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Dihydromyricetin attenuated Ang II induced cardiac fibroblasts proliferation related to inhibitory of oxidative stress

Qiuyi Song[1], Lulu Liu[1], Jin Yu, Jingyao Zhang, Mengting Xu, Linlin Sun, Huiqin Luo, Zhaosong Feng, Guoliang Meng*

Department of Pharmacology, School of Pharmacy, Nantong University, Key Laboratory of Inflammation and Molecular Drug Target of Jiangsu Province, Nantong, Jiangsu, China

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ABSTRACT

Dihydromyricetin (DMY) is one of the most important flavonoids in vine tea, which showed several pharmacological effects. However, information about the potential role of DMY on angiotensin II (Ang II) induced cardiac fibroblasts proliferation remains unknown. In the present study, cardiac fibroblasts isolated from neonatal Sprague-Dawley rats were pretreated with different concentrations of DMY (0–320 μM) for 4 h, or DMY (80 μM) for different time (0–24 h), followed by Ang II (100 nM) stimulation for 24 h, Then number of cardiac fibroblasts and content of hydroxyproline was measured. The level of cellular reactive oxygen species, malondialdehyde (MDA), activity of superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) were also evaluated. Expression of type I, type III collagen, α-smooth muscle actin (α-SMA), $p22^{phox}$ (one vital subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase), SOD and thioredoxin (Trx) were detected with real time PCR or/and western blot. We found that pre-incubation with DMY (20 μM, 40 μM, 80 μM) for 4 h, 12 h or 24 h attenuated the proliferation of cardiac fibroblasts induced by Ang II. Expression of type I and type III collagen, as well as α-SMA were inhibited by DMY at both mRNA and protein level. DMY also significantly decreased cellular reactive oxygen species production and MDA level, while increased the SOD activity and T-AOC. DMY suppressed $p22^{phox}$, while enhanced antioxidant SOD and Trx expression in Ang II stimulated cardiac fibroblasts. Thus, dihydromyricetin attenuated Ang II induced cardiac fibroblasts proliferation related to inhibitory of oxidative stress.

1. Introduction

Myocardial fibrosis mostly results from long-time overload of the heart and is regarded as an independent risk factor for adverse cardiovascular events (Peng et al., 2016; Zhang et al., 2012). Cardiac fibroblasts are the major source of extracellular matrix in myocardium, and they play an important role in the pathogenesis of cardiac fibrosis (Chen et al., 2012). Previous study suggested that excessive activation of cardiac fibroblasts (including proliferation, collagen synthesis and transformation) contributed to occurrence and development of myocardial fibrosis (Fan and Guan, 2016). However, there are no specific methods to attenuate myocardial fibrosis in the clinical setting.

Several factors, such as mechanical stress, electrical signal coupling, inflammatory factors, oxidative stress and so on, are involved in myocardial fibrosis (Kurose and Mangmool, 2016; Patel and Mehta, 2012). These pathological stimulations disorder the sympathetic nervous system and renin angiotensin aldosterone system, then accelerate the proliferation of cardiac fibroblasts and promote collagen synthesis (Lopez et al., 2015; Yong et al., 2015). Moreover, excessive reactive oxygen species due to oxidative stress is one of the most dominant factors to induce myocardial fibrosis (Kong et al., 2014). A major source of reactive oxygen species in myocardium is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase contains multiple subunits and p22phox was the key one (Gray and Jandeleit-Dahm, 2015). There is also a complex antioxidant system to combat oxidative stress, including antioxidant enzymes and substances. The main antioxidant enzymes contain superoxide dismutase (SOD) and thioredoxin (Trx) (Ladjouzi et al., 2015; Kikusato et al., 2015). All the antioxidants, coordinating and maintaining a dynamic balance with oxidative injury, scavenge reactive oxygen species, reduce oxidative stress and eliminate oxidative damage in the body.

Ampelopsis grossedentata (Hand-Mazz) W.T.wang is a species of plant mainly distributed in southern China. Its tender stems and leaves are widely used as Vine tea. It has been used for herbal tea and

* Corresponding author.
E-mail address: mengguoliang@ntu.edu.cn (G. Meng).
[1] These authors equally contributed to this work.

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traditional Chinese medicine for over hundreds of years, with effect of lowing blood pressure, antibacterial, anti thrombosis, antioxidant, anti-inflammatory and anti alcoholism (He et al., 2003; Huang et al., 2013; Murakami et al., 2004; Ni et al., 2012; Shen et al., 2012; Zeng et al., 2006). Dihydromyricetin (DMY) and myricetin are two most important flavonoids in vine tea. It was reported that DMY significantly decreased reactive oxygen species formation in H_2O_2-stimulated human umbilical vein endothelial cells and lipopolysaccharide-treated RAW264.7 macrophages (Hou et al., 2015; Wang et al., 2016). One latest research found that DMY abolished reactive oxygen species and glutathione production in HepG2 Cells (C Xie et al., 2016; L Xie et al., 2016). Moreover, DMY seems to be cardioprotective, as it attenuated reactive oxygen species production by rat primary cardiomyocytes in adriamycin-induced cardiotoxicity model (Zhu et al., 2015). Our previous study also suggested that DMY inhibited angiotensin II (Ang II)-induced cardiomyocyte hypertrophy via an anti-oxidative manner (Meng et al., 2015a). However, there is no information about the potential role of DMY in Ang II induced cardiac fibroblasts proliferation until now.

The aim of the present study was to examine whether DMY attenuated cardiac fibroblasts proliferation and to elucidate the possible role and detailed mechanism of oxidative stress in this protective effect.

2. Materials and methods

2.1. Cell culture and treatment

The experiment was conducted according to NIH Guidelines for Care and Use of Laboratory Animals and was approved by the Institutional Animal Ethical Committee of Nantong University (approval no. NTU-20150305). Sprague-Dawley rats of 1- to 3-d-old were provided by Experimental Animal Center of Nantong University (Nantong, China). Hearts were removed from newborn rats immediately after euthanized by decapitation. The ventricles were separated from the atria, trisected and digested with 0.25% trypsin (Beyotime, Shanghai, China) at 37 °C for 7–10 cycles until completely digested. After digestion, all supernatants except the first one were collected. Fetal bovine serum (FBS, Hyclone labs, Logan, UT) of 10% was added into dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) to stop digestion. The cell suspension, re-suspended with DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime, Shanghai, China), was incubated at 37 °C for 4 h in a humidified 5% CO_2 incubator to separate fibroblasts from cardiomyocytes. The cells from 5 rats were planted in a 6-well plate or other culture plate with equal area for the first subculture. Confluent cardiac fibroblasts were treated with trypsin and sub-cultured, and cells of second or third passages were applied in our further experiments. The phenotype of cardiac fibroblasts was characterized with α-SMA and vimentin specific immunofluorescence staining (SFig. 1). The purity of cardiac fibroblast in the present study was more than 95%. After the culture medium was changed into DMEM supplemented with 0.5% FBS for 24 h, serum-starved cells were pre-incubated with DMY ((2R,3R)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one, $C_{15}H_{12}O_8$, PubChem CID: 161557, Standard Center of China, Beijing, China, chemical structure in Fig. 1) at different concentrations (0, 1 μM,10 μM, 20 μM, 40 μM, 80 μM, 160 μM, and 320 μM) for different times (0, 1 h, 2 h, 4 h, 12 h or 24 h), (Meng et al., 2015a) followed by Ang II (Sigma-Aldrich, St. Louis, MO; 100 nM) (Zhou et al., 2016) stimulation for an additional 24 h as previous study. The culture medium without DMY was used as a vehicle control.

2.2. Cell number and cytotoxicity assay

Cell number was measured by Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) assay to estimate proliferation of cardiac fibroblasts according to the manufacturer's directions. Briefly, cardiac fibroblasts (1×10^4) were seeded in a 96-well plate and exposed to Ang II (100 nM, 24 h) after pretreatment with DMY or vehicle (DMEM) for different times, followed by addition of 10 μl 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) mixture, which was similar to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and was reduced by some dehydrogenases to form aurantius formazan. The cells were then incubated at 37 °C for 1 h in the incubator. The absorbance was measured in a microplate reader (Biotek Instruments, Winooski, VT) at a wavelength of 450 nm (C Xie et al., 2016; L Xie et al., 2016). Cell numbers were represented as optical density (OD) value.

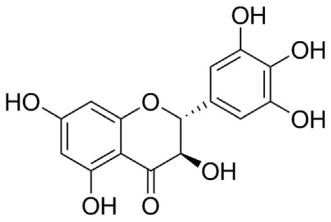

Fig. 1. Chemical structure of dihydromyricetin (DMY).

Lactate dehydrogenase (LDH) level released from cells was detected by LDH-Cytotoxic Assay Kits (Roche Diagnostics, Mannheim, Germany) to assess cytotoxicity. The detailed protocol was according to the manufacturer's directions. The medium without cells was served as a blank control. The LDH content in the medium was calculated based on the absorbance which was normalized with the blank control. The average LDH level was normalized by OD value representing cell numbers.

2.3. Determination of hydroxyproline in culture medium

After treatment, hydroxyproline content in cell culture medium for evaluating collagen production was measured according to the protocol provide by the manufacturers (Jiancheng Bioengineering Institute, Nanjing, China). The medium without cells was served as a blank control. The hydroxyproline content in the medium was calculated based on the absorbance which was normalized with the blank control. The average hydroxyproline content was normalized by OD value representing cell numbers.

2.4. Measurement of malondialdehyde (MDA), SOD, and total antioxidant capacity (T-AOC)

Cell precipitate of cardiac fibroblasts was re-suspended in lysis buffer containing 20 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA, 150 mmol/l NaCl, 20 mmol/l NaF, 3 mmol/l Na_3VO_4, 1 mmol/l PMSF, with 1% Triton X-100 and protease inhibitor cocktail and further lysed on ice for 30 min. After centrifugation at 12, 000g for 5 min, the supernatants were collected. The protein was applied for MDA measurement and western blot. The level of MDA in cardiac fibroblast was measured by Lipid Peroxidation and Assay Kits (Beyotime, Shanghai, China) with thiobarbituric acid (TBA) method and was expressed as μmol/g·protein.

Protein, extracted from cells with homogenate in PBS, was applied for SOD activity and T-AOC measurement. SOD activity was assessed by SOD Assay Kits (Beyotime, Shanghai, China) with nitroblue tetrazolium (NBT) method and was expressed as units/mg·protein. T-AOC was detected by T-AOC Assay Kits (Beyotime, Shanghai, China) with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) method and was expressed as mmol/g·protein. All the detections were performed according to the manufacturer's instructions.

2.5. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted using Trizol reagent (Takara, Kyoto,