Phytomedicine 34 (2017) 106–114

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Original Article

Tilianin pretreatment prevents myocardial ischemia-reperfusion injury via preservation of mitochondrial function in rat heart

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ARTICLE INFO

Keywords:
Tilianin
MRI
Mitochondria
Apoptosis
Protective effect
Mechanisms

ABSTRACT

Background: Tilianin has been demonstrated to exert protective effects on the heart against ischemia-reperfusion (I/R) injury, yet whether it is beneficial to the mitochondria during myocardial I/R is unclear.
Methods: In this study, we demonstrated that pretreatment with Tilianin dose-dependently raised the levels of ATP of the myocardium, and protected the microstructures and functions of mitochondria in rats. Furthermore, the cytoprotective effect of Tilianin has been confirmed *in vivo* and in the H9c2 cardiomyoblast cell line with enhancing activities of the mitochondria, controlling the levels of Ca^{2+} and reactive oxygen species (ROS), and inhibiting the expression of caspase-3 and AIF in cytoplasm.
Conclusions: In conclusion, the study suggests that Tilianin may be of clinical value for the protective effects of cardiomyocytes and mitochondria by inhibiting myocardium energy metabolism and apoptosis during myocardial ischemia-reperfusion injury (MIRI).

Introduction

MIRI contributes to a series of severe adverse cardiovascular outcomes after myocardial ischemia, cardiac surgery, or circulatory arrest (Brevoord et al., 2012; Liu et al., 2013). Developing novel drugs or interventions to improve the clinical outcomes of patients with I/R is a worldwide unmet medical need.

Discovering new biologically active compounds for cardiovascular disease treatment from natural products, especially from herbs, has been continuously attractive for scientists. *Dracocephalum* is a large genus of the Lamiaceae family that includes about 70 species (rarely semishrubs) (Kakasy, 2006). Among them, the most extensively studied species is *Dracocephalum moldavica* L., which is traditionally used in the ethnomedicine of European countries for the treatment of hypertension and heart diseases. In Iran, this plant known as Badershoo has traditionally been used as a cardiotonic agent in folk medicine. In China, *D. moldavica* L. has been used to treat cardiovascular diseases in the Uyghur community for over 800 years (Kang et al., 2013; L., 2008). Previous pharmacological studies (Cao et al., 2011, 2013; Mailudemu Maimaituxun et al., 2009; Tian et al., 2012; Xing et al., 2013; Yang et al., 2010) have confirmed its antioxidative (Wang et al., 2011; Yuan et al., 2010b), hepatoprotective, antibiosis, anti-inflammatory, and cardioprotective properties (Najafi et al., 2009). Further studies focused on separating and analyzing the phytochemical compounds of *D. moldavica* L. revealed that the major active components from the plant abstract were derivatives of flavonoids, triterpenoids, steroids, penylpropanoids, iridoids, and polysaccharides (Fan et al., 2013; Yang et al., 2013). Importantly, phytochemical and pharmacodynamics studies showed that the major flavonoid in *D. moldavica* L. was separated and identified as Tilianin (Yuan et al., 2010a). Tilianin has been proved to possess pharmacological activities: it could induce nitric oxide synthase (iNOS) expression and production of nitric oxide (NO), and may act as an anti-inflammatory agent (Nam et al., 2006). Another study revealed that Tilianin showed an obvious protective effect on MIRI rats (Guo et al., 2015).

However, the mechanism of the protective effect of Tilianin to the

Abbreviations: I/R, ischemia-reperfusion; ROS, reactive oxygen species; MIRI, myocardial ischemia-reperfusion injury; mPTP, mitochondrial permeability transition pore; SD, Sprague-Dawley; CsA, Cyclosporine A; ECG, Electrocardiogram; LAD, left arterial descending; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; H/R, hypoxia/reoxygenation

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http://dx.doi.org/10.1016/j.phymed.2017.08.007
Received 30 December 2016; Received in revised form 9 April 2017; Accepted 6 August 2017

heart remains unclear. During the I/R process, ATP depletion as a result of ischemic hypoxia leads to the excessive production of free radicals from the mitochondrial respiratory chain during reperfusion. Such reperfusion-stimulated extensive oxidative damage is believed to be the main trigger for myocardial cell damage and death in the reperfusion injury (Turrens et al., 1991;). In addition, impaired mitochondria release ROS, which may be proposed to activate the mitochondrial permeability transition pore (mPTP) and membrane potential falling rapidly in adjacent mitochondria, so as to trigger a new round of ROS burst. The burst out of ROS may activate the Ca^{2+} overload, ultimately lead to the mitochondrial membrane structure damaged. So the mitochondria is not only the main organelles of ROS produced and released, but also is the main target of ROS causing oxidative damage (Halestrap et al., 2004). In recent years, it has found that AIF, an apoptosis promoting protein which located in mitochondria, is involved in a Caspase independent process induced apoptosis. In stress conditions, mitochondrial AIF is released in cytoplasm and transferred to the nucleus to hydrolyze DNA. It was also reported that AIF plays an important role after hypoxia-reoxygenation: the increase of ROS, Ca^{2+} content and mitochondrial membrane function can activate AIF on membrane (Halestrap et al., 2004).

Based on this, the current study aims to evaluate the mitochondrial protective effect of Tilianin on the myocardium in a rat model of myocardial I/R injury, as well as an *in vitro* model of H9c2 cardiomyoblast cell line. The potential mechanism underlying Tilianin-mediated cytoprotection of MIRI-injured myocardium was investigated.

Materials and methods

Animal myocardial I/R model and drug administration

Healthy male adult Sprague-Dawley (SD) rats (weighing 250–300 g, 2 months of age) were procured from the Experimental Animal Centre of XinJiang Medical University (Certificate Number: syxk2003-0001). They were kept at 25 °C in a well ventilated animal house under 12 h light/dark cycle, maintained in compliance with the guidelines of the animal ethics committee of the Institute. The rats were divided into 7 groups: sham-operation group (Sham, $n = 10$); I/R model group (IR, $n = 10$); three Tilianin-treated groups (T-H, 5 mg/kg, $n = 10$; T-M, 2.5 mg/kg, $n = 10$; and T-L, 1.25 mg/kg, $n = 10$) (Guo et al., 2015); the propranolol group (Prop, 25 mg/kg) and the Cyclosporine A group (CsA, 20 mg/kg). Tilianin was obtained from XingJiang Institute of Materia Medic, China with purity of 98%. The Propranolol group was used as the positive control meanwhile the T-H\M\L group and the Prop group were administered by gavage once a day for 7 days. mPTP opening inhibitors CsA was administered by tail intravenous injection before reperfusion, and standard rat chow or water *ad libitum*. The sham group and IR group were fed with vehicle (distilled water) by oral gavage once a day for 7 days, along with standard rat chow and water, *ad libitum*. The treatment schedule did not cause any change in food and water intake patterns. Seven days after drug gavage, the animals were fasted overnight and were then forced fed for the last time. The animal study was performed in conformance with the guidelines of National Institutes of Health.

The *in vivo* myocardial I/R model was modified from a previous study (Qiao et al., 2011). Electrocardiogram (ECG) was continuously monitored throughout the experiment. Briefly, rats were anesthetized with 25% urethane (0.5 ml•100 g^{-1}, intraperitoneally). The rats were intubated and mechanically ventilated with air using a rodent respirator. A left thoracotomy was performed and the pericardium was opened to expose the heart. The left arterial descending (LAD) coronary artery was ligated 2 mm from its origin by a 5–0 silk suture with a traumatic needle. The ECG S-T elevation was obserbated for a myocardial ischemia signs of success. After a 30-min ischemia, the myocardium was reperfused by releasing the snare gently for a period of 2 h. In reperfusion, 50% or more of the ST elevation was dropped, and T gradually was restored. The sham control animals were subjected to the entire surgical procedure described above, except for the LAD ligation and release. After reperfusion, the blood from abdominal aorta was taken, standing for 30 min, and centrifuged (0–4 °C, 3000 rpm, 10 min). The supernatant fluid was stored in −80 °C refrigerator, prepared for the detection of serology indexes. The heart was quickly removed, and washed with cold physiological salty water to clean the blood inside the heart. The ischemic tissue in left ventricle was cut off to be cryopreserved standby for later histological examinations.

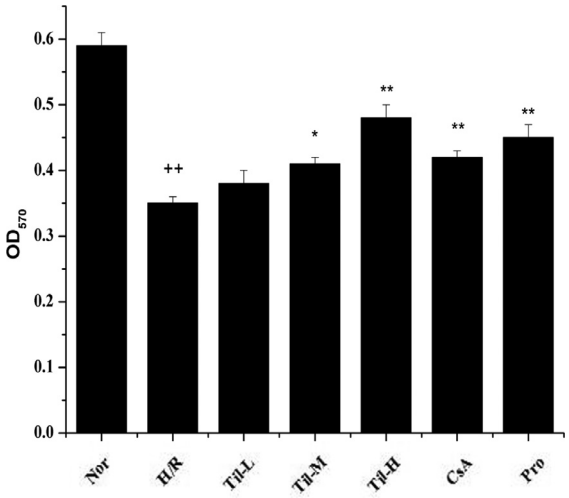

Fig. 1. Tilianin increases myocardium activity in H/R H9c2 ($n = 3$). All data were expressed as Means ± SD. Compared with Nor group, ++ $P < 0.01$; compared with the H/R group, * $P < 0.05$, ** $P < 0.01$ and compared with the CsA group, # $P < 0.05$.

Cell culture and drug administration

H9c2 rat cardiac cell line was from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China, and cultured in Dulbecco's modified eagle medium (DMEM) from Gibco, USA, supplemented with 10% fetal bovine serum (FBS) from Gibco, USA. The cell was kept at 37 °C in a humidified atmosphere consisting of 5% CO_2. Cells were exposed to 1% O_2 (hypoxia) for 3 h in the culture medium deprived of serum followed by reoxygenation under normoxic conditions in complete culture medium for 2 h to mimic MIRI condition.

Cells were divided into 6 groups: the normal group (Nor, $n = 10$); the H/R model group (H/R, $n = 10$); three Tilianin-treated groups (Til-H, $n = 10$; Til-M, $n = 10$, Til-L, $n = 10$) treated Tilianin in three doses: 20 μg ml^{-1}, 10 μg ml^{-1}, and 5 μg ml^{-1} (Fig. S1); and the Propranolol group (0.6 μg ml^{-1}, Pro) as the positive control of Tilianin. The mPTP opening inhibitors—Cyclosporine A group (0.24 μg ml^{-1}, CsA) were fed before reoxygenation, and the other drugs were fed before hypoxia.

Cellular activity detection

Mitochondrial suspension was prepared from the cardiac tissue by the mitochondrial isolation kit. 50 μl of mitochondria suspension was added to the microporous enzyme label plate, then 20 μl of MTT was added and mixed at 37 °C for 30 min. After 20 min 50 μl of isopropyl alcohol was added for detection using an enzyme-linked immune detector at 570 nm.

Preparation of mitochondria

Mitochondria were isolated from the cardiac tissue or the H9c2 cells using a Tissue/Cell Mitochondria Isolation Kit following the manufacturer's recommendations from the Applygen Technologies Inc. After centrifugation, the pellet was kept as the mitochondria and resuspended