ISCHEMIA/REPERFUSION INJURY

Irisin protects mitochondria function during pulmonary ischemia/reperfusion injury

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Limb remote ischemic preconditioning (RIPC) is an effective means of protection against ischemia/reperfusion (IR)–induced injury to multiple organs. Many studies are focused on identifying endocrine mechanisms that underlie the cross-talk between muscle and RIPC-mediated organ protection. We report that RIPC releases irisin, a myokine derived from the extracellular portion of fibronectin domain–containing 5 protein (FNDC5) in skeletal muscle, to protect against injury to the lung. Human patients with neonatal respiratory distress syndrome show reduced concentrations of irisin in the serum and increased irisin concentrations in the bronchoalveolar lavage fluid, suggesting transfer of irisin from circulation to the lung under physiologic stress. In mice, application of brief periods of ischemia preconditioning stimulates release of irisin into circulation and transfer of irisin to the lung subjected to IR injury. Irisin, via lipid raft–mediated endocytosis, enters alveolar cells and targets mitochondria. Interaction between irisin and mitochondrial uncoupling protein 2 (UCP2) allows for prevention of IR-induced oxidative stress and preservation of mitochondrial function. Animal model studies show that intravenous administration of exogenous irisin protects against IR-induced injury to the lung via improvement of mitochondrial function, whereas in UCP2-deficient mice or in the presence of a UCP2 inhibitor, the protective effect of irisin is compromised. These results demonstrate that irisin is a myokine that facilitates RIPC-mediated lung protection. Targeting the action of irisin in mitochondria presents a potential therapeutic intervention for pulmonary IR injury.

INTRODUCTION

Acute lung injury (ALI) is commonly encountered in hospital and outpatient settings and is associated with a high rate of morbidity and mortality. Many medical conditions, such as pulmonary embolism, thrombosis, acute respiratory distress syndrome (ARDS), and cardiopulmonary bypass surgery, can cause ischemia/reperfusion (IR)–induced lung injuries (1, 2). In infants born with neonatal respiratory distress syndrome (NRDS), insufficiency of pulmonary surfactant production and structural immaturity increase the susceptibility of the lung to IR injury (3). IR injury is often associated with activation of cellular inflammatory and oxidative stresses. IR-induced increase in reactive oxygen species (ROS) (4–6), in particular, can disrupt mitochondrial function and plasma membrane integrity and thus contribute to alveolar damage, lung edema, and hypoxemia that accompany ALI (7, 8). Preservation of mitochondrial function under IR stress represents a potential therapeutic target for treatment of ALI.

Mild exercise training has demonstrated beneficial effects in preventing pulmonary IR injury (9) and is associated with the reduced mortality in ARDS (10). Limb remote ischemic preconditioning (RIPC), defined as intermittent periods of ischemia applied at a remote limb muscle before prolonged IR injury of a target organ, is a noninvasive intervention that has substantial benefits in protection against tissue injuries (11). Humoral signaling pathways involving cross-talk between skeletal muscle and the target organ have been hypothesized to explain the beneficial effects of RIPC on ALI (12). However, the underlying mechanisms remain largely unknown.

Because of the unique vascular supply and the continuous physiologic demand for oxygen uptake and gas exchange, the lung is especially vulnerable to IR injury. Mitochondrial dysfunction is a key factor in lung IR injury (13, 14). Irisin, a type 1 membrane protein encoded by the Fndc5 gene, participates in mitochondrial biogenesis and oxidative metabolism (15, 16). Here, we tested the hypothesis that irisin functions as a myokine to protect against mitochondrial dysfunction during IR-induced pulmonary injury. We provide evidence that RIPC increases irisin in the bloodstream, and under IR stress, irisin transfers to injured alveolar cells. Through interaction with mitochondrial uncoupling protein 2 (UCP2), a homolog of UCP1, which is predominantly expressed in the lung tissue (17, 18), irisin preserves mitochondrial function and prevents IR-induced lung injury. We also found that intravenous administration of exogenous irisin can ameliorate IR-induced pulmonary injury in mouse models, and the pulmonary protective function of irisin is compromised in mice with genetic ablation of UCP2.

RESULTS

RIPC causes increase of irisin in the bloodstream and transfer to injured alveolar cells

We used Western blots to quantify the changes in serum concentration of irisin after IR-induced lung injury in a mouse model. Specificity of the irisin antibody was tested in fig. S1. Injury to the left lung was established by ischemia for 1 hour followed by reperfusion for another 1 hour in mice. As shown in Fig. 1A, mice subjected to RIPC, consisting of five cycles of 5-min ischemia/5-min reperfusion applied to the hindlimb, showed a time-dependent increase of irisin in the serum. RIPC rapidly raised irisin concentrations in the bloodstream (immediately after RIPC), and they reached about threefold of the basal level at 1 hour after RIPC (Fig. 1A). In mice subjected to lung IR injury, the serum concentration of irisin was significantly reduced when compared with sham-treated mice (Fig. 1B; P < 0.05). Moreover, under IR condition, the RIPC-induced

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increase in serum irisin was reduced, indicating the possibility of irisin transfer from the serum to the injured lung.

In addition to Western blotting, we followed the protocol of Albrecht et al. (19) and established a label-free liquid chromatography–mass spectrometry (LC-MS) method to quantify the time-dependent changes in the serum concentration of irisin in mice subjected to IR and RIPC. The LC-MS demonstrated the presence of a short peptide, FIQEVNTTTR, which is present in irisin (fig. S2). Figure 1C showed that the irisin concentration in mouse serum is decreased upon lung IR injury. Although RIPC could initially raise the concentration of irisin, a time-dependent decline was observed (Fig. 1C).

Immunohistochemical (IHC) staining was used to quantify the changes in irisin protein expression in mouse lung tissue (Fig. 1D). IR injury resulted in accumulation of irisin in the lung, and there was a progressive increase of irisin protein in the injured lung tissue after RIPC treatment. RIPC applied to sham-treated mice did not induce irisin uptake into the alveolar cells (fig. S3). The reciprocal relationship of time-dependent decline of irisin in the serum (Fig. 1C) and concurrent accumulation of irisin in the lung tissue (Fig. 1D and fig. S4) support the notion IR makes irisin transfer from blood circulation to the injured lung tissue, which may have physiological consequences.

Infants with NRDS show reduced irisin concentrations in their serum

We conducted a pilot human study to establish the relationship between irisin and NRDS in newborn patients (see table S1). A total of 15 controls and 7 NRDS patients were enrolled in this study. The serum samples and characteristics of control infants and NRDS patients
the lack of irisin expression in the lung under normal conditions (Fig. 1A). Using a protein liquid-chip assay, we found that the serum irisin concentrations derived from NRDS and recovery infants were considerably lower than those of healthy infants (Fig. 2A). On average, the serum of healthy infants contained 1113 ± 183 pg/ml irisin, the serum of patients with NRDS contained 331 ± 63 pg/ml irisin, and that of patients with NRDS after recovery from lung injury contained 553 ± 90 pg/ml irisin (Fig. 2B).

Using the LC-MS method, we determined the serum concentrations of irisin in these infant populations (Fig. 2C; n = 6 in each group, partial samples from the chip study). Similar to the protein liquid-chip assay, there were significantly lower amounts of irisin in serum derived from NRDS patients compared with controls (P < 0.05). Although NRDS infants who recovered from lung injury appeared to have increased concentrations of irisin in the serum, because of the limited number of patient samples, we could not determine the statistical significance among the NRDS patients during acute illness and after recovery (Fig. 2C).

We also measured the irisin concentrations in bronchoalveolar lavage fluid (BALF) from the NRDS infants and found higher concentrations than those present in the serum (Fig. 2, D and E). Considering the lack of irisin expression in the lung under normal conditions (fig. S1), this indicates that irisin could transfer to lung from circulation in the condition of NRDS. Together, these findings suggest that the serum concentration of irisin may be a potential marker for human pulmonary diseases and that therapeutic means of increasing irisin concentrations in the serum may benefit treatment of lung injuries.

**RIPC-mediated pulmonary protection is replicated by application of exogenous irisin**

Previous studies demonstrated protective effects of RIPC against IR injury to the lung (20, 21). Because irisin is an important humoral factor released by skeletal muscle during RIPC, we also tested whether the protective effect of RIPC could be simulated by the application of exogenous irisin. Studies from other investigators have reported the basal serum concentrations of irisin to be in a range of 5 to 15 ng/ml in mice (22, 23). On the basis of this and our observation of about threefold increase in irisin after RIPC (Fig. 1A), we treated the mice with intravenous administration of irisin (1 µg/kg) given by bolus injection immediately after lung ischemia. Assuming a 2-ml blood volume with a mouse of 20-g body weight, intravenous administration of 1 µg/kg should increase irisin in the circulation by ~20 ng/ml and about two- to threefold increase over the basal concentration, as we observed with RIPC.

We found that implementation of RIPC improved the survival of mice that were subjected to IR treatment. Similarly, mice receiving irisin treatment also showed improvement in survival rate after IR treatment (Fig. 3A). To quantify the effect of irisin and RIPC on lung edema, we measured the wet/dry lung weight ratio after completion of the survival experiment. Exogenous irisin administration reduced edema in mice subjected to lung IR injury, to a similar degree as RIPC (Fig. 3B). Hypoxemia is another hallmark associated with lung injury. The arterial blood gas analysis showed that PaO2 was decreased and PaCO2 was increased in mice subjected to lung IR injury, but both irisin administration and RIPC ameliorated these hallmarks of lung function (Fig. 3, C and D). Moreover, both RIPC and exogenous irisin caused comparable reduction of proinflammatory cytokine concentrations, interleukin-1β (IL-1β) in Fig. 3E and IL-6 in Fig. 3F, in serum derived from mice subjected to lung IR injury. These results demonstrate that

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**Table 1. The characteristics of patients included in the study.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Gender (male/female)</th>
<th>Gestational age (weeks)</th>
<th>Birth weight (kg)</th>
<th>5-min Apgar score</th>
<th>SpO2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>7/8</td>
<td>33.5 ± 3.3</td>
<td>3.12 ± 0.12</td>
<td>9.3 ± 0.2</td>
<td>96.1 ± 1.8</td>
</tr>
<tr>
<td>NRDS</td>
<td>7</td>
<td>3/4</td>
<td>31.6 ± 4.8</td>
<td>2.65 ± 0.29*</td>
<td>6.9 ± 0.3*</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05, versus control. †P < 0.05, versus neonatal respiratory distress syndrome (NRDS) infants after recovery.
systemic administration of irisin has beneficial effects in preservation of lung function after IR injury.

Irisin targets mitochondria to ameliorate apoptosis of lung epithelial cells during IR injury

Using IHC staining, we found that the intravenously administered exogenous irisin could target IR-injured lung tissue but not the sham-operated lung tissue (Fig. 4A). Irisin staining was not visible in lung tissue derived from sham-operated mice (Fig. 4B, top). Alveolar epithelial cells took up endogenous irisin after IR injury because the irisin signal was mostly surrounded by AT1a, an alveolar type I epithelial cell marker (Fig. 4B, middle). Intravenous administration of exogenous irisin (1 µg/kg) increased accumulation of irisin in the alveolar cells after IR injury (Fig. 4B, bottom).

As a key factor in lung IR injury, mitochondria dysfunction causes cellular energy depletion, ROS generation, and mitochondria-induced cellular apoptosis (13, 14). Previous studies established a link between irisin and the metabolic function of mitochondria in multiple tissues (24). We found that mouse lung tissue subjected to IR injury showed enhanced dihydroethidium (DHE) fluorescence intensity, indicative of increased ROS, whereas irisin treatment significantly reduced ROS in IR-injured lung tissue (Fig. 4C; P < 0.05). A biochemical assay showed that IR injury to the lung caused release of cytochrome c from mitochondria, and systemic administration of exogenous irisin could reduce the release of cytochrome c (Fig. 4D). The effect of irisin in prevention of apoptotic cell death was evident in immunoblotting of caspase 9, a key enzyme involved in execution of apoptosis. Figure 4E showed that caspase 9 was activated in IR-treated lung (represented by the cleavage product of caspase 9), and administration of irisin reduced caspase 9 activity in IR-injured lung. Using Seahorse measurement of the mitochondrial metabolic function, we found that the oxygen consumption rate (OCR) of the basal respiration and adenosine 5′-triphosphate (ATP) production were decreased in IR-injured lung tissue, and irisin increased OCR and ATP production in lung tissue derived from IR-treated mice (fig. S5). Overall, these results suggest that irisin-mediated pulmonary protection involves mitochondria-dependent apoptosis.

In addition to the mouse studies, we conducted experiments with three different lung epithelial cell lines, including A549 cells (a human lung epithelial cell line), RLE-6TN cells (a rat lung alveolar epithelial cell line), and BEAS-2B cells (a human lung bronchus epithelial cell line).
These lung cells do not express endogenous irisin (fig. S1), thus allowing convenient assessment of the exogenous irisin on the mitochondria function under stress conditions. Figure 5A showed that A549 cells subjected to anoxia/reoxygenation (AR; 2-hour anoxia and 2-hour reoxygenation) could take up exogenous irisin in a time-dependent manner. Similar observations were made with RLE-6TN and BEAS-2B cells (fig. S6 and movies S1 to S3).

Because entry of exogenous irisin was only observed in lung tissues or cells subjected to either IR or AR, recognition of stress-induced extracellular signal plus receptor-mediated endocytosis are most likely required for penetration of irisin into the injured alveolar cells. We conducted confocal microscopic imaging of AR-treated A549 cells and tested the effects of several pharmacological agents that disrupt endocytic pathways for uptake of irisin into cells. As shown in Fig. 5B, AR treatment stimulated irisin uptake into A549 cells. Addition of either chloropyrazine (CPZ), a known inhibitor of clathrin-mediated endocytosis (25), or dimethylacetamide (DMA), a known inhibitor of large endocytic uptake pathway (26), did not affect uptake of irisin into A549 cells. However, the application of 50 μM nystatin, a known inhibitor of lipid raft–mediated endocytosis (27), blocked the uptake of irisin into A549 cells after AR treatment. This result supports a role for lipid raft–mediated endocytosis in facilitating entry of irisin into cells that are exposed to IR stress.

Confocal microscopy imaging showed that the exogenous irisin could target mitochondria in A549 cells, as shown by colocalization of MitoTracker Deep Red and fluorescein isothiocyanate (FITC)–labeled irisin (green, Fig. 5C). As a control, denatured irisin (boiled for 10 min at 100°C) did not show intracellular localization (Fig. 5C).

**Fig. 4.** Irisin protected against oxidative stress and apoptosis in IR-injured lung tissue. (A) Immunostaining showed that irisin was detected in the mouse lung tissue after IR injury with irisin treatment but not in sham-operated animals (scale bars, 50 μm). (B) Lung tissue sections were immunostained for irisin (green) and AT1a (red, alveolar type I epithelial cell marker) (scale bars, 20 μm). (C) The extent of reactive oxygen species (ROS) production in mouse lung tissue was determined by dihydroethidium staining (*P < 0.05 versus sham group and #P < 0.05 versus IR group; n = 5; scale bars, 100 μm). (D) Assessment of cytochrome c (cyc c) in mitochondria and cytosol derived from lung tissue subjected to IR (*P < 0.05 versus sham and #P < 0.05 versus IR; n = 4). COX IV, cyclooxygenase IV; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (E) Caspase 9 activation in lung tissue subjected to IR (*P < 0.05 versus sham and #P < 0.05 versus IR; n = 4).
entry in A549 cells under AR conditions. Similar observations were made with the RLE-6TN and BEAS-2B cells (fig. S7). Irisin targeting mitochondria significantly reduced ROS in AR-injured cells (Fig. 5D; P < 0.05). Increased release of cytochrome c was observed in A549 cells after AR treatment, and irisin could reduce cytochrome c release (Fig. 5E). Moreover, similar to what we observed with the IR-treated lung tissue, the activation of caspase 9 activity in AR-treated A549 cells was reduced by the addition of irisin (Fig. 5F).

In fig. S8, we showed that cultured lung epithelial cells, including A549, RLE-6TN, and BEAS-2B, displayed reduced viability after AR treatment, and incubation with exogenous irisin could improve their survival. In addition, AR-induced increases in lactate dehydrogenase (LDH) activity in culture medium derived from all three types of lung epithelial cells were reduced when irisin was present in the solution.

Irisin interacts with UCP2 to preserve mitochondrial function during IR
UCPs control mitochondrial function in several tissues (28), and irisin has been found to modulate UCP1 expression and act on the mitochondrial biogenesis in white adipose tissue (29). As compared with the other members of the UCP family, UCP2 is the predominant isoform expressed in lung tissue (17, 28, 29) and shares 55 to 60% homology with UCP1 (17, 18). We found that IR-treatment reduced the expression of UCP2 protein in mouse lung tissue, and administration of exogenous irisin could partially restore UCP2 expression (Fig. 6A). As a result, AR-induced increases in lactate dehydrogenase (LDH) activity in culture medium derived from all three types of lung epithelial cells were reduced when irisin was present in the solution.

To understand the potential role of UCP2 in irisin-mediated protection of mitochondrial function, we used confocal microscopy.
to examine the subcellular distribution of irisin and UCP2. Using FITC-labeled irisin, we observed a high degree of colocalization between FITC-irisin and Cy3-UCP2 in lung epithelial cells subjected to AR treatment, although there was no colocalization in control cells (Fig. 6C and fig. S9). The colocalization of irisin and UCP2 was lost if we used denatured irisin. We next performed coimmunoprecipitation studies and found that lung epithelial cells subjected to AR with supplementation of exogenous irisin displayed physical interaction between irisin and UCP2; the interaction was no longer noted in control cells with irisin treatment or AR-treated cells without irisin (Fig. 6D). To examine whether irisin treatment could prevent degradation of UCP2 in response to AR, we treated A549 cells with cycloheximide (CHX, 10^{-5} M) to inhibit protein synthesis. As shown in Fig. 6E, in the presence of CHX, the half lifetime for UCP2 was increased from 1.5 ± 0.14 hours under control conditions to 3.51 ± 0.21 hours when irisin (0.1 μg/ml) was present in the culture medium. On the basis of these results, we conclude that functional interaction between irisin and UCP2 could protect UCP2 from degradation. These effects likely underlie the irisin-mediated amelioration of IR-induced oxidative stress in alveolar epithelial cells and improvement of mitochondrial function associated with pulmonary injury.

Genetic ablation of UCP2 abolishes the pulmonary protective function of irisin

To further elucidate the physiological role of UCP2 and its interaction with irisin in pulmonary protection, we used a mouse model with genetic ablation of Ucp2 (30, 31). The Ucp2^{−/−} mice are viable, thus enabling us to examine their physiological function under stress conditions. In normal physiologic conditions, there are no significant differences in ROS and inflammation between wild-type (WT) and Ucp2^{−/−} mice (fig. S10). Moreover, the Ucp2^{−/−} mice do not show abnormal lung structure under normal conditions (Fig. 7A, left).

IR-induced lung injury was more severe in Ucp2^{−/−} mice than in WT mice, with increased hemorrhage and inflammatory cell infiltration observed in the interstitium and alveoli of the Ucp2^{−/−} mice (Fig. 7, A and B). When exogenous irisin was intravenously administered to the Ucp2^{−/−} mice, we observed no significant difference in the targeting of irisin to the injured alveolar cells between WT and Ucp2^{−/−} mice.
and 15 fields of vision in each slide were chosen for histopathological assessment (lung injury score) of pulmonary tissue for each group is summarized. The staining for each group was repeated at least five times, and 15 fields of vision in each slide were chosen for histopathological assessment (*P < 0.05 versus WT mice under same conditions and #P < 0.05 versus IR group in WT mice). (C) Immunohistochemical staining shows targeting of irisin to the injured alveolar cells in WT and Ucp2−/− mice (scale bars, 50 μm). (D) Lung edema, determined by wet/dry ratio in WT and Ucp2−/− mice after IR injury (*P < 0.05 versus WT mice under same conditions and #P < 0.05 versus IR group in WT mice; n = 5). Measurement of PaO2 (E) and PaCO2 (F) in WT and Ucp2−/− mice after IR injury (*P < 0.05 versus WT mice under same conditions and #P < 0.05 versus IR group in WT mice; n = 5).

DISCUSSION

Data presented in this study support the concept that circulating irisin alleviates lung IR injury through preservation of mitochondrial function. Although lung alveolar cells do not express endogenous irisin, irisin can transfer from blood circulation to the injured alveoli after IR treatment. In human infants with NRDS, the serum concentrations of irisin are lower than those present in control newborns. Ischemic preconditioning applied to the limb muscle ameliorates lung IR injury through elevation of irisin in circulation. Systemic administration of exogenous irisin protein protects the lung from IR injury in mice through reduction of ROS activity and mitochondria-dependent apoptosis. Biochemical and cell imaging studies show that irisin interacts with UCP2 in mitochondria to preserve its function in controlling oxidative stress associated with lung injury. Mice with knockout of UCP2 display increased susceptibility to lung IR injury relative to the WT littersmates, and the protective effect of irisin is compromised in the Ucp2−/− animals. On the basis of these data, we conclude that a functional interaction between irisin and UCP2 contributes to maintenance of pulmonary integrity under pathologic conditions of excessive oxidative stress.

Skeletal muscle has recently been recognized as an endocrine organ that not only contains metabolically important molecules but also communicates with other tissues through the secretion of hormones, known as myokines, that are released into circulation during or immediately after physical activity (33). Irisin is an exercise-induced hormone secreted by skeletal muscle that drives brown fat-like conversion of white adipose tissue, which improves systemic metabolism by increasing energy expenditure (29). Because of these properties, irisin has emerged as an appealing therapeutic target for treatment of metabolic diseases (29, 34–36). However, published research on irisin...
faced several controversies regarding the method for detection/quantification of irisin protein expression and deciphering the long-term versus acute action of irisin in physiological or pathological settings (19, 37, 38). Here, we used four complementary methods, including Western blotting, IHC staining, protein liquid-chip assay, and LC-MS, for quantification and subcellular localization of irisin in lung epithelial cells and tissues under stress conditions. We present data to show that human and mouse lung epithelial cells do not express endogenous irisin protein but can take up irisin from the extracellular environment under AR or IR conditions.

RIPC as a noninvasive intervention can be readily applied in a hospital setting or at home as a prophylactic means to protect against injury to the lung or other organs via different humoral factors (39). We showed that RIPC increased irisin in the bloodstream, whereas IR would make irisin transfer from blood circulation to the injured lung tissue. On the basis of the reciprocal relationship of time-dependent decline of irisin in the serum and the concurrent accumulation of irisin in mice subjected to RIPC and lung IR injury, we conclude that transfer of irisin from the bloodstream to the injured lung tissue underlies the pulmonary protective function of irisin. Under IR condition, we show that injury to the lung can stimulate transfer of endogenous irisin from blood circulation to alveolar cells. However, the amount of endogenous irisin accumulation inside the cells is unlikely to be sufficient for pulmonary protection during IR injury, especially in NRDS infants. Thus, systemic administration of exogenous irisin would supplement the endogenous irisin and offer a potential treatment modality for NRDS.

Mitochondrial dysfunction is a key factor in lung IR injury, and impaired mitochondrial integrity may predispose cells to energy depletion, free radical generation, and eventual cell death (13). One contributing factor for ALI is the excessive ROS generation (1, 6, 7), which could damage the alveolar-capillary interaction and disrupt integrity of the lung epithelia, leading to lung edema (40). Irisin participates in mitochondrial biogenesis and oxidative metabolism (29). Although previous studies by other investigators have

Fig. 8. Irisin inhibitor eliminated the protective effect of irisin on IR-injured lung. (A) Effect of irisin and genipin administration on lung edema in IR mice. Arterial blood samples were drawn from individual mice, and plasma PaO₂ (B) and PaCO₂ (C) were measured; n = 5 for control, 4 for genipin alone, 4 for IR, 4 for (IR + irisin), and 4 for (IR + irisin + genipin) (*P < 0.05 versus control and #P < 0.05 versus irisin-treated lung IR mice). (D) Effect of genipin on lactate dehydrogenase (LDH) concentration in supernatant from A549 cells after AR with or without irisin administration (*P < 0.05 versus control cells and #P < 0.05 versus irisin-treated AR cells; n = 4). (E and F) Effect of genipin on mitochondria-mediated apoptosis in A549 cells after AR, with or without irisin administration. Cyc c release (E) was expressed as the ratio of cyc c in cytosol and mitochondria, and caspase 9 activity (F) was expressed as the ratio of active caspase 9 and GAPDH (*P < 0.05 versus control cells and #P < 0.05 versus irisin-treated AR cells; n = 4).
demonstrated the myokine function of circulating irisin in regulating lipogenesis (41, 42), little is known on the role of irisin in pulmonary protection. Here, we present evidence that IR-induced elevation of ROS and inflammatory factors (such as IL-1β and IL-6) can be suppressed by irisin. We found that systemic administration of exogenous irisin before IR injury can preserve lung function and prevent lung edema. Targeting of exogenous irisin to the injured alveolar cells requires recognition of injury-associated extracellular signal plus vesicle- or receptor-mediated endocytosis. Through the use of pharmacological agents that disrupt endocytic pathways, we provide evidence that lipid raft–mediated endocytosis plays an important role in facilitating the entry of irisin into the lung epithelial cells under IR stress conditions. Thus, irisin can potentially be used as a prophylactic agent in the medical settings of anticipated lung injury.

The protective effect of irisin on ALI involves UCP2-related preservation of mitochondria function. Mitochondrial UCP plays important roles in regulating cellular metabolic function and its response to physiologic or pathologic stresses (43–47). There are three UCP subtypes: UCP1, UCP2, and UCP3 (46). Irisin has previously been shown to up-regulate UCP1 expression in adipocytes (48). UCP2 shares 55% to 60% similarity with UCP1, and they have a comparable activity (17, 18, 45). Besides the spleen, the lung has the highest expression of UCP2 (17). UCP2 has been suggested to participate in attenuation of the ROS production (49–51) and to inhibit ROS-mediated apoptosis in lung epithelial cells (52). Our present study showed colocalization of irisin and UCP2 in mitochondria. The irisin/UCP2 interaction was able to prevent IR-induced degradation of UCP2 protein, thus facilitating the protection of lung mitochondria. A role of UCP2 on the protection of lung injury was further confirmed in the in vivo experiment, where knockout of UCP2 abolished the protective effect of irisin on lung and mitochondrial function during IR injury. Moreover, pharmacological interventions with genipin to inhibit UCP2 further support a role of irisin–UCP2 interaction in preservation of lung function under stress conditions.

The current study has several limitations. First, our pilot study with the human NRDS infants only included a limited number of serum and BALF samples. With the LC-MS and protein liquid–chip assay methods for quantification of irisin, we should be able to expand the sample size to help establish a causative relationship between the changes in irisin expression and pulmonary function in human diseases. Moreover, genome sequencing may be used to identify the genetic basis for the reduced irisin circulation in the bloodstream of human patients with pulmonary dysfunction. Second, our present study focused on the role of irisin in preservation of mitochondria function in the lung tissue, but IR-induced oxidative stress can also affect the integrity of the plasma membrane. We recently showed that MG53, a skeletal muscle–derived secretary protein, is a key component of the cell membrane repair machinery and plays a protective role after lung IR injury (53). Elevation of ROS in the cytosol can inhibit MG53’s function in cell membrane repair (54). Thus, IR-induced ROS elevation could, in principle, cause defective cell membrane repair and contribute to lung dysfunction associated with IR injury. Whether irisin can ameliorate cell membrane repair is another topic for future study. Third, irisin circulation in the bloodstream is regulated by exercise (37). Exercise shares some similarities with RIPC, and RIPC could be used as an adjunct to exercise for pulmonary protection (55). Further research will need to expand on the mechanisms that underlie the potential synergy between exercise training and RIPC in modulation of irisin secretion to treat pulmonary injury.

The identified functional interaction between irisin and UCP2 at the interface of mitochondria has important implications for cell signaling and the stress response. Therapeutic targeting of irisin/UCP2 to modulate mitochondria-dependent ROS signaling may have translational value for treating ALI or other human diseases associated with IR tissue injuries. Additional studies to elucidate the molecular motifs that directly control irisin/UCP2 interaction or the mechanisms that facilitate anchoring of irisin to mitochondria can advance our understanding of the biology of irisin and its translational applications. Because controlled elevation of irisin in the bloodstream has therapeutic benefits in treating ALI, development of noninvasive physiologic approaches, such as RIPC or pharmacological interventions that enhance secretion or ectodomain shedding of irisin from the fibronectin domain–containing 5 protein (FNDC5), may offer therapeutic potential for human diseases associated with oxidative stress.

MATERIALS AND METHODS

Study design

In light of the effect of RIPC on pulmonary protection, our study was designed to determine whether irisin, a myokine, could replicate RIPC’s effect. The experiments were carried out in mice with pulmonary IR injury and three different lung epithelial cell lines. To show the role of UCP2 in irisin-mediated protective action, we also used Ucp2−/− mice and a UCP2 inhibitor, genipin. Equal numbers of male and female mice were used for experimentation. Whenever possible, littermate control animals were used for comparison without randomization. To assess the clinical relevance of our findings, we conducted a pilot human study to establish the relationship between irisin and NRDS in newborn patients. Serum and BALF concentrations of irisin were determined by a protein liquid–chip assay and LC-MS method. Investigators for the survival study and lung injury scoring were blinded to the group assignment and treatment.

Clinical study of NRDS samples

Seven newborns with NRDS were recruited from the neonatal intensive care unit (NICU) at Daping Hospital, Third Military Medical University (Chongqing, China). Samples of serum and BALF from NRDS patients during acute illness and after recovery were collected. In addition, 15 control blood samples were collected from neonatal jaundice patients in the NICU at Daping Hospital, excluding those with NRDS, neonatal pneumonia, meconium aspiration syndrome, or other respiratory diseases. The inclusion criteria and NRDS diagnosis were described in a previous study (56). The irisin concentration in serum or BALF was measured by an irisin-specific LumineX bead-based multiplex detection system (Merck Millipore) and LC-MS. Because of the limited volume and quality of collected serum samples, six samples in each group were measured by LC-MS. All procedures were conducted in compliance with protocols approved by the Ethics Committee of Daping Hospital, Third Military Medical University, and informed consent was received from all participants’ legal representatives. Details of all participants are presented in tables S1 and S2.

LC-MS test

Irisin measurement by LC-MS was performed as previously described (19, 37, 57). The serum or BALF samples (50 μL) from control or NRDS newborns were collected for SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained by Coomassie, and the fragments were excised from the 10- to 15-kDa region. After destaining, dehydrating,
resuspending, and desalting, the gel pieces were digested by trypsin. The digested peptides were eluted for LC-MS by an linear ion trap (LTQ) Orbitrap Velos Pro mass spectrometer and an LC pump (Thermo Fisher Scientific). Peptides were separated using a 90-min gradient of 4 to 50% acetonitrile in 0.1% formic acid with a flow rate of 0.5 μL/min. For each analysis, we loaded 5 μl onto the column. The MS data were generated in the LTQ linear ion trap. The full-scan MS spectra were recorded at a resolution of 100,000× with a scan range from 300 to 1800 m/z (mass/charge ratio) in the LTQ and acquired in the Orbitrap at m/z 604. The tryptic peptide (“F IQEVTNTR”) was chosen from the central portion after collision-induced dissociation. The intensity of the y-ion series for peptides corresponds to the irisin sequence (fig. S2), confirming that these ions can be used for identification and quantification of irisin.

Mouse lung IR model
UCP2 knockout mice with C57BL/6j background and C57BL/6j WT mice of both sexes, weighing 20 to 25 g, were purchased from the Model Animal Research Center of Nanjing University in Nanjing, China. The UCP2 knockout mice, as a global knockout model, were created as described previously (30, 31). Mice were randomized to the sham-operated group and lung IR group, anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and placed on a heating pad to maintain their body temperature. The right femoral arteries and veins of mice were then catheterized with polyethylene tubing for arterial blood sampling and irisin (or saline as control) injection. After intubation via tracheotomy, mice were connected to a Harvard ventilator (Hugo Sachs Elektronik) with an inspiratory pressure of 7 ml/kg. The respiratory rate was set at 100 breaths per minute. After thoracotomy, the left hilum of the IR group was clamped for 1 hour and released; reperfusion proceeded for 1 hour. IR groups were divided into the following groups: IR control group, RIPC group, and irisin treatment group. In RIPC group, RIPC was induced by five episodes of 5-min ischemia of the femoral artery with a small vascular clamp to occlude arterial blood flow followed by releasing the clamp for 5-min reperfusion, then 1-hour ischemia immediately, and then reperfusion. In the irisin treatment group, irisin was given by bolus injection immediately after lung ischemia. After reperfusion, blood samples were obtained immediately for arterial blood gas analysis (Gem primer 3000, Instrumentation Laboratory) or enzyme-linked immunosorbent assay (ELISA).

This study was approved by the Research Council and Animal Care and Use Committee of Daping Hospital, Third Military Medical University. All experiments conformed to the guidelines of the American Association for the Accreditation of Laboratory Animal Care.

Survival analysis
After 1-hour reperfusion, mice were ventilated and observed for additional time to evaluate the survival rate. The surviving animals were euthanized by overdosage of sodium pentobarbital after 1-hour observation. The definition of death was a combination of (i) cardiac arrest or cessation of regular cardiac activity, (ii) the collapse of the left atrium, and (iii) brief clonus activity (58). A researcher blinded to the group assignment (all groups were undergoing a thoracotomy) would record the time of death for survival analysis. Left lung tissues were taken for histological analysis or other experiments.

Wet/dry lung weight ratio
At the time of death, the left lungs of mice were taken and weighed to determine the wet weight, then placed in an oven at 80°C for 24 hours, and reweighed to determine the dry weight for calculation of the wet/dry weight ratio.

IL-1β and IL-6 concentrations in serum by ELISA
After 1-hour reperfusion, blood samples were collected from right femoral arteries by catheterizing, kept at room temperature for clotting, and centrifuged at 3000g for 15 min to obtain serum. The IL-1β and IL-6 concentrations were detected by ELISA (Jiancheng).

Histological study
Lung tissues, cleared of blood with phosphate-buffered saline (PBS) and kept in 4% paraformaldehyde for 1 to 2 days at 4°C, were embedded in paraffin, sectioned, and mounted on slides. After deparaffinizing and rehydrating by xylene and different concentrations of ethanol, the sections were stained with hematoxylin solution for 10 min, washed with running water for 5 min, soaked in 1% acid alcohol for 1 min, and counterstained with eosin Y solution for 1 min; alternatively, the sections were treated with 0.1% Triton X-100 and 10 mM sodium citrate (pH 6.0) and blocked with 5% goat serum in PBS for 1 hour at 37°C for immunohistochemistry. The tissues were incubated with rabbit anti-irisin antibody (1:200; Phoenix Pharmaceuticals) overnight at 4°C.

DHE staining
Superoxide production in the mouse lung tissue was evaluated with the fluorescent dye DHE (Beyotime). Frozen section of the left lung tissue isolated from sham-treated and IR mice with vehicle (saline) or irisin treatment was stained with DHE (10−4 M) for 20 min. After the tissues were washed, images were taken with a fluorescence microscope at an excitation wavelength of 490 nm and an emission wavelength of 590 nm. All sections were processed under the same conditions. Settings for image acquisition were identical for all sections (exposure time, 30 ms). The DHE fluorescence intensity was quantified by ImageJ (National Institutes of Health).

OCR assay
To assess the OCR, lung tissues were obtained from mice after IR or irisin treatment and rinsed briefly in Seahorse XF medium supplemented with 1 mM sodium pyruvate, 2 mM l-glutamine, and 10 mM glucose to clear any blood. Lung tissues were cut into small pieces (2 to 5 mg), placed in the center of each well of an XF24 Islet Capture Microplate, and covered with a customized nylon mesh by an islet capture screen insert tool. The OCR was measured at 37°C in a Seahorse XF24 extracellular flux analyzer (Agilent) to assess effects of oligomycin (10 μg/ml), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (16 μM), and rotenone/antimycin A (3:12 μM) on OCR. Once the XF experiment was completed, the lung tissue was homogenized to determine the protein concentration for normalization. Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (HyClone Pierce). The production of ATP and basal respiration rate were determined by observing how the OCR changes in response to drugs that modulate mitochondrial activity (59).

AR-induced injury in lung epithelial cells
Lung epithelial cell lines were obtained from the American Type Culture Collection. A549, a human lung epithelial cell line (60), was cultured in 10% fetal bovine serum (FBS) Dulbecco’s modified Eagle’s medium/F-12 culture medium. BEAS-2B, a clonal cell line derived from human lung bronchus epithelium, was cultured in bronchial epithelial cell basal medium supplemented with BEGM kit (Lonza/Clonetics Corporation).
munoprecipitates were suspended in sample buffer, boiled for 10 min, and subjected to immunoblotting with the irisin antibody. To determine the specificity of the bands found on the immunoblots, IgG (negative control) and irisin antibody (positive control) were used as the immunoprecipitants, instead of the UCP2 antibody.

**Statistical analysis**
The data are expressed as means ± SEM. Comparisons within groups were made by analysis of variance (ANOVA) for repeated measures, and comparisons among groups were made by ANOVA with Holm-Sidak test. The product limit (Kaplan-Meier) estimate of cumulative survival was assessed with the log-rank test to evaluate significance of differences. A value of \( P < 0.05 \) was considered significant. All original data and \( P \) values are provided in table S3.

**SUPPLEMENTARY MATERIALS**
www.sciencetranslationalmedicine.org/cgi/content/full/9/418/eaao6298/DC1

Fig. S1. Specificity of irisin antibody and absence of endogenous irisin expression in lung epithelial cells.

Fig. S2. Mass spectrogram profile of irisin peptide.

Fig. S3. Irisin expression in lung tissues from sham-treated mice after RIPC.

Fig. S4. Irisin expression in mouse lung tissues at different time points after RIPC, with or without IR injury.

Fig. S5. Colocalization of irisin and UCP2 in lung epithelial cells.

Fig. S6. Basal OCR of lung tissue from IR-injured mice.

Fig. S7. Localization of irisin in mitochondria of cultured lung epithelial cells.

Fig. S8. Measurement of cell viability and LDH concentration in culture medium derived from lung epithelial cells.

Fig. S9. Time-dependent accumulation of exogenous irisin in RLE-6TN and BEAS-2B cells.

Fig. S10. Basal UCP2 expression, inflammation markers, and amount of ROS in WT and AS/G reporter mouse lung epithelial cells.

Fig. S11. Movie S1. Uptake of exogenous irisin into BEAS-2B cells.

Fig. S12. Movie S2. Uptake of exogenous irisin into RLE-6TN cells.

Fig. S13. Movie S3. Uptake of exogenous irisin into A549 cells.

**REFERENCES AND NOTES**


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Irisin protects mitochondria function during pulmonary ischemia/reperfusion injury

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Protecting the lungs with irisin

Limb remote ischemic preconditioning, or subjecting a limb to temporary periods of brief ischemia, is a method that can be used to protect the lungs from ischemia/reperfusion damage due to an expected stressor such as surgery. Chen et al. investigated the mechanism by which this occurs and determined that remote ischemic preconditioning releases irisin, a myokine that targets mitochondria and blocks some of the deleterious effects of oxidative stress. The authors showed that this can occur naturally, as when infants with neonatal respiratory distress syndrome show transfer of endogenous irisin from the circulation into the lungs, or it can be induced therapeutically, as the authors demonstrated by treating mouse models with exogenous irisin, which protected their lungs from ischemia/reperfusion injury.