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Original article

Ursolic acid prevents endoplasmic reticulum stress-mediated apoptosis induced by heat stress in mouse cardiac myocytes

Ying Yang [a,1], Changwu Li [a,1], Xi Xiang [b], Zhaolai Dai [a], Jianyu Chang [c], Ming Zhang [a], Hong Cai [a], Hua Zhang [a], Meijia Zhang [b], Yuming Guo [a,*], Zhenlong Wu [a,*]

[a] State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, 100193, PR China
[b] State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing, 100193, PR China
[c] College of Veterinary Medicine, China Agricultural University, Beijing 100193, PR China

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ABSTRACT

Heat stress causes serious physiological dysfunction of cardiac myocytes and is associated with several types of cardiovascular diseases. However, the underlying mechanisms and therapeutic strategies to alleviate heat stress-induced myocardial damage are not available. The objective of this study was to (1) investigate the functional role of endoplasmic reticulum (ER) stress-mediated apoptosis in heat exposure-induced myocardial damage, and (2) to evaluate the effects of ursolic acid on the myocardial apoptosis as well as the underlying mechanisms in mouse cardiac myocytes. We show here that heat stress-induced apoptosis is predominantly mediated by the activation of PERK-eIF2α-CHOP unfolded protein response which up-regulates the protein expression of Puma, and by the modulation of cellular redox state. Intriguingly, the myocardial apoptosis is markedly attenuated by ursolic acid treatment. Mechanistically, the protective effects of ursolic acid are mediated, at least partly, by reestablishing the intracellular redox state and inducing the expression of the anti-apoptotic protein Mcl-1, which, in turn, inactivating CHOP-induced Puma up-regulation. The striking finding that ursolic acid has both anti-apoptotic and antioxidative activities against ER stress-associated myocardial damage suggests that supplementation of ursolic acid might be a potential strategy to reduce the detrimental effects of heat stress in cardiomyocytes.

1. Introduction

The endoplasmic reticulum (ER) is classically recognized as an organelle for protein synthesis and maturation as well as the regulation of intracellular Ca^{2+} homeostasis [1,2]. The accumulation of unfolded proteins in the ER lumen or depletion of Ca^{2+} from the ER lumen activates a signaling cascade known as the unfolded protein response (UPR) in response to endogenous or exogenous stresses, such as nutrition limitation, oxidative stress, hypoxia, virus infection or high temperature exposure (collectively called ER stress) [3,4]. This response protects the cells from ER stress by an increased ability of protein folding, the degradation of misfiled proteins, and the inhibition of global protein synthesis [3,5]. However, the excessive or extensive stress from which the cell cannot recover leads to apoptotic cell death [2,3,6].

Accumulating evidence indicates that the ER stress-mediated apoptotic cell death plays a critical role in cardiovascular diseases, such as myocardial infarction, cardiac dysfunction and heart failure [7,8]. Apoptosis triggered by heat stress has been reported to cause cardiac dysfunction and even cardiac failure in human and animal models through largely unknown mechanisms [7,9–11]. Recent studies show that heat stress induces myocardial injury concomitant with an increased free radicals [12] and a disruption of intracellular calcium homeostasis [11] which are important UPR triggers. These results indicate that the ER signaling pathway might be associated with heat stress and contribute to the apoptotic cell death. Moreover, practical strategies to alleviate heat stress-induced myocardial damage are not available yet.

Ursolic acid (3-β-hydroxy-urs-12-en-28-olic acid), a pentacyclic triterpenoid compound widely distributed in apple peels, herb medicines and many other edible plants [13], is gaining more and more attention due to its multiple beneficial effects, such as antioxidative activity [14,15], anti-inflammatory response [16] and anti-glycative effects in animal models [17,18]. Recent study demonstrates that ursolic acid increases the production of atrial natriuretic peptide and thus exerts cardiovascular protection in isolated atria [19]. However, it is unknown whether ursolic acid has a protective effect on heat stress-induced cellular damage in cardiac myocytes. The aim of this study was 1) to investigate functional role of ER stress-mediated apoptosis

* Corresponding authors at: Department of Animal Nutrition and Feed Science, China Agricultural University, Beijing, 100193, PR China. Tel./fax: +86 10 62731003.
E-mail addresses: guoym9899@yahoo.com.cn (Y. Guo), bio2046@hotmail.com (Z. Wu).
[1] These authors contributed equally to this work.

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in heat exposure-induced myocardial damage, and 2) to investigate the beneficial effects of ursolic acid on heat stress-induced apoptosis as well as the underlying mechanisms in mouse cardiac myocytes.

2. Materials and methods

2.1. Animals

Eight-week-old male ICR mice were maintained on a standard laboratory diet with free access to tap water. After a 1-week adaptation period, the mice were randomly assigned into 4 treatment groups with ten mice each. The mice in control group were reared under a normal ambient temperature (21 ± 0.5 °C). Mice in the remaining groups were orally administered with ursolic acid (0, 20 or 40 mg/kg body weight/day) once daily for 2 days in a volume of 0.5 ml of phosphate-buffered saline, and then were put in a hot chamber at high temperature (41 ± 0.5 °C) for 2 h to induce heat stress. The same volume of phosphate-buffered saline was applied to the mice in the control treatment group. Blood and left ventricular tissue samples were immediately collected after heat exposure and frozen at −80 °C for later analysis. All experiments were approved by the Animal Care and Use Committee of the China Agricultural University which conforms to the Guide for the Care and Use of Laboratory Animals. Ursolic acid was purchased from the National Pharmaceutical Engineering Center for Solid Preparation Chinese Herbal Medicine (Beijing, China).

2.2. Haemodynamic analysis of cardiac function

Analysis of left ventricular performance was evaluated using the method as previously described [20,21]. Briefly, mice were anesthetized with intraperitoneal injection of chloral hydrate (400 mg/kg) and a micro-tip catheter transducer system (SPR671, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle under pressure control. After stabilization for 15–20 min, the pressure signal was continuously recorded using an AcqKnowledge pressure conductance system (Version 3.8.1, BioPAC system) coupled with a transducer control unit (TC-510, Millar Instruments, Houston, TX), stored, and displayed on a computer. The heart rate, the left ventricular systolic and end-diastolic pressure were measured, and the maximal slope of systolic pressure increment (+dP/dt) and diastolic pressure increment (−dP/dt) were calculated.

2.3. Plasma troponin I and lactate dehydrogenase determination

The biochemical indicators of myocardial injury were assessed at the end of experiment by determinations plasma troponin I and the activity of lactate dehydrogenase. Plasma troponin I levels were detected using the mouse cardiac troponin I ELISA assay kit (Life Diagnostics, West Chester, PA). Plasma lactate dehydrogenase (LDH) activities were determined using the method as described [22].

2.4. Detection of apoptotic cell death

For the detection of apoptotic cardiomyocytes, paraffin-embedded heart tissues were sectioned into 4 μm thin sections and immunohistochemistry assay was performed as previously described [23]. TUNEL staining was performed by using In Situ Apoptosis Detection Kit (Roche Applied Science, Rockford, IL) according to the manufacturer's instructions. The sections were subsequently stained with anti-alpha-sarcomeric actinin (anti-SA) (clone EA-53; Sigma-Aldrich) primary antibodies and then with FITC conjugated secondary antibodies (Molecular Probes, Invitrogen CA). Nuclei were stained with Hoechst 33342 (Sigma-Aldrich). The apoptotic cells were expressed as percentage of the number of TUNEL and SA positive cells divided by the total number of SA positive cells per field. All of the images of tissues were taken by fluorescent microscopy (Axiovert 200, Zeiss, Germany).

2.5. Small interfering RNA (siRNA) injection

Specific siRNAs targeting CHOP and Mcl-1 mRNA as described previously [24,25] or non-targeting control siRNA were obtained from Dharmacon Research (Lafayette, CO) and applied to mice according to the method reported [26]. Briefly, 5 μg of synthetic siRNA dissolved in 0.1 ml of RNase-free phosphate-buffered saline was rapidly injected into the tail vein. The negative control siRNA in the same volume of phosphate-buffered saline was injected into the tail veins of mice in control. Mice injected with CHOP siRNA or negative siRNA were subjected to heat temperature (41 °C, 2 h) 48 h after siRNA injection and left ventricular tissues were harvested for Western blot analysis. In another experiment, mice injected with Mcl-1 siRNA or negative siRNA were subjected to normal temperature control or subjected to heat stress (41 °C, 2 h) following ursolic acid treatment (0 and 40 mg/kg/day, 2 days). Left ventricular tissues were collected for apoptosis determination and Western blot analysis.

2.6. Western blot analysis

Whole cell lysate was prepared from mouse ventricles as described previously [27]. Briefly, ventricular tissue was lysed in an ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM protease inhibitor cocktail and protein phosphatase inhibitor (Roche Applied Science, Rockford, IL) and then were sonicated for 15 s at 4 °C. Cell lysate was centrifuged at 12,000 *g* for 15 min at 4 °C to remove cellular debris. The protein concentration was determined using BCA protein assay kit (Applygen Technologies Inc., Beijing, China). Equal amounts of protein (30 μg) were separated on 12% SDS–PAGE gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc, Hercules, CA). The membranes were blocked in 5% skimmed milk solution at room temperature for 1 h, and then were incubated with diluted primary antibody. Antibodies against CHOP, Puma, Bad, Bcl-XL, and Bcl-XS were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against PERK, p-PERK, eIF2α, p-eIF2α, IRE-1, JNK, p-JNK, ATF6, cleaved caspase 3, cleaved caspase 9, cleaved PARP, Mcl-1 and Bcl-2 were purchased from Cell Signaling Technology, Inc (Danvers, MA). Blots were stripped and re-probed with anti-β-actin antibody (Abcam, Cambridge, MA) to demonstrate equal loading. After incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, the chemiluminescent signal was detected by using the Super Enhanced Chemiluminescence Kit (Applygen Technologies Inc., Beijing, China). Quantification of band density was determined using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

2.7. Measurements of mitochondrial cytochrome c release into cytosol

Measurements of cytochrome c release from the mitochondrial space to the cytosol were achieved by sub-cellular fractionation and Western blotting as described previously with some modifications [28]. Briefly, ventricular tissue was lysed in 50 μl of ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 12,000 *g* for 4 min at 4 °C to obtain the supernatants (cytosolic extracts free of mitochondria). Proteins were separated on 15% SDS–PAGE gels, analyzed by using anti-cytochrome c antibody (7H8.2C12, from Pharmingen, San Diego, CA).

2.8. Measurement of tissue lipid peroxidation

Heart tissue was homogenized in RIPA homogenizing buffer. The level of lipid peroxidation was estimated by the thiobarbituric acid test [29]. Homogenate was centrifuged at 1600 *g* for 10 min at 4 °C