Irisin protects against endothelial injury and ameliorates atherosclerosis in apolipoprotein E-Null diabetic mice

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1. Background

Cardiovascular disease (CVD) is the leading complication of diabetes and accounts for up to 70% of mortality in patients with diabetes [1,2]. Atherosclerosis, which constitutes the most important contributor to cardiovascular disease, involves an ongoing inflammatory process and oxidative stress in arterial wall. The inflammatory factors and oxidative stress promote macrophages and T lymphocytes infiltration, foam cell formation, extracellular matrix degradation and vascular smooth muscle proliferation leading to atherosclerotic plaque formation. Emerging data support the concept that endothelial injury is an early marker of atherosclerotic vascular disease [3], and has been shown to contribute to atherogenesis [4–6]. Approaches that protect against endothelial injury may be therapeutic for atherosclerosis.

Irisin is a cleaved and secreted fragment of the plasma membrane protein fibronectin type III domain-containing protein 5 (FNDC5), and mainly secreted by skeletal muscle [7]. The circulating irisin converts white fat into the more thermogenic beige fat [7], a process known as “browning”. Overexpression of
this myokine was sufficient to promote energy expenditure and alleviate insulin resistance in C57/BL mice [7]. Actually, some studies showed that circulating irisin levels were significantly lower in type 2 diabetic patients [8,9]. Moreover, one study showed that plasma irisin levels are negatively associated with homocysteine levels [10], and another study revealed that decreased plasma levels of irisin seem to be associated with urinary albumin excretion in type 2 diabetes [11]. Recently, our and other studies suggested that circulating irisin concentrations are positively associated with endothelial function in type 2 diabetes [11,12], and in non-diabetic patients [13]. Furthermore, one in vitro study showed that irisin can attenuate endothelial cells apoptosis induced by high glucose [14]. Taken together, these data indicate that irisin may be associated with angiopathy in diabetes. However, the potential role of irisin on atherosclerosis has not been investigated. Therefore, we hypothesized that irisin can protect against endothelial injury and ameliorate atherosclerosis in diabetic conditions. In the present study, we assessed whether irisin has endothelium-protective and anti-atherosclerotic effects in vivo and in vitro experiments in diabetic conditions and investigated the possible mechanisms-involved.

2. Materials and methods

The experiments conformed to the National Institutes of Health Guidelines for the Use of Laboratory Animals. All animal experimental protocols were approved by the animal ethics committee of the Wuhan General Hospital of Guangzhou Command (reference number: WZEA2012008). Thirty nine male 4-week-old apoE−/− mice (The Jackson Laboratory, USA) were housed in a specific–pathogen-free environment with unrestricted access to water. The mice were fed with a high-fat diet (HFD: 45% kcal fat, 35% kcal carbohydrates, and 20% kcal protein) from 4-week-old to the end of the study. After 4 weeks of high-fat diet feeding, a total of twenty six mice were rendered diabetic by five daily intraperitoneal injections of streptozotocin (STZ, Sigma, US) at a dose of 50 mg/kg. The other mice received the vehicle citrate buffer alone (non-diabetic, n = 13). Diabetic animals were further divided into 2 groups (13 mice in each group) matching serum glucose and body weight, then treated with irisin (Phoenix Pharmaceuticals, US) or normal saline (NS), respectively. For diabetic + irisin group, each mouse received tail vein injection of irisin (2 µg per mouse in a total of 100 µL NS) twice a week, and for both diabetic + NS and non-diabetic groups, each mouse received an equivalent volume of NS by tail vein injection for 12 weeks. The serum glucose and body weight were followed weekly for the 3 groups.

After 4 weeks or 12 weeks of irisin or NS intervention, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight) and euthanized for endothelial function assessment (n = 3 mice in each group at 4th week) or for blood tests and histological examination (at 12th week) (n = 10 mice in each group), respectively. The serum concentrations of insulin and irisin were measured with ELISA kits (insulin: EMD Millipore Corporation, USA; irisin: Phoenix Pharmaceuticals, USA). All procedures were performed as the manufacturers’ instructions. Measurements were performed in duplicate.

2.1. Glucose and insulin tolerance tests

The intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) was performed before and after irisin or NS treatment in 3 groups (n = 13 mice before treatment and n = 10 mice after treatment), respectively. For IPGTT, mice were fasted overnight, followed by an intraperitoneal injection of 2 g/kg glucose. For ITT, mice were fasted for 5 h, followed by an intraperitoneal injection of 0.075 units/kg insulin. Blood samples were obtained by tail-bleeding and blood glucose level was checked by a portable glucose meter (Johnson & Johnson, USA) before and after glucose or insulin injection, respectively. The incremental area under the curve (AUC) over the basal value of IPGTT during the time period of 120 min was calculated using the trapezoidal rule and used as an index of glucose tolerance. The incremental area below the basal value of ITT during the time period of 90 min was calculated using the trapezoidal rule and used as an index of insulin tolerance.

2.2. Endothelial function assessment in mice

The thoracic aortas were cleaned of connective tissue under the stereo-microscope (Olympus, Japan) and cut into 4 mm rings immediately after euthanasia. Aortic rings were mounted on wire hooks, suspended in organ chambers containing Krebs buffer kept at 37 °C and aerated with 95% O2. The wire hooks were connected to force transducers (JH-2, Chengdu TME Technology Co., Ltd, China) to record changes of tension by a Data Acquisition System (BL-420S, Chengdu TME Technology Co., Ltd, China). After equilibration for 1 h at a preload tension of 0.5 g, the rings were precontracted with norepinephrine (NE, 10−6 mol/L). Once a steady-state was achieved, vasodilation responses were evaluated by cumulative concentration–response curves to acetylcholine (ACh, 10−9 to 10−4 mol/L) and sodium nitroprusside (SNP, 10−5 to 10−4 mol/L).

In ex vivo experiments, thirty apoE−/− male mice (4-week-old, weighting 18–20 g) were divided into 6 groups with 5 mice in each group. After the mice were euthanized, the aortic rings were prepared and the aortic rings in each group were then incubated in the following medium: (1) Normal glucose group (NG): glucose 5.5 mmol/L in Krebs’ solution, (2) High glucose group (HG): glucose 20 mmol/L in Krebs’ solution, (3) Irisin group (Irisin): irisin 100 ng/ml in Krebs’ solution with glucose 20 mmol/L, (4) Compound C group (Compound C, an AMPK inhibitor): Compound C 500 µmol/L + irisin 100 ng/ml in Krebs’ solution with glucose 20 mmol/L, (5) LY group (LY294002, a PI3K inhibitor): LY294002 10 µmol/L + irisin 100 ng/ml in Krebs’ solution with glucose 20 mmol/L, (6) L-NAME group (L-NAME, an eNOS inhibitor): L-NAME 500 µmol/L + irisin 100 ng/ml in Krebs’ solution with glucose 20 mmol/L. The endothelium-dependent and endothelium-independent vasodilation was measured.

2.3. Quantification of atherosclerotic plaques

To evaluate the atherosclerotic plaque in apoE−/− mice, two complementary approaches were used (en face and cross sections analyses). After irisin or NS treatment for 12 weeks, whole aortas (n = 3 in each group) were dissected and stained with Oil Red O (Sigma, USA) for plaque en face area analysis. The extent of atherosclerosis was expressed as the percent of the lesion area extending from the ascending aorta to the iliac bifurcation. To quantify luminal cross-sectional area involved by atherosclerotic plaque, 8 sections obtained every 20 µm from the abdominal aorta (n = 7 mice in each group) were stained with hematoxylin and eosin, and analyzed by Image-Pro Plus 6.0 (Media Cybernetics, USA).

2.4. Cell culture

Primary HUVECs were purchased from ScienCell (USA). Cells were seeded at a density of 5 × 104/cm2 flask in M199 (Gibco, USA), supplemented with 20% fetal bovine serum (FBS, Gibco, USA), 10 µg/ml heparin, 1% penicillin/streptomycin, and 50 µg/ml.
endothelial cell growth factor (ECGF, Sigma, USA). The cultures were maintained at 37 °C with a 5%CO2 atmosphere and the media were refreshed every third day. Endothelial cells of the third to fifth passages were used for experiments. Cells were starved in M199 containing 1% FBS for 20 h to synchronize before cytokines treatment.

2.5. Gene silencing

A synthetic small interfering RNA (siRNA) was used to knock-down the expression of adenosine monophosphate-activated protein kinase (AMPK) and endothelial nitric oxide synthase (eNOS) in HUVECs. The siRNA oligonucleotides against AMPK (AMPK-siRNA), eNOS (eNOS-siRNA) and scrambled control (Scr-siRNA) were transfected into cells by using TransMessenger Reagent (Qiagen, Canada) according to the manufacturer’s instructions. A successful knockdown of AMPK and eNOS genes was determined by RT-PCR and Western blot analyses.

2.6. Analysis of endothelial cell apoptosis in vivo and in vitro

Endothelial cell apoptosis in aortas was detected by double-stain with terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) and anti-CD31, and HUVECs apoptosis was evaluated by TUNEL single-staining. HUVECs were divided into 6 groups and pretreated with inhibitors against AMPK (Compound C, 500 μmol/L), PI3K (LY294002, 10 μmol/L) or eNOS (l-NAME, 500 μmol/L) for 30 min before irisin (100 ng/ml) was added. The cells were then cultured for 48 h with media containing 5.5 mmol/L glucose (NG group) or 20 mmol/L glucose (HG group). Moreover, to verify the role of AMPK or eNOS in endothelial cells apoptosis, HUVECs were transfected with Scr-siRNA, AMPK-siRNA or eNOS-siRNA for 24 h, followed by cultured in normal (5.5 mmol/L) or high (20 mmol/L) glucose for 48 h in the presence or absence of irisin (100 ng/ml). After treatment, the cells were double-stained with Annexin V- FITC/PI (BD, USA) and analyzed by flow cytometry.

2.7. Western blot analyses

Proteins extracted from the aortas of apoE−/− mice and cultured HUVECs were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin (BSA) for 1 h at room temperature, the membranes were incubated with primary antibodies including P-AMPKα, AMPK, P-Akt, Akt, P-eNOS (Serine 1177), eNOS, Bcl-2 and Bax (all the primary antibodies were purchased from CST, USA) overnight at 4 °C. The membranes were washed several times in TBST and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. The specific reactions were revealed with the enhanced chemiluminescence detection system (Perkin–Elmer, USA).

2.8. Real-time PCR (RT-PCR)

To determine the expression levels of inflammatory cytokines in aortas and antioxidant enzymes in cultured HUVECs, the interleukin (IL)-10, IL-6, tumor necrosis factor-alpha (TNF-α), intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) in aortas, and monocyte chemoattractant protein-1 (MCP-1), glutathione peroxidase type 1 (GPX-1), catalase (CAT) and superoxide dismutase (SOD) in HUVECs were measured by quantitative RT-PCR. Total RNA was extracted from thoracic aortas (n = 7 mice in each group) or cultured HUVECs with Trizol reagent (Sigma, USA) according to the manufacturer’s instructions. The mRNA was then reverse-transcribed to cDNA using iScript™ cDNA Synthesis kit (Bio-Rad, USA). Relative changes in mRNA levels among groups were determined with ΔΔCt method.

2.9. Detection of reactive oxygen species (ROS) generation in HUVECs

Intracellular ROS generation was detected by using the peroxide-sensitive fluorescent probe 2’, 7’-dichlorofluorescein diacetate (DCFH-DA). HUVECs were seeded in 96-well plates. As described above, HUVECs were divided into 6 groups and treated with compound C, LY294002, l-NAME or irisin for 48 h, and then the cells were incubated with DCFH-DA (10 μmol/L per well) for 30 min at 37 °C. After that, the cells were washed with phosphate buffered saline (PBS) and imaged by fluorescence microscope. The fluorescence of DCFH-DA was measured by fluorescence plate reader Victor3TM (Perkin–Elmer, USA).

2.10. Statistical analysis

Data are expressed as mean ± SD. Differences between 2 groups were tested with unpaired Student’s t test. Data of multiple groups were compared using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) t-test. Statistical significance was defined as p < 0.05. All analyses were performed with SPSS 19.0 (IBM, USA).

3. Results

3.1. The effects of irisin on metabolic characteristics of ApoE−/− mice

Previous study showed that even moderately increased levels of circulating irisin potently increase energy expenditure, reduce body weight and improve diet-induced insulin resistance [7]. Thus, the effects of irisin on metabolic characteristics were detected in mice in the present study. To explore the effects of irisin on insulin sensitivity in STZ-induced diabetic mice, IPGTT and ITT was performed before and 12 weeks after irisin or NS treatment. Firstly, to determine the half-life of circulating irisin, 10 male 10-week old C57BL/6 mice were purchased, then each mouse received tail vein injection of irisin (2 μg per mouse in a total of 100 μl NS) for one time according to literature [7], and the circulating irisin levels were measured with ELISA kits (irisin: Phoenix Pharmaceuticals, USA) at day 0, 1, 2, 3, 4, 5, and day 6 by tail venous. Since the serum concentrations decreased 21% at day 3, and 60% at day 4, we injected irisin twice a week for mice (Supplementary Fig. 1A). So far, receptor(s) for irisin has not been identified yet. According to the results (Supplementary Fig. 1A), the plasma half-life of intravenously injected irisin is surprisingly long, longer than 4 days, despite the findings showing irisin is a bioactive molecule probably acting via specific receptors. Such, further study for its receptor(s) are needed. Secondly, before irisin or NS treatment, diabetes displayed a significant decreased glucose and insulin tolerance when compared with non-diabetes (p < 0.05) (Supplementary Fig. 1B and C, Supplementary Fig. 2A and B). After 12 weeks of irisin or NS treatment, irisin treated mice showed increased glucose and insulin tolerance when compared with mice treated with NS, but which was still lower than non-diabetic mice (p < 0.05) (Supplementary Fig. 1D and E, Supplementary Fig. 2C and D). Thirdly, compared with non-diabetic mice, fasting insulin was significantly decreased before irisin or NS treatment, and further decreased at 12th week in diabetic mice (p < 0.05), but no difference was observed in fasting insulin between the diabetic groups at both two time points (Supplementary Fig. 1E and F). As shown in Table 1, irisin treated mice also exhibited decreased fasting plasma glucose and...
glycosylated hemoglobin (HbA1c), but had no notable effects on body weight at the end of study (p > 0.05 vs. diabetic + NS group).

3.2. Irisin improved endothelial dysfunction in ApoE \(^{-/-}\) diabetic mice

Endothelial dysfunction is an early marker of atherosclerotic vascular disease [3]. We next examined the effects of irisin on endothelial function at 4th week after irisin or NS treatment in mice. The results showed that diabetes decreased the endothelium-dependent vasodilation in response to ACh, while irisin treatment markedly improved the vasodilation in diabetic condition (82.43 ± 4.79% vs. 61.54 ± 4.62% vasodilation at 10 \(^{-5}\) mmol/L ACh, 5 aortic rings from each mouse, p < 0.05 vs. diabetic + NS group) (Fig. 1A). In contrast, the endothelium-independent vasodilation in response to SNP did not differ among the 3 groups (Fig. 1B). To explore the possible mechanisms-involved, proteins extracted from thoracic aortas after 12 weeks of irisin or NS treatment were analyzed by Western blot. The eNOS phosphorylation was significantly lower in diabetic group than that in non-diabetic group, and irisin treatment apparently increased eNOS phosphorylation compared with the diabetic + NS (Fig. 1C and D). Nitric oxide (NO), produced by eNOS, plays a pivotal role in regulating endothelial cell function, apoptosis, and exhibits athero-protective effects [3]. The

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**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic (n = 10)</th>
<th>Diabetic + NS (n = 10)</th>
<th>Diabetic + irisin (n = 10)</th>
</tr>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.1 ± 1.8†</td>
<td>25.6 ± 2.5</td>
<td>26.2 ± 2.3</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>12.35 ± 0.74†</td>
<td>28.50 ± 2.43</td>
<td>21.48 ± 2.75*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.8 ± 0.7†</td>
<td>13.5 ± 1.3</td>
<td>10.1 ± 1.1*</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.95 ± 0.24†</td>
<td>0.42 ± 0.11</td>
<td>0.52 ± 0.12</td>
</tr>
<tr>
<td>Irisin (ng/ml)</td>
<td>105.3 ± 6.9†</td>
<td>72.1 ± 10.6</td>
<td>189.6 ± 15.2**</td>
</tr>
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</table>

HbA1c indicates glycosylated hemoglobin. Values are mean ± SD and analyzed by ANOVA. *p < 0.05, **p < 0.01, compared with diabetic + NS group. |p| < 0.05, ||p| < 0.01, compared with other two groups.

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**Fig. 1.** Irisin improved endothelial dysfunction in apoE \(^{-/-}\) diabetic mice. (1) ApoE \(^{-/-}\) mice were treated with irisin or normal saline (NS) for 4 weeks. Aortic rings were collected for endothelial function assessment after euthanasia. A: Endothelium-dependent vasodilation to acetylcholine (ACh). B: Endothelium-independent vasodilation to sodium nitroprusside (SNP). (The data represent mean ± SD. n = 3 mice, 15 aortic rings/group, *p < 0.05 vs. Non-diabetic or diabetic + NS). (2) C: Western blot analyses of protein from the thoracic aortas of mice (n = 7 mice in each group) at 12th week after irisin or NS treatment. D: Relative PKA, AMPK, Akt and eNOS protein levels in the aortas for Western blot (The data represent mean ± SD. n = 7 mice in each group, *p < 0.05, **p < 0.01). (3) E and F: Aortic rings from apoE \(^{-/-}\) mice without treatment (5 mice in each group) were incubated in different media. Endothelium-dependent vasodilation to Ach (E) and endothelium-independent vasodilation to SNP (F) was detected. NG indicates normal glucose group (5.5 mmol/L); HG, High glucose group (20 mmol/L) (The data represent mean ± SD. n = 10 aortic rings/group, *p < 0.05, compared with other groups, **p < 0.01, compared with NG group).
serum concentrations of NO decreased in diabetic mice and irisin treatment significantly increased the NO levels after irisin or NS treatment (Supplementary Fig. 3A), which are consistent with eNOS phosphorylation levels in aortas of 3 groups. The phosphorylation of eNOS can be regulated by several kinases [15,16]. Thus, we examined the changes in the levels of PKA, AMPK, and Akt in aortas. Irisin treatment for 3 months increased AMPKα and Akt phosphorylation when compared with the diabetic + NS group, and did not influence the protein expression of PKA (Fig. 1C and D).

To determine whether changes in the levels of AMPK, Akt and eNOS phosphorylation are responsible for the improvement of endothelial function in irisin treatment group, we performed ex vivo intervention experiments of irisin, high glucose, compound C, LY294002 and L-NAME on aortic rings from apoE⁻/⁻ male mice. The concentration-dependent vasodilation in response to ACh was significantly decreased in the HG group compared with NG group (38.56 ± 3.63% vs. 95.52 ± 3.14% vasodilation at 10⁻⁴ mmol/L ACh, n = 10 aortic rings in each group, p < 0.01 vs. NG group), while irisin attenuated the decrease of endothelial function induced by high glucose (87.43 ± 3.79% vs. 38.56 ± 3.63% vasodilation at 10⁻⁴ mmol/L ACh, n = 10 aortic rings in each group, p < 0.05 vs. HG group), which was still lower than that in NG group (95.52 ± 3.14% vasodilation at 10⁻⁴ mmol/L ACh, p < 0.05). The protective effect of irisin on endothelial dysfunction was abolished by compound C or LY294002 or L-NAME (Fig. 1E). In contrast, the vascular endothelium-independent vasodilation did not differ among different groups (Fig. 1F).

### 3.3. Irisin decreased endothelial cell apoptosis in vivo and in vitro

Apoptosis of endothelial cells has been suggested to play a role in atherosclerosis [17]. Thus, we tested the apoptosis of endothelial cells in vivo and in vitro in the present study. TUNEL staining with double-stain was applied to identify the apoptotic cells in aortic cross sections of apoE⁻/⁻ mice treated with irisin or not (Fig. 2A). The percentage of TUNEL-positive cells within the endothelium was significantly increased in diabetic mice, and irisin treatment significantly decreased apoptotic endothelial cells (22.51 ± 1.92% in diabetic + irisin group vs. 42.83 ± 3.07% in diabetic + NS group, p < 0.01) (Fig. 2B). As expected, the electron microscopy also showed apoptotic profiles with karyopyknosis in some of endothelial cells and disconnection from the underlying internal elastic lamina (IEL) in diabetic + NS group, while most of endothelial cells covered well on the surface of luminal wall in diabetic + irisin group (Fig. 2C). For the in vitro experiments, first of all, we investigated the concentration-dependent and time-dependent responses for irisin in the phosphorylation of eNOS (Ser1177) by Western blot, and the results showed that the best concentration of irisin for eNOS activation was 100 ng/ml, and the best time was 30 min (Supplementary Fig. 4). Using this intervention condition, the in vitro experiments were performed. TUNEL staining exhibited that exposure of endothelial cells to high glucose (20 mmol/L) for 48 h resulted in a significant increase in apoptosis when compared with NG group (25.96 ± 3.46% vs. 6.23 ± 1.61%, p < 0.01 vs. NG group) (Fig. 3A and B), and irisin reduced high glucose-induced...
endothelial cell apoptosis (20.60 ± 2.79%, p < 0.05 vs. HG group). The protective role of irisin on endothelial apoptosis was blocked by the addition of compound C, LY294002 or L-NAME. Moreover, the suppression of AMPK and eNOS expression by siRNA significantly restrained the effect of irisin on endothelial apoptosis compared with Scr-siRNA transfection (p < 0.05) (Fig. 4A, B, D and E). In addition, Western blot analysis of apoptotic proteins (Bcl-2 and Bax) in cultured endothelial cell is consistent with these findings (Figs. 3C, 4C and F). Taken together, these data suggest that irisin has protective effect of endothelial cell damage induced by diabetes, which may be mediated by the AMPK-PI3K-Akt-eNOS signaling pathway.

3.4. Irisin stimulated AMPK-PI3K-Akt-eNOS signaling in cultured endothelial cells

Our in vivo and ex vivo experiments showed that AMPK-PI3K-Akt-eNOS signaling was involved in the protective effect of irisin on endothelium. We next explored the effects of irisin on AMPK-PI3K-Akt-eNOS pathway in vitro. Firstly, our results showed that high glucose inhibited Akt and eNOS phosphorylation, and irisin (100 ng/ml) increased Akt and eNOS phosphorylation in high glucose condition in HUVECs (Supplementary Fig. 5A and B), and the stimulatory effects of irisin on Akt and eNOS phosphorylation were blocked by LY294002 or L-NAME. Furthermore, the concentrations of NO in culture media by different treatment were consistent with the changes of eNOS phosphorylation (Supplementary Fig. 3B). The levels of PKA phosphorylation did not change among different treatment groups (Supplementary Fig. 5C). Secondly, PI3K activity can be regulated by AMPK in endothelial cells [20,21]. We found that irisin stimulated the phosphorylation of AMPKz, Akt and eNOS against high glucose (Fig. 5A, B and C), and knock-down of the AMPK by siRNA can inhibit these effects of irisin. These findings indicated that irisin could activate AMPK-PI3K-Akt-eNOS signaling pathway in endothelial cells.

3.5. Irisin alleviated atherosclerotic lesion formation in ApoE−/− diabetic mice

Endothelial injury is the early pathophysiologic change of atherosclerosis, approaches that protect against endothelial injury may be therapeutic for atherosclerosis. Therefore, we investigated whether irisin alleviate atherosclerosis besides protecting vascular endothelium. For this purpose, en face and cross sections analyses were performed in atherosclerotic plaque. By Oil Red O staining (Fig. 6A), we found that diabetes significantly increased atherosclerotic plaque area, and plaque area of en face sections were significantly decreased in irisin-treated mice when compared with diabetic + NS group (22.57 ± 2.17% vs. 35.09 ± 2.38%, p < 0.05) (Fig. 6B). Similarly, as compared with NS treatment, irisin decreased plaque area of cross sections (16.87 ± 1.85% vs. 33.46 ± 5.77%, p < 0.05 vs. diabetic + NS group) (Fig. 6C and D).

Endothelial injury decreases NO production, leading to inflammatory lesion in aortas[18,19]. In our in vivo study, irisin protected against endothelial injury and increased serum NO levels in diabetic mice. We next questioned whether irisin could reduce the inflammatory lesion in aortas. Therefore, identification of macrophages and T lymphocytes in atherosclerotic plaque was performed with anti-CD68 and anti-CD90 staining, respectively (Fig. 7A). Quantification of CD68-and CD90-positive area revealed that diabetes significantly increased inflammatory infiltration within plaque, and irisin treatment significantly reduced inflammatory...
infiltration within plaque when compared with diabetic + NS group (CD68: 35.37 ± 3.59% vs. 50.02 ± 5.73%, p < 0.05; CD90: 22.52 ± 4.43% vs. 34.09 ± 4.08%, p < 0.05, vs. diabetic + NS) (Fig. 7B).

In addition, the inflammatory cytokines in aortas were assessed by quantitative RT-PCR. As shown in Fig. 7C, irisin treatment significantly down-regulated the mRNA expression levels of IL-6, TNF-α, ICAM-1, VCAM-1 and MCP-1, but not that of IL-10 (p > 0.05).

Fig. 4. Reduction of high glucose-induced apoptosis by irisin was blocked by AMPK or eNOS gene silencing. The apoptosis of cultured HUVECs in high glucose with or without irisin after knockdown of AMPK or eNOS expression by small interfering RNA (siRNA) (Scr-siRNA indicates scramble siRNA). A and D: HUVECs apoptosis detected by Annexin V-FITC/PI staining (A: siRNA for eNOS; D: siRNA for AMPK). B and E: the percentage of apoptotic cells (B: after knockdown for eNOS; E: after knockdown for AMPK). C and F: the expressions of apoptotic proteins by Western blot analysis (C: after knockdown for eNOS; F: after knockdown for AMPK). Values are mean ± SD (each experiment repeated 6 times). *p < 0.05, **p < 0.01.

Fig. 5. Irisin stimulated AMPK-PI3K-Akt-eNOS signaling in endothelial cells cultured with high glucose. HUVECs were cultured in media containing normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 20 mmol/L) with or without irisin after knockdown of AMPK expression by small interfering RNA (siRNA) (Scr-siRNA indicates scramble siRNA). AMPK (A), Akt (B) and eNOS (C) phosphorylation were detected by Western blot. Values are mean ± SD (each experiment repeated 6 times). *p < 0.05, **p < 0.01.
3.6. Irisin inhibited oxidative stress in vivo and in vitro

Our previous studies have revealed that high glucose increased ROS generation in endothelial cells, resulting in endothelial injury [20–23]. In the present study, we found that irisin protected against endothelial injury in vivo and in vitro. Therefore, we investigated the effect of irisin on oxidative stress. The results showed that high glucose-induced reduction in total antioxidant capacity was significantly improved by irisin treatment in mice (Supplementary Fig. 6A). In addition, a 2.85-fold increase in superoxide production was observed in aortic ring segments isolated from apoE−/− diabetic mice, and treatment with irisin significantly prevented aortic superoxide overproduction (Supplementary Fig. 6B). We also measured intracellular ROS in cultured endothelial cells incubated with high glucose in the presence or absence of irisin. High glucose-induced ROS generation in endothelial cells was dramatically increased compared with NG group (p < 0.01), whereas irisin treatment markedly inhibited it (p < 0.01 vs. HG group) (Supplementary Fig. 7A and B). Moreover, the inhibitory effect of irisin on ROS generation was abolished by compound C or LY294002 or L-NAME. On the other hand, the mRNA expression levels of GPX-1, CAT and SOD were reduced in high glucose-treated endothelial cells, and irisin treatment significantly up-regulated antioxidant enzymes expression (p < 0.05), and the effect could also be blocked by compound C or LY294002 or L-NAME (Supplementary Fig. 7C).

4. Discussion

In the present study, we have demonstrated for the first time that systemic administration of irisin protected against endothelial injury and ameliorated atherosclerosis induced by diabetes in the apoE−/− mice. Such benefit is related to an activation of AMPK-PI3K-Akt-eNOS signaling pathway and inhibition of oxidative stress by irisin. Thus, irisin can be considered as a potential therapeutic for diabetic angiopathy.

Atherosclerosis is considered as an inflammatory disease [24]. Inflammatory process participates in all stages of atherosclerosis [24,25]. The endothelium plays a crucial role in regulating inflammatory process [26]. Endothelial injury has been shown to promote atherosclerosis initiation and progression [27]. Numerous studies have evaluated interventions to improve endothelial dysfunction and many of these are known to limit atherosclerotic vascular disease [28–30]. The present study showed that diabetes reduced endothelium-dependent vasodilation and increased endothelial apoptosis in apoE−/− mice, while treatment with irisin predominantly improved endothelial dysfunction and reduced endothelial apoptosis. Moreover, our data demonstrated that irisin decreased the plaque area and the infiltrating macrophages and T lymphocytes in the plaques, and down-regulated the mRNA expression of inflammatory cytokines in the aortas. Also, our in vitro experiments suggested that irisin decreased endothelial cell apoptosis induced by high glucose. Moreover, the irisin concentration 100 ng/ml used...
for the cell culture experiments is very similar to that of plasma levels of mice (Table 1), indicating that endogenous irisin circulating in the blood has a very significant role in regulating endothelial function and development of atherosclerotic lesions. Taken together, these results suggested that irisin could protect against endothelial injury, consequently ameliorate inflammatory reaction and atherosclerosis.

Numerous studies have shown that inflammation results in insulin resistance, and the improvement of insulin sensitivity alleviates endothelial dysfunction and atherosclerosis [31–34]. In this study, glucose and insulin tolerance tests in STZ-induced apoE−/− diabetic mice fed by high fat diet displayed impairment in both glucose tolerance and insulin sensitivity when compared with non-diabetic mice, and irisin treatment for 12 weeks significantly improved insulin and glucose tolerance when compared with diabetic without irisin, suggesting that irisin can improve insulin resistance in STZ-induced apoE−/− diabetic mice. As a result, the fasting glucose and HbA1c in apoE−/− diabetic mice significantly decreased in irisin treatment group, which is consistent with the previous study in insulin resistance mice [7]. Therefore, the improvement of plasma glucose and insulin sensitivity can partially explain the endothelium-protective as well as anti-atherosclerotic effects of irisin. However, our ex vivo intervention experiments on aortic rings from apoE−/− male mice showed that irisin can reverse the impairment of endothelium-dependent vasodilation induced by high glucose, indicating that irisin may improve endothelial dysfunction by direct effect on endothelium independent of directly ameliorating diabetic control.

We next questioned whether oxidative stress involved in the endothelium-protective and anti-atherosclerotic effects of irisin in diabetic conditions. Many studies have revealed that exposure of endothelial cells to high glucose apparently increases the generation of ROS [35,36], which inhibits production of NO by blocking eNOS phosphorylation and activates the apoptotic pathways implicated in endothelial cells. This contributes to endothelial damage and accelerates atherosclerosis. Moreover, hyperglycemia blunted the defense of antioxidant enzymes, thus allowing excessive intracellular ROS accumulation resulting in endothelial damage. Our results showed that high glucose decreased eNOS phosphorylation, NO production and increased ROS generation, consequently endothelial injury and atherosclerosis were deteriorated, and irisin can partially reverse these reactions. We found that irisin prevented ROS overproduction in aortic ring segments and suppressed high glucose-induced ROS generation in HUVECs. Furthermore, irisin up-regulated the mRNA expression of antioxidant enzymes (GPX-1, CAT and SOD) in HUVECs, consequently endothelial injury and atherosclerosis were ameliorated. At least in part, we can conclude that irisin improves diabetes-induced endothelial dysfunction and apoptosis through (eNOS−NO−ROS) pathway.
The molecular signal pathways for the protective effect of irisin on artery are unclear. It has been proven that AMPK-PI3K-Akt-eNOS pathway plays an important role in prevention of endothelial injury [20,21,37]. Therefore, we examined the contribution of the AMPK-PI3K-Akt-eNOS pathway to the protective effects of irisin in high glucose condition. Firstly, irisin stimulated phosphorylation of AMPK, Akt and eNOS in aortas of apoE−/− mice, and consequently serum NO concentrations increased in irisin treated mice. Secondly, the improvement of endothelial function of irisin from ex vivo intervention experiments on aortic rings was abolished by addition of inhibitors for AMPK, Akt and eNOS. Thirdly, irisin reversed the decreased phosphorylation of AMPK, Akt and eNOS, and production of NO in cultured endothelial cells, and the effect of irisin on eNOS phosphorylation and NO production could also be blocked by compound C, LY294002 and l-NAME. Fourthly, knockdown of eNOS expression by siRNA restrained the protective effect of irisin on endothelial apoptosis. These results suggested that the AMPK-PI3K-Akt-eNOS pathway is involved in the endothelium-protective effects of irisin. However, very recently, Haibo Song et al. found that treating HUVeCs with irisin (20 nmol/L) had no effect on Akt signaling [14]. Possibly, the concentration of irisin was not enough to activate Akt signaling in HUVeCs.

Some limitations should be mentioned here. Firstly, the relationship between cardiovascular disease and diabetes has been mechanistically demonstrated in a recent report focusing on intracellular calcium [38]. However, extracellular calcium ions in endothelium were not measured in the present study. Secondly, we did not explore the Akt/mammalian target of rapamycin (mTOR) pathway, because some studies showed that Akt/mTOR pathway was involved in cardiovascular diseases [39,40]. Thirdly, it is established that calcium/calmodulin-dependent kinase IV (CaMKIV) and adrenergic system are associated with the regulation of vascular tone [41,42]. In the present study, we did not investigate the effect of irisin on the association between CaMKIV, adrenergic system and vascular tone. Fourthly, we did not measure blood pressure or serum lipids, thus, the changes of blood pressure and serum lipids before and after irisin treatment could not be evaluated. Finally, we can not provide a better imaging of the endothelial layer (i.e. using en face preparations of the vessels). Therefore, some further studies are needed.

In summary, by studying apoE−/− diabetic mice treated with irisin, our results showed that irisin may play a crucial role in protecting against endothelial injury and alleviating atherosclerosis. The in vivo, ex vivo and in vitro experiments established that the protective effects of irisin on endothelium are through the activation of AMPK-PI3K-Akt-eNOS signaling pathway. Our study suggests that irisin could be therapeutic for the treatment of atherosclerotic vascular diseases in diabetes mellitus.

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**Conflict of interest**

None.

**Author's contributions**

Guangda Xiang performed research design; Junyan Lu and Min Liu conducted the animal experiment and the data analysis; Wen Mei and Lin Xiang performed the ex vivo and in vitro experiments; Jing Dong conducted the manuscript writing.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2015.10.020.

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