Effect of hesperetin on isoprenaline-induced hypertrophy of H9C2 cardiomyocytes

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ABSTRACT

An experiment was carried out to investigate the effect of hesperetin on isoproterenol-induced H9C2 cardiomyocyte hypertrophy and its possible mechanism. For this aim, H9C2 cardiomyocytes were coincubated with different concentrations of hesperetin (0.125, 0.25, 0.5, 1μmol/L) and isoproterenol to detect the changes in the area of H9C2 cells, the expression of cardiac hypertrophy marker β-MHC mRNA and autophagy marker LC3II; H9C2 cells were coincubated with hesperetin and isoproterenol at the optimal intervention concentration, and the intervention time was set to 6h, 12h, 24h, respectively, and the changes of H9C2 cell area, β-MHC mRNA and LC3II expression were detected. Results showed that hesperetin could reduce isoproterenol-induced H9C2 cardiomyocyte area enlargement; hesperetin can reduce β-MHC mRNA expression in isoproterenol-induced H9C2 cardiomyocyte; hesperetin can increase the expression level of LC3II in isoproterenol-induced H9C2 cardiomyocyte. Conclusion Hesperetin may improve the hypertrophy of H9C2 cardiomyocytes induced by isoproterenol by activating autophagy.

Introduction

Myocardial hypertrophy is an adaptive compensatory response of the heart to pathological stimuli, mainly characterized by enhanced protein synthesis, increased cardiomyocyte size, and thickening of the ventricular wall, which are the major risk factors promoting arrhythmias and heart failure. Neuroendocrine-cytokine system activation further stimulates myocardial remodeling, resulting in irreversible processes such as myocardial apoptosis and myocardial fibrosis, which gradually progress to decompensated heart failure. Therefore, it is significant to find potential effective targets of intervention to inhibit or even reverse myocardial hypertrophy clinically (1).

Autophagy is a catabolic mechanism that is highly conserved in eukaryotes and mammals, and in recent years, cardiomyocyte autophagy has been considered to make a significant contribution to regulating cardiac hypertrophy. However, there is still controversy as to whether cardiomyocyte autophagy plays a beneficial or aggravating role in cardiac hypertrophy, which is considered to be a cardiomyocyte protective process on the one hand and a deleterious mechanism leading to the pathological progression of cardiac hypertrophy on the other (2).

Hesperidin is a dihydroflavonoid widely presented in lemons, citrus and other plants. Previous studies have demonstrated its various biological activities such as anti-inflammatory, anti-aging, immunomodulatory, and oxygen-free radical removal. It was reported that hesperidin contributes to reducing myocardial ischemia-reperfusion injury by inhibiting excessive autophagy(3-4). Myocardial hypertrophy is caused by a series of neuroendocrine factors in addition to pressure and volume load, and evidence has been reported in the literature that hesperidin can inhibit pressure overload-induced myocardial remodeling. In the study, we applied hesperidin to intervene in isoproterenol-stimulated H9C2 cardiomyocytes and proposed to confirm that hesperidin inhibits myocardial hypertrophy by regulating autophagy at the ex vivo level.

Materials and Methods

Cell source and reagents

H9C2 cardiomyocytes were provided by Wuhan Procell Life Science&Technology Co., Ltd. Hesperidin (98.75% purity) was purchased from Wuhan Promote Biological Co., Ltd. DMEM culture medium, fetal bovine serum, trypsin, penicillin/streptomycin double antibodies were purchased from Gibco Life Technologies. DMSO was purchased from Sigma, and the rest were domestic analytical purity.

Experimental groups

The experimental groups were as follows.

1/ Grouping according to concentration gradient: control group, Isoproterenol (ISO) group, ISO+0.125μmol/L hesperidin group, ISO+0.25μmol/L hesperidin group, ISO+0.5μmol/L hesperidin group, ISO+1μmol/L hesperidin group. 2/ Grouping according to intervention time: ISO group for 6h, ISO +0.25μmol/L hesperidin group for 6h,

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ISO group for 12h, ISO+0.25μmol/L hesperidin group for 12h, ISO group for 24h, ISO + 0.25μmol/L hesperidin group for 24h.

Experimental techniques

The experimental techniques were as follows.

1/ Observation of the surface area of individual H9C2 cells by immunofluorescence staining(4,5): The cells were fixed at room temperature by 4% paraformaldehyde and heated in the antigen repair solution at 95 EDG C for 10 min. Then coverslips were carefully placed in a water bath by small forceps and the surface of the slides on which the cells were growing were noted. After that, samples were incubated using phosphate buffer solution for 10 min, followed by an antibody permeant wash using Triton X-100 to determine the optimal ratio of Triton X-100 for each protein. Cells were treated with 1% BSA + 22.50 mg/ml glycine and incubated for 1 h at room temperature using diluted antibodies in a wet box. Second antibodies were fully dissolved with 1% BSA for 1 h in full light-proof incubation. Then cells were stained with 0.1 μg/ml of DAPI staining solution.

2/ Quantitative Real-time PCR: Expression of β-MHC mRNA, a marker of cardiomyocyte hypertrophy(6).

3/ Western-blot detection on the expression of autophagy marker protein LC3II (7):

Protein extraction

tissues were extracted into tissue homogenate, and fine cells were collected by centrifugation at 1000 rpm for 5 min in a high-speed centrifuge, washed twice by pre-chilled 1×PBS, then 1 ml of ice-cold RIPA protein lysis buffer was added, homogenized with gentle shaking at 4 EDG C, put in an ice bath for 45 min, and centrifuged at 12000 rpm for 20 min. Subsequently, the supernatant was collected as total protein and stored in an ultra-low temperature refrigerator at -80EDG C for backup.

Protein quantification (Bradford test)

10ul of protein sample was diluted 10 times and added to Coomassie Brilliant Blue G250 staining solution, and the OD value of the mixture was measured at room temperature after leaving for 30 minutes at 37EDG C. Then the protein content of the sample was calculated according to the measurement results and the standard curve prepared.

Protein SDS-PAGE gel electrophoresis preparation and electrophoresis

a. SDS-PAGE gel preparation: SDS-PAGE separation gel and SDS-PAGE concentration gel used here were 10% and 5%, respectively. The glass plate was cleaned and dried to align with the clamps and stuck vertically on the shelf, ready for gel filling. Various reagents in proportion were added and mixed in a 50 mL centrifuge tube, then 10% SDS-PAGE separation gel was slowly added along the glass plate to a height of 6.5 cm with a 1.5 cm height reserved for filling the concentrated gel, closed with water on the gel, and left to stand at room temperature for about 60 minutes until the gel was completely polymerized. After the gel was completely solidified, the water was poured out and blotted dry with absorbent paper. Then the 5% SDS-PAGE gel concentration was poured into the remaining space and the comb was inserted horizontally into the gel concentration, left at room temperature for 30-60 min until the gel polymerizes, and removed from the shelf for spare after the gel concentration was fully solidified.

b. Protein denaturation and electrophoresis: Protein samples were mixed with the loading buffer in the ratio of 5:1 and denatured in a boiling water bath for 10 minutes. The gel was fixed on the electrophoresis device, and the appropriate amount of electrophoresis buffer was added to the electrophoresis tank, and the comb was removed. Then the microsampler was loaded in a predetermined sequence (50 μg/hole) and 120 V regulated electrophoresis is performed until bromophenol blue was left to terminate electrophoresis.

Transmembrane

The gel was removed after electrophoresis and put into the transfer buffer to equilibrate for 20 min. PVDF membrane with the general size of gel was immersed in anhydrous methanol for 15s, transferred to double-distilled water for 2 minutes when the membrane became translucent, and then placed in the transfer buffer for 5-10 min for equilibration. PVDF membrane was covered on the gel with air bubbles driven out, then a layer of thick filter paper and another sponge pad was covered on the PVDF membrane to drive out the air bubbles and merge the clamps. Remarkably, the whole operation needs to be performed in the electro-transfer solution. The clamps were placed in the electrotransfer bath and an ice-cold electrotransfer solution was added. Then the transfer device was placed in an ice box and transferred at 100V for 60 min.

Blocking and antibody incubation

PVDF membrane was rinsed 3 times on a shaker with TBST solution for 5 min each, transferred to a plate containing blocking solution (TBST solution with 5% skimmed milk powder), and blocked on a shaker for 2 h at room temperature and overnight at 4 EDG C. Then the blocking solution was poured and discarded after blocking. The membrane was washed 3 times with TBST solution for 5 min each. The diluted primary antibody was incubated with the blocking PVDF membrane for 2 hours at room temperature. The membrane was washed 3 times with TBST solution for 10 min each. The second antibody diluted with the blocking solution was added and incubated with PVDF membrane for 2 hours at room temperature.

ECL (Thermo Fisher)

The luminescent solution was prepared in the dark room, and the dried PVDF membrane protein surface was fully contacted with the luminescent solution. Subsequently, the membrane was put into the chemiluminescent imaging analyzer for imaging and analyzed by the image analysis software. Using β-actin as an internal control, the ratio of the target protein to β-actin was used to represent the relative protein expression level and calculate the expression amount.

Semi-quantitative analysis of assay samples

The software was used to perform grayscale scanning of the experimental results. The amount of each sample protein (grayscale value) was divided by the amount of the internal reference protein to correct for the error. The resulting value was the relative amount of each sample protein (relative grayscale value) after the internal reference correction. Therefore, the relative grayscale value

of each sample protein used for comparative analysis can obtain the results of the actual variation of the target protein content among samples.

Statistical methods

SPSS25.0 statistical software is used for analysis, and all data were expressed as mean ± standard deviation (x ± SD), where t-test is used for comparison between two groups; multi-factor ANOVA is used for comparison between multiple groups, and P value< 0.05 is considered statistically significant.

Results

Effects of hesperidin at different concentrations on ISO-induced hypertrophy of H9C2 cardiomyocytes

With ISO (10 μmol /L)(8) stimulation (683.6±19.2 v.s. 991.9±54.30 μm2, p<0.01), both H9C2 cell area increased (683.6±19.2v.s.991.9±54.30 μm2, p<0.01) and the expression of β-MHC mRNA raised significantly
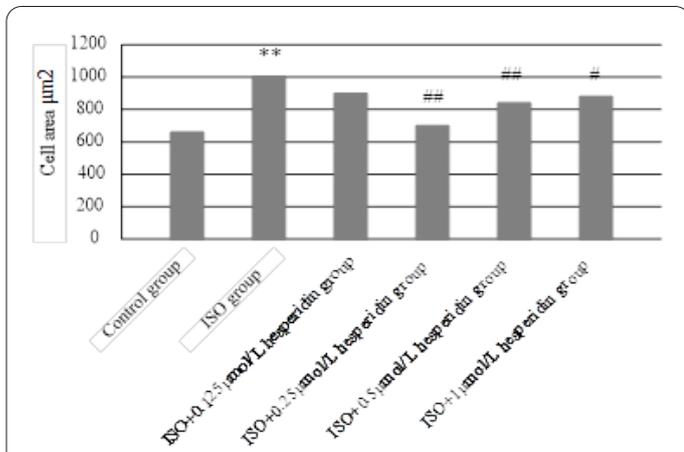

Figure 1. Changes in cell area after stimulation with different concentrations of hesperidin. Note: * p<0.05, compared with the control group; ** p<0.01, compared with the control group. ## p<0.05, compared with ISO group; ## p<0.01, compared with ISO group.
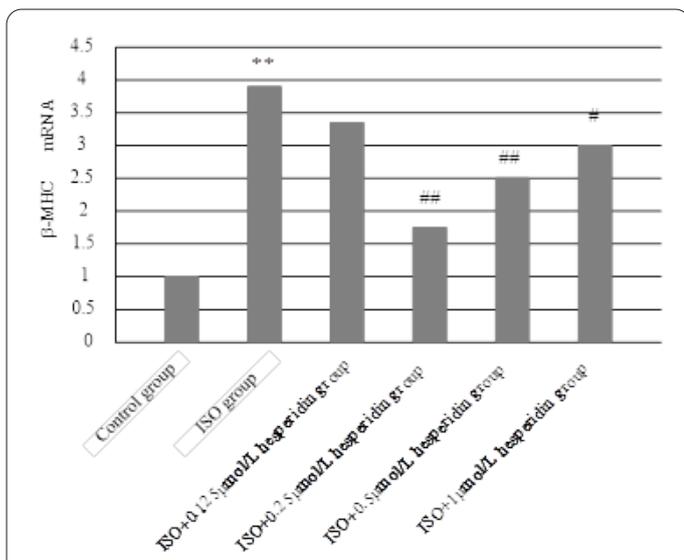

Figure 2. Changes in expression of β-MHC mRNA after stimulation with hesperidin at different concentrations. Note: * p<0.05, compared with the control group; ** p<0.01, compared with the control group. ## p<0.05, compared with ISO group; ## p<0.01, compared with ISO group.

(0.99±0.21v.s.3.92±0.75, p<0.01). And 0.125 μmol/L hesperidin could somewhat inhibit cellular hypertrophy and reduce the expression of β-MHC mRNA without a statistical difference (p>0.05). In addition, the remaining concentrations of hesperidin significantly reduce the increase of cell area induced by ISO stimulation (p<0.05) (Fig. 1) and inhibits the expression of β-MHC mRNA (p<0.05) (Fig. 2). The cell hypertrophy improvement (including cell area and β-MHC mRNA) is the most significant at the hesperidin intervention concentration of 0.25 μmol/L. (Fig. 1 and Fig. 2)

Effect of hesperidin on ISO stimulation-induced hypertrophy in H9C2 cardiomyocytes at different intervention times

After hesperidin intervention for 6h, it can be found that the increase in β-MHC mRNA expression and cell area of cardiomyocytes induced by ISO stimulation was inhibited (p<0.05). After hesperidin intervention for 12h, it can be seen that both the increase of β-MHC mRNA expression and cell area increase in cardiomyocytes induced by ISO stimulation could be significantly improved (p<0.01); and after hesperidin intervention for 24h, the increase in β-MHC mRNA expression and the cell area
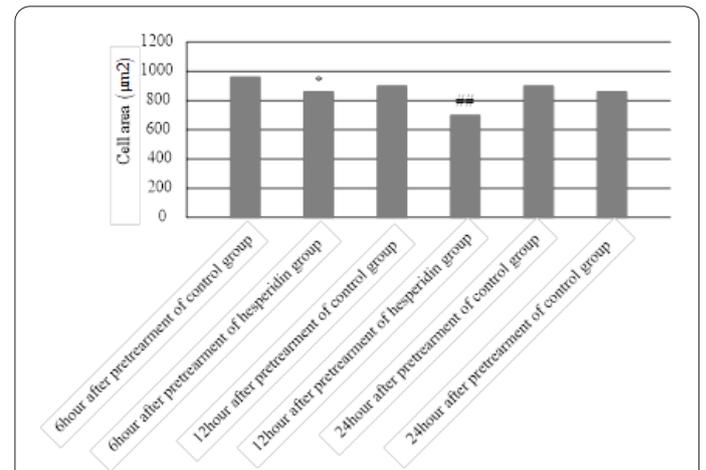

Figure 3. Effect of different intervention times on cardiomyocyte area. Note: *p<0.05, compared with ISO control group for 6h; ##p<0.01, compared with ISO control group for 12h.
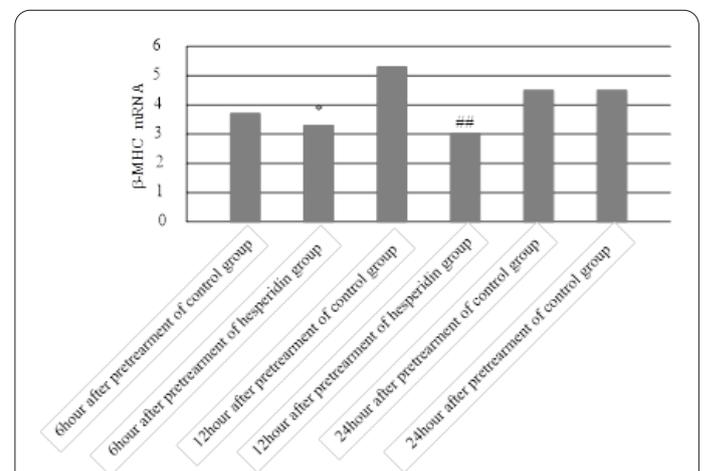

Figure 4. Effect of different intervention times on β-MHC mRNA expression in cardiomyocytes. Note: *p<0.05, compared with ISO control group for 6h; ##p<0.01, compared with ISO control group for 12h.

increase in H9C2 cardiomyocytes are not significantly inhibited (p>0.05). (Figures 3 and 4).

Regulation of autophagy in H9C2 cardiomyocytes by hesperidin

LC3II expression is reduced in H9C2 cells after ISO stimulation (p<0.05) and significantly increased after hesperidin intervention. And in different concentration gradients (p<0.05), we can observe the most significant increase in expression at 0.25 μmol/L of hesperidin; and in different intervention time trials, the most significant increase in LC3II expression can be observed in the intervention for 12h (p<0.05). (Figures 5 and 6).

Discussion

Chronic heart failure, one of the two major challenges in the cardiovascular field in the 21st century, is the end-stage manifestation and the leading cause of death in cardiovascular disease. Myocardial hypertrophy is the main compensatory mechanism for increased cardiac afterload, with or without ventricular dilation, which is mainly characterized by cardiomyocyte hypertrophy and myocardial fibrosis, without an increase in the number of cardiomyocytes. Its enlarged and increased nuclei and mitochondria lag behind the fibrosis of the myocardium, leading to myocardial insufficiency and subsequent progression to cardiomyocyte death. The decrease in cardiomyocytes results in a decrease in overall myocardial contractility, which eventually leads to an irreversible stage of heart failure. Neurohumoral mechanisms are involved in the development of the pathophysiology of cardiac hypertrophy, including increased sympathetic excitability and activation of the renin-angiotensin-aldosterone system. In the study, isoproterenol stimulation is used to induce the cardiomyocyte hypertrophy model(9), ISO group shows a remarkable increase in cell volume and a significant upregulation of β-MHC mRNA expression compared to the control group, consistent with previous studies. ISO can activate β-adrenergic receptors, which may follow pathways including the well-established classical pathway like the G protein-AC-cAMP-PKA pathway, as well as non-classical pathways such as PI3K/Akt and Ras/ Raf/ MEK/ERK pathways, but the exact mechanisms of which are not fully appreciated(10).

Autophagy is a metabolic process in which cells degrade cell contents and maintain cell viability through lysosomes. In general, vesicles with a bilayer membrane structure wrap around cell contents to form autophagosomes and then fuse with lysosomes to form autophagic lysosomes, ultimately degrading cell contents through various enzymes. Autophagy is an essential way to control cell quality, whereby cells meet metabolic needs and remove and renew cellular contents and organelles. It is found that the level of autophagy in cardiomyocytes increases significantly after hypoxia, which facilitates to the removal of damaged organelles from damaged cells(11). Evidence from recent in vivo and ex vivo studies in animals has shown that cardiomyocyte autophagy decreases in the hypertrophic phase of the myocardium and increases in the heart failure phase after chronic pressure overload(12). There are also studies reporting that astragaloside prevents stress stress-induced myocardial hypertrophy by activating autophagy and reducing inflammation(13-14). The study finds
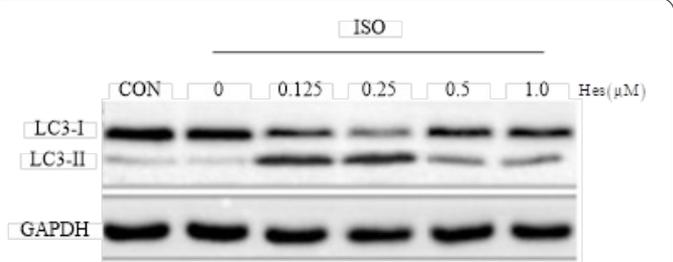

Figure 5. Changes in LC3II expression after hesperidin stimulation with different concentrations.

Figure 6. Changes in LC3II expression after hesperidin stimulation at different times.

a considerable reduction in LC3II expression in a model of cardiomyocyte hypertrophy induced by ISO stimulation, which is consistent with previous studies.

Hesperidin is one of the most common flavonoids in citrus, deriving from glycosidase-mediated hydrolysis of hesperidin and neohesperidin. Some studies found that hesperidin inhibited stress stress-induced myocardial hypertrophy, fibrosis and dysfunction in mice model of stress stress-induced cardiac remodeling.[14-15]In this study, we find that hesperidin is able to inhibit myocardial hypertrophy induced by ISO stimulation without concentration and time dependence, and the results showed that 0.25 μmol /L hesperidin could maximally inhibit myocardial remodeling after 12 h of action on cardiomyocytes, and possibly there is an optimal concentration and effective time for hesperidin intervention. And it has been demonstrated that hesperidin is able to improve glucose uptake partly by inhibiting autophagy and attenuating the impaired energy metabolism in early Alzheimer's disease, thus reducing neuronal damage (15). In this study, however, hesperidin is capable of significantly increasing the expression of LC3II, suggesting activation of autophagy in cardiomyocytes, and the activation is most evident at a concentration of 0.25 μmol /L and 12 h of action on cardiomyocytes.

Hesperidin may reverse myocardial hypertrophy by activating autophagy and promises to be a new clinical drug for the treatment of heart failure.

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