Magnesium lithospermate B improves myocardial function and prevents simulated ischemia/reperfusion injury-induced H9c2 cardiomyocytes apoptosis through Akt-dependent pathway

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Abstract

Ethnopharmacological relevance: Magnesium lithospermate B (MLB), an active polyphenol acid of Radix Salviae Miltiorrhiza (Danshen), showed a wide range of pharmacological activities in cardiovascular diseases. However, its role in protection against ischemia/reperfusion injury in H9c2 cardiomyocytes has not been elucidated. This study was aimed to investigate the protective effect and potential molecular mechanisms of MLB on apoptosis in H9c2 cardiomyocytes in vitro.

Materials and methods: We tested cell viability, shortening amplitude, necrosis, apoptosis, and the expression levels of Akt, phosphorylated Akt, Bcl-2 and Bax protein after they were subjected to global 30-min ischemia and 180-min reperfusion.

Results: Pretreatment with MLB markedly increased cell viability and while reducing evidence of necrosis and apoptosis in H9c2 cardiomyocytes. In addition, the expression of Bcl-2 and Bax protein was modulated. The results also showed that MLB significantly increased phosphorylation of Akt and that this phosphorylation can be partially inhibited by phosphoinositide 3-kinase/Akt inhibitor. Furthermore, MLB improved MI/R-induced myocardial contractile function.

Conclusion: Our results showed that MLB prevents IR-induced myocardial damage by reducing necrosis and apoptosis in H9c2 cardiomyocytes and improving myocardial function in rat hearts.

1. Introduction

Myocardial ischemia/reperfusion (MI/R) injury, a general health problem, is due to blood restoration after a critical period of coronary artery obstructions. It associates with a series of clinical problems such as thrombolysis, angioplasty, and coronary bypass surgery (Yellon and Hausenloy, 2007). It is widely accepted that MI/R injury is a pathological process that results in extensive cardiomyocytes death (Zhao et al., 2000; Yucel et al., 2011).

Cardiomyocytes death involves apoptotic and necrotic cell death, and apoptosis is a significant cellular mechanism responsible for MI/R injury in myocardium (Buja and Entman, 1998; Freude et al., 2000). Thus, therapeutic strategies aim at preventing or delaying cardiomyocytes apoptosis and death may be a reasonable choice for the treatment of related heart disease, especially on MI/R injury (Hausnutter and Izumo, 2000). In the past decades, many traditional Chinese medicines (TCM) have been claimed to be useful for the control of problems due to reperfusion and associated pathologies (Dong et al., 2011; Hwa et al., 2012). Therefore, searching for anti-apoptotic compounds with minimal side effects from natural sources like herbs or plants probably represent an ideal strategy to develop safe and effective agents for MI/R injury treatment.

Radix Salviae miltiorrhiza [Danshen (Labiatae)], an important herb in Oriental medicine, has been used extensively for the treatment of coronary artery diseases, angina pectoris, myocardial infarction and cerebrovascular diseases (Zhou and Ruigrok, 1990; Fong et al., 2011; Chiu et al., 2012). Magnesium lithospermate...
2.1. Materials

MLB (MLB, Fig. 1), an active polyphenol acid extracted from the dried root of Danshen, was known to have antioxidative and antifibrotic effects (Hur et al., 2010; Paik et al., 2011). A wide range of pharmacological activities of MLB in cardiovascular diseases had been reported in the past decades (Chen and Wang, 2006; Hur et al., 2008). In our previous study, the heart protective effect of MLB on a rat model of MI/R injury had been well investigated (Wei et al., 2013). We found MLB decreased myocardial infarct size, alleviated histopathological damage. However, whether MLB has a protective effect against cardiomyocytes apoptosis was poorly understood in vitro. Thus, we investigated the cardioprotective role of MLB against H9c2 cardiomyocytes apoptosis induced by simulated ischemia and reperfusion (SI/R) injury. The present study was designed to examine the effect of MLB treatment on H9c2 cardiomyocytes necrosis and apoptosis and its effects on contractile function.

Finally, it is of importance to explore the action mechanism of MLB as a novel therapeutics for MI/R treatment. It is well known that the PI3K/Akt is a powerful survival signaling pathway in many systems. Inhibition of PI3K accelerated apoptosis, and activation of Akt blocked apoptosis (Hemmings and Restuccia, 2012; Prasad et al., 2012). Activation of the PI3K/Akt pathway may be useful to promote cardiomyocytes survival in the damaged heart. We hypothesized that MLB might inhibit cell apoptosis caused by SI/R in H9c2 cardiomyocytes in vitro through activating PI3K/Akt signaling pathway.

2. Materials and methods

2.1. Materials

MLB (molecular formula: C_{36}H_{28}MgO_{16}; molecular weight: 740.90, purity >95%) was provided by Xi’an Honson Biotechnology Co., Ltd (Xian, China). Dubbeco’s modified Eagle’s medium (DMEM) was obtained from Gibco Co. (Grand Island, NY). Fetal bovine serum (FBS) was provided by Hangzhou Sijiqing Biological Engineering Materials Co., Ltd (Hangzhou, China). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide], type I collagenase and western blot reagents were purchased from Sigma Co. (St. Louis, MO). The kits for determination of lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB) were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Anti-Phospho-Akt, anti-Akt, anti-Bax, anti-Bcl-2 and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology Co. (CA, USA). The fluorescent kit for Hoechst 33258 was obtained from Roche Co. (Mannheim, Germany). LY-294002 was obtained from Calbiochem Novabiochem Co. (San Diego, USA). The caspase-3 assay kit was purchased from Chemicon International Co. (Temecula, CA, USA).

2.2. Cell culture

Rat H9c2 cardiomyocytes cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The H9c2 cells were maintained in DMEM supplemented with 10% fetal calf serum at 37 °C in CO2 incubation. The medium was replaced every 2–3 days, and cells were subcultured or subjected to experimental procedures at 80–90% confluence.

2.3. Simulated ischemia/reperfusion injury model

The oxygen and glucose deprived (OGD) technique was based on a previously described protocol (Rizvi et al., 2010). In our study, the OGD injury was produced by incubating with blank solution and exposed to a hypoxic environment of 95% N2 and 5% CO2 in airtight gas chambers at 37 °C for 2 h (Billups-Rothenberg, USA). After OGD treatment, cells were removed from the gas chamber, and the OGD solution was replaced with warmed culture medium for 24 h (recovery period) in a CO2 incubator at 37 °C. After 24 h cultured, H9c2 cardiomyocytes were used in subsequent experiments. H9c2 cardiomyocytes were randomly divided into seven groups: (1) Control group without any treatment; (2) SI/R group (model group), which was cultured under OGD for 2 h and then under recovery conditions for 24 h; (3–5) SI/R+MLB groups, which were pretreated with MLB at concentrations of 20, 40 or 60 μg/ml for 24 h before OGD; (6) SI/R+MLB+LY group, pretreated with MLB (60 μg/ml) for 24 h before OGD, and then cardiomyocytes were pretreated with LY-294002 (PI3K inhibitor, 10 μM) 1 h before OGD. (7) SI/R+LY group, cardiomyocytes were only pretreated with LY-294002 1 h before OGD.

2.4. Cell viability assays

Cell viability was determined by the MTT assay. H9c2 cardiomyocytes were seeded at a density of 4 × 10^4 cells/well in 96-well plates. After different treatment, 20 μl of the MTT solution (5 mg/ml) was added into each well and made the final concentration of 5 mg/ml for 2 h at 37 °C. After that, the medium was removed and DMSO (150 μl) was added into each well. The optical density (OD) was determined spectrophotometrically at 490 nm with a microplate reader (Infinite M200 PRO, Switzerland) and the H9c2 cardiomyocytes survival ratio were expressed as a percentage of the control.

2.5. LDH and CK-MB activities assays

The activities of LDH in the cultured supernatant, CK-MB activities in H9c2 cardiomyocytes were measured with a microplate reader (Model 550, USA) using diagnostic kits, according to the manufacturer’s instructions, respectively.

2.6. Annexin-V/PI assay

In brief, H9c2 cardiomyocytes were collected, washed with calcium-free PBS, resuspended with binding buffer and incubated with Annexin-V at room temperature in the dark for 10 min. Then the H9c2 cardiomyocytes were centrifuged and resuspended with binding buffer. PI was added to the resuspended cardiomyocytes before they were analyzed with a flow cytometer (Becton Dickinson, USA).

Fig. 1. Chemical structure of magnesium lithospermate B (MLB). Molecular formula: C_{36}H_{28}MgO_{16}; molecular weight: 740.90.
2.7. Hoechst 33258 staining assay

Briefly, H9c2 cardiomyocytes were washed in ice-cold PBS, fixed in 10% neutral buffered formalin for 10 min at room temperature, and washed again in ice-cold PBS. Then, the H9c2 cardiomyocytes were exposed to Hoechst 33258 (2 μg/ml in PBS) and incubated for 20 min at room temperature. The H9c2 cardiomyocytes were then washed three times in PBS and examined under a fluorescence microscope with an appropriate filter.

2.8. Caspase-3 activity assay

Caspase-3 activity was measured using a colorimetric activity assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer’s protocol. In brief, control or SI/R treated H9c2 cardiomyocytes were lysed in ice-cold lysis buffer, placed on ice for 30 min, and then centrifuged at 4 °C for 15 min at 16,000 rpm. After determining the protein concentration, the supernatant was incubated with the caspase-3 substrate (Ac-DEVD-pNA) on a 96-well-plate. The activity of caspase-3 was determined using a Microplate Reader at 405 nm.

2.9. Western blot analysis

The whole protein 50 μg was resolved on a 15% SDS-polyacrylamide gel. The fractionated proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membranes and probed with rabbit polyclone anti-phosphor-Akt, anti-Akt, anti-Bcl and anti-Bax at overnight 4 °C followed by incubation with the corresponding secondary antibodies at room temperature for 2 h. The blots were visualized with ECL-Plus reagent (GE Healthcare, Piscataway, NJ). In some immunoblotting experiments, the blots were reprobed with an anti-β-actin antibody to control for the protein loading.

2.10. Animal myocardial ischemia/reperfusion injury model

Adult male Sprague-Dawley rats (250 ± 20 g) were supplied by the animal research center at Fourth Military Medical University, Xi'an, China. The MI/R model was produced as described previously (Zhu et al., 2008). Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally). Myocardial ischemia was produced by exteriorizing the heart with a left thoracic incision followed by making a slipknot (6–0 silk) around left anterior descending coronary artery. After 30 min of ischemia, the slipknot was released and followed by 3 h of reperfusion. Rats were randomized into 3 groups: (1) Sham group, rats underwent the same surgical procedures with the exception of left anterior descending coronary artery occlusion; (2) MI/R+vehicle group, MI/R rats received the same volume of saline alone; (3) MI/R+MLB (60 mg/kg) group, MI/R rats received MLB 60 mg/kg. The dose mentioned in previous report was used in this study (Wei et al., 2013). Drug was administered via intravenously injection within 5 min at the beginning of reperfusion.

2.11. Cardiac function assay

Cardiac function was continuously monitored before and during the entire reperfusion procedure. A fluid filled-latex balloon connected to a pressure transducer was inserted into the left ventricle via a small incision in the left atrium and inflated to set a left ventricular end-diastolic pressure (LVEDP) (5–10 mmHg) using a hemodynamic analysis system (Taimeng Co., Chengdu, China) for the recording of functional parameters, such as heart rate (HR), left ventricular systolic pressure (LVSP), and the rate in rise and fall of ventricular pressure (± dp/dtmax).

2.12. Statistical analysis

Data were presented as mean ± S.D. and analyzed by Graphpad Prism software version 5.01. One-way ANOVA followed by Tukey’s test was applied for the statistical analysis of the parameters. Value of at P < 0.05 was considered statistically significant.

3. Results

3.1. Effects of MLB on cell viability

Cell viability was shown in Fig. 2 using the MTT assay. We first examined the viability of H9c2 cardiomyocytes after treatment with MLB (0.64–400 μg/ml) in Fig. 2A. From this test, we found that MLB at concentrations 16–80 μg/ml did significantly affect cell viability. On the basis of the results of the previous step, four concentrations (20, 40, 60 and 80 μg/ml) were selected for further investigate (Fig. 2B). After SI/R, there were only 39.7 ± 4.2% (P < 0.05, Fig. 2B) viable H9c2 cardiomyocytes as compared to
3.3. Effects of MLB on cell apoptosis

In control group, most H9c2 cardiomyocytes were viable. SI/R stimulation induced apoptotic damage in H9c2 cardiomyocytes compared to control group (15.73 ± 0.77%, P < 0.05, Fig. 4B and F). While treated with MLB, the apoptotic H9c2 cardiomyocytes were decreased markedly (to 6.95 ± 1.05%, P < 0.05, Fig. 4E and F) compared to SI/R group.

Nuclear morphological change was observed by Hoechst 33258 staining, which illustrated that the control cells exhibited uniformly dispersed chromatin, normal organelles and intact cell membranes (Fig. 5A). After SI/R injury, cardiomyocytes featured typical characteristics of apoptosis, including shrinkage of the nuclei, chromatin condensation and the appearance of a few apoptotic bodies (Fig. 5B). However, with MLB pretreatment, the morphological changes were significantly attenuated, and the number of cells with nuclear condensation and fragmentation was significantly decreased (Fig. 5E).

3.4. Effects of MLB on caspase-3 activity

To further characterize the inhibitory effect of MLB on myocardial cell apoptosis, we examined whether MLB could inhibit caspase-3 activity. As illustrated in Fig. 6, SI/R resulted in a noticeable increase in caspase-3 activity compared with that of control group (153.1 ± 10.2, P < 0.05, Fig. 6), while 60 μg/ml MLB reduced the level of caspase-3 significantly compared with that of SI/R group (119.9 ± 7.6, P < 0.05, Fig. 6). However, compared with the MLB-treated group, treatment with MLB plus LY-294002 markedly increased active caspase-3 production (129.1 ± 21, P < 0.05, Fig. 6).

3.5. Intracellular mechanisms of the anti-apoptosis by MLB

The phosphorylation of Akt (p-Akt) expression level was determined by western blot analysis (Fig. 7A). The H9c2 cardiomyocytes were subjected to SI/R in different time (including 10 min, 30 min, 6 h, 12 h, 24 h and 48 h). At 48 h the expression level of p-Akt decreased to the lowest. The effect of MLB on p-Akt expression under SI/R condition was investigated then (Fig. 7C). In SI/R group, the ratio of p-Akt to Akt was markedly increased (144.4 ± 7.6, P < 0.05, Fig. 7D) compared with control group (100.8 ± 3.6, P < 0.05, Fig. 7D); however, MLB (60 μg/ml) pretreatment for 24 h before OGD significantly increased p-Akt level compared with SI/R group (335.9 ± 29.0, P < 0.05, Fig. 7D). Western blot analysis showed that PI3K inhibitor LY-294002 (10 μM) 1 h before OGD inhibited the increase of p-Akt level induced by MLB pretreatment and almost restored p-Akt level to basal level (115.9 ± 10.1, P < 0.05, Fig. 7D). It was demonstrated that PI3K/Akt signaling pathway involved in the anti-apoptotic effect of MLB.

Furthermore, SI/R potently slightly increased Bcl-2 expression (138.3 ± 4.8 vs 102.0 ± 3.9, P < 0.05, Fig. 8B) and promoted Bax expression (170.9 ± 4.8 vs 99.2 ± 4.3, P < 0.05, Fig. 8C) in SI/R-injured H9c2 cardiomyocytes compared with control group, however, MLB (60 μg/ml) pretreatment for 24 h before OGD remarkably changes the effects of SI/R on Bcl-2 and Bax expressions (324.8 ± 9.4, P < 0.05, Fig. 8B; 89.2 ± 1.9, P < 0.05, Fig. 8C). LY-294002 (10 μM) 1 h before OGD significantly reversed the expressions of Bcl-2 and Bax of MLB (198.4 ± 7.1, P < 0.05, Fig. 8B; 152.3 ± 2.2, P < 0.05, Fig. 8C), which confirmed the anti-apoptotic effect of MLB on SI/R-induced apoptosis.

3.6. Effect of MLB on the cardiac function

Before ischemia, there were no apparent differences in baseline parameters between all experimental groups. However, when

3.2. Effect of MLB on cell necrosis

The release of LDH and CK-MB was used as an index of cardiomyocytes necrosis. LDH and CK-MB release in model group (464.7 ± 35.4 U/L and 23.80 ± 4.18 U/ml, respectively, P < 0.05, Fig. 3A and B) was higher than that in control group (149.9 ± 33.8 U/L and 9.81 ± 1.94 U/ml, respectively, P < 0.05, Fig. 3A and B), and significant decrease of LDH and CK-MB activities were observed in the MLB groups (to 287.6 ± 41.3 U/L and 12.18 ± 2.21 U/ml, respectively, P < 0.05, Fig. 3A and B) than that in model group.

the control ones. MLB (20, 40 and 60 μg/ml) prevented H9c2 cardiomyocytes from SI/R-induced damage, restoring H9c2 cardiomyocytes survival to 76.0 ± 3.3% (P < 0.05, Fig. 2B). Then, the three concentrations (20, 40 and 60 μg/ml) were therefore used in subsequent in vitro studies.
compared with the control group, the MI/R injury led to left ventricular dysfunction characterized by a significant decrease in HR, LVSP, and \( \pm dp/dt_{\text{max}} \) in the MI/R group. MLB treatment resulted in the partial prevention of reperfusion damage by increasing LVSP, \( \pm dp/dt_{\text{max}} \) and HR as compared with MI/R group. The LVEDP was not significantly changed (Table 1).

4. Discussion

There have been a number of studies illustrating the benefits of MLB treatment in cardiovascular diseases, and evidence points to the possibility that MLB may act on multiple targets leading to a concerted positive effect. However, the particular pathway and underlying mechanisms have yet to be elucidated.

Accumulating evidence indicates that apoptosis plays an important role in many cardiovascular diseases, including MI/R injury (Freude et al., 2000; Zhao et al., 2000). Blocking the apoptosis process could be prevent the loss of contractile cells, minimize cardiac injury induced by MI/R injury and therefore, slow down or even prevent the occurrence of heart failure (Haunstetter and Izumo, 2000). No previous studies have investigated the direct effects of MLB on the cardiomyocytes under SI/R conditions. In the present study, we investigated the possible molecular mechanisms underlying the effects of MLB to protect H9c2 cardiomyocytes against SI/R-induced apoptosis.

It is generally believed that MI/R plays a primary role in the loss of cardiomyocytes; therefore, preventing necrosis and apoptosis from progressing during MI/R injury may help to slow down or even prevent the occurrence and development of heart failure. As such, it is very important to find a molecular target that is
involved in protecting cardiomyocytes against MI/R-induced necrosis and apoptosis. We monitored the cardiomyocytes survival rate through MTT assay. The percentage of the H9c2 cardiomyocytes survival was decreased in model group and MLB protected cells in a dose-dependent manner by attenuating H9c2 cardiomyocytes necrosis as displayed by reduced LDH and CK-MB release into the culture medium. As is known, LDH and CK-MB are expressed constitutively in mammalian cells and affluent in endochylema. They cannot shuttle across cytoplasmic membranes in the normal physiological state. But, when cell is damage or dead, LDH and CK-MB are released out of cell (Gurgun et al., 2008). So the LDH and CK-MB levels in the culture media are measured to represent the extent of H9c2 cardiomyocytes injury induced by SI/R. More importantly, the present study has demonstrated that MLB improved heart contractile function during reperfusion via increase in ± dp/dt_max, and it also limited the decline of LVSP and the elevation of LVEDP in rats. Based on these results, we demonstrated that MLB protected H9c2 cardiomyocytes from SI/R-induced injury by having a significant role in the improvement of cardiomyocytes contractile function and promotion of anti-necrotic and anti-apoptotic effects after SI/R injury.

The anti-apoptotic effects were also confirmed by the results of Annexin-V/PI staining. The results strongly suggested that MLB might exert a protective effect against the SI/R induced cytotoxicity and apoptosis. Furthermore, we also demonstrated that MI/R led to a significant increase in Hoechst 33258-positive H9c2 cardiomyocytes, while incubating with MLB before SI/R for 24 h significantly decreased SI/R-induced apoptosis. Cardiomyocytes apoptosis is one of the major contributors to the development of myocardial infarcts (Gottlieb et al., 1994; Blalik et al., 1997), which is related to the pathogenesis of heart failure. Caspase-3 is thought to be activated during the final step of the proapoptotic signaling pathway in many cell lines. Inhibition of caspase activity has been shown to attenuate both MI/R injury and apoptosis in cardiomyocytes (Gustafsson et al., 2002; Borutaite and Brown, 2003). In our experiments, we found that MLB can upregulate Caspase-3 level, inhibiting H9c2 cardiomyocytes apoptosis. We believe that the anti-apoptotic effect of MLB may be mediated by Caspase-3.

On the basis of obtaining results that MLB protected H9c2 cardiomyocytes against apoptosis induced by SI/R, further investigation was performed with focus on the possible mechanisms involved in the anti-apoptotic effects of the MLB. The PI3K/Akt is a powerful survival signaling pathway in many systems (Hemmings and Restuccia, 2012). Activated PI3K and Akt are each sufficient to protect cardiomyocytes against apoptosis, and activation of Akt blocked apoptosis (Jie et al., 2012). Activation of PI3K/Akt pathway may be useful to promote cardiomyocytes survival in the damaged heart (Ravingerova et al., 2012). The phosphorylated Akt level in cardiomyocytes treated with MLB were further elevated, suggesting that the anti-apoptotic effect of MLB is Akt dependent. Meanwhile, Akt exerts its protective effects through phosphorylation of diverse target molecular such as Bcl-2 family. Previous studies have also indicated that an increase in proapoptotic Bax family proteins and a decrease in anti-apoptotic Bcl-2 family proteins are involved in the process of apoptosis (Produit-Zengaffinen et al., 2009). Thus, the
ratio of Bcl-2 and Bax may be a critical factor in the cellular threshold for apoptosis. In the present study, MLB treatment upregulated the expression of Bcl-2 and downregulated the expression of Bax. These changes caused an increased in the Bcl-2/Bax ratio. Based on these results, it could be reasonably speculated that MLB might attenuate apoptosis. In addition, it is well known that caspase-3 activity can be induced by the proapoptotic Bax and inhibited by the anti-apoptotic Bcl-2. Bax can neutralize Bcl-2 actions by forming heterodimers with Bcl-2 (Hasenjager et al., 2004; Abdel-Latif et al., 2009). Our data showed that MLB inhibited the cell apoptosis through suppressing Bax expression, and increasing Bcl-2 synthesis so as to maintain Bcl-2/Bax balance. Moreover, to dissect the role of MLB in protecting cardiomyocytes against SI/R-induced injury, LY-294002, a PI3K/Akt inhibitor, was used in our present study. The data demonstrated that LY-294002 blocked MLB elicited cardioprotection, inhibited the phosphorylation of Akt, decreased the Bcl-2/Bax ratio. Therefore, our findings showed the crucial participation of the PI3K/Akt pathway in MLB-induced survival signaling in H9c2 cardiomyocytes.

5. Conclusion

In conclusion, we have revealed a novel function of MLB by demonstrating its significant role in the regulation of cardiomyocytes contractile function and promotion of anti-necrotic and anti-apoptotic effects following SI/R injury. Our data have also shown that increased expression of p-Akt, Bcl-2 and decrease of Bax and
caspase-3 play an essential role in the cardioprotective effect of MLB. These beneficial effects were reversed by LY-294002. We could conclude that MLB possessed anti-apoptosis against SJ/R injury on cardiomyocytes via activation of Akt-dependent pathway. The present study may provide important insights for the understanding of the molecular mechanisms involved in the cardioprotective effect of MLB.

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