Article

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Irisin Treatment Prevents Isoproterenol-Induced Cardiac Fibrosis in Mice

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Background: Cardiac fibrosis is a pathophysiological process that occurs as the end stage of cardiovascular diseases. Irisin is a myokine secreted mainly by skeletal muscle exerting pleiotropic effects. Previous studies found altered irisin levels in patients with cardiovascular diseases and irisin has been shown to preserve cardiac function after ischemia-reperfusion injury in mice. This study aimed to explore whether pretreatment with irisin prevents cardiac fibrosis induced in mice through a single injection of the beta-adrenergic agonist isoproterenol at a high dose.

Methods: The cardiac fibrosis model was obtained through a single intraperitoneal administration of 160 mg/kg isoproterenol [ISO] in young C57BL/6J mice. Before ISO injection, mice were pretreated with irisin 100 µg/kg/week [irisin-ISO] or saline [veh-ISO] for 4 weeks. A third group of mice received saline for 4 weeks without ISO injection [CTRL].

Results: The mice pretreated with irisin recovered faster than vehicle-treated mice after acute ISO stimulation, as measured by behavioral test. Twenty-four hours after ISO treatment, the serum levels of Troponin I were significantly lower in the group of mice pretreated with irisin compared with veh-ISO mice ($p = 0.0117$). Moreover, the expression of atrial natriuretic peptide ($p = 0.0197$) and alpha-smooth muscle actin ($p = 0.0261$) mRNAs in cardiac tissue of veh-ISO mice were 10- and 15-fold higher than CTRL mice, respectively, while pretreatment with irisin maintained their expression at control levels. Interestingly, 7 days after ISO, the expression of alpha-smooth muscle actin mRNA was still significantly lower in the irisin-ISO group than in the veh-ISO group ($p = 0.0145$). Moreover, we found increased cardiac hypertrophy, measured as heart-weight/tibia-length ratio, in veh-ISO mice versus CTRL mice ($p = 0.0312$) which was fully prevented in irisin-ISO mice ($p = 0.0258$). The cardiac fibrosis score assessed by Masson's trichrome staining was significantly lower in irisin-ISO mice versus veh-ISO mice ($p = 0.0261$). Notably, some mitochondrial genes, previously identified as controlled by irisin, were markedly increased in the early phase following ISO, whereas irisin maintained their expression similar to controls.

Conclusion: Our results demonstrate the beneficial effect of irisin in preventing isoproterenol-induced cardiac hypertrophy and fibrosis.

Keywords: irisin; isoproterenol; b-adrenergic cardiac fibrosis; αSMA

Introduction

Heart failure is a clinical syndrome that is a major cause of morbidity and mortality worldwide and whose prevalence is steadily increasing [1]. It is characterized by a set of symptoms caused by cardiac abnormalities that mainly affect patients with cardiovascular disease. If patients experience pressure overload, this triggers pathological hypertrophy, characterized by an increase in ventricular wall thickness [2]. During the process of cardiac hypertrophy, collagen synthesis is activated in fibroblasts as a protective mechanism to replace dead cardiomyocytes. However, as contraction efficiency decreases, excessive collagen accumulation causes the onset of cardiac fibrosis, aggravating contractile function and proper perfusion of other organs [2].

Regular physical activity has been recognized as an effective non-pharmacological therapy for the treatment of cardiovascular disease. It alleviates risk factors for heart failure and reduces mortality in patients with cardiovascular disease [3,4]. Specifically, cardiac rehabilitation performed by exercise has been found to be effective in im-

proving left ventricular function after myocardial infarction [5,6]. Although the benefits of exercise on cardiac function have been well-known for decades, the molecular mechanisms involved in this process have long remained unclear. A recent finding have shown that irisin, a myokine produced by skeletal muscle tissue during physical exercise, plays an essential role in the pathophysiology of heart failure [7]. An *in vitro* study demonstrated that irisin treatment significantly reduced the increase in cardiomyocyte size caused by angiotensin II [8]. A study in mice showed that angiotensin II-induced cardiac interstitial fibrosis was reduced by pretreatment with irisin [9]. As a proposed mechanism of action in this elegant study, the authors showed that irisin treatment inhibited cardiac fibrosis by blocking angiotensin II-mediated generation of reactive oxygen species, which in turn would activate transforming growth factor beta 1 (TGFβ1) and Smad2/3 signaling, resulting in increased collagen synthesis and transformation of fibroblasts to myofibroblasts in cardiac tissue [9]. Moreover, the treatment in mice with recombinant irisin for 4 weeks attenuated cardiac hypertrophy caused by transverse aortic constriction (TAC) [8]. Additionally, the mouse model overexpressing the irisin precursor, Fibronectin type III domain-containing protein 5 (FNDC5), after undergoing TAC intervention, showed a reduction in heart weight/tibia length ratio and levels of hypertrophic markers, such as atrial natriuretic peptide (ANP), suggesting that the FNDC5/irisin system may contribute to preserving cardiac function [7]. To confirm this, the authors also showed that FNDC5 knock-out mice exhibited severe heart failure after TAC surgery, accompanied by eccentric cardiac hypertrophy, fibrosis, and cardiac dysfunction [7]. Mechanistically, the authors showed that irisin protects against cardiac hypertrophy triggered by pressure overload by inducing protective autophagy in the myocardium [7].

The TAC surgical model is widely used in cardiovascular research to study the mechanisms of heart failure and to evaluate potential therapeutic interventions [10]. Although this model is valuable for translational cardiovascular research, it has the disadvantage of inducing systemic effects beyond the heart, such as inflammatory response, and also leads to a high mortality rate. Stimulation with β-agonists such as isoproterenol (ISO) is another routine method used to experimentally induce cardiac hypertrophy and fibrosis in animal models [11,12]. Previous studies have shown that ISO administered in rodents at a high dose induced necrosis of cardiomyocytes, followed by fibrosis [11,13]. The pretreatment with irisin in mice, administered according to a protocol that mimics moderate physical activity, i.e., intermittently (once a week) for 4 weeks, has been shown to be successful in recapitulating the positive effects of the exercise on bone, brain, and skeletal muscle [14–17].

Given this premise, the rationale of our study was to evaluate whether pretreatment with irisin could prevent cardiac fibrosis caused by high-dose ISO administration in mice. Moreover, the mouse model used in the present study mimics the common condition of acute β-adrenergic stimulation, such as that experienced in humans from challenging life events or daily stressors. Therefore, a potential clinical application of these findings could lead to the use of irisin treatment in the prevention of cardiac fibrosis, especially for those patients unable to engage in physical activity.

Materials and Methods

Mice and Experimental Design

4-month-old C57BL6 male and female mice (n = 40), with body weight 25.83 $\pm$ 3.89 grams, were purchased from Charles River, Wilmington, Massachusetts, USA and were randomly assigned to three groups: control mice injected with vehicle [CTRL] (N = 12), mice injected with vehicle and then subjected to ISO treatment [veh-ISO] (N = 12), and mice treated with irisin and then subjected to ISO [irisin-ISO] (N = 16) treatment (Fig. 1A). The sample size was determined based on statistical power analysis using the G-power3 calculator, with an effect size of 0.25, type I error = 0.05 and test power = 80% based on our previous studies [14–17]. To account for allocation bias, such as the day on which animals were treated or their cage location, we used the method of randomization within blocks. More specifically, we divided the experiment into smaller sub-experiments, and treatments were randomized to the experimental units within each block. Individual animals were randomly assigned to experimental blocks from a group homogeneous in age and sex, and treatments and analyses were performed by blinded researchers. Our study examined male and female animals, and similar findings were reported for both sexes. Mice were housed in conventional cages in temperature-controlled facilities on a 12-h light/dark cycle on a standard diet and were weighed once a week.

Treatments of Mice

Mice were treated by subcutaneous injection (s.c.) once a week for 4 weeks with physiologic solution sterilized by 0.22 µ filtration [veh-ISO group] (N = 12) or with 100 µg/kg rec-irisin (AG-40B-0128-C010, Adipogen International, San Diego, CA, USA) [irisin-ISO group] (N = 16). A third group of mice was used as a control and received physiologic solution by subcutaneous (s.c.) injection once a week for 4 weeks without receiving ISO injection at the end of 4 weeks [CTRL group] (N = 12). Based on the results of a previous study [13], the cardiac fibrosis model was obtained through a single intraperitoneal injection (i.p.) of isoproterenol hydrochloride (HCL) (U26G021, Sigma-Aldrich, Merck, St. Louis, MO, USA) in Dulbecco's phosphate-buffered saline (DPBS; 2676168, Sigma-Aldrich, Merck) (160 mg/kg), 24 hours after the last injection of irisin or vehicle. Criteria for determining the

success of the mouse model were evaluated in the veh-ISO versus CTRL mouse group by measuring serum Troponin I levels and heart weight/tibia length ratio after 24 hours and 7 days following ISO injection, respectively.

To ensure the welfare of the mice, a trained researcher skilled in animal behavior arranged for pain relief before performing the invasive experimental procedure of injection with isoproterenol. To this end, we included a pretreatment with tramadol hydrochloride 0.05 mg/kg for pain relief, injected subcutaneously 30 min prior to isoproterenol treatment [18]. Control mice [CTRL] were treated with Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, Merck) at an equivalent volume. To assess the recovery from the acute effects of isoproterenol, we did not find it appropriate to use the manual mouse grimace scale (MGS) assessment because neither change in whisker position or swelling of the nose or cheeks, nor narrowing of the orbits were detected [19]. Instead, we measured the time to recovery in motor activity [20] and the quality of recovery using an activity score (good, average, poor) related to movement, eating, or drinking, as previously described [13]. To assess the motor activity, the Open Field Test (OFT) was carried out by a trained researcher. The OFT was conducted in a polycarbonate, transparent cage with bedding on the bottom, similar to mice home cages (37 × 21 × 18 cm). Mice were placed individually in the center of the cage and enabled to explore the environment.

As shown in the experimental design (Fig. 1A), a subset of each subgroup of mice (n = 17) was sacrificed 24 h after administration with ISO injection for blood and molecular biology analysis. The other subset of mice (n = 23) was sacrificed 7 days after ISO injection, then hearts were weighed and collected for molecular and histological analysis. Following the procedure approved by the Italian Health Authority (12/2022-PR), all mice were euthanized in the home cage using 100% carbon dioxide and infused into the cage for 2 min through the automatic watering system. After 2 min of gas flow, the gas was turned off and the mice remained in the carbon dioxide-filled cage for 3 min; then the mice were laid supine, stimulated by pinching their feet, and observed for any signs of resuscitation [21].

Intracardiac Blood Collection

Twenty-four hours after ISO injection, a subset of each subgroup of mice (n = 17) was anesthetized before cardiac puncture with 100 mg/kg ketamine (cat. n. BP736, Sigma-Aldrich, Merck, Darmstadt, Germany) and 10 mg/kg xylazine (cat. n. 1720407, Sigma-Aldrich, Merck, Darmstadt, Germany) according to the UCSF guidelines for rodent anesthesia [22]. After confirming that the mice were deeply anesthetized by checking for the absence of the pedal reflex by pinching their feet, a trained researcher located the xiphoid process and inserted the needle into the left ventricle of the heart, with the plunger pulled slightly to create a negative pressure. Once the heart was successfully punctured, blood automatically began to fill the syringe due to the negative pressure. Once the steady flow of blood into the needle was confirmed, the needle hub was stabilized to draw the required amount of blood (about 0.8–1 mL of blood). Then, mice were euthanized using 100% carbon dioxide, as described in the paragraph above. The collected blood was released into a tube after removing the needle from the syringe to avoid hemolysis. Before centrifugation at 1000 ×g for 20 min at 23 °C, blood samples were allowed to clot for 30 min at room temperature. Then, the samples were stored at –80 °C until analysis.

ELISA Assay

A mouse cardiac troponin I (cTnI) enzyme-linked immunosorbent assay (ELISA) Kit (cat. N. A79005, Antibodies, Cambridge, UK) was used to determine cTnI concentrations in serum as instructed by the manufacturer. Serum samples were collected 24 h after ISO injection by intracardiac puncture and diluted two-fold in supplied diluent. A standard curve was generated using serial dilutions of the calibration standards supplied, to calculate cTnI concentrations in pg/mL. For each biological replicate, we performed three independent technical replicates. Optical densities were measured at 450 nm using a spectrophotometer (EONC, Eon, BioTek, Winooski, VT, USA) at the end of the assay.

Tissue Harvesting and Histological Analysis

Seven days after the ISO injection, mice were euthanized with CO_2. Experimental procedures have been carried out following the standard biosecurity and institutional safety procedures. Hearts were excised and weighed, the apex was removed, and the remaining organ was fixed with 4% paraformaldehyde (STBJ6640, Sigma-Aldrich, St. Louis, MO, USA) overnight, then incubated for 1 h and 30 minutes in a 15% sucrose solution in DPBS and stored in 70% ethanol. Subsequently, heart samples were dehydrated in an increasing gradient of ethanol and embedded in paraffin. Five-µm sections were cut with a standard microtome (RM-2155 Leica, Leica Biosystem, Heidelberg, Germany), dewaxed, and rehydrated in an ethanol gradient. Sections were stained with Masson's trichrome kit (cat. N. HT15, Sigma-Aldrich, St. Louis, MO, USA). All reagents were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Masson's trichrome was used to analyze and score fibrosis, using a scoring scale based on the severity of cardiac fibrosis: 0 (none), 1 (mild), 2 (moderate) to 3 (severe), as previously described [13]. Scores were obtained from 10 fields per section, considering 3 sections at the mid-heart level per animal by the blinded researcher. Images were captured using an Aperio scanner (CS2, Leica Biosystem, Heidelberg, Germany) and processed for the area of fibrosis quantification using ImageScope 12.4.6 software (Leica Biosystem, Heidelberg, Germany).

Table 1. Primer sequences.

Gene name	Forward (5′–3′) and template	Reverse (5′–3′) and template
Gapdh	ACACCAGTAGACTCCACGACA	ACGGCAAATTCAACGGCACAG
Beta-2-Microglobulin	TGCTATCCAGAAAACCCCTCA	TTTCAATGTGAGGCGGGTGG
αSMA	CAGCCATCTTTCATTGGGATG	TGGTACCCCCTGACAGGAC
ANP	CTGATGGATTTCAAGAACCTGCT	TCTCAGAGGTGGGTTGACCT
IL33	TGCTACTACGCTACTATGAG	TGTGAAGGACGAAGAAGG
Sirt1	TCGGCTACCGAGGTCCATA	CGCTTTGGTGGTTCTGAAAGG
PGC1α	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTTC
Tfam	TAGGCACCGTATTGCGTGAG	CAGACAAGACTGATAGACGAGGG

Gapdh, glyceraldehyde-3-phosphate dehydrogenase; *αSMA*, alpha-smooth muscle actin; *ANP*, atrial natriuretic peptide; *IL33*, interleukine-33; *Sirt1*, sirtuin 1; *PGC1α*, peroxisome proliferator-activated receptor-gamma coactivator 1-alpha; *Tfam*, mitochondrial transcription factor A.

Real-Time Polymerase Chain Reaction (RT-PCR)

Heart samples were homogenized with Ultra-Turrax T8 (Ika, Staufen im Breis-gau, Germany). Total RNA from heart tissue was extracted using spin columns (172031538, Qiagen, Hilden, Germany). Reverse transcription was performed by iScript Reverse Transcription Supermix (64505402, Bio-Rad Laboratories, Hercules, CA, USA) in the thermocycler (T100, My cycler; Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR on the CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA, USA) was performed by SsoFast EvaGreen Supermix (batch 64596675, Bio-Rad Laboratories, Hercules, CA, USA) for 40 cycles (denaturation 95 °C for 5 s; annealing/extension 60 °C for 10 s) after an initial 30-s phase for enzyme activation at 95 °C. The primers used were designed with Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers span an exon–exon junction. The primer sequences are described in Table 1. The geometric mean of two housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and *beta-2-Microglobulin* (*B2M*)) was used to normalize the data. For each biological replicate, we performed three independent technical replicates, and quantitative measures were calculated by the $2^{-\Delta\Delta CT}$ method and expressed as relative fold change from control.

Statistics

Analysis of sample distribution was performed by the D'Agostino & Pearson normality test. Parameters were expressed as the median and interquartile range (IQR) from max to min, with all data points shown, by using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA). For values that passed the normality test, we performed a One-way analysis of variance (ANOVA) with Tukey's multiple comparisons tests, whereas for non-normal distributed values we performed the Kruskal-Wallis test with two-group comparison by Dunn's multiple comparisons tests. Every comparison was done among three groups: the control group injected with vehicle [CTRL] and the mice groups pretreated with irisin [irisin-ISO] or saline solution [vehicle-ISO] before ISO injection. To achieve 80% power and type I error = 0.05, we considered a group size of 4–5 mice for some measurements, such as behavioral test analysis, and gene expression analysis in cardiac tissue. Instead, group sizes were increased for ELISA assay and histological tissue analysis, having predicted and accepted smaller effect sizes as biologically significant. Differences were considered significant at $p < 0.05$.

Results

Effect of Irisin Pretreatment on the Recovery of Mice after High Dose of Isoproterenol

To investigate whether intermittent irisin treatment can prevent isoproterenol-induced cardiac hypertrophy, we pre-treated mice with irisin 100 µg/kg/week [irisin-ISO] or saline [veh-ISO] for 4 weeks and then performed a single intraperitoneal injection of ISO at high dose (160 mg/kg) (Fig. 1A). To ensure the animal welfare, we have monitored the body weight during the experiment that resulted unchanged among the animal groups (Fig. 1B).

ISO effect was evident from 5 minutes after injection when mice ceased moving and increased respiration rate. We measured the time to recovery in motor activity (Fig. 1C) and the quality of recovery using an activity score [good, average, poor] related to movement, eating, or drinking by the Open Field Test (OFT) (Fig. 1D). No significant change was observed between the group of mice pretreated with irisin for 4 weeks [irisin-ISO] and the group pretreated with saline [veh-ISO]. However, irisin-treated mice showed faster recovery, although not reaching significance ($p = 0.0857$) (Fig. 1C), suggesting that the pretreatment may have improved the response to the acute effects of ISO. In addition, we did not observe any differences in body weight between the two groups of ISO-treated mice (Fig. 1E).

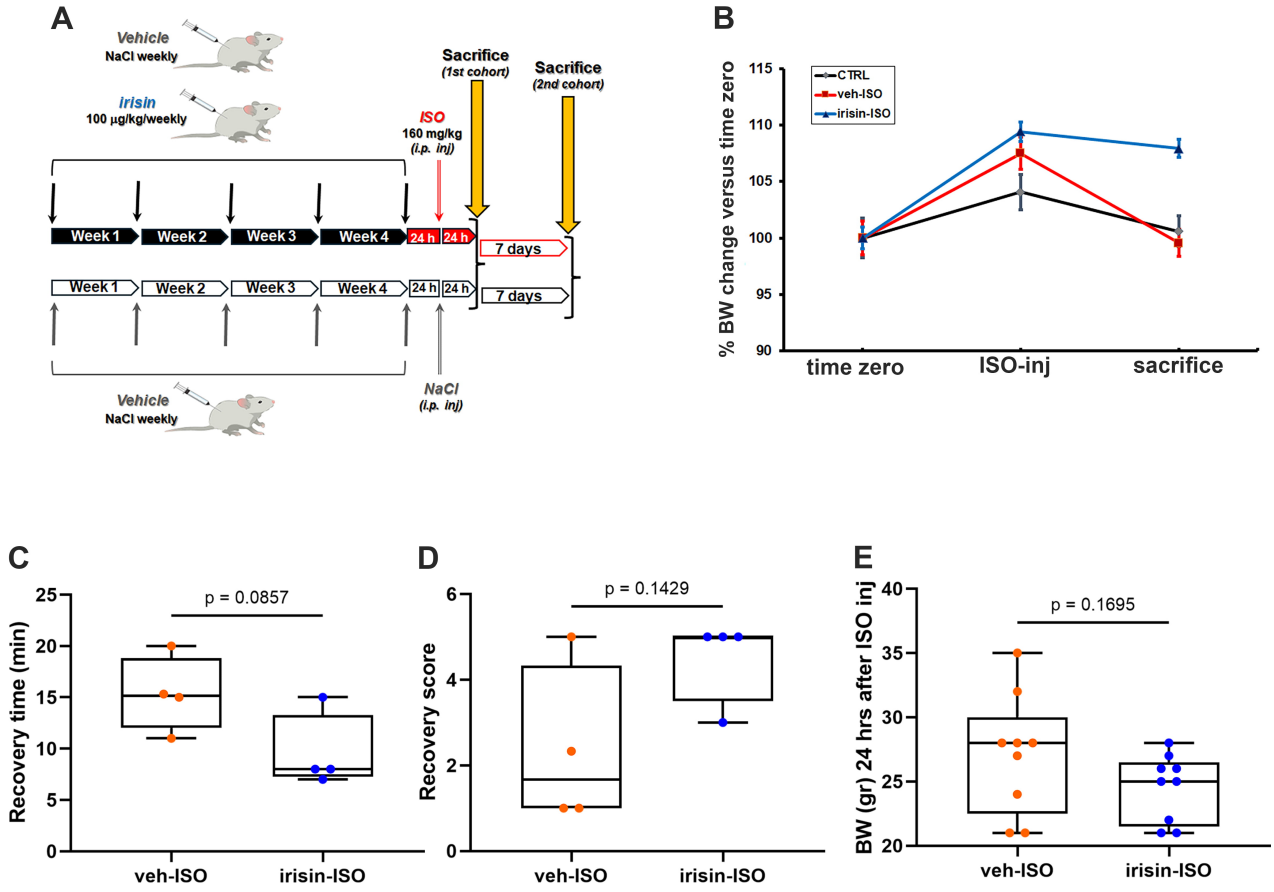

Fig. 1. Effect of irisin pretreatment on the recovery of mice after ISO injection. (A) Experimental design and timeline of the study. The figure was drawn in part using images from Servier Medical Art (https://smart.servier.com/), licensed under a Creative Commons Attribution 4.0 Unported License. (B) Percentage of body weight changes versus time zero at the time points of isoproterenol (ISO) injection and sacrifice in control mice [CTRL] and in mice treated with vehicle [veh-ISO] or irisin [irisin-ISO] for 4 weeks before ISO injection. (C) Measurements of the time to recovery and (D) the quality of recovery by the Open Field Test (OFT) after ISO injection in veh-ISO (n = 4) and irisin-ISO (n = 4) mice. (E) Body weight (BW) measurement 24 hours after ISO injection in veh-ISO (n = 9) and irisin-ISO (n = 9) mice. Shapiro-Wilk test followed by Mann-Whitney test (C,D) and unpaired *t*-test (E) were performed. Data are presented as box-and-whisker with median and interquartile ranges, from max to min, with all data points shown. *p*-value as indicated.

Pretreatment with Irisin Prevents Dysregulation of Molecules Involved in ISO-Induced Cardiac Failure

Twenty-four hours after ISO treatment, the increase of serum cardiac troponin I (cTnI), a biomarker of cardiomyocyte necrosis, was not observed in irisin-pretreated mice ($p = 0.0117$) (Fig. 2A).

Gene expression analysis on heart tissue showed that mRNA levels of *ANP*, a molecular marker for myocardial hypertrophy, was 10-fold higher in the veh-ISO group than in the control group ($p = 0.0197$), whereas in irisin-ISO mice its expression was comparable to the controls (Fig. 2B). Additionally, the expression of alpha-smooth muscle actin (*αSMA*) was 15-fold higher in veh-ISO mice than controls ($p = 0.0261$), but its increase was prevented in mice pretreated with irisin (Fig. 2C). In contrast, no significant change was observed in the expression of the anti-inflammatory interleukin-33 (*IL33*), although an increasing trend was noted in ISO-treated mice compared with controls that nearly borders on significance in the veh-ISO group ($p = 0.0605$) (Fig. 2D).

Irisin Pretreatment Prevents Cardiac Hypertrophy and Fibrosis Induced by a Single Dose of Isoproterenol

To study whether irisin prevents ISO-induced cardiac fibrosis we performed Masson's trichrome staining and found increased collagen deposition in veh-ISO mice. This effect was not observed in the irisin-ISO group, resulting comparable to the control group (Fig. 3A). Cardiac fibrosis was quantified by scoring according to severity: 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The obtained scores showed that fibrosis was significantly greater in ISO-treated mice than control mice [CTRL] ($p = 0.0131$) and it was fully prevented in irisin-ISO versus veh-ISO mice (p

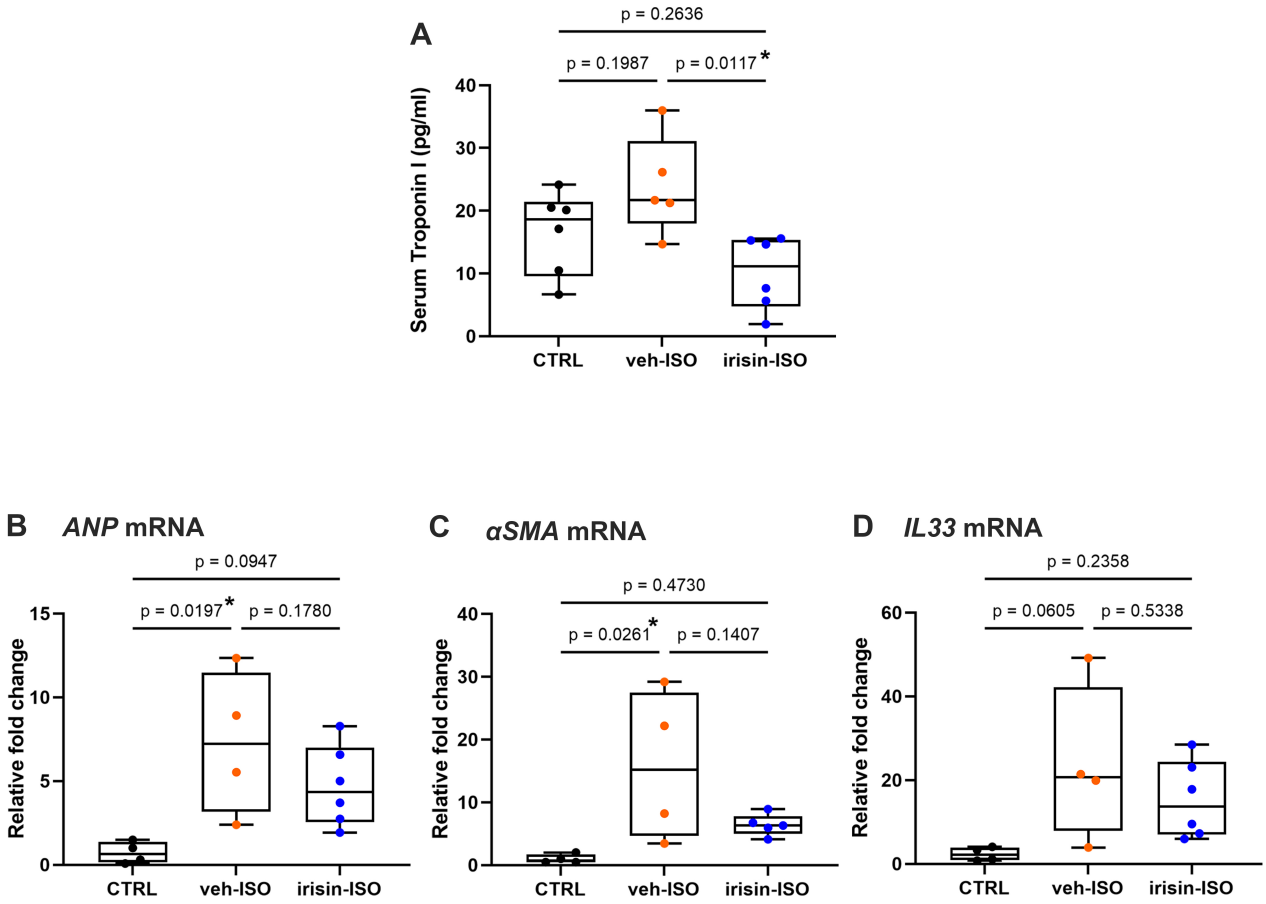

Fig. 2. Irisin prevents dysregulation of molecules involved in ISO-induced cardiac failure. (A) Concentration of serum cardiac troponin I (cTnI) determined by enzyme-linked immunosorbent assay (ELISA) assay in CTRL (n = 6), veh-ISO (n = 5), and irisin-ISO (n = 6) mice 24 hours after ISO injection. (B–D) Quantification of mRNA expression in heart tissue for atrial natriuretic peptide (*ANP*), *αSMA*, and *IL33* in CTRL (n = 4), veh-ISO (n = 4), and irisin-ISO (n = 5–6, one sample is missing from Fig. 2C) mice 24 hours after ISO injection. Shapiro-Wilk test followed by One Way analysis of variance (ANOVA) with Tukey's multiple comparisons tests were performed. Data are presented as box-and-whisker with median and interquartile ranges, from max to min, with all data points shown. *$p < 0.05$.

= 0.0261) (Fig. 3B). Moreover, to quantify cardiac hypertrophy we measured heart-weight/tibia-length ratio, and we found that ISO injection induced a 30% increase in veh-ISO mice compared with CTRL mice ($p = 0.0312$), whereas this effect was not present in mice pretreated with irisin [irisin-ISO] (Fig. 3C). Furthermore, we observed that ISO treatment did not change the spleen-weight/tibia-length ratio (Fig. 3D), thus implying the absence of systemic inflammation in mice.

Interestingly, 7 days after ISO administration, while *ANP* returned to control levels (Fig. 4A), *αSMA* expression in the veh-ISO group was still high, albeit not significantly, whereas it was maintained at control levels by irisin ($p = 0.0145$) (Fig. 4B).

Irisin Pretreatment Protects against Mitochondria Dysregulation Caused by Cardiac Hypertrophy

Since after cardiac damage, the mitochondrial bioenergetics must adjust according to hypertrophic phenotype, it has been reported that mitochondrial activity can be increased at the onset of cardiac hypertrophy [23]. Therefore, we sought to investigate whether mitochondria key factors genes were affected by ISO treatment. Our results showed that some mitochondrial genes, previously identified as controlled by irisin in skeletal muscle cell and tissue [24–26], namely sirtuin 1 (*Sirt1*) ($p < 0.001$), peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (*PGC1α*) ($p < 0.05$), and mitochondrial transcription factor A (*Tfam*) ($p < 0.01$), were markedly increased in the early phase following ISO injection (24-hrs) in the heart tissue of veh-ISO group compared to CTRL (Fig. 5A–C). However, in mice pretreated with irisin, their expression remained similar to control levels, suggesting that irisin com-

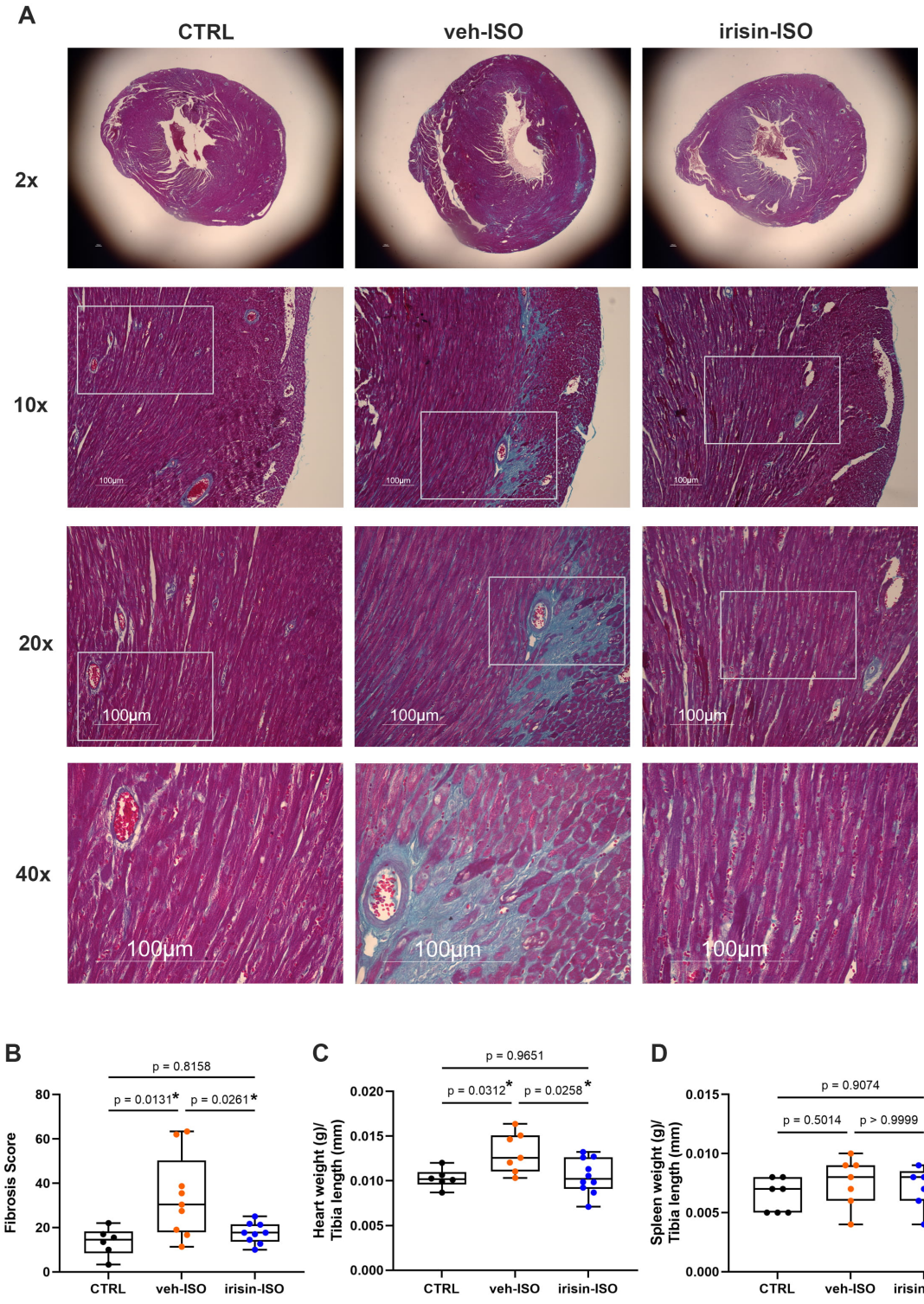

Fig. 3. Irisin prevents cardiac hypertrophy and fibrosis induced by ISO. (A) Photomicrographs of Masson's trichrome staining on sections of heart tissue harvested from CTRL, veh-ISO, and irisin-ISO mice 7 days after ISO injection, at different magnifications as indicated. The white boxes in 10× and 20× indicate the specific areas of photomicrographs in 20× and 40×, respectively. Scale bars = 100 μm. (B) Quantitative assessments of fibrosis score in CTRL (n = 6), veh-ISO (n = 9), and irisin-ISO (n = 9) mice. (C) Quantitative assessments of heart-weight/tibia-length ratio in CTRL (n = 6), veh-ISO (n = 7) and irisin-ISO (n = 10) mice. (D) Quantitative assessments of spleen-weight/tibia-length ratio in CTRL (n = 7), veh-ISO (n = 7), and irisin-ISO (n = 9) mice. Shapiro-Wilk test followed by One Way ANOVA with Tukey's multiple comparisons tests were performed (B,C). Shapiro-Wilk test followed by Kruskal-Wallis with Dunn's multiple comparison tests were performed (D). Data are presented as box-and-whisker with median and interquartile ranges, from max to min, with all data points shown. *$p < 0.05$.

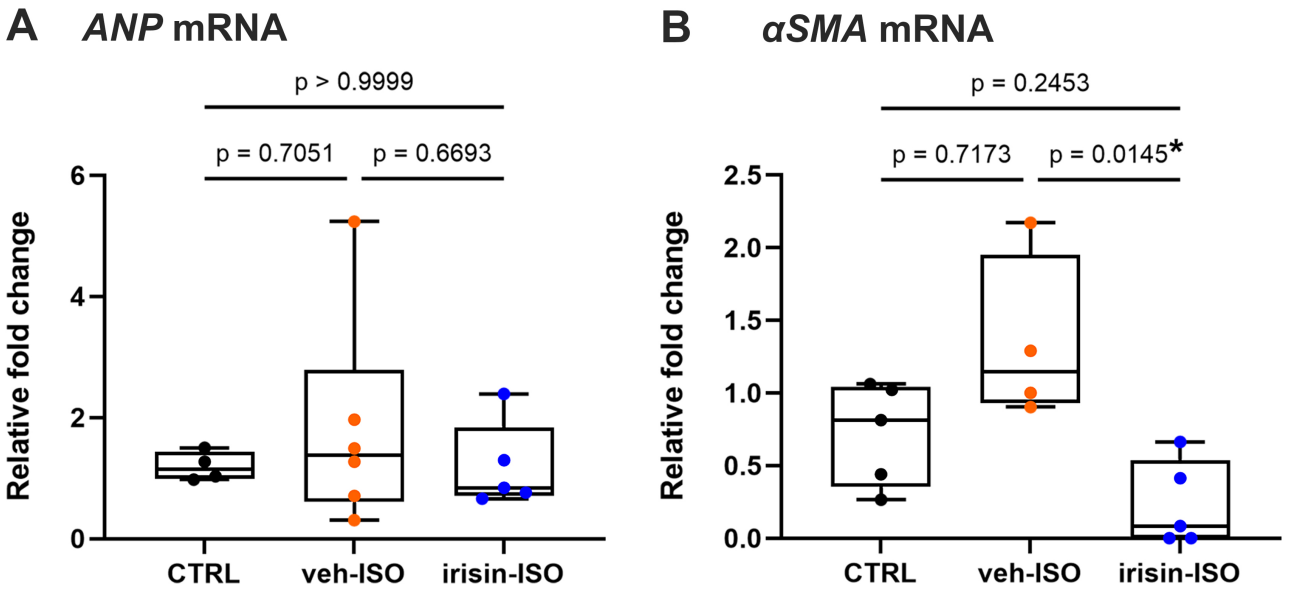

Fig. 4. Irisin prevents α*SMA* increase 7 days after ISO injection. (A) Quantification of mRNA expression in heart tissue for *ANP* in CTRL (n = 4), veh-ISO (n = 6), and irisin-ISO (n = 5) mice 7 days after ISO injection. (B) Quantification of mRNA expression in heart tissue for α*SMA* in CTRL (n = 5), veh-ISO (n = 4), and irisin-ISO (n = 5) mice 7 days after ISO injection. Shapiro-Wilk test followed by One Way ANOVA with Tukey's multiple comparisons tests (A) and Kruskal-Wallis with Dunn's multiple comparison tests were performed (B). Data are presented as box-and-whisker with median and interquartile ranges, from max to min, with all data points shown. *$p < 0.05$.

pletely protected against the induction of increased energy demand caused by cardiac hypertrophy. Interestingly, we observed that the upregulation of mitochondrial genes in the early phase of ISO-induced cardiac hypertrophy was completely blunted when the disease progressed to cardiac fibrosis at 7 days. Confirming this, the expression of *Sirt1* ($p < 0.0001$), *PGC1*α ($p < 0.05$), and *Tfam* ($p < 0.001$) was significantly reduced in veh-ISO mice after 7 days compared with the veh-ISO group after 24 hours (Fig. 5A–C). Noteworthy, the expression of *PGC1*α in irisin-ISO mice at 7 days appeared to have an increasing trend compared with the veh-ISO group at the same time point (Fig. 5B), suggesting a potential stimulation of irisin on mitochondriogenesis independently of cardiac hypertrophy.

Discussion

In the present study, we demonstrated that treatment with systemic irisin prevents cardiac hypertrophy and fibrosis induced by acute stimulation with ISO. The fibrotic phenotype of the murine heart observed in vehicle-treated mice before ISO administration was not observed in irisin-treated mice (Fig. 6). This effect of irisin in inhibiting cardiac tissue fibrosis is also evidenced by its ability to maintain αSMA expression at control levels in both early and late phases post-ISO injection and to preserve normal mitochondria gene expression after the acute phase of heart injury.

Our present data demonstrate that irisin has a strong impact in preventing cardiac damage in a mouse model of myocardial fibrosis caused by high-dose ISO administration. In agreement with this, a recent study supported a significant protective effect of irisin on myocardial fibrosis in a mouse model of experimental myocardial infarction obtained by ligation of the left anterior descending coronary artery [27]. The authors demonstrated that FNDC5 knockout mice have increased myocardial collagen production and reduced cardiac function, indicating that FNDC5/irisin plays a key role in the heart [27]. As a matter of fact, they also showed that resistance exercise stimulated the endogenous production of irisin in the heart tissue, which inhibited cardiomyocyte apoptosis and alleviated myocardial fibrosis by decreasing the expression of αSMA, metalloproteases, and collagen type I and III [27].

The main cause of myocardial fibrosis is an imbalance in myocardial collagen homeostasis characterized by a pronounced accumulation of extracellular matrix proteins in the cardiac tissue leading to the onset of cardiac pathological conditions [28]. A key cellular event driving the fibrotic response following cardiomyocyte death is the differentiation of cardiac fibroblasts into secretory and contractile cells, termed myofibroblasts. Although the acquisition of the myofibroblast phenotype is physiological in some healing processes, the excessive myofibroblast activity, resulting in increased secretion and stiffening of the extracellular matrix, dramatically hampers the normal physiology of or-

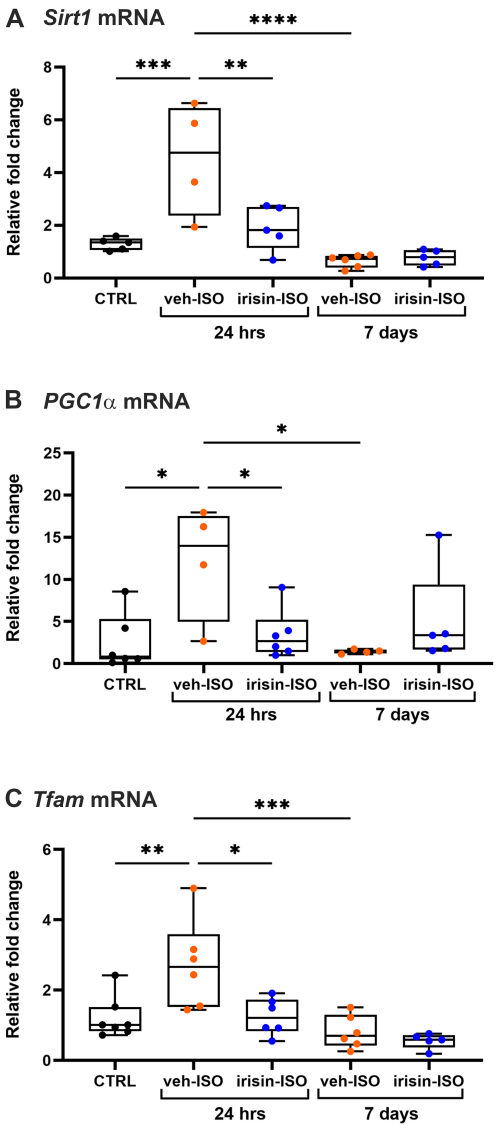

Fig. 5. Irisin prevents mitochondria dysregulation induced by cardiac hypertrophy. (A) Quantification of mRNA expression in heart tissue for *Sirt1* at different time points in veh-ISO (n = 4) and irisin-ISO (n = 5) mice 24 hours after ISO, and in veh-ISO (n = 6) and irisin-ISO (n = 5) mice 7 days after ISO, compared with CTRL (n = 5). (B) Quantification of mRNA expression in heart tissue for *PGC1α* at different time points in veh-ISO (n = 4) and irisin-ISO (n = 6) mice 24 hours after ISO, and in veh-ISO (n = 4) and irisin-ISO (n = 5) mice 7 days after ISO, compared with CTRL (n = 6). (C) Quantification of mRNA expression in heart tissue for *Tfam* at different time points in veh-ISO (n = 6) and irisin-ISO (n = 6) mice 24 hours after ISO, and in veh-ISO (n = 6) and irisin-ISO (n = 5) mice 7 days after ISO, compared with CTRL (n = 7). Shapiro-Wilk test followed by One Way ANOVA with Tukey's multiple comparisons tests were performed. Data are presented as box-and-whisker with median and interquartile ranges, from max to min, with all data points shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

gans whose functioning relies on contractility, such as in the heart [29]. Activated myofibroblasts are the main source of extracellular matrix proteins in fibrotic heart tissue [28]. The trans-differentiation from fibroblastic to myofibroblastic phenotype is routinely proved by the expression αSMA. Therefore, high expression of αSMA in cardiac tissue is considered a marker of cardiac fibrosis [29].

Our study revealed that irisin pretreatment prevented the ISO-induced 15-fold upregulation of *αSMA* in cardiac tissue that occurred in mice pretreated with vehicle. These results demonstrate that irisin completely inhibited differentiation toward the fibrotic phenotype, likely contributing to cardiomyocyte survival, as evidenced by irisin-mediated blockade of troponin I increase and *ANP* mRNA increase. Interestingly, the expression of *αSMA*, which remained elevated up to 7 days after ISO treatment, was still markedly maintained at control levels in irisin-pretreated mice, indicating that the effect of irisin persists even in a later phase after the acute event. In contrast, regarding *ANP* expression, our data showed that it returned to basal levels after 7 days following ISO administration. This temporal regulation of *ANP* was not unexpected and in line with previous studies demonstrating its increase in acute volume or pressure overload [30,31] as well as in chronic patterns of hypertension [32]. Specifically, it was shown that after ISO injection-induced myocardial infarction in rats, *ANP* mRNA gradually increased to its peak within 3 days after ISO injection [30].

Cardiomyocytes are highly metabolically demanding cells, using mitochondria as the primary source of energy [33]. In human studies, it has been reported that patients with heart failure have abnormal mitochondrial energetics [34]. *In vitro* studies in murine cardiomyocytes have shown that mitochondria can generate reactive oxygen species responsible for the hypertrophic effect of β-adrenergic agonists [35]. Our data showed that the expression of key mitochondrial genes, previously identified as controlled by irisin [24–26], namely *Sirt1*, *PGC1α*, and *Tfam*, were markedly increased 24 hours after ISO injection, however, this upregulation was not observed in mice pretreated with irisin. These data lead us to speculate that irisin pretreatment may have protected against the abnormal mitochondrial biogenesis caused by cardiac hypertrophy. It has been previously shown that to compensate for the abnormal generation of reactive oxygen species by mitochondria [36], PGC-1α becomes activated through SIRT1-mediated deacetylation to induce reactive oxygen species (ROS) detoxifying enzymes, such as glutathione peroxidase 1 and superoxide dismutase 2 [37]. Therefore, it is plausible to assume that the increased expression level of PGC-1α leads, in turn, to upregulation of its downstream transcription factor, Tfam, a key marker of mitochondriogenesis [38]. However, the significance of increased Tfam expression in heart disease is debated. Indeed, an elegant study has shown that overexpression of Tfam protects against fatal heart failure in

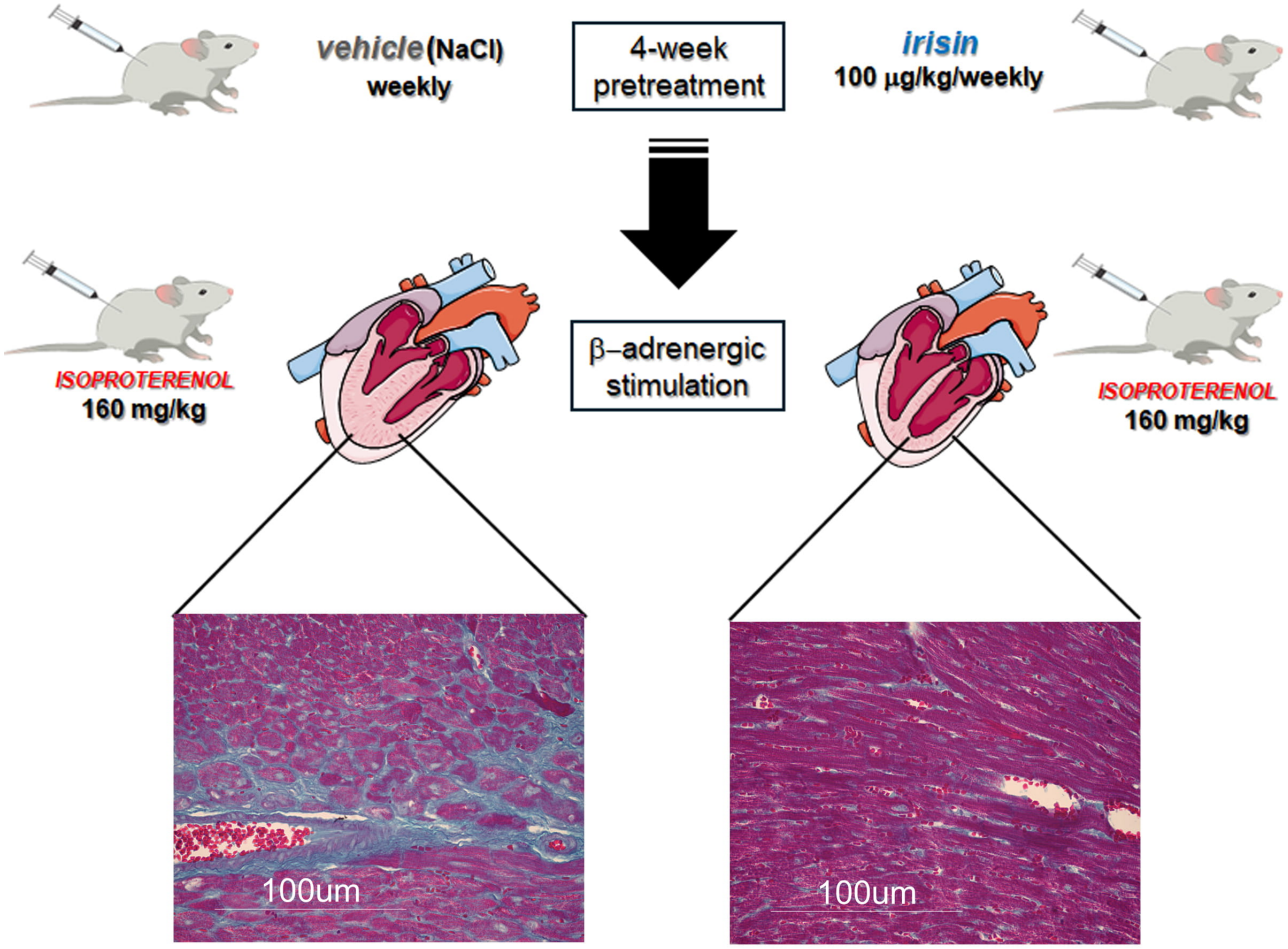

Fig. 6. Schematic representation of the effect of irisin in preventing cardiac fibrosis caused by acute isoproterenol stimulation. The figure was drawn in part using images from Servier Medical Art (https://smart.servier.com/), licensed under a Creative Commons Attribution 4.0 Unported License.

myocardial infarction. Although the increased Tfam expression would be indicative of increased numbers of mitochondria, this effect is not able to fully compensate for the damage to cardiac tissue in terms of hypertrophy and fibrosis [23]. In our study, we observed a rapid increase in the expression of *Sirt1*, *PGC1*α, and *Tfam* after administration with ISO, which did not occur in irisin-pretreated mice. This might suggest that irisin preserved the viability of cardiomyocytes, and thus also their mitochondrial integrity. However, a limitation of our study is that we did not provide a molecular mechanism by which irisin is able to prevent the increase in energy demand, hence *de novo* synthesis of mitochondria. We can only speculate that this is not necessary because the cardiac cells survived the acute ISO-induced insult. Moreover, another limitation of our study is that we have not quantified reactive oxygen species production in cardiac tissue after ISO treatment. This specific investigation could clarify *in vivo* if irisin blocks the production of reactive oxygen species, and hence the requirement for mitochondria, as previously observed in cardiomyocytes *in vitro* [9].

Conclusion

As shown in previous studies demonstrating that irisin could be a therapy for cardiac injury in the mouse model of transverse aortic constriction, the added value of our study is to demonstrate that pretreatment with irisin prevents cardiac fibrosis caused by isoproterenol administration. This mouse model offers, in addition to the advantage of not causing systemic inflammation that could be a confounding factor, the advantage of recapitulating the common condition of acute stress that leads to small cardiac necrosis, often silent and undiagnosable, which over time can lead to the fatal event of myocardial infarction. Future directions of research include the potential translation of irisin treatment in preventing cardiac fibrosis caused by β-adrenergic stimulation. A possible clinical application of our findings could lead to the use of exercise-mimetic irisin, especially for patients with cardiovascular disease who are unable to engage in physical activity.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

Conceptualization, CS, RZ, MG, and GC; methodology, CS, RZ, PP, MD, and AO; software, CS; validation, CS, RZ, MG, and GC; formal analysis, RZ and ST; resources, MG; data curation ADS, GC, and SC; statistical analysis and revision, LZ; writing—original draft preparation, CS, RZ and GC; writing—review and editing, MG; supervision, GC; funding acquisition, MG. All authors have been involved in the drafting and critical revision of the manuscript, and have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal study protocol was approved by the Italian Health Authority [12/2022-PR]. The Care and Husbandry of animals were performed in accordance with European Directives no. 2010/63 and with the Italian Regulatory system [D.L. vo n. 26, March 4th, 2014]. All parts of this study concerning animal care were approved by the Animal Welfare Agency [OPBA] of the University of Bari "Aldo Moro", Bari [Italy].

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Conflict of Interest

The authors declare no conflict of interest.

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