Inhibition of CaMKII Attenuates Mitochondrial Oxidative Stress and Improves Cardiac Function after Ischemia-Reperfusion Injury in Isolated Hearts

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Abstract

Objective: To investigate the role and potential mechanism of calcium/calmodulin-dependent protein kinase II (CaMKII) in isolated heart ischemia-reperfusion (IR) injury. Methods: Using a rat isolated heart ischemia-reperfusion (IR 45 min/120 min) model, 40 rats were randomly divided into control group (Control), KN-93 drug control group (2.5 mol/L KN93), IR group, and CaMKII specific inhibitor KN-93 intervention ischemia-reperfusion group (KN-93+IR). Left ventricular cardiac function, lactate dehydrogenase (LDH) activity and cardiac troponin (cTnI) content in coronary effluent, and myocardial infarct size were used to evaluate the degree of cardiac injury. Western blot was used to detect the expression of CaMKII phosphorylation (p-CaMKII), CaMKII oxidation (ox-CaMKII), and PLN phosphorylation (p-PLN) proteins. Kit detection was used for mitochondrial superoxide dismutase (SOD) activity and malondialdehyde (MDA) content. Results: Compared with the Control group, all indices in the KN-93 group showed no significant changes; the IR group exhibited decreased cardiac function and mitochondrial SOD activity ($P<0.01$), while myocardial infarct size, LDH activity and cTnI content in coronary effluent, expression of p-CaMKII, ox-CaMKII, and p-PLN, and mitochondrial MDA content were all significantly increased ($P<0.01$); KN-93 intervention in the IR group significantly improved cardiac function ($P<0.01$), increased mitochondrial SOD activity, and reduced myocardial infarct size, LDH activity, cTnI content, expression of p-CaMKII, ox-CaMKII, and p-PLN, as well as mitochondrial MDA content ($P<0.01$). Conclusion: CaMKII participates in isolated heart ischemia-reperfusion injury, and inhibition of CaMKII can alleviate isolated heart ischemia-reperfusion injury by reducing mitochondrial oxidative stress.

Full Text

Inhibition of CaMKII Alleviates Myocardial Ischemia-Reperfusion Injury by Reducing Mitochondrial Oxidative Stress in Isolated Perfused Rat Hearts

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Abstract

Objective: To investigate the role of calcium/calmodulin-dependent protein kinase II (CaMKII) in myocardial ischemia-reperfusion (IR) injury in isolated perfused rat hearts and explore the underlying mechanisms. **Methods:** An ischemia-reperfusion model was established using isolated rat hearts perfused with Krebs-Henseleit solution. Forty rats were randomly divided into four groups: control group, KN-93 drug control group (2.5 mol/L KN-93), IR group, and CaMKII-specific inhibitor KN-93 intervention group (KN-93+IR). Myocardial injury was evaluated by left ventricular cardiac function, lactate dehydrogenase (LDH) activity and cardiac troponin I (cTnI) content in coronary effluent, and myocardial infarct size. Western blotting was used to detect the expression of phosphorylated CaMKII (p-CaMKII), oxidized CaMKII (ox-CaMKII), and phosphorylated PLN (p-PLN). Mitochondrial superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured using assay kits. **Results:** Compared with the control group, the KN-93 group showed no significant changes in any parameters. The IR group exhibited decreased cardiac function and mitochondrial SOD activity ($P<0.01$), while myocardial infarct size, LDH activity and cTnI content in coronary effluent, expression of p-CaMKII, ox-CaMKII, and p-PLN, and mitochondrial MDA content were all significantly increased ($P<0.01$). KN-93 intervention in the IR group significantly improved cardiac function ($P<0.01$), increased mitochondrial SOD activity, and reduced myocardial infarct size, LDH activity, cTnI content, expression of p-CaMKII, ox-CaMKII, and p-PLN, as well as mitochondrial MDA content ($P<0.01$). **Conclusion:** CaMKII participates in isolated heart ischemia-reperfusion injury, and inhibiting CaMKII can alleviate ischemia-reperfusion injury by reducing mitochondrial oxidative stress.

Keywords: calcium/calmodulin-dependent protein kinase II; myocardial ischemia-reperfusion injury; mitochondria; superoxide dismutase; malondialdehyde

Introduction

Myocardial ischemia-reperfusion is an inevitable process in the surgical treatment of cardiovascular diseases such as thrombolytic therapy for acute myocardial infarction, coronary angioplasty, and cardiac arrest surgery [1]. Current research on the mechanisms of myocardial ischemia-reperfusion injury has primarily focused on calcium overload, oxidative stress, apoptosis, inflammatory response, mitochondrial dysfunction, and autophagy [2-8]. Despite extensive literature reporting these mechanisms, clinical translation remains limited, with low application value and unsatisfactory therapeutic outcomes.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase with four isoforms (, , , and), with the isoform being predominantly expressed in the heart [9-11]. CaMKII can be activated through autophosphorylation or oxidation at the M281/282 site by reactive oxygen species (ROS) [12-14]. Activated CaMKII phosphorylates L-type calcium channels, sarcoplasmic reticulum ryanodine receptors (RyR), and calcium pumps (SERCA2a), thereby regulating intracellular calcium changes in cardiomyocytes [15-18]. The specific CaMKII inhibitor KN-93 competitively binds to the calmodulin (CaM) binding domain of CaMKII, thereby inhibiting CaMKII activity [1]. According to previous literature, CaMKII participates in myocardial injury mainly by regulating L-type calcium channels and sarcoplasmic reticulum function, which exacerbates intracellular calcium overload [15-18]; however, its role in mitochondrial function, particularly in regulating mitochondrial redox reactions, has not been reported. This study employed an isolated heart ischemia-reperfusion model to investigate the effects of CaMKII inhibition by KN-93 on mitochondrial oxidative stress damage.

Materials and Methods

1.1 Materials Forty healthy male Sprague-Dawley (SD) rats (body weight 250-300 g) were purchased from the Laboratory Animal Center of the Fourth Military Medical University. Triphenyltetrazolium chloride (TTC) was purchased from Sigma, and KN-93 was purchased from Tocris; its concentration was determined based on literature reports [19]. Lactate dehydrogenase assay kits, SOD assay kits, and MDA assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. Antibodies against phosphorylated CaMKII, PLN, CaMKII, and GAPDH were purchased from Cell Signaling Technology. Phospho-specific antibody for PLN-Thr17 was purchased from Badrilla, and oxidized CaMKII antibody was purchased from Millipore. The normal perfusion solution, Krebs-Henseleit (KH) solution, contained (mmol/L): NaCl 118, KCl 4.7, KH PO 1.2, MgSO 1.2, CaCl 1.25, glucose 11, and NaHCO 25. The KH solution was bubbled with mixed gas (95% O /5% CO , v/v) for 45 minutes before the experiment and throughout the procedure. The perfusion solution pH was maintained at 7.35-7.45, and temperature was kept at 37°C.

1.2 Experimental Methods 1.2.1 Experimental Groups and Model Preparation

SD rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg) and heparin (500 U/kg). After adequate anesthesia, the chest was quickly opened along both sides of the xiphoid process to expose the heart, which was immediately removed and placed in ice-cold KH solution. The aortic root was rapidly cannulated onto a Langendorff perfusion apparatus using 0-gauge silk suture, with retrograde perfusion from the aortic ostium at a perfusion pressure of 80 mmHg. A latex balloon was inserted into the left ventricle through the mitral valve, and cardiac function was recorded using Labchart 7 software. The balloon volume was adjusted to maintain left ventricular end-diastolic pressure (LVEDP) at 0-5 mmHg, and the heart was allowed to stabilize for 15 minutes. After equilibrium, hearts were randomly divided into four groups (n=10): control group (Control), KN-93 drug control group (KN-93), ischemia-reperfusion group (IR), and KN-93 intervention ischemia-reperfusion group (KN-93+IR). The control group received normal perfusion for 165 minutes. The KN-93 group was perfused with KH solution containing 2.5 mol/L KN-93 for 6 minutes, followed by normal KH solution for 159 minutes. The IR group underwent 45 minutes of ischemia and 120 minutes of reperfusion. The KN-93+IR group was treated with KH solution containing 2.5 mol/L KN-93 for 1 minute before ischemia, subjected to 45 minutes of ischemia, treated again with KN-93-containing KH solution for 5 minutes, and then reperfused with normal KH solution for 114 minutes.

1.2.2 Cardiac Function Monitoring

Throughout the entire experiment, Labchart 7 software was used to monitor left ventricular pressure (LVP), left ventricular systolic peak pressure (LVPSP), and left ventricular end-diastolic pressure (LVEDP). Left ventricular developed pressure (LVDP) was calculated as the difference between LVPSP and LVEDP. LVDP and LVEDP were used as indicators for evaluating cardiac function.

1.2.3 Myocardial Infarct Size Measurement

At the end of reperfusion, hearts were immediately stored at -20°C for 60 minutes. The hearts were then cut perpendicular to the long axis into six continuous slices of approximately 1 mm thickness. The slices were placed in a 24-well plate containing 1% TTC solution and incubated at 37°C in a constant-temperature water bath for 15 minutes in the dark, followed by fixation in 4% paraformaldehyde for 2 days. Images were captured using a digital camera, and infarct size was calculated using Image J software. White areas represented infarcted myocardium, while red areas represented normal myocardium. The infarct size percentage was calculated as (infarct area/total myocardial area) × 100%.

1.2.4 LDH Activity and cTnI Measurement

Coronary effluent was collected during the first 10 minutes of reperfusion. LDH activity and cTnI content were measured strictly according to the instructions

provided with the LDH assay kit and cTnI detection kit from Nanjing Jiancheng Bioengineering Institute. The absorbance values of coronary effluent from each group were read and converted to corresponding LDH activity and cTnI content values.

1.2.5 Western Blot Analysis

Left ventricular myocardial tissue of equal weight was obtained from each group and lysed on ice for 30 minutes in a mixture containing protease inhibitors, phosphatase inhibitors, and RIPA lysis buffer (strong). The lysate was centrifuged at 12,000 g for 20 minutes at 4°C, and the supernatant was collected for BCA protein quantification. Proteins were separated on 5% stacking gel and 10% separating gel, then transferred to PVDF membrane at 90 V for 70 minutes. The membrane was blocked with 5% skim milk in TBST at room temperature for 90 minutes, then incubated overnight at 4°C with primary antibodies against p-CaMKII, ox-CaMKII, CaMKII, p-PLN, PLN (1:1000), and GAPDH (1:4000). The next day, the membrane was washed six times with TBST (5 minutes each) and incubated with HRP-conjugated secondary antibody (1:5000) at room temperature for 90 minutes, followed by six additional TBST washes (5 minutes each). After ECL development and exposure, the bands were scanned and analyzed using Image J software for grayscale values, with GAPDH serving as the internal reference.

1.2.6 Mitochondrial SOD Activity and MDA Content Measurement

After reperfusion, mitochondrial fractions were extracted from myocardial tissue as follows: 40 mg of myocardial tissue from each group was placed in a 1.5 mL centrifuge tube containing 500 L ice-cold PBS, minced on ice, and centrifuged at 800 g for 60 seconds at 4°C. The supernatant was discarded, and the pellet was resuspended in 500 L mitochondrial isolation reagent containing PMSF, homogenized on ice, and centrifuged at 1000 g for 5 minutes at 4°C. The supernatant was transferred to another tube and centrifuged at 12,000 g for 15 minutes at 4°C, and the resulting pellet was collected as mitochondria. Mitochondrial MDA content (nmol/mg) and SOD activity (U/mg) were measured according to the instructions provided with the mitochondrial MDA and SOD assay kits from Nanjing Jiancheng Bioengineering Institute.

1.3 Statistical Analysis Data are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 13.0 software. One-way ANOVA was used for inter-group comparisons, and Tukey' s test was used for pairwise comparisons. $P<0.05$ was considered statistically significant.

Results

2.1 KN-93 Improved Cardiac Function in Isolated Hearts Subjected to Ischemia-Reperfusion As shown in [Figure 1: see original paper], LVDP

and LVEDP remained unchanged throughout the perfusion period in both the control and KN-93 groups, with no significant difference between these two groups ($P>0.05$). Compared with the control group, LVDP was significantly decreased and LVEDP was significantly increased in the IR group after 45 minutes of ischemia followed by reperfusion ($P<0.01$). In contrast, the KN-93+IR group showed significantly increased LVDP and decreased LVEDP compared with the IR group ($P<0.01$).

2.2 KN-93 Reduced Myocardial Infarct Size and cTnI Release in Isolated Hearts Subjected to Ischemia-Reperfusion As shown in [Figure 2: see original paper], no infarcted areas were observed in the control and KN-93 groups, and LDH activity and cTnI content in coronary effluent were very low. Compared with the control group, myocardial infarct size was significantly increased, and LDH activity and cTnI content in coronary effluent were markedly elevated in the IR group ($P<0.01$). The KN-93+IR group exhibited significantly reduced myocardial infarct size and decreased LDH activity and cTnI content compared with the IR group ($P<0.01$).

2.3 KN-93 Inhibited CaMKII and PLN Activity in Isolated Hearts Subjected to Ischemia-Reperfusion Injury Western blot results shown in [Figure 3: see original paper] demonstrated that compared with the control group, the KN-93 group showed no significant changes in ox-CaMKII, p-CaMKII, and p-PLN levels ($P>0.05$). In the IR group, expression of ox-CaMKII and p-CaMKII was significantly increased, and phosphorylation of its substrate PLN was also markedly elevated ($P<0.01$). In contrast, the KN-93+IR group showed significantly reduced expression of ox-CaMKII, p-CaMKII, and p-PLN compared with the IR group ($P<0.01$).

2.4 KN-93 Attenuated Mitochondrial Oxidative Stress in Isolated Hearts Subjected to Ischemia-Reperfusion Injury As shown in [Figure 4: see original paper], mitochondrial SOD activity and MDA content showed no significant changes in the KN-93 group compared with the control group ($P>0.05$). The IR group exhibited significantly decreased mitochondrial SOD activity and increased MDA content ($P<0.01$). Compared with the IR group, the KN-93+IR group showed significantly increased mitochondrial SOD activity and decreased MDA content ($P<0.01$).

Discussion

Myocardial reperfusion is currently the most effective treatment for ischemic heart disease; however, reperfusion itself causes damage to ischemic myocardium, increases apoptosis and necrosis, induces cardiac dysfunction and malignant arrhythmias, and severely affects patient prognosis. In recent years, the important role of CaMKII in cardiovascular diseases has become a research

hotspot [20-22]. Numerous studies have reported that CaMKII participates in the pathogenesis and progression of myocardial ischemia-reperfusion injury, myocardial infarction, heart failure, and diabetic cardiomyopathy [23-27], with mechanisms primarily focused on CaMKII-induced exacerbation of intracellular calcium overload. However, its role in myocardial oxidative stress has been less extensively reported.

This study employed an isolated heart ischemia-reperfusion model to examine clinically relevant indicators reflecting cardiac function, including cardiac systolic and diastolic function, myocardial infarct size, and LDH activity and cTnI content in coronary effluent to evaluate the extent of myocardial injury. We also measured CaMKII phosphorylation and oxidation expression and changes in phosphorylation levels of its substrate PLN to reflect CaMKII activity, thereby assessing the role of CaMKII in the process of cardiac ischemia-reperfusion.

Our experimental results confirmed that KN-93 intervention in IR hearts significantly improved cardiac function, reduced myocardial infarct size, and decreased LDH activity and cTnI content in coronary effluent, consistent with numerous previous reports [4, 19]. These findings demonstrate that CaMKII inhibition significantly alleviates myocardial injury. Additionally, the IR group showed significantly increased CaMKII phosphorylation and oxidation expression, along with elevated phosphorylation of its substrate PLN, whereas KN-93-mediated CaMKII inhibition markedly reduced both CaMKII phosphorylation and oxidation as well as PLN phosphorylation. This indicates that KN-93 can simultaneously inhibit both activation modes of CaMKII to attenuate IR-induced myocardial injury.

Mitochondria serve as the primary energy-producing organelles in cardiomyocytes and play a crucial role in myocardial energy metabolism. Myocardial ischemia-reperfusion disrupts mitochondrial structure and oxidative phosphorylation, increases mitochondrial oxidative stress damage, leads to massive accumulation of oxygen free radicals, reduces ATP synthesis, opens mitochondrial permeability transition pores, and initiates apoptosis [28-29]. Therefore, inhibiting mitochondrial oxidative stress can effectively alleviate cardiac ischemia-reperfusion injury. SOD activity reflects the capacity of mitochondria to scavenge oxygen free radicals, while MDA content indirectly reflects the degree of lipid peroxidation. Our experimental results demonstrated that after isolated heart ischemia-reperfusion, mitochondrial SOD activity was significantly decreased while MDA content was markedly increased, indicating enhanced mitochondrial oxidative stress. KN-93 intervention in IR hearts increased mitochondrial SOD activity and decreased MDA content, suggesting that CaMKII inhibition can reduce mitochondrial oxidative stress.

In summary, this study further clarifies that CaMKII participates in cardiac ischemia-reperfusion injury and demonstrates that KN-93-mediated inhibition of CaMKII phosphorylation and oxidation can ameliorate cardiac injury by reducing mitochondrial oxidative stress, providing new experimental evidence for the role of CaMKII in cardiac ischemia-reperfusion injury. Future work should

further elucidate the distinct roles and molecular mechanisms of CaMKII phosphorylation and oxidation in mediating mitochondrial oxidative stress, which may provide novel insights for cardioprotection against ischemia-reperfusion injury.

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