

Luteolin Protects Against Isoproterenol-Induced Cardiac Hypertrophy by Modulating AMPK/HO-1-Mediated Oxidative Stress and Inflammatory Responses

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Abstract

Luteolin is a naturally occurring flavonoid with antioxidant and anti-inflammatory properties; however, its molecular regulation of pathological cardiac hypertrophy remains incompletely understood. Here, we investigated the effects and mechanisms of luteolin in an isoproterenol (ISO)-induced model of cardiac hypertrophy using male Sprague-Dawley rats and H9c2 cardiomyoblasts. Luteolin treatment attenuated ISO-induced cardiac hypertrophy, ventricular wall thickening, cardiomyocyte enlargement, collagen deposition, and the expression of hypertrophic markers. In parallel, luteolin reduced inflammatory cytokine production and oxidative stress in both animal and cellular models. Mechanistic experiments demonstrated that luteolin restored AMP-activated protein kinase (AMPK) phosphorylation and enhanced heme oxygenase-1 (HO-1) expression. Pharmacological inhibition and siRNA-mediated silencing of AMPK or HO-1 partially abolished the protective effects of luteolin against hypertrophic responses, inflammation, and oxidative injury. Time-course analysis further indicated that AMPK activation preceded HO-1 induction, supporting a potential AMPK/HO-1 signaling cascade. Collectively, these findings reveal that luteolin protects against β -adrenergic stress-induced cardiac remodeling through regulation of inflammatory and oxidative responses and identify AMPK/HO-1 signaling as an important molecular mechanism underlying its cardioprotective effects.

1. Introduction

Cardiac hypertrophy is an adaptive response that initially helps maintain cardiac performance under physiological or pathological stress. Persistent exposure to pathological stimuli, however, including hypertension, aortic stenosis, and sustained neurohumoral activation, drives the transition to pathological cardiac hypertrophy (PCH).¹ Unlike physiological growth, PCH is characterized by maladaptive myocardial remodeling, increased cardiac mass, reactivation of the fetal gene program, and enhanced protein synthesis, ultimately contributing to cardiac dysfunction. As an important antecedent of heart failure and sudden cardiac death, PCH represents a substantial clinical burden.² Although numerous regulators of cardiac hypertrophy have been identified, the molecular networks that determine the progression from adaptive growth to maladaptive remodeling remain incompletely understood.

The pathogenesis of PCH reflects interacting inflammatory and oxidative processes. Activation of pro-inflammatory signaling and increased production of cytokines and chemokines contribute to pathological remodeling.^{3,4} Elevated levels of interleukin (IL)-6, IL-1 β , IL-18, and tumor necrosis factor- α (TNF- α) can aggravate cardiac hypertrophy and promote progression toward heart failure,^{5–7} and several anti-inflammatory interventions have shown antihypertrophic effects in experimental models.^{8–10} Oxidative stress is likewise a central feature of PCH, and antioxidant interventions have produced beneficial effects in multiple models of cardiac hypertrophy.^{8,11,12} Because inflammatory and redox pathways can reinforce one another during myocardial remodeling, bioactive compounds capable of modulating both processes are of particular mechanistic interest.

Luteolin (3',4',5,7-tetrahydroxyflavone) is a dietary flavone that occurs naturally in fruits, vegetables, and culinary herbs.¹³ As a food-derived bioactive polyphenol, luteolin has attracted interest because of its antioxidant and anti-inflammatory activities and its reported cardiovascular effects.^{13,14} However, its actions during sustained β -adrenergic stimulation, a well-established experimental trigger of hypertrophic remodeling, remain incompletely defined. Importantly, the oral bioavailability of luteolin is limited, and exposure to purified luteolin in experimental models cannot be assumed to represent habitual dietary intake.¹³ Mechanistic preclinical studies are therefore needed to establish biological plausibility while keeping dose-exposure and nutritional translation distinct.

AMP-activated protein kinase (AMPK) is a central cellular energy sensor and a major regulator of metabolic homeostasis.^{15,16} Beyond its metabolic functions, AMPK modulates inflammatory signaling and cellular redox balance.¹⁶ Heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme degradation, is an inducible cytoprotective protein with antioxidant and anti-inflammatory activities.^{17,18} Activation of AMPK/HO-1 signaling has been implicated in a range of protective biological responses.^{19,20} The present study therefore evaluated luteolin in an isoproterenol-induced rat model of cardiac hypertrophy and in H9c2 cardiomyoblasts and used pharmacological inhibition and small interfering RNA-mediated knockdown to assess the contribution and potential signaling relationship of AMPK and HO-1. By integrating an *in vivo* hypertrophic phenotype with cell-based pathway perturbation, we sought to provide mechanistic evidence relevant to the evaluation of luteolin as a food-derived bioactive compound while avoiding inference beyond the experimental exposure studied.

2. Materials and Methods

Materials

Isoproterenol (ISO; cat. no. HY-B0468), Cell Counting Kit-8 (CCK-8; cat. no. HY-K0301), Compound C (cat. no. HY-13418A), and zinc protoporphyrin IX (ZnPPIX; cat. no. HY-101193) were obtained from MedChemExpress (Monmouth Junction, NJ, USA). Luteolin (purity $\geq 99\%$; cat. no. L107412) was obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM; cat. no. 11965092) was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and fetal bovine serum (FBS; cat. no. 04-001-1A) was obtained from Biological Industries (Beit Haemek, Israel). The lactate dehydrogenase (LDH; cat. no. A020-2-2), malondialdehyde (MDA; cat. no. A003-1-2), and superoxide dismutase (SOD; cat. no. A001-3-2) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The dihydroethidium (DHE) assay kit (cat. no. G1023) was obtained from Servicebio (Wuhan, China). The dichlorodihydrofluorescein diacetate (DCFH-DA) reactive oxygen species assay kit (cat. no. S0033S), DAPI staining solution (cat. no. C1005), F-Actin-Tracker Green-488 (cat. no. C2201S), and enhanced bicinchoninic acid protein assay kit (cat. no. S0022) were obtained from Beyotime Institute of Biotechnology (Shanghai, China).

Stock solutions of luteolin, Compound C and ZnPPIX were prepared in dimethyl sulfoxide and stored at -80°C . The final dimethyl sulfoxide concentration was maintained below 0.1% (v/v) in all cell-culture

groups. ISO was freshly prepared in sterile water or phosphate-buffered saline before use.

Animals and experimental protocol

Male Sprague-Dawley rats (7–8 weeks old; mean body weight, approximately 200 g) were obtained from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). All animal procedures were approved by the Animal Care and Use Committee of Chongqing Medical University and were conducted in accordance with national and institutional guidelines for the care and use of laboratory animals. The rats were housed with ad libitum access to food and water under controlled conditions ($22 \pm 2^\circ\text{C}$; relative humidity, $55 \pm 5\%$; 12-h light/dark cycle). Thirty rats were randomly allocated to three groups ($n = 10/\text{group}$): control, ISO and ISO+luteolin. Rats in the ISO group received ISO (5 mg/kg/day, subcutaneously).^{21,22} Rats in the ISO+luteolin group received ISO at the same dose together with luteolin (100 mg/kg/day, by oral gavage).²³ Control rats received equivalent volumes of 0.9% saline by the corresponding routes. The concurrent preventive intervention was continued for 14 days.

The study was designed, conducted and reported in accordance with the ARRIVE 2.0 guidelines. The total duration of the in vivo experiment was 14 days, from the first ISO administration to the terminal procedure. The health and behavior of all animals were monitored at least once daily throughout this period by trained personnel, and body weight, food and water intake, posture, coat condition, respiratory pattern and spontaneous activity were recorded. Prespecified humane endpoints, at which an animal would have been euthanized before the scheduled end of the study, were as follows: loss of $> 20\%$ of the initial body weight; inability to access food or water; persistent labored or agonal respiration; sustained immobility or hypothermia with unresponsiveness to gentle stimulation; or any other sign of severe, unrelievable pain or distress as judged by the attending veterinarian.

A total of 30 rats were used ($n = 10$ per group). All 30 animals survived to the scheduled end of the 14-day intervention; no animal reached a humane endpoint, no animal was euthanized before the scheduled end point and no animal was found dead, and therefore no cause of unscheduled death is reportable. All 30 rats were euthanized at the scheduled end point as planned and all were included in the analysis; no animal and no data point was excluded. The group size ($n = 10$ per group) was based on previous studies that used the same ISO-induced cardiac hypertrophy model;^{21–23} no a priori statistical power calculation was performed.

Animal welfare measures included group housing under the controlled environmental conditions described above with nesting and enrichment material, ad libitum access to standard chow and water, and an acclimatization period of at least 7 days before the start of the experiment. Subcutaneous injection and oral gavage were performed by trained personnel using appropriately sized needles and flexible gavage tubes in order to minimize handling stress and discomfort, and the doses and the 14-day treatment duration were kept to the minimum required to establish the hypertrophic phenotype. No surgical procedure was performed and no procedure was expected to cause more than transient

discomfort; analgesics were therefore not administered. All procedures that could cause discomfort, including echocardiography and the terminal procedure, were performed under anesthesia.

At the end of the 14-day intervention, and immediately after the echocardiographic examination, all rats received an intraperitoneal injection of an overdose of sodium pentobarbital (150 mg/kg). Once a deep surgical plane of anesthesia had been confirmed by the loss of the corneal and pedal withdrawal reflexes and by the absence of any response to a firm toe pinch, the thorax was opened and terminal blood collection followed by exsanguination was performed by cardiac puncture as a secondary physical method, after which the heart was rapidly excised. Death was subsequently confirmed by the permanent cessation of the heartbeat and of spontaneous respiration, together with exsanguination and the removal of vital organs.

Echocardiography

At the end of the 14-day intervention, rats were anesthetized with isoflurane in oxygen (4% for induction in a chamber and 2% for maintenance via a nose cone) and placed in the supine position on a heated platform; the depth of anesthesia was monitored by loss of the pedal withdrawal reflex. Transthoracic echocardiography was performed using a Vevo 3100 LT Imaging System equipped with a 21-MHz transducer (FUJIFILM VisualSonics, Inc., Toronto, ON, Canada). Left ventricular anterior wall thickness, left ventricular posterior wall thickness and left ventricular internal diameter were measured during diastole and systole from M-mode images.²⁴ Echocardiographic acquisition and analysis were performed by experienced investigators who were blinded to group allocation. Each measurement was averaged over three consecutive cardiac cycles.

Heart weight-to-body weight ratio

Blood samples and heart tissues were collected during the terminal procedure described above.²⁴ Hearts were rinsed with freshly prepared phosphate-buffered saline, blotted dry and weighed. The heart weight-to-body weight ratio (mg/g) was calculated as an index of cardiac hypertrophy.

H9c2 cell culture and treatment

Rat H9c2 cardiomyoblasts were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂. For experiments, cells were cultured for 24 h and then serum-starved in DMEM for 4 h. Cells were subsequently treated with ISO (10 µM), luteolin (10 µM), Compound C (25 µM) and/or ZnPPiX (10 µM), as indicated, for 24 h. For gene-silencing experiments, cells were transiently transfected with siRNAs targeting AMPK or HO-1 using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The siRNAs were synthesized by Beijing Tsingke Biotechnology Co., Ltd. (Beijing, China).²⁵ The siAMPK sequences were 5'-AAGUCAAACAGCUCUAUCGUTT-3' (sense) and 5'-ACGAUAGAGCUGUUUGAACUUTT-3' (antisense). The siHO-1 sequences were 5'-GGAGGUGAAUUGUUCGACUTT-3' (sense) and 5'-

AGUCGAACAAUUCACCUCCTT-3' (antisense). Knockdown efficiency was verified by western blot analysis 48 h after transfection (Figure S1a and b). AICAR (cat. no. HY-13417; MedChemExpress), a canonical AMP-activated protein kinase activator, was used at 1 mM as a pharmacological positive control and was added together with ISO for 24 h. For the time-course experiments, cells were treated with luteolin alone and harvested at 0, 0.5, 1, 2, 4, 8, 12 and 24 h for western blot analysis of phosphorylated AMPK, total AMPK and HO-1. Three or more independent experiments were performed for each condition.

CCK-8 assay

H9c2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well, cultured for 24 h and treated as described above.²⁵ Following 24 h of treatment, 10 μ L of CCK-8 reagent was added to each well, and the plates were incubated for 2 h at 37°C in the dark. Absorbance at 450 nm was measured using a microplate spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Histological analysis

Left ventricular tissues were collected during the terminal procedure described above, rinsed with cold phosphate-buffered saline and fixed in 4% neutral-buffered formaldehyde for 24 h before paraffin embedding. Sections (4 μ m) were stained with hematoxylin and eosin to evaluate myocardial morphology. Masson's trichrome staining was performed to assess collagen deposition. Five randomly selected fields from five sections per heart were examined using a microscope (Nikon Corporation, Tokyo, Japan).

Wheat germ agglutinin staining

Paraffin-embedded sections were deparaffinized and rehydrated before incubation with wheat germ agglutinin (5 μ g/mL) for 1 h at 37°C in a humidified, light-protected chamber. The sections were washed three times with phosphate-buffered saline and counterstained with DAPI. At least five randomly selected fields per specimen were imaged using a fluorescence microscope (Nikon Corporation, Tokyo, Japan) and analyzed.

Measurement of reactive oxygen species

Fresh heart tissues were embedded in optimal cutting temperature compound and cut into 8- μ m cryosections. The sections were incubated with DHE (10 μ M) for 20 min at 37°C in a humidified, light-protected chamber, washed twice with phosphate-buffered saline and counterstained with DAPI. Whole-slide images were acquired using a Panoramic MIDI fluorescence slide scanner (3DHISTECH Ltd., Budapest, Hungary) equipped with a Plan-Apochromat 20 $\times$ objective at a level-0 spatial resolution of 0.27381 μ m/pixel and were stored in MRXS format; the SpOrange (DHE) channel was acquired at 8-bit depth using an exposure time of 80 ms, an excitation intensity of 100% and a digital gain of 0, and flat-field correction and intensity compensation were applied automatically by the scanner during acquisition. Myocardial DHE fluorescence was quantified from original level-0 MRXS whole-slide images. DAPI-positive nuclear regions were defined using a fixed threshold of > 40/255, applied identically to all

samples, and mean DHE fluorescence intensity was measured within the nuclear mask across the whole tissue section. No DHE thresholding or background subtraction was performed. Representative fields were selected objectively as regions with nuclear DHE fluorescence intensity closest to the corresponding whole-slide mean and were confined to a single original camera field of view to avoid stitching boundaries. Uniform linear brightness/contrast adjustment was used only for figure presentation.

Measurement of LDH, SOD and MDA

LDH, SOD and MDA levels were measured using the corresponding commercial assay kits according to the manufacturers' instructions.²⁶ Absorbance was measured using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Enzyme-linked immunosorbent assay

The concentrations of IL-1 β , IL-6 and TNF- α in serum and cell-culture supernatants were measured using the corresponding enzyme-linked immunosorbent assay kits according to the manufacturers' instructions. Absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Measurement of H9c2 cell surface area

At the end of treatment, H9c2 cells were washed three times with phosphate-buffered saline, fixed with 4% neutral-buffered formaldehyde on ice for 10 min and permeabilized with 1% Triton X-100 at 37°C for 10 min. Following three additional washes, the cells were incubated with F-Actin-Tracker Green-488 (5 μ g/mL) for 20 min at 37°C in a humidified, light-protected chamber and counterstained with DAPI. Images were acquired using a fluorescence microscope (Nikon Corporation, Tokyo, Japan). Cell surface area was quantified using ImageJ software in at least 50 cells randomly selected from five fields per specimen.

Western blot analysis

Total protein was extracted from heart tissues or cells using radioimmunoprecipitation assay buffer supplemented with phenylmethylsulfonyl fluoride. Protein concentrations were determined using the bicinchoninic acid assay. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 for 2 h and incubated overnight at 4°C with primary antibodies against GAPDH, β -actin, ANP, BNP, β -MHC, AMPK, phosphorylated AMPK and HO-1. The membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (Beyotime Institute of Biotechnology, Shanghai, China),²⁷ and band densities were quantified using ImageJ software.

Reverse transcription-quantitative PCR

Total RNA was extracted from heart tissues and cells using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).²⁸ cDNA was synthesized using the PrimeScript Reverse Transcription Kit, and quantitative PCR was performed using SYBR Premix Ex Taq (Takara Bio, Inc., Shiga, Japan). The real-time PCR primers are shown in Supplementary Table S1. Reactions were performed in triplicate in 96-well plates for 40 cycles at 95°C for 15 sec and 60°C for 60 sec.²⁹ Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.3 (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the mean $\pm$ SD. For in vivo studies, the individual animal was considered the experimental unit; for in vitro studies, an independently performed cell-culture experiment was considered the experimental unit. Normality and homogeneity of variance were assessed using the Shapiro-Wilk and Brown-Forsythe tests, respectively. Comparisons among three or more groups were performed using one-way ANOVA followed by Tukey's multiple-comparisons test when the assumptions were satisfied. Appropriate non-parametric tests were used when these assumptions were not met. Exact P values are reported where practicable, and $P < 0.05$ was considered to indicate a statistically significant difference. No data were excluded unless a prespecified technical exclusion criterion was met.

3. Results

Luteolin attenuates ISO-induced cardiac hypertrophy

After 14 days of ISO administration, heart size and the heart weight-to-body weight ratio were significantly increased compared with the control group. Both changes were attenuated by luteolin treatment (Fig. 1a and b). Echocardiographic assessment showed that luteolin reduced the ISO-induced increases in left ventricular anterior and posterior wall thickness and partially restored left ventricular internal diameter (Fig. 1c-i).

Wheat germ agglutinin staining demonstrated a significant increase in cardiomyocyte cross-sectional area in ISO-treated rats compared with controls, which was attenuated by luteolin (Fig. 2a and c). Hematoxylin and eosin staining and Masson's trichrome staining were used to evaluate myocardial morphology and collagen deposition, respectively (Fig. 2b). Luteolin ameliorated ISO-induced interstitial congestion, myocardial edema, myofiber disruption and collagen deposition (Fig. 2b and d). Consistent with these histological findings, ISO increased the protein and mRNA expression levels of the hypertrophic markers ANP, BNP and β -MHC, whereas luteolin significantly reduced their expression (Fig. 2e-k).

Luteolin attenuates inflammation and oxidative stress in ISO-treated rats

Serum IL-1 β , IL-6 and TNF- α levels were significantly increased in the ISO group compared with the control group. Luteolin treatment significantly reduced the circulating concentrations of all three cytokines (Fig. 3a-c).

DHE fluorescence was increased in myocardial tissue from ISO-treated rats, indicating enhanced reactive oxygen species generation; this increase was attenuated by luteolin (Fig. 3d and e). ISO also increased MDA and LDH levels and reduced SOD activity, whereas luteolin partially normalized these indices (Fig. 3f-h). In addition, myocardial AMPK phosphorylation and HO-1 protein expression were reduced following ISO treatment and restored by luteolin (Fig. 3i-k).

Luteolin attenuates ISO-induced hypertrophy in H9c2 cells

Luteolin at concentrations of 5, 10 and 20 μ M did not significantly affect H9c2 cell viability, whereas 50 and 100 μ M luteolin significantly reduced viability (Fig. 4a). In ISO-treated cells, 10 and 20 μ M luteolin produced comparable effects on viability and were more protective than 5 μ M luteolin (Fig. 4b). Therefore, 10 μ M luteolin was selected for subsequent experiments.

Exposure to ISO for 24 h increased H9c2 cell surface area, whereas luteolin significantly attenuated this hypertrophic response (Fig. 4c and d). ISO also increased the protein and mRNA expression levels of ANP, BNP and β -MHC, and these changes were significantly reduced by luteolin treatment (Fig. 4e-k).

Luteolin attenuates inflammatory mediator release and oxidative stress in ISO-treated H9c2 cells

Exposure to ISO for 24 h increased IL-1 β , IL-6 and TNF- α levels in the cell-culture supernatant, whereas luteolin significantly reduced the release of these inflammatory mediators (Fig. 5a-c). ISO also increased intracellular reactive oxygen species, LDH release and MDA levels and reduced SOD activity; luteolin attenuated each of these changes (Fig. 5d-h). Furthermore, ISO reduced AMPK phosphorylation and HO-1 expression, both of which were restored by luteolin (Fig. 5i-k).

Luteolin sequentially activates AMPK and induces HO-1, and a pharmacological AMPK activator reproduces the protective effects of luteolin

To clarify the temporal relationship between AMPK activation and HO-1 induction, H9c2 cells were treated with luteolin (10 μ M) and analyzed over a 24-h time course. AMPK phosphorylation increased progressively, becoming significantly elevated above baseline within 30 min and reaching a maximum at approximately 4 h; thereafter it declined slowly but remained significantly higher than baseline at 24 h. In contrast, HO-1 protein accumulated more slowly, showing no significant change at 0.5 or 1 h, becoming significantly elevated from 2 h onwards and peaking at approximately 12 h (Figure S1c-e). This temporal dissociation, in which the rise in AMPK phosphorylation both begins and peaks before HO-1 induction, is consistent with AMPK acting upstream of HO-1.

To determine whether direct pharmacological AMPK activation is sufficient to reproduce the effects of luteolin and to provide a positive control for the pathway, ISO-treated H9c2 cells were exposed to the

canonical AMPK activator AICAR (1 mM). AICAR reduced the protein and mRNA expression of ANP, BNP and β -MHC, increased AMPK phosphorylation and HO-1 expression, and attenuated the release of IL-1 β , IL-6 and TNF- α as well as reactive oxygen species accumulation and MDA levels, while restoring SOD activity. The magnitude of these effects was comparable to that of luteolin, with no significant difference between the luteolin and AICAR groups for any measured endpoint (Figure S2). These findings indicate that AMPK activation is sufficient to attenuate ISO-induced hypertrophy, inflammation and oxidative stress in H9c2 cells, and establish a pharmacological positive control for the AMPK/HO-1 pathway.

Pharmacological inhibition of AMPK or HO-1 partially attenuates the effects of luteolin

To evaluate the involvement of AMPK/HO-1 signaling, H9c2 cells were treated with Compound C or ZnPPiX together with luteolin and ISO. Dose-response experiments showed that 25 μ M Compound C and 10 μ M ZnPPiX did not significantly affect the viability of ISO-treated H9c2 cells; these concentrations were therefore used in subsequent experiments (Fig. 6a and b).

Compound C and ZnPPiX partially reversed the luteolin-mediated reductions in cell surface area and the protein and mRNA expression levels of hypertrophic markers (Fig. 6c-k). Western blot analysis confirmed efficient gene silencing: compared with the corresponding untransfected control, transfection with si-AMPK markedly reduced AMPK protein expression and transfection with si-HO-1 markedly reduced HO-1 protein expression in H9c2 cells (Figure S1a and b). Consistent with the pharmacological findings, siRNA-mediated knockdown of AMPK or HO-1 partially attenuated the protective effects of luteolin in ISO-treated H9c2 cells (Figure S3).

Compound C and ZnPPiX also partially reversed the effects of luteolin on IL-1 β , IL-6, TNF- α , LDH and MDA levels, reactive oxygen species accumulation and SOD activity (Fig. 7a-h). Compound C suppressed luteolin-induced increases in both AMPK phosphorylation and HO-1 expression, whereas ZnPPiX reduced HO-1 expression without altering AMPK phosphorylation (Fig. 7i-k). Similarly, siRNA-mediated knockdown of AMPK or HO-1 partially attenuated the anti-inflammatory and antioxidant effects of luteolin (Figure S4). These findings support the involvement of AMPK/HO-1 signaling in the cellular effects of luteolin against ISO-induced hypertrophy, inflammation and oxidative stress.

4. Discussion

The present study demonstrated that luteolin attenuated ISO-induced cardiac hypertrophy in rats and reduced hypertrophic growth in H9c2 cardiomyoblasts. These effects were accompanied by lower circulating pro-inflammatory cytokine levels, reduced oxidative injury and restoration of AMPK phosphorylation and HO-1 expression. In H9c2 cells, pharmacological inhibition or siRNA-mediated knockdown of AMPK or HO-1 partially attenuated the antihypertrophic, anti-inflammatory and antioxidant effects of luteolin. Moreover, inhibition of AMPK reduced HO-1 expression, whereas inhibition of HO-1 did not alter AMPK phosphorylation, supporting a potential AMPK-to-HO-1 signaling relationship.

Pathological cardiac hypertrophy is characterized by increased cardiomyocyte size, reactivation of fetal gene expression and progressive structural remodeling.^{30,31} ANP and BNP are established markers of myocardial stress, while β -adrenergic stimulation and pressure overload induce β -MHC expression as part of the fetal gene program.^{32–34} In the present study, luteolin reduced cardiomyocyte size, collagen deposition and the expression of ANP, BNP and β -MHC in vivo and in vitro. These findings indicate that luteolin limits multiple features of the hypertrophic phenotype under sustained β -adrenergic stimulation.

Oxidative stress is a major contributor to pathological cardiac remodeling. Excessive production of reactive oxygen and nitrogen species can activate hypertrophic kinases and transcription factors, promote lipid peroxidation and impair cardiomyocyte function.^{35–42} Consistent with this concept, ISO increased reactive oxygen species generation, MDA and LDH levels and reduced SOD activity in the present study. Luteolin attenuated these changes in both the rat model and H9c2 cells. The partial loss of these antioxidant effects following AMPK or HO-1 inhibition further supports the involvement of this pathway in the redox-regulatory actions of luteolin.

Inflammatory signaling also contributes to the development and progression of cardiac hypertrophy.⁴³ Cardiomyocytes and non-myocyte populations can release IL-1 β , IL-6, IL-18 and TNF- α in response to pathological stress, thereby promoting myocardial remodeling and functional deterioration.^{44–46} In the present study, luteolin reduced circulating cytokine levels in ISO-treated rats and decreased inflammatory mediator release from ISO-treated H9c2 cells. These effects were partially attenuated by pharmacological inhibition or siRNA-mediated knockdown of AMPK or HO-1, suggesting that AMPK/HO-1 signaling contributes to the anti-inflammatory effects of luteolin.

AMPK regulates cellular energy balance, mitochondrial function, redox homeostasis and inflammatory signaling.^{47–51} HO-1 is an inducible cytoprotective enzyme that limits oxidative and inflammatory injury and may function downstream of AMPK, potentially through nuclear factor erythroid 2-related factor 2-dependent transcription.^{52–56} In the present study, ISO reduced AMPK phosphorylation and HO-1 expression, whereas luteolin restored both. Compound C suppressed luteolin-induced HO-1 upregulation, while ZnPPiX did not affect AMPK phosphorylation. Together with the siRNA data, these observations support, but do not definitively establish, a signaling hierarchy in which AMPK acts upstream of HO-1. Rescue experiments and direct assessment of nuclear factor erythroid 2-related factor 2 activation will be required to confirm this relationship.

Consistent with this hierarchy, a time-course analysis showed that luteolin induced AMPK phosphorylation before HO-1 accumulation (Figure S1c-e), and the AMPK activator AICAR, used as a pharmacological positive control, reproduced the antihypertrophic, anti-inflammatory and antioxidant effects of luteolin and comparably increased AMPK phosphorylation and HO-1 expression (Figure S2). Nevertheless, this evidence remains associative. We did not demonstrate direct binding of luteolin to AMPK or to an upstream kinase such as liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase β (CaMKK β), and we cannot exclude that the effects of luteolin arise, in part, from direct reactive oxygen species scavenging or from parallel activation of nuclear factor erythroid 2-related factor

2 or SIRT1 signaling, each of which can independently induce HO-1 and modulate cardiomyocyte redox and inflammatory status. It also remains possible that HO-1 and its downstream products feed back to influence AMPK activity under some conditions. Definitive establishment of the pathway will therefore require direct target-engagement studies together with epistasis or rescue experiments.

Inflammation and oxidative stress can reinforce one another during pathological remodeling. Pro-inflammatory cytokines may stimulate NADPH oxidase-dependent reactive oxygen species generation, whereas oxidative stress can further amplify inflammatory signaling.^{57–61} The concurrent reductions in inflammatory mediators and oxidative injury observed with luteolin are therefore biologically plausible and may jointly contribute to attenuation of the hypertrophic response. Nevertheless, the present data do not establish whether one process is primary or whether the effects arise exclusively from cardiomyocytes rather than other cardiac cell types.

Several limitations should be acknowledged. First, H9c2 cardiomyoblasts do not fully reproduce the phenotype of adult cardiomyocytes, contractile apparatus and hypertrophic signaling; accordingly, our in vitro findings should be interpreted as a cardiomyoblast model of the hypertrophic response rather than as definitive evidence of adult cardiomyocyte behavior, and where we refer to cardiomyocyte hypertrophy in the context of H9c2 experiments this qualification applies. Second, all pathway-perturbation experiments, including Compound C, ZnPPiX, siRNA knockdown, the AICAR positive control and the time-course analysis, were performed exclusively in H9c2 cells; the in vivo data therefore remain correlative, and our study does not establish that the in vivo effects of luteolin depend on AMPK/HO-1 signaling. Future work incorporating cardiomyocyte-specific genetic or pharmacological manipulation of AMPK or HO-1 in vivo will be required to confirm the mechanism at the whole-animal level. Third, only male rats were studied, in keeping with prior isoproterenol models and to limit the confounding influence of estrous-cycle-related hormonal fluctuations; however, because pathological cardiac hypertrophy also affects females and because sex hormones can modulate both AMPK and HO-1 signaling, our findings may not generalize to females, and dedicated sex-balanced studies are needed. Fourth, the AICAR positive control was applied only in H9c2 cells and was not validated in vivo; no positive-control group was included in the rat experiments, and the sufficiency of direct pharmacological AMPK activation to attenuate ISO-induced cardiac hypertrophy therefore remains to be confirmed at the whole-animal level. We consider these open questions important priorities for follow-up investigation.

Conclusion

Luteolin attenuated isoproterenol-induced cardiac hypertrophy in rats and H9c2 cardiomyoblasts by suppressing inflammatory responses and oxidative stress. The beneficial effects of luteolin were associated with restoration of AMPK phosphorylation and HO-1 expression, and pathway intervention experiments supported the involvement of AMPK/HO-1 signaling in these protective responses. These findings provide new mechanistic insights into the regulation of cardiac remodeling by a bioactive flavonoid and suggest that modulation of cellular stress-response pathways contributes to the biological actions of luteolin.

Luteolin attenuated isoproterenol-induced cardiac hypertrophy in rats and hypertrophic responses in H9c2 cardiomyoblasts, accompanied by lower inflammatory and oxidative stress indices and restoration of AMPK phosphorylation and HO-1 expression. Pharmacological inhibition and small interfering RNA-mediated knockdown in H9c2 cells support, but do not establish in vivo, a functional contribution of AMPK/HO-1 signaling to these effects. From a food and nutrition perspective, these findings provide mechanistic preclinical evidence for luteolin as a dietary bioactive with potential relevance to cardiac remodeling. However, the experimental exposure should not be interpreted as equivalent to habitual dietary intake. In vivo pathway validation, dose-exposure and bioavailability studies, sex-balanced experiments, and more physiologically relevant cardiomyocyte models are needed before nutritional or clinical implications can be defined.

Abbreviations

AICAR

5-aminoimidazole-4-carboxamide ribonucleoside

AMPK

AMP-activated protein kinase

ANP

atrial natriuretic peptide

BCA

bicinchoninic acid

BNP

B-type natriuretic peptide

β -MHC

β -myosin heavy chain

CaMKK β

calcium/calmodulin-dependent protein kinase kinase β

CCK-8

Cell Counting Kit-8

DAPI

4',6-diamidino-2-phenylindole

DCFH-DA

dichlorodihydrofluorescein diacetate

DHE

dihydroethidium

DMEM

Dulbecco's modified Eagle's medium

ELISA

enzyme-linked immunosorbent assay

FBS

fetal bovine serum
GAPDH
glyceraldehyde-3-phosphate dehydrogenase
H&E
hematoxylin and eosin
HO-1
heme oxygenase-1
HW/BW
heart weight-to-body weight ratio
IL
interleukin
ISO
isoproterenol
LDH
lactate dehydrogenase
LKB1
liver kinase B1
LVAW
left ventricular anterior wall thickness
LVID
left ventricular internal diameter
LVPW
left ventricular posterior wall thickness
MDA
malondialdehyde
NADPH
nicotinamide adenine dinucleotide phosphate
NF- κ B
nuclear factor- κ B
Nrf2
nuclear factor erythroid 2-related factor 2
PBS
phosphate-buffered saline
PCH
pathological cardiac hypertrophy
ROS
reactive oxygen species
RT-qPCR
reverse transcription-quantitative PCR
SD

standard deviation
siRNA
small interfering RNA
SIRT1
sirtuin 1
SOD
superoxide dismutase
TNF- α
tumor necrosis factor- α .

Declarations

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Statements

Conflict of Interest: The authors declare that they have no conflicts of interest.

Ethical Review: All animal procedures were reviewed and approved by the Animal Care and Use Committee of Chongqing Medical University (Approval No. IACUC-CQMU-2021-1885). The procedures were conducted in accordance with applicable national and institutional guidelines for the care and use of laboratory animals. The study was designed and reported in accordance with the ARRIVE 2.0 guidelines, and a completed ARRIVE checklist accompanies the submission.

Informed Consent: Not applicable. The study did not involve human participants or identifiable human data.

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AI Use Disclosure

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Figures

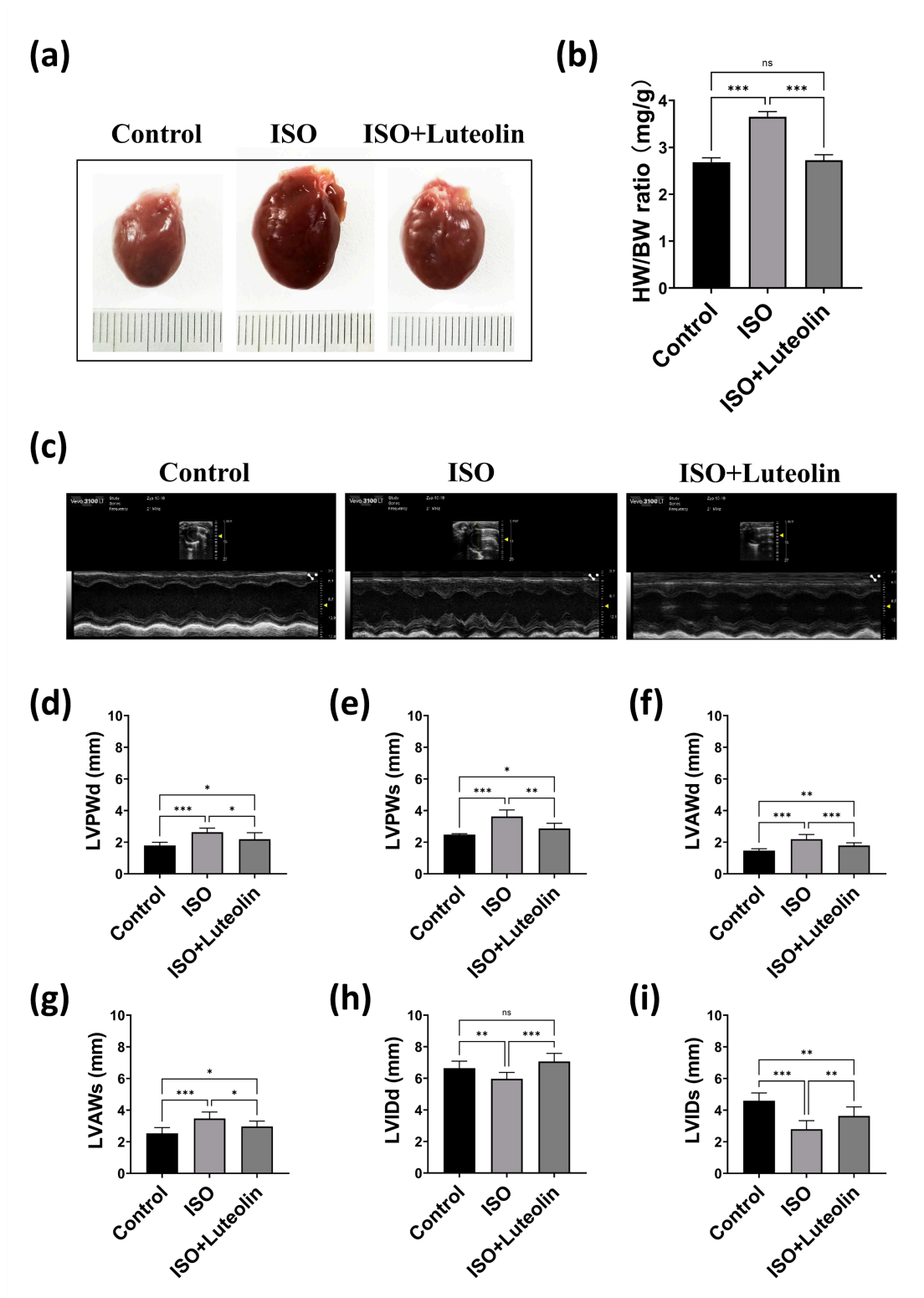


Figure 1

Luteolin attenuates ISO-induced cardiac hypertrophy in rats. (a) Representative gross images of the hearts. (b) Heart weight-to-body weight ratio. (c) Representative M-mode echocardiographic images. (d and e) Left ventricular posterior wall thickness at end-diastole (LVPWd) and end-systole (LVPWs). (f and g) Left ventricular anterior wall thickness at end-diastole (LVAWd) and end-systole (LVAWs). (h and i) Left ventricular internal diameter at end-diastole (LVIDd) and end-systole (LVIDs). To facilitate direct

comparison, panels (d-i) are plotted on a common vertical-axis scale (n = 10/group). Data are presented as the mean $\pm$ SD. ns, not significant; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

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Figure 2

Effects of luteolin on myocardial morphology and hypertrophic markers.(a) Representative wheat germ agglutinin-stained sections. (b) Representative hematoxylin and eosin- and Masson's trichrome-stained sections. Red arrows indicate interstitial congestion, blue arrows indicate myocardial edema and yellow arrows indicate myofiber disruption. (c) Cardiomyocyte cross-sectional area. (d) Collagen volume fraction. (e-k) Protein and mRNA expression levels of the hypertrophic markers ANP, BNP and β -MHC (n = 10/group). Data are presented as the mean $\pm$ SD. *** $P < 0.001$.

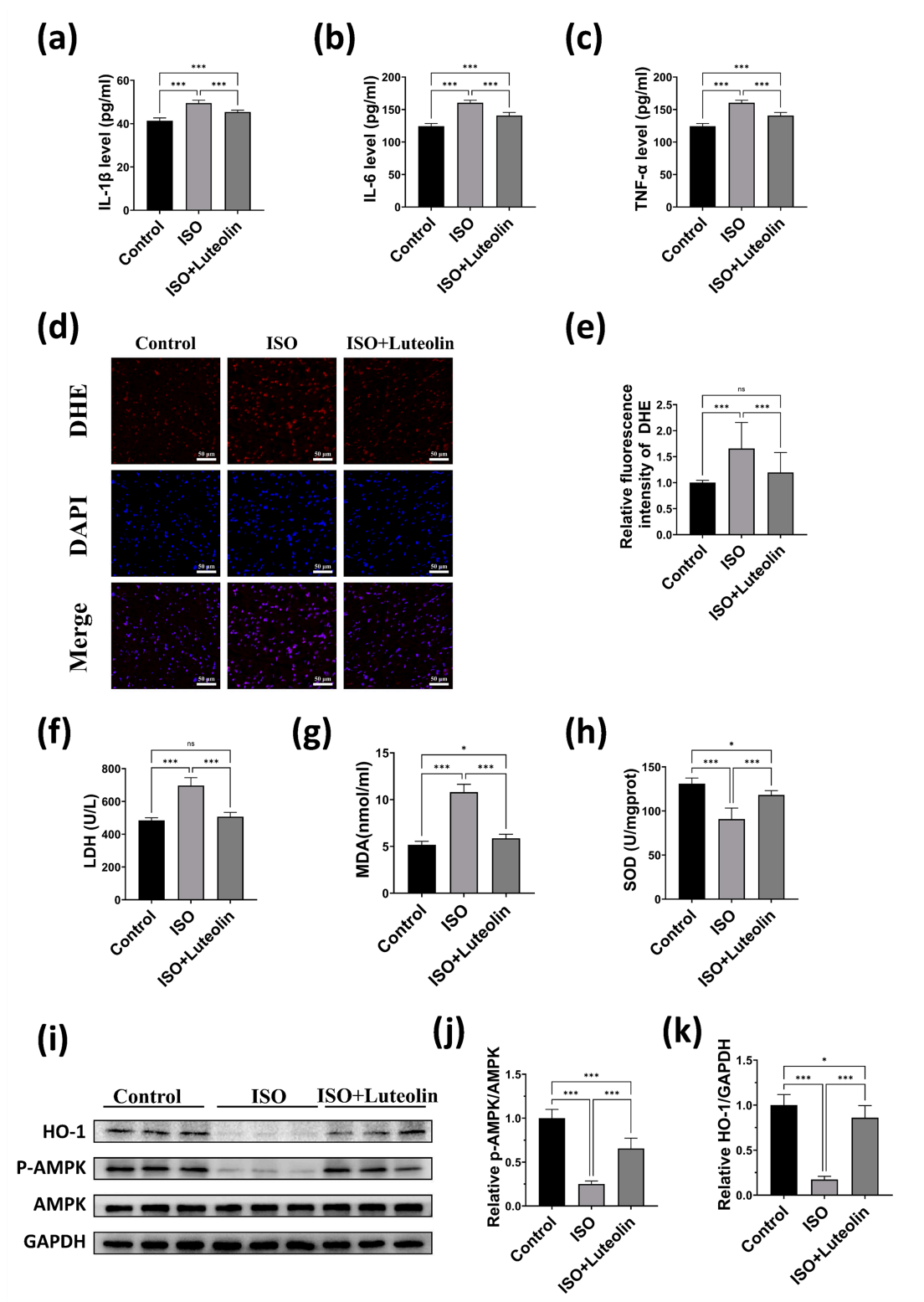


Figure 3

Luteolin attenuates inflammation and oxidative stress in ISO-treated rats. (a-c) Serum IL-1 β , IL-6 and TNF- α levels measured by enzyme-linked immunosorbent assay. (d) Representative DHE fluorescence images of myocardial reactive oxygen species. (e) Quantification of DHE fluorescence intensity. (f-h) Serum LDH and MDA levels and SOD activity. (i) Representative western blots of HO-1, phosphorylated

AMPK and total AMPK. (j and k) Quantification of phosphorylated AMPK/AMPK and HO-1/GAPDH (n = 10/group). Data are presented as the mean $\pm$ SD. ns, not significant; * $P < 0.05$ and *** $P < 0.001$.

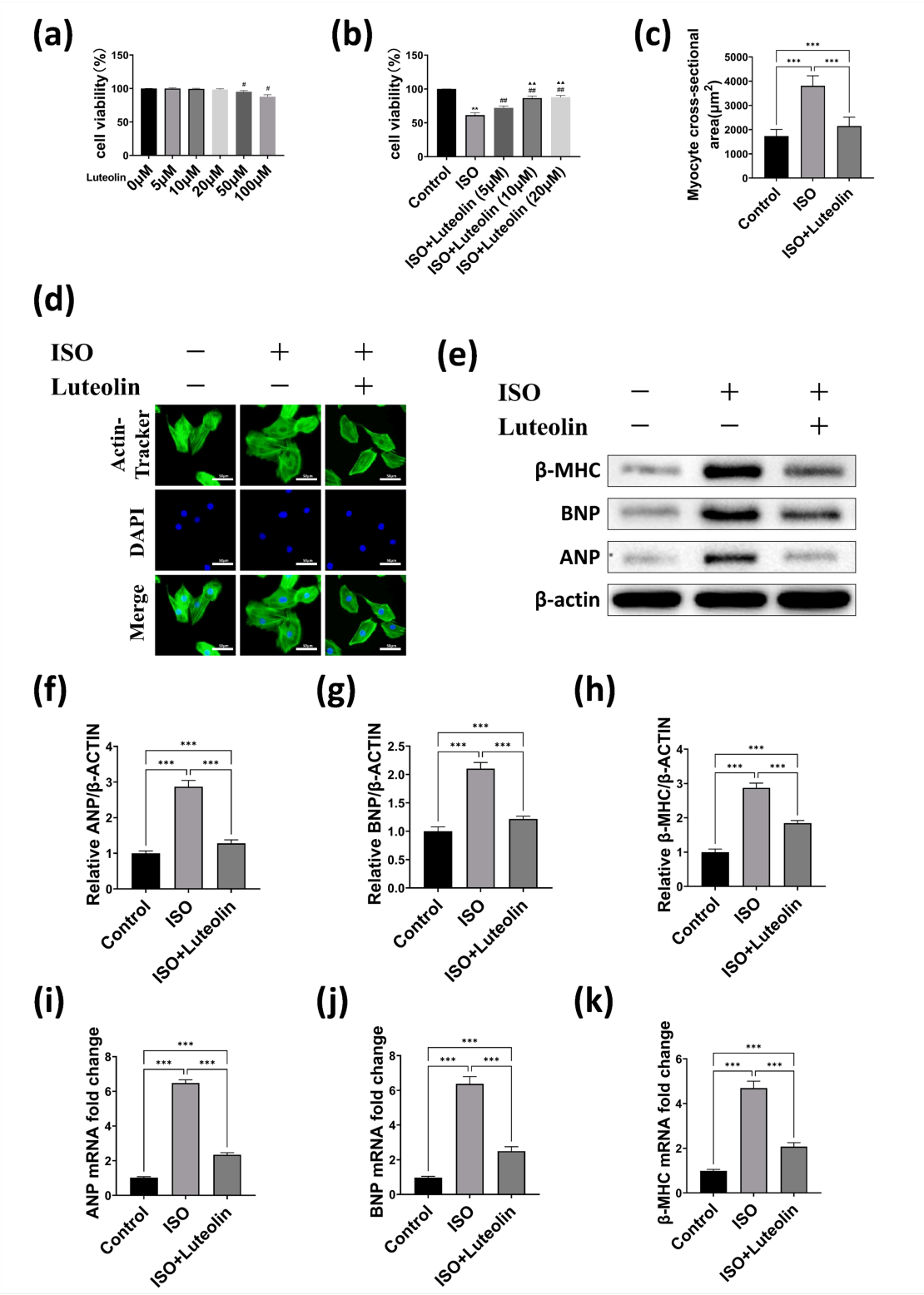


Figure 4

Luteolin attenuates ISO-induced hypertrophy in H9c2 cells.

(a and b) CCK-8 assays used to select the luteolin concentration. (c) Quantification of H9c2 cell surface area. (d) Representative F-actin fluorescence images. (e) Representative western blots of ANP, BNP and β -MHC. (f-h) Quantification of ANP/GAPDH, BNP/GAPDH and β -MHC/GAPDH. (i-k) Relative mRNA expression levels of ANP, BNP and β -MHC (n = 10 independent experiments). Data are presented as the mean $\pm$ SD. ns, not significant; * P < 0.05 and *** P < 0.001. # P < 0.05 vs. 0 μ M group (in a). ** P < 0.01 vs. control group (in b). ## P < 0.01 vs. ISO group (in b). $\blacktriangle$ P < 0.01 vs. ISO+luteolin (20 μ M) group (in b).

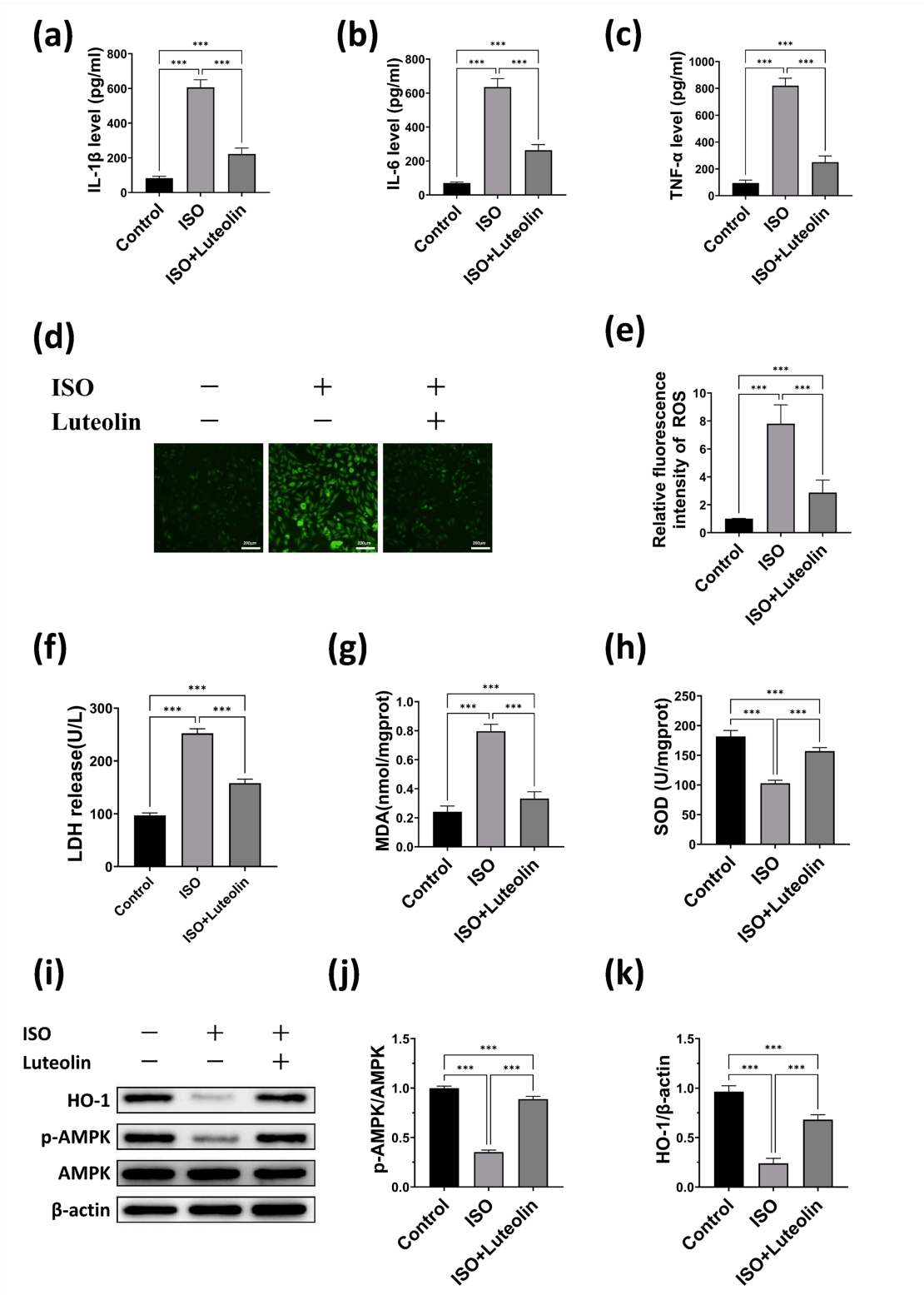


Figure 5

Luteolin attenuates inflammatory mediator release and oxidative stress in ISO-treated H9c2 cells.

(a-c) IL-1 β , IL-6 and TNF- α levels in the cell-culture supernatant measured by enzyme-linked immunosorbent assay. (d) Representative DCFH-DA fluorescence images. (e) Quantification of relative reactive oxygen species fluorescence intensity (n = 6 independent experiments). (f) LDH levels in the cell-culture supernatant. (g) Intracellular MDA levels. (h) Intracellular SOD activity. (i) Representative western blots of phosphorylated AMPK, total AMPK, HO-1 and β -actin. (j and k) Quantification of phosphorylated AMPK/AMPK and HO-1/ β -actin (n = 10 independent experiments unless otherwise indicated). Data are presented as the mean $\pm$ SD. ** P < 0.01 and *** P < 0.001.

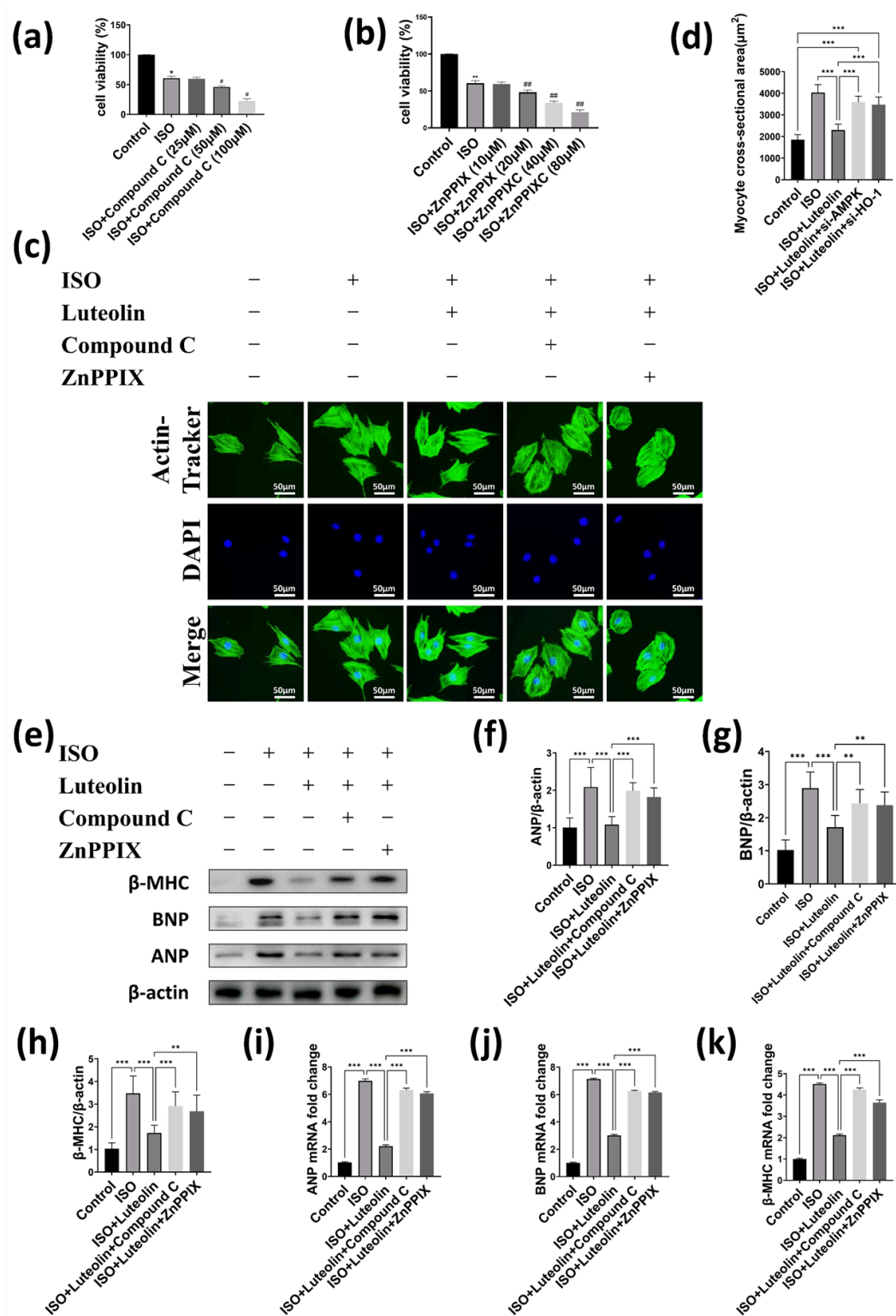


Figure 6

Inhibition of AMPK or HO-1 partially attenuates the protective effects of luteolin in ISO-treated H9c2 cells.

(a and b) CCK-8 assays used to select the concentrations of Compound C and ZnPPiX. (c) Representative F-actin fluorescence images. (d) Quantification of H9c2 cell surface area. (e)

Representative western blots of ANP, BNP and β -MHC. (f-h) Quantification of ANP/GAPDH, BNP/GAPDH and β -MHC/GAPDH. (i-k) Relative mRNA expression levels of ANP, BNP and β -MHC (n = 10 independent experiments). Data are presented as the mean $\pm$ SD. * P < 0.05, ** P < 0.01 and *** P < 0.001. # P < 0.05 vs. the control group and ## P < 0.05 vs. the ISO group (in a). ** P < 0.01 vs. control group (in b). ## P < 0.01 vs. ISO group (in b).

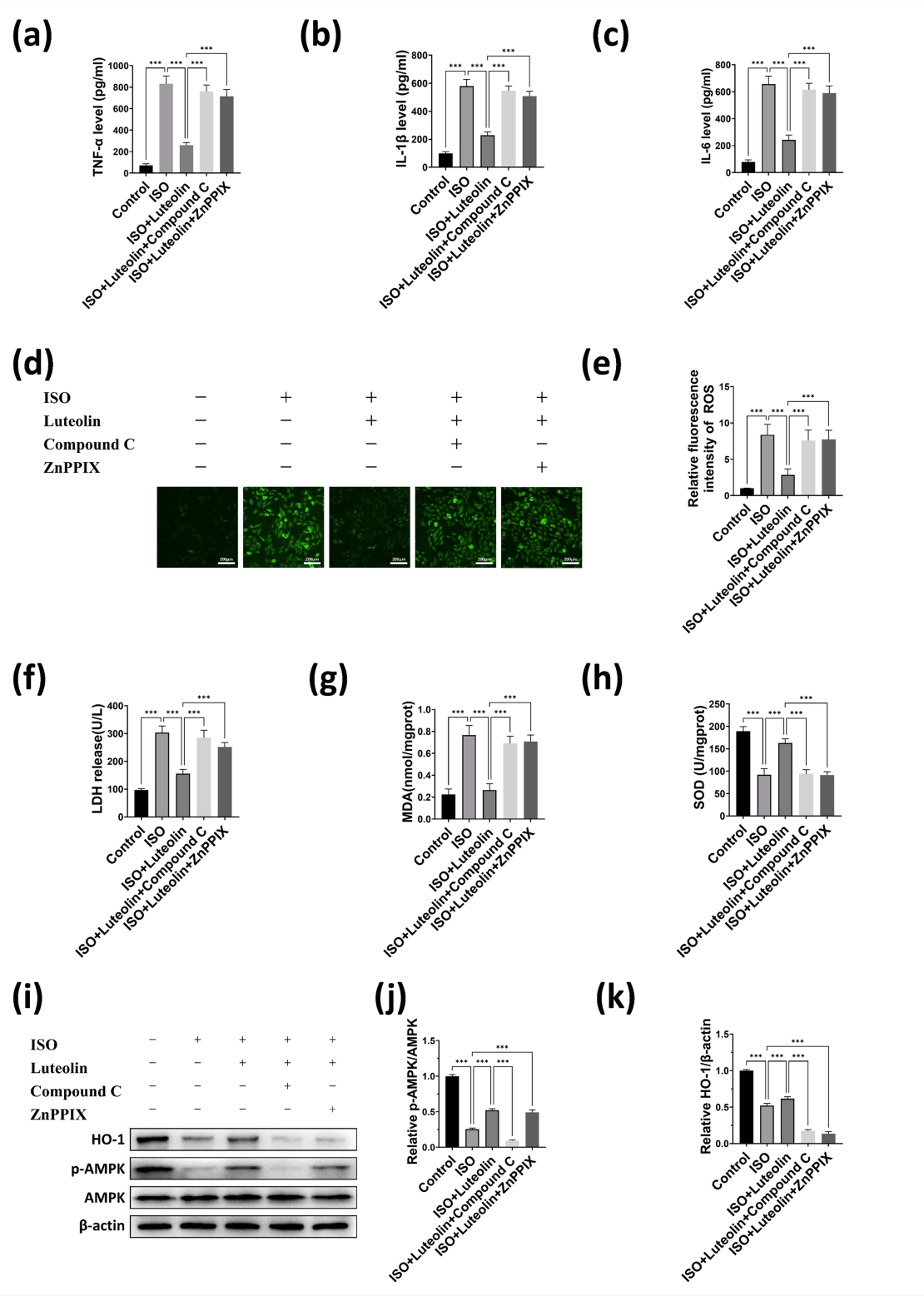


Figure 7

Inhibition of AMPK or HO-1 partially attenuates the anti-inflammatory and antioxidant effects of luteolin in ISO-treated H9c2 cells.

(a-c) IL-1 β , IL-6 and TNF- α levels in the cell-culture supernatant. (d) Representative DCFH-DA fluorescence images. (e) Quantification of relative reactive oxygen species fluorescence intensity. (f) LDH levels in the cell-culture supernatant. (g) Intracellular MDA levels. (h) Intracellular SOD activity. (i) Representative western blots of phosphorylated AMPK, total AMPK, HO-1 and β -actin. (j and k) Quantification of phosphorylated AMPK/AMPK and HO-1/ β -actin (n = 10 independent experiments). Data are presented as the mean $\pm$ SD. ** P < 0.01 and *** P < 0.001.

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