Glycogen Synthase Kinase 3β Inhibition Protects the Cardiomyocytes from Anoxia/Reoxygenation Injury by Modulating Inflammatory Response and Reducing the Opening of mPTP

Yi Cheng¹, Jun Ma¹, Fu-Shan Xue², and Xin-Long Cui²

ABSTRACT

Background: Phosphorylation of glycogen synthase kinase 3β (GSK-3β) is crucial in multiple cardioprotective signaling pathways. But the downstream mechanism is unclear. Considering GSK-3β is the key regulator of nuclear factor-κB (NF-κB) and mitochondrial permeability transition pore (mPTP) is a critical determinant of lethal reperfusion injury, we assessed the mechanism of postconditioning with the selective inhibitor of GSK-3β associated with modulating inflammatory response and reducing the opening of mPTP.

Methods: After cultured for 72 hours, neonatal rat cardiomyocytes were randomly divided into 3 groups according to random number table: Sham group, anoxia/reoxygenation injury group (AR group) and GSK-3β inhibitor TDZD-8 postconditioning group (TDZD-8 group). At the end of the experiment, the lactate dehydrogenase (LDH) release rate, myocardial apoptosis and the supernatant concentrations of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured. The NF-κBp65 and phosphorylated NF-κBp65Ser536 in all samples were assessed by western-blotting technique. The mitochondrial membrane potential was measured by rhodamine 123 staining flow cytometry and confocal laser scanning microscope.

Results: We discovered that postconditioning with GSK-3β inhibitor TDZD-8 could significantly protect against cardiomyocyte anoxia/reoxygenation injury, as shown by reducing the early apoptosis and LDH release rate. And this cardioprotective method could significantly attenuate inflammatory response to the anoxia/reoxygenation injury, as evidenced by decreasing the activity of NF-κB and levels of inflammatory cytokines, such as IL-6 and TNF-α. Furthermore, it could significantly reduce the irreversibly high level opening of the mPTP, as evidenced by increasing the mitochondrial membrane potential of anoxia/reoxygenation cardiomyocyte.

Conclusions: Our current results indicated that postconditioning with GSK-3β inhibitor TDZD-8 significantly attenuated the systemic inflammatory and reduced the opening of mPTP response to cardiomyocytes anoxia/reoxygenation injury.
Coronary heart disease is the leading cause of death worldwide. After an acute myocardial infarction, restoration of blood flow with the use of primary percutaneous coronary intervention (PCI) or thrombolytic therapy is the most effective strategy for reducing the size of a myocardial infarct and improving the clinical outcome. However, reperfusion itself has been proved to cause irreversible myocardial damage, termed "ischemia and reperfusion injury" (IRI), which is closely related to the lethal ventricular arrhythmia, apoptosis, ventricular remodeling and cardiac dysfunction (1). Studies in animal models of acute myocardial infarction suggest that IRI accounts for up to 50% of the final size of a myocardial infarct (2). As a result, cardioprotection is a broad term that refers to all strategies aiming at the attenuation of injurious results of myocardial IRI. Although the ischemia preconditioning remains the most effective cardioprotective method, its clinical application has been limited by the requirement of intervention before the onset of acute myocardial ischemia, which is impracticably in the setting of acute myocardial infarction. Ischemia postconditioning can be triggered during the clinically applicable period of reperfusion. However, it has the similar limitation as ischemia preconditioning is still an invasive protocol. Since cardioprotective drugs can be administered after ischemia, pharmacological postconditioning may be of the strongest clinical applicability.

It has been demonstrated that endogenous inflammatory response is a vital factor in formation and progression of myocardial IRI (3), and regulation of inflammatory response is proved to afford cardioprotection (4-7). Glycogen synthase kinase 3β (GSK-3β) is a multifunctional serine/threonine kinase (Ser/Thrkinase), which was initially thought just to be the limiting enzyme in glycogen synthesis. But now it is confirmed that GSK-3β plays important roles in cell metabolism, growth, differentiation and apoptosis (8-11). It is a vital component of several signal transduction pathways with the ability to phosphorylate the substrates (12, 13). It has been proved that GSK-3β plays a critical role in the activity of nuclear factor-κB (NF-κB) (14, 15) which is traditionally used as an indicator of inflammatory gene expression in cells (16, 17). Since then, much attention has been paid to its role in inflammatory diseases. Moreover, previous works have shown that much endogenous and exogenous cardioprotection plays efficacy by depressing the activation of GSK-3β (18-20), whereas the precise downstream mechanism is unclear. Mitochondrial permeability transition pore (mPTP) is a nonselective high conductance channel between the inner and outer membranes of the mitochondria. It is definitely established that the proof of mPTP plays important roles in mitochondrial homeostasis and in many aspects of cell physiology, especially for heart function. The high level irreversible opening of mPTP is another important mechanism of IRI. Past and recent researches definitely established that mPTP is the ultimate determinant of myocardial cell apoptosis (21-23) and it is the key mechanism of many different types of cardioprotective methods, such as preconditioning, postconditioning and some pharmacological postconditioning (24-27).

The clues mentioned above suggest that the downstream mechanisms of GSK-3β may be related with the inflammatory response and mPTP. Therefore, this randomized experiment was designed to assess the cardioprotection and mechanisms of postconditioning with TDZD-8, a non-adenosine triphosphate (ATP) competitive inhibitor of GSK-3β, in an in vitro rat cardiomyocyte anoxia/reoxygenation injury model.

**MATERIALS AND METHODS**

**Primary Culture of Neonatal Rat Cardiac Ventricular Myocytes**

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Cardiac ventricular myocytes were prepared from 1-2-day-old Sprague-Dawley rats. The hearts were removed and ventricles were minced in calcium-free Hanks’ buffer. Tissue fragments were digested by 0.125% trypsin and 0.1% collagenase (Gibco, USA) and purified by differential velocity adherent technique for 1 hour combined with 5-bromodeoxyuridine (5-BrdU) treatment. The nonadherent myocytes were then plated in culture medium contained the antimitotic 5-BrdU (0.1 mmol/l) to inhibit fibroblast growth in addition to 5% fetal bovine serum (FBS) (28).
In Vitro Rat Cardiomyocyte Anoxia/Reoxygenation Injury Model

In vitro rat cardiomyocyte anoxia/reoxygenation injury model was established by an AnaeroPack system. After cardiomyocytes were cultured for 72 hours, the medium used to grow the cardiomyocytes was replaced with glucose-free Dulbecco’s modified eagle’s medium (DMEM) before the cells were exposed to hypoxic stress. After 6 hours of exposure to hypoxia, the medium was replaced with 5% FBS-containing DMEM (reoxygenation medium) (29).

Experimental Protocols

Based on the different treatments, the cardiomyocytes were randomly divided into 3 groups according to random number table: Sham group, the cardiomyocytes were cultured as normal but only adding the same concentration of dimethyl sulfoxide (DMSO) as the other groups 6 hours prior to detection; anoxia/reoxygenation injury group (AR group), the reoxygenation medium contained DMSO with the same concentration as the other groups; GSK-3β inhibitor TDZD-8 postconditioning group (TDZD-8 group), the reoxygenation medium contained 10 μM TDZD-8 (dissolved in DMSO in advance) (protocol is shown in Figure 1).

Measurement of Lactate Dehydrogenase Release Rate

The cardiomyocytes injury was evaluated by measuring lactate dehydrogenase (LDH) release rate with the LDH Release Assay Kit (Beyotime Biotechnology, China). The experiment was performed as per manufacturer’s instructions. The intensity of the red color formed in the assay and measured at a wavelength of 490 nm was proportional to LDH activity and to the number of damaged cells. The data were normalized to the activity of LDH released from glutamate-treated culture media (100%) and expressed as a percentage of this control.

Assessment of Apoptosis

Myocardial apoptosis was detected using the dead cell apoptosis Kit with Annexin V/propium iodide (Annexin V/PI) for flow cytometry (Life Technologies, USA). The cardiomyocytes that had been treated as mentioned above were harvested by 0.25% trypsin- ethylenediaminetraacetic acid (EDTA), washed twice with cold phosphate buffer solution (PBS) and then resuspended in 1 × binding buffer at a concentration of 1 × 10^6 cell/ml. To a 100-μl of the cell suspension, 5 μl of fluorescein isothiocyanate (FITC)-conjugated annexin V and 5 μl of propidium iodide (50 μg/ml) were added. After 15 minutes incubation in the dark at room temperature, the cells were analyzed within 1 hour with a flow cytometer. Annexin V- FITC selectively passed through the plasma membranes of apoptotic cells and stained them with green fluorescence. Necrotic cells were stained fluorescent red with propidium iodide. The percentages of unchanged, apoptotic and necrotic cells for each sample were estimated. The experiment was repeated three times.

Phosphorylation of NF-κBp65

Protein was extracted from cardiomyocytes with ice cold lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10% glycerol, 137 mM sodium chloride, 20 mM Tris, pH 7.4). Proteins (50 μg) were subjected to 12% SDS- polyacrylamide gel electrophoresis (PAGE) electrophoresis and transferred to polyvinylidene difluoride (PVDF) nitrocellulose membrane. The blots were reacted with anti-bodies for NF-κBp65 and phosphorylated NF-κBp65, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies.

Measurements of Supernatant Inflammatory Cytokines

At 6 hours of reoxygenation, 1 ml cell culture su-
pernatan was collected to be centrifuged at 1, 500 revolutions per minute (rpm) for 5 minutes. The supernatants were collected and stored at -80°C until future analysis. TNF-α and IL-6 in cell culture supernatants were measured by specific enzyme-linked immunosorbent assays kits (ELISAs) (eBioscience, USA), following the manufacturer's instructions.

Measurement of Mitochondrial Membrane Potential
The mitochondrial membrane potential was measured by rhodamine 123 (Sigma-Aldrich, USA) staining flow cytometry and confocal laser scanning microscope. By flow cytometry, cells were digested by 0.25% trypsin after washed twice with warm PBS. Cells should be dispersed into a single cell suspension lightly. After cells were collected by centrifugation, they were stained with 10 μg/ml rhodamine 123 for 20 minutes and analyzed by BD FACSAria II System. By confocal laser scanning microscope, the myocardial cell culture supernatant was replaced with 10 μg/ml rhodamine 123 at 37 °C for 15 minutes. Mitochondrial fluorescence intensity was assessed by LeicaTCS-SP5 System. Images were obtained during baseline and at periodic intervals. Intensity values were corrected for background. Stage positions were programmed using Image-Pro Plus 6.0.

Statistical Analysis
Statistical analysis of data was performed with SPSS (version 16.0, SPSS Inc, Chicago, IL, USA). All parametric data from the different study groups were tested for normality using the Shapiro-Wilk test. The data were also tested for homogeneity of variance using the Levene’s median test. If the data were normally distributed and had homogeneous variance, they were expressed as means ± SD. One-way analysis of variance (ANOVA) was used to compare the data between groups. Tukey’s multiple comparison test was used for post hoc multiple comparisons. The data of inhomogeneous variance were compared using the Kruskal-Wallis test and Mann-Whitney U test as necessary. The criterion for rejection of the null hypothesis was P<0.05 for all tests.

RESULTS

LDH Release Rate
At the end of reoxygenation, the LDH release rate was (5.8±1.2)% , (26.8±2.0)% , and (18.7±3.0)% in the Sham, AR, and TDZD-8 groups, respectively. As compared to the Sham group, the LDH release rates were significantly increased in the AR and TDZD-8 groups. As compared to the AR group, the LDH release rate was decreased in the TDZD-8 group.

Myocardial Apoptosis
The early apoptosis was (1.3±0.4)% , (17.7±1.6)% , and (10.4±2.5)% in the Sham, AR, and TDZD-8 groups, respectively. As compared to the Sham group, the early and late apoptosis were significantly higher in the AR and TDZD-8 groups. As compared to the AR group, the early apoptosis was significantly lower in the TDZD-8 group (Figure 2 and Table 1).

Phosphorylation of NF-κBp65 and Levels of Inflammatory Cytokines
Both NF-κBp65, phosphorylated NF-κBp65 and the supernatant concentrations of TNF-α and IL-6 were significantly increased in the AR and TDZD-8 groups than those in the Sham group. As compared to the AR group, the phosphorylated NF-κBp65 and the supernatant concentrations of TNF-α and IL-6 were significantly de-
Mitochondrial Membrane Potential

By flow cytometry, the mitochondrial membrane potential was 100% (61.8 ± 3.5)%, and (75.0 ± 4.5)% in the Sham, AR, and TDZD-8 groups, respectively (Figure 4). By confocal laser scanning microscope, the mitochondrial membrane potential was 100%, (40.3 ± 3.7)%, and (55.7 ± 3.8)% in the Sham, AR, and TDZD-8 groups, respectively (Figure 5). As compared to the Sham group, the mitochondrial membrane potential was significantly decreased in the AR and TDZD-8 groups. As compared to the AR group, the mitochondrial membrane potential was increased in the TDZD-8 group (Table 3).

DISCUSSION

The main aims of this study were to determine the cardioprotective effect and mechanisms of the pharmacological postconditioning with the selective GSK-3β inhibitor TDZD-8 in the cardiomyocyte anoxia/reoxygenation injury model in vitro.

GSK-3β is a Ser/Thr kinase that was originally found as a kinase inhibiting glycogen synthase (14, 15). However, it is now known as a multifunctional Ser/Thr kinase that plays important roles in cell metabolism, growth, differentiation and apoptosis (8-11). GSK-3β is a vital component of several signal transduction pathways with the ability to phosphorylate the substrates (12, 13).

The first two studies implicating GSK-3β in regulating pathologic processes in the heart were published a decade ago and identified GSK-3β as a negative regulator of the hypertrophic response in cardiomyocytes in culture (32, 33). Then many researches paid attention on the role of GSK-3β on myocardial protection. Recent studies have found that GSK-3β is where cardiomyocyte damage and eventually death converge in the process of myocardial ischemia and phosphorylation of GSK-3β is crucial in multiple cardioprotective signaling pathways (18-20). But the downstream mechanism is unclear.

In 2000, Hoeflich et al. (14) reported that GSK-3β germline KO is lethal early in development due to massive hepatic apoptosis that was traced to the failure to recruit cytoprotective NF-kB signaling in response to TNF-α in the
KO. Then Schwabe et al. (15) found that, when the cells were treated with the GSK-3β inhibitor lithium chloride (LiCl), the transcription activity of NF-κB was decreased significantly. The research revealed that GSK-3β activates NF-κB by phosphorylation of four potential phosphorylation sites of NF-κB. And these findings are the basis for the theory that GSK-3β may play a key role in inflammation. It has been demonstrated that inflammation plays a pivotal role in the pathogenesis of IRI (3), and treatment modulating inflammatory response can attenuate the IRI (4-7). So we hypothesized that GSK-3β participate in the pathophysiological process of IRI by the potential to modulate proinflammatory gene expression. And our results confirmed that the active GSK-3β can amplify the inflammatory response to promote IRI and inhibition of GSK-3β could protect the cardiomyocytes from IRI through modulating inflammatory response.

The mPTP is a nonselective channel of the inner mitochondrial membrane (24). Irreversibly high level opening of the channel collapses the mitochondrial membrane potential than uncoupling oxidative phosphorylation, resulting in ATP depletion and cell death (25, 35). During myocardial ischemia, the mPTP remains closed, only to open within the first few minutes after myocardial reperfusion in response to mitochondrial oxidative stress, Ca²⁺ overload, restoration of a physiologic pH, and ATP depletion (36, 37). Therefore, the mPTP is a critical determinant of lethal reperfusion injury, and as such it is the foremost important new target for cardioprotection. And recent researches have confirmed that inhibiting mPTP opening at reperfusion could protect against IRI (38, 39). Furthermore, it is thought that mPTP is the ultimate determinant of myocardial cell apoptosis (21-23) and it is the key mechanism of many different types of cardioprotective methods (24-27). So we speculated the mechanism of cardioprotection from the inhibitor of GSK-3β which is highly possible associated with reducing the irreversibly high level opening of mPTP and activating the apoptotic pathways in turn that aggravates myocardial IRI. The data from both flow cytometry and confocal laser scanning microscope indicated that mPTP maybe the downstream of GSK-3β in IRI. And postconditioning with the inhibitor of GSK-3β can attenuate the IRI injury through reducing the irreversibly high level opening of mPTP and activating the apoptotic pathways in turn that aggravates myocardial IRI.

It is considered that both the mitochondrial dysfunction and inflammation are the most important factors in the pathogenesis of ischemia reperfusion injury (40). Mitochondrial dysfunction promotes the cellular irreversible damage (21-23). The inflammatory response leads to microcirculation dysfunction (41-43) and tissue damage (44-47). However, the relationship between the inflammatory response and mitochondrial dysfunction in the IRI process has not been revealed. Since the endosymbiont hypothesis was proposed (48), it is speculated that there must be an unrevealed relation between inflammatory response and mitochondria. In 2010, Zhang et al. (49) reported that mitochondrial DNA may act as a danger-associated molecular pattern or alarmin after shock, contributing to the initiation of systemic inflammatory response syndrome. And then, Zhou et al. (50) reported that inflammasome activation was suppressed when mitochondrial activity was dysregulated by inhibition of the voltage-dependent anion channel. This indicates that NLRP3 inflammasome senses mitochondrial dysfunction and may explain the frequent association of mitochondrial damage with inflammatory diseases. These studies propose a new understanding of mitochondria, the role of promoting the inflammatory response. Consequently, we speculated that there are some potential interaction between mPTP and the inflammatory response for the mechanism of TDZD-8.

In summary, this experiment demonstrated that inhibitor of GSK-3β postconditioning significantly protected against cardiomyocyte anoxia/reoxygenation injury by modulating inflammatory response and reducing the opening of mPTP.

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References


