RESEARCH

FNDC5/Irisin counteracts lipotoxic-induced apoptosis in hypoxic H9c2 cells

Isabel Moscoso^{1,2,*}, María Cebro-Márquez^{1,*}, Moisés Rodríguez-Mañero^{2,3}, José Ramón González-Juanatey^{1,2,3} and Ricardo Lage^{1,2}

¹Cardiology Group, Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), Universidade de Santiago de Compostela and Health Research Institute, University Clinical Hospital of Santiago de Compostela, Santiago de Compostela, Spain

²Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares (CIBERCV), Madrid, Spain

³Department of Cardiology and Coronary Unit, University Clinical Hospital of Santiago de Compostela, Santiago de Compostela, Spain

Correspondence should be addressed to I Moscoso or R Lage: imosgal@gmail.com or rlagef@gmail.com

*(I Moscoso and M Cebro-Márquez contributed equally to this work)

Abstract

Irisin is a newly identified adipokine critical to modulate body metabolism, fatty acid metabolism and oxidative stress; recent evidence suggests a cardioprotective role in ischemic injury. Loss of cardiomyocytes during acute myocardial infarction is strongly associated with energetic changes and lipotoxic-induced apoptosis. Our aim was to study FNDC5/irisin's potential protective role against hypoxia and lipotoxicity, both related with myocardial infarction environment. H9c2 cells were treated with palmitate and/or irisin in normoxic/hypoxic conditions. Cell viability and apoptosis were assessed by MTT assay and annexin V/PI staining. Immunoblotting was used to confirm apoptotic cascade regulation. Irisin counteracts lipotoxic-induced apoptosis in hypoxic cardiomyoblasts by activating Akt signaling pathway suggesting the potential therapeutic role of irisin in ischemic heart disease.

Key Words

- ▶ ischemic heart disease
- ▶ apoptosis
- ▶ irisin
- ▶ lipotoxicity

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Introduction

Continuous cardiac contractile activity is a strictly controlled process that entails a high-energy demand to generate the necessary mechanical force but also to maintain cellular homeostasis. Energy demand is supported mainly through fatty acids (FAs) oxidation (Lopaschuk *et al.* 2010). Nevertheless, due to lack of myocardial oxygen supply, abrupt metabolic changes are produced after a coronary occlusion, compensatory adrenergic response and fatty acid overload turn FA metabolism deleterious (Geerling *et al.* 2014). Increased delivery, uptake or decreased FA turnover lead to myocardial lipid accumulation (Wende & Abel 2010) and lipotoxicity (Wende & Abel 2010). Myocardial lipotoxicity is associated to endoplasmic reticulum stress and

mitochondrial dysfunction, altered calcium homeostasis, ceramide synthesis, oxidative stress, contractile dysfunction and apoptosis (Wende & Abel 2010).

Irisin is a small polypeptide hormone cleaved from the fibronectin type III domain containing 5 (FNDC5) (Bostrom *et al.* 2012), is regulated by exercise and associated to healthy metabolic effects (Bostrom *et al.* 2012, Huh *et al.* 2012). Higher levels of FNDC5 expression were reported in brain, skeletal and cardiac muscle (Varela-Rodriguez *et al.* 2016). A growing body of evidence suggests a strong association between irisin and cardiovascular health (Lecker *et al.* 2012, Emanuele *et al.* 2014). Low levels of circulating irisin have been associated with unfavorable lipid profile (Oelmann *et al.* 2016),

reduced aerobic performance (Lecker *et al.* 2012), increased risk of heart failure (HF) (Emanuele *et al.* 2014) and endothelial dysfunction (Lu *et al.* 2015). In addition to risk factors, recent studies establish a close relationship between irisin and cardiovascular disease (Lecker *et al.* 2012, Emanuele *et al.* 2014, Kuloglu *et al.* 2014, Xiang *et al.* 2014, Oelmann *et al.* 2016). Elderly that maintain elevated circulating irisin levels, despite the age-associated decline (Huh *et al.* 2012, 2014), showed lower rate of infarction (Emanuele *et al.* 2014), whereas unusually young infarcted individuals showed lower levels of irisin. Significantly low levels of irisin have been described in patients with coronary artery disease (CAD), which is negatively associated with the severity of artery disease (Deng 2016), as well as myocardial infarction (MI) (Kuloglu *et al.* 2014, Anastasilakis *et al.* 2017). Data from murine models showed that cardiac FNDC5 expression is regulated in infarcted animals (Kuloglu *et al.* 2014). Also, irisin attenuates ischemia/reperfusion (I/R)-associated cytotoxicity in rat cardiomyoblasts and that preconditioning prevents I/R-induced mitochondrial dysfunction, decreasing infarct size (Zhao *et al.* 2016, Wang *et al.* 2017, 2018). In addition, irisin overexpression leads to mitochondrial overdrive and increases reactive oxygen species (Ho *et al.* 2018). Based on previous studies where irisin levels are decreased in a lipotoxic context and its protective role against ischemic injury is also described, we hypothesized that irisin might directly protect cardiomyocyte against lipotoxic-induced apoptosis.

Materials and methods

Cell culture and reagents

H9c2 cells were obtained from ATCC. H9c2 cells were chosen because these cells maintain electrical and hormonal signal pathway elements of adult cardiac cells (Hescheler *et al.* 1991), in addition to sensitivity to hypoxia and energetic similarities to primary cardiomyocytes (Kuznetsov *et al.* 2015). H9c2 were cultured in 0.1% gelatin-coated plates with DMEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 UI/mL penicillin, 100 µg/mL streptomycin) and 2 mM L-glutamine, in a 5% CO₂ atmosphere at 37°C. H9c2 were seeded at least 24 h before treatments unless otherwise indicated and the experimental procedures were conducted upon reaching 80% confluence.

Cells were treated with palmitate (Sigma-Aldrich) to trigger *in vitro* lipotoxicity (Wende & Abel 2010),

5 nM irisin (Sigma-Aldrich) and/or 20 µM specific Akt inhibitor (10-NCP) (Merck). Palmitate was prepared by conjugation with fatty acid-free bovine serum albumin by dissolving in ethanol and diluting 100-fold in an aqueous 2% bovine serum albumin (BSA) solution to achieve selected concentration, and palmitate solution was freshly prepared for each experiment.

Hypoxia induction

H9c2 were cultured in a hypoxic chamber (Baker Ruskinn's InvivoO₂200, Bridgewater) under lower oxygen concentration (0.1% O₂, 5% CO₂ and N₂ balance) than oxygen demand for 24 h to mimic pathophysiologic ischemic environment (Lage *et al.* 2015).

Cell viability and apoptosis

Cell viability and apoptosis was measured using the MTT assay (Sigma-Aldrich) and FITC Annexin-V-FLUOS staining kit (Roche Diagnostics) according to the manufacturer's protocol. Briefly, H9c2 cells were seeded at a density of 5000 cells/well in 96-well plates. Cells were treated with 250 µM or 500 µM palmitate and/or 5 nM irisin for 24 h under normoxic and hypoxic conditions. Later, MTT (0.5 mg/mL) was added and incubated at 37°C for 4 h. Formazan crystals were solubilized with DMSO and isopropanol (1:1). The optical density (OD) was measured at a wavelength of 570 nm and 690 nm using an automated microplate reader. Otherwise, H9c2 cells were seeded at a density of 5 × 10⁵ cells/well in six-well plates; cells were then treated for 24 h, collected by trypsinization and centrifuged at 300g for 5 min for flow cytometry. Following suspension in binding buffer, cells were labeled with Annexin-V-FITC and propidium iodide (PI) according to the manufacturer's instructions. FITC and PI were measured by flow cytometry analysis of 5000-gated cells using FACScan and CellQuestPro software from Becton Dickinson. Cell populations were defined as viable (AV−/PI−), apoptotic (AV+) or necrotic (PI+) cells.

Real-time quantitative PCR

RNA was isolated from cell culture or tissues using TRI Reagent® (Sigma-Aldrich). First-strand cDNA was synthesized from 1 µg total RNA using RevertAid First-Strand cDNA Synthesis kit (Thermo Scientific). The resulting cDNA was subjected to real-time PCR using FastStart Universal SYBR Green Master (Rox) (Roche Molecular

Biochemicals) was performed using specific primers (FNDC5, Fw: GAGGTGCTGATCATCGTCGT, Rv: GAGCAAGCACTGAAAGGGTTT; 36B4, Fw: GGCG TCCTCATTAGAGTGACA, Rv: TAGTTGGACTTCCA GGTCGC). All reactions were carried out in an Eppendorf Realplex thermocycler. Sample values were standardized against an indicated housekeeping gene.

Western blot

H9c2 cells were homogenized in ice-cold lysis buffer containing 50 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EGTA, 1 mmol/L EDTA, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, 0.27 mol/L sucrose, 0.1% 2-mercaptoethanol and 1× Complete Protease Inhibitor Cocktail (Roche Diagnostics) for 1 h. Homogenates were centrifuged at 21,100 *g* for 15 min at 4°C; supernatants were removed, and frozen at -20°C. Cells lysates (15 µg) were subjected to 13% acrylamide SDS-PAGE gels, transferred on a PVDF membrane as previously described. Membranes were blocked for 1 h in TBS-Tween (TBST: 50 mmol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl and 0.1% Tween) containing 3% BSA and probed with specific antibodies against Akt, phosphorylated Akt, Bax, phosphorylated Bad, Caspase-3 (Cell Signaling), FNDC5 (Abcam), B-actin (Sigma-Aldrich) and α -tubulin (Santa Cruz). Detection of proteins was performed using horseradish peroxidase-conjugated secondary antibodies

Anti-Rabbit IgG (Cell Signaling) and Anti-Mouse IgG (Dako EnVision system, peroxidase) and an enhanced chemiluminescence reagent Pierce-ECL Western blotting substrate (Thermo Scientific).

Representative blots correspond to unedited images of full radiographic film detection. Each band is accompanied by its respective load control except for the ratio between cleaved and uncleaved Caspase 3 form. In fact, although Akt and phosphoAkt was represented as a ratio, both load controls were included since samples should be incubated in individual membranes (for accuracy).

Statistical analysis

Data are represented as mean $\pm$ S.E.M. (standard error of the media). Statistical significance was determined by Mann-Whitney test, ANOVA followed by Tukey's or Bonferroni's *post hoc* test or one-way ANOVA followed by Tukey's, *post hoc* test determined using GraphPad Prism 6 Software. $P < 0.05$ was considered significant.

Results

Decreased FNDC5 expression in hypoxic cardiomyoblasts

H9c2 cells exposed to hypoxia showed a time-dependent decrease in FNDC5 mRNA and protein levels (Fig. 1A and B).

Figure 1

FNDC5 expression in H9c2 cells under hypoxic conditions. (A) FNDC5 mRNA levels measured by real-time PCR in normoxic and hypoxic conditions. Data are expressed as mean $\pm$ S.E.M. ($n = 3$), P value was determined by two-way ANOVA followed by Bonferroni's *post hoc* test. (B) FNDC5 protein levels measured by Western blot in normoxic and hypoxic conditions. Data are expressed as mean $\pm$ S.E.M. ($n = 3$), P value was determined by two-way ANOVA followed by Bonferroni's *post hoc* test. (C) Annexin V flow cytometry assay under normoxic and hypoxic conditions. Data are expressed as mean $\pm$ S.E.M. ($n = 4$). $*P < 0.05$ vs control; $**P < 0.01$ vs control; $***P < 0.001$ vs control. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0123>.

Flow cytometry study showed that lower expression of FND5 after 24h of hypoxia is accompanied by a significant increase in H9c2 cell apoptosis (Fig. 1C and D).

Irisin prevents lipotoxic-induced apoptosis under hypoxic conditions

Since lipotoxicity exacerbates cellular damage under ischemic conditions, we aimed to show if irisin supplementation exerts any protective effect against lipotoxic-induced apoptosis in hypoxic H9c2 cells. Despite the significant decrease in FDNC5 expression, our data failed to show a protective effect of irisin against hypoxia in the absence of lipotoxicity (data not shown). Also, our data showed that under normoxic conditions, irisin supplementation has no effect on viability, regardless of lipotoxicity (Fig. 2A). Nevertheless, our results clearly demonstrated that in hypoxic conditions, irisin supplementation improves cell viability (0.39 ± 0.04 vs 0.63 ± 0.04) and counteracts lipotoxic-induced apoptosis (0.68 ± 0.05 vs 0.47 ± 0.08) (Fig. 2B and C).

Irisin prevents lipotoxic-induced increase of pro-apoptotic Bcl-2 family members under hypoxic conditions

Pro-apoptotic Bcl-2 family protein expression was measured by Western blot analysis. As expected, lipotoxicity triggered a significant increase in Bax levels (0.99 ± 0.09 vs 3.69 ± 0.91) and Caspase-3 activity (0.007 ± 0.004 vs 0.99 ± 0.205) as well as a significant decrease in phosphorylated Bad levels (1 ± 0.16 vs 0.12 ± 0.02). Our data showed that irisin supplementation prevents all the above-described lipotoxic-induced pro-apoptotic changes in Bax levels (3.69 ± 0.91 vs 1.53 ± 0.18), Caspase-3 activity (0.99 ± 0.205 vs 0.31 ± 0.01) and phosphorylated Bad levels (0.12 ± 0.02 vs 0.31 ± 0.09) (Fig. 3A and B).

Akt mediates irisin cardioprotective effects against lipotoxicity

Irisin supplementation stimulated a significant increase of pAkt/Akt ratio in palmitate-treated group

Figure 2

Irisin prevents lipotoxic-induced H9c2 cells apoptosis under hypoxic conditions. (A) MTT cell viability assay in H9c2 cells treated with palmitate in normoxic and hypoxic conditions ($n = 4$). (B) Annexin V flow cytometry assay of lipotoxic-induced apoptosis under normoxic and hypoxic conditions. Histogram represents the viable, apoptotic and necrotic rate of H9c2 cells relative to control group. Data are expressed as mean $\pm$ s.e.m. ($n = 4$). (C) Representative density plots images of Annexin V-FITC/propidium iodide double staining flow cytometry in normoxic and hypoxic conditions. * $P < 0.05$ vs control; ** $P < 0.01$ and *** $P < 0.001$; ### $P < 0.001$ vs palmitate. P values determined by two-way ANOVA followed by Tukey's *post hoc* test. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0123>.

Figure 3

Caspase-3 and Bcl-2 family proteins expression in irisin and/or palmitate H9c2-treated cells under hypoxic conditions. (A) Histogram represents mean $\pm$ S.E.M. relative to control group ($n = 3$). Caspase-3 is represented as cleaved to full-length form. (B) Representative Western blots are shown for H9c2 cells exposed to palmitate/irisin. White spaces indicate splicing of cropped lines from different parts of the same gel or from different gels. α -Tubulin is shown as a loading control. Band intensity was quantified regarding Caspase 3 or α -Tubulin expression. ** $P < 0.01$ vs control; *** $P < 0.001$ vs control and # $P < 0.05$ vs palmitate. P values determined by one-way ANOVA followed by Tukey's *post hoc* test.

(14.24 ± 6.26 vs 45.67 ± 16.51) (Fig. 4A and B). To determine if increased Akt activity mediates cardioprotective role of irisin, annexin V staining flow cytometry assay was performed in palmitate/irisin treated cells co-treated with 10-NCP (Fig. 4C and D). Akt inhibition counteracts anti-apoptotic irisin effects ($P: 0.50 \pm 0.04$ vs P/I: 0.38 ± 0.04 vs P/I/10-NCP: 0.54 ± 0.06).

Discussion

As it is widely accepted that maintaining regular physical activity reduces the risk of cardiovascular events, it is also known that exercise-based cardiac rehabilitation improves myocardial remodeling, recurrence and all-cause mortality (Suaya *et al.* 2009, Hammill *et al.* 2010, Goel *et al.* 2011, Lawler *et al.* 2011). Boström *et al.* identified a new potential molecular mechanism by which physical activity improves overall metabolism by increasing irisin levels.

Beyond the general metabolic improvement, cumulative evidence to date established a strong relationship between irisin and cardiovascular health. Curiously, there seems to be a threshold in both cases, as well as excessive exercise after MI has been related with adverse cardiac remodeling and scar formation (Kloner & Kloner 1983), excessive irisin levels have been related with increased oxidative stress and cardiomyocyte apoptosis (Ho *et al.* 2018). These observations are in accordance with recent clinical results, which conclude that elevated serum concentrations of irisin are associated with the development of major adverse cardiovascular events in patients with established CAD after revascularization (Aronis *et al.* 2015) and also related with adverse cardiovascular outcomes in both acute heart failure (Shen *et al.* 2017) and infarcted patients (Hsieh *et al.* 2018). Although the relationship and benefit of irisin on the myocardium is demonstrated, there remains some controversy mainly based on the experimental approaches (inadequate doses, short experimental groups, clinical confounding variables) and technical limitations such as antibody specificity and poor accuracy of ELISA kits (Jedrychowski *et al.* 2015), but also neither it can be ruled out that irisin may have the double-edged effect.

Our data placed the focus on the role that irisin might play against lipotoxic injury associated to ischemic events. Recent study of the FNDC5 gene expression profile in different tissues in adult rats showed that cardiac muscle is one of the tissues with the highest expression of irisin (Varela-Rodriguez *et al.* 2016). Our data demonstrated for the first time that FNDC5 expression, both mRNA and protein, is downregulated in a time-dependent manner in hypoxic H9c2 cells. Also, our data showed that lower levels of irisin observed after 24 h of hypoxia are accompanied to increased apoptosis. Decreased cardiomyocyte irisin expression has been previously described in murine model of MI (Kuloglu *et al.* 2014). Suppression of uncoupled activity of irisin in an energy-depleting context such as lack of oxygen sounds coherent. Curiously, in the MI murine model, it was found that irisin serum expression is decreased but it is accompanied with an increase in the surrounding connective tissue amount in which irisin expression is increased, suggesting that the maintenance of physiological irisin concentrations around cardiac muscle might be important during MI. Reduction of *in vitro* reperfusion injury (Zhao *et al.* 2016) and infarct size in *in vivo* models, together with the potential existence of an irisin-specific receptor (Xie *et al.* 2015) strongly suggest that myocardium might respond to circulating irisin levels and that there are cardiovascular benefits beyond energy homeostasis.

Figure 4

Akt inhibition counteracts irisin effects in lipotoxic-induced H9c2 cells apoptosis under hypoxic conditions. (A) Histogram represents mean $\pm$ s.e.m. relative to control group ($n = 3$). Akt is represented as phosphorylated to non-phosphorylated form. P values determined by one-way ANOVA followed by Tukey's *post hoc* test. (B) Representative Western blots are shown for H9c2 cells exposed to palmitate/irisin. White spaces indicate splicing of cropped lines from different parts of the same gel or from different gels. α -Tubulin is shown as a loading control. Band intensity was quantified regarding α -Tubulin expression. (C) Annexin V flow cytometry assay of H9c2 cells treated with palmitate, irisin and/or 10-NCP under hypoxic conditions. Histogram represents the viable, apoptotic and necrotic rate of H9c2 cells relative to control group. Data are expressed as mean $\pm$ s.e.m. ($n = 4$). P values determined by two-way ANOVA followed by Tukey's *post hoc* test. (D) Representative density-plot images of Annexin V-FITC/propidium iodide double staining flow cytometry. *** $P < 0.001$ vs control; # $P < 0.05$ vs palmitate; ### $P < 0.001$ vs palmitate; \$ $P < 0.05$ and \$\$\$ $P < 0.001$ vs palmitate/irisin. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0123>.

Sudden myocardial metabolic changes, due to lack of oxygen supply and compensatory adrenergic response (Geerling *et al.* 2014) finally trigger a lipotoxic environment (Wende & Abel 2010), central in MI cardiomyocyte apoptosis. In addition to previously described benefits in reperfusion injury, our data show a protective role of irisin in this context.

Previous studies showed that irisin induces proliferation in endothelial (Song *et al.* 2014) and neuronal (Moon *et al.* 2013) cell lines. However, increased proliferative activity was described at pharmacological but not physiological concentrations (Moon *et al.* 2013). According to previous studies (Xie *et al.* 2015), our data demonstrated that irisin supplementation failed to increase cell proliferation at physiological concentrations (5 nM) (data not shown) but also, flow cytometry analysis confirmed that the observed increased viability is associated with the prevention of lipotoxic-induced apoptosis in hypoxia. Anti-apoptotic activity of irisin was also confirmed through immunoblot studies that evidenced the regulation of apoptotic pathways. Increased phosphorylation of pro-apoptotic Bcl-2 family protein Bad (inactive form) accompanied by a drop in Bax and Caspase-3 expression. Since Akt is involved in a wide range of cellular processes including apoptosis and regulation of myocardial metabolism by irisin (Xie *et al.* 2015), our data showed that selective pharmacological Akt inhibition counteracted the

anti-apoptotic role of irisin, demonstrating that activation of Akt signaling pathway is implicated in protective role of irisin.

One of the highlights of this study is that irisin expression and function seems to be related to pathophysiological environment. Protective role of irisin against lipotoxic injury, only in hypoxic conditions, may be related to metabolic shift in conditions of deficient oxygen supply (Lopaschuk *et al.* 2010) and metabolic effects of irisin depend on substrate availability (Vaughan *et al.* 2015). Pathophysiological response to acute MI is associated with a sudden increase in circulating FA simultaneously to increased FA translocation and intramyocardial lipid accumulation (Lopaschuk *et al.* 2010) that contributes to cardiomyocyte apoptosis (Wende & Abel 2010). For that reason, it is essential to address and counteract the deleterious effects of FA overload, especially in ischemic conditions. Our data indicate that irisin counteracts lipotoxic-induced injury and suggest that differential FNDC5 expression might occur as a coordinated response or as a consequence of the context of lack of oxygen and lipid overload to modulate fatty acid oxidation. In this sense, decreased myocardial expression of FNDC5 might reduce fatty acid oxidation to avoid oxygen consumption beyond its possibilities and oxidative stress; more studies are required to assess this possibility.

Figure 5

Irisin effect on H9c2 treated with FA under normoxic/hypoxic conditions. H9c2 cells exposed to hypoxia showed a significant decrease in *FNDc5* mRNA levels. Irisin supplementation counteracts lipotoxic-induced apoptosis in hypoxic cardiomyoblast mediated by Akt. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0123>.

Undoubtedly, there are certain considerations to take into account in our study. Cardiomyoblasts cell line could not be compared with animal models or primary cultures. However, we aimed for this model because it allows us to work with an energetically differentiated myocardial cell line, closest to adult cardiomyocytes and avoid external conditioning of *in vivo* models. Another issue to highlight is that contrary to previous data (Zhao *et al.* 2016), our data failed to show any cardioprotective role in hypoxic conditions, in the absence of lipotoxicity. In this sense, previous positive studies were carried out considering reperfusion injury but also using pharmacological concentrations of irisin (Zhao *et al.* 2016), while in our model irisin concentration is closer to physiological concentrations. This might be the reason why prevention of damage is not effective in this model; however, our data are consistent with the previously described increase of irisin expression in surrounding connective tissue since increased local levels of irisin might be enough to reduce cardiomyocyte apoptosis during MI. New studies that locally overexpress *FNDc5*, near to the ischemic region, in murine models of infarction, are needed to solve this question. Another outstanding question is that we did not observe changes in irisin effects in normoxia, unlike in a hypoxic environment. In this sense, it seems that irisin might play a different role depending on the pathophysiological state.

Despite experimental and technical limitations, a huge number of studies showed that irisin plays a central role in cardiovascular health and key issues remain unresolved by the scientific community. Literature, together with the data obtained in this study, clearly demonstrate that myocardial expression of *FNDc5* is regulated in pathophysiological conditions, showing a direct protective role of irisin against lipotoxicity in

addition to previously described reperfusion injury, but also strongly suggest that local increase, closest to infarcted area, might be an adaptive response to reduce ischemic damage (Fig. 5).

Conclusion

Our *in vitro* model mimicking a lipotoxic context characteristic of hyperlipidemic states in ischemic events shows that irisin counteracts lipotoxic-induced apoptosis in hypoxic cardiomyoblast in an Akt-dependent manner. Undoubtedly, future in-depth studies in animal models of acute MI are needed to address the mechanism as well as functional significance of differential *FNDc5* expression under pathophysiological conditions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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