

Original Article

# Ginsenoside Rb1 protects cardiomyocytes against CoCl<sub>2</sub>-induced apoptosis in neonatal rats by inhibiting mitochondria permeability transition pore opening

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**Aim:** To investigate whether mitochondria permeability transition pore (mPTP) opening was involved in ginsenoside Rb1 (Gs-Rb1) induced anti-hypoxia effects in neonatal rat cardiomyocytes *ex vivo*.

**Methods:** Cardiomyocytes were randomly divided into 7 groups: control group, hypoxia group (500 μmol/L CoCl<sub>2</sub>), Gs-Rb1 200 μmol/L group (CoCl<sub>2</sub> intervention+Gs-Rb1), wortmannin (PI3K inhibitor) 0.5 μmol/L group, wortmannin+Gs-Rb1 group, adenine 9-β-D-arabinofuranoside (Ara A, AMPK inhibitor) 500 μmol/L group, and Ara A and Gs-Rb1 group. Apoptosis rate was determined by using flow cytometry. The opening of the transient mPTP was assessed by using co-loading with calcein AM and CoCl<sub>2</sub> in high conductance mode. Expression of GSK-3β, cytochrome c, caspase-3 and poly (ADP-ribose) polymerase (PARP) was measured by using Western blotting. ΔGSK-3β was defined as the ratio of p-Ser9-GSK-3β to total GSK-3β.

**Results:** CoCl<sub>2</sub> significantly stimulated mPTP opening and up-regulated the level of ΔGSK-3β. There was a statistically significant positive correlation between apoptosis rate and mPTP opening, between apoptosis rate and ΔGSK-3β, and between mPTP opening and ΔGSK-3β. Gs-Rb1 significantly inhibited mPTP opening induced by hypoxia (41.3%±2.0%, *P*<0.001). Gs-Rb1 caused a 77.3%±3.2% reduction in the expression of GSK-3β protein (*P*<0.001) and a significant increase of 1.182±0.007-fold (*P*=0.0001) in p-Ser9-GSK-3β compared with control group. Wortmannin and Ara A significantly inhibited the effect of Gs-Rb1 on mPTP opening and ΔGSK-3β. Gs-Rb1 significantly decreased expression of cytochrome c (66.1%±1.7%, *P*=0.001), caspase-3 (56.5%±2.7%, *P*=0.001) and cleaved poly ADP-ribose polymerase (PARP) (57.9%±1.4%, *P*=0.001).

**Conclusion:** Gs-Rb1 exerted anti-hypoxia effect on neonatal rat cardiomyocytes by inhibiting GSK-3β-mediated mPTP opening.

**Keywords:** ginsenoside Rb1; cardiomyocytes; hypoxia; apoptosis; mitochondria permeability transition pore; glycogen synthase kinase-3β; cytochrome c; caspase 3; wortmannin

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## Introduction

By inducing cardiomyocyte apoptosis, hypoxia is an important factor involved in ischemic heart disease. Thus, improving the anti-hypoxia capability of cardiomyocytes has been an important issue within the cardiovascular field. Ginsenoside-Rb1 (Gs-Rb1), a main component of Ginsenosides extracted from Ginseng (the root of *Panax ginseng* CA Meyer, family Araliaceae, a traditional medicine in Asian countries), has been reported to inhibit ischemic/reperfusion injury as a result of myocardial infarction *in vivo*<sup>[1–4]</sup>. Gs-Rb1 may also protect

neonatal rat cardiomyocytes from CoCl<sub>2</sub>-induced apoptosis depending on whether the PI3K or AMPK signaling cascade results in GLUT4 translocation *ex vivo*<sup>[5]</sup>.

The mitochondrial permeability transition pore (mPTP) is a mega-channel with permeability to all molecules of less than 1.5 kDa. The opening of the mPTP is often a common cause of cardiac cell apoptosis in numerous cardiac diseases. It may lead to cardiomyocyte apoptosis and/or necrosis<sup>[6–8]</sup> by activating mitochondrial membrane depolarization, which has been indicated as a major contributor to ischemic/reperfusion injury<sup>[9]</sup>. Inhibition of mPTP opening by glycogen synthase kinase-3β (GSK-3β) or melatonin for example<sup>[10, 11]</sup>, may prevent cell apoptosis, which further implies that mPTP is a critical site for intervention<sup>[6–8]</sup>. To our knowledge, the anti-hypoxia

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capability of Gs-Rb1 through inhibiting mPTP opening has not been reported. It is well known that the mitochondria-mediated intrinsic pathways take part in the ischemic/reperfusion apoptosis of cardiomyocytes, during which cytochrome *c* release, caspase activation and poly ADP-ribose polymerase (PARP) cleavage play important roles in the apoptosis process. However, whether those proteins in hypoxic cardiomyocytes were influenced by Gs-Rb1 has not been reported.

GSK-3 $\beta$ , one of two GSK-3 isoforms, has a relatively higher activity than the other isoform in cardiac myocytes and is thought to be the crucial regulatory target of mPTP. In other words, the activity of GSK-3 $\beta$  is a threshold determinant for mPTP opening in cardiomyocytes apoptosis<sup>[12, 13]</sup>. However, whether the Gs-Rb1 effect in inhibiting mPTP opening, is mediated by GSK-3 $\beta$  remains unclear. Thus, we investigated whether Gs-Rb1 influenced the expression of GSK-3 $\beta$  and whether a correlation existed between GSK-3 $\beta$  and mPTP opening. In addition, considering that the anti-hypoxia ability of Gs-Rb1 is mediated by both phosphoinositide 3-kinase (PI3K) and AMP-activated protein kinase (AMPK) signaling<sup>[5]</sup>, we further evaluated whether the mechanisms contributing to adjust both mPTP opening and the activity of GSK-3 $\beta$  were mediated by both PI3K and AMPK signaling *ex vivo*.

## Materials and methods

This study was performed in strict accordance with the institutional recommendations for animal care of China Medical University (CMU) and approved by the local animal research committee. All Wistar rats were purchased from the Laboratory Animal Center of CMU [SCXK (Liao) 2003-0009].

### Cardiomyocytes culture

As in our previous study<sup>[5]</sup>, cardiomyocyte cultures were prepared from hearts of 1-3-d-old rats. In brief, ventricles had been separated and cut into 1 mm<sup>3</sup> pieces and digested (37 °C, 10 min, two to three such cycles to achieve full digestion) with phosphate-buffered saline (PBS) containing 0.08% trypsin and 0.05% collagenase II (Gibco). Next, the cell suspension was collected, centrifuged (100 $\times$ g, 10 min) and suspended in Dulbecco's modified Eagle's media (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 0.1 mmol/L bromodeoxyuridine in 100 mL culture dishes (37 °C, 5% CO<sub>2</sub>, 95% O<sub>2</sub>; NuAire 8000 Autoflow CO<sub>2</sub> Incubator; Thermo Electron Corporation, Franklin, MA) for 90 min to lyse fibroblasts. Finally, cardiomyocytes were seeded on culture flasks at a density of 1.0 $\times$ 10<sup>5</sup> cells per milliliter and incubated for 3 d.

### CoCl<sub>2</sub>-induced chemical hypoxia and Gs-Rb1 intervention

According to our previous experimental results<sup>[5]</sup>, 500  $\mu$ mol/L CoCl<sub>2</sub>, 200  $\mu$ mol/L Gs-Rb1, 500  $\mu$ mol/L adenine 9- $\beta$ -D-arabinofuranoside (Ara A, AMPK inhibitor) and 0.5  $\mu$ mol/L wortmannin (PI3K inhibitor) were freshly prepared. Cells were randomly divided into seven groups as follows: control group (normal oxygen group), hypoxia group (500  $\mu$ mol/L CoCl<sub>2</sub> intervention), Gs-Rb1 group (200  $\mu$ mol/L Gs-Rb1 and

500  $\mu$ mol/L CoCl<sub>2</sub> intervention), W-1 group (0.5  $\mu$ mol/L wortmannin and 500  $\mu$ mol/L CoCl<sub>2</sub> intervention), W-2 group (0.5  $\mu$ mol/L wortmannin intervention basing on 200  $\mu$ mol/L Gs-Rb1 and 500  $\mu$ mol/L CoCl<sub>2</sub> intervention), A-1 group (500  $\mu$ mol/L Ara A and 500  $\mu$ mol/L CoCl<sub>2</sub> intervention) and A-2 group (500  $\mu$ mol/L Ara A intervention basing on 200  $\mu$ mol/L Gs-Rb1 and 500  $\mu$ mol/L CoCl<sub>2</sub> intervention). Before the above intervention, all cardiomyocytes were synchronized and then incubated, *ex vivo*, in L-DMEM supplemented with 10% fetal bovine serum and administrated with fresh CoCl<sub>2</sub>, Gs-Rb1, wortmannin or Ara A for 12 h.

### Flow cytometry

The above cells were adjusted to 1.0 $\times$ 10<sup>6</sup>/mL in each sample and then were analyzed by flow cytometry (FCM). Briefly, in a double variance scatterplot, single positive (FITC<sup>+</sup>/PI) populations were referred to as apoptotic cells, and the quadrant percentage was regarded as the apoptosis ratio (AR). Annexin V FITC/PI kits were from Boehringer Mannheim biological technology Ltd.

### Immunocytochemistry for GSK-3 $\beta$ and p-Ser9-GSK-3 $\beta$ (phosphorylation at Ser 9)

The immunofluorescence staining for GSK-3 $\beta$  and p-Ser9-GSK-3 $\beta$ , after being fixed in PBS (pH 7.2) with 4% paraformaldehyde (PFA) for 20 min at 4°C, was performed with primary antibodies against GSK-3 $\beta$  and p-Ser9-GSK-3 $\beta$  (Sigma) at a dilution of 1:200 (overnight at room temperature), and secondary antibodies FITC-conjugated goat anti-Rabbit IgG (Invitrogen) at a dilution of 1:500 for 45 min. Quality analysis was performed via SCION image software. Every cell at  $\times$ 400 (five sections was chosen in each group) was calculated according to the formula [(mean gray value-Min)/(Max-Min) $\times$ 100].

### Western blotting analysis of GSK-3 $\beta$ , caspase-3, PARP and cytochrome *c*

Western blotting was used to evaluate the expression of GSK-3 $\beta$ , caspase-3, PARP and cytochrome *c* in each group. Total protein was extracted with ice-cold lysis buffer and centrifugation (4 °C, 12 000 $\times$ g, 10 min). A 20  $\mu$ L protein solution for each sample was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. Afterwards, the membrane was incubated with specific primary antibodies against GSK-3 $\beta$  (1:1000 dilution), p-Ser9-GSK-3 $\beta$  (1:1000 dilution), p-T216-GSK-3 $\beta$  (phosphorylation at Tyr 216; 1:1000 dilution), caspase-3 (1:1000 dilution), PARP (1:1000 dilution) and cytochrome *c* (1:1000 dilution) overnight at 4 °C and then with HRP-conjugated secondary antibody (1:2000 dilution) for 2 h. Integral optical density (IOD) was detected via SCION image software. Relevant band intensities were quantified after normalization to the amount of  $\beta$ -actin protein. To identify the exact effect of different interventions,  $\Delta$ GSK-3 $\beta$ , expressed as the inactive form, was analyzed and defined as  $\Delta$ GSK-3 $\beta$ = p-Ser9-GSK-3 $\beta$ /total GSK-3 $\beta$ <sup>[14, 15]</sup>.

### Determination of mPTP opening

According to the previous report<sup>[7]</sup>, the opening of the transient mPTP was directly assessed by co-loading with calcein AM and CoCl<sub>2</sub> in high conductance mode. The principle idea revolved around the fact that calcein AM is permeable to intact membranes but not to intact mitochondrial membranes and that mPTP opening leads to the exit of calcein in high conductance mode. Thus, the condition allows for monitoring of calcein fluorescence in mitochondria of intact cells. In view of hypoxia mode induced by CoCl<sub>2</sub>, the present study did not add CoCl<sub>2</sub> intervention any longer than previous studies. In brief, cardiomyocytes were loaded for 15 min with 1 μmol/L calcein AM in working solution<sup>[7]</sup> at room temperature and then washed free of calcein and CoCl<sub>2</sub>. The rate of calcein AM loading and exit was measured by recording the fluorescence signal every 5 min using Turner Quantech Digital Filter Fluorometer (excitation filter NB490 and emission filter SC515) and calculated as a percent change to maximal fluorescence signal. In addition, the present study recorded the fluorescence signal for 30 min, keeping in accordance with hypoxia intervention time according to our preliminary experiments.

### Data analysis

All experiments were repeated at least three times, independently. All data in this study are presented as mean±SD. Statistical analysis was performed using analysis of variance with one-way ANOVA and linear regression analysis.  $P < 0.05$  were considered significant.

## Results

### Apoptosis rate

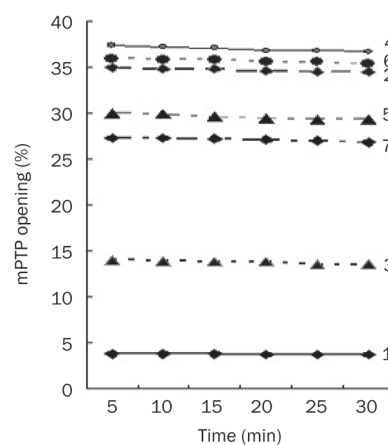
The apoptosis rate was 3.3%±0.1% in the control group; 39.2%±0.1% in hypoxia group; 14.9%±0.4% in Gs-Rb1 200 μmol/L treatment group; 40.7%±1.5% in 0.5 μmol/L wortmannin group; 36.7%±0.6% in wortmannin+Gs-Rb1 group; 41.3%±0.7% in Ara A 500 μmol/L group; and 29.6%±0.6% in Ara A+Gs-Rb1 group. Gs-Rb1 protected cardiomyocytes from hypoxia-induced apoptosis, which was in agreement with our previous study.

### mPTP opening

In each group, mPTP opening was not significantly changed at different time points during 30 min. Therefore, all data were presented as the mean in each group for comparison. mPTP opening was 3.6%±0.2%, 33.4%±0.9%, 13.8%±0.5%, 36.9%±0.6%, 30.0%±1.1%, 35.1%±0.8%, and 26.1%±0.9% in the control group, hypoxia group, Gs-Rb1 group, wortmannin group, wortmannin+Gs-Rb1 group, Ara A group, and Ara A+Gs-Rb1 group, respectively (Figure 1).

### The correlation between apoptosis rate and ΔGSK-3β and/or mPTP opening

There were significant positive correlations between apoptosis rate and mPTP opening (Figure 2A), between AR and ΔGSK-3β (Figure 2B), and between mPTP opening and ΔGSK-3β (Figure 2C).



**Figure 1.** Inhibitory effect of ginsenoside (Gs) Rb1 on mitochondria permeability transition pore (mPTP) opening from 5 to 30 min. (1) control group; (2) simple hypoxia group; (3) Gs-Rb1 200 μmol/L treatment group; (4) 0.5 μmol/L wortmannin group; (5) wortmannin+Gs-Rb1 group; (6) Ara A 500 μmol/L treatment group; (7) Ara A+Gs-Rb1 group.

### Immunofluorescence staining for GSK-3β protein and p-Ser9-GSK-3β protein

A positive expression of GSK-3β protein (Figure 3A) and p-Ser9-GSK-3β protein (Figure 3B) was observed in the cytoplasm of cardiomyocytes in each group. Gs-Rb1 significantly decreased the expression of GSK-3β induced by hypoxia. But the inhibitory effect of Gs-Rb1 was reversed by wortmannin and Ara A.

### Influence of hypoxia on mPTP opening and GSK-3β protein

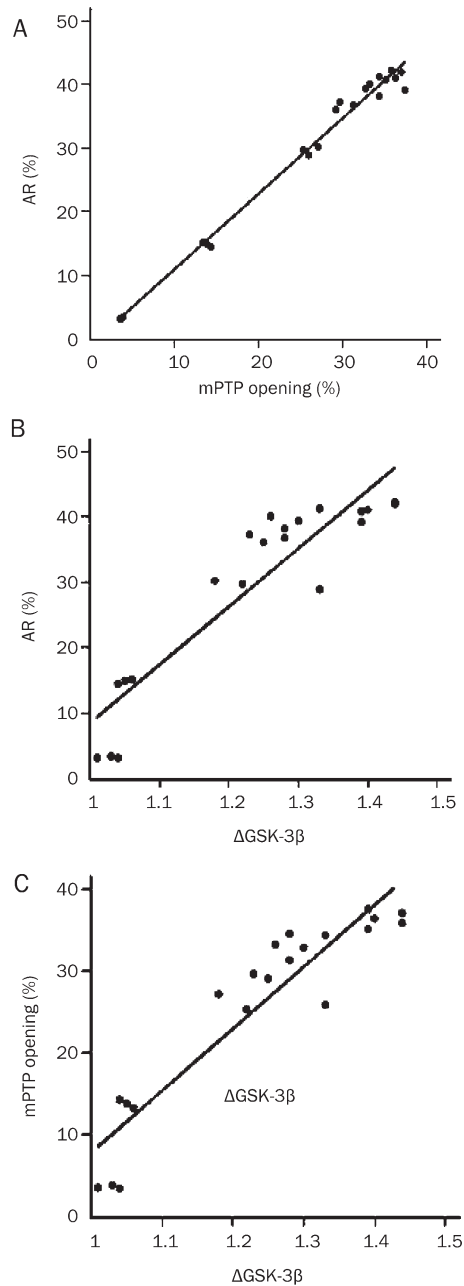
mPTP was not opened in normoxia cardiomyocytes and was significantly opened after CoCl<sub>2</sub>-induced hypoxia (9.25±0.95-fold,  $P < 0.001$ ; Figure 1, 4). Compared with the control group, hypoxia caused significant increase in GSK-3β protein (2.326±0.245-fold,  $P < 0.001$ ; Figure 5Aa) and its phosphorylation of Ser9 (*ie*, p-Ser9-GSK-3β, 1.602±0.115-fold,  $P < 0.001$ ; Figure 5Ab) but not T216 (*ie* p-T216-GSK-3β,  $P > 0.05$ ; Figure 5Ac), and significantly up-regulated ΔGSK-3β up to 1.25±0.01-fold ( $P < 0.001$ ; Figure 5B).

### Effect of Gs-Rb1 on mPTP opening

Gs-Rb1 significantly inhibited mPTP opening (41.3%±2.0%,  $P < 0.001$ ) induced by hypoxia; but the difference between Gs-Rb1 group and the control group was significant ( $P < 0.001$ , Figure 4).

### Effect of Gs-Rb1 on the expression of GSK-3β protein

Gs-Rb1 intervention caused a 77.3%±3.2% reduction in the expression of GSK-3β protein ( $P < 0.001$ ) and a significant increase of 1.182±0.007-fold ( $P < 0.0001$ ) in p-Ser9-GSK-3β, except for p-T216-GSK-3β ( $P > 0.05$ ) in hypoxic cardiomyocytes. Gs-Rb1 significantly decreased ΔGSK-3β (82.0%±1.7%,  $P < 0.001$ ) induced by hypoxia, but the level of ΔGSK-3β in Gs-Rb1 group was still higher than that in the control group ( $P < 0.001$ , Figure 5).



**Figure 2.** The correlation among apoptosis rate (AR),  $\Delta$ GSK-3 $\beta$  and/or mPTP opening. (A) The correlation between AR and mPTP opening ( $r=0.993$ ,  $P<0.001$ ); (B) The correlation between AR and  $\Delta$ GSK-3 $\beta$  ( $r=0.917$ ,  $P<0.001$ ); (C) The correlation between mPTP opening and  $\Delta$ GSK-3 $\beta$  ( $r=0.931$ ,  $P<0.001$ ).

#### Effect of wortmannin on mPTP opening and GSK-3 $\beta$ protein

Wortmannin 0.5  $\mu$ mol/L significantly increased the mPTP opening and the level of  $\Delta$ GSK-3 $\beta$  compared with control group ( $P<0.001$ ). Gs-Rb1 inhibited the effects of wortmannin (Figure 1, 4, 5).

#### Effect of Ara A on GSK-3 $\beta$ protein and mPTP opening

Ara A significantly increased the opening of mPTP and the level of  $\Delta$ GSK-3 $\beta$  ( $P<0.001$ ) compared with control group. Gs-Rb1 inhibited the effects of Ara A (Figure 1, 4, 5).

#### Expression of cytochrome c, caspase-3 and PARP

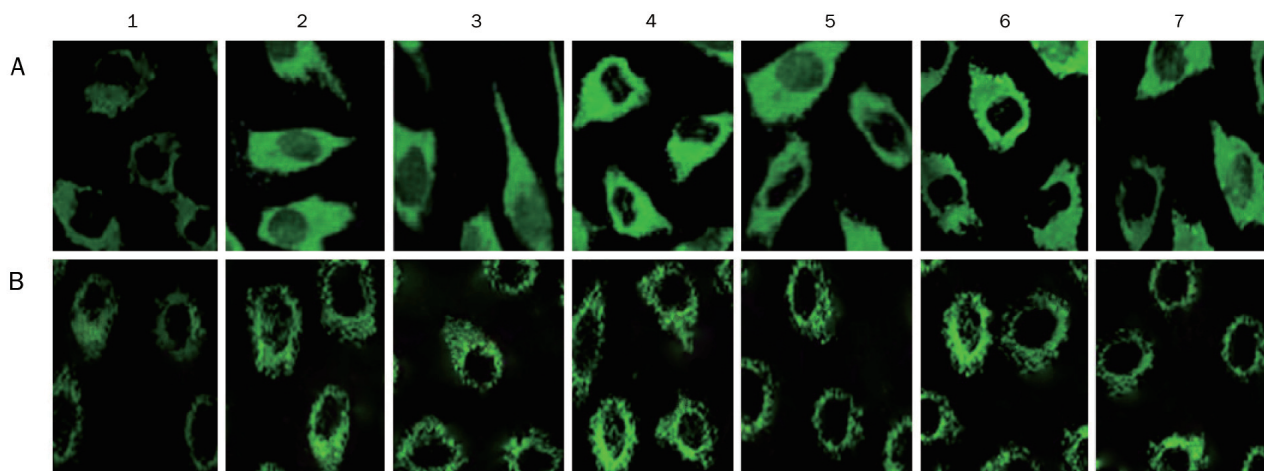
Compared to the hypoxia group, Gs-Rb1 significantly decreased expression of cytochrome c ( $66.1\pm 1.7\%$ ,  $P=0.001$ ), caspase-3 ( $56.5\pm 2.7\%$ ,  $P=0.001$ ) and cleaved PARP ( $57.9\pm 1.4\%$ ,  $P=0.001$ ), all of which were significantly diminished by wortmannin and Ara A. However, there was no difference in total PARP content between the three groups ( $P>0.05$ , Figure 6).

#### Discussion

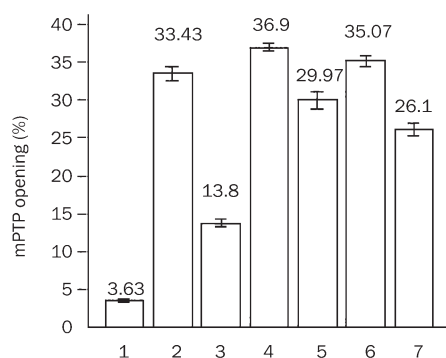
Our previous study demonstrated that Gs-Rb1, by improving glucose uptake, plays an important role in protecting neonatal rat cardiomyocytes from  $\text{CoCl}_2$ -induced apoptosis *ex vivo*<sup>[5]</sup>. This role may be controlled by the translocation of GLUT-4 mediated by both AMPK and/or PI3K signaling cascade. However, the full set of anti-apoptotic mechanisms of Gs-Rb1 remains unclear. Therefore, the questions of whether the effect of Gs-Rb1 was mediated by inhibiting mitochondrial permeability transition pore (mPTP), what role glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) plays in the mPTP opening, and whether the AMPK and PI3K signaling cascade mediated those roles were investigated in the present study.

It is well known that mitochondria play a key role in determining cell fate by controlling the balance between the survival signal and death signal<sup>[6-8]</sup>. The mPTP, being composed of the voltage dependent anion channel (VDAC, in the outer mitochondrial membrane), the adenine nucleotide translocase (ANT, in the inner mitochondrial membrane) and the phosphate carrier (PiC), has been an important mediator and end effector in mitochondria-mediated death pathways in cardiomyocytes as a result of ischemic/reperfusion injury<sup>[6-9, 16-21]</sup>. Some studies have shown that there is a strong negative correlation between cardiomyocyte survival and the fraction of depolarized mitochondria (*ie* mPTP opening)<sup>[22]</sup> and have suggested that the mPTP, closed under physiological conditions, is opened due to cellular stress. A previous study showed that the mPTP remains closed throughout ischemia<sup>[23]</sup>; however, we found that  $\text{CoCl}_2$  may result in the opening of mPTP, which plays a key role in hypoxia-induced apoptosis of cardiomyocytes *ex vivo*. The reason for these differences could be due to experimental methods, particularly the hypoxia model. The mechanisms of mPTP opening mediating cell apoptosis may involve the collapse of the membrane potential, uncoupling of the respiratory chain, efflux of cytochrome c,  $\text{Ca}^{2+}$  overload, or other pro-apoptotic factors<sup>[6-11, 17-22, 24]</sup>. The fact that Gs-Rb1 inhibited the opening of mPTP suggests that the effect of Gs-Rb1 protecting neonatal rat cardiomyocytes from hypoxia-induced apoptosis was performed by virtue of inhibiting mPTP opening. However, it is unknown how Gs-Rb1 modulates mPTP opening.



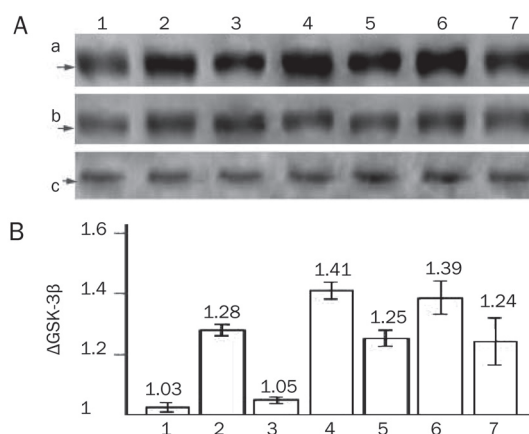


**Figure 3.** Fluorescent immunocytochemical staining for GSK-3 $\beta$  protein in cardiomyocytes. Green fluorescent grains appeared in the cell plasma of cardiomyocytes (400 $\times$ ). (A) GSK-3 $\beta$  protein; (B) p-Ser9-GSK-3 $\beta$ ; (1) control group; (2) simple hypoxia group; (3) Gs-Rb1 200  $\mu$ mol/L treatment group; (4) 0.5  $\mu$ mol/L wortmannin group; (5) wortmannin+Gs-Rb1 group; (6) Ara A 500  $\mu$ mol/L treatment group; (7) Ara A+Gs-Rb1 group.



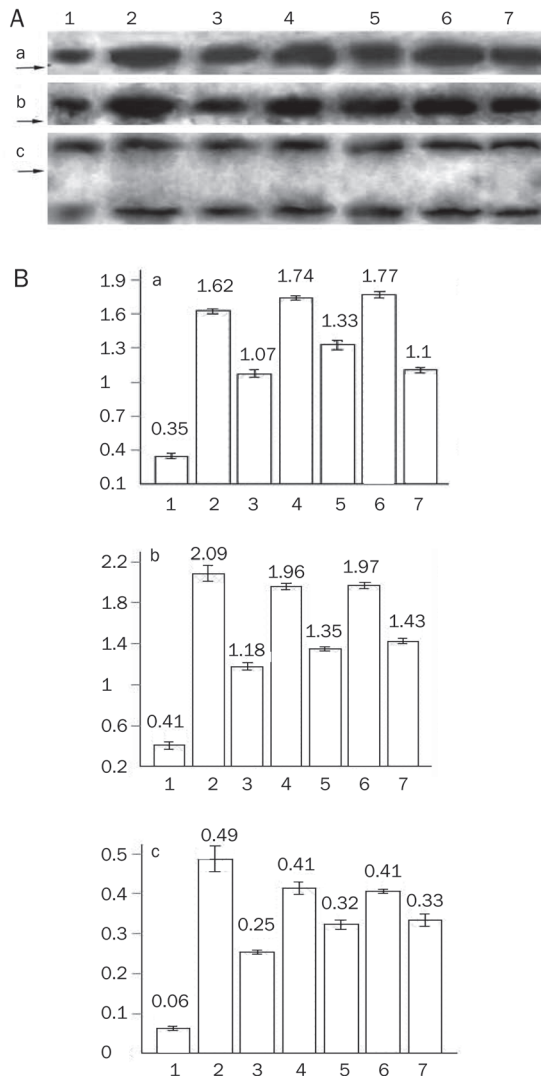
**Figure 4.** Inhibitory effect of Gs-Rb1 on mPTP opening and GSK-3 $\beta$  activity in each group. (1) control group; (2) simple hypoxia group; (3) Gs-Rb1 200  $\mu$ mol/L treatment group; (4) 0.5  $\mu$ mol/L wortmannin group; (5) wortmannin+Gs-Rb1 group; (6) Ara A 500  $\mu$ mol/L treatment group; (7) Ara A+Gs-Rb1 group.

GSK-3 $\beta$ , as the exact regulatory target of mPTP opening, was viewed as a possible therapeutic target for cardiomyocyte protection<sup>[12, 13]</sup>. The mechanisms that cause GSK-3 $\beta$  to provoke mPTP opening is not fully understood; some evidence to date, however, suggests that preservation of hexokinase-II in the mPTP complex, inhibition of cyclophilin-D-ANT binding, inhibition of p53 and inhibition of ANT in the mitochondria contribute to these mechanism<sup>[10, 25]</sup>. The positive correlation between hypoxia apoptosis and GSK-3 $\beta$  in our study supported previous conclusions<sup>[12]</sup>. It is noteworthy that the expression of GSK-3 $\beta$ , being significantly increased by hypoxia, may be significantly inhibited by Gs-Rb1 to the extent shown in the present results. The results suggested that the high activity of GSK-3 $\beta$  mediates hypoxia cardiomyocyte injury, in agreement with previous studies<sup>[25]</sup>. Therefore, we suggested that the effect of Gs-Rb1, inhibiting expression of GSK-3 $\beta$ , may be an important mechanism in improving the



**Figure 5.** Western blotting results of GSK-3 $\beta$ , p-Ser9-GSK-3 $\beta$  and p-T216-GSK-3 $\beta$  in different groups. (A) Representatives of Western blotting. (a) GSK-3 $\beta$  protein; (b) p-Ser9-GSK-3 $\beta$ ; (c) p-T216-GSK-3 $\beta$ . (B)  $\Delta$ GSK-3 $\beta$  values. (1) control group; (2) simple hypoxia group; (3) Gs-Rb1 200  $\mu$ mol/L treatment group; (4) 0.5  $\mu$ mol/L wortmannin group; (5) wortmannin+Gs-Rb1 group; (6) Ara A 500  $\mu$ mol/L treatment group; (7) Ara A+Gs-Rb1 group.

survival of hypoxia cells. GSK-3 $\beta$  phosphorylation is a crucial step in suppressing mPTP opening for protecting cardiomyocytes from apoptosis<sup>[10]</sup>. In the present study, we found both hypoxia and Gs-Rb1 significantly modulated the phosphorylation of GSK-3 $\beta$  at serine9 but not on T216 site; in addition, the expression level of p-Ser9-GSK-3 $\beta$  was significantly increased in the Gs-Rb1 group than in the hypoxia group. All of the above showed that Gs-Rb1 may down-regulate the activation of GSK-3 $\beta$  via phosphorylation at its serine9 site and that the phosphorylation of serine216 seems to play an insignificant role. Thus, we concluded that the phosphorylation at Ser9 may be a key step in preventing the activity of GSK-3 $\beta$ . In a



**Figure 6.** Western blotting results of cytochrome *c*, caspase-3, and PARP. (A) Representatives for Western blotting. (a) Cytochrome *c* protein; (b) caspase-3; (c) PARP (upper for total PARP 116 kDa, lower for cleaved PARP 85 kDa). (1) control group; (2) simple hypoxia group; (3) Gs-Rb1 200 μmol/L treatment group; (4) 0.5 μmol/L wortmannin group; (5) wortmannin+Gs-Rb1 group; (6) Ara A 500 μmol/L treatment group; (7) Ara A+Gs-Rb1 group.

word, the effect of Gs-Rb1 works in association with GSK-3β inactivation to inhibit cardiomyocyte apoptosis. However, further investigations of how Gs-Rb1 inhibits mPTP opening via GSK-3β are required.

Akt is one of the best described survival kinases. It is activated by receptor ligands, and its activation preserves mitochondrial integrity and protects cardiomyocytes against necrosis and apoptosis<sup>[12, 26, 27]</sup>. The role of PI3K-PKB/Akt via its downstream target, GSK-3β, mediates the convergence of myocyte protection signaling<sup>[12, 26, 27]</sup>. Many studies have suggested that PI3K/Akt may inhibit mPTP opening through inhibiting GSK-3β activity<sup>[26-31]</sup>. In the present study, we found that the effect of Gs-Rb1, which inhibits the activity of GSK-3β

and mPTP opening, was at least partially mediated by PI3K-PKB/Akt. According to the results, the influence of Gs-Rb1 on mPTP opening or GSK-3β activity is partially abrogated by PI3K activity inhibitors, which suggests that the PI3K-PKB/Akt-GSK-3β pathway is an important one in anti-hypoxia apoptosis of Gs-Rb1 *ex vivo*. However, it is just partly responsible for the efficacy of Gs-Rb1, in accordance with our previous study<sup>[5]</sup>. The mechanisms responsible for Gs-Rb1 controlling GSK-3β activity and mPTP opening via the PI3K/Akt pathway have not been fully elucidated in the present study. There exists, however, accumulating evidences that multiple Akt target molecules are recruited through both transcriptional<sup>[32-34]</sup> and post-transcriptional<sup>[35, 36]</sup> mechanisms to directly impinge upon and protect mitochondria<sup>[27]</sup>. Akt, once activated at the plasma membrane, may translocate to sub-cellular compartments, such as the nucleus and the mitochondria, and further preserve mitochondrial integrity in cardiomyocytes<sup>[37]</sup>, regulate the expression level of Bcl-2 family proteins in cardiomyocytes<sup>[38]</sup>, phosphorylate directly GSK-3β at Ser9, increase cellular hexokinase<sup>[37, 39, 40]</sup>, inhibit cytosolic Ca<sup>2+</sup> overload and so on. Therefore, we suggested that the effect mediated by Gs-Rb1, inhibiting GSK-3β activity and mPTP opening, may be performed via these pathways too, which is adjusted by PI3K/Akt. The exact mechanisms, however, remain to be determined.

A growing body of literature indicates that AMP-activated protein kinase (AMPK) may protect cardiomyocytes from ischemic/reperfusion by modulating energy generating metabolic pathways<sup>[41, 42]</sup>, enhancing glucose uptake and glycolysis<sup>[43, 44]</sup>, stimulating the oxidation of FFAs (free fatty acid)<sup>[45]</sup> and so on. Our previous result suggested that AMPK activity is an important regulator in Gs-Rb1 anti-hypoxia effect, which may be performed via the translocation and the expression of GLUT-4<sup>[5]</sup>. Some previous studies showed that the AMPK pathway associates with inactivation of GSK-3β by Ser9 phosphorylation, which improves mitochondrial dysfunction<sup>[46-48]</sup>. Our present study supports the conclusion that Gs-Rb1 may phosphorylate the Ser9 site (non Ser216 site) of GSK-3β through the AMPK pathway and then further inhibit the opening of mPTP. In summary, the effect of Gs-Rb1 on hypoxia cardiomyocytes can be partly mediated by the AMPK-GSK-3β-mPTP system, in which Ser9 phosphorylation of GSK-3β may play a key role. However, whether the AMPK effect of Gs-Rb1 mediating GSK-3β was performed by other signaling pathways needs further investigation.

Previous studies demonstrated that ischemia may lead to the release of cytochrome *c* from mitochondria into cytosol<sup>[9, 49-53]</sup>, which was shown in the present study. Here we showed that Gs-Rb1 can significantly inhibit cytochrome *c* release to the cytoplasm, PARP cleavage and caspase-3 activation in hypoxia cardiomyocytes, which may be partly inhibited by wortmannin or Ara A. It appears that mPTP opening is a main mechanism mediating cytochrome *c* release<sup>[17, 20, 54]</sup>; then cytochrome *c* release and PARP cleavage activate the caspase cascade, setting apoptosis in motion<sup>[54]</sup>; thus, the effects of Gs-Rb1 were evident in those pathways at least. However,

whether other pathways take part in mediating the Gs-Rb1 effect has been unknown. Overall, the mitochondrial pathway is an important one in Gs-Rb1 helping cardiomyocytes resist hypoxia apoptosis<sup>[55,56]</sup>.

In the present study, we found that neither Ara A nor wortmannin completely inhibited the activation of GSK-3 $\beta$  and the opening of mPTP mediated by Gs-Rb1, which suggested that other pathways exist that help to control the effect of Gs-Rb1 on GSK-3 $\beta$  and mPTP. Some studies have shown that the activation of intracellular kinase cascades, especially PKC<sup>[57]</sup>, PKG<sup>[58]</sup> and mTOR/p70s6K<sup>[59-61]</sup>, plays a key role in preventing cardiomyocytes from ischemic/reperfusion injury. However, whether these signal transduction networks, together with AMPK and PI3K pathways, modulate the Gs-Rb1 inhibition of mPTP opening requires further study.

The present data strongly suggest that inhibiting mPTP opening is an important mechanism by which Gs-Rb1 protects cardiomyocytes from hypoxia-induced apoptosis; this is partially controlled by the downregulation of GSK-3 $\beta$ . In addition, the GSK-3 $\beta$  and/or the mPTP effects of Gs-Rb1 may be partly regulated by both the AMPK and PI3K signaling pathways; however, it is unclear whether there are other pathways, particularly death receptor pathways, taking part in the effects of Gs-Rb1 anti-apoptosis. In conclusion, our findings highlight a novel anti-apoptosis mechanism of Gs-Rb1 and suggest the importance to further ascertain the exact mechanism of how Gs-Rb1 improves hypoxic cardiomyocytes in resisting apoptosis *in vivo*.

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### Author contribution

Hong-liang KONG and Zhan-quan LI designed the experiments; Hong-liang KONG, Yao FU, Tong LI and Hui-jun LI performed the experimental protocols; Zhan-quan LI, Hong-liang KONG, Ying-jun ZHAO and Shu-mei ZHAO contributed new analytical tools and reagents; Hong-liang KONG and Yao FU analyzed data; Hong-liang KONG, Shu-mei ZHAO and Li ZHU wrote the paper.

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