Irisin improves endothelial function in obese mice through the AMPK–eNOS pathway

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Running title: Irisin improves endothelial function
Abstract

Irisin is a novel hormone secreted by myocytes. Lower levels of irisin are independently associated with endothelial dysfunction in obese subjects. The objective of this study was to explore whether irisin exerts a direct vascular protective effect on endothelial function in high-fat diet-induced obese mice. Male C57BL/6 mice were given chow or a high-fat diet with or without treatment with irisin. Aortic endothelial function was determined by measuring endothelium-dependent vasodilatation (EDV). Nitric oxide (NO) in the aorta was determined. The effect of irisin on the levels of AMPK, Akt and eNOS phosphorylation in endothelial cells was determined. Human umbilical vein endothelial cells (HUVECs) were used to study the role of irisin in the AMPK–eNOS pathway. Acetylcholine-stimulated EDV was significantly lower in obese mice compared with control mice. Treatment of obese mice with irisin significantly enhanced EDV and improved endothelial function. This beneficial effect of irisin was partly attenuated in the presence of inhibitors of AMPK, Akt and eNOS. Treatment of obese mice with irisin enhanced NO production, and phosphorylation of AMPK, Akt and eNOS in endothelial cells. These factors were also enhanced by irisin in HUVECs in vitro. Suppression of AMPK expression by siRNA blocked irisin-induced eNOS and Akt phosphorylation and NO production. We have provided the first evidence that irisin improves endothelial function in aortas of high-fat diet induced obese mice. The mechanism for this protective effect is related to the activation of the AMPK–eNOS signaling pathway.

Keywords: irisin, endothelial function, nitric oxide, obesity.
New & Noteworthy

Irisin improved endothelial function in aortas of high-fat-diet-induced obese mice.

The mechanism for the protective effect of irisin is related to activation of the AMPK–eNOS signaling pathway.
Introduction

Obesity is an ever-increasing worldwide healthcare challenge and a major independent risk factor for cardiovascular disease (10, 18, 20). Endothelial dysfunction, an early step in the pathogenesis of atherosclerosis, is considered one of the main reasons for cardiovascular disease in obese people (1, 22). Numerous mechanisms have been proposed to explain this pathological alteration, such as reduced nitric oxide (NO) production and low NO bioavailability induced by excessive free fatty acid (FFA) production from adipose tissue (3, 9, 12). Increased inflammation and superoxide production can also lead to increased destruction of NO and endothelial dysfunction in obesity (3, 12).

Irisin is a novel hormone secreted by myocytes, and is a cleavage product of fibronectin type III domain containing 5, which is produced in response to the activation of peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α) (2). It has been proposed that irisin mediates the beneficial effects of exercise on metabolism, inducing the browning of adipocytes and thermogenesis by stimulating uncoupling protein 1 expression in vitro and in vivo (2, 6, 15). Overexpression of irisin by adenoviral vector induced weight loss and improved glucose metabolism in mice fed a high-fat diet (2). Circulating irisin is significantly lower in individuals with type 2 diabetes and obesity compared with controls (4, 14, 27). Because of the relationship between irisin and observed improvements in systemic metabolism, irisin is now regarded as an appealing therapeutic target for metabolic diseases such as type 2 diabetes and obesity.

Recently, the relationship between irisin and cardiovascular disease has gained increasing attention. Xiang et al (26) found that circulating irisin levels were reduced in
newly diagnosed type 2 diabetic patients and were positively associated with endothelium-dependent vasodilation. We have also found that serum irisin levels were lower in non-hypertensive, non-diabetic obese subjects compared with lean healthy controls (8). Lower levels of irisin are independently associated with endothelial dysfunction (8). These findings suggest that irisin may be involved in the regulation of endothelial function in obesity. However, it is still not known whether irisin exerts a direct vascular protective effect on endothelial function in obesity. Therefore, the objectives of this study were to explore whether irisin could improve endothelial function in obesity and if so, to investigate the potential mechanisms involved. We used an established mouse model of obesity induced by a high-fat diet for this study.

2. Materials and Methods

2.1 Materials

Phenylephrine, acetylcholine (ACh), sodium nitroprusside (SNP), API-2/triciribine and nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma (St. Louis, MO, USA). Compound C was from Merck (Darmstadt, Germany). Recombinant irisin and the irisin assay kit were purchased from Phoenix Pharmaceuticals, Inc (Burlingame, CA, USA). Adiponectin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and highly sensitive C-reactive protein (hs-CRP) assay kits were obtained from Uscn Life Science Inc. (Wuhan, China). NO and malondialdehyde (MDA) assay kits were purchased from Beyotime Biotechnology (Beijing, China). Antibodies (AMPK, phosphorylated (p)-AMPK, Akt, p-Akt, eNOS, p-eNOS and β-actin) for western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA,
USA). SiRNA duplex oligonucleotides used to knockdown AMPK expression were designed from human cDNAs encoding AMP kinase (AMPK). The siRNA sequences targeting AMPK were 5′-UGCCUACCAUCUCAUAAUAdTdT-3′ (sense) and 5′-UAUUAUGAGAUGGUAGGCAdTdT-3′ (antisense) (20). Other reagents were purchased from Beijing General Chemical Reagent Factory (Beijing, China).

2.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell (Carlsbad, CA, USA), and cultured in endothelial basal medium (EBM-2; cc-3202, Lonza Group, Ltd., Basel, Switzerland) supplemented with 2% fetal bovine serum and various endothelial cell growth factors at 37°C in a humidified 95% O₂/5% CO₂ incubator. All cells in the experiment were used within 4–6 passages, at 80–90% confluence.

2.3 Experimental animals

Four-week-old male C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). Mice were housed under standard laboratory conditions and maintained at a controlled room temperature and humidity on a 12-h light/dark cycle. Mice were acclimatized to the laboratory environment for 1 week prior to the experiment. All experimental protocols were approved by the local ethics committee for animal studies and the mice were handled following the committee’s “Principles of Laboratory Animal Care”.

Mice were randomly assigned to one of four groups: a normal control (NC) group; an irisin (NC-irisin) group; an obesity (OB) group; and an obesity plus irisin (OB-irisin) group. Mice in the NC and NC-irisin groups were fed a regular diet (330 kcal 100 g⁻¹) and mice in
the other two groups were fed a high-fat diet (50.10% fat, mainly saturated; 493 kcal 100 g⁻¹) (9). Mice in the NC-irisin and OB-irisin groups were given irisin (0.5 μg/g/day) (28) by intraperitoneal injection daily in the morning for 8 weeks. The other two groups were given the same amount of physiological saline in the same manner. Body weight and food intake were monitored weekly.

2.4 Determination of blood parameters
After 8 weeks, the mice were euthanized with 3% isoflurane. Blood samples were collected and stored at −80°C until analysis, as described below. Plasma glucose levels were measured using the glucose oxidase method. Plasma FFA level and serum lipid profiles including total cholesterol (TC), triglyceride (TG), and malonic dialdehyde (MDA) were measured using colorimetric assays. Serum levels of irisin, insulin, TNF-α, IL-6, adiponectin and hs-CRP were measured by ELISA.

2.5 Evaluation of endothelial function
Thoracic aortas of mice were obtained and equilibrated in Krebs–Henseleit bicarbonate buffer (K-H solution) for measurement of endothelial function. Aortic vascular reactivity was determined as described previously (22, 23). Each thoracic aorta vascular ring was cut into 2-mm ring segments and immersed in ice-cold oxygenated K-H solution (95% oxygen and 5% carbon dioxide). All rings were equilibrated for 60 min with a resting tension of 0.75 g. Subsequently, all rings were first contracted with KCl (60 mM) and rinsed several times in K-H solution. Phenylephrine (1 μM) was then added to produce a steady contraction. ACh
(10^{-9}-10^{-5} \text{ M}) or SNP (10^{-9}-10^{-5} \text{ M}) was added to the organ bath to measure endothelium-dependent vasodilatation (EDV) or endothelium-independent vasodilatation (EIV) responses after a stable contraction was achieved. Changes in isometric tone of the rings were recorded using a data acquisition system (PowerLab; AD Instruments, Castle Hill, Australia). Aortic rings were also incubated with NG-nitro-L-arginine methyl ester (L-NAME 10^{-4} \text{ M}, Sigma) for 30 min followed by determination of EDV.

In a separate set of experiments, aorta vascular rings from animals in the OB-Irisin group were incubated with the following specific inhibitors of candidate pathways for 60 min: (1) 20 µM of an AMPK inhibitor, compound C, which competes with ATP-binding sites; (2) 5 µM of an Akt inhibitor, API-2/triciribine, which selectively inhibits Akt1/2/3 activation without inhibiting PI3K, followed by precontraction and determination of EDV.

2.6 Total NO and superoxide anion production measurement

After the organ bath study, total NO production (nitrite and nitrate) by aortic rings was determined using a modified Griess reaction method as described previously (11, 13). Briefly, after ACh (10^{-5} \text{ M}) was added, 100 µl of K-H solution was taken from the organ bath and mixed with an equal volume of modified Griess reagent (1% sulfanilamide, 0.1% naphthylethlenediamine dihydrochloride, and 2% phosphoric acid). After 10 min of incubation at room temperature, the NO concentration was spectrophotometrically determined at 540 nm according to the manufacturer’s instructions. The values were calculated per amount of dry weight in the tissue.

In the cell culture experiment, HUVECs were cultured in 6-well plates, and experiments were performed 24 h after the cells reached confluence. HUVECs were
serum-starved for 3 h, then incubated with irisin (50 nM) and modified Griess reagent. After 10 min of incubation at room temperature, the absorbance of the resultant medium was determined at 540 nm using a spectrophotometer and total NO production calculated from an assay standard curve.

Aortic superoxide anion production was measured using the lucigenin chemiluminescence method as described previously (7). Superoxide production was expressed as counts per minute per milligram aortic tissue.

2.7 Western blot analysis

Equal amounts of total protein extract from tissue samples and endothelial cells were electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, probed with different primary antibodies of anti-AMPK (1:1000 dilution), anti-p-AMPK(Thr 172) (1:1000), anti-Akt (1:1000), anti-p-Akt (Ser 473) (1:1000), anti-eNOS (1:1000) and anti-p-eNOS (Ser 1177) (1:1000) and incubated with the appropriate secondary antibodies. The intensity of the protein bands was quantified using a pro-plus 5.0 image-processing system.

2.8 Ex vivo culture of mouse aortic rings.

Mouse thoracic aortic rings (2 mm in length) from obese mice were dissected in sterile phosphate buffered saline and incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, plus 100 IU/mL penicillin and 100 mg/mL streptomycin. Irisin (50 nM) (15), compound C (20 μM), API-2/triciribine (Akt inhibitor, 5 μmol/L) and L-NAME (10^{-4} M) were individually added into the culture medium. After incubation for 60 min, the rings were transferred to a chamber filled with fresh K-H solution for measuring
2.9 Statistical analysis

All data were analyzed statistically using the SPSS 13.0 statistical package (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed by one-way ANOVA or two-way ANOVA, as appropriate. A $P$-value of <0.05 was considered statistically significant.

3. Results

3.1 Biometric and blood parameters

After 8 weeks of experimental intervention, mice in the OB group exhibited increased body weight compared with age-matched control mice in the NC group ($P$<0.05). Body weight was slightly lower in the OB-irisin group compared with the OB group ($P$<0.05). As expected, treatment of mice with recombinant irisin significantly increased serum irisin levels in both control and obese mice ($P$ <0.01). Serum levels of insulin and TG and plasma levels of glucose and FFA were higher in the OB group compared with the NC group ($P$ <0.01). Treatment of obese mice with irisin improved glucose and lipid metabolism ($P$ <0.01). Serum levels of TNF-$\alpha$, IL-6, hs-CRP and MDA increased and serum adiponectin levels decreased significantly in the obese group compared with the control group ($P$<0.05). Treatment of obese mice with irisin reduced serum levels of TNF-$\alpha$, IL-6, hs-CRP and MDA, and increased serum adiponectin levels ($P$<0.05). There were no significant differences in these parameters between the NC and NC-irisin groups (Table 1).

3.2 Effect of irisin on endothelial function

ACh-stimulated concentration-dependent vasorelaxation of aortic strips was lower in obese mice compared with control mice. SNP-stimulated concentration-dependent vasorelaxation
remained unchanged in these vessels. These results indicate that endothelial function was impaired in the obese mice. Treatment of obese mice with irisin significantly enhanced ACh-induced vasorelaxation and improved endothelial function (Figure 1A). This beneficial effect of irisin was partly attenuated in the presence of compound C, or API-2/triciribine (Figure 1C). When the aortic rings were incubated with the NO-synthase inhibitor L-NAME, ACh-induced concentration-dependent vasorelaxation was abolished in all groups (Figure 1A).

3.3 Effect of irisin on total NO production in endothelial cells

To further explore the possibility that irisin may increase endothelium-derived NO production, we measured total NO production in the K-H solution from the organ bath (Figure 1D). NO production was significantly reduced in the OB group compared with the control group ($P<0.05$). NO production from the OB-irisin group showed a substantial increase compared with the OB group ($P<0.05$). NO production in the NC-irisin group was also increased compared to that of the NC group ($P<0.05$).

3.4 Effect of irisin on aortic superoxide anion production.

We observed an increase in superoxide anion production in the OB group compared with the control group ($p < 0.05$). However, irisin treatment decreased superoxide anion production ($p < 0.05$; Figure 2).

3.5 Effect of irisin on AMPK, Akt and eNOS phosphorylation in endothelial cells

To investigate the mechanism that modulates endothelial function in obese mice treated with irisin, the total and phosphorylated AMPK, Akt and eNOS in endothelial cells were determined. As shown in Figure 4, the OB groups showed reduced phosphorylation of AMPK,
Akt and eNOS ($P<0.05$). However, irisin treatment enhanced phosphorylation of AMPK, Akt and eNOS in obese mice ($P<0.05$; Figure 3).

### 3.6 Effect of irisin on endothelial function on aortic rings in vitro

ACh caused a concentration-dependent vascular relaxation in all the aortic rings. Pre-incubation of aortic rings from obese mice with irisin significantly increased concentration-dependent vascular relaxation due to ACh. This beneficial effect of irisin on ACh-induced EDV in obese mice was partly attenuated in the presence of compound C, API-2/triciribine or L-NAME (Figure 4).

### 3.7 Effect of irisin on NO production in HUVECs in vitro

As shown in Figure 5, irisin induced NO production in a dose-dependent (Figure 5A) and time-dependent (Figure 5B) manner. Treatment with L-NAME (an eNOS inhibitor) abolished irisin-induced NO production. Suppression of AMPK expression by siRNA blocked irisin-induced NO production (Figure 5B).

### 3.8 Effect of irisin on AMPK, Akt and eNOS expression in HUVECs

Having demonstrated that irisin induces EDV in a NO-dependent manner, we further investigated whether irisin induces endothelial cell NO production via AMPK-Akt-eNOS signaling *in vitro*. HUVECs were incubated with irisin and NO production, and the phosphorylation of AMPK, Akt and eNOS was examined. Incubation of the HUVECs with irisin (50 nM) (15) significantly enhanced phosphorylation of AMPK, Akt and eNOS in a time dependent manner (Figure 6).

To obtain direct evidence supporting a causative role of AMPK activation and irisin-induced NO production, HUVECs were transfected with siRNA specifically targeting
AMPK for 48 h and then incubated with irisin (50 nM) for 60 min. The results showed that suppression of AMPK expression by siRNA blocked irisin-induced eNOS and Akt phosphorylation (Figure 7).

4. Discussion

In this study, we have demonstrated for the first time that irisin improves endothelial function in aortas of high-fat diet-induced obese mice. The mechanism for this protective effect of irisin appeared to be related AMPK–eNOS signaling pathway activation. These results suggest that irisin plays an important role in modulating endothelial function in vivo in obesity.

Irisin, a novel PGC-1α-dependent signaling protein, promotes brown-like fat development and thermogenesis in white adipose tissue both in vitro and in vivo. It has been suggested that irisin may become a new therapeutic target for the prevention and treatment of obesity and other related metabolic disorders (19). Two recent studies have focused on the potential relationship between circulating irisin levels and endothelial function. One study found that circulating irisin levels were reduced in newly diagnosed type 2 diabetic patients and positively associated with endothelium-dependent vasodilation (25). Our previous study found that the circulating irisin level was also reduced in non-hypertensive, non-diabetic obese subjects compared with lean healthy controls (8). This lower level of irisin was independently associated with endothelial dysfunction in the obese state (8). These findings indicate that circulating irisin levels are associated with endothelial dysfunction, and irisin may be involved in the regulation of endothelial function. However, we still do not know whether irisin exerts a direct vascular protective effect on endothelial function in obesity. In
In the current study, we found that treatment of obese mice with irisin significantly enhanced ACh-induced vasorelaxation and improved endothelial function. These results indicate that irisin may be a promising agent possessing the ability to improve endothelial function in obesity.

It is established that endothelial-dependent vasodilation in the aorta is mainly related to endothelial-derived NO induced by eNOS phosphorylation (5). In our study, NO levels from the OB-irisin group were substantially increased compared with those from the OB group. Irisin also induced NO production in a dose-dependent and time-dependent manner in HUVECs. Treatment with L-NAME abolished irisin-induced NO production and blocked irisin-mediated aortic ring vasorelaxation. Together, these results clearly demonstrate that irisin is a novel endothelium-dependent and NO-mediated vasorelaxive agent.

Having demonstrated that irisin induces endothelial-dependent vasodilation in a NO-mediated fashion, we further investigated the underlying mechanisms responsible for this beneficial effect. It has already been demonstrated that high FFA levels released from excess white adipose tissue could impair eNOS phosphorylation through inhibiting the Akt signaling pathway in obesity (24). Because irisin has the ability to turn white adipocytes into brown-like adipocytes, it may also reduce circulating FFA levels from adipose tissue. In our study, we found that irisin treatment reduced serum levels of FFA and enhanced phosphorylation of Akt and eNOS in obese mice. Further studies found that the effect of irisin on ACh-induced vascular relaxation in obese mice was significantly attenuated when Akt and eNOS inhibitors were added. Furthermore, pre-incubation of aortic rings from obese mice with irisin significantly increased endothelial function in vitro and this beneficial effect of
Irisin on endothelial-dependent vasodilation in obese mice was partly attenuated upon co-incubation with Akt inhibitor. These results suggest that irisin could improve endothelial function via the Akt–eNOS pathway in obesity.

Adiponectin has been shown to have protective effects on endothelial function by activating the AMPK–Akt–eNOS pathway (17). In the current study, we found that treating obese mice with irisin increased serum adiponectin levels and enhanced AMPK phosphorylation. This beneficial effect of irisin was partly attenuated in the presence of an AMPK inhibitor. To determine whether irisin could directly activate AMPK independently of adiponectin, we incubated HUVECs with irisin and found that irisin could also increase AMPK phosphorylation. Suppression of AMPK expression by siRNA blocked irisin-induced Akt and eNOS phosphorylation and abolished irisin-induced NO production. These results suggest that irisin may improve endothelial function by activating the AMPK–eNOS pathway in an adiponectin-dependent and independent manner.

Adipose-derived FFA and inflammation-induced reactive oxygen species can quench NO to form peroxynitrite and reduce NO bioavailability. In our study, treatment of obese mice with irisin reduced serum levels of hs-CRP and MDA, and also reduced superoxide anion production by the aorta. This indicates that irisin may improve endothelial function by reducing inflammation and oxidative stress. However, whether irisin has direct anti-oxidative and anti-inflammatory effects has not been elucidated and should be clarified in future studies.

In conclusion, this study found that irisin improved endothelial function in aortas of high-fat-diet-induced obese mice. The mechanism for the protective effect of irisin is related
to activation of the AMPK–eNOS signaling pathway. These results suggest that irisin plays an important role in modulating endothelial function in obesity, and may have important clinical implications in the prevention and treatment of cardiovascular disease in obesity.

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Conflict of Interest

The authors declare no conflict of interest


14. **Liu JJ, Wong MD, Toy WC, Tan CS, Liu S, Ng XW, Tavintharan S, Sum CF, Lim SC.** Lower circulating irisin is associated with type 2 diabetes mellitus. *J Diabetes*


20. **Rokholm B, Baker JL, Sorensen TI.** The levelling off of the obesity epidemic since


**Figure legends**

Figure 1. Effect of irisin on endothelial function (A-C) and total NO production (D) of the endothelium from the studies groups. Ach-stimulated concentration-dependent vasorelaxation of aortic strips was lower in obese mice compared with control mice (A). Treatment of obese mice with irisin rejection significantly enhanced Ach-induced vasorelaxation and improved endothelial function (A). When the aortic strips from irisin-treated obese mice were incubated with the compound C (AMPK inhibitor) and API-2/triciribine (Akt inhibitor), this beneficial effect of irisin was partly attenuated (C). No significant differences in endothelium-independent vasodilatation were found between the different groups (B). Data are shown as mean ± SD. n= 6-7 mice per group. *P < 0.05 vs NC group. #P < 0.05 vs OB group. &p < 0.05 vs other groups. NC, normal control mice; OB, obese mice; NC-irisin, irisin-treated control mice; OB-irisin, irisin-treated obese mice.

Figure 2. Effect of irisin on aortic superoxide anion production. n=5 mice per group. *P < 0.05 vs NC group; #P <0.05 vs OB group. NC, normal control mice; OB, obese mice; NC-irisin, irisin-treated control mice; OB-irisin, irisin-treated obese mice.

Figure 3. Effect of irisin on AMPK, Akt and eNOS phosphorylation in the aorta rings of obese mice. n=5 mice per group. *P < 0.05 vs NC group; #P <0.05 vs OB group. NC, normal control mice; OB, obese mice; NC-irisin, irisin-treated control mice; OB-irisin, irisin-treated obese mice.
Figure 4. Effect of irisin on endothelial function in aortic rings in vitro. Pre-incubation of aortic rings from obese mice with irisin significantly increased concentration-dependent vascular relaxation to ACh. This beneficial effect of irisin on ACh-induced EDV in obese mice was partly attenuated in the presence of compound C, API-2/triciribine or L-NAME. n = 5-6 mice per group. *P < 0.05 vs NC group. #P < 0.05 vs OB group. &P < 0.05 vs irisin group.

NC, normal control mice; OB, obese mice.

Figure 5. Effect of irisin on total NO production in human umbilical vein endothelial cells (HUVEC). (A) HUVEC incubation with irisin (10 nM, 20 nM, 50 nM, 100 nM) for 60 min. (B) HUVEC incubation with irisin (50 nM) for 15 min, 30 min, 60 min, 120 min. Treatment with L-NAME and SiRNA targeting the AMPK abolished irisin-induced NO production. n = 5-6 for each experiment. *P < 0.05 vs control, #P < 0.05 vs 100 nM (A) or 120 min (B)

Figure 6. Effect of irisin on AMPK, Akt and eNOS phosphorylation in the human umbilical vein endothelial cells in different time. irisin (50 nM) significantly enhanced phosphorylation of AMPK, Akt and eNOS in a time dependent manner. n = 5 for each experiment. *P < 0.05 vs control.
Figure 7. Effect of AMPK SiRNA on Akt and eNOS phosphorylation in the human umbilical
vein endothelial cells. Suppression of AMPK expression by siRNA blocked irisin-induced
eNOS and Akt phosphorylation. n = 5 for each experiment. *P < 0.05 vs NC, #P < 0.05 vs
irisin.
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Data are shown as mean ± SD *P<0.01 vs. NC group. #P<0.01 vs. OB group. n=6-8 mice in each group. FFA, free fatty acids; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; Hs-CRP, high-sensitive C reaction protein; MDA, malondialdehyde;