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Protection of Ursolic Acid on Myocardial Ischemia/Reperfusion Injury in H9c2 Cells

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Abstract: To investigate the effects of ursolic acid (UA) against myocardial ischemia-reperfusion in H9c2 cells, cells were divided into six groups: control group, myocardial ischemia reperfusion group, UA (10, 20, or 40 $\mu\text{mol} \cdot \text{L}^{-1}$) group, and nimodipine positive control group (5 $\mu\text{mol} \cdot \text{L}^{-1}$). The activity of cell proliferation was determined by CCK-8, and the flow cytometer analysis was employed to determine Ca^{2+} concentration, reactive oxygen species (ROS) production and cell apoptosis after 6 h when the drugs were given before the hypoxia reoxygenation of cells. The results showed that the UA treatment protected ischemia/reperfusion (I/R) injured cells from apoptosis remarkably via attenuating the release of cytokines and chemokines, Ca^{2+} overload and ROS generation. Furthermore, western blot showed that UA could significantly induced SLC8A2, RyR2 and RyR1 activity. Compared with the I/R group, the activity of Bcl-2 also increased significantly, and the activity of cleaved Caspase-3, Cyt-c, p-ERK 1/2 and p-p38 all decreased. These results suggest that UA has dose-dependent protective effects against myocardial I/R injury in H9c2 cells. Therefore, these findings provided evidence that UA could protect H9c2 cells from I/R injury, and the pro-proliferation and anti-apoptotic effects of UA might be mediated through decrease of ROS generation by Ca^{2+} content by activation of ERK 1/2 and p38 pathways.

Keywords: ursolic acid; myocardial ischemia/reperfusion injury; apoptosis; reactive oxygen species; Ca^{2+}

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熊果酸对心肌缺血再灌注损伤的 H9c2 细胞的保护作用

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摘要: 为研究熊果酸(UA)对 H9c2 细胞心肌缺血/再灌注的影响,将细胞分为对照组、心肌缺血再灌注组(I/R)、

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熊果酸组(10,20,40 $\mu\text{mol} \cdot \text{L}^{-1}$)和尼莫地平阳性对照组(5 $\mu\text{mol} \cdot \text{L}^{-1}$). 在细胞缺氧和复氧之前给药 6 h 后, 经 CCK-8 检测细胞的增殖能力,采用流式细胞术检测钙离子(Ca^{2+})摩尔浓度、活性氧(reactive oxygen species,ROS)的产生及细胞凋亡情况. 结果发现,UA 对心肌缺血/再灌注细胞损伤的细胞具有明显的保护作用,其主要通过抑制细胞中细胞因子和趋化因子的释放,进而抑制 I/R 受损细胞的凋亡. 蛋白质免疫印迹实验结果显示,与 I/R 组相比,UA 能够显著诱导细胞中 SLC8A2,RyR1,RyR2 及 Bcl-2 的表达,抑制剪切型 Caspase-3,Cyt-c,p-ERK1/2 和 p-p38 蛋白的表达,且随着 UA 浓度升高趋势愈明显,表明 UA 对 H9c2 细胞的 I/R 损伤的保护作用具有浓度依赖性.UA 促进细胞增殖和对心肌缺血/再灌注损伤的 H9c2 细胞保护作用可能与细胞中 Ca^{2+} 摩尔浓度及 ROS 值降低有关,其主要机制可能通过 ERK1/2 和 p-38 信号通路介导. 该研究能为 UA 保护 H9c2 细胞免受 I/R 损伤提供依据.

关键词: 熊果酸; 心肌缺血/再灌注损伤; 细胞凋亡; 活性氧; 钙离子

The definition of myocardial ischemia/reperfusion (I/R) injury is that ischemic myocardium re-stores blood flow after reperfusion, with its structural damage, cell death, infraction extend and further damage of the heart function which can affect the prognosis of patients with myocardial infarction^[1]. The pathogenesis of myocardial I/R injury is complex, including oxygen free radicals, calcium overload, and inflammatory medium. Ursolic acid (UA), a pentacyclictriterpenoid carboxylic acid, mainly exists in the bearberries, *Prunella vulgaris* and *Ilex rotunda* medicinal herbs and other plants^[2]. It is known that UA has a wide range of biological activities, such as good oxidation resistance, anti-inflammatory, cytotoxic, anti-apoptosis, anti-tumor and other pharmacological effects^[3-4]. UA has been shown to suppress tumorigenesis^[5] and inhibit tumor promotion^[6-7]. UA has also been reported to induce apoptosis in a wide variety of cancer cells through inhibition of DNA replication^[8], activation of caspases^[9], or induction of Ca^{2+} release^[10]. In the current report, we construct I/R model and study the protection effects of UA in myocardial I/R damage.

1 Materials and Methods

1.1 Cell Culture and Treatment

The H9c2 cells were purchased from American Type Culture Collection and Cultured in DMEM supplemented with 10% FBS at 37 °C in an atmosphere of 5% CO_2 and 95% air.

In order to study effects of UA, H9c2 cells were treated with or without various concentrations of UA. The cells were divided into six groups: normal group (A group), I/R group (B group), UA (10, 20, 40 $\mu\text{mol} \cdot \text{L}^{-1}$)-treated I/R group (C group, D group, E group), and nimodipine (5 $\mu\text{mol} \cdot \text{L}^{-1}$)-treated I/R positive group (F group). Cells were treated with UA and nimpdipine before hypoxia and reoxygenation.

I/R of H9c2 myocardial cells were cultured in ischemia buffer (1.13 mmol $\cdot \text{L}^{-1}$ CaCl_2 , 5 mmol $\cdot \text{L}^{-1}$ KCl, 0.3 mmol $\cdot \text{L}^{-1}$ KH_2PO_4 , 0.5 mmol $\cdot \text{L}^{-1}$ MgCl_2 , 0.4 mmol $\cdot \text{L}^{-1}$ MgSO_4 , 128 mmol $\cdot \text{L}^{-1}$ NaCl, 4 mmol $\cdot \text{L}^{-1}$ NaHCO_3 , 10 mmol $\cdot \text{L}^{-1}$ HEPES ((4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid), pH=6.8) and placed into a hypoxic chamber (37 °C, 1% O_2 , 20% CO_2 and 79% N_2) for 1 h, followed by reperfusion for 3 h under normal culture conditions^[11].

1.2 CCK-8 Assay

H9c2 cells were plated at a density of 3.0×10^3 cells/well in 96-well plates, and the cell viability was assessed by the CCK-8 assay (Dojindo, Japan). Briefly, cells were treated with 10, 20, or 40 $\mu\text{mol} \cdot \text{L}^{-1}$ UA or 5 $\mu\text{mol} \cdot \text{L}^{-1}$ nimodipine before constructed hypoxia and reoxygenation model and then incubated at 37 °C with the DMEM medium for 6 h. Each well of the plate was added 10 μL CCK-8 solution and cultured for 1 h in the incubator. The absorbance was measured at a test wavelength of 450 nm.

1.3 Assay of Apoptosis

To detect early stages of apoptosis, an annexin-V/propidium iodide staining kit (Roche) was used according to the manufacturer's instructions. Briefly, I/R cells were washed with PBS(phosphate buffered solution) and incubated with annexin-V/PI for 15 min at 4 °C, prior to analysis by a flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

1.4 Assay of Intracellular Ca^{2+} Detection

Ca^{2+} has been recognized as the second messenger in the signal transduction. Briefly, cells were incubated on 6-well plates, loaded with the fluorescent Ca^{2+} probe Fluo-3-AM ($10 \mu\text{mol} \cdot \text{L}^{-1}$) in the PBS buffer, washed and examined the fluorescence intensity (F) of single cell using flow cytometry. Then sample aspiration was added 1% Triton X-100 for 30 min at room temperature and $1 \text{ mmol} \cdot \text{L}^{-1}$ CaCl_2 for 1-10 min, and the F_{\max} value was detected. The F_{\min} was measured at wavelength of 488 nm after added with $10 \text{ nmol} \cdot \text{L}^{-1}$ EDTA for 1-10 min. Ca^{2+} concentration was achieved by the equation:

$$\text{Ca}^{2+} = \frac{K_d(F - F_{\min})}{F_{\max} - F}, \text{ where } K_d = 390 \text{ nmol} \cdot \text{L}^{-1}.$$

1.5 Measurement of Intracellular ROS

Intracellular reactive oxygen species (ROS) levels were examined using DCFH-DA. Cells were seeded in 6-well plates and loaded with DCFH-DA ($10 \mu\text{mol} \cdot \text{L}^{-1}$) in a loading buffer for 20 min at 37 °C. ROS fluorescence intensity was determined by flow cytometry with excitation at 480 nm and emission at 525 nm. ROS positive cells have strong green fluorescence, corresponding to FL1 detection channel BD flow cytometry.

1.6 Western Blot Analysis

To determine the levels of protein expression, whole cell extracts were prepared in lysis buffer. At the end of incubation, the medium was removed and H9c2 cells were harvested and washed briefly with PBS and immediately lysed in a buffer containing $20 \text{ mmol} \cdot \text{L}^{-1}$ Tris-HCl. The total proteins were quantified using the BCA protein assay kit. Loading buffer was added to cytosolic extracts and boiling for about 5 min, and the same amounts of supernatant from each sample were separated by 10% SDS PAGE and transferred to a nylon membrane.

After blocking with 5% skim milk for 1.5 h in fresh block buffer, the membrane was probed with anti-SLC8A2, anti-RyR2, anti-RyR1, anti-Bcl-2, anti-cleaved Caspase-3, anti-Cyt-c, anti-p-ERK 1/2, anti-ERK 1/2, anti-p-p38, anti-p38 and anti-GADPH in freshly prepared TBS-T with milk overnight at 4 °C. The membranes were washed for 3 times with TBS-T and then incubated with secondary anti-mouse or anti-rabbit antibodies at room temperature for 2 h. The immunoreactive signals were visualized by using an enhanced chemiluminescence (ECL) system and glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as the corrected protein for total protein.

1.7 Statistical Analysis

The experiments were repeated three times in six replicate samples. Data were expressed as mean \pm SD. Statistical significance was determined using Graph Pad Prism V5.0 software (GraphPad, La Jolla, CA). Differences were performed by caspase-tailed t test and one-way ANOVA, and the level of significance was set at $P < 0.05$ *, $P < 0.01$ * *. Note: in the following figure, “##” representative $P < 0.01$, when compared with the control groups; “*” representative $P < 0.05$ and “* *” representative $P < 0.01$, when compared with the I/R group.

2 Results and Analysis

2.1 UA Increases Cell Proliferation of I/R H9c2 Cells

H9c2 cell line, treated with various concentrations (10 , 20 or $40 \mu\text{mol} \cdot \text{L}^{-1}$) of UA, were then

constructed I/R cells. After cultured for 6 h, the optical density value of cells was detected, shown in figure 1.

According to CCK-8 assay, the result showed that the cell proliferation rate of I/R cells was significantly decreased compared with control group, while that of I/R cells treated with UA was significantly increased compared with that of I/R cells without UA treatment.

2.2 UA Decreases Cell Apoptosis of H9c2 I/R Cells

The aim of this study was to investigate whether UA can enhance the sensitivity of H9c2 cells. Cardiomyocyte apoptosis is potentially important in many disorders. To evaluate the effects of UA on myocardial apoptosis, cell apoptosis was determined by flow cytometry. Effects of different concentrations of ursolic acid on cell apoptosis in I/R H9c2 cells were measured by flow cytometry, shown in figure 2.

The result showed that the cell apoptosis in I/R model was significantly increased compared with the control group ($P<0.01$); after treated with UA ($10, 20$, or $40\text{ }\mu\text{mol}\cdot\text{L}^{-1}$) and nimodipine ($5\text{ }\mu\text{mol}\cdot\text{L}^{-1}$), the apoptosis rate of mycocardial I/R cells were decreased compared with I/R cells without UA treatment ($P<0.05^*$, $P<0.01^{**}$).

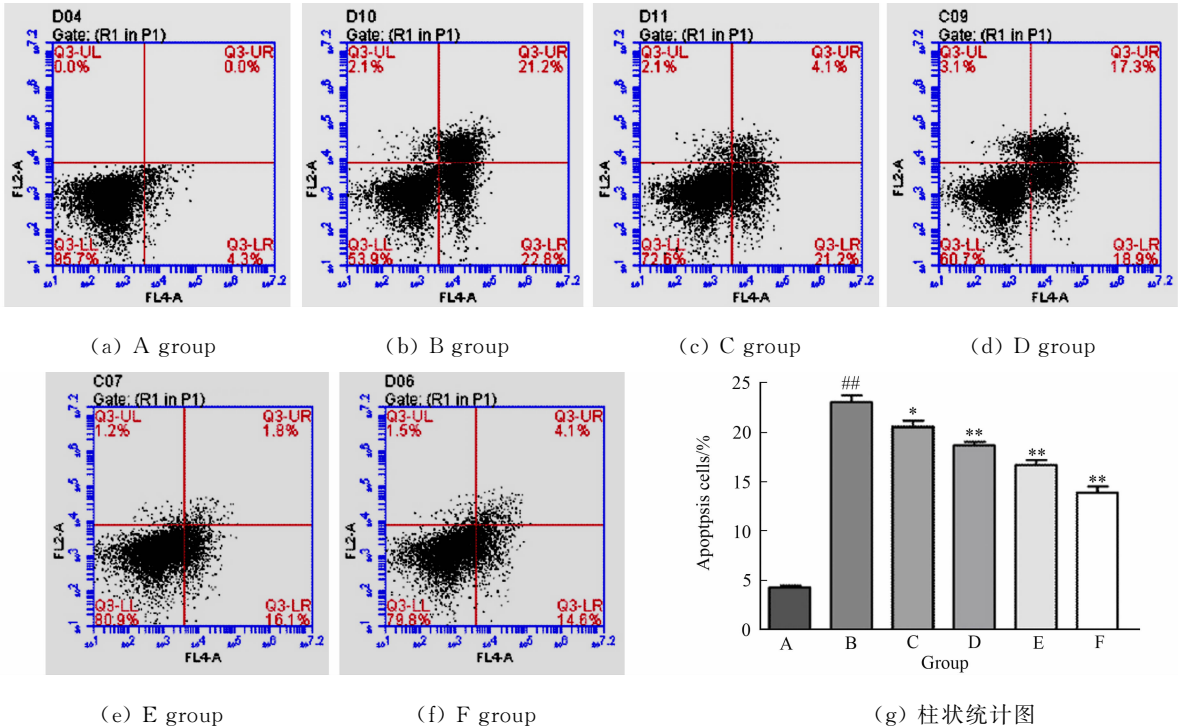


Fig. 2 Effects of different concentrations of ursolic acid on cell apoptosis in I/R H9c2 cells measured by flow cytometry

图2 不同摩尔浓度的熊果酸对 I/R 细胞凋亡的影响通过流式细胞术测量 H9c2 细胞

2.3 UA Decreases ROS and Ca²⁺ Level in H9c2 I/R Cells

Mechanisms of cell toxicity particularly involving in oxidative stress are strictly linked to a deregulation of cytosolic free calcium concentration. Since intracellular Ca²⁺ generation is a powerful signal for ROS production, which directly results in cell death, the ROS and Ca²⁺ levels were therefore determined in I/R H9c2 cells. The ROS and Ca²⁺ contents were significantly increased in I/R group compared with control group, while those were dramatically decreased in mycocardial I/R cells with UA ($10, 20$ or $40\text{ }\mu\text{mol}\cdot\text{L}^{-1}$) or nimodipine ($5\text{ }\mu\text{mol}\cdot\text{L}^{-1}$) treatment compared with I/R group

without UA and nimodipine treatment (figures 3 and 4).

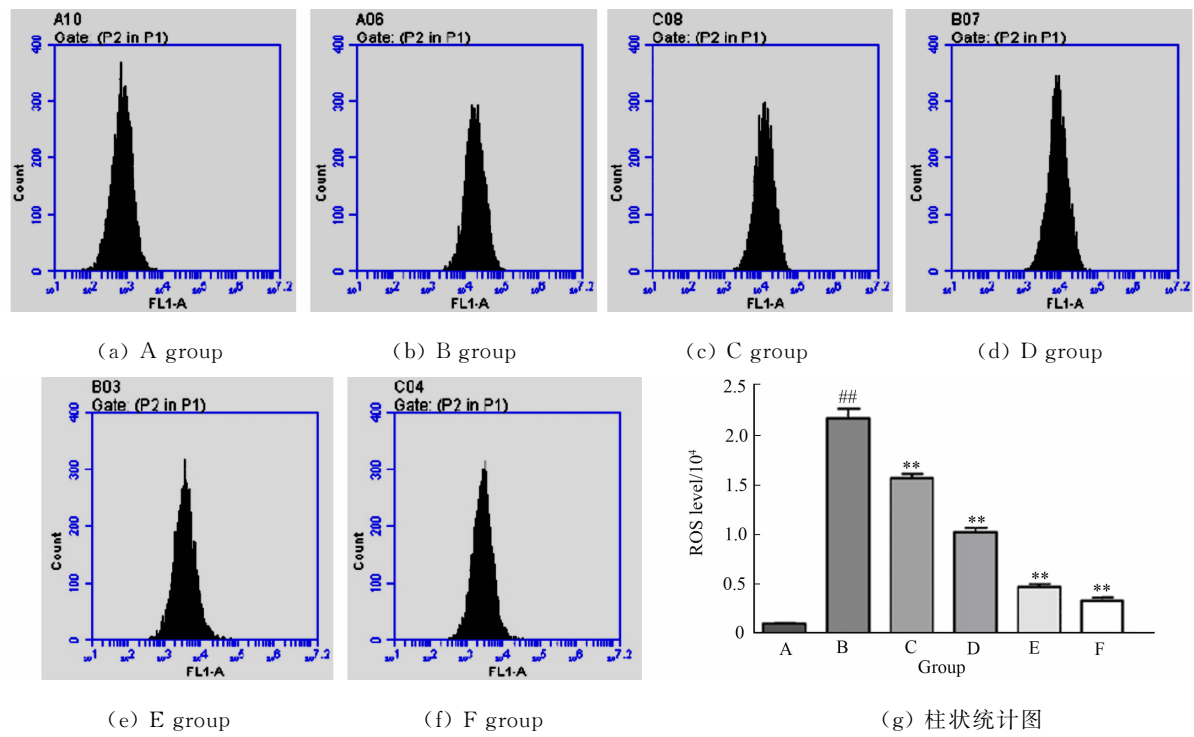


Fig. 3 Concentration-dependent effect of ursolic acid on ROS level in I/R H9c2 cells

图 3 熊果酸摩尔浓度依赖性对 I/R H9c2 细胞中 ROS 水平的影响

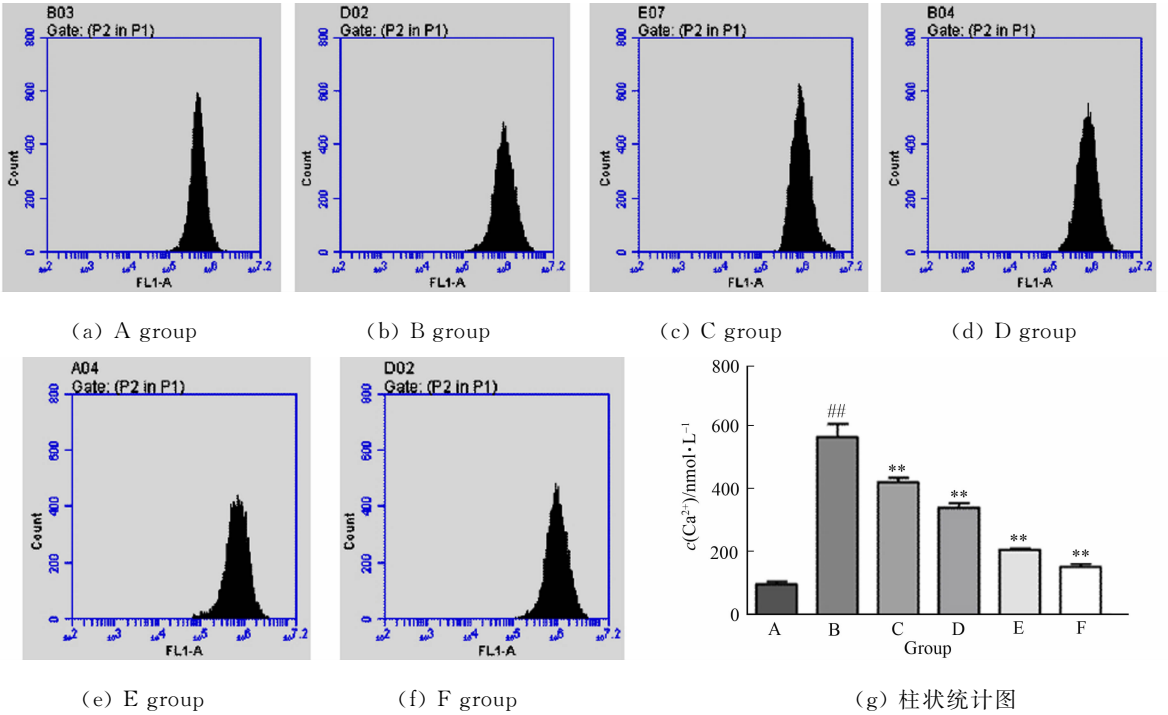


Fig. 4 Concentration-dependent effect of ursolic acid on Ca²⁺ content in I/R H9c2 cells

图 4 熊果酸摩尔浓度依赖性对 I/R H9c2 细胞中 Ca²⁺ 摩尔浓度的影响

2.4 UA Regulates Expression of Proteins Associated With Ca²⁺ Transduction and Apoptosis

I/R can execute apoptosis by activating mitochondrial pathway. In order to detect the mechanism of UA on I/R cells. Western blot analysis was performed in H9c2 cells pre-incubated with different concentrations of UA. SLC8A2, RyR2 and RyR1 are proteins which related to Ca²⁺ transduction. Western blot results showed that the expression levels of SLC8A2, RyR2 and RyR1 were significantly

decreased in I/R model cells compared with control cells.

After treated with UA (10, 20 or 40 $\mu\text{mol} \cdot \text{L}^{-1}$) or nimodipine (5 $\mu\text{mol} \cdot \text{L}^{-1}$), the expression levels of SLC8A2, RyR2 and RyR1 in I/R model cells were significantly increased compared with the I/R model cells without UA and nimodipine treatment. We also detected the effects of UA on expression of Bcl-2, cleaved Caspase-3, Cyt-c, p-ERK 1/2, ERK 1/2, p38 and p-p38.

The results showed that the apoptosis factor Bcl-2 expression in I/R model cells treated with UA (10,20, or 40 $\mu\text{mol} \cdot \text{L}^{-1}$) or nimodipine (5 $\mu\text{mol} \cdot \text{L}^{-1}$) was increased and the expression of cleaved Caspase-3, Cyt-c, p-ERK 1/2 and p-p38 in those were significantly down-regulated compared with the I/R model cells without UA and nimodipine treatment (Figure 5).

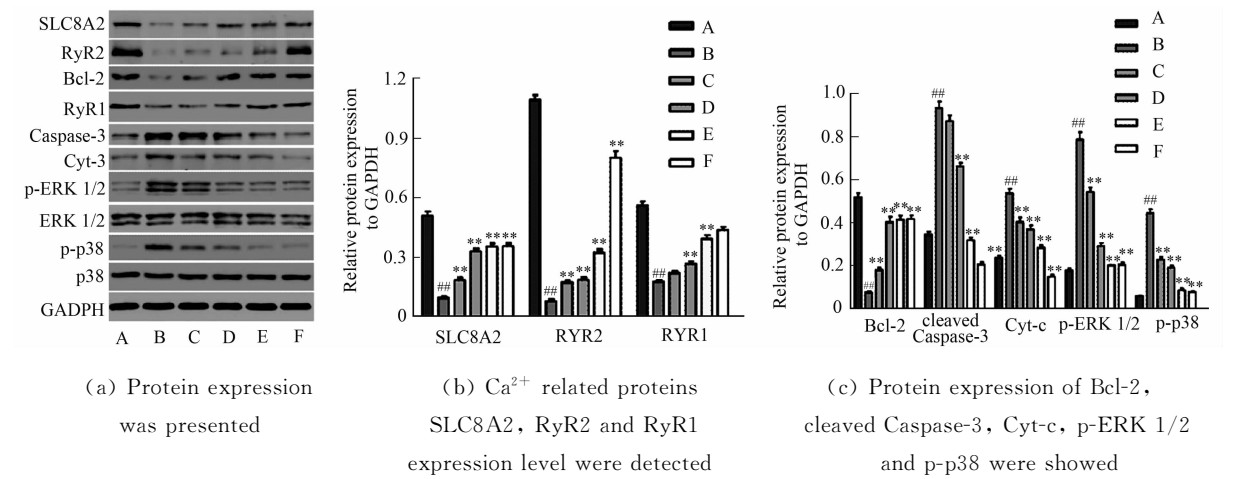


Fig. 5 Effects of different concentrations of ursolic acid on protein expression in human I/R H9c2 cells

图 5 不同浓度的熊果酸对人 I/R H9c2 细胞中蛋白质表达的影响

3 Discussion

Myocardial ischemia-reperfusion injury (MIRI) typically arises in patients presenting with myocardial infarction. The process of myocardial infarction can itself induce further cardiomyocyte death, a phenomenon known as myocardial reperfusion injury^[12]. Ursolic acid is an anti-inflammatory and anti-proliferative triterpene that has been shown to interfere with cytokine signaling pathway. In this study, the effects of different concentrations of UA on the functions of I/R cells H9c2 were investigated. Our results indeed demonstrated that cell proliferation in I/R cells was significantly increased with the dose dependent of UA. And UA can also inhibit cell apoptosis in a dose-dependent manner when compared with I/R H9c2 cells. This result revealed that UA had the dose-dependent protection against MIRI in H9c2 cells. In line with our findings that UA could protect against hypoxia/reoxygenation-induced cell apoptosis in H9c2 cells *in vitro* and also against I/R-induced injury in rat hearts *ex vivo*^[13].

It is well known that mitochondrial respiration attenuates the generation of reactive oxygen species (ROS)^[14]. Ischaemia is thought to contribute to the damage during reperfusion^[15]. Increases in cellular Ca^{2+} and ROS, initiated in ischaemia and then amplified upon reperfusion, are thought to be the main causes of reperfusion injury. And mitochondria are as targets for the damaging action of both ROS and calcium^[16]. Chen, *et al*^[17] has shown that pre-incubation with UA produced a transient increase in the mitochondrial membrane potential in H9c2 cells, which was accompanied by increases in mitochondrial ROS production. Consist with the previous study, our results found that UA could reduce ROS level and the content of Ca^{2+} , which reveal that UA can increase the body's ability to remove free radicals and decrease the Ca^{2+} content.

How UA regulated the activation of related proteins is not clear. SLC8A2 (sodium/calcium exchanger, member 2) is a protein that relates to calmodulin binding and calcium. An analysis of SLC8A2 has been performed in glioma previously^[18]. RyR2 (Ryanodine Receptor 2 (cardiac)) is an intracellular Ca^{2+} release channel that includes calcium ion binding and enzyme binding^[19]. An important paralog of this gene is RyR1. Western blot showed that Ca^{2+} related proteins SLC8A2, RyR2 and RyR1 expression were up-regulated after UA treatment when compared with I/R group. Anti-apoptotic protein Bcl-2 has been reported to prevent apoptosis and I/R injury^[20]. Cytochrome C (Cyt-c) released from mitochondria requires a caspase-step process and often during the early stages of apoptosis^[21]. Cyt-c has two cellular functions: respiration and caspase activation, resulting in apoptosis^[22]. Previous study showed that blockade of Caspase-3 can ameliorate reperfusion injury by upregulating Bcl-2 and without affecting cytochrome c release^[17]. However, in the present study, the apoptosis related factor Bcl-2 was up-regulated; cleaved Caspase-3 and Cyt-3 factors were down-regulated after treated with UA in I/R cells. ERK 1/2 can be activated by I/R, and they may play important roles in the evolution of ischemic injury^[23]. p38 mitogen-activated protein kinase in myocardial I/R injury associated with ischemic preconditioning^[24].

In the present study, UA significantly decreased the phosphorylation of ERK 1/2 and p38 in I/R H9c2 cells. These data indicated that UA protected cells from apoptosis via the inhibition of ERK 1/2 and p38 activation. However, vitexin protects brain against cerebral I/R injury through upregulating p-ERK 1/2 and downregulating p-p38^[17]. Taken together, these data suggest that the role of ERK 1/2 and p38 may differential presence in the I/R injury model, and the mechanisms need further investigation.

Overall, our results suggest that pro-proliferation and anti-apoptotic effects of UA may be mediated through inhibition of ROS level and Ca^{2+} content and activation of ERK 1/2 and p38 signaling.

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