Original Paper

Luteolin Enhances Sarcoplasmic Reticulum Ca^{2+} -ATPase Activity through p38 MAPK Signaling thus Improving Rat Cardiac Function after Ischemia/Reperfusion

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Key Words

Luteolin • Ischemia/reperfusion • Systolic/diastolic function • p38 MAPK pathway • SERCA2a

Abstract

Background/Aims: A major challenge for current therapeutic strategies against ischemia/reperfusion (I/R) is the lack of effective drugs. Considering luteolin enhances the activity of sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA2a) to improve the systolic/diastolic function of rat hearts and cardiomyocytes during the I/R process, we studied the regulatory function of the p38 MAPK pathway in this protective mechanism. **Methods:** Isolated cardiomyocytes and perfused hearts were separately divided into five groups and used to investigate I/R. The phosphorylation of p38 and phospholamban (p-PLB), the levels and activity of SERCA2a and the levels of proteins related to apoptosis were measured. Apoptotic cells were assessed using the TUNEL assay. Single-cell shortening, Ca^{2+} transients, and the decay of the mitochondrial membrane potential ($\Delta\psi\text{m}$) were detected. **Results:** The p38 MAPK pathway was activated during the I/R process, and inhibiting it with SB203580 promoted p-PLB, which enhanced the activity of SERCA2a and relieved the calcium overload to promote the recovery of the $\Delta\psi\text{m}$ and reduce cardiomyocyte apoptosis in I/R. Luteolin also suppressed the activation of the p38 MAPK pathway and showed cardioprotective effects during I/R injury. **Conclusions:** We conclude that luteolin enhances SERCA2a activity to improve systolic/diastolic function during I/R in rat hearts and cardiomyocytes by attenuating the inhibitive effects of the p38 pathway on p-PLB.

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Introduction

Ischemic heart disease has serious impacts on the health of humans worldwide and will continue to do so in the future [1]. The most efficient method of reducing mortality

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in patients suffering from myocardial ischemia is to achieve emergency reperfusion treatment by thrombolysis or opening the occluded vessels. Under these circumstances, the mortality from acute myocardial infarction is inversely related to myocardial salvage achieved by reperfusion [2]. However, considerable evidence gained from research from fundamental studies and clinical practice has demonstrated that reperfusion itself damages the myocardium, which is known as ischemia/reperfusion (I/R) injury. I/R injury includes reperfusion arrhythmia, myocardial systolic/diastolic dysfunction and no reflow, which is known to influence the effects of reperfusion therapy and is associated with higher mortality or worse prognosis. Therefore, in order to achieve the satisfactory therapeutic effects of myocardial ischemia, it is crucial to investigate the pathogenesis of I/R injury and search for new drugs.

Researchers discovered many mechanisms in I/R injury [3-6], calcium overload is the main cause of I/R injury [7-9], but concrete evidence of the mechanisms involved has not yet been obtained. Calcium overload has many causes that lead to an increasing concentration of intracellular Ca^{2+} , resulting in structural damage, functional or metabolic disorders, and sometimes cell apoptosis and death. The expression and activity of sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA2a) is the main factor involved in calcium handling, as its dysfunction impairs myocardial systolic/diastolic function [10]. Phospholamban (PLB), a phosphoprotein that is mainly expressed in ventricular myocytes, is tightly related to SERCA2a activity; thus, it indirectly regulates the sarcoplasmic reticulum (SR) absorption of calcium during diastole [11, 12]. In its dephosphorylated state, PLB inhibits SERCA2a function, leading to reduced Ca^{2+} re-uptake into the SR and cardiac contractility. By contrast, the phosphorylated form eliminates this inhibition.

The mitogen-activated protein kinase (MAPK) family includes extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38-MAPKs [13]. Many studies have reported that JNK and ERK participate in myocardial I/R injury and pathological remodeling. In our previous studies, we also found that the JNK and ERK1/2 pathways were important in regulating cardiomyocyte contractility and decreasing apoptosis. The ERK1/2-PP1a pathway regulates the expression of phosphorylated PLB and SERCA2a [14]. The p38 MAPK pathway is another important pathway of the MAPK family, and p38 MAPK pathway inhibition could alleviate cardiomyocyte I/R injury and develop cardio-protective effects [15, 16]. In a recent randomized phase 2 trial in non-ST-segment elevation myocardial infarction, Newby et al. observed that the use of a novel p38 mitogen-activated protein kinase inhibitor, losmapimod, was well tolerated in non-ST-segment elevation myocardial infarction patients and might improve outcomes after acute coronary syndromes [17]. Kaikkonen et al. reported that negative p38 α enhances SERCA2a function to improve cardiomyocyte contractility [18]. However, the exact mechanisms of the p38 MAPK pathway interacting with SERCA2a need to be clarified.

Luteolin (3',4',5',7'-tetrahydroxyflavone, Lut) is a natural, soluble flavone that is found in high levels in many plants, such as vegetables, fruits and herbs, and it exerts anti-tumor, anti-inflammatory and anti-apoptotic activities [19-21]. Our preliminary experiments found that luteolin suppressed p38 MAPK activation when a pretreatment was administered before I/R. Due to the lack of effective solutions or drugs in clinical practice to alleviate I/R injury, we need to elucidate the relationship between the p38 MAPK pathway and SERCA2a, as well as the possible regulatory mechanism of luteolin pretreatment in the process of myocardial I/R.

The aim of this study was to use luteolin and a p38 mitogen-activated protein kinase inhibitor (SB203580) to determine whether SERCA2a is involved in and affects the negative cardiac function induced by p38 MAPK during myocardial I/R injury. The results of this study may lead to potential therapeutic targets for the prevention and treatment of myocardial I/R injury. Thus, we may clarify the mechanism underlying the cardio-protection of luteolin, which could be an effective drug for clinical application.

Materials and Methods

Animals and reagents

The experimental procedures on the animals were approved by the Xuzhou Medical University Animal Ethics Committee (permit number CMCACUC 2011-04-167). The animals were kept in a room with a controlled ambient temperature ($22 \pm 2^\circ\text{C}$) and humidity ($50\% \pm 10\%$), with food and water ad libitum. All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of Xuzhou Medical University. Thirty clean-grade adult male Wistar rats (220–250 g, Animal Breeding Center of Xuzhou Medical University) were randomly assigned to the following groups (6 rats per group): 1. DMSO (DMSO); 2. I/R (I/R); 3. luteolin+I/R (Lut+I/R); 4. SB203580+I/R (SB+I/R); and 5. SB203580+luteolin+I/R (SB+Lut+I/R). DMSO was used to dissolve luteolin (purity>98%, Fluka, Milwaukee, WI, USA) and SB203580 (Cell Signaling Technology, Beverly, MA, USA) to a final concentration of <0.01% diluted with perfusate or culture medium, which had no effect on the heart [22]. All groups received DMSO.

Isolation of the cardiomyocytes

Single ventricular myocytes were obtained from the isolated rat hearts using type II collagenase (Gibco, Grand Island, NY, USA) as previously described [14, 23–25]. The cells were suspended 3 times with Krebs-bicarbonate solution (pH 7.2) containing (mM) 85 KCl, 15 NaCl, 5 MgSO_4 , 30 KH_2PO_4 , 5 sodium pyruvate, 5 creatine, 20 taurine, 2 L-glutamic acid, 20 HEPES, 20 glucose, 0.2 CaCl_2 and 0.5 EGTA, and was bubbled with 100% O_2 at 37°C . More than 80% of the viable cardiac myocytes were quiescent after resuspension. The isolated cardiomyocytes were cultured subsequently in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% penicillin-streptomycin at a density of 2×10^4 cell in 12-well culture plates that were placed in an incubator (Heraeus, Hanau, Hesse-Darmstadt, Germany) under 95% O_2 and 5% CO_2 at 37°C .

Simulated I/R protocol and drug treatment

After the cardiac myocytes were equilibrated for 1 h, they were treated as follows: DMSO, the myocytes were incubated with DMSO alone for 18 h; I/R, the myocytes were treated with DMSO for 13 h, and then they were subjected to simulated ischemia for 3 h by substituting the cell medium for an 'ischemic buffer' containing (mM): 24 NaHCO_3 , 118 NaCl, 1.0 NaH_2PO_4 , 2.5 CaCl_2 , 2 H_2O , 20 sodium lactate, 1.2 MgCl_2 , 16 KCl and 102 deoxyglucose (pH 6.2) as described previously [26]. Next, the cells were incubated in a tri-gas incubator that was adjusted to 1% O_2 and 5% CO_2 at 37°C . After the simulated ischemia, the ischemic buffer was replaced by normal medium under normoxic conditions, and the cells were cultured for 2 h to simulate reperfusion. In the Lut+I/R group, before the simulated I/R, the myocytes were pretreated with luteolin (8 μM) for 12 h; in the SB+I/R group, the myocytes were treated with SB203580 (10 μM) for 1 h before the simulated I/R; in the SB + Lut + I/R group, the myocytes were pretreated with SB203580 for 1 h before the luteolin treatment, the same as Lut + I/R.

Isolated perfused heart preparation

Global I/R of the rat hearts was performed *ex vivo* according to our previous procedure [14]. Sodium heparin (1000 U/kg) and sodium pentobarbital (150 mg/kg) was administered to the experiment animals, then the rat hearts were excised and perfused, by Langendorff, with Krebs-Henseleit (KH) buffer solution containing (mM) 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4 , 1.2 MgSO_4 , 25 NaHCO_3 , 11 glucose and 1.25 CaCl_2 , which was bubbled with 95% O_2 and 5% CO_2 at 37°C to maintain a pH of 7.4. The perfusion pressure was fixed at 80 mmHg.

After the hearts were equilibrated, the different groups were treated as follows: DMSO, the hearts were perfused with DMSO and KH buffer for 3.5 h; I/R, the isolated hearts were perfused with KH buffer for 1 h and then subjected to 30 min without the KH solution to simulate ischemia, which was followed by reperfusion with KH solution for 2 h; Lut + I/R, the isolated hearts were perfused with the KH buffer for 30 min before pretreatment with luteolin (40 μM) for 30 min, and then the I/R procedure was applied; SB + I/R, SB203580 (10 μM) was administered to the isolated hearts for 30 min, which had been treated with KH buffer for 30 min, followed by the I/R procedure; and SB + Lut + I/R, the hearts were administered SB203580 (10 μM) for 30 min before treatment with luteolin and then were treated as the Lut + I/R group.

Assessment of apoptosis

Apoptotic cells were assessed using a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit (Roche, Basel, Kanton Basel-Stadt, Switzerland). After reperfusion, the isolated rat hearts were immersed in 4% paraformaldehyde overnight before being paraffin-embedded for sectioning. At least 3 slices and >10 fields were observed randomly, with each viewed field containing >50 cells from each group, by light microscopy (20× objective). Moreover, the nuclei of the cardiac myocytes were stained by 4', 6-diamidino-2-phenylindole (DAPI). Only the nuclei of the apoptotic cells were stained brown by the TUNEL method, whereas all of the nuclei were stained blue with DAPI. The percentage of apoptotic cardiac myocytes was the TUNEL-positive cardiomyocytes / the total number of counted cells.

Measurement of cell shortening and re-lengthening

Only distinct edges of rod-shaped cardiomyocytes were used to evaluate contractile function. The mechanical properties of single cardiomyocytes were examined using the SoftEdge MyoCam® system (IonOptix, Milton, MA, USA) [27]. Briefly, under an inverted microscope (40× objective; IonOptix, Milton, MA, USA), the cardiac myocytes were stimulated with an electrical field with a frequency of 0.5 Hz. At least 30 cardiomyocytes in each group were detected. A single myocyte was shown on the monitor of a computer using an IonOptix MyoCam camera. The IonOptix SoftEdge software was used to image cell shortening and re-lengthening. The following parameters were recorded to measure these changes: the resting cell length, the peak shortening amplitude (PS), the time-to-peak shortening (TPS), the time-to-90% re-lengthening (TR90), and the maximum velocity of shortening and re-lengthening ($\pm$ dL/dt). The contraction of the myocyte had to be stable for at least 5 min before the above indices were recorded.

Intracellular Ca^{2+} transient measurements

The intracellular Ca^{2+} changes reflect the Ca-handling. Intracellular calcium was detected using a dual-excitation fluorescence photomultiplier system (IonOptix, Milton, MA, USA) [28]. Briefly, the myocytes were loaded with a Fura-2/AM (0.5 μM) solution for 10 min, and the fluorescence signal of Fura-2 was detected using an IonOptix fluorescence system (IonOptix, Milton, MA, USA). After subtracting the background fluorescence, the 340 nm and 380 nm ratio was analyzed offline using the SoftEdge MyoCam® system (IonOptix, Milton, MA, USA). The fluorescence emission was detected from 480 to 520 nm, and the quantitative change in Fura-2 fluorescence intensity (FFI) was obtained from the FFI ratio at the 2 wavelengths. Regarding the baseline FFI, the ΔFFI (340/380) reflected the resting intracellular Ca^{2+} level, the electrical stimulation-increased intracellular Ca^{2+} levels and the intracellular Ca^{2+} transient attenuation rate. The single exponential Tau was presented as an indicator of intracellular Ca^{2+} clearance.

Detection of the decay of the mitochondrial membrane potential ($\Delta\psi\text{m}$)

Cardiac mitochondria were isolated from rats using the method previously described by Palmer et al. [29] Briefly, mitochondria were isolated by differential centrifugation from heart homogenates. Proteolytic treatment of the homogenate with Nagarse (3 mg/g tissue) was used to release the mitochondria from myofibrils. All steps of the isolation were performed on ice or at 4°C.

The differences of $\Delta\psi\text{m}$ were detected by JC-1 staining (Kaiji Bio, Nanjing, Jiangsu, China), which is a sensitive fluorescent probe and a cationic lipid fluorescent dye according to its dual emission characteristics. JC-1 spontaneously forms complexes (JC-1 aggregation) at high $\Delta\psi\text{m}$ in normal cells and emits an intense red fluorescence. However, JC-1 remains in the monomeric form at low $\Delta\psi\text{m}$ in apoptotic cells and emits only green fluorescence. A prepared mitochondria suspension extracted from fresh myocardial tissues was incubated in JC-1 solution (5 $\mu\text{g}/\text{ml}$) for 20 min at 37°C before being rinsed twice with JC-1 buffer. The ratio of the red and green fluorescence was calculated to give a $\Delta\psi\text{m}$ value. The percentage of cells with low mitochondrial $\Delta\psi\text{m}$ was measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). The red fluorescence intensity was detected in the FL-1 channel and the green in the FL-2 channel, with the Q3 region enclosing the low mitochondrial $\Delta\psi\text{m}$ population.

Measurement of SERCA2a activity

SERCA vesicles were obtained as previously described for the measurement of its activity [30], which was detected using an inorganic phosphorus colorimetric method with an Ultramicro-ATPase assay kit (Jiancheng, Nanjing, Jiangsu, China). Briefly, this test includes an enzymatic and phosphorus reaction based

on the theory that ATP is decomposed into ADP and Pi by ATPase. The activity of the ATPase was measured by the quantity of Pi, and the amount of Pi decomposed by ATPase per mg of tissue protein per h ($\mu\text{mol Pi/mg prot/h}$) was regarded as one unit of ATPase activity.

Western blot analysis

After the simulated I/R process, the cardiomyocytes were washed in ice-cold PBS and the total protein extracts were obtained by lysis in RIPA buffer supplemented with protease inhibitors. After 10 min, the insoluble material was discarded by centrifugation at $14,000 \times g$ for 10 min at 4°C , and the protein concentration was detected by a Bradford assay (Bio-Rad, Hercules, CA, USA). The total protein ($20 \mu\text{g}$) was separated by 8-12% SDS-PAGE, followed by electrophoretic transfer to PVDF membranes. The blocking of non-specific binding was performed using 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) by incubating the membranes for 2-3 h at room temperature. The membranes were incubated with primary antibodies targeting p38 MAPK, p-p38 MAPK, PLB, p-PLB (Cell Signaling Technology, Beverly, MA, USA), SERCA2a (Abcam, Cambridge, Cambridgeshire, England), Bcl-2, Bax, cleaved caspase-3 (Santa Cruz Biotech, Santa Cruz, CA, USA), caspase-3 (CST, Beverly, MA, USA) or β -actin (Zhongshan, Zhongshan, Guangdong, China) overnight at 4°C . The blots were washed three times in TBST and were incubated with the corresponding secondary antibody (Zhongshan, Zhongshan, Guangdong, China) for 1 h at room temperature. The bands were detected using chemiluminescence luminol reagents (Santa Cruz Biotech, Santa Cruz, CA, USA). The films were scanned as images, and the grey value of the bands was measured using Image J 3.0 (National Institutes of Health, Bethesda, MD, USA). The optical density of the bands in the control group was taken as one arbitrary densitometry unit.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 5.0, and the data are shown as the mean $\pm$ SEM. Significant differences were calculated by one- or two-way ANOVA, followed by a Bonferroni post hoc correction between all group comparisons. $P < 0.05$ was considered statistically significant.

Results

Effect of luteolin on the expression of p-p38 MAPK, p-PLB and SERCA2a protein expression and SERCA2a activity after I/R

Western blotting indicated that, when compared with the DMSO group, the p38 MAPK pathway was markedly activated in the I/R group ($P < 0.001$), while p-PLB and SERCA2a protein expression was decreased ($P < 0.001$). By contrast, the activation of the p38 MAPK pathway was suppressed in the SB + I/R, Lut + I/R and SB + Lut + I/R groups in comparison with the I/R group ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). However, p-PLB and SERCA2a protein expression was partially reversed in the SB+I/R group ($P < 0.05$) and in the Lut + I/R and SB + Lut + I/R groups (both $P < 0.01$). There was no significant difference between the SB + I/R and SB + Lut + I/R groups ($P > 0.05$; Fig. 1a, b, c).

After the simulated I/R process, the activity of SERCA2a markedly decreased compared with the DMSO group ($1.93 \pm 0.12 \mu\text{mol Pi/mg/h}$ vs $6.90 \pm 0.26 \mu\text{mol Pi/mg/h}$, $P < 0.01$). The SB+I/R group showed increased activity ($3.43 \pm 0.27 \mu\text{mol Pi/mg/h}$ vs $1.93 \pm 0.12 \mu\text{mol Pi/mg/h}$, $P < 0.05$), and the activity of the Lut + I/R and SB + Lut + I/R groups was significantly increased compared with the DMSO group (4.87 ± 0.27 and $5.47 \pm 0.29 \mu\text{mol Pi/mg/h}$ vs $1.93 \pm 0.12 \mu\text{mol Pi/mg/h}$, all $P < 0.01$). The SB + Lut + I/R group had a higher SERCA2a activity than the Lut + I/R group, but this difference was not significant (Fig. 1d).

Effects of luteolin on I/R-induced apoptosis

Compared with the DMSO group, I/R clearly accelerated the apoptosis of cardiomyocytes ($25.63 \pm 0.59\%$ vs $8.57 \pm 0.38\%$, $P < 0.01$). The SB+I/R group partially decreased apoptosis ($20.17 \pm 0.46\%$ vs $25.63 \pm 0.59\%$, $P < 0.05$) compared with the I/R group. There was no difference between the effect in the Lut+I/R and SB+Lut+I/R groups ($14.37 \pm 0.41\%$ vs $12.73 \pm 0.44\%$, $P > 0.05$) on I/R-induced apoptosis (Fig. 2a, b, c).

Fig. 1. Effects of different treatments on p-p38 MAPK, p-PLB and SERCA2a protein levels were detected by western blot analysis and SERCA2a activity in isolated I/R cardiomyocytes. (a) The expression of p-p38 MAPK following I/R and the effects of luteolin on the activation of p38 MAPK. (b) Representative images of the immunoreactivity and the quantitative analysis of the protein levels of p-PLB by western blotting. (c) Images of immunoreactivity and the quantitative analysis of the protein levels of SERCA2a by western blotting. (d) Effects of the various treatments on the activity of SERCA2a. The results are expressed as the mean $\pm$ SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus DMSO; # $P < 0.05$, ## $P < 0.01$ versus I/R; % $P < 0.05$ versus Lut (40 μ M) + I/R; \$ $P < 0.05$, \$\$ $P < 0.01$ versus SB (10 μ M) + I/R.

Fig. 2. Effects of different treatments on apoptosis, detected by TUNEL and DAPI staining, in isolated I/R myocardium. After a 2 h reperfusion, the heart tissue was sectioned for analysis of the anti-apoptotic effects of SB203580 and luteolin by TUNEL staining. The cells were examined by microscopy (200 $\times$ magnification). (a) TUNEL staining. (b) DAPI staining. Only the nuclei of the apoptotic cardiomyocytes were stained brown by the TUNEL method (as indicated by the arrow), whereas all nuclei were stained blue with DAPI. (c) Quantitative analysis of the TUNEL-positive cells from the different groups. The results are expressed as the mean $\pm$ SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$ versus DMSO; # $P < 0.05$ versus I/R; % $P < 0.05$ versus Lut (40 μ M) + I/R; \$ $P < 0.05$ versus SB (10 μ M) + I/R.

Effect of luteolin on I/R-induced changes in apoptotic protein expression

After the I/R process, the levels of Bax and cleaved caspase-3 were increased compared with the DMSO group ($P < 0.001$), and the levels of Bcl-2 and caspase-3 were decreased, which were associated with contraction ($P < 0.001$). However, the expression of these proteins, which was induced by the I/R injury, was partially reversed in the SB+I/R group

Fig. 3. Effects of different treatments on the expression of Bax, Bcl-2, and cleaved caspase-3 in the isolated I/R cardiomyocytes. (a) Images of the immunoreactivity and the quantitative analyses of the protein levels of Bax by western blotting. (b) Images of immunoreactivity and the quantitative analyses of the protein levels of Bcl-2 by western blotting. (c) Images of immunoreactivity and the quantitative analyses of the protein levels of caspase-3 and cleaved caspase-3. The results are expressed as the mean $\pm$ SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus DMSO; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus I/R; % $P < 0.05$, %% $P < 0.01$ versus Lut (8 μ M) + I/R; \$ $P < 0.05$, versus SB (5 μ M) + I/R.

($P < 0.05$) and the Lut + I/R and SB + Lut + I/R groups (both $P < 0.01$). However, no significant difference was found between the Lut + I/R and SB + Lut + I/R groups (Fig. 3a, b, c).

Effect of luteolin on shortening/re-lengthening and the intracellular calcium transient of cardiomyocytes subjected to I/R

After the simulated I/R, the shortening/re-lengthening (Fig. 4a) and the intracellular calcium transients (Fig. 4b) of the cardiomyocytes were measured. There was no significant difference among all groups in resting cell length, TPS and baseline FFI ($P > 0.05$). I/R markedly decreased the peak shortening ($P < 0.001$), $\pm$ dl/dt ($P < 0.001$) and the Δ FFI ($P < 0.001$) and prolonged the TR90 ($P < 0.001$) and the Tau ($P < 0.001$) compared with the DMSO group. In comparison with the I/R group, pretreatment with SB203580 alone reversed the above indices ($P < 0.05$), as did pretreatment with luteolin alone and pretreatment with SB203580 plus luteolin ($P < 0.01$). The pretreatment with SB203580 plus luteolin seemed to be better than the pretreatment with luteolin alone, but this difference was not significant ($P > 0.05$).

Influence of luteolin on $\Delta\Psi_m$ of the different groups

After the simulated I/R process, $\Delta\Psi_m$ clearly decreased ($7.58 \pm 0.55\%$ vs $30.83 \pm 0.73\%$, $P < 0.01$). The SB + I/R group exhibited increased $\Delta\Psi_m$ compared with the DMSO group ($21.63 \pm 0.76\%$ vs $30.83 \pm 0.73\%$, $P < 0.05$). The SB + Lut + I/R group had a higher $\Delta\Psi_m$ than the Lut+I/R group, but this difference was not significant ($14.00 \pm 0.70\%$ vs $16.43 \pm 0.72\%$, $P > 0.05$) (Fig. 5).

Fig. 4 (a) Effects of different treatments on the systolic/diastolic indices in isolated I/R cardiomyocytes. 1. Resting cell length; 2. Peak shortening; 3. TPS; 4. +dL/dt; 5. -dL/dt; 6. TR90. (b) Effects of the groups on the calcium transient in the isolated I/R cardiomyocytes. 1. Resting intracellular [Ca²⁺]; 2. Δ intracellular [Ca²⁺] in response to electrical stimuli; 3. Tau. The results are expressed as the mean ± SEM, n = 30. *P < 0.05, **P < 0.01, ***P < 0.001 versus DMSO; #P < 0.05, ##P < 0.01 versus I/R; %P < 0.05 versus Lut (8 μM) + I/R; \$P < 0.05, \$\$P < 0.01 versus SB (5 μM) + I/R.

Fig. 5. Effects of different treatments on ΔΨm on isolated I/R cardiomyocytes. The red fluorescence intensity was detected by the FL-1 channel and the green by the FL-2 channel, with the Q3 region containing the low ΔΨm mitochondria population. The results are expressed as the mean ± SEM, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001 versus DMSO; #P < 0.05, ##P < 0.01 versus I/R; %P < 0.05 versus Lut (40 μM) + I/R; \$P < 0.05, versus SB (10 μM) + I/R.

Fig. 6. Lut enhances SERCA2a activity and inhibits apoptosis through p38 MAPK signaling. Lut, like the p38-activated protein kinase inhibitor SB203580, suppresses the activation of p38 MAPK after I/R, which increases p-PLB and enhances the activity of SERCA2a, resulting in relief of the calcium overload and a strengthening of the calcium transient. Mitochondrial membrane potential and functional recovery after p38 MAPK pathway inhibition by Lut reduce the apoptosis of the cardiomyocytes.

Discussion

The mechanism of the p38 MAPK pathway on SERCA2a after I/R

Myocardial I/R injury is a common and complex clinical phenomenon for which calcium overload is not only one of the mechanisms but also the result of a pathological process in I/R injury that causes cardiomyocyte death. As a critical SR protein regulating calcium release, storage and reuptake by the cardiomyocytes, SERCA2a is vital for maintaining intracellular calcium at a steady state to regulate cardiac systolic/diastolic function [31]. Increasing evidence shows that the expression and activity of SERCA2a decreases markedly after I/R, although the mechanism is unclear. The I/R process induces the dephosphorylation of PLB, which inhibits SERCA2a activity and causes an imbalance between SR Ca^{2+} uptake and release, promoting mitochondrial calcium overload and leading to a considerable loss of cardiomyocytes.

In the initial part of this experiment, we found that the p38 MAPK pathway was markedly activated during I/R, which increased the expression of p-p38 MAPK; pretreatment with Lut before I/R suppressed the activation of the p38 MAPK pathway, which decreased p-p38 MAPK expression. These results showed that the cardioprotective effects of Lut were closely associated with the p38 MAPK pathway. The same result was found by Yu et al.; their study showed that I/R injury significantly enhanced p-p38 MAPK, p-ERK1/2 and p-JNK expression levels, and Lut pretreatment (10, 40, or 70 mg/kg) in rats significantly inhibited p-p38 MAPK, which was dependent on the concentration of Lut. [32]

Our data provide crucial evidence of the mechanism of p38 MAPK on SERCA2a activity after I/R. The p38 MAPK pathway was obviously activated after I/R and induced a diastolic intracellular calcium increase, but it did not change the systolic intracellular calcium, which resulted in diastolic calcium overload accompanied by a decrease in p-PLB, SERCA2a levels, SERCA2a activity and cardiomyocyte contractility. However, pretreatment with SB203580 before I/R suppressed the activation of the p38 MAPK pathway, and these I/R-induced changes were partially reversed, from which we inferred that the p38 MAPK pathway participates in the process of I/R. Inhibiting the activation of p38 MAPK increased the phosphorylation of PLB and the natural protein of SERCA2a, leading to a loss of the inhibition of SERCA2a and an enhancement of SERCA2a activity, which increased the release and reuptake of SR calcium to alleviate the calcium overload, thereby improving the myocardial excitation-contraction coupling. Thus, PLB is the main target of the p38MAPK pathway to regulate SERCA2a activity.

The cardio-protection of Lut is produced by regulating the p38 MAPK pathway and then interacting on SERCA2a

The protective effects of Lut on cardiovascular disease have been widely studied. Hertog et al. [33] found that routine consumption of flavonoids such as Lut reduces the incidence

of myocardial infarction. Lut is cardioprotective against I/R injury, which is mediated by the PI3K/Akt, JNK, ERK and caspase pathways [34]. In previous experiments, we found that Lut had anti-apoptotic effects and improved myocardial contractile function after I/R through the PI3K/Akt, ERK1/2 and JNK pathways and that SERCA2a protein expression was significantly reduced in a rat myocardial I/R model but rebounded significantly after Lut pretreatment [23-24]. However, the effect of Lut on the I/R cardiomyocyte calcium transient and the mechanism of its regulation of SERCA2a need elucidation. Our recent work demonstrated that Lut presented a similar function as SB203580, which, when administered before I/R, downregulated p-p38 MAPK and reversed the negative inotropic effect and the negative chronotropic calcium transient of I/R. PLB is the key protein regulated by Lut, which enhances the activity of SERCA2a and accelerates calcium recycling and release from the SR. This, in turn, lowers the diastolic calcium concentration and relieves the calcium overload to encourage excitation-contraction coupling in the myocardium. These effects improve the systolic/diastolic function in the I/R rat myocardium.

An excess of calcium accumulating in the mitochondria causes an overload, which damages the respiratory chain, decreases ATP synthesis, increases the release of ROS, and promotes the formation of the mitochondrial permeability transition pore, resulting in mitochondrial dysfunction, cell apoptosis and finally death [35]. Our results confirm that the destruction of the mitochondrial transmembrane potential and the increased apoptosis and necrosis of cardiomyocytes during I/R can be prevented or reduced by SB203580 or Lut, which was mediated by suppressing the activation of the p38 MAPK pathway.

In conclusion, from the above results, an activated p38 MAPK pathway after I/R is involved in regulating SERCA2a activity and the calcium transients, resulting in not only poor cardiac systolic/diastolic function but also cardiomyocyte apoptosis and necrosis. The cardioprotective effect of Lut against I/R injury is closely associated with the p38 MAPK pathway, which operates in two ways (Fig. 6). First, like the p38-activated protein kinase inhibitor SB203580, Lut suppresses the activation of p38 MAPK after I/R, which increases p-PLB and enhances the activity of SERCA2a, resulting in relief of the calcium overload and a strengthening of the calcium transient, thereby promoting myocardial excitation-contraction coupling and showing positive inotropic and positive chronotropic actions. Second, Lut promotes mitochondrial membrane potential and functional recovery, reducing the apoptosis and necrosis of the cardiomyocytes. These two aspects directly or indirectly improve the cardiac systolic/diastolic function of I/R hearts. Similar to the function of p38-activated protein kinase inhibitor, Lut may have a potential value in the treatment of ischemic heart disease.

Further, this study and our previous studies demonstrate that the protective effect of Lut arises from regulating the PI3K/Akt and MAPK pathways; these pathways increase p-PLB expression, which relieves the inhibition of SERCA2a. Therefore, it is possible for Lut to target PLB through different pathways to protect the myocardium against I/R injury by enhancing SERCA2a activity, which would thereby improve the prognosis and long-term survival of patients suffering from I/R injury if these effects can be translated to humans.

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Disclosure Statement

The authors declare that they have no competing interests

References

- Moran AE, Forouzanfar MH, Roth GA, Mensah GA, Ezzati M, Murray CJ, Naghavi M: Temporal trends in ischemic heart disease mortality in 21 world regions, 1980 to 2010: the Global Burden of Disease 2010 study. *Circulation* 2014;129:1483-1492.
- Cabades A, Valencia J, Rueda J, Echanove I, Sanjuan R, Cebrian J, Gonzalez-Hernandez E, Cardona J, Colomina F, Frances M, Ortolá V, Sogorb F: Evolution in the management of acute myocardial infarction in the autonomous community of valencia (Spain): ten years of the primvac registry (1995-2004). *Int J Biomed Sci* 2010;6:87-95.
- Luo F, Shi J, Shi Q, Xu X, Xia Y, He X: Mitogen-Activated Protein Kinases and Hypoxic/Ischemic Nephropathy. *Cell Physiol Biochem* 2016;39:1051-1067.
- Yang D, Zhang P, Wang T, Gao L, Qiao Z, Liang Y, Yu B: SalA attenuates ischemia/reperfusion-induced endothelial barrier dysfunction via down-regulation of VLDL receptor expression. *Cell Physiol Biochem* 2014;33:747-757.
- Xu Z, Yu J, Wu J, Qi F, Wang H, Wang Z, Wang Z: The Effects of Two Anesthetics, Propofol and Sevoflurane, on Liver Ischemia/Reperfusion Injury. *Cell Physiol Biochem* 2016;38:1631-1642.
- Chen Z, Zhang X, Liu Y, Liu Z: Morphine Postconditioning Protects against Reperfusion Injury via Inhibiting JNK/p38 MAPK and Mitochondrial Permeability Transition Pores Signaling Pathways. *Cell Physiol Biochem* 2016;39: 61-70.
- Talukder MA, Zweier JL, Periasamy M: Targeting calcium transport in ischaemic heart disease. *Cardiovasc Res* 2009;84:345-352.
- Fauconnier J, Roberge S, Saint N, Lacampagne A: Type 2 ryanodine receptor: a novel therapeutic target in myocardial ischemia/reperfusion. *Pharmacol Ther* 2013;138:323-332.
- Mozaffari MS, Liu JY, Abebe W, Baban B: Mechanisms of load dependency of myocardial ischemia reperfusion injury. *Am J Cardiovasc Dis* 2013;3:180-196.
- Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R: Calcium and apoptosis: ER-mitochondria Ca^{2+} transfer in the control of apoptosis. *Oncogene* 2008;27:6407-6418.
- Eisner D, Bode E, Venetucci L, Trafford A: Calcium flux balance in the heart. *J Mol Cell Cardiol* 2013;58:110-117.
- Bers DM: Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol* 2008;70:23-49.
- Wada T, Penninger JM: Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 2004;23:2838-2849.
- Wu X, Xu T D, Li D Y, Zhu SS, Chen QP, Hu WJ, Pan DF, Zhu H, Sun H: ERK/PP1a/PLB/SERCA2a and JNK Pathways Are Involved in Luteolin-Mediated Protection of Rat Hearts and Cardiomyocytes following Ischemia/Reperfusion. *Plos One* 2013;8:e82957.
- Bogoyevitch MA, GillespieBrown J, Ketterman AJ, Fuller SJ, BenLevy R, Ashworth A, Marshall CJ, Sugden PH: Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart - p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ Res* 1996;79:162-173.
- Kumphune S, Chattipakorn S, Chattipakorn N: Role of p38 inhibition in cardiac ischemia/reperfusion injury. *European Journal of Clinical Pharmacology* 2012;68:513-524.
- Newby LK, Marber MS, Melloni C, Sarov-Blat L, Aberle LH, Aylward PE, Cai G, de Winter RJ, Hamm CW, Heitner JF, Kim R, Lerman A, Patel MR, Tanguay JF, Lepore JJ, Al-Khalidi HR, Sprecher DL, Granger CB: Losmapimod, a novel p38 mitogen-activated protein kinase inhibitor, in non-ST-segment elevation myocardial infarction: a randomised phase 2 trial. *Lancet* 2014;384:1187-1195.
- Kaikkonen L, Magga J, Ronkainen VP, Koivisto E, Perjes A, Chuprun JK, Vinge LE, Kilpio T, Aro J, Ulvila J, Alakoski T, Bibb JA, Szokodi I, Koch WJ, Ruskoaho H, Kerkela R: p38 alpha regulates SERCA2a function. *J Mol Cell Cardiol* 2014;67:86-93.
- Ko WC, Shih CM, Lai YH, Chen JH, Huang HL: Inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure-activity relationships. *Biochem Pharmacol* 2004;68:2087-2094.
- Sun W, Zhang HD, Mao L, Mao CF, Chen W, Cui M, Ma R, Cao HX, Jing CW, Wang Z, Wu JZ, Tang JH: Luteolin Inhibits Breast Cancer Development and Progression In Vitro and In Vivo by Suppressing Notch Signaling and Regulating MiRNAs. *Cell Physiol Biochem* 2015;37:1693-1711.

- 21 Zhang BC, Zhang CW, Wang C, Pan DF, Xu TD, Li DY: Luteolin Attenuates Foam Cell Formation and Apoptosis in Ox-LDL-Stimulated Macrophages by Enhancing Autophagy. *Cell Physiol Biochem* 2016;39:2065-2076.
- 22 Wu Q, Zhao Z, Sun H, Hao YL, Yan CD, Gu SL: Oestrogen changed cardiomyocyte contraction and beta-adrenoceptor expression in rat hearts subjected to ischaemia-reperfusion. *Exp Physiol* 2008;93:1034-1043.
- 23 Qi LL, Pan HJ, Li DY, Fang F, Chen D, Sun H: Luteolin improves contractile function and attenuates apoptosis following ischemia-reperfusion in adult rat cardiomyocytes. *Eur J Pharmacol* 2011;668:201-207.
- 24 Fang F, Li D Y, Pan HJ, Chen D, Qi LL, Zhang RQ, Sun H: Luteolin Inhibits Apoptosis and Improves Cardiomyocyte Contractile Function through the PI3K/Akt Pathway in Simulated Ischemia/Reperfusion. *Pharmacology* 2011;88:149-158.
- 25 Pan H, Li D, Fang F, Chen D, Qi L, Zhang R, Xu T, Sun H: Salvianolic acid A demonstrates cardioprotective effects in rat hearts and cardiomyocytes after ischemia/reperfusion injury. *J Cardiovasc Pharmacol* 2011;58:535-542.
- 26 Das A, Smolenski A, Lohmann S M, Kukreja RC: Cyclic GMP-dependent protein kinase I alpha attenuates necrosis and apoptosis following ischemia/reoxygenation in adult cardiomyocyte. *J Biol Chem* 2006;281:38644-38652.
- 27 Doser TA, Turdi S, Thomas DP, Epstein PN, Li SY, Ren J: Transgenic overexpression of aldehyde dehydrogenase-2 rescues chronic alcohol intake-induced myocardial hypertrophy and contractile dysfunction. *Circulation* 2009;119:1941-1949.
- 28 Moshal KS, Tipparaju SM, Vacek TP, Kumar M, Singh M, Frank IE, Patibandla PK, Tyagi N, Rai J, Metreveli N, Rodriguez WE, Tseng MT, Tyagi SC: Mitochondrial matrix metalloproteinase activation decreases myocyte contractility in hyperhomocysteinemia. *Am J Physiol Heart Circ Physiol* 2008;295:H890-897.
- 29 Palmer JW, Tandler B, Hoppel CL: Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J Biol Chem* 1977;252:8731-8739.
- 30 Pei JM, Kravtsov GM, Wu S, Das R, Fung M L, Wong TM: Calcium homeostasis in rat cardiomyocytes during chronic hypoxia: a time course study. *Am J Physiol Cell Physiol* 2003;285:C1420-1428.
- 31 Kamkin A, Kiseleva I, Theres H, Eulert-Grehn JJ, Wagner KD, Scholz H, Vetter R: Enhanced L-type calcium currents in cardiomyocytes from transgenic rats overexpressing SERCA2a. *Exp Clin Cardiol* 2010;15: e109-115.
- 32 Yu D, Li M, Tian Y, Liu J, Shang J: Luteolin inhibits ROS-activated MAPK pathway in myocardial ischemia/reperfusion injury. *Life Sciences* 2015; 122:15-25.
- 33 Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D: Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* (London, England) 1993; 342:1007-1011.
- 34 Xu TD, Li DY, Jiang DH: Targeting Cell Signaling and Apoptotic Pathways by Luteolin: Cardioprotective Role in Rat Cardiomyocytes Following Ischemia/Reperfusion. *Nutrients* 2012; 4:2008-2019.
- 35 Wang C, Youle RJ: The role of mitochondria in apoptosis. *Annu Rev Genet* 2009;3:95-118.