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Puerarin protects against heart failure induced by pressure overload through mitigation of ferroptosis

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ABSTRACT

Heart failure (HF) is the end stage of cardiovascular disease and is characterized by the loss of myocytes caused by cell death. Puerarin has been found to improve HF clinically, and animal study findings have confirmed its anti-cell-death properties. However, the underlying mechanisms remain unclear, especially with respect to the impact on ferroptosis, a newly defined mechanism of iron-dependent non-apoptotic cell death in HF. Here, ferroptosis-like cell death was observed in erastin- or isoprenaline (ISO)-treated H9c2 myocytes in vitro and in rats with aortic banding inducing HF, characterized by reduced cell viability with increased lipid peroxidation and labile iron pool. Interestingly, the increased iron overload and lipid peroxidation observed in either rats with HF or H9c2 cells incubated with ISO were significantly blocked by puerarin administration. These results provide compelling evidence that puerarin plays a role in inhibiting myocyte loss during HF, partly through ferroptosis mitigation, suggesting a new mechanism of puerarin as a potential therapy for HF.

1. Introduction

As the final stage of cardiovascular diseases, heart failure (HF) is endangering the life and health of 22.5 million people around the world, and this number is increasing at a rate of 2 million per year. Thus, the prevention and treatment of HF is currently one of the most important topics in the medical community. Under hemodynamic stress such as high blood pressure, compensated cardiac cells lead to myocardial hypertrophy, the most important pathological characteristic of HF, causing progressive cell loss and finally advancing to HF. Several kinds of cell death have been proved to be involved in cell loss, such as apoptosis, necrosis and autophagy [1]. As a new form of regulated cell death (RCD), ferroptosis was defined by Dixon in 2012. Different from other major forms of RCD, ferroptosis, characterized by cell volume shrinkage and mitochondrial membranes thickening, is mediated by iron-dependent lipid peroxide accumulation [2]. Ferroptosis was initially observed in cancer cells expressing oncogenic Ras and then discovered in other diseases, such as Huntington's disease and tubular failure [3], but few studies on the role of ferroptosis in cardiovascular disease have been reported. Nevertheless, the study of iron homeostasis and myocardial injury has a long history. The term iron overload cardiomyopathy has been introduced to describe a secondary form of cardiomyopathy resulting from the accumulation of iron in the myocardium, mainly because of genetically determined disorders of iron metabolism or multiple transfusions [4]. The clinical use of doxorubicin is limited by its cardiotoxicity. The possible involvement of iron in doxorubicin-induced cardiotoxicity became evident from studies in which iron chelators were shown to be cardioprotective [5]. Nitenberg et al. demonstrated that abnormal myocardial iron status may exist in diabetic patients with HF, and chelation therapy can improve the prognosis of coronary microvascular adaptation [6]. A recent study by Lapenna et al. [7] demonstrated that the levels of low-molecular-weight iron (LMWI), a redox-active catalytic form of iron, as well as the levels of lipid and protein oxidation, were higher in the hearts of aged rabbits than in those of young adult control rabbits. The above and other research results indicate that ferroptosis, characterized by altered iron status with catalytic LMWI burden and related cardiac oxidative stress, might be underlying mechanisms for cell death during cardiac dysfunction. However, iron pools as expression of iron status and related oxidative stress have not yet been well investigated in pressure-overload-induced HF, and the further exploration of effective therapies that specifically target ferroptosis

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in HF may represent a new and useful management strategy.

Puerarin, one of the most abundant phytoestrogens with antioxidant and other properties, has been approved by the State Food and Drug Administration in China as a therapeutic agent for clinical therapy in cardiovascular and other diseases [8]. Numerous clinical trials have established the beneficial effects of puerarin on patients with HF, but the detailed mechanisms remain unclear. Our previous study showed puerarin exerting protective effects against cardiomyocyte hypertrophy and apoptosis by restoration of autophagy in rats with myocardial hypertrophy induced by pressure overload [9], which could block the progression to HF. Based on the antioxidant properties of puerarin, the details of this agent in cell death mechanisms are worthy of further exploration, especially in relation to ferroptosis.

2. Methods

2.1. Reagents

Isoprenaline(ISO), puerarin, chloroquine(CQ), propidium iodide (PI) solution and MTT assay kit were purchased from Sigma Aldrich(USA). Ferrostatin-1(Fer-1) and Z-VAD-FMK were purchased from Selleck Chemicals (USA). The hematoxylin and eosin (HE) kit was obtained from Baso (Zhuhai, China). Puerarin for injection was purchased from Zhenyuan Pharmaceutical Co., Ltd (Zhejiang, China), and the Bradford assay kit was obtained from Bio-Rad(USA). The primary antibody against Nox4 was from Sigma Aldrich(USA), while those against glutathione peroxidase 4 (GPX4), ferritin heavy chain 1(FTH1) and GAPDH were from Cell Signaling Technology(USA). The thiobarbituric acid reactive substances (TBARS) assay kit was from R&D Systems (USA), the VECTASTAIN ABC kit was from Vector Laboratories Inc. (USA), and the primary rabbit antibody against 4-hydroxy-*trans*-2-nonenal (4-HNE) was from Abcam Inc.(USA).

2.2. Animal model

The animal experiments conformed to the Guide for the Care and Use of Laboratory Animals (US and National Institutes of Health). Male Sprague Dawley rats weighing 80–100 g were used to make the HF model induced by descending aortic banding (AB) procedure [9]. Rats receiving a similar procedure except for the arterial ligation were defined as the sham-operated (SO) group. After the procedure, echocardiography was immediately applied to confirm the arterial banding. Rats receiving subcutaneous injections of low- or high-dose puerarin (100mg/kg/day and 200mg/kg/day, respectively) after the AB procedure were respectively defined as the Pue1 and Pue2 groups. An equal volume of normal saline was injected into the rats of the SO and AB groups.

2.3. Cell culture and treatment

H9c2 cardiac myoblast cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Upon reaching 50–60% confluence, the cells were treated with ISO or erastinalone, or in combination with puerarin (10, 20 and 40 μM, respectively, dissolved in dimethyl sulfoxide [DMSO]).

2.4. Cytotoxicity assays

Cell viability in vitro was determined using the in MTT kit as per the manufacturer's instructions. For the detection of cell death in vivo, cardiac sections of rats were incubated with 5μg/mL PI for 30 min and then imaged using an inverted fluorescence microscope.

2.5. Lipid peroxidation assay

Lipid peroxidation in cultured cell lysates was determined using the classical assay of measurement of the rate of production of TBARS, expressed as pmol/mg protein.

2.6. Immunohistochemistry for 4-HNE in vivo

Formaldehyde-fixed and paraffin-embedded sections were incubated in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity and then incubated overnight at 4 °C with primary rabbit antibody against 4-HNE. For antigen retrieval, the sections were immunostained using the VECTASTAIN ABC kit following the manufacturer's specifications. Diaminobenzidine was used for staining development, and the sections were counterstained with hematoxylin.

2.7. Iron assay

A fluorescence technique with the Fe sensor calcein was applied to detect the labile iron pool (LIP). Cells were washed with PBS and then treated with Chelex-100. Subsequently, 100 μL of calcein-AM solution (final concentration of 30 μM) was incubated with the cells for 30 min at 37 °C. After removing the excess calcein-AM with PBS, the fluorescence ($\lambda_{ex} = 450$ nm, $\lambda_{em} = 515$ nm) was monitored. The results were expressed as fold change. For labile iron measurements in vivo, LMWI was determined using the sensitive iron colorimetric detector ferene S as previously described [7]. The absorbance values of the ferene S–iron complex at 594 nm were then recorded spectrophotometrically against an appropriate blank, and the results were calculated as nmol iron/mg protein using a molar extinction coefficient of 35,500 [10].

2.8. Western blotting

Western blotting was performed as previously described [9]. Briefly, after collection of the supernatants of the tissue or cell lysates, protein samples (20–25 mg) were separated by sodiumdodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed with primary antibodies overnight. Diluted secondary antibodies were used to detect the corresponding primary antibodies. Further analysis was carried out using Image Pro Plus v6.0 (Media Cybernetics, Carlsbad, CA, USA) to quantify the protein bands.

2.9. Echocardiography

Chloral hydrate was used to anesthetize the rats. An experienced technician blinded to the study groups then used an IE33 echocardiographic system (Philips Medical Systems, Nederland BV) to perform the transthoracic two-dimensionally guided M-mode echocardiography every week after the procedure.

2.10. Histological analysis

Sections were stained with H&E and examined under a light microscope (AMG EVOS FL), and the myocyte area was measured with Image Pro Plus v6.0.

2.11. Transmission electron microscopy

Harvested cardiac tissues were stained en bloc with 2% uranyl

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