Effect of Ligustrazine on Myocardial Ischemia/reperfusion Injury Through Upregulating UCP3

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Research

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Abstract

Background: The study aimed to investigate whether ligustrazine, a traditional Chinese medicine, could attenuate myocardial ischemia/reperfusion (I/R) injury and explore the potential mechanism.

Methods: 32 Sprague-Dawley rats were divided equally into four groups: sham operation (S); I/R; I/R + ligustrazine preconditioning (Lig); I/R + ligustrazine preconditioning + mitochondrial permeability transition pore (mPTP) opener lonidamine (LND) (Lig + LND). Myocardial I/R model was established and ligustrazine was administered intraperitoneally 5 min prior to ischemia, LND was administered intraperitoneally 10 min prior to reperfusion. The infarct area (IA) was measured by Evans blue staining, where levels of Myocardial injury markers, malondialdehyde (MDA), superoxide dismutase (SOD), adenosine triphosphate (ATP) were detected. RT-PCR and Western Blot were adopted to measure the uncoupling protein 3 (UCP3) expression.

Results: Compared to I/R group, the IA/area at risk (AAR) in Lig, and Lig + LND groups were significantly decreased, UCP3 levels of mRNA and protein were increased ($P < 0.05$). Compared to Lig group, the IA/AAR in Lig + LND group was significantly increased ($P < 0.05$). Ligustrazine pretreatment increased SOD, ATP activity, and decreased cardiac troponin I (cTnI), creatine kinase-muscle/brain (CK-MB), lactate dehydrogenase (LDH) and MDA activities. The effect of ligustrazine was reversed by LND.

Conclusion: The present results indicated that ischemic preconditioning of ligustrazine might protect myocardium against I/R injury through upregulating UCP3 expression.

Introduction

Acute myocardial infarction is one of the major causes of morbidity and mortality in humans [1], and revascularization by thrombolysis or percutaneous coronary intervention (PCI) remain the most effective therapy to salvage ischemic myocytes and improve outcomes [2]. However, further myocardial damage occurs following reperfusion, namely myocardial ischemia/reperfusion (I/R) injury [3]. Therefore, finding novel therapeutic strategies and targets to lessen the myocardial I/R injury is a vital research topic in the field of cardiology.

Previous studies showed that the pathological mechanisms of myocardial I/R injury were associated with a number of factors, including reactive oxygen species (ROS), mitochondrial injury, increased inflammation, intracellular calcium overload, myocardial necrosis, and apoptosis [4, 5]. Mitochondria play an important role in ROS generation, energy metabolism, and cell survival. Mitochondria produce more than 90% of adenosine triphosphate (ATP) in cardiomyocyte. Preservation of mitochondrial structural and functional integrity is critical for tolerance to myocardial I/R damage and improvement of cardiac function [6].

Uncoupling proteins (UCPs) are integral membrane proteins and belong to the subfamily of mitochondrial anion carriers. UCP3 is the product of adjacent genes located on human chromosome 11 related to

diabetes and obesity [7]. Previous study found that UCP3 plays a crucial role in protection against myocardial I/R damage [6]. Therefore, UCP3 could become a new therapeutic target for cardioprotection against I/R injury.

As a traditional Chinese medicine, ligustrazine is also known as 2,3,5,6-tetramethylpyrazine and can be used to remove blood stasis, activate blood circulation, expand blood vessels, and relieve pain [8]. Ligustrazine is extracted from the Umbelliferae Szechwan lovage rhizome, and its molecular formula is $C_8H_{12}N_2$ [9]. In China, it is widely used in patients with ischemic cardiovascular and cerebrovascular diseases [8, 10]. The physiological mechanism of ligustrazine may include improving energy metabolism and protecting mitochondria, reducing the inflammatory reaction, scavenging oxygen free radicals, mitigating cell damage, and inhibition of apoptosis [11–13]. In the present study, we assessed whether ligustrazine preconditioning plays myocardial protection against I/R injury, and, more importantly, explored the interaction between ligustrazine and UCP3 expression.

Materials And Methods

Animals

Adult Sprague-Dawley (SD) rats (260 ± 40 g) were from Animal Lab Center of Zhejiang Academy of Medical Sciences (SCXK 2019 – 0002, Zhejiang, China), and lived in a specific pathogen-free (SPF) lab. The rats were maintained under standard laboratory conditions and natural light-dark photoperiod. All animal operation rules and experiment procedure were permitted and approved by the animal ethics committee. SD rats were randomly divided into 4 groups, 8 in each group: sham operation (S) group; I/R group; I/R + ligustrazine preconditioning (Lig) group; I/R + ligustrazine preconditioning + mitochondrial permeability transition pore (mPTP) opener lonidamine (LND) (Lig + LND) group.

S group: SD rats underwent entire surgical procedures, but the left anterior descending coronary artery (LAD) was not ligated. I/R group: SD rats received saline intraperitoneally (i.p.) 5 min before ischemia and 10 min before reperfusion (10 mg/kg and 50 mg/kg saline) and underwent 30 min of ischemia followed by 2 h of reperfusion. Lig group: SD rats were treated with 10 mg/kg ligustrazine i.p. (5 min before ischemia), plus 50 mg/kg saline i.p. (10 min before reperfusion) and underwent 30 min of ischemia followed by reperfusion. Lig + LND group: SD rats were treated with 10 mg/kg ligustrazine i.p. (5 min before ischemia), plus 50 mg/kg LND i.p. (10 min before reperfusion) and underwent 30 min of ischemia followed by reperfusion.

Ischemia-reperfusion model

SD rats were anesthetized by 2% pentobarbital (i.p.), and were fixed in the supine position and connected to animal electrocardiogram (ECG). Heart rate, arrhythmias and ST-segment change were recorded by limb II of the ECG. After anesthesia, rats were intubated with endotracheal tubes and mechanically ventilated with oxygen-enriched room air. The rat heart was exposed from left side through incising the third and fourth ribs. Then, the pericardium was sheared gently and the LAD ligated with 5/0 silk suture.

Except the ligation of LAD was not conducted, the same surgical procedures were performed in S group rats. Both ST-segment elevation, R wave broadening on the ECG and a turning in the color of the cardiac apex to purple white could confirm cardiac ischemia. Reperfusion was achieved with the snare loosening for 120 min after occlusion of 30 min. Blood samples were collected from abdominal aorta after reperfusion, centrifuged at 3600 r/min (4 °C, 10 min) in a high-speed refrigerated centrifuge. Plasma samples were extracted and saved at -80 °C for uniform measurement.

Myocardial injury marker analysis

The plasma levels of, cardiac troponin I (cTnI), creatine kinase-muscle/brain (CK-MB), and lactate dehydrogenase (LDH) released were measurements of the degree of cardiac injury. cTnI, CK-MB, and LDH kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were adopted to measure the plasma levels.

Determination of SOD, MDA and ATP in myocardial tissues

After reperfusion, the left ventricular tissue was obtained and immediately stored at -80 °C. Heart tissues were homogenized with physiological saline at a 1:9 ratio (w/v, heart: saline) and centrifuged (3000 r/min, 15 min). The supernatant was applied to detect the activities of superoxide dismutase (SOD) and malondialdehyde (MDA) in myocardial tissues using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The ATP content was detected using an ATP Detection kit (Beyotime Biotech Inc, Shanghai, China). The specific operations were strictly followed by the instructions of the kits.

Measurement of UCP3 mRNA Expressions by RT-PCR

Total RNA was extracted using a TRIzol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) and converted into cDNA. Quantitative polymerase chain reaction (qPCR) was utilized to measure the mRNA levels of UCP3. The relative levels of UCP3 mRNA were standardized through β -actin gene as reference. The primers used for PCR were listed in Table 1.

Table 1
RT-PCR primer sequences.

Gene name	Primer sequence	Size (bp)
UCP3	F: 5'-3'GTGCTCGGTACCATCCTGACTA	163
	R: 5'-3'TGGAGTGGTCCGTTTCCTTTG	
β -actin	F: 5'-3'TGTGCCCATCATGAGGGTTAC	150
	R: 5'-3'ATGTCACGCACGATTTCCT	

Western blotting analysis

The UCP3 protein levels were analyzed by western blotting analysis. Frozen heart tissues were weighed and homogenized in the RIPA lysis buffer containing a cocktail of protease and phosphatase inhibitors using automatic tissue homogenizers. Homogenized samples were centrifuged at 12,000 g for 30 min to attain supernatant and recentrifuged to attain a clear lysate and all procedures were given out at 4 °C according to the producer's instructions. Protein extracts were separated by electrophoresis on SDS-PAGE and then moved onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skimmed milk blocking buffer at indoor temperature for 1 h and incubated with primary (anti-UCP3) antibody overnight (18 h) at 4 °C. Afterwards, the membranes were washed and incubated with the secondary antibody. The protein bands were visualized with ECL plus reagent. β -Actin proteins were adopted as loading and internal controls. The data are exhibited as the grayscale proportion of the object protein to β -actin.

Measurement of area at risk and infarction area

LAD was ligated again at the original location and 2 ml of 2% Evans blue dye (Sigma, USA) was injected into the aorta to map the normally perfused region of the heart after 120 min reperfusion. The normal myocytes were displayed as blue region, while the ischemic area was displayed as the non-blue region. The heart was rapidly excised and frozen (30 min, -20 °C) and then cut transversely into five slices. Each piece of rat heart was 2 mm thick. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) (Sigma, USA) in pH 7.4 buffer for 15 min at 37 °C to differentiate infarct area (IA) from ischemia zone, followed by fixation for 24 h in 10% formaldehyde. The IA cannot take up TTC stain and remain pale, while the viable tissue in ischemia area was identified as red staining by TTC. Morphometric measurements of the area at risk (AAR) and IA in each slice were analyzed with an image analysis software (Image-Pro plus 6.0). The proportion of AAR vs. left ventricle (LV) (AAR/LV) and IA vs. AAR (IA/AAR) were calculated.

Statistical analysis

All values are reported as means $\pm$ standard deviations (SD). Differences among experimental groups were determined by one-way analysis of variance (ANOVA), the LSD t test was performed for pairwise comparisons, using GraphPad Prism version 5 software. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of ligustrazine on infarct size

Myocardial ischemia was not found in the hearts of the S group. No difference in AAR/LV was found between I/R, Lig, and Lig + LND groups ($P > 0.05$). Compared to I/R group, the IA/AAR in Lig, and Lig + LND groups were significantly decreased ($P < 0.05$). Compared to Lig group, the IA/AAR in Lig + LND group was significantly increased ($P < 0.05$, Table 2, Fig. 1).

Table 2
The comparison of AAR/LV and IA/AAR in 3 groups.

	I/R	Lig	Lig + LND
AAR/LV, %	48.1 ± 5.4	49.5 ± 3.7	48.9 ± 5.1
IA/AAR, %	49.5 ± 4.6	34.0 ± 4.4*	40.2 ± 4.2*#
Note: * $P < 0.05$, vs. I/R group; # $P < 0.05$, vs. Lig group.			
AAR, area at risk; LV, left ventricle; IA, infarct area; I/R, ischemia/reperfusion; Lig, ligustrazine; LND, lonidamine.			

Changes of UCP3 levels of mRNA and protein in the myocardial tissue

The UCP3 levels of mRNA and protein were increased in I/R, Lig, and Lig + LND groups compare to those in S group ($P < 0.05$). Compared with I/R group, UCP3 mRNA and protein levels were increased in Lig, and Lig + LND groups ($P < 0.05$), whereas UCP3 mRNA and protein levels were similar between Lig, and Lig + LND groups ($P > 0.05$, Table 3, Fig. 2).

Table 3
UCP3 mRNA transcription levels and protein levels in 4 groups

	S	I/R	Lig	Lig + LND
UCP3 mRNA	1.03 ± 0.13	1.39 ± 0.25*	1.86 ± 0.32*#	1.66 ± 0.29*#
UCP3 protein	0.289 ± 0.081	0.401 ± 0.127*	0.553 ± 0.092*#	0.510 ± 0.081*#
Note: * $P < 0.05$, vs. S group; # $P < 0.05$, vs. I/R group.				
UCP3, uncoupling protein 3.				

Effect of ligustrazine on plasma levels of cTnI, CK-MB, and LDH

Compared with S group, plasma, cTnI, CK-MB, and LDH levels of I/R, Lig, and Lig + LND groups were significantly increased ($P < 0.05$). Compared with I/R group, plasma cTnI, LDH, and CK-MB levels of Lig group and plasma LDH, and CK-MB levels of Lig + LND group were significantly decreased ($P < 0.05$). plasma LDH, and CK-MB levels in Lig group were significantly lower than those in Lig + LND group ($P < 0.05$, Table 4).

Table 4
Plasma cTnl, LDH, and CK-MB levels in 4 groups

	S	I/R	Lig	Lig + LND
cTnl, ng/mL	1.0 ± 0.4	24.8 ± 12.0*	15.7 ± 4.4* [#]	20.0 ± 3.8*
LDH, U/L	375.8 ± 42.2	3944.5 ± 311.6*	3140.1 ± 416.4* [#]	3505.3 ± 410.1* ^{#a}
CK-MB, U/L	37.5 ± 8.8	133.6 ± 15.5*	92.3 ± 10.8* [#]	107.1 ± 9.9* ^{#a}
Note: * <i>P</i> < 0.05, vs. S group; # <i>P</i> < 0.05, vs. I/R group; a <i>P</i> < 0.05, vs. Lig group.				
cTnl, cardiac troponin I; LDH, lactate dehydrogenase; CK-MB, creatine kinase-muscle/brain.				

Effect of ligustrazine on SOD activity, MDA and ATP contents in myocardial tissues

Compared with S group, the activity of SOD in the myocardial tissue of the rats in I/R, Lig, and Lig + LND groups decreased significantly, the content of MDA increased significantly, and the content of ATP decreased significantly (*P* < 0.05). Compared with I/R group, the activity of SOD in Lig, and Lig + LND groups increased significantly, the content of MDA decreased significantly, and the content of ATP increased significantly (*P* < 0.05). The content of MDA in Lig group was significantly lower than that in Lig + LND group (*P* < 0.05), the content of ATP in Lig group was significantly higher than that in Lig + LND group (*P* < 0.05, Table 5).

Table 5
The activity of SOD, and contents of MDA, and ATP in 4 groups

	S	I/R	Lig	Lig + LND
SOD, U/mg prot	57.3 ± 7.7	27.6 ± 7.3*	48.1 ± 6.6* [#]	42.1 ± 6.0* [#]
MDA, nmol/mg prot	2.7 ± 0.7	6.2 ± 0.6*	3.9 ± 0.6* [#]	5.0 ± 0.4* ^{#a}
ATP, %	100.0 ± 0.0	36.2 ± 4.3*	64.8 ± 9.1* [#]	55.3 ± 6.9* ^{#a}
Note: * <i>P</i> < 0.05, vs. S group; # <i>P</i> < 0.05, vs. I/R group; a <i>P</i> < 0.05, vs. Lig group.				
SOD, superoxide dismutase; MDA, malondialdehyde; ATP, adenosine triphosphate.				

Discussion

This study was conducted with a view to investigate whether ligustrazine preconditioning protects the myocardial tissues in I/R injury rats via regulating UCP3 expression. Evidences show that ligustrazine pretreatment has involved in the protection against myocardial I/R injury of rat heart through reduced IA, myocardial injury markers, and MDA, and increased concentrations of SOD, and ATP. The effect of ligustrazine were reversed by the mPTP opener LND (IA/AAR 34.0 ± 4.4 vs. 40.2 ± 4.2). This study, in

particular, focuses on UCP3, which has been previously reported to be upregulated when myocardial I/R damage. Observations indicate that ligustrazine pretreatment could up-regulate the expression of UCP3 mRNA and protein, and the LND could not affect the expression of UCP3. This finding may contribute to the development of novel clinical strategies to protect myocardium from I/R injury.

In the experiments of myocardial I/R injury, IA is a vital indicator assessing the degree of myocardial damage. And the levels of plasma myocardial injury markers can effectively reflect acute myocardial damage. Huang et al. confirmed that pretreatment with *Salvia miltiorrhiza* and ligustrazine significantly decreased myocardial infarct size, creatine kinase, LDH and MDA levels. The effect of *Salvia miltiorrhiza* and ligustrazine may reduce I/R injury in cardiomyocytes and inhibit apoptosis in rats by activating the Akt-eNOS signaling pathway and down-regulating the expression levels of proapoptotic factors [14]. Experiment data suggest that ligustrazine preconditioning alone can also decrease IA, plasma cTnI, CK-MB, and LDH levels, which confirmed that ligustrazine preconditioning can effectively protect myocardial I/R injury. In addition, there is evidence showing that mPTP opening LND can inhibit the cardioprotective effect of ligustrazine preconditioning against I/R damage. These findings suggest that ligustrazine preconditioning may potentially have effect on attenuating myocardial I/R injury via the prevention of mPTP opening.

Due to the large energy requirements, mitochondria are highly enriched in myocardium and are particularly vital to the heart [15]. During I/R injury, the available oxygen of myocardial tissues is deficient, resulting in depletion of myocardial ATP, and the generation of large amounts of ROS exaggerates development of myocardial damage [16, 17]. Numerous researches have indicated that the myocardial oxidative stress indexes such as SOD and MDA were changed remarkably after I/R [18]. MDA is one of the key markers of oxidant-mediated lipid peroxidation that occurs as a result of the damaging effects of oxidative stress and antioxidant status. SOD is commonly considered as a cellular ROS scavenger and represents the degree of neutrophil infiltration [19, 20]. This study found that ligustrazine preconditioning upregulated the expressions of UCP3 mRNA and proteins, enhanced the activities of cardiac SOD and ATP, suppressed the content of MDA to protect mitochondrial function and reduce oxidative stress, while the presence of mPTP opening LND weakened the effect.

Ligustrazine, a traditional Chinese medicine extracted from the Umbelliferae *Szechwan lovage* rhizome, has been previously reported in significantly improvement of cardiac and cerebral blood flow by scavenging oxygen free radicals, protecting mitochondria function, improving energy metabolism, alleviating the inflammatory reaction, and inhibition myocardial apoptosis [11, 12, 21]. *Salvia miltiorrhiza* and ligustrazine may protect cardiomyocytes after I/R through the activation of the Akt serine/threonine kinase (Akt)-endothelial nitric oxide synthase (eNOS) signaling pathway and downregulation of the caspase-3 expression [14]. Chen et al. found that the effect of ligustrazine could increase the expression levels of heme oxygenase-1 to attenuate myocardial I/R injury in rats [22]. Ligustrazine also showed protective effects in ischemic brain injury and acute kidney injury after I/R. Ligustrazine plays protective roles via activating the PI3K/Akt pathway and inhibiting nucleotide-binding oligomerization domain-containing 2 (NOD2) mediated inflammation [23, 24]. Furthermore, Han et al. showed that ligustrazine

could suppress the increased striatal concentrations of neurotransmitters and neurological deficits induced by cerebral I/R injury in rats [25].

The development and evolution of myocardial I/R injury is a complicated process. The opening of the mPTP at the outset of reperfusion is a crucial determinant of myocardium following I/R damage [26, 27]. Pharmacological mPTP opening inhibition at the outset of reperfusion has been declared to reduce myocardial infarction size in rat models of I/R injury [28]. Previous study revealed that mitochondrial UCP3 plays an essential role in cardioprotection against I/R damage as a component of the cellular antioxidant defense program [29]. Safari et al. showed that the expression of UCP3 protein was markedly up-regulated in the ischemic myocardium area early after acute myocardial I/R, and it was also increased both in the ischemic area of the left ventricle and in non-ischemic area of the right ventricle [30]. In the experimental mice model by Ozcan et al., in contrast to wild-type (WT) mouse hearts under I/R conditions, UCP3 knockout (UCP3^{-/-}) mice were found to have poorer ability of recovery in left ventricular function, twofold larger infarcts, higher incidence of arrhythmias, lower ATP content, and more ROS. The cardioprotection of ischemic preconditioning (IPC) was abolished in UCP3^{-/-} mice. It can be observed that lacking of UCP3, myocardial vulnerability would be increased when undergoing I/R injury in hearts. The mechanisms of UCP3-mediated cardioprotection contain maintaining myocardial high-energy phosphates and suppressing detrimental ROS generation in the context of I/R injury [6]. In the present study, ligustrazine mitigated I/R injury, decreased infarction size, increased the levels of SOD, ATP, mRNA and protein expressions of UCP3, while mPTP opener LND reversed the effect. Therefore, ligustrazine may induce cardioprotective effects by preventing mPTP opening and upregulating expression of UCP3.

There are several limitations in the present study should be considered when interpreting results. First, only one dose of ligustrazine was adopted in this study, the study did not investigate effects of different doses of ligustrazine preconditioning on myocardial I/R. Second, the present study just focused on short-term outcomes and myocardial injury markers after I/R injury, there are no data involving long-term survival rate and heart function in rats. Further researches are needed to investigate the effects of different ligustrazine doses in long-term outcomes after myocardial I/R injury.

Conclusions

In conclusion, this study provides the first evidence that ligustrazine preconditioning can protect the myocardial tissues in I/R injury by decreasing infarct size, preventing mPTP opening, enhancing mitochondrial energy metabolism and alleviating oxidative stress through upregulating UCP3 expression. Further studies are required to confirm whether ligustrazine can be used in clinical practices.

Abbreviations

I/R

ischemia/reperfusion; mPTP:mitochondrial permeability transition pore; LND:lonidamine; IA:infarct area; MDA:malondialdehyde; SOD:superoxide dismutase; ATP:adenosine triphosphate; UCP3:uncoupling

protein 3; AAR:area at risk; cTnI:cardiac troponin I; CK-MB:creatine kinase-muscle/brain; LDH:lactate dehydrogenase; PCI:percutaneous coronary intervention; ROS:reactive oxygen species; SD:Sprague-Dawley; SPF:specific pathogen-free; LAD:left anterior descending coronary artery; ECG:electrocardiogram; qPCR:quantitative polymerase chain reaction; PVDF:polyvinylidene difluoride; TTC:triphenyltetrazolium chloride; LV:left ventricle; SD:standard deviations; ANOVA:one-way analysis of variance; NOD2:nucleotide-binding oligomerization domain-containing 2; IPC:ischemic preconditioning.

Declarations

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Authors' contributions

KF Zhu and SZ Wu contribute equally to this article. All authors contributed to the design and interpretation of the study and to further drafts.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Institutional Animal Care and Use Committee (IACUC-20040009) at Animal Lab Center of Zhejiang Academy of Medical Sciences.

Consent for publication

Not applicable.

Competing interests

All authors declared no competing interests.

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Figures

Figure 1

Effect of ligustrazine on infarct size during ischemia and reperfusion. (A) Representative photographs of myocardial sections stained by Evan’s blue dye and triphenyltetrazolium chloride. Blue area indicates normal myocytes, red area indicates area at risk (AAR), and white area indicates infarct area (IA). (B) The ratio of AAR to the left ventricular area (AAR/LV). (C) The ratio of IA to AAR (IA/AAR). Values are expressed as mean ± SD. *P < 0.05.

(A)

(B)

(C)

Figure 2

Effect of ligustrazine preconditioning on the expression of UCP3 mRNA and proteins. (A-B) Protein levels of UCP3 were determined by western blotting. (C) mRNA transcription levels of UCP3 were determined by RT-PCR. Values are expressed as mean $\pm$ SD. * $P < 0.05$.