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Original article

Imperatorin protects H9c2 cardiomyoblasts cells from hypoxia/reoxygenation-induced injury through activation of ERK signaling pathway

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A B S T R A C T

Background: Imperatorin is a compound found in plants and has been widely used in Chinese medicine for many years. It has many pharmacological effects, including the recently reported anti-apoptotic function, however, the mechanism largely remains unclear. This study is aimed to elucidate the mechanism of Imperatorin's anti-apoptotic function.
Methods: A model of hypoxia and reoxygenation (H/R) treated h9c2 cardiomyoblasts was successfully constructed. The cells were treated with H/R condition, and followed by adding Imperatorin alone, Imperatorin with ERK inhibitor and/or ERK inhibitor alone, to examine the cell viability by Cell Counting Kit-8 assay, cell apoptosis rate by flow cytometry, and ERK expression by Western-blot under different conditions.
Results: The results showed that imperatorin exerted protective effect on h9c2 cells from H/R injure. It was also found that it not only increased cell viability but also reduced the apoptotic rate for H/R treated h9c2 cells. The experiments also demonstrated that imperatorin could upregulate the expression levels of both ERK1 and ERK2, which is a key step in ERK signaling pathway activation.
Conclusions: These findings provided evidence that imperatorin could increase the cell viability and lower apoptotic rate in H/R treated h9c2 cells, and could also enhance the expression of ERK1/ERK2, demonstrating imperatorin's protective effect on H/R injured h9c2 cells through ERK signaling pathway.
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1. Introduction

Inefficient vascular supply and the resultant reduction in tissue oxygen tension often lead to neovascularization in order to satisfy the needs of the tissue. The reliance of many cells on aerobic respiration as a mandatory energy source requires a variety of responses to oxygen lack or "hypoxia" (Darby and Hewitson, 2016). Both hypoxia (lack of oxygen relative to metabolic needs) and reoxygenation (reintroduction of oxygen to hypoxic tissue) are important in human pathophysiology because they occur in a wide variety of important clinical conditions. Prominent examples of tissue hypoxia that predispose to injury during reoxygenation include circulatory shock, myocardial ischemia, stroke, and transplantation of organs (Martindale and Holbrook, 2002; Wenger, 2002; Brady et al., 2006). At the cellular level, hypoxia activates numerous major signaling pathways, resulting in changes in gene expression, which influence the cellular ability to survive or die. Severe hypoxia, occurring at partial pressure of oxygen below 20 mmHg, impairs cellular energy production and ion homeostasis, leading to cell injury and cell death. A lower degree of hypoxia, defined as between 50 and 100 mmHg, may activate mechanisms that could produce cellular phenotype more resistant to acute severe oxidative stress (Zweier and Talukder, 2006). This phenomenon is one of the most important components of different forms of ischemic heart diseases which include myocardial infarction also. Cellular models of hypoxia-reoxygenation (HR) have provided useful tools for the study of reactive species mediated mechanisms of

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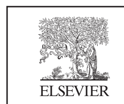

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cellular dysfunction in ischemia-reperfusion injury (Watkins et al., 1995). H9c2 cells are derived from embryonic rat heart and are generally accepted to be a good model for cardiomyoblast cells. These cells have been successfully implemented to study mechanisms of cellular and cardiac protection (Ekhterae et al., 1999; Ranki et al., 2002).

The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in the regulation of various cellular responses, such as cell proliferation, differentiation, and apoptosis (Cross et al., 2000; Pearson et al., 2001). The extracellular signal-regulated kinase (ERK) is a subfamily member of MAPKs which is activated by an upstream kinase called MAPK/ERK kinase (MEK) in response to growth stimuli. The ERK pathway mediates a number of cellular fates including growth, proliferation, and survival (Sun et al., 2015; Peti and Page, 2013). It has been found that H/R induces activation of MEK/ERK and PI3K/Akt survival signaling pathways through a PKC-dependent mechanism (Sung et al., 2007), suggesting These pathways may be responsible for the repair process during ischemia/reperfusion, but the mechanism remains not very clear. Later it was also found that the PI3K/Akt pathway was required for hypoxia-induced expression of the transcription factor hypoxia-inducible factor (HIF)-1alpha and Vascular endothelial growth factor (VEGF), whereas the MEK/ERK pathway was required only for VEGF in laser-induced rat choroidal neovascularization (CNV) (Wang et al., 2015; Yang et al., 2009).

Many compounds found in plants have been widely used in traditional Chinese medicines to treat various chronic diseases (Wang et al., 2009a,b), for example, Imperatorin. Imperatorin is noted as a constituent in many traditional medicaments, especially in Traditional Chinese Medicine. Imperatorin, known as 8-isopentenyloxypsoralen or 9-(3-methylbut-2-enyloxy)-7H-furo [3,2-g] chromen-7-one, is the main composition of the dried root or rhizome of Radix Angelicae Dahuricae. It has several pharmacological effects, such as anti-inflammation and tumor inhibition (Yine et al., 2015; Garcia-Argaez et al., 2000; Ban et al., 2003), inhibiting either vesicular stomatitis virus-pseudo typed or gp160-enveloped recombinant HIV-1 infection (Sancho et al., 2004). Evidence also indicates that imperatorin is a calcium channel blocker and develops favorable vasodilator effect (He et al., 2007; Zhang et al., 2012). However, there are few reports about the protective effect on cerebral ischemia by this furanocoumarins. In this report, the studies showed that imperatorin can protect h9c2 cells from H/R injure. It can increase cell viability and reduce the apoptotic rate for H/R treated h9c2 cells. It also demonstrated that imperatorin can upregulate the expression level of ERK1/2, which is a key step in ERK signaling pathway activation. This suggested that imperatorin's protective effect is through the ERK signaling pathway.

2. Methods

2.1. Cell culture

The H9c2 cardiomyocyte line was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Upon receipt the culture were visually examined for macroscopic evidence of any microbial contamination. Decontaminate by dipping in or spraying with 70% ethanol, and all of the operations from this point on were carried out under strict aseptic conditions. Aseptically remove all of the shipping medium and wash cells 3–4 times with phosphate-buffered saline (PBS) buffer, each time 2–3 ml. Then add 1.5 mL 0.25% (w/v) Trypsin-EDTA solution to suspend the cells. Centrifuge the cells at 1000 rpra for 5 min, and re-suspend cells with high glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin. The cells were maintained in an incubator containing 5% CO_2 at 37 °C. When cells reached 90% confluence and are actively proliferating, it is time to subculture.

To freeze the h9c2 cells for storage, the cells were transferred to the freezing media containing 90% FBS and 10% dimethyl sulphoxide (DMSO) and the cell concentration was adjusted to 2×10^6/mL freezing media. The procedure was to keep the cells at 4 °C for 30 min, followed 30 °C min at −20 °C, and finally transferred to −80 °C or into liquid N_2 for storage.

2.2. Hypoxia/reoxygenation construction

H9c2 cardiomyocytes were grown in DMEM supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin at 37 °C for 24 h. After the cells were washed with PBS buffer twice, high glucose DMEM medium was changed to DMEM with no glucose to mimic ischemia, which was pre-saturated with 95% CO_2 and 5% N_2. The H9c2 cardiomyocytes were cultured in a hypoxia chamber, saturated with 5% CO_2/95% N_2 for 6 h. After that, the medium was replaced with high glucose medium (High glucose 10% FBS DMEM) and incubated for reoxygenation in a chamber containing 5% CO_2 at 37 °C for 6 h.

2.3. H9c2 cells grouping

The cells were randomly selected to form 5 groups, each group 10 wells. In the C group, cells were cultured in high glucose DMEM supplemented with 10% FBS in an incubator with 5% CO_2 for 12 h; In the H/R treatment group (H/R), cells underwent the 6 h hypoxia and then 6 h reoxygenation procedure; in the imperatorin treatment group (IM), 20 μM imperatorin was added into the media and then the cells underwent the 6 h hypoxia and then 6 h reoxygenation procedure; the fourth group is the imperatorin and inhibitor treatment group (IM + PD), in which 20 μM imperatorin and 10 μM ERK inhibitor PD98059 were added into the media and then the cells underwent the 6 h hypoxia and then 6 h reoxygenation procedure; the last group is the inhibitor group (PD), in which 10 μM ERK inhibitor PD98059 was added into the media and then the cells underwent the 6 h hypoxia and then 6 h reoxygenation procedure.

2.4. Cell viability analysis

Cell viability was determined with Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan). Briefly, H9c2 cells were plated on 48-well plates at a density of 1×10^6 cells/mL, with six duplicate wells in each group, each well 200 μl. 20 μl of CCK-8 solution was added to each well containing 200 μl of DMEM/F12 medium. The absorbance at 490 nm of each well was monitored using a micro-well plate reader (Multiscan MK3; Thermo Labsystems, Waltham, MA, USA) after a 4 h incubation at 37 °C.

2.5. Determination of apoptosis

The rates of cell apoptosis were analyzed by flow cytometry (Becton Dickinson) following fluorescein isothiocyanate-conjugated annexin V (FITC-annexin V) and propidium iodide (PI) staining. The staining procedure was performed according to the instructions of the annexin V-FITC Apoptosis Detection Kit (BioVision). H9c2 cells were re-suspended after centrifugation to a concentration of 1×10^6 cells/mL. 100 μl cells were transferred to 5 ml tubes, followed by adding 5 μl FITC annexin V and 5 μl PI. The cell suspension was gently vortexed and incubated at room temperature for 15 min, shielded from light, and then 400 μl Annexin V binding buffer was added. Flow cytometry was performed as soon as possible (within 1 h). In the annexin V-PI

co-labelling method, the annexin V-negative/PI-negative fraction represented viable cells, the annexin V-positive/PI-negative fraction represented early apoptotic cells, and the annexin V-positive/PI-positive fraction represented late apoptotic and dead cells.

2.6. Western blot

H9c2 Cells were incubated with 0–250 μM of IGOB131 acids for 12 and 24 h at 37 °C in a humidified 5% CO_2 incubator before they were collected and lysed. The protein concentration was estimated with BCA assay (Olsen and Markwell, 2007). Total protein (50–60 μg) was separated by SDS-PAGE using a 12% polyacrylamide gel. β-actin (Cell Signaling Technology, USA) served as an internal control. The proteins in the gel were transferred to a PVDF membrane which was then blocked with 5% skim milk in PBST (0.05% v/v Tween-20 in PBS, pH 7.2) for 1 h or at 4 °C overnight. Membranes were incubated with specific antibodies against ERK1/2 (1:1000) and β-actin (1:1000), respectively, at 4 °C overnight. Membranes were washed in PBST for 10 min three times between each step. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). The relative expression of ERK1 and ERK2 was quantified densitometrically using the software LabWorks 4.6, and calculated according to the reference bands of β-actin.

2.7. Statistical data analysis

Statistical analysis was performed with the program SPSS statistical software, version 19.0 (IBM Corp., Armonk, NY). Data were expressed as the mean ± standard deviation (SD). Single data points were presented in some graphs. Statistical analyses were performed using one way Analysis of variance (ANOVA) followed by two tailed student *t*-test (*T*-test) with equal variances assumption. Statistical significance was assumed at $p < 0.05$ and $p < 0.01$ respectively.

3. Results

3.1. Effects of imperatorin on cell viability of H9c2 cells after H/R treatment

H9c2 cells in the C group were under normal culture condition without H/R treatment. Cells in all other four groups were treated with 6 h hypoxia followed 6 h reoxygenation. Table 1 clearly showed that the cell viabilities in all other four groups were significantly lower than C group ($p < 0.01$). This confirmed that our H/R modeling with h9c2 cells was successful.

Comparing to H/R group, IM group which were treated with imperatorin after H/R, showed both significant higher OD_{450nm} and cell viability ($p < 0.01$), which suggested that imperatorin has protective effect on H/R injured H9c2 cells and could increase the cell viability. However, for IM + PD group and PD group, neither of them showed significant difference in OD_{450nm} or cell viability than H/R group. This demonstrated that the protective effect of imperatorin on H/R injured H9c2 cells was inhibited by the ERK inhibitor PD98059, therefore the protective effect of imperatorin on H/R injured H9c2 cells is through ERK signaling pathway.

3.2. Effects of imperatorin on H9c2 cells apoptosis induced by H/R treatment

The method of TUNEL was used to determine the percentage of apoptotic cells in all five groups. Table 2 clearly showed that the cell apoptotic rates in all four H/R treated groups were significantly higher than the C group ($p < 0.01$), which also confirmed that our H/R modeling on h9c2 cells was successful.

Comparing to H/R group, IM group which were treated with imperatorin after H/R, showed a significant lower apoptotic rate ($p < 0.01$), which suggested that imperatorin can inhibit the h9c2 cells apoptosis induced by H/R treatment. However, for IM + PD group and PD group, neither of them showed significant difference in the apoptotic rate comparing to the H/R group. This demonstrated that the protective effect of imperatorin on H/R induced apoptosis in H9c2 cells was inhibited by the ERK inhibitor PD98059, which indicated that the protective effect of imperatorin on H/R injured H9c2 cells is through ERK signaling pathway.

3.3. Effects of imperatorin on expression of ERK1/2 in H/R treated H9c2 cells

ERK1/2 expression is an important biomarker in the ERK signaling pathway. Protein expression levels were examined by western blot analysis to investigate if H/R treatment and imperatorin alter the expression of ERK1/2. As shown in Fig. 1, as well as in Fig. 2, comparing to the expression of ERK1/2 of h9c2 cells in the C group, the expression of both ERK1 and ERK2 in the four H/R treated groups, all exhibited a significantly higher level ($p < 0.01$ for H/R, IM and IM + PD groups, $p < 0.05$ for PD group), confirming the H/R treatment did activate the ERK signaling pathway.

Comparing to H/R group, group IM showed significant higher expression levels in both ERK1 and ERK2 ($p < 0.01$). This suggested that imperatorin can promote the expression of ERK1/2 in H/R treated h9c2 cells. However, ERK1/2 expression in IM + PD group did not show significant difference comparing to that in the H/R group. When comparing to the IM group, the expression levels of ERK1/2 in both IM + PD and PD groups were significantly lower ($p < 0.01$). This demonstrated that the enhancing effect of imperatorin on ERK1/2 expression in H9c2 cells was inhibited by the ERK inhibitor PD98059, which indicated that the protective effect of

Table 1
Imperatorin's effect on viability of H/R injured h9c2 cells.

Group	OD_{450nm}
C	1.68 ± 0.03
H/R	$0.86 \pm 0.05^{**}$
IM	$1.47 \pm 0.11^{**,\#\#}$
IM + PD	$1.05 \pm 0.21^{**,\#,\star\star}$
PD	$0.76 \pm 0.15^{**,\star\star}$

*, ** indicate that comparing to the C group, the difference is significant; $^{*}p < 0.05$; $^{**}p < 0.01$.
#, ## indicate that comparing to the H/R, the difference is significant; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$.
★, ★★ indicate that comparing to the IM, the difference is significant; $^{\star}p < 0.05$; $^{\star\star}p < 0.01$.

Table 2
Imperatorin's effect on apoptotic rate of H/R injured h9c2 cells.

Group	Apoptotic rate (%)
C	0.045 ± 0.010
H/R	$0.426 \pm 0.023^{**}$
IM	$0.242 \pm 0.017^{**,\#\#}$
IM + PD	$0.433 \pm 0.031^{**,\star\star}$
PD	$0.446 \pm 0.026^{**,\star\star}$

*, ** indicate that comparing to the C group, the difference is significant; $^{*}p < 0.05$; $^{**}p < 0.01$.
#, ## indicate that comparing to the H/R, the difference is significant; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$.
★, ★★ indicate that comparing to the IM, the difference is significant; $^{\star}p < 0.05$; $^{\star\star}p < 0.01$.

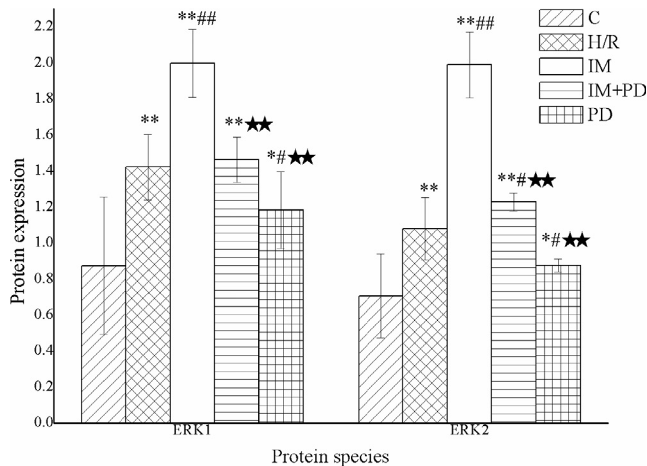

Fig. 1. The protein expression level of ERK1 and ERK2 in the H9c2 cells of each of the five groups. The characters * and ** represent *P < 0.05* and *P < 0.01 respectively, comparing to the C group; # and ## represent P < 0.05* and *P < 0.01 respectively, comparing to the H/R group; ★ and ★★ represent P < 0.05* and *P < 0.01 respectively, comparing to the IM group.*

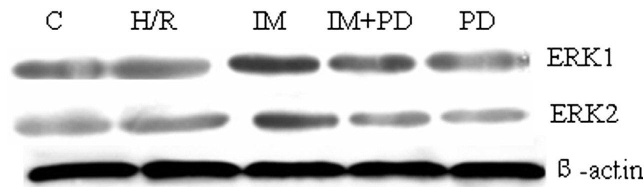

Fig. 2. SDS gel comparing the expression level of ERK1 and ERK2 in the H9c2 cells of each of the five groups. β-actin served as an internal control.

imperatorin on H/R injured H9c2 cells is through ERK signaling pathway.

4. Discussion

The present study showed that imperatorin exerted protective effect on h9c2 cells from H/R injure. It could increase cell viability and also reduce the apoptotic rate for H/R treated h9c2 cells. We further demonstrated that imperatorin could upregulate the expression levels of both ERK1 and ERK2, suggesting that imperatorin's protective effect on h9c2 cardiomyoblasts is associated with its anti-apoptosis function through ERK signaling pathway.

The activation of the ERK pathway is critical for cells' protection from hypoxia-induced apoptosis. The ERK pathway, defined by Extracellular Signal-regulated Kinase-1 (ERK1) and ERK2, functions in cellular proliferation, differentiation, and survival, and its inappropriate activation is a common occurrence in human cancers (Luo et al., 2016; Liu et al., 2015; Wortzel and Seger, 2011). As one of four Mitogen-Activated Protein Kinase (MAPK) signaling pathways, the ERK phosphorylation cascade's importance in intracellular signaling has been compared to the role of the Krebs cycle in energy metabolism (Pagès et al., 1999). Our observation that imperatorin can protect H/R injured h9c2 cells through ERK pathway revealed a novel mechanism of its therapeutic effect. Our results are in accordance with other researchers' reports, for example, Wang et al. report that imperatorin protects neuronal cells from apoptosis induced by hypoxia re-oxygenation (Wang et al., 2013). Interestingly, it was also reported that imperatorin induces Mcl-1 degradation to release Bak and Bax to trigger the intrinsic apoptosis pathway to induce apoptosis of the multidrug-resistant liver cancer cells (Li et al., 2014). Bcl-2 family proteins are downstream proteins of ERK signaling pathway, so taken together, now we have a more complete picture of imperatorin's beneficial effects in anti-apoptosis and cancer therapy.

On the other hand, there was still a notable limitation in our study. The H9C2 cell line we chose in our study is not a perfect mimic of human cardiomyocytes and does not undergo spontaneous contraction, sarcomeric structure, and is not identical in its gene expression profile to human cardiomyocytes. Thus the cell apoptosis of H9C2 cell line in our present study might not represent the actual condition of human cardiomyocytes. To this end, we intend to establish an animal model to further confirm our results, which in under way.

Imperatorin has been used in Traditional Chinese medicine for thousands of years, and in recent years many scientific researches have reported various pharmacological effects of imperatorin in different cell lines and animal models. It has high value in new drug target designing in treating cancer, inflammation, infection and others. For this purpose, it would be worth to search for imperatorin interacting proteins in host and investigate their interaction, with structural modeling studies and biophysics studies. This will be another important and beneficial research area in imperatorin.

5. Conclusion

In summary, this report provides evidence that imperatorin could increase the cell viability and lower apoptotic rate in H/R treated h9c2 cells, and could also enhance the expression of ERK1/ERK2, demonstrating imperatorin's protective effect on H/R injured h9c2 cells through ERK signaling pathway.

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