conditions [13-15]. The study found that the PK2 protein also plays an important role in the development of cardiovascular disease and that PK2 can protect the damaged cardiomyocytes [16]. However, the role and specific mechanism of PK2 in arrhythmias have not been elucidated. Therefore, this study established a rat model of ischemia-reperfusion arrhythmia and analyzed the effect of PK2 on arrhythmia after pretreatment and the related mechanisms.

Materials and methods

Experimental animals

60 healthy male Sprague-Dawley rats, 2 months old, SPF grade, body weight (250±20) g, were purchased from the experimental animal center of this unit, and fed in the SPF animal experiment center, at a temperature of 21±1°C and a relative humidity of 50-70% under constant temperature and constant humidity conditions, and ensuring a 12 hour day/night cycle.

Main materials and instruments

PK2 was purchased from Sigma (USA). Sodium pentobarbital and lidocaine were purchased from Shanghai Zhaohui Pharmaceutical Co., Ltd. The PVDF membrane was purchased from Pall Life Sciences, the Western blot related chemical reagent was purchased from Shanghai Biyuntian Biotechnology Co., Ltd., the ECL reagent was purchased from Amersham Biosciences, the rabbit anti-human Caspase-3 monoclonal antibody, and the goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody was purchased from Cell signaling Corporation of the United States. The SOD activity detection kit was purchased from the Nanjing Jiancheng Bioengineering Research Institute. The surgical microscopy equipment was purchased from the Suzhou Medical Instrument Factory. The Medlab-U/4CS biosignal acquisition and processing system was purchased from Nanjing Meiyi Technology Co., Ltd. The MD automatic microplate reader was purchased from United States Molecular Device.

The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (USA). The 1752 spectrophotometer was purchased from Shanghai Optical Instrument Factory. The SAR1000 animal breathing machine was purchased from Shanghai Yuyan Scientific Instrument Company. Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd.

Grouping and processing of experimental animals

60 rats were randomly and equally divided into three groups: a sham group; an I/R group; and a PK2 group, in which PK2 pretreatment was given before the model establishment, and 100 nM PK2 was administered by gavage before the model preparation once a day for a total of 2 weeks.

Preparation of the rat model of ischemia-reperfusion arrhythmia

According to the literature [17], the experimental group was fasted for 12 hours before the modeling. The rats were anesthetized through an intraperitoneal injection of 10% chloral hydrate, fixed on the operating table, connected to the Medlab-U/4CS biosignal, intubated, and connected to the animal ventilator. We then opened the chest layer by layer and exposed the heart. In the trunk of the coronary vein, we found the lower edge of the left atrial appendage of the pulmonary artery cone, about 1-2 mm. We use a non-invasive suture to suture at the anterior descending branch of the left coronary artery to prepare for the acute myocardial ischemia. The lead electrocardiogram of the II lead was connected, and after ligating for 30 minutes, the ligature was removed and reperfusion was performed for 60 min. In the sham group, we only opened the chest without any ligation.

Record the incidence of arrhythmia

The rate of arrhythmia, and the incidences of VPB and VT were recorded using ECG monitoring.

VA score

According to the literature [18], the VA score was performed. 0 was defined as no VA or VPB fewer than 5 times. 1 was defined as VPB only
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Specimen collection

The negative aorta collection method was used to collect the abdominal aorta blood samples from the rats in the vacuum biochemical tube for 12 hours after model establishment. The cells were allowed to stand at room temperature for 30 minutes. After the blood was coagulated, it was centrifuged at 4°C at 3600 rpm for 10 min, and the supernatant was collected and stored in a -20°C refrigerator for further use. The heart tissues of each group were collected and stored in a refrigerator at -80°C.

ELISA detection of SP secretion

The serum of each group was collected for an analysis of the changes of SP by ELISA according to the ELISA kit’s instructions. The linear regression equation of the standard curve was calculated according to the concentration of the standard and the corresponding optical density (OD) value, and the corresponding sample concentration was calculated on the regression equation according to the OD value of the sample.

Analysis of SOD activity

The changes in the SOD activity in the heart tissue of each group were examined according to the kit’s instructions. The tissue protein was extracted and washed in a 95°C water bath. After 40 min, it was taken out and rinsed with cold water. After cooling, it was centrifuged at 4000 rpm for 10 minutes. The tissue homogenate was incubated with 2', 7'-dichlorofluorescein diacetate (DCF-DA) for 15 min at 37°C, centrifuged at 10,000 rpm for 15 min, and resuspended in a sterile PBS phosphate buffer and incubated for 60 min at 37°C. The level of ROS was measured using a spectrophotometer.

Western blot analysis of the caspase 3 protein expression

The heart tissue was extracted as follows: liquid nitrogen grinding of the tissue, add lysate, lyse cells on ice for 15~30 min, disrupt the cells by 5 s×4 times, centrifuge at 4°C, 10,000×g for 15 min, transfer the supernatant to a new tube. The protein was quantified and stored at -20°C for the western blot experiments. The isolated protein was electrophoresed on a 10% SDS-PAGE, was transferred to a PVDF membrane using a semi-dry transfer method, blocked with 5% skim milk powder for 2 h, and incubated with a primary antibody 1:1000 diluted caspase 3 monoclonal antibody at 4°C overnight. After PBST washing, 1:2000 goat anti-rabbit secondary antibody was added and incubated in the dark for 30 min followed by PBST washing, chemiluminescence color development for 1 min and X-slice exposure imaging. The X-film and strip density measurements were separately scanned using a protein image processing system software and Quantity One software. The experiment was repeated four times (n=4).

Real-time PCR detection of Bcl-2 and Bax expressions

Under sterile conditions, the myocardial tissue mRNA was extracted using Trizol reagent, and cDNA was synthesized by PCR according to the relevant primers (Table 1). Real-time PCR was used to detect the expression of the target gene with reaction conditions as follows: 52°C 1 min, 90°C 30 S, 58°C 50 S, 72°C 35 S, for a total of 35 cycles. Bcl-2 and Bax: 55°C 1 min, 90°C 30 S, 62°C 50 S, 72°C 35 S, for a total of 35 cycles. Fluorescence quantitative PCR reactor analysis software was used to collect relevant data. According to the internal reference GAPDH, the standard cycle number (CT) of the

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADPH</td>
<td>AGTGCCAGCCTCGTCTCATAG</td>
<td>CGTTGAACTTGCCGTGGTAG</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>GATATCGGCCAGGTAGTTGA</td>
<td>ATGTCGTCCAGGAGTTGTCTGT</td>
</tr>
<tr>
<td>Bax</td>
<td>TCGTCTGCGCAGCAAGATA</td>
<td>GTCAAGGTATGAGTTG</td>
</tr>
</tbody>
</table>

and more than 5 times; 2 was VT with less than 1 min; 3-5 was increased according to the number of VT times and time, and 6 was defined as death or high frequency and time trembling.
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Table 2. Analysis of the arrhythmia indexes in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham group</th>
<th>I/R group</th>
<th>PK2 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrhythmia (%)</td>
<td>0</td>
<td>100*</td>
<td>60.0*  #</td>
</tr>
<tr>
<td>VPB (%)</td>
<td>0</td>
<td>90.0*</td>
<td>50.0*  #</td>
</tr>
<tr>
<td>VT (%)</td>
<td>0</td>
<td>75.0*</td>
<td>40.0*  #</td>
</tr>
</tbody>
</table>

Compared with the sham group, *P<0.05; compared with the I/R group, #P<0.05.

standard was calculated, and a standard curve was drawn. The quantitative analysis was analyzed using the 2-ΔCt method.

Statistical analysis

All data were processed using SPSS 19.0 software. The measurement data were expressed as the mean ± standard deviation (SD). The comparisons of multiple groups of samples were performed using one-way ANOVA, and the comparisons between groups were performed using an LSD test. P<0.05 was considered a statistically significant difference.

Results

Analysis of the arrhythmia indexes in rats

The changes in the arrhythmia indexes after 12 hours of model establishment were analyzed. The results showed that compared with the sham group, the incidence of arrhythmia was significantly increased in the I/R group, and the incidences of VPB and VT were increased (P<0.05). However, after PK2 pretreatment, the incidences of arrhythmia, VPB, and VT was significantly reduced compared with the I/R group (P<0.05). The change was more significant with an increase in the dosage (P<0.05) (Table 2).

The effect of PK2 on the VA score of arrhythmia in rats with ischemia-reperfusion

The VA scores of the rats in each group were analyzed after 12 hours. The results showed that, compared with the sham group, the VA scores of the rats in the I/R group were significantly increased (P<0.05). However, after PK2 pretreatment, the VA scores were significantly decreased compared with the I/R group (P<0.05) (Figure 1).

The effect of PK2 on SP secretion in rats with ischemia-reperfusion arrhythmia

The effect of PK2 on SP secretion in rats with ischemia-reperfusion arrhythmia was analyzed by ELISA. The results showed that SP secretion was significantly decreased in the I/R group compared with the SP secretion in the sham group (P<0.05). However, after PK2 pretreatment, the SP substance secretion was significantly increased compared with the I/R group (P<0.05) (Figure 2).
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Table 3. The effect of PK2 on the oxidative stress in the myocardial tissue of each group

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham group</th>
<th>I/R group</th>
<th>PK2 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS</td>
<td>51±11</td>
<td>192±22'</td>
<td>101±18'×,#</td>
</tr>
<tr>
<td>SOD</td>
<td>163±23</td>
<td>67±15'</td>
<td>107±21'×,#</td>
</tr>
</tbody>
</table>

Compared with the sham group, *P<0.05; compared with the I/R group, #P<0.05.

The effects of PK2 on SOD and ROS

The effects of PK2 on the oxidative stress index in rats with ischemia-reperfusion arrhythmia were analyzed. The ROS production in the myocardial tissue of rats with arrhythmia caused by I/R was significantly increased, but the SOD content was decreased. Compared with the sham group, the differences were statistically significant (P<0.05). However, the PK2 pretreatment significantly inhibited the production of ROS and increased the content of SOD. Compared with the I/R group, the difference was statistically significant (P<0.05) (Table 3).

Effect of PK2 on caspase 3 expression

A western blot analysis of the effect of PK2 on Caspase 3 in the myocardial cells of rats with ischemia-reperfusion arrhythmia showed that I/R caused a significant increase in caspase 3 expression in the myocardial tissue of rats with arrhythmia, but the pretreatment of PK2 in I/R rats significantly inhibited the expression of caspase 3 (Figure 3).

The effects of PK2 on the expressions of Bcl-2 and Bax in cardiomyocytes

Real-time PCR was used to analyze the effect of PK2 on the expressions of Bcl-2 and Bax in the cardiomyocytes of rats with ischemia-reperfusion arrhythmia. The expression of Bcl-2 was decreased and the expression of Bax was increased in rat myocardial tissue. The difference was statistically significant compared with the sham group (P<0.05). However, the pretreatment of PK2 significantly promoted Bcl-2 expression and decreased Bax expression compared with the I/R group (P<0.05) (Figure 4).

Discussion

Myocardial ischemia-reperfusion arrhythmia causes channel opening due to transient blood flow interruption, and the reperfusion of blood flow can cause mechanical recanalization, leading to abnormalities in the myocardial biochemistry, electrophysiological function, etc., leading to arrhythmia [19]. The occurrence of ventricular arrhythmias such as VPB and VT can cause cardiac arrest, cardiogenic shock, and even sudden death [16]. Therefore, finding a drug that is effective for myocardial ischemia-reperfusion arrhythmia is beneficial for clinical treatment and has become a hot spot of concern.

In this study, a rat model of arrhythmia induced by myocardial ischemia-reperfusion injury was established. It was confirmed that myocardial ischemia-reperfusion can cause arrhythmia, leading to a significantly increased VA score, VPB, and VT. Pretreatment with PK2 can reduce the incidence of myocardial ischemia-reperfusion arrhythmia, reduce the VA score, and decrease VPB and VT, suggesting that PK2 has
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Under the action of the endogenous antioxidant system, ROS and SOD are in equilibrium. In the normal physiological state, the antioxidant enzyme SOD maintains important functions, such as scavenging oxygen free radicals such as ROS in the body, thereby preventing and alleviating tissue damage [20]. Myocardial ischemia-reperfusion injury is an oxygen-forming reaction of cardiomyocytes or necrotic cardiomyocytes, which are often caused by ischemia, during reperfusion and H$_2$O$_2$ in the blood, generating oxygen free radicals, which can further damage the myocardium and trigger arrhythmia, and a redox balance disorder leads to an increase in arrhythmia [21, 22]. Bcl-2 and Bax are important regulatory proteins of cell apoptosis. Bcl-2 has anti-apoptotic effect, but Bax is a protein that induces apoptosis. The imbalance of expression leads to cell apoptosis. Caspase 3 is an important member in the Caspase family and plays an important role in inducing cardiomyocyte apoptosis, leading to myocardial cell damage [23, 24]. The endogenous neuropeptide SP substance, which is present in sensory motor nerve fibers, regulates cardiomyocyte protein kinase, which can exert negative chronotropic and inotropic effects on the myocardium. The increased expression can inhibit cardiomyocyte apoptosis and further protect the myocardium [25]. This study analyzed the mechanism of PK2 and confirmed that pretreatment with PK2 can significantly reduce the incidence of myocardial ischemia-reperfusion arrhythmia by increasing SOD activity, reducing ROS, increasing SP expression, inhibiting caspase 3 expression, increasing Bcl-2 expression, decreasing Bax expression, and thus inhibiting cell apoptosis, thereby exerting a protective effect on the myocardium and reducing the occurrence of arrhythmia. In further research, it is proposed to further analyze the relevant pathways and mechanisms of the possible effects of PK2.

Conclusion

PK2 can inhibit the apoptosis of rat cardiomyocytes, increase the expression of SP substance, reduce the occurrence of ischemia-reperfusion arrhythmia, and then protect the myocardium by regulating the oxidation/antioxidant balance.

Disclosure of conflict of interest

None.

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