Irisin protects cardiomyocytes against hypoxia/reoxygenation injury via attenuating AMPK mediated endoplasmic reticulum stress

Rongchuan Yue1,2,3,6, Mingming Lv1,6, Meide Lan1,6, Zaiyong Zheng1, Xin Tan1, Xuemei Zhao3, Yulong Zhang5, Jun Pu1, Lei Xu1,4, & Houxiang Hu1,2,6

Endoplasmic reticulum (ER) stress plays a central role in myocardial ischemia/reperfusion (I/R) injury. Irisin has been reported to have protective properties in ischemia disease. In this study, we aimed at investigating whether irisin could alleviate myocardial I/R injury by ER stress attenuation. The in vitro model of hypoxia/reoxygenation (H/R) was established, which resembles I/R in vivo. Cell viability and apoptosis were estimated. Expressions of cleaved caspase-3, cytochrome c, GRP78, pAMPK, CHOP, and eIF2α were assessed by western blot. Our results revealed that pre-treatment with irisin significantly decreased cytochrome c release from mitochondria and caspase-3 activation caused by H/R. Irisin also reduced apoptosis and increased cell viability. These effects were abolished by AMPK inhibitor compound C pre-treatment. Also, GRP78 and CHOP expressions were up-regulated in the H/R group compared to the control group; however, irisin attenuated their expression. The pAMPK level was significantly decreased compared to the control, and this effect could be partly reversed by metformin pre-treatment. These results suggest that ER stress is associated with cell viability decreasing and cardiomyocytes apoptosis induced by H/R. Irisin could efficiently protect cardiomyocytes from H/R-injury via attenuating ER stress and ER stress-induced apoptosis.

Cardiovascular disease is one of the most prevalent diseases in developed and developing countries, with high morbidity and mortality rates1. It has been demonstrated that cardiomyocyte apoptosis is involved in cardiac dysfunction and chronic heart diseases2,3, such as heart failure, ischemia/reperfusion injury, myocardial infarction, and dilated cardiomyopathy.

Ischemia/reperfusion-injury induced by coronary revascularization therapy has been a significant focus of medical research because of its long-term impact on cardiac dysfunction4,5. There is growing evidence that mitochondrial dysfunction plays an essential role in cell injury induced by myocardial ischemia/reperfusion and reactive oxygen species. The release of apoptosis-inducing factor (AIF), cytochrome c, and the decrease of membrane ΔΨ are all mechanisms that lead to mitochondrial dysfunction and apoptotic cell death6–8. AMP-activated protein kinase (AMPK) is activated to protect cardiomyocytes from I/R injury via regulating mitochondrial function and endoplasmic reticulum homeostasis9,10.

Previous studies reported that endoplasmic reticulum (ER) stress-associated apoptosis is involved in I/R injury. Therefore, it is possible to alleviate I/R injury via attenuating ER stress11,12. For example, antioxidants, such as pineal secreted hormone melatonin and artificial pigment lycopene can protect primary cultured neonatal cardiomyocytes against hypoxia/reoxygenation (H/R) injury by attenuating ER stress and maintaining

1Department of Cardiology, Affiliated Hospital of North Sichuan Medical College, No. 63, Wenhua Road, Shunqing District, Nanchong 637000, Sichuan, People’s Republic of China. 2Academician Workstation, Affiliated Hospital of North Sichuan Medical College, Nanchong 637000, Sichuan, People’s Republic of China. 3Cardiovascular Research Center, Affiliated Hospital of North Sichuan Medical College, Nanchong 637000, People’s Republic of China. 4Department of Cardiology, Central Hospital of Guangyuan, No. 16, Jing Alley, Lizhou District, Guangyuan 628000, Sichuan, People’s Republic of China. 5Anesthesiology Department, Affiliated Hospital of North Sichuan Medical College, Nanchong 637000, People’s Republic of China. 6These authors contributed equally: Rongchuan Yue, Mingming Lv and Meide Lan. *email: 670405651@qq.com; hhxiang17@163.com
mitochondrial functions\textsuperscript{6,13,14}. In addition, irisin has also been shown to reduce oxidative stresses and apoptosis in different models\textsuperscript{13,15,16}.

Irisin, a type I membrane protein encoded by the FNDCS5 (fibronectin domain-containing 5 protein) gene, participates in mitochondrial biogenesis and oxidative metabolism\textsuperscript{15,17}. Recent evidence has indicated that irisin could induce white adipose tissue's browning, which could be used as a therapeutic tool for metabolic disorders\textsuperscript{8,19}. Moreover, the systemic administration of irisin ameliorated atherosclerosis in an apoE\textsuperscript{−/−} diabetic mouse model and protected against endothelial injury, indicating that irisin could benefit atherosclerotic vascular diseases\textsuperscript{20}.

While irisin's lipogenic effect may correlate with its chronic actions\textsuperscript{17,21–23}, no study tests its acute effect on the heart. Therefore, we tested the hypothesis that irisin functions as a myokine to alleviate I/R injury by attenuating ER stress and ER stress-associated apoptosis.

**Materials and methods**

**Cell culture.** Primary cultures of ventricular cardiomyocytes were prepared from neonatal C57BL/6 mice (Animal Center of the North Sichuan Medical College, Nanchong, China) according to the method in our previous study with few modifications\textsuperscript{6}. After being disinfected with 70% ethanol, the neonatal mice were euthanized by decapitation; and the heart was quickly removed and placed in an ice-cold PBS buffer. The heart was washed with ice-cold PBS buffer 3 times and then cut into small pieces in dissociation buffer (containing 0.1% trypsin and 99.9% PBS). After, it was digested for 8 min. Next, the supernatant was transferred to a centrifuge tube, mixed with 2 ml fetal bovine serum, and preserved at 4 °C. The remaining tissue was digested in a solution containing 0.08% collagenase and 99.92% M199 medium (HyClone SH30253.01B). After digestion at 37 °C for 30 min, the cell suspension was mixed with the supernatants, filtrated with a 200X screen, and centrifuged at 300×g for 5 min. Next, after washing the precipitate with PBS buffer twice, the dissociated cells were replated in a culture flask at 37 °C for 1 h to enrich the culture's cardiomyocytes. The cells were collected in a medium consisting of M199 supplemented with 50 units/ml penicillin/streptomycin, 10% fetal bovine serum, and 0.1 mM B-2-mercaptoethanol (BrdU). After 24 h, replace the BrdU-free DMEM medium. BrdU is commonly used to block the mitosis of non-myocyte cells in primary cardiomyocyte culture. Previous research indicated that BrdU could reduce glycogen which means it may have an impact on AMPK\textsuperscript{24}. To make its influence less obvious, the control groups were settled set up and all groups were done under the same conditions in the experiment.

**Hypoxia/reoxygenation treatment to cardiomyocytes.** The hypoxia/reoxygenation model was established according to our previously published method, with few modifications\textsuperscript{25,26}. We started experiments after being cultured for 48–72 h and pretreated with different agents. For simulation of ischemia, the cardiomyocyte medium was replaced with the DME without glucose and serum, which was flushed with a gas mixture (95% N2 and 5% CO2) for 15 min. The cardiomyocytes were incubated in a modular incubator chamber with a gas mixture of 95% N2 and 5% CO2 at 37 °C for 4 h. After hypoxia incubation, the cells were provided with a serum-free medium and then restored to 95% air and 5% CO2 to reoxygenation for 8 h.

**Cell viability and lactate dehydrogenase (LDH) release assay.** Cell viability was assessed by using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, CK04) according to the manufacturer's protocol\textsuperscript{27,28}. About 0.5 × 10\textsuperscript{4} cells were seeded onto 96-well plates and then pretreated with different agents. Following hypoxia/reoxygenation exposure, 10 μl CCK-8 was added to each well. Then the cells were incubated at 37 °C for 1 h. After incubation, the OD value at 450 nm was determined using an Infin ite\textsuperscript{®} 200 Microplate Reader (Tecan, M200).

**Quantitative real-time PCR.** Total RNA was extracted from cultured cardiomyocytes with Trizol reagent (Invitrogen, Green Island, NY). Reverse transcription was performed with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The transcript level of mRNA was determined with Bio-Rad CFX96 Real-time PCR system-C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Each real-time PCR (20 μl total volume) contained 1 μl of template DNA (100 mg) or cDNA, 10 μl Ex Taq, 1 μl of each of the forward and reverse primers, and 7 μl of sterile deionized water. The samples were denatured by heating at 95 °C for 3 min, followed by 39 cycles of amplification and quantification (95 °C for 15 s and 58 °C for 15 s, respectively) and by a final extension cycle (72 °C, 90 s). The primers used for amplification were (from 5’to 3’): CHOP-F: TATTCATCCCAGGAGAC, CHOP-R:CTGTCTCCTTTCTCTTATGC, Grp78-F:TTCAGCCTATTCGAGAAACTCT, Grp78-R:TTTTCTGTATCTTCTTCTAGAG, sXbp-1-F:CCT TGTGTTGAGCGAGAC, sXbp-1-R:CCTGCACTGCTGGGCGAC, GAPDH-F:ACCCCAAGTATTCTCACTCC, GAPDH-R:TGTTCGCGGTTGTTCTGACG. The mRNA levels were quantified first against GAPDH levels in the same sample, then normalized to the control group, set up as onefold. These experiments were repeated six times.

**Western blot analysis.** Neonatal mice cardiomyocytes were cultured at a cell density of 1.2 × 10\textsuperscript{4} in 60-mm plastic culture dishes. Western blot analysis\textsuperscript{27,28} was performed on cultured cardiomyocytes from the different groups. After being washed twice with the ice-cold PBS, the cells were lysed at 4 °C for 10 min with RIPA buffer (Beyotime, Nanjing, China) containing a cocktail of protease inhibitors (Roche, USA), then centrifuged at 20,000×g and 4 °C for 20 min. To analyze the mitochondrial and cytosol cytochrome c protein levels respectively, the mitochondrial extracts were isolated from the cultured cardiomyocytes using the Mitochondria Isolation Kit (Beyotime) according to our previous experiments\textsuperscript{6}. COX IV was used as the loading control for mitochondrial
Detection of apoptotic cardiomyocytes. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was used to detect the apoptotic cardiomyocytes according to our previous experiments by using an in situ cell death detection kit (Roche, USA). After being rinsed twice for ice PBS, the cardiomyocytes slides were fixed in 4% paraformaldehyde at room temperature for 30 min, followed by permeabilization with 0.1% TritonX-100 in 0.1% sodium citrate for 10 min at room temperature. Then, a 30 µl TUNEL reaction mixture was added to the sample and incubated in a humidified chamber for 60 min. DAPI was used to label nuclei. After cardiomyocytes were mounted in a mounting medium, apoptotic cells images were obtained using a Zeiss confocal laser scanning microscope (Carl Zeiss, LSM 510 Meta Confocal Laser Scanning Microscope).

Statistical analysis. All experimental data are expressed as the mean ± standard error of the mean (SEM), and each experiment was performed a minimum of 3 times. Raw data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). Data were also analyzed using a one-way analysis of variance (ANOVA). A two-tailed P < 0.05 was taken to indicate a statistically significant difference.

Ethics statement. This study was performed strictly with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Ethical and Welfare Committee of North Sichuan Medical College. This study was approved by the Medical Ethics Committee.

Result

The protective effects of irisin on H/R-injury. The percentage of survived cardiomyocytes was proportional to the measured cell viability. Here we evaluated the effect of irisin on cell viability to investigate its potential protective effect on cardiomyocytes undergoing H/R injury. For that, cardiomyocytes pretreated with irisin underwent H/R treatment. We found that pre-treatment with irisin could successfully attenuate the decrease in cell viability caused by the H/R treatment (Fig. 1A). Moreover, cell injury was quantified by measuring the LDH levels in the extracellular medium. Irisin pre-treatment significantly reduced the LDH release in H/R-treated cardiomyocytes (Fig. 1B), demonstrating the protective effects of irisin against H/R-injury.

Irisin reduces H/R-induced apoptosis of cultured cardiomyocytes. We investigated the effect of irisin on the H/R-induced apoptosis in cultured cardiomyocytes (Fig. 2A). More TUNEL positive cells (green) were observed in H/R-treated cultures (Fig. 2A1). Quantitative analysis also revealed that H/R increased TUNEL positive apoptotic cells compared to cells under regular conditions. The pre-treatment with irisin reduced the number of TUNEL-positive cells compared to the H/R group (Fig. 2A2). The Annexin V/PI analysis results with flow cytometry confirmed that irisin reduced H/R-induced apoptosis (Fig. 2B).

Irisin blocks the mitochondrial apoptotic pathway. Mitochondrial dysfunction may initiate apoptosis by releasing pro-apoptotic factors, such as cytochrome c, from the mitochondrial intermembrane space into the cytoplasm to trigger apoptosis via a caspase-3-dependent pathway. Accordingly, changes in mitochondrial and cytosol cytochrome c were measured by immunoblotting. As shown in Fig. 3A, H/R treatment caused the release of cytochrome c from mitochondria, and irisin administration could reduce the release of cytochrome c from mitochondria to the cytosol. The effect of irisin in preventing apoptotic cell death was evident in the immunoblotting of caspase 3, a key enzyme involved in the execution of apoptosis. Cardiomyocytes with H/R treatment showed a significant increase in caspase-3 activity. In contrast, pre-treatment with irisin significantly inhibited the H/R-induced activation of caspase-3 (Fig. 3B), suggesting that the irisin-reduced H/R-induced apoptosis was mediated through the blockade of caspase-3-dependent cardiomyocytes apoptosis.
**H/R resulted in excessive ER stress and CHOP upregulation.** GRP78/BiP is a marker of ER stress. Excessive ER stress results in the over-expression of GRP78 and thus leads to upregulation of pJNK, Caspase-12, and CHOP, which could induce cell apoptosis. In our experiment, H/R induced a significant upregulation of GRP78, and this effect could be attenuated by irisin pre-treatment (Fig. 4A). Consistently with this phenomenon, CHOP expression in the H/R group was significantly up-regulated compared to the control and irisin groups. On the other hand, irisin pre-treatment showed the ability to reduce CHOP expression induced by H/R (Fig. 4B). Meanwhile, we find that the level of spliced Xbp-1 mRNA in cardiomyocytes was increased in H/R-treated cells and irisin significantly reversed the increase (Fig. 4C). The phosphorylation level of eIF2α was increased in H/R-treated cardiomyocytes compared to the control group, whereas irisin ameliorated these changes (Fig. 4D).

**Irisin blunted H/R induced apoptosis via an AMPK-mediated pathway.** Notably, the percentage of TUNEL-positive cells to total cells in the H/R group increased significantly compared to the control group. The pre-treatment partly blunted apoptosis with either irisin or AMPK-activator metformin (Fig. 5A). Consistent with the change of the apoptotic index in different groups, the activity of caspase-3 in the H/R group significantly increased compared to the control group, and this change was attenuated by either irisin or metformin pre-treatment (Fig. 5B). Similarly, cell viability in the H/R group significantly decreased compared to the control group, and this change was attenuated by either irisin or metformin pre-treatment (Fig. 5C). Moreover, LDH levels of the extracellular medium in the H/R group significantly increased compared to the control group, and this change was attenuated by either irisin or metformin pre-treatment (Fig. 5D). The effect of irisin on the apoptotic index (Fig. 5A), caspase-3 activity (Fig. 5B), cell viability (Fig. 5C), and LDH release (Fig. 5D) in the H/R group was abolished by AMPK-inhibitor Compound-C pre-treatment.

**H/R resulted in AMPK inactivation, and irisin showed similar effects on H/R cardiomyocytes as metformin.** As an energy metabolism-regulating kinase, AMPK is activated by either increasing its phosphorylation or by up-regulating the expression of total AMPK. Expression of total AMPK and phosphorylated AMPK was detected using western blot (Fig. 6). In our experiment, there was an evident decrease in phosphorylated AMPK expression in the H/R group compared to the control group, and there was no difference in the expression of total AMPK between the different groups. Irisin had the same effect on increasing AMPK phosphorylation in cardiomyocytes exposure to H/R as AMPK activator metformin, and this phenomenon could be obliterated by AMPK-inhibitor Compound-C pre-treatment.

**Irisin showed its protective effects against H/R injury-induced ER stress in an AMPK-dependent manner.** To investigate whether AMPK associated signaling pathways were involved in irisin’s protective effects, cardiomyocytes were pretreated with AMPK inhibitor compound C before irisin pre-treatment and exposure to H/R. Then, we compared the expression of GRP78, CHOP, sXbp-1, and the phosphorylation of eIF2α between different groups to explore the underlying relationship among irisin, ER stress, and AMPK signaling pathways. There was no difference in GRP78, CHOP, and sXbp-1 expression and eIF2α phosphorylation among groups not exposed to H/R. As depicted in Fig. 7A, irisin had the same effect as AMPK activator metformin in decreasing the GRP78 expression compared to the H/R group, and compound C pre-treatment could abrogate this protection. In line with the change of GRP78, CHOP expression was significantly up-regulated in the H/R group, irisin or metformin pre-treatment decreased CHOP expression levels compared to the H/R group (Fig. 7B). sXbp-1 expression was significantly up-regulated in the H/R group, irisin or metformin pre-treatment decreased sXbp-1 expression levels compared to the H/R group (Fig. 7C). The phosphorylation of eIF2α was significantly up-regulated in the H/R group, irisin or metformin pre-treatment decreased eIF2α phos-
Phosphorylation levels compared to the H/R group (Fig. 7D). Finally, we observed that compound C pre-treatment markedly blocked irisin's effect on cardiomyocytes undergoing H/R.

**Discussion**

In the present study, by using a stimulated hypoxia/reoxygenation model, we verified that apoptosis was essential to H/R injury, and H/R injury-induced apoptosis was closely associated with ER stress. Moreover, we also demonstrated that ER stress was, at least partly, regulated by AMPK. We first showed that irisin protected cardiomyocytes from H/R injury via attenuating ER stress in an AMPK-dependent manner. Therefore, AMPK-mediated ER stress might be a new target pathway for exploring pharmacological treatment for I/R-induced myocardium injury.\(^3,36,37\).
Figure 3. Irisin inhibited H/R-induced cytochrome c release and caspase-3 activation in cardiomyocytes. (A) Assessment of cytochrome c (Cyt c) in mitochondria and cytosol derived from cardiomyocytes subjected to H/R. COX IV: Cytochrome c oxidase subunit IV; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. (B) Caspase-3 activation in cardiomyocytes subjected to H/R. *P < 0.05 versus control group, #P < 0.05 versus H/R group, n = 6.

Figure 4. H/R treatment resulted in excessive ER stress and CHOP upregulation. The GRP78, CHOP, sXbp-1, and eIF2α expressions were assessed by PCR or Western blot analysis. (A) Effects of irisin on H/R induced GRP78 mRNA (A1) and protein (A2) expression. (B) Effects of irisin on H/R induced CHOP mRNA (B1) and protein (B2) expression. (C) Effects of irisin on H/R induced sXbp-1 mRNA expression. (D) Effects of irisin on H/R induced eIF2α phosphorylation. *P < 0.05 versus control group, #P < 0.05 versus H/R group, n = 6.
ER stress is an innate adaptive mechanism existing in different species. Mild ER stress presents a protective effect against injury factors. In contrast, prolonged stress shows lethal damage to cells or organs. In mild ER stress, GRP78 (glucose-regulated protein 78) released its transducers, including IRE-1, PERK, ATF-6, and allow them to dimerize activation or move to other locations, then triggering the downstream signal pathways to hold ER homeostasis by decreasing the accumulating of unfolded proteins and promoting unfolded proteins to fold. There is a conversion from pro-surviving pathways to the pro-apoptotic pathways under severe injury in different degrees of stress. ER stress-mediated intrinsic apoptosis pathways involve several proteins, including the transcription factor CHOP, phosphorylated JNK, and the ER-resident cysteine protease caspase-12. Besides, ER stress-associated apoptosis is characterized by upregulation of CHOP, increased phosphorylated JNK, and cleaved caspase-12. Each of them could activate the downstream pro-apoptotic signal transducers, leading to caspase-3 activation and terminal apoptosis.

In our experiment, we detected the expression of GRP78 and CHOP to investigate the influence of irisin on H/R-induced ER stress. Western blot revealed that H/R exposure induced upregulation of GRP78 compared to the control group, suggesting that H/R injury induces excessive ER stress. In addition, increased CHOP expression confirmed that H/R injury activated ER stress-mediated CHOP apoptotic signal. Studies demonstrated that ER stress-associated apoptosis is closely related to both H/R injury and CHOP is usually considered downstream of ER stress-induced apoptosis. Furthermore, Runtao et al. showed that ischemia/reperfusion injury induces excessive ER stress and activates the downstream pro-apoptotic signal pathways. Therefore, ischemic post-conditioning could protect the myocardium from I/R injury by attenuating ER stress-associated apoptosis. Here, we show for the first time that irisin could protect cardiomyocytes against H/R injury by attenuating ER stress.

As an energy metabolic kinase, AMPK activation significantly reduced the percentage of apoptotic cardiomyocytes and the activity of the apoptotic effectors of ER stress in a cardioplegia-induced H/R injury model.
Figure 6. H/R treatment led to AMPK inactivation, and irisin showed the same effects on H/R cardiomyocytes as metformin. AMPK activation was estimated by detecting the phosphorylated AMPK. Irisin attenuated the inactivating of AMPK induced by H/R exposure and compound C pretreat abolished this effect. *P < 0.05 versus control group, #P < 0.05 versus H/R group, $P < 0.05 versus Irisin + H/R group, n = 6. p-AMPK phosphorylated AMPK, t-AMPK total AMPK.

Figure 7. Irisin showed its protective effects against H/R injury-induced ER stress in an AMPK-dependent manner. AMPK activator metformin showed the same effect as irisin in attenuating ER stress induced by H/R exposure. AMPK potent inhibitor compound C pre-treatment abolished cardioprotection of irisin on H/R cardiomyocytes. *P < 0.05 versus control group, #P < 0.05 versus H/R group, $P < 0.05 versus Irisin + H/R group, n = 6.
Shintani et al. also found that nonfatal damage to cells could reduce the energy consumption and AMPK activation, which shows a protective effect on cell injury via increasing stress tolerance. Additionally, using a heart failure model in dogs, Sasaki and Asanuma discovered that metformin and another AMPK activator (AICAR) have equivalent effects of decreasing cardiomyocytes apoptosis and improving cardiac function in failing dog hearts. Interestingly, treatment with compound C inhibited the effects of metformin and AICAR, suggesting the primary role of AMPK activation in reducing apoptosis and preventing heart failure. Therefore, AMPK might be a new target for pharmacological potential in reducing cardiomyocyte H/R injury or myocardium I/R injury.

Although there is much evidence about the protective effect of AMPK activation, there was no research suggesting that irisin could protect the myocardium by activating AMPK and attenuating ER stress.

AMPK activator metformin and AMPK inhibitor compound C was used in our experiment to investigate the possible role of AMPK in the cardioprotection by irisin. Our experiment testified that irisin has the same effect as metformin to activate AMPK and decrease the expression of GRP78 and CHOP in a stimulated H/R model. Our results showed that compared to the compound C pre-treatment group, irisin's effects on H/R cardiomyocytes disappeared, suggesting that the cardioprotection of irisin was partly mediated by AMPK activation and AMPK might be an upstream regulator of stress and ER-stress-associated apoptosis. The beneficial effects of irisin's activation of AMPK-related pathways on cells have been widely explored. In the human placenta, irisin can induce trophoblast differentiation via AMPK activation and improve trophoblast functions. In addition, irisin also protects against pressure overload-induced cardiac hypertrophy via activating AMPK-ULK1 signaling. In diabetic mice, irisin attenuates myocardial ischemia/reperfusion injury through the AMPK pathway. Similarly, in the setting of IR injury and hyperglycemia stress, irisin can increase the survival rate of cardiomyocytes by activating the AMPK pathway. Our study suggests that AMPK was responsible for mitochondrial dysfunction-induced energy metabolic disorders and involved in regulating ER stress signal pathway. However, whether irisin activates AMPK directly or through some intermediate receptor, has not yet been determined. Previous study has shown that irisin can downregulate ATP levels in HSMCs, triggering AMPK phosphorylation. The majority of ATP is generated in mitochondria by oxidative phosphorylation. Uncoupling proteins on the mitochondria can release the coupling relationship between oxidation and phosphorylation in part of the normal respiratory chain, hindering the normal production of ATP. Studies have confirmed that interaction between irisin and UCP2 (an uncoupling protein) allows for the prevention of IR-induced injury to the lung via improvement of mitochondrial function. Besides, irisin can increase the ROS level in L6 cells, and using the ROS scavenger resulted in a decrease in AMPK phosphorylation. Their research further demonstrated that calcium may play an upstream of ROS in irisin-mediated signaling. These results indicate that irisin increases AMPK phosphorylation through the Ca2+/ROS pathway in the skeletal muscles. It is worth noting that cardiac muscle exercise produces more irisin over time than skeletal muscle. Importantly, the study revealed the presence of yet-to-be-identified irisin receptor(s) on the H9C2 cell membrane. Through this receptor, irisin may activate several downstream signaling pathways, such as the PI3K-AKT pathway. Similarly, they found that irisin also can increase Ca2+ in cardiomyocytes. However, they did not verify the above experimental results of irisin in animal models.

Conclusion

In conclusion, our data revealed that ER stress plays an essential role in cell death during H/R and that AMPK activation is protective. Irisin has a potent effect in protecting cardiomyocytes from H/R injury by significantly attenuating excessive ER stress-induced apoptosis in an AMPK-mediated manner.

Data availability

All data generated or analyzed during this study are included in this published article.

Received: 10 August 2021; Accepted: 19 April 2022

Published online: 06 May 2022

References

1. Virani, S. S. et al. Heart disease and stroke statistics-2020 update: A report from the American Heart Association. Circulation 141, e139–e596. https://doi.org/10.1161/CIR.0000000000000757 (2020).
2. Sanchis, D., Llovera, M., Ballester, M. & Comella, J. X. An alternative view of apoptosis in heart development and disease. Cardiovasc. Res. 77, 448–451. https://doi.org/10.1093/cvr/cvo074 (2008).
3. Takeamura, G., Kanoh, M., Minatoguchi, S. & Fujitani, H. Cardiomyocyte apoptosis in the failing heart: A critical review from a molecular and cellular perspective. Arch. Biochem. Biophys. 507, 107–114. https://doi.org/10.1016/j.abb.2015.06.005 (2015).

4. Binder, A. et al. Myocardial protection from ischemia–reperfusion injury post coronary revascularization. Expert Rev. Cardiovasc. Ther. 10, 1045–1057. https://doi.org/10.1586/14779072.2015.1070669 (2012).
5. Yue, R. et al. Lycopene protects against hypoxia/reoxygenation-induced apoptosis by preventing mitochondrial dysfunction in primary neonatal mouse cardiomyocytes. PLoS ONE 7, e50778. https://doi.org/10.1371/journal.pone.0050778 (2012).
6. Luo, T. et al. PD150606 protects against ischemia/reperfusion injury by preventing mu-calpain-induced mitochondrial apoptosis. Arch. Biochem. Biophys. https://doi.org/10.1016/j.abb.2015.06.005 (2015).
7. Yue, R. et al. Mitochondrial DNA oxidative damage contributes to cardiomyocyte ischemia/reperfusion-injury in rats: Cardioprotective role of hypoxene. J. Cell Physiol. 230, 2128–2141. https://doi.org/10.1002/jcp.22941 (2015).
8. Yeh, C. H., Chen, T. P., Wang, Y. C., Lin, Y. M. & Fang, S. W. AMP-activated protein kinase activation during cardioplegia-induced hypoxia/reoxygenation injury attenuates cardiomyocytic apoptosis via reduction of endoplasmic reticulum stress. Mediators Inflamm. 2010, 130636. https://doi.org/10.1155/2010/130636 (2010).
9. Matsui, Y. et al. Distinct roles of autophagy in the heart during ischemia and reperfusion: Roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. Circ. Res. 100, 914–922. https://doi.org/10.1161/01.RES.0000261924.76669.36 (2007).
11. Doroudgar, S. & Glembotski, C. C. New concepts of endoplasmic reticulum function in the heart: Programmed to conserve. J. Mol. Cell. Cardiol. 55, 85–91. https://doi.org/10.1016/j.yjmcc.2012.10.006 (2013).

12. Ray, D., Mukherjee, S., Falchi, M., Bertelli, A. & Das, D. K. Amelioration of myocardial ischemic reperfusion injury with Calendula officinalis. Carr. Pharm. Biotechnol. 11, 849–854. https://doi.org/10.2174/13892010793261971 (2010).

13. Lee, Y. M. et al. Protective effects of melatonin on myocardial ischemia/reperfusion injury in vivo. J. Pinel Res. 33, 72–80. https://doi.org/10.1034/j.1600-079x.2002.01869.x (2002).

14. Xu, J. et al. Lycopene protects against hypoxia/reoxygenation injury by alleviating ER stress induced apoptosis in neonatal mouse cardiomyocytes. PLoS ONE 10, e0136443. https://doi.org/10.1371/journal.pone.0136443 (2015).

15. Zhu, D. et al. Irisin improves endothelial function in type 2 diabetes through reducing oxidative/nitrative stresses. J. Mol. Cell. Cardiol. 87, 138–147. https://doi.org/10.1016/j.yjmcc.2015.07.015 (2015).

16. Park, M. J., Kim, D. I., Choi, J. H., Heo, Y. R. & Park, S. H. New role of irisin in hepatocytes: The protective effect of hepatic steatosis in vitro. Cell. Signal. 27, 1831–1839. https://doi.org/10.1016/j.cellsig.2015.04.010 (2015).

17. Lee, P. et al. Irisin and FGE21 are cold-induced endocrine activators of brown fat function in humans. Cell Metab. 19, 302–309. https://doi.org/10.1016/j.cmet.2013.12.017 (2014).

18. Jeremic, N., Chaturvedi, P. & Tyagi, S. C. Browning of white fat: Novel insight into factors, mechanisms, and therapeutics. J. Cell. Physiol. 232, 61–68. https://doi.org/10.1002/jcp.25450 (2017).

19. Luo, Y. et al. Disordered metabolism in mice lacking irisin. Sci. Rep. 10, 17368. https://doi.org/10.1038/s41598-020-74588-7 (2020).

20. Lu, J. et al. Irisin protects against endothelial injury and ameliorates atherosclerosis in apolipoprotein E-Null diabetic mice. Atherosclerosis 243, 438–448. https://doi.org/10.1016/j.atherosclerosis.2015.10.020 (2015).

21. Bostrom, P. et al. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 481, 463–468. https://doi.org/10.1038/nature10777 (2012).

22. Villarroya, F. Irisin, turning up the heat. Cell Metab. 15, 277–278. https://doi.org/10.1016/j.cmet.2012.02.010 (2012).

23. Perez-Sotoelo, D. et al. Lack of adipocyte-Fndc5/irisin expression and secretion reduces thermogenesis and enhances adipogenesis. Sci. Rep. 7, 16289. https://doi.org/10.1038/s41598-017-16602-z (2017).

24. Lokuta, A., Kirby, M. S., Gaa, S. T., Lederer, W. J. & Rogers, T. B. On establishing primary cultures of neonatal rat ventricular myocytes for analysis over long periods. J. Cardiovasc. Pharmacol. 14.x (1994).

25. Guo, J. et al. Protective effects of melatonin on myocardial ischemia/reperfusion injury in vivo. J. Cell. Biochem. 110, 589–597. https://doi.org/10.1002/jcb.22781 (2010).

26. Minamino, T., Komuro, I. & Kitakaze, M. Endoplasmic reticulum stress as a therapeutic target in cardiovascular disease. J. Mol. Cell. Cardiol. 45, 1151–1157. https://doi.org/10.1016/j.yjmcc.2007.04.006 (2007).

27. Galluzzi, L. et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ. 16, 1093–1107. https://doi.org/10.1038/cdd.2009.44 (2009).

28. Malhi, H. & Kaufman, R. J. Endoplasmic reticulum stress in liver disease. J. Hepatol. 54, 795–809. https://doi.org/10.1016/j.jhep.2010.11.005 (2011).

29. Groenendyk, J., Sreenivasasaih, P. K., de Kim, H., Agellon, L. B. & Michalak, M. Biology of endoplasmic reticulum stress in the heart. Circ. Res. 107, 1185–1197. https://doi.org/10.1161/CIRCRESAHA.110.227819 (2010).

30. Zou, X. J., Yang, L. & Yao, S. L. Endoplasmic reticulum stress and C/EBP homologous protein-induced Bax translocation are involved in angiotensin II-induced apoptosis in cultured neonatal rat cardiomyocytes. Exp. Biol. Med. 237, 1341–1349. https://doi.org/10.1258/ebm.2012.012041 (2012).

31. Wu, X. D. et al. Hypoxia preconditioning protects microvascular endothelial cells against hypoxia/reoxygenation injury by attenuating endoplasmic reticulum stress. Apoptosis 18, 85–98. https://doi.org/10.1007/s10495-012-0766-6 (2013).

32. Gan, R. et al. Post-conditioning protecting rat cardiomyocytes from apoptosis via attenuating calcium-sensing receptor-induced endo(sarco)plasmic reticulum stress. Mol. Cell. Biochem. 361, 123–134. https://doi.org/10.1007/s11010-011-0996-7 (2012).

33. Shintani, Y. et al. TLR9 mediates cellular protection by modulating energy metabolism in cardiomyocytes and neurons. Proc. Natl. Acad. Sci. USA 110, 5109–5114. https://doi.org/10.1073/pnas.1219243110 (2013).

34. Sasaki, H. et al. Metformin prevents progression of heart failure in dogs: Role of AMP-activated protein kinase. Circulation 119, 2568–2577. https://doi.org/10.1161/CIRCULATIONAHA.108.798561 (2009).

35. Drewlo, S. et al. Irisin induces trophoblast differentiation via AMPK activation in the human placenta. J. Cell. Physiol. 235, 7146–7158. https://doi.org/10.1002/jcp.29663 (2020).

36. Li, R. L. et al. Irisin alleviates pressure overload-induced cardiac hypertrophy by inducing protective autophagy via mTOR-independent activation of the AMPK-ULK1 pathway. J. Mol. Cell. Cardiol. 121, 242–255. https://doi.org/10.1016/j.yjmcc.2018.07.230 (2018).

37. Xin, C. et al. Irisin attenuates myocardial ischemia/reperfusion injury and improves mitochondrial function through AMPK pathway in diabetic mice. Front. Pharmacol. 11, 565160. https://doi.org/10.3389/fphar.2020.565160 (2020).

38. Fan, J. et al. Protective effects of irisin on hypoxia-reoxygenation injury in hyperglycemia-treated cardiomyocytes: Role of AMPK pathway and mitochondrial protection, J. Cell. Physiol. 235, 1165–1174. https://doi.org/10.1002/jcp.29030 (2020).

39. Huh, J. Y. et al. Exercise-induced irisin secretion is independent of age or fitness level and increased irisin may directly modulate muscle metabolism through AMPK activation. J. Clin. Endocrinol. Metab. 99, E2154-2161. https://doi.org/10.1210/jc.2014-1437 (2014).
50. Chen, K. et al. Irisin protects mitochondria function during pulmonary ischemia/reperfusion injury. Sci. Transl. Med. https://doi.org/10.1126/scitranslmed.aao6298 (2017).

51. Lee, H. J. et al. Irisin, a novel myokine, regulates glucose uptake in skeletal muscle cells via AMPK. Mol. Endocrinol. 29, 873–881. https://doi.org/10.1210/me.2014-1353 (2015).

52. Aydin, S. et al. Cardiac, skeletal muscle and serum irisin responses to with or without water exercise in young and old male rats: Cardiac muscle produces more irisin than skeletal muscle. Peptides 52, 68–73. https://doi.org/10.1016/j.peptides.2013.11.024 (2014).

53. Xie, C. et al. Irisin controls growth, intracellular Ca\textsuperscript{2+} signals, and mitochondrial thermogenesis in cardiomyoblasts. PLoS ONE 10, e0136816. https://doi.org/10.1371/journal.pone.0136816 (2015).

Author contributions
Conceived and designed the experiments: R.Y., H.H., X.L. and L.M. Performed the experiments: R.Y., X.L., L.M., Z.Z., T.X., X.Z. and M.L. Analyzed the data: Z.Y. and P.J. Contributed reagents/materials/analysis tools: R.Y., H.H., P.J. and Z.Y. Wrote the paper: R.Y., H.H., and L.M. All authors reviewed the manuscript.

Funding
Grant support was obtained from the major projects of the Sichuan Provincial Health Commission (21ZD004), Science and Technology Department of Sichuan Province Project (2021YJ0210), Research Project Foundation of Affiliated Hospital of North Sichuan Medical College (2021ZXK002; 2021YS006; 2021LC011), and Science and Technology Strategic Special Cooperation between City and University in Nanchong (18SXHZ0545; 19SXHZ0218;19SXHZ0217; 19SXHZ0193).

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to L.X. or H.H.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2022