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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 Miltiorrhizae* (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 after 2-h simulated ischemia and 24-h simulated reperfusion in H9c2 cardiomyocytes. We further observed the contractile function in hearts 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 I/R-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 (Haunstetter 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 miltiorrhizae [Danshen (*Labiatae*)], an important herb in Oriental medicine, has been used extensively for the treatment of coronary artery diseases, angina pectoris, myocardial infarction and celebrovascular diseases (Zhou and Ruigrok, 1990; Fong et al., 2011; Chiu et al., 2012). Magnesium lithospermate

Abbreviations: SI/R, simulated ischemia/reperfusion; MLB, magnesium lithospermate B; LDH, lactate dehydrogenase; CK-MB, creatine phosphokinase-MB; ELISA, enzyme-linked immunosorbent assay; OGD, oxygen and glucose deprived; MI/R, myocardial ischemia/reperfusion

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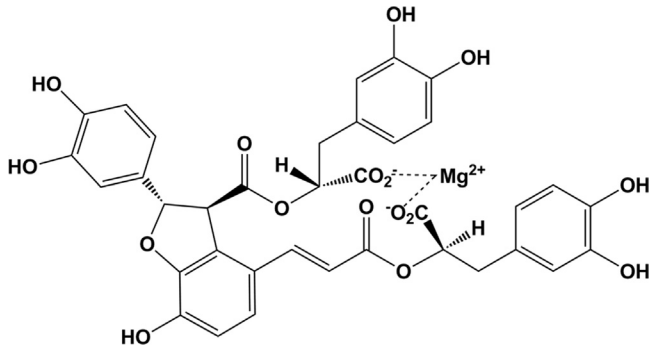

Fig. 1. Chemical structure of magnesium lithospermate B (MLB). Molecular formula: $C_{36}H_{28}MgO_{16}$; molecular weight: 740.90.

B (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 induce 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 (Xi'an, China). Dulbecco'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 Hoechest 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 with10% fetal calf serum at 37 °C in CO_2 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% N_2 and 5% CO_2 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 CO_2 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).