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Graphical abstract

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Exercise hormone irisin mitigates endothelial barrier dysfunction and microvascular leakage related diseases

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Declaration of Interests
The authors have declared that no conflict of interest exists
Abstract

Increased microvascular leakage is a cardinal feature of many critical diseases. Regular exercise is associated with improved endothelial function and reduced risk of cardiovascular disease. Irisin, secreted during exercise, contributes to many health benefits of exercise. However, the effects of irisin on endothelial function and microvascular leakage remain unknown. In this study, we found that irisin remarkably strengthened endothelial junctions and barrier function via binding to integrin αVβ5 receptor in LPS-treated endothelial cells. The beneficial effect of irisin was associated with suppression of the Src-MLCK-β-catenin pathway, activation of the AMPK-Cdc42/Rac1 pathway and improvement of mitochondrial function. In preclinical models of microvascular leakage, exogenous irisin improved pulmonary function, decreased lung edema and injury, suppressed inflammation, and increased survival. In ARDS patients, serum irisin levels were decreased and inversely correlated with disease severity and mortality. In conclusion, irisin enhances endothelial barrier function and mitigates microvascular leakage related diseases.

Keywords: irisin; endothelial permeability; microvascular leakage; integrin αVβ5 receptor; Src; AMPK; mitochondrial function
Introduction

Microvascular leakage is a pivotal pathological process in many diseases, such as asthma, sepsis, acute respiratory distress syndrome, anaphylaxis and diabetic retinopathy. Increased endothelial permeability is main cause of microvascular leakage (1). On one hand, endotoxin, inflammatory factors and neutrophils facilitate endothelial myosin light chain (MLC) phosphorylation to combine with actin and induce the phosphorylation of β-catenin and the separation of VE-cadherin from the cytoskeleton, resulting in disruption of adhesion junctions between endothelial cells (2, 3). On the other hand, Rho GTPase family members dynamically regulate intercellular junctions and cytoskeletal remodeling via the formation of cortical actin. Under disease conditions, decreased activation of Cdc42 and Rac1 and increased activation of Rho lead to cytoskeletal remodeling, resulting in endothelial cell shrinkage, intercellular broadening and ultimately increased vascular permeability (4, 5). Additionally, mitochondrial dysfunction, including decreased mitochondria quantity, imbalanced mitochondrial dynamics, mitochondrial fragmentation, respiratory chain inhibition and massive reactive oxygen species (ROS) generation, directly damages endothelial cells and increases pulmonary endothelial permeability (6-8).

It has been widely reported that regular exercise can improve endothelial function and slow the progression of atherosclerosis (9). Irisin, a newly identified hormone secreted by skeletal muscle during exercise, was initially discovered as a myokine responsible for browning white fat (10). Subsequent studies have shown that irisin is implicated in type 2 diabetes, obesity, aging and mitochondrial function (11). A recent study verified that integrin αVβ5 is the receptor of irisin in osteocytes and fat cells (12). Vascular integrins are major mediators of endothelial adhesion to extracellular matrix (13). Src, downstream pathway of vascular integrins, directly alters the structure of the endothelial barrier by phosphorylation of MLCK, β-catenin and focal adhesion (14). Several studies have reported that the effects of irisin in improved energy metabolism are associated with activation of adenosine monophosphate activated protein kinase (AMPK), the central metabolic sensor (15, 16). Interestingly, in addition to regulating energy metabolism, AMPK activation can also strengthen the aggregation of microtubules and myosin to protect vascular barrier function (17-19). Previous
studies have shown that mitochondrial ATP generation regulates endothelial cytoskeletal remodeling by Rac activation (20). However, the role of irisin in microvascular endothelial permeability remained unknown. We therefore hypothesized that irisin strengthened endothelial junctions and barrier function via binding to integrin αVβ5 receptor, further inhibiting the P-Src (Y416)/P-MLCK(Y464)/P-β-catenin(Y142) pathway and activating the AMPK-mitochondria-Cdc42/Rac1 pathway in endothelial cells. The main purpose of this study was to explore whether irisin benefits the endothelial barrier function. Additionally, this study also sought to clarify the therapeutic effect of irisin on microvascular leakage related diseases.
Results

Irisin strengthened endothelial junctions and barrier function.

Phalloidin and VE-cadherin were stained to assess cytoskeletal remodeling and adherens junction integrity in endothelial cells, respectively. These results showed that LPS induced massive formation of actin stress fibers and intercellular gaps due to cell contractions in HMVECs. These changes were largely reduced by irisin treatment (Figures 1A-B). Meanwhile, transwell permeability assays were performed to verify endothelial cell permeability. Irisin treatment significantly decreased the LPS-induced increase in FITC-labeled albumin in HMVECs (Figure 1C). Additionally, endothelial cell permeability was assessed by TER in endothelial cell monolayers. TER measurement and determination of the maximum TER relative to baseline revealed that 10 nM Irisin significantly increased the TER in HMVECs (Figure 1D). Moreover, irisin treatment largely reversed the decreasing trend in TER after LPS challenge in HMVECs (Figure 1E). The similar results were showed in HUVECs. Irisin decreased the formation of actin stress fibers and enhanced the VE-cadherin and β-catenin mediated adherens junction (Figure 1F). Besides, the irisin-treated HUVECs showed increasing trend in TER and decreasing endothelial cell permeability (Figures 1G-J).

Exogenous irisin administration alleviated microvascular leakage related diseases

At 24 h after LPS administration intratracheally and 21 h after CLP operation, serum irisin levels were decreased in LPS- and CLP-treated mice compared with control mice, while exogenous irisin administration significantly increased serum irisin levels (p<0.05, Figure 2A). TEM analysis showed distinct increases in joint gaps between pulmonary microvascular endothelial cells, whereas Irisin treatment abolished this change to a great extent (Figure S1). Levels of total cells and total proteins in the BALF increased greatly after LPS administration intratracheally, while exogenous irisin treatment immediately or 6 hours after LPS administration intratracheally showed significant reductions. (Figures 2B-C). H&E staining of the lung tissues showed a mass of alveolar hemorrhage, inflammatory cell infiltration and alveolar wall thickening after LPS administration. Irisin treatment immediately or 6 hours after LPS administration significantly alleviated these changes. Consistent with these histological changes, irisin treatment significantly decreased the ALI scores and water content compared
with the control-treated animals in the above models (Figures 2D-F). Arterial blood gas analysis showed irisin treatment immediately or 6 hours after LPS administration reversed the decrease in PaO$_2$ and increase in PaCO$_2$ levels at 24 h after LPS administration intratracheally (Figures 2G-H). Meanwhile, we found that irisin neutralizing antibody pretreatment further increased the levels of total cells and proteins in the BALF, aggravated tissue damage and decreased PaO$_2$ after LPS administration (Figures 2B-H). To further verify the protective effect of irisin, we used clp-, gut IR- and aged rat liver IR-induced ALI models. The results showed that irisin significantly relieved liver tissue damage and reduced the levels of total cells, total proteins and inflammatory cytokines after LPS administration, CLP, gut IR and aged rat liver IR induction. Meanwhile, low concentration of irisin treatment (50 μg/kg) showed limited effects after CLP operation (Figures 2I-M, figure S2 and figure S3B-G). In addition, mice treated with 250 μg/kg exogenous irisin had a higher survival rate than normal saline-treated mice after CLP operation, although there was no difference in weight loss between untreated and treated mice (Figures 2N and S3A).

Blood cell analysis revealed a significant increase in inflammatory cell number in BALF 24 h after LPS treatment. Administration of irisin significantly reduced WBC, lymphocyte, monocyte and neutrophil counts in the BALF (Figures S4A-D). MPO immunostaining also showed a lower percentage of MPO-positive cells in the lungs of irisin-treated mice compared with vehicle-treated mice (Figures S4E-F). The levels of TNF-α, MIP-2 and CIRP were determined in the BALF; irisin treatment markedly reduced the increases in the levels of these inflammatory factors after LPS administration (Figures S4G-I). The serum levels of TNF-α, MIP-2 and CIRP showed similar results, and serum IL-10 levels largely increased after irisin treatment in LPS-treated mice (Figures S4J-M).

**Irisin enhanced endothelial cell barrier function via binding to integrin αVβ5 receptor and suppression of the P-Src (Y416)/P-MLCK(Y464)/P-β-catenin(Y142) pathway.**

Immunofluorescent staining showed an observable co-localization of irisin and integrin αvβ5 proteins in irisin treated-HMVECs (Figure 3A). CO-IP analysis demonstrated that irisin could directly bind to the integrin αVβ5 receptor in the lung (Figure 3B). Cilengitide trifluoroacetate, an inhibitor of integrin αVβ5, is used to further confirm whether irisin enhanced endothelial
cell barrier via integrin αVβ5. We found that cilengitide trifluoroacetate alone induced obvious gap formation. Meanwhile, cilengitide trifluoroacetate abolished the protective function of irisin in decreasing albumin permeability and intercellular gaps area in HMVECs (Figures 3C-E). Additionally, Consistent with the in vitro experiments, the cilengitide-treated mice lost therapeutic effects of irisin in reducing exudation of proteins and cells and water content in the lungs (Figures 3F-H). Western blot showed that irisin significantly decreased the phosphorylation of Src, MLCK and β-catenin at Tyr416, Tyr464, Tyr142, respectively, after LPS treatment (Figures 3I-L). Meanwhile, cilengitide abolished the protective role of irisin in decreasing P-Src, P-MLCK and P-β-catenin (Figures 3M-P).

**Irisin restored endothelial barrier function by activation of AMPK-Rac1/cdc42 Signaling.**

AMPK phosphorylation at Thr172 was downregulated after LPS challenge, whereas irisin treatment significantly increased the activation of AMPK in HMVECs. Additionally, despite the reduction of Rac1 and cdc42 activation after LPS administration, irisin treatment markedly increased activation of both Rac1 and cdc42 in HMVECs (Figures 4A-D). Furthermore, cilengitide trifluoroacetate markedly reversed the increased activation of AMPK after irisin administration (Figures 4E-F). To further determine the relationship between AMPK phosphorylation and activation of Rac1 and cdc42, AMPK siRNA was transfected. The protective effects of irisin in upregulating the activation of Rac1 and cdc42 were abolished in HMVECs transfected with AMPK siRNA at 2 h and 8 h after LPS treatment (Figures 4G-I). Fluorescence staining showed that HMVECs transfected with AMPK siRNA markedly increased the formation of actin stress fibers and intercellular gaps compared with normal HMVECs after LPS and irisin treatment (Figures 4J-K). Similarly, AMPK siRNA-treated HMVECs exhibited lower TER and higher FITC-labeled albumin permeability in contrast to nonspecific siRNA-treated HMVECs (Figures 4L-M).

**Compound C abolished the protective effects of irisin on LPS-induced microvascular leakage.**

A prominent increase in AMPK phosphorylation at Thr172 was observed in irisin-treated mice after LPS treatment, which completely abolished the reduction in LPS-treated mice (Figures 5A-B). Administration of compound C, an AMPK inhibitor, decreased the activation of Rac 1 after irisin treatment in LPS-induced microvascular leakage (Figures 5C-D). Meanwhile, total
cell and protein levels in the BALF were dramatically increased after compound C treatment (Figures 5E-G). Additionally, mice that received compound C showed more serious histological changes, higher water content, lower PaO$_2$ levels and higher PaCO$_2$ levels compared with the control-treated mice (Figures 5H-L). Moreover, ATP production was significantly increased after irisin treatment, while compound C reversed this change in LPS-induced microvascular leakage (Figure 5M).

**Irisin protected mitochondrial function in endothelial cells.**

Mitochondrial dysfunction of vascular endothelial cells is a pivotal pathologic mechanism of endothelial cell hyperpermeability. The expression of mitochondrial biogenesis-related peroxisome proliferative activated receptor-γ (PPARγ) coactivator 1α (PGC-1α) and mitochondrial transcription factor (Tfam) were decreased after LPS challenge in HMVECs. Irisin treatment significantly upregulated PGC-1α and Tfam expression (Figures 6A-C). Furthermore, HMVECs transfected with AMPK siRNA showed marked reduction of PGC-1α and Tfam expression at 2 h and 8 h after LPS and irisin treatment (Figures 6D-E). To determine the overall number of mitochondria, mitotracker staining of live HMVECs was performed. Irisin markedly restored the LPS-induced decrease in mitochondria number. However, AMPK siRNA treatment significantly abolished the protective function of irisin in mitochondrial biogenesis (Figures 6F-G). Additionally, ATP biosynthesis-related protein ATP synthase β (ATPB) and mitophagy-related PTEN-induced putative kinase 1 (PINK-1) expression levels were increased after irisin administration (Figures 6H-J). Consistent with Western blot results, ATP levels were significantly increased in irisin-treated HMVECs 2 h and 8 h after LPS challenge (Figure 6K). Furthermore, significant increases in uncoupling protein (UCP) 2 expression were found after irisin treatment (Figures 6L-M). ROS generation (detected by DHE staining) showed that irisin treatment markedly decreased ROS fluorescence intensity in LPS-treated HMVECs (Figures 6N-O). Meanwhile, irisin markedly inhibited the expression of mitochondrial fission-related proteins dynamin related protein 1 (drp-1) and fission 1 (Fis-1) in LPS-treated HMVECs (Figures S5).

**Serum irisin levels were decreased and negatively correlated with disease severity and mortality in ARDS patients.**
Blood samples from 60 ARDS patients and 60 healthy volunteers were collected, patient demographics and serum irisin levels were measured (Table S1). As shown in Figure 7A, serum irisin levels were decreased in ARDS patients compared to healthy volunteers (p<0.05). Additionally, serum irisin levels were negatively correlated with APACHE II scores ($R^2=0.1336$, $p=0.004$, Figure 7B). Meanwhile, there is a weak correlation between serum irisin and SOFA scores ($R^2=0.0687$, $p=0.045$, Figure 7C). The survival analysis showed that patients with serum irisin levels $\geq 2.75$ ng/ml had a lower mortality (Figure 7D). The univariate analysis showed hypertension, SOFA score at admission, APACHEII score at admission, and serum irisin levels were associated significantly with 28-day mortality of ARDS patients. However, in the multivariate analysis, APACHEII score at admission [hazard ratio (HR): 1.375, 95% CI: 1.009-1.874, $p=0.044$] and serum irisin levels [HR: 0.153, 95% CI: 0.024-0.961, $p=0.045$] were independently associated with 28-day mortality of ARDS patients (Table S2).
Discussion

In the present study, we found that low serum irisin was associated with worse outcomes in ARDS patients, and exogenous irisin protected against endothelial barrier dysfunction and microvascular leakage related diseases via binding to integrin αVβ5 receptor, further inhibiting the P-Src (Y416)/P-MLCK(Y464)/P-β-catenin(Y142) pathway, activating the AMPK-Cdc42/Rac1 pathway and improving mitochondrial function in endothelial cells (Figure 8). Irisin may, therefore, assist with the urgent medical need for preventing or minimizing ARDS and other microvascular leakage related diseases.

Irisin, mainly secreted by the skeletal muscle during exercise, was initially discovered as a myokine responsible for the browning of white fat and thermogenesis in 2012 (10). Subsequent studies have shown that irisin regulates glucose/lipid metabolism and has antioxidant functionality in type 2 diabetes (21, 22). Additionally, irisin has shown protective effects on mitochondrial function in ischemia/reperfusion injury (23). Irisin therefore is anticipated to provide solutions for energy metabolism-related problems. A previous study compared lung injury before and after irisin administration in LPS-treated mice (24). In this study, we found that irisin remarkably strengthened endothelial junctions and barrier function via binding to integrin αVβ5 receptor in LPS-treated human endothelial cells. Serum irisin levels were decreased and negatively correlated with disease severity and mortality in ARDS patients, suggesting that irisin levels may predict the severity and prognosis of ARDS. More importantly, irisin showed dramatic therapeutic effects in multiple animal models of microvascular leakage related diseases, suggesting a novel treatment approach for endothelial barrier dysfunction and microvascular leakage related diseases. Additionally, we found that irisin neutralizing antibody pretreatment increased the levels of total cells and proteins in the BALF, aggravated tissue damage and decreased PaO2 after LPS administration, suggesting that endogenous irisin plays an important role in regulating endothelial barrier function.

The destruction of endothelial barrier integrity is pivotal in the pathogenesis of ARDS, sepsis, anaphylaxis and other diseases (25). The mechanisms underlying this increased endothelial permeability have multiple suggested hypotheses. First, endotoxin can directly cause endothelial cell shrinkage and indirectly damage vascular endothelial cells by activating
inflammatory cells, toxic oxygen free radicals, peroxides, proteolytic enzymes and cytokines (1). Second, endotoxin and inflammatory factors cause MLC phosphorylation and joining with actin. Neutrophils induce the phosphorylation of β-catenin and the separation of VE-cadherin from the cytoskeleton, thereby weakening tight junctions between endothelial cells (2, 3). Moreover, the damage of endothelial progenitor cell (EPC) repair function in circulation results in the destruction of endothelial permeability (26).

Vascular integrins are major mediators of endothelial adhesion to extracellular matrix (13). SFK, especially Src, plays an important role in increasing permeability of endothelial cells under inflammatory conditions. Tyr416 (catalytic subunit localization) phosphorylation of Src enhances Src activity. Previous study has proved that Src directly alters the structure of the endothelial barrier by phosphorylation of MLCK, β-catenin and focal adhesion (14). Src deficient mice and inhibition of Src activity can reduce the degree of cerebral edema during stroke (27, 28). Interestingly, a recent study verified that integrin αVβ5 is the receptor of irisin in osteocytes and fat cells (12). However, whether irisin affects endothelial barrier function remains unclear. In this study, we found that irisin significantly decreased the activation of Src and inhibited the phosphorylation of MLCK, β-catenin. Irisin might enhanced endothelial barrier function via suppression of P-Src (Y416)/P-MLCK(Y464)/P-β-catenin(Y142) pathway.

AMPK is a central metabolic sensor regulating energy metabolism and mitochondrial function (19). Meanwhile, it has been shown that AMPK activation can also protect vascular barrier function by strengthening endothelial intercellular junctions and cytoskeletal remodeling (18). Interestingly, several studies have reported that the regulatory role of irisin in energy metabolism is associated with activation of AMPK (15, 29). In the present study, irisin treatment significantly increased the activation of AMPK in endothelial cells. Meanwhile, administration of compound C or AMPK siRNA abolished the protective function of irisin in microvascular leakage in both in vitro and in vivo experiments. Our study indicates that irisin treatment might restore endothelial barrier function via activation of AMPK.

The Rho GTPase family, including Rho, Rac1 and cdc42, regulates endothelial intercellular junctions and cytoskeletal remodeling (30). It has been shown that Rac 1 maintains tight junctions of endothelial cells via the formation of cortical actin in its GTP-bound state, i.e., the
active state (20, 31). Conversely, the GDP-bound state is associated with vascular leakage (32). AMPK activation can strengthen the aggregation of microtubules and myosin to protect vascular barrier function (18). In our study, we found that the activation of Rac1 and cdc42 was increased after irisin administration. However, AMPK siRNA transfection reversed the increased activation of Rac1 and cdc42 in irisin-treated HMVECs. Our results demonstrate that irisin may protect endothelial barrier function through activation of the AMPK-Rac1/cdc42 pathway.

Mitochondria regulate ATP synthesis, ROS production, apoptosis stimulation and aging (33). Mitochondrial dysfunction is an important cause of endothelial barrier dysfunction (34). Maintaining sufficient quality and quantity of mitochondria in endothelial cells is an essential requirement for endothelial barrier integrity (6). Mitochondrial biogenesis is responsible for the generation of mitochondria, which is regulated by PGC-1α and its downstream target Tfam (35, 36). Many studies have shown that PGC-1α expression is regulated by AMPK activation (37, 38). Furthermore, ATP facilitates Rac activation and cortactin formation to exert endothelial barrier protection (8, 20). ATP synthase β is a key enzyme in the process of ATP synthesis. Previous studies have shown that irisin protects mitochondrial function in instances of ischemia/reperfusion injury (23). Our study revealed that irisin treatment reversed the decreased expression of PGC-1α and TFAM after LPS treatment, which was abolished by transfection with AMPK siRNA. Mitotracker staining showed that irisin treatment significantly increased mitochondria numbers. Additionally, irisin restored ATPB expression and ATP levels after LPS administration. Taken together, our results reveal that irisin may facilitate endothelial barrier function via facilitating AMPK/PGC-1α signaling-dependent mitochondrial biogenesis and increasing ATP production.

Certain limitations should be considered regarding this study. On the one hand, the present study focused on the role of irisin in endothelial barrier function in lung injury. The effects of irisin on lung epithelial cells and any other pathogenic mechanisms require further exploration. On the other hand, although exogenous irisin protects against microvascular leakage by restoring lung endothelial barrier function, the therapeutic effects identified here are only based on basic experiments, and prospective clinical studies are needed.
In conclusion, Irisin mitigates endothelial barrier dysfunction and microvascular leakage related diseases via binding to integrin αVβ5 receptor, further inhibiting the P-Src (Y416)/P-MLCK(Y464)/P-β-catenin(Y142) pathway, activating the AMPK-Cdc42/Rac1 pathway and improving mitochondrial function in endothelial cells. Low serum irisin was associated with worse outcomes in ARDS patients, and exogenous irisin protected against microvascular leakage related diseases Irisin may, therefore, assist with the urgent medical need for preventing or minimizing microvascular leakage related diseases.
Methods
Detailed methods are provided in the online supplement.

Patients
This study included 60 adult ARDS patients (age ≥ 18 year) admitted to the First Affiliated Hospital of Xi’an Jiaotong University. ARDS was defined as having PaO2/FiO2 ≤ 300 mmHg, acute pulmonary infiltrates identified on chest X-ray or computed tomography and mechanical ventilation with a positive end-expiratory pressure (PEEP) of at least 5 cmH2O (39). The severity of ARDS was assessed using the Acute Physiology and Chronic Health Evaluation II (APACHE II) score and the Sequential Organ Failure Assessment (SOFA) score. Patient survival was monitored for 28 days after admission. Sixty healthy volunteers who underwent routine physical examination were included as healthy controls. The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi’an Jiaotong University. All study participants provided informed consent in accordance with the Declaration of Helsinki.

Experimental animals
Experiments were performed on male wild-type C57BL/6 J mice (aged 6-8 weeks, weighing 20–25 g) and male Sprague-Dawley rats (weighing 500–650 g, aged 22 months). All animal experiments were performed in accordance with the guidelines of the China Council on Animal Care and Use and approved by the Institutional Animal Care and Use Committee of the Ethics Committee of Xi’an Jiaotong University Health Science Center, China (approval number: 2017-564). All animal experiments conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. In this study, all the animals were anesthetized by inhaling 3% isoflurane. Euthanasia was conducted by exsanguination and cervical dislocation under deep anaesthesia with isoflurane in all animal experiments.

Statistical analysis
All measurement data are expressed as the means ± standard error (SEM). The t-test was used to analyze the differences between two groups and one-way ANOVA was used to analyze the differences among three or more groups. Spearman’s correlation coefficient (ρ) was used to analyze associations between two parameters. Kaplan-Meier curves were used for survival
analysis and log-rank testing for difference analysis. All analyses were conducted with data statistics software SPSS 18.0. P < 0.05 represented a significant difference.

**Study approval**

The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi’an Jiaotong University.
**Author Contributions**

Bi J participated in the research design, performed most experiments, statistical analysis and paper writing; Zhang J and Ren Y, participated in the animal studies and western blot analysis. Du Z and Zhang Y participated in the ELISA, statistical analysis and participated in the cell culture and immunofluorescence. Liu C, Zhang L, Wang Y and Shi Z collected the serum from patients and analyzed human data. Wu Z and lv Y assisted with the design of the study. Wu R designed and supervised the study and revised the manuscript. All authors have read and agreed with the submission of the manuscript.

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30. Cullere X, et al. Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange


Figure 1. Irisin strengthened endothelial barrier function and reduced LPS-induced endothelial cell hyperpermeability. HMVECs and HUVECs were treated with 10 nM irisin immediately after 500 ng/ml LPS administration. (A) Phalloidin and VE-cadherin staining for assessing cytoskeletal remodeling and adherens junction integrity in HMVECs 2 h after LPS and irisin treatment (scale bar = 20 μm); * in the Phalloidin and VE-cadherin staining represents gaps between the cells. (B) Gap areas in the total areas; (C) Relative permeability of FITC-labeled albumin 2 h after LPS and irisin treatment in HMVECs; (D) Transendothelial electrical resistance (TER) in irisin- and control-treated human microvascular endothelial cells (HMVECs); (E) TER in irisin- and control-treated HMVECs after 500 ng/ml LPS administration; (F) Phalloidin (scale bar = 10 μm), VE-cadherin and β-catenin (scale bar = 5 μm) staining for assessment of cytoskeletal remodeling and adherens junction integrity in HUVECs 2 h after LPS and irisin treatment; Arrows in the VE-cadherin/β-catenin staining represents gaps between the cells. (G) Transendothelial electrical resistance (TER) in irisin- and control-treated human umbilical vein endothelial cells (HUVECs); (H) Maximum TER relative to baseline; (I) TER in irisin- and control-treated HUVECs after 500 ng/ml LPS administration; (J) Relative permeability of FITC-labeled albumin 2 h after LPS and irisin treatment in HUVECs; n=6 per
group, mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the LPS group. The t-test was used to analyze the differences between two groups and one-way ANOVA was used to analyze the differences among three or more groups.

Figure 2. Exogenous irisin administration alleviated microvascular leakage related diseases. Irisin was given by intravenous administration (250 μg/kg, a single dose) immediately or 6 h after LPS administration intratracheally (2 mg/kg), and immediately after CLP operation. Irisin-neutralizing antibody was administrated by intravenous injection in mice (50 μg/kg, a single dose) 24 h before LPS administrated intratracheally. Vehicle group of mice was given equivalent amounts of saline. At 24 h after LPS administrated intratracheally or 21 h after CLP operation, lung tissue, BALF and arterial blood samples were collected. (A) Serum irisin levels; (B, C) Total cells and protein levels in bronchoalveolar lavage fluid (BALF) in LPS-induced lung microvascular leakage; (D) Water content of lungs; (E) Hematoxylin and eosin (H&E) staining in LPS-induced lung microvascular leakage (scale bar = 50 μm); (F) ALI scores; (G) At 24 h after
LPS administration, arterial blood was obtained from the abdominal aorta, and PaO$_2$ was assessed via blood gas analyzer; (H) PaCO$_2$ levels; (I) H&E staining of lung in CLP-induced sepsis; (J) ALI score; (K) Water content; (L, M) Total cells and protein levels in BALF in CLP-induced sepsis; (N) 7-day survival study in CLP-induced sepsis; Kaplan-Meier curves were used for survival analysis and log-rank testing for difference analysis. High irisin represents a dose of 250 μg/kg, Low irisin represents a dose of 50 μg/kg; n=6 per group, mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the LPS or CLP group; One-way ANOVA was used to analyze the differences between groups.

Figure 3. Irisin enhanced endothelial cell barrier function via binding to integrin αVβ5 receptor and suppression of P-Src (Y416)/P-MLCK(Y464)/P-β-catenin(Y142) pathway. Irisin (250 μg/kg, iv) and cilengitide trifluoroacetate (20 mg/kg, iv) were given by intravenous administration immediately after LPS administration intratracheally (2 mg/kg). Vehicle group of mice was given equivalent amounts of saline. Lungs are harvested 24 h after LPS administration. HMVECs were treated with 10 nM irisin and 20 μM cilengitide trifluoroacetate immediately after 500 ng/ml LPS administration. (A) Immunofluorescence co-localization of intergrin αVβ5 and irisin at 2h after LPS and irisin treatment in HMVECs (scale bar = 10 μm). (B) CO-IP of irisin and integrin αVβ5 at 24 h after LPS-induced microvascular leakage. (C) Relative
permeability of FITC-labeled albumin 2 h after LPS, irisin and cilengitide trifluoroacetate treatment in HMVECs; (D) Phalloidin and VE-cadherin staining for assessing cytoskeletal remodeling and adherens junction integrity (scale bar = 10 μm); * in the Phalloidin and VE-cadherin staining represents gaps between the cell. (E) Gap areas in the total areas; (F, G) Total cells and protein levels in bronchoalveolar lavage fluid (BALF) in LPS-induced microvascular leakage; (H) Water content of lungs in LPS-induced microvascular leakage; (I-L) Western blot analysis of phosphorylation of Src, MLCK and β-catenin at Tyr416, Tyr464, Tyr142, respectively. *P < 0.05 versus the sham group, #P < 0.05 versus the LPS or CLP group. (M-P) Western blot analysis of phosphorylation of Src, MLCK and β-catenin after treatment with cilengitide trifluoroacetate. *P < 0.05 versus the LPS group, #P < 0.05 versus the irisin group; n=6 per group, mean ± SEM; One-way ANOVA was used to analyze the differences between groups.

Figure 4. Irisin restored endothelial barrier function by activation of AMPK-Rac1/cdc42 signaling. HMVECs were treated with 10nM irisin immediately after 500 ng/ml LPS administration. (A-D) Western blot analysis of activation of adenosine monophosphate-activated protein kinase (AMPK), and Rac 1 and cdc42 activation assays in irisin- and control-treated HMVECs; (E-F) Western blot analysis of activation of AMPK in cilengitide trifluoroacetate- and control-treated HMVECs after 500 ng/ml LPS and 10nM irisin
administration; (G-I) Rac 1 and cdc42 activation assays in irisin- and LPS-treated HMVECs transfected with nonspecific siRNA or AMPK siRNA. (J) Phalloidin and VE-cadherin staining in HMVECs (scale bar = 10 μm); (K) Gap areas in the total areas; (L) TER; (M) Relative permeability of FITC-labeled albumin; * in the Phalloidin and VE-cadherin staining represents gaps between the cells; n=6 per group, mean ± SEM, *P < 0.05 versus the sham group or the LPS + irisin group; The t-test was used to analyze the differences between two groups and one-way ANOVA was used to analyze the differences among three or more groups.

Figure 5. Compound C abolished the protective effects of irisin on LPS-induced microvascular leakage. Mice were treated with irisin (iv, 250 μg/kg, a single dose) and compound C (2 mg/kg, an AMPK inhibitor) immediately after LPS administration. Vehicle group of mice was given equivalent amounts of saline. 24 h after LPS treatment, lung tissues, BALF and arterial blood samples were collected. (A, B) Western blot analysis of the expression of AMPK and AMPK phosphorylation at Thr172; (C,D) Rac 1 activation assays; (E-G) The total cells, total protein levels and WBC numbers in BALF, respectively; (H) H&E staining (scale bar = 20 μm); (I) ALI score; (J) Water content in lungs; (K)PaO2; (L) PaCO2; (M) lung ATP concentration in LPS-induced microvascular leakage. n=6 per group, mean ± SEM, *P < 0.05 versus the sham group, #P < 0.05 versus the LPS group; One-way ANOVA was used to analyze the differences between groups.
Figure 6. Irisin protected mitochondrial function in endothelial cells to restore endothelial barrier integrity. HMVECs were treated with 10 nM irisin immediately after 500 ng/ml LPS administration. (A-C) Western blot analysis of the expression of peroxisome proliferative activated receptor-γ (PPARγ) coactivator 1α (PGC-1α) and mitochondrial transcription factor (Tfam) in HMVECs 2 h and 8 h after LPS treatment; (D, E) Western blot analysis of PGC-1α expression in irisin- and LPS-treated HMVECs transfected with nonspecific siRNA or AMPK siRNA; (F, G) MitoTracker Red CMXRos fluorescence staining and fluorescence intensity of HMVECs 2 h after LPS, irisin and AMPK siRNA treatment (scale bar = 20 μm); (H-J) Western blot analysis of the expression of ATP synthase β (ATPB) and PTEN-induced putative kinase 1 (PINK-1) in HMVECs; (K) ATP concentration 2 h and 8 h after irisin and LPS treatment in HMVECs. (L, M) Western blot analysis of uncoupling protein (UCP) 2 expression in HMVECs; (N, O) DHE fluorescence staining and its fluorescence intensity of HMVECs at 2 h after LPS and irisin.
treatment (scale bar = 20 μm); n=6 per group, mean ± SEM, *P < 0.05 versus the sham group or LPS + irisin group, #P < 0.05 versus the LPS group; One-way ANOVA was used to analyze the differences between groups.

Figure 7. Serum irisin levels were decreased and negatively correlated with disease severity and mortality in ARDS patients. Blood samples from 60 ARDS patients and 60 healthy volunteers were collected, and serum irisin levels were measured. (A) Serum irisin levels in ARDS patients and healthy volunteers; The t-test was used to analyze the differences between two groups; (B) Correlation analysis of serum irisin and APACHE II scores; (C) Correlation analysis of serum irisin and SOFA scores; Spearman’s correlation coefficient (ρ) was used to analyze associations between two parameters; (D) Patients were divided into a high irisin group and low irisin group based on the median of irisin concentration and followed for 28 days after admission to the hospital to assess survival; Kaplan-Meier curves were used for survival analysis and log-rank testing for difference analysis; *P < 0.05 compared to healthy volunteers.
Figure 8. Exercise hormone irisin mitigates endothelial barrier dysfunction and microvascular leakage related diseases.