Original Article
Curcumin protects H9c2 cardiomyocyte against ischemia/reperfusion injury through inactivation of glycogen synthase kinase-3

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Abstract: Curcumin, a polyphenolic compound derived from turmeric, has a protective potential on cardiovascular system. Glycogen synthase kinase-3 (GSK-3) is a multifunctional serine/threonine kinase, which has been demonstrated to play a role in cardioprotection. The present study was aimed to determine the effect of curcumin against ischemia/reperfusion (I/R) injury in cardiomyocyte and its underlying mechanisms involving the role of GSK-3. In this study, an in vitro I/R model was simulated in H9c2 cardiomyocytes. The cell viability, release of lactate dehydrogenase (LDH), cell apoptosis and mitochondrial membrane potential (MMP) were examined in the culture of H9c2 cardiomyocytes following I/R injury. The levels of GSK-3 expression and it's phosphorylation of tyrosine and serine were also evaluated by Western blotting analysis. The results showed that curcumin increased the cell viability, and decreased the release of LDH and cell apoptosis in the culture of H9c2 cells exposed to I/R. Furthermore, curcumin treatment significantly suppressed the loss of MMP in cardimyocytes exposed to I/R. In addition, I/R injury markedly increased both the activated and inactivated phosphorylation of GSK-3 in H9c2 cells. Curcumin significantly reduced the activated phosphorylations of GSK-3 at tyrosine residues and increased the inactivated phosphorylations of GSK-3 at serine residues. Our findings indicate that curcumin has protective effect against I/R injury in cardiomyocyte, which may partly be mediated by the inactivation of GSK-3 via promoting phosphorylations at serine residues and dephosphorylations at tyrosine residues.

Keywords: Curcumin, ischemia/reperfusion, cardiomyocyte, glycogen synthase kinase-3

Introduction

Perinatal asphyxia contributes to one million newborn deaths per year globally [1]. Hypoxemia-related cardiovascular dysfunction is estimated to occur in 50% to 80% of asphyxiated neonates and may lead to significant morbidity and mortality [2]. Compared with adult myocardium, the immature myocardium of neonates is more vulnerable to hypoxia/reoxygenation or ischemia/reperfusion (I/R) injury. Currently, limited treatment is available to protect neonatal myocardium from I/R injury.

Curcumin is a polyphenol responsible for the yellow color of the curry spice turmeric. It has been used in a variety of diseases in traditional medicine for hundreds of years. Molecular studies to understand the therapeutic effects of curcumin have been extensively performed during the past two decades, particularly in the treatment of cancer [3]. Recently, there is growing evidence that curcumin has a protective role in many cardiovascular diseases, such as adriamycin-induced cardiotoxicity, diabetic cardiovascular complications, and myocardial infarction [4, 5]. However, little is focused on I/R injury of neonatal cardiomyocyte.

A number of intracellular signaling elements have been proposed to be involved in cardioprotection against I/R injury. Recent studies have revealed that glycogen synthase kinase-3β (GSK-3β) plays a critical role in cardioprotection during I/R [6, 7]. The aim of our study was to explore the effect of curcumin against I/R injury in neonatal cardiomyocyte. The relationship between I/R and GSK-3 expression and its modulation by curcumin was also evaluated.

Materials and methods

Cell culture and simulated I/R

Embryonic rat heart derived H9c2 cardiac myoblast cell line was obtained from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO), supplemented with 10% fetal bovine serum and 100 units penicillin-streptomycin at 37°C in a humidified 5% CO_2-95% room air atmosphere. I/R was simulated according to the published method [8]. Briefly, cultured cells were washed twice with PBS and incubated in standard Tyrode solution containing 140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, and 5.8 mM glucose (pH 7.4) for 30 min before the experiments. Then, the cells were exposed to a simulated ischemia solution (glucose-free Tyrode solution containing 10 mM 2-deoxy-d-glucose and 10 mM sodium dithionite) for 90 min followed by 30 min of reperfusion with the normal Tyrode solution (I/R model) or to normal Tyrode solution for 120 min (normal control). Curcumin was applied at the onset of reperfusion for 30 min.

Cell viability assay

Stock solutions of curcumin (Sigma) were prepared in dimethyl sulfoxide (DMSO, Sigma). The final concentration of DMSO in the medium was always ≤0.1%. To test the cytotoxicity of curcumin itself, 1-40 μM of curcumin was added to H9c2 cell cultures for 30 min and 24 h. To investigate the effect of I/R with or without curcumin on cell proliferation, cells were cultured with fresh complete medium for another 24 h after I/R. The viability of cells was determined by the MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide) assay. Briefly, H9c2 cells were incubated in 24-well plates at $5×10^4$ cell/well for 24 h prior to the individual treatment. At the appropriated time, 50 μl of MTT (5 mg/ml) was added to the cells and incubated at 37°C for 3 h. The MTT solution was carefully removed and 1 ml of DMSO was added to each well to dissolve the blue formazan crystals. The optical density was measured at 490 nm wavelengths using universal microplate reader Elx800 (Bio-tek Instruments). The absorbance values were normalized by assigning the value of the control line in the medium without drug to 1.0 and the value of the no-cell control to 0. Mean values were calculated from three independent experiments.

Measurement of LDH activity

After different treatments, the supernatant of culture medium was collected. The LDH (lactate dehydrogenase) activity of culture supernatant was detected by commercially available kit according to the manufacturer's instruction.

Apoptosis detection by flow cytometry

Apoptosis was measured using Annexin V-FITC/PI detection kit (Invitrogen, USA) following the manufacturer's protocol. In brief, cells were harvested, washed with PBS three times, and resuspended in binding buffer. Then, cells were incubated with Annexin V-FITC/PI and analyzed by flow cytometry using a bicolor FACS analysis (Beckman Coulter). The percentage of cells with Annexin V-positive staining was calculated.

Measurement of mitochondrial membrane potential

The change in mitochondrial membrane potential (MMP, Δψm) in H9c2 cells was observed flow cytometrically by using JC-1 assay kit (Invitrogen) according to the kit directions. Briefly, cells were harvested, washed with PBS twice, labeled with 2 μM of JC-1 for 20 min at 37°C, and washed with assay buffer. Then cells were resuspended in 500 μL assay buffer and analyzed on a flow cytometer using 488 nm excitation with 530 nm and 585 nm band pass emission filters.

Western blotting

Cells were harvested and lysed in modified protein lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.02% sodium azide) added with 1% proteinase inhibitor cocktail (Sigma). The protein concentration was measured by the Bradford method. Equal amounts of sample lysates were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk in TBST buffer (20 mM Tri-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) and incubated overnight at 4°C with the antibodies against GSK-3α/β (1:500, mouse mAb, Santa Cruz), pTyr-GSK-3α/β (1:400, rabbit pAb, Santa Cruz), pSer-GSK-3α/β (1:400, rabbit pAb, Santa Cruz), and β-actin (1:1000, mouse mAb, Santa Cruz), respectively. The membrane was washed with
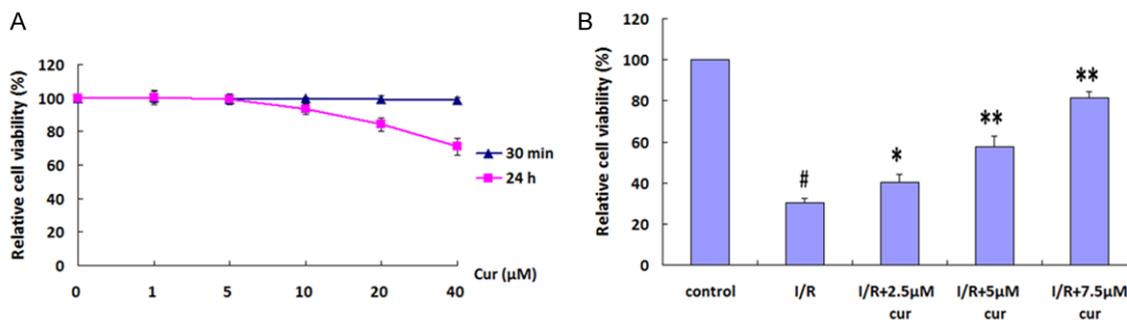
Figure 1. Effect of curcumin on viability in H9c2 cardiomyoblast cells. A. The cytotoxic effect of curcumin (1-40 μM) was evaluated in H9c2 cells. B. Effect of different concentrations of curcumin (2.5, 5 and 7.5 μM) on viability in H9c2 cells exposed to I/R injury. Curcumin was applied at the onset of reperfusion for 30 min, and then cells were cultured with fresh complete medium for another 24 h after I/R. The results are represented as mean ± SEM of three independent experiments. #P<0.01 versus control group; *P<0.05 versus I/R group; **P<0.01 versus I/R group.
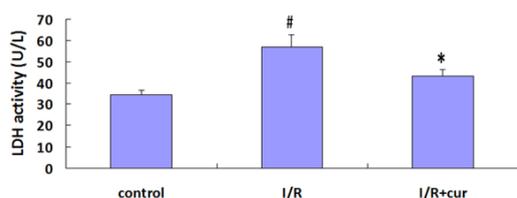
Figure 2. Effects of curcumin on LDH release in H9c2 cells exposed to I/R injury. The results are represented as mean ± SEM of three independent experiments. #P<0.01 versus control group; *P<0.05 versus I/R group.

TBST buffer and incubated with appropriate secondary antibodies. The protein bands were visualized using the enhanced chemiluminescence kits (Pierce). Signal data was normalized for β-actin bands and a mean value was calculated from three independent experiments.

Statistical analysis

Each experiment was performed at least three times, and the results were presented as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences between means. All tests were two-tailed, and P<0.05 was considered to be significant.

Results

Curcumin increased cell viability of H9c2 cells with I/R injury

Initially, we determined the cytotoxicity of curcumin itself. H9c2 cells were treated with dif-

ferent concentrations of curcumin (1-40 μM) for 30 min (cultured with fresh complete medium for another 24 h before MTT assay) and 24 h. For 30 min treatment, curcumin did not cause any cytotoxicity. For 24 h treatment, however, curcumin (≥10 μM) inhibited cell proliferation in a dose dependent manner (**Figure 1A**). For assessment of protective potential of curcumin against I/R, different concentrations of curcumin (2.5, 5 and 7.5 μM) were applied at the onset of reperfusion for 30 min, and then cells were cultured with fresh complete medium for another 24 h after I/R. We found that curcumin treatment increased the viability of H9c2 cells after I/R injury in a concentration dependent manner (**Figure 1B**). Therefore, 7.5 μM of curcumin was applied in the followed experiments.

Curcumin decreased the release of LDH in H9c2 cells induced by I/R

We detected the release of LDH in the culture mediums for further determine the protective effect of curcumin. We found that I/R injury obviously increased the release of LDH in H9c2 cells. However, treatment with curcumin significantly attenuated the leakage of LDH when compared with untreated I/R cells (**Figure 2**).

Curcumin attenuated cell apoptosis induced by I/R

To determine whether curcumin prevents I/R injury could depend in part on apoptosis, we used flow cytometry to stain for the apoptotic marker annexin V. We found that I/R injury
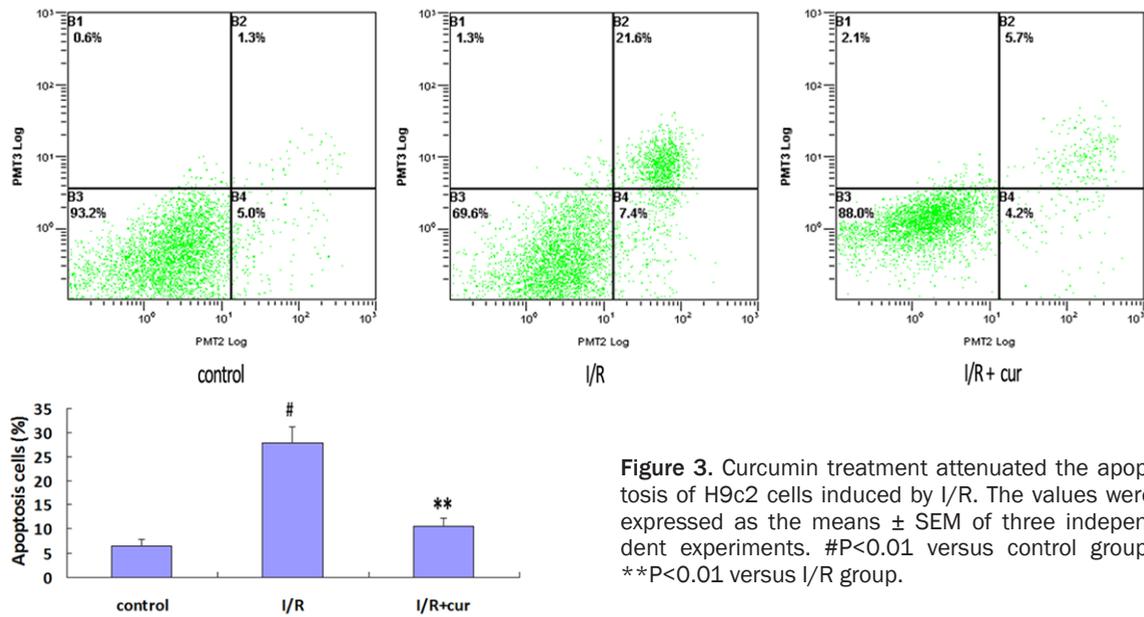
Figure 3. Curcumin treatment attenuated the apoptosis of H9c2 cells induced by I/R. The values were expressed as the means ± SEM of three independent experiments. #P<0.01 versus control group; **P<0.01 versus I/R group.
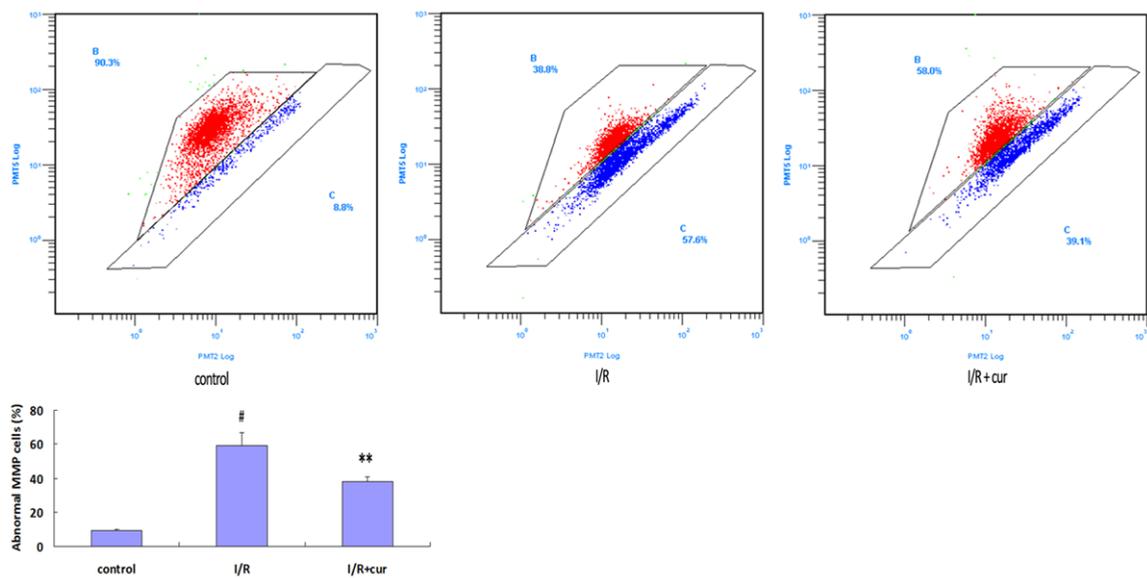
Figure 4. Effects of curcumin on the mitochondrial membrane potential (MMP) in H9c2 cells exposed to I/R. Cells were labeled with JC-1 reagent for 20 minutes. After washing, cells were analyzed on a flow cytometer using 488 nm excitation with 530 nm and 585 nm bandpass emission filters. A dot plot of red fluorescence (PMT5) resolved live cells with intact mitochondrial membrane potential from apoptotic and dead cells with lost mitochondrial membrane potential (PMT2). The values were expressed as the means ± SEM of three independent experiments. #P<0.01 versus control group; **P<0.01 versus I/R group.

resulted in a significant increase in the number of apoptotic cells, as compared to control. However, compared with I/R group, rate of apoptotic cells in I/R+cur group was significantly decreased (**Figure 3**).

Curcumin suppressed the loss of MMP in I/R cells

To get a better insight into the mechanism of curcumin preventing I/R injury, we measured
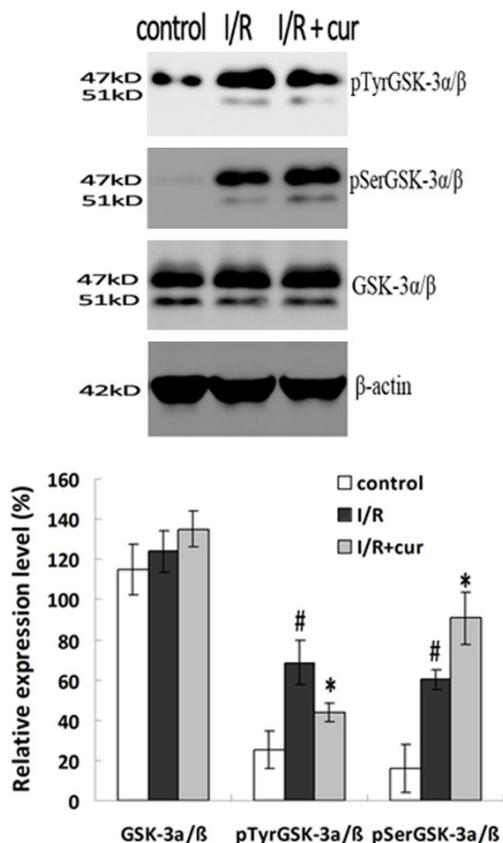
Figure 5. Effects of curcumin on the expression of GSK-3, pTyr-GSK-3 and pSer-GSK-3 in H9c2 cells exposed to I/R by Western blotting. Values were means ± S.E.M. for 3 individual experiments. #P<0.01 versus control group; *P<0.05 versus I/R group.

the change in MMP using JC-1 assay by cytometry. The majority (>90%) of control H9c2 cells maintained normal MMP. About 60% of cells injured by I/R had reduced MMP. Only <40% of cells treated with curcumin during reperfusion had reduced MMP (**Figure 4**). These results show that I/R injury caused obvious loss of MMP, while the loss of MMP in I/R cells could be partly restored by curcumin treatment.

Curcumin decreased pTyr-GSK-3 and increased pSer-GSK-3 expression

Recent evidence suggests that GSK-3 plays a key role in cardioprotection. Therefore, expression levels of GSK-3, pTyr-GSK-3 and pSer-GSK-3 were evaluated by Western blotting analysis. Compared with normal control, the relative expression levels of both pTyr-GSK-3 and pSer-GSK-3 in I/R cells were significantly

increased. However, there was no significant difference of GSK-3 expression between I/R and control cells. There was still no significant difference of GSK-3 expression between I/R+cur and I/R cells. Compared with I/R cells, the expression of pTyr-GSK-3 in I/R+cur cells was significantly decreased, but the expression of pSer-GSK-3 in I/R+cur cells was significantly enhanced (**Figure 5**).

Discussion

The poor consequences of neonatal asphyxia are not only caused by hypoxia but also the resultant I/R injury [9]. Myocardial injury is unfortunately common and contributes significantly to the morbidity and mortality associated with this condition [10]. Currently, the management of neonatal asphyxia and its related complications includes oxygen management, precise fluid and blood pressure support, and cooling [11]. But an ideal supportive regimen for newborns has yet to be established.

Curcumin is the active component in turmeric-a spice that has been extensively used as a culinary agent and a home remedy to prevent and treat many diseases for hundreds of years. Recently, the protective potential of curcumin on the cardiovascular system has been shown in animal models and in human subjects [4, 5]. In the present study, we have provided evidence that treatment with curcumin significantly attenuate the I/R-induced injury of H9c2 cell line derived from embryonic rat heart, as reflected by reducing the release of LDH and increasing cell viability. Our results suggest that treatment with curcumin could protect neonatal cardiomyocyte against the I/R-induced injury.

There is evidence that apoptosis may play a pivotal role in the I/R-induced injury [12]. On the other hand, curcumin has been described as modulator of apoptosis [3]. Therefore, cell apoptosis was determined in this study. We found that I/R could cause an obvious cardiomyocyte apoptosis using flow cytometric analysis. Furthermore, we have confirmed that treatment with curcumin could attenuate the I/R-induced apoptosis. These results suggest that the cardioprotective role of curcumin against the I/R-induced injury might be due to its anti-apoptotic effect.

It was reported that loss of ΔΨm is an early sign of apoptosis and change of mitochondrial function is an important event in the I/R-induced injury [13, 14]. To get a better insight into the mechanism of curcumin preventing the I/R-induced injury, we measured the change of ΔΨm using JC-1 assay by cytometry. In this study, we have shown that I/R-induced injury caused an obvious loss of ΔΨm in cardiomyocyte, while the loss of ΔΨm in I/R-injured cells could be partly reversed by curcumin treatment. Our results demonstrated that cardioprotective effect of curcumin on the I/R-induced injury may also be due to its role on prevention of mitochodrial damage.

GSK-3 is a multifunctional serine (Ser)/threonine kinase known to play a key role in the regulation of metabolism, cell proliferation, cell differentiation, and apoptosis [15]. Two isoforms of GSK-3, GSK-3α and GSK-3β, have been identified with high homology and similar biochemical functions. The actions of GSK-3 are often regulated by phosphorylation. The activity of GSK-3 is activated by phosphorylation of tyrosine (Tyr) 216 in GSK-3β and Tyr279 in GSK-3α; but nevertheless, its activity is inactivated by phosphorylation of Ser9 in GSK-3β and Ser21 in GSK-3α. That is to say, these phosphorylations of Ser and Tyr are important gating switches for regulating the activity of GSK-3. Recent studies have revealed that GSK-3β may be involved in the I/R-induced injury in various tissues. Previous studies indicated that inhibition of GSK-3β may exert protective effects against I/R-induced injury in heart [7], kidney [16], intestine [17], brain [18] and liver [19].

In this study, we performed Western blotting analysis using 3 antibodies which can simultaneously recognize the two isoforms of GSK-3, and their activated or inactivated forms, respectively. Our Western blotting analysis showed that I/R injury markedly increased both the activated and inactivated phosphorylation of GSK-3. Therefore, we speculated that Dual phosphorylation of GSK-3 may be involved in I/R injury in cardiomyocyte. Moreover, we found that curcumin treatment significantly reduced the activated phosphorylations of GSK-3 at tyrosine residues. The inactivated phosphorylations of GSK-3 at serine residues were evidently increased after curcumin treatment. However, the expression levels of the total GSK-3 protein were not affected by curcumin treatment.

These results suggest that curcumin is an inhibitor of GSK-3, it plays its roles through promoting phosphorylations of GSK-3 at serine residues and dephosphorylations of GSK-3 at tyrosine residues.

Previous data have demonstrated that the activation of GSK-3 can promote the opening of the mitochondrial permeability transition pore (mPTP) [20]. The disturbance of ion homeostasis resulting from ATP depletion following the opening of the mPTP can lead to apoptotic and necrotic cell death [7]. Therefore, cellular damage may be alleviated by converging on the mPTP through GSK-3 inhibition, which in turn inhibits its opening.

In summary, the protective effect of curcumin against I/R injury in cardiomyocyte is likely to be mediated by the inactivation of GSK-3 and, at least in part, subsequent inhibition of the opening of mPTP, which in turn inhibits cell apoptosis. Further studies defining the role of mPTP and its interaction with GSK-3 at the time of reperfusion in curcumin-induced cardioprotection may be needed.

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Disclosure of conflict of interest

None.

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