Endothelial Uncoupling Protein 2 Regulates Mitophagy and Pulmonary Hypertension During Intermittent Hypoxia

Maria Haslip, Iva Dostanic, Yan Huang, Yi Zhang, Kerry S. Russell, Michael J. Jurczak, Praveen Mannam, Frank Giordano, Serpil C. Erzurum, Patty J. Lee

Objectives—Pulmonary hypertension (PH) is a process of lung vascular remodeling, which can lead to right heart dysfunction and significant morbidity. The underlying mechanisms leading to PH are not well understood, and therapies are limited. Using intermittent hypoxia (IH) as a model of oxidant-induced PH, we identified an important role for endothelial cell mitophagy via mitochondrial uncoupling protein 2 (Ucp2) in the development of IH-induced PH.

Approach and Results—Ucp2 endothelial knockout (VE-KO) and Ucp2 Flox (Flox) mice were subjected to 5 weeks of IH. Ucp2 VE-KO mice exhibited higher right ventricular systolic pressure and worse right heart hypertrophy, as measured by increased right ventricle weight/left ventricle plus septal weight (RV/LV+S) ratio, at baseline and after IH. These changes were accompanied by increased mitophagy. Primary mouse lung endothelial cells transfected with Ucp2 siRNA and subjected to cyclic exposures to CoCl₂ (chemical hypoxia) showed increased mitophagy, as measured by PTEN-induced putative kinase 1 and LC3BII/I ratios, decreased mitochondrial biogenesis, and increased apoptosis. Similar results were obtained in primary lung endothelial cells isolated from VE-KO mice. Moreover, silencing PTEN-induced putative kinase 1 in the endothelium of Ucp2 knockout mice, using endothelial-targeted lentiviral silencing RNA in vivo, prevented IH-induced PH. Human pulmonary artery endothelial cells from people with PH demonstrated changes similar to Ucp2-silenced mouse lung endothelial cells.

Conclusions—The loss of endothelial Ucp2 leads to excessive PTEN-induced putative kinase 1–induced mitophagy, inadequate mitochondrial biosynthesis, and increased apoptosis in endothelium. An endothelial Ucp2–PTEN-induced putative kinase 1 axis may be effective therapeutic targets in PH. (Arterioscler Thromb Vasc Biol. 2015;35:1166-1178. DOI: 10.1161/ATVBAHA.114.304865.)

Key Words: autophagy ▪ endothelium ▪ hypertension, pulmonary ▪ mitochondrial degradation ▪ mitochondrial uncoupling protein 2 ▪ PTEN-induced putative kinase

Pulmonary hypertension (PH) is a progressive and incurable disorder associated with the remodeling of lung vessels, with subsequent right heart failure and early death. Therapies and our molecular understanding of PH remain limited. Dysregulated angiogenesis, growth factor induction, and pulmonary artery smooth muscle cell (PASMC) apoptosis resistance have been invoked as important pathogenetic mechanisms. Recently, the role of the mitochondria in altering PASMCs and pulmonary artery endothelial cells (PAECs) to an apoptosis-resistant phenotype has gained attention. There are many different rodent models for PH, both genetic and pharmacological, including intermittent hypoxia (IH). We selected IH as a model of oxidant-induced vascular injury, which is thought to be a common mechanism underlying PH-associated disorders, such as parenchymal lung disease, obstructive sleep apnea, and heart failure, as well as idiopathic pulmonary arterial hypertension (PAH). Uncoupling protein 2 (Ucp2) belongs to a family of anion transporters that are localized on inner mitochondrial membrane and dissipate proton gradient originated from mitochondrial electron transport chain. Thus far, 5 Ucp family members have been discovered. Ucp1, known as thermogenin, is expressed in brown adipose tissue and dissipates the proton gradient into heat. Ucp2 is widely expressed in the central nervous system, pancreas, liver, and lungs and known to regulate fatty acid metabolism, mitochondrial calcium uptake, and mitochondrial reactive oxygen species (ROS) production. Ucp2 has been studied primarily in the central nervous system and pancreas, where its role was established in neurotransmission, synaptic plasticity, and neurodegenerative processes, as well as in insulin resistance. Recently, Ucp2 knockout mice were found to have worse hypoxia-induced PH, and the authors attributed the mechanism to cellular dysfunction in PASMCs, such as endoplasmic reticulum stress,
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Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Endothelial-Specific Ucp2 Knockout Mice Have Increased Right Ventricular Systolic Pressure and Right Ventricular Hypertrophy After IH

Global Ucp2 knockout mice have been already reported to be more susceptible to chronic hypoxia–induced PH.21,22 To specifically determine the role of endothelial Ucp2 in regulating right ventricular (RV) systolic pressures (RVSPs), a measurement of pulmonary artery pressures, and RV remodeling in response to IH, we generated mice with endothelial-specific knockout of the Ucp2 gene by crossing Ucp2 Flox mice (Flox)30 and mice containing VE-cadherin driving the transcription of Cre recombinase (VE-KO). Figure I in the online-only Data Supplement shows the absence of Ucp2 expression in ECs isolated from total lungs of VE-KO mice. Animals were subjected to 5 weeks of IH, and RVSP was measured. Figure 1A demonstrates that VE-KO mice have significantly higher RVSP even at baseline compared with Flox mice. Moreover, RVSPs markedly increased after IH in VE-KO mice compared with RVSPs in Flox mice. We also determined that VE-KO mice have significantly increased RV remodeling, as measured by the ratio of RV weight/left ventricle (LV) plus septal weight (RV/LV+S), compared with Flox mice (Figure 1B). These data suggest that endothelial Ucp2 is an important determinant of PH at baseline and after IH.

Ucp2 VE-KO Mice Demonstrate Increased Endothelial Apoptosis in Lungs at Baseline and After 1 Week of IH

We costained lung sections from experimental mice for terminal deoxynucleotidyl transferase dUTP nick-end labeling staining, a marker for apoptosis, and von Willebrand factor, an EC marker. After 5 weeks of IH, we did not detect apoptosis in ECs of Flox or VE-KO mice (Figure IIA, IIB, and IID in the online-only Data Supplement). However, VE-KO mice showed increased EC apoptosis at baseline (Figure IIC in the online-only Data Supplement), which may account for their baseline changes in RVSP. Given that early endothelial apoptosis has been described as a hallmark of PH31,32

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>Atg5</td>
<td>autophagy protein 5</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>IH</td>
<td>intermittent hypoxia</td>
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<tr>
<td>MLEC</td>
<td>mouse lung endothelial cell</td>
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<tr>
<td>PAEC</td>
<td>pulmonary artery endothelial cell</td>
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<td>PAH</td>
<td>pulmonary arterial hypertension</td>
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<td>PASMC</td>
<td>pulmonary artery smooth muscle cell</td>
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<td>PGC1α</td>
<td>peroxisome proliferator–activated receptor γ coactivator 1-α</td>
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<tr>
<td>PH</td>
<td>pulmonary hypertension</td>
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<tr>
<td>Pink1</td>
<td>PTEN-induced putative kinase 1</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RVSP</td>
<td>right ventricular systolic pressure</td>
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<td>Ucp2</td>
<td>uncoupling protein 2</td>
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Figure 1. Pulmonary hypertension evaluation in uncoupling protein 2 Flox and VE-KO mice at room air and after 5 weeks of intermittent hypoxia (IH). A. Right ventricle systolic pressure was measured by direct catheter insertion. B. Right ventricle hypertrophy, as measured by right ventricle (RV) weight divided by left ventricle (LV)+septal weight (RV/LV+S). Mouse hearts were dissected into RV and LV with septum. Each part was weighed, and a RV/LV+S ratio was calculated for each animal; *P<0.05; n=8 to 11 in each group.
and that later stages of PH may be associated with apoptosis resistance, we exposed mice to 1 week of IH and performed terminal deoxynucleotidyl transferase dUTP nick-end labeling and von Willebrand factor staining, which showed increased IH-induced endothelial apoptosis in VE-KO mice compared with Flox mice (Figure III in the online-only Data Supplement). These data suggested that endothelial apoptosis may precede the development of overt PH by weeks.

**Ucp2 VE-KO Mice Have Increased Mitophagy and Autophagy Markers in Lungs**

Given that IH is known to generate endothelial oxidant injury and we recently reported that lung ECs undergo autophagy and mitophagy in response to oxidant injury, we hypothesized that the absence of endothelial Ucp2 will alter autophagy and mitophagy. Immunoblotting of the mitochondrial fraction of total lung protein lysates prepared by differential centrifugation (Materials and Methods in the online-only Data Supplement) revealed significantly increased Pink1 and Parkin levels in VE-KO mice at baseline (room air) and after IH (Figure 2A and 2B). IH significantly increased the mitochondrial content of Pink1 and Parkin in both VE-KO mice and Flox animals. At baseline, VE-KO had increased autophagy, as measured by the LC3BII/LC3BI ratio, compared with Flox animals (room air). IH increased autophagy in both Flox and VE-KO animals (Figure 2C). Similar to the VE-KO mouse, global Ucp2 knockout mice demonstrated higher baseline Pink1 and Parkin levels in the mitochondrial fraction and an increased ratio of LC3BII/LC3BI in total lung lysates compared with control mice (Figure IV A–IVC in the online-only Data Supplement). These results suggest that the loss of EC Ucp2 phenocopies the global loss of Ucp2 and is sufficient to cause baseline increases in lung mitophagy, autophagy, and PH.

**Mouse Lung ECs Lacking Ucp2 Demonstrate Increased Mitophagy and Autophagy Flux**

To more specifically investigate the mitophagy and autophagy in lung ECs, we isolated primary mouse lung ECs (MLECs) from the lungs of Flox and VE-KO mice. For all assays, we used MLECs between passages 5 and 8, during which the time cellular senescence does not seem to play a significant role (Figure V in the online-only Data Supplement). Our protein data showed that Ucp2 silencing in wild-type (WT) MLECs resulted in increased baseline levels of mitophagy- and autophagy-associated proteins (Pink1 and LC3BII/I ratios, respectively), whereas a major mitochondria biogenesis regulator, peroxisome proliferator–activated receptor γ coactivator 1-α (PGC1α), was significantly lower after Ucp2 silencing (Figure 3A). Moreover, mitochondrial mass in WT MLECs, as assessed by MitoTracker Green FM staining and detected by flow cytometry, was significantly lower in cells transfected with Ucp2 siRNA (Figure 3B). Similarly, MLECs isolated directly from the lungs of VE-KO mice before and after IH also had less mitochondrial mass compared with MLECs from Flox animals (Figure 3C). This suggests that the accelerated removal of damaged mitochondria in MLECs is not compensated by increased mitochondrial biosynthesis. We also transfected cells with ptfLC3 plasmid that contained LC3 tagged with green and red fluorescent proteins. When autophagy flux starts, LC3 localizes to the lysosomes, and only red fluorescent proteins can be detected because green fluorescent proteins are sensitive to degradation by the acidic environment of the lysosome. Figure 3D shows that MLECs transfected with Ucp2 siRNA have baseline increased autophagy flux.
which we refer to as hyperactive autophagy) compared with MLECs transfected with scrambled siRNA.

As additional confirmation, WT MLECs transfected with Ucp2 siRNA had significantly lower mitochondrial membrane potential, as measured by MitoTracker Red CMXRos, compared with control cells both in room air and after CoCl₂ IH (Figure 4A). Mitochondrial membrane potential of ECs isolated from the lungs of VE-KO mice also demonstrated significantly lower mitochondrial potential compared with Flox MLECs in room air and after 5 weeks of IH (Figure 4B).

Figure 4C shows that Ucp2 silencing increased WT MLEC apoptosis in room air and after IH, suggesting that increased autophagy/mitophagy can lead to MLEC apoptosis, an important early mechanism of PH. It has been shown that IH is associated with excessive mitochondrial ROS production that can induce autophagy. To determine whether hyperactive autophagy in MLECs in the absence of Ucp2 is driven by elevated ROS, we assessed mitochondrial ROS content in MLECs with and without Ucp2 siRNA. Figure 4D shows no significant difference in mitochondrial ROS production between MLECs with and without Ucp2 siRNA at baseline. However, CoCl₂ IH stimulates a significant increase in mitochondrial ROS levels in Ucp2 siRNA–transfected MLECs. These data suggest that Ucp2 silencing increased baseline mitophagy in MLECs without increasing mitochondrial ROS production.

**Ucp2 Silencing Mediates Mitochondrial Changes and Apoptosis via Calcium in MLECs**

Increased baseline mitophagy appears to be associated with decreased mitochondrial membrane potential in WT MLECs transfected with Ucp2 siRNA and in MLECs isolated from VE-KO mice, Figure 3). Although a direct effect of Ucp2 loss may be increased mitochondrial membrane potential, calcium levels are also important modulators of membrane potential. We determined whether calcium-dependent mechanisms mediate Ucp2-silencing effects on mitochondria and apoptosis in MLECs. MLECs were transfected with Ucp2 siRNA and subjected to CoCl₂ IH. Figure 5A shows that BAPTA-AM eliminated Ucp2 siRNA–induced apoptosis after CoCl₂ IH. BAPTA-AM also reversed the effects of Ucp2 siRNA on mitochondrial mass and potential in MLECs (Figure 5B and 5C). Of note, BAPTA-AM alone did not significantly change apoptosis, mitochondrial mass, or mitochondrial potential in...
MLECs. These data suggested that the effects of Ucp2 silencing on mitochondria and apoptosis may be calcium mediated.

Ucp2 Silencing Increases Autophagy Protein 5 Cleavage in MLECs
Calpains are calcium-dependent proteases that can cleave autophagy protein 5 (Atg5), a major component of autophagosome formation, leading to increased autophagy and apoptosis.\(^{40}\) Figure 6 shows that Ucp2 siRNA–treated WT MLECs have significantly increased cleaved Atg5 compared with control (sc siRNA) cells. Therefore, Ucp2 silencing may alter calcium signaling, leading to calpain-induced Atg5 cleavage, which results in increased autophagy and apoptosis in MLECs.

Pink1 Silencing Partially Reverses Hyperactive Autophagy in MLECs
To determine whether mitophagy-associated protein, Pink1, mediates the hyperactive autophagy associated with Ucp2 silencing, we transfected MLECs with both Ucp2 and Pink1 siRNAs before CoCl\(_2\) IH. Transfection efficiency of simultaneous transfections with Ucp2 and Pink1 siRNAs is shown in Figure VIII in the online-only Data Supplement. Figure 7A and 7B show that Pink1 silencing reduced the hyperactive autophagy associated with Ucp2 silencing in MLECs. We quantified autophagy in MLECs by counting the percentage of cells labeled with the red fluorescent protein in Figure 7B. We also evaluated the mitophagy rate by cotransfjecting cells with mitochrondria-targeted mKeima-Red plasmid.\(^{41}\) The fluorescent protein Keima has an excitation spectrum that changes with pH. A neutral pH is associated with a short wavelength (\(\approx 440\) nm), whereas an acidic pH, as found in lysosomes, leads to a longer wavelength (\(\approx 550\) nm). Keima containing plasmid tagged with a mitochondria-localizing peptide is a mode of detecting mitophagy flux via 550 nm/440 nm ratios. This ratio determines the amount of mitochondrion outside (440 nm) and inside (550 nm) lysosomes, which reflects the mitophagy rate. Figure 7C shows that MLECs transfected with Ucp2 siRNA have increased mitophagy flux at baseline that is not changed by CoCl\(_2\) IH but significantly reduced by Pink1 silencing. Moreover, Pink1 silencing in MLECs that had been transfected with Ucp2 siRNA restored mitochondrial potential (Figure 8A) and partially reduced apoptosis, as well as ROS production (Figure 8C and 8D). We confirmed our MLECs in CoCl\(_2\) IH results by showing similar changes in mitochondrial potential in MLECs isolated from Flox and VE-KO mice before and after IH (Figure 8B). To further confirm our results in vivo, we initially constructed an all-cell-targeting Pink1 silencing lentiviral construct (Lenti-Pink1 miRNA) for intranasal delivery, per our recent reports of intranasal lentiviral delivery.\(^{34,35,42}\) We first confirmed adequate Pink1 silencing in the mitochondria of lung lysates after WT mice were given intranasal Lenti-Pink1 miRNA (Figure 9A). We then subjected WT and global Ucp2 KO mice to 5 weeks of IH and isolated the mitochondrial fraction from lung lysates. Pink1 silencing restored mitochondrial potential (Figure 9B) and

Figure 4. Mouse lung endothelial cells (MLECs) after uncoupling protein 2 (Ucp2) silencing and kept in room air or challenged with CoCl\(_2\) intermittent hypoxia (IH). Scrambled siRNA (Sc) was used as a negative control. A, Mitochondrial membrane potential as assessed by fluorescence-activated cell sorter (FACS) in room air or after CoCl\(_2\) IH. B, Mitochondrial membrane potential as assessed by FACS in ECs isolated from Flox and VE-KO lungs in room air and after 5 weeks of IH. C, Apoptosis as assessed by annexin-propidium iodide FACS in room air or after CoCl\(_2\) IH. D, Mitochondrial reactive oxygen species (ROS) production as assessed by FACS in room air or after CoCl\(_2\) IH; \(P<0.05\).
mitochondrial mass (Figure 9D) in the lungs of KO mice. Of note, in MLECs in which Ucp2 was silenced, Pink1 silencing did not restore mitochondrial mass (Figure 9C). These data suggest that Pink1 silencing has different effects on mitochondrial mass in vitro (MLECs) versus in vivo, which may be because of the contributing role of other cells, as proposed in the Discussion section.

**Endothelial Pink1 Silencing in Ucp2 Knockout Mice Prevents IH-Induced PH**

To demonstrate the specific effect of endothelial Pink1 silencing in vivo, we delivered intranasal endothelial-specific Pink1 silencing RNA (VE-Lenti-Pink1 miRNA) to WT and global Ucp2 knockout mice before 5 weeks of IH. We recently demonstrated endothelial specificity and effective Pink1 silencing in endothelium using intranasal VE-Lenti-Pink1 miRNA.35 Figure 10 demonstrates a significant decrease in RVSP and RV/LV+S ratio in Ucp2 knockout mice treated with VE-Lenti-Pink1 miRNA compared with mice treated with control lentivirus. Of note, WT mice treated with VE-Lenti-Pink1 miRNA showed an increase in RVSP compared with control virus–treated mice (Figure 10A). This may be because of the fact that Pink1 silencing in WT endothelium leads to a phenotype similar to Ucp2 silencing, such as increased apoptosis (Figure 8C), decreased mitochondrial potential (Figure 9B), and mitochondrial mass (Figure 9C). These data suggest that endothelial Pink1 is an important determinant of the Ucp2 knockout PH phenotype in vivo.

**PAECs From Patients With PAH Demonstrated Decreased Ucp2 and PGC1α, as well as Increased Pink1 Protein Expression**

PAECs were obtained from normal lung and 4 PAH-explanted donor lungs, as we recently described.43 Immunoblotting for...
Pink1, PGC1α, Atg5, and Ucp2 proteins in total lysates from PAECs showed significantly increased Pink1, Atg5 cleavage (the relative amount of truncated Atg in comparison with Atg), as well as decreased PGC1α and Ucp2 protein expression in patients with PAH compared with normal donors (Figure 11). These data parallel our findings in IH and in Ucp2 siRNA–treated MLECs.

Discussion

The overall goal of our study was to determine the role of EC Ucp2 and identify previously unrecognized mechanisms, whereby Ucp2 deficiency or silencing results in PH. We also demonstrated the relevance of EC Ucp2 and mitophagy to human PH. Unlike previous reports of Ucp2 in PASMCs and chronic hypoxia-induced PH, we studied IH as a general model for oxidant-induced PH, which is thought to be a common mechanism underlying both PH and PAH in a variety of disease states. IH has been reported to lead to pulmonary vascular and systemic hypertension. However, the severity of the vascular changes varies. For example, IH in mice leads to milder changes. Campen et al demonstrated increased RV/LV+septal weight ratio in C57Bl6 mice subjected to 5 weeks of IH to levels similar to what we found in VE-KO mice. Fagan showed an increase in RVSP in WT mice after IH (29.5±0.6 mm Hg in normoxia versus 36±0.9 mm Hg after IH). Our findings were slightly different (23.6±0.7 mm Hg in normoxia versus 25.7±0.8 mm Hg after IH) that can be explained by differences in the length of IH exposure (24 h/d by Fagan versus 10 h/d in our experiments) and the fact that our mice were floxed for Ucp2, which may have some effect on the phenotype. IH has been proposed as an animal model for PH development as a response to cyclic hypoxia-reoxygenation. However, others demonstrated that the development of PH in response to IH was predominantly observed in transgenic mice. Similarly, our control Flox mice did not show significant increases in RVSP or RV hypertrophy, as measured by the RV/LV+septal weight ratio, whereas VE-KO mice demonstrated both higher RVSP and RV hypertrophy. These results seem to suggest that flox mice are partially protected against PH development, which requires future investigation.

![Figure 7. PTEN-induced putative kinase 1 (Pink1) knockdown in uncoupling protein 2 (Ucp2)–silenced mouse lung endothelial cells (MLECs) and kept in room air or challenged with CoCl2 intermittent hypoxia (IH). A, Autophagy levels as assessed by immunofluorescent ptfLC3 plasmid. Original magnification of all photomicrographs, ×40. B, Quantification of autophagy as a percent of red fluorescent protein (RFP) positive cells. C, Quantification of mitophagy as a 540 nm/440 nm fluorescence ratio. Mitophagy in MLECs treated with 30-μmol/L CCCP for 16 hours is used as a positive control. Data presented as a percent of mitophagy undergoing cells relative to positive control; *P<0.05.](http://atvb.ahajournals.org/)**
We detected apoptosis in endothelium after 1 week of IH but not after 5 weeks, which is the time point when physiological evidence of PH, such as increased RVSP and RV hypertrophy, is most evident. These data are consistent with reports that early, wide-spread EC apoptosis eventually selects for ECs with hyperproliferative and apoptosis-resistant phenotypes. We speculate that ECs transition to an apoptosis-resistant phenotype between 1 to 5 weeks of IH. Apoptosis analyses at multiple time points may address this issue. Yamagi-Kegan et al recently demonstrated EC apoptosis during early time points of inducible PH and increased remodeling near the end. Interestingly, VE-KO mice had detectable endothelial apoptosis even at baseline, which may account for their baseline increases in RVSP and RV hypertrophy, which will become the basis of future studies.

Our data show that the loss of EC Ucp2 leads to increased Pink1 expression, mitophagy, and apoptosis in mouse ECs and that PAH in people is associated with similar changes in ECs. On the basis of these data, we propose the following mechanism for IH-induced PH: decreased Ucp2 in endothelium results in increased mitophagy–autophagy without adequate replacement of healthy, newly synthesized mitochondria, which leads to increased endothelial loss, at least in part via apoptosis and, ultimately, PH. VE-KO mice allowed us to evaluate the specific role of ECs in vivo. Previous reports show that endothelial events likely precede PH-associated vasculopathy. We demonstrate that VE-KO mice develop PH, even at baseline, and that their lungs have increased mitophagy-associated proteins, Pink1 and Parkin, suggesting higher baseline mitophagy than control mice. Markers of macroautophagy, LC3BII/LC3BI, were also increased in lungs of VE-KO mice. We found parallel findings in MLECs in which Ucp2 was silenced and in lungs of global Ucp knockout mice. Increased autophagy has been recently identified by Long et al in the pathogenesis of monocrotaline-induced PH in rats. They showed that blocking autophagy with chloroquine, an inhibitor of autophagic protein degradation, led to decreased proliferation and increased apoptosis of rat PASMCs. Mitophagy is a specific type of autophagy in which damaged mitochondria are removed.

Mitophagy and mitochondrial biogenesis represent 2 coordinated processes that determine cellular dysfunction and disease. To maintain a healthy cellular environment, a balance between removal of damaged mitochondrion and biosynthesis of new mitochondria is necessary. To evaluate mitochondrial balance, we determined the expression of PGC1α and mitochondrial mass. PGC1α is a major transcription factor that controls mitochondrial biogenesis through the coactivation of many nuclear and nonnuclear receptors. We evaluated mitochondrial mass not only in WT MLECs with silenced Ucp2
but also ex vivo in MLECs isolated from lungs of VE-KO mice. Ryan et al.\(^5\) reported that female rats developed monocrotaline-induced PH through PGC1\(^{\alpha}\). The same authors showed earlier that mitochondrial fragmentation, increased proliferation, and impaired apoptosis in PASMCs were in the basis of PH development.\(^5\)

Similar mechanisms may be responsible for IH-induced PH in VE-KO mice and ECs. Xu and Erzurum\(^5\) introduced the concept of increased glycolysis in the ECs of patients with PAH, potentially because of decreased mitochondrial function and increased ROS production. The historical work by Warburg,\(^5\) showing a predominance of glycolysis compared with respiration in tumors, was expanded by Tuder et al.\(^3\) into the pathogenesis of PH. Condello et al.\(^5\) demonstrated that tumor cells often have fewer, smaller, more fragile, and highly pleomorphic mitochondria than their normal counterparts. Decreased quantities of effective mitochondria in ECs from Ucp2 knockout mice could potentially lead to a glycolytic phenotype, resulting in PH.

To verify our in vivo findings, we developed a chemical IH in vitro model. We avoided using gaseous hypoxia, which necessitates serum starvation, because serum deprivation activates autophagy. Gaseous hypoxia also requires at least 2 to 4 hours before the cell culture media attains hypoxic conditions. To mimic our in vivo model of IH, in which cycling between hypoxia and normoxia occurs within minutes, we used the rapid anoxic/hypoxic conditions achieved by chemical methods. We instituted chemical IH using CoCl\(_2\) after comparing the signaling protein profiles of various conditions (intermittent hypoxia and sustained hypoxia) with that of in vivo profiles. The signaling results from chemical IH most closely paralleled in vivo IH. We analyzed autophagy and mitophagy using complementary assays. In addition to calculating LC3BII/LC3BI ratios, we detected autophagy flux with pmtLC3 plasmid transfection, as others have described.\(^5\) To evaluate the mitophagy rate, we used a relatively new approach by cotransfecting cells with mitochondrial-targeted mKeima-red plasmid. mKeima protein has a different excitation wavelength depending on the acidity of the environment, such as the acidic environment of the lysosomal compartment.\(^4\) Ucp2-silenced MLECs had significantly higher 540 nm/440 nm ratios than the WT cells, suggesting increased mitophagy rates. Interestingly, CoCl\(_2\) IH induced autophagy but not mitophagy in WT MLECs. These data suggest that the increased baseline autophagy markers detected after Ucp2 silencing may be primarily due to increased mitophagy rather than autophagy.

Although a direct effect of Ucp2 loss is expected to be increased mitochondrial membrane potential,\(^3\) there may be counterbalancing, adaptive effects of Ucp2 deficiency that occur in ECs. We found that excessive apoptosis, loss of mitochondrial mass, and mitochondrial potential in Ucp2-silenced MLECs after CoCl\(_2\) IH can be prevented by a calcium chelator, BAPTA-AM. Calcium homeostasis is vital for normal cell physiology, and its disruption can lead to heart failure.
diabetes mellitus, and neuronal degeneration. Under physiological conditions, there is constant crosstalk between endoplasmic reticulum, which releases calcium, and mitochondria, which uptakes calcium. Ucp2 has been shown to play a role in mitochondrial uptake of intracellular calcium. We postulate that the absence of Ucp2 during IH in ECs results in intracellular calcium accumulation, which leads to mitochondrial depolarization and apoptotic cell death. The precise mechanisms of calcium release and the molecular sequence of events leading to calcium-induced apoptosis merit future investigations. We invoke a potential role for increased cleaved Atg5, which is regulated by calcium-sensitive proteases and has been shown to cause both excessive autophagy and apoptosis. Yousefi et al reported that increased Atg5 cleavage by calcium-sensitive calpains represented the point at which beneficial autophagy becomes excessive or detrimental apoptosis. Shi et al linked calcium influx, Atg5 cleavage by calpains and mitochondrial potential disruption with increased apoptosis. Dromparis et al demonstrated that calcium dysregulation plays a role in PH development in Ucp2 knockout mice, but their findings were in PASMCs. Our data point to a role for Ucp2 in cleavage of Atg5 in human and mouse lung ECs.

Others have reported that decreasing Ucp2 expression with antisense oligonucleotides increased mitochondrial membrane potential and ROS production. These apparent discrepancies may be because of the specific cells used for the experiment. For example, Duval et al used a CRL-2181 cell line, which originates from lymph nodes and is immortalized by SV40. In our study, we used primary MLECs. We also studied mitochondrial potential and ROS production in both MLECs and PASMCs (not shown). Unlike MLECs, which showed decreased mitochondrial potential with Ucp2 silencing, PASMCs transfected with Ucp2 siRNA demonstrated...
mitochondrial hyperpolarization. Moreover, mitochondrial and autophagy-related protein expression and autophagy flux (p62LC3 plasmid) and mitophagy flux (mKeima plasmid) were different in PASMCs (not shown). Therefore, the effect of Ucp2 on mitochondrial membrane potential is likely cell type specific.

Finally, we invoked a functional role for Pink1, a mediator of mitophagy, in Ucp2 deficiency–induced PH. In vivo Pink1 silencing, specifically in endothelium, may restore the balance between removal of nonfunctional mitochondrial and biosynthesis of healthy mitochondria. With Pink1 silencing, we observed mitochondrial potential restoration in both in vitro and in vivo models. We recently reported that the absence of Pink1 leads to increased apoptosis, defective mitochondrial division, and increased susceptibility during acute hyperoxia and lipopolysaccharide-induced inflammation in WT MLECs. Our current studies show that Pink1 silencing in MLECs in the absence of Ucp2 seems to have a beneficial effect, which we speculate may be because of the restoration of the balance between mitochondrial and the presence of healthy mitochondrial.65 In our model of Ucp2 deficiency, increased baseline mitochondrial likely has a detrimental effect because mitochondrial biosynthesis is decreased, the net effect being inadequate mitochondrial replenishment (as evidenced by the decrease in mitochondrial mass detected in Ucp2-deficient ECs). When Pink1 was silenced, mitochondrial mass was restored in vivo only, which also suggests some degree of specificity of Pink1-induced mitochondrial effects and the requirement for alternative cell types in modulating mitochondrial mass. Given that smooth muscle cell proliferation is regulated differently by ECs of different sources and the importance of PASMCs in PH pathogenesis, specific EC–PASMC interactions involving Ucp2–Pink1 are likely. A more precise understanding of these cell–cell interactions, and the importance of PASMCs in PH pathogenesis, specific EC–PASMC interactions involving Ucp2–Pink1 are likely.

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Disclosures

None.

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**Significance**

Our studies link endothelial cell uncoupling protein 2 to mitophagy/autophagy and mitochondrial biogenesis. In addition, we identified endothelial cell mitophagy as a critical, initiating event in the development of pulmonary hypertension. Moreover, we found that PTEN-induced putative kinase 1 silencing reversed uncoupling protein 2 deficiency–induced pulmonary hypertension. Our studies also show that endothelial cells from human pulmonary hypertension lungs exhibit changes in uncoupling protein 2-PTEN-induced putative kinase 1-mitochondrial biogenesis that parallel our mouse studies, thus expanding the clinical relevance of these molecules as potential biomarkers and therapeutic targets in pulmonary hypertension.
Endothelial Uncoupling Protein 2 Regulates Mitophagy and Pulmonary Hypertension During Intermittent Hypoxia
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Supplemental figure I. RT-PCR data for Ucp2 and 18S genes expression in cells isolated from VE-Ucp2KO and Flox mice. Single cell suspensions were prepared from whole lungs and separated on autoMACS PRO after consecutive incubations with CD45 and CD31 beads. Endothelial cells were selected as CD45 negative and CD31 positive fraction (CD45-CD31+). Data demonstrate the absence of Ucp2 expression in the endothelial fraction.
Supplemental figure II. Apoptosis in endothelium assessed by TUNEL staining in lung sections from Flox and VE-KO mice at baseline and after 5 weeks of IH. Original magnification of all photomicrographs: ×40. Blue – DAPI, Green – TUNEL staining, Red – von Willebrand factor, an endothelial marker. A. Flox mice at room air. B. Flox mice after 5 weeks of IH. C. VE-KO mice at room air. D. VE-KO mice after 5 weeks of IH. Arrows indicate merging of green and red fluorescence, indicating apoptotic endothelium.
Supplemental figure III. Apoptosis in endothelium assessed by TUNEL staining in lung sections from Flox and VE-KO mice after 1 week of IH. Original magnification of all photomicrographs: ×40. Blue – DAPI, Green – TUNEL staining, Red – von Willebrand factor, an endothelial marker. A. Flox mice. B. VE-KO mice. Arrows indicate merging of green and red fluorescence, indicating apoptotic endothelium.
Supplemental figure IV. Immunoblots of lung lysates for proteins associated with mitophagy/autophagy from WT and Ucp2 KO mice. A. Pink1 protein in the mitochondrial fraction of total lung lysates. B. Parkin protein in the mitochondrial fraction of total lung lysates. C. LC3BII/LC3BI ratio in total lung lysates. *p<0.05
Supplemental figure V. Q-PCR data for RNA isolated from MLEC passage 3 to passage 8. Cellular senescence markers p16 and p19 were evaluated. Data is presented as $2^{-\Delta\Delta C_T}$ relative to 18S RNA at passage 3. n=4, *p<0.05
Supplemental figure VI. A time course for chemical intermittent hypoxia in mouse lung endothelial cells and Ucp2 and pAKT/AKT protein expressions analysed. Cells were incubated with CoCl$_2$ containing media for 15 min followed by reoxygenation in normal media for 45 min (15’-45’) for 6 cycles or 5 min CoCl$_2$ followed by 20 min reoxygenation (5’-20’) for 6 cycles or 6 h of continuous CoCl$_2$-containing media. *p<0.05 relative to control.
Supplemental figure VII. Protein expression in total mouse lung at room air and after 5 weeks of intermittent hypoxia (IH). The time point for chemical IH in vitro was selected based on similarities of Ucp2 and AKT expression in cells and lungs. *p<0.05
Supplemental figure VIII. mRNA silencing efficiency after simultaneous transfection with Ucp2 and Pink1 siRNAs. For control a double dose of scrambled siRNA (sc siRNA) was transfected. Experiments were run in triplicate. Data is presented as $2^{-\Delta\Delta Ct}$ for each mRNA relative to 18S and sc siRNA. Error bars represent modified standard deviations for $2^{-\Delta\Delta Ct}$ per Applied Biosystems guide.
Materials and Methods.

Materials.

CoCl₂ was purchased from Sigma Aldrich (St. Louis, MO), CCCP (carbonyl cyanide m-chlorophenyl hydrzone) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), MitoTracker Green FM (M7514), MitoSox Red (M36008) and MitoTracker® Red CMXRos (M7512), DMEM/F15, OptiMEM media, Penicillin/Steptomycin and TripLE cell dissociation reagent were purchased from Life Technologies (USA). BAPTA-AM was purchased from Abcam (Cambridge, USA). FuGene6 was purchased from Promega (Madison, WI). FITC AnnexinV Apoptosis Detection kit (#556547) was from BD Pharmingen (San Diego, CA). In Situ Cell Death Detection kit was from Roche Diagnostics (Indianapolis, USA). EDTA-free protease inhibitor tablets were from Roche Diagnostics (Mannheim, Germany). BCA protein assay, RIPA lysis buffer and Super Signal Femtoluminescence detection kit were from Thermo Scientific (Rockford, IL). Precast protein Tris-HCl gels, Trans Blot Turbo Transfer system and PVDF containing Trans Blot Turbo Transfer kit were purchase from BioRad Laboratories (Hercules, CA). EBM-2 bullet kit for human endothelial cells was purchased from Lonza (Cologne, Germany), fibronectin was from Calbiochem (San Diego, CA). Nucleofector transfection kit for primary endothelial cells was purchased from Lonza (Cologne, Germany). All restriction endonucleases were purchased from New England Biolabs, Ipswich, MA, USA.

Antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): β actin (sc-47718), HSP60 N-20 (sc-1052), PGC1α (sc-sc-13067); Cell Signaling Technology (Beverly, MA); LC3B (#2775); Parkin (#4211); Pink1 (AM6406a and Atg5 (AP1812a) were from Abgent (San Diego, CA). Antibodies for FACS were purchased from ED Biosciences. von Willebrand Factor (vWF) antibodies were purchased from DAKO (Carpinteria, CA, USA). Secondary antibodies for immunofluorescence (donkey anti-rabbit Alexa Fluor 555) were purchased from Life Technologies (USA).

Pink1 (sc-44599) and Ucp2 (sc-42683) siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). scrambled (sc) siRNA was from .TransIT-TKO transfection reagent (#MIR 2150) was purchased from Mirus Bio LLC (Madison, WI). pflLC3 plasmid (#21074) was purchased from Addgene (Cambridge, MA). MT-mKeima-Red (AM-V0251) was from Medical and Biological Laboratories International (Way Woburn, MA). pcDNA6.2-GW/EGFP-miR plasmid for lentivirus construction was from Invitrogen, Grand Island, NY, USA.

Animals.

Ucp2 KO mice were obtained from Dr. Tamas Horvath (Yale University, New Haven, CT, USA). Wild type littermates were used as control mice. Animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Yale University.

Ucp2 Flox mice were provided by Dr. Bradford Lowell (Harvard Medical School, MA, USA). Endothelial Ucp2 knockout mice (Ucp2 VE-KO) were created by crossing Ucp2 Flox (Ucp2 Flox) (1) mice with mice containing VE-cadherin gene driving Cre recombinase (Jackson Laboratories, Maine).
Intermittent Hypoxia.

10 week old Flox and VE-KO mice were placed into a plexiglass chamber connected to OxyCycler Models A420OC and AT42CO (BioSpherix, NY, USA). Intermittent Hypoxia (IH) was run for 1 or 5 weeks according to previously published with slight modifications: O₂ nadir=5%, 6 min cycles, 10h/day (2). Age matched VE-KO and Flox mice were kept in room air as controls. Both groups of mice were fed a standard rodent chow and had free access to water. During the IH exposure, systemic blood pressure was measured weekly in the IH and room air control groups using CODA2 (Kent Scientific Corp.) mouse tail cuff system as previously described (3, 4). After 1 or 5 weeks mice were subjected to right ventricular systolic pressure (RVSP) measurements, and euthanized. Hearts were harvested, the right ventricle was dissected from left ventricle and septum. Subsequently, both parts were weighed and RV/(LV+S) ratio was calculated. Lung sections were preserved for protein and RNA analyses.

Verification of endothelial Ucp2 knockdown in VE-KO Ucp2 mice.

Endothelial (CD31+, CD45⁻) and cells were isolated using Miltenyi Biotec magnetic columns or Dynabead Tosyl–activated Dynabeads and the autoMACS pro system using manufacturer-supplied antibodies, according to the manufacturers’ instructions. Lungs were harvested from mice, sliced to small pieces in PBS buffer on ice, and filtered through 70-µm nylon meshes to make a single cell suspensates. Cell labeling with magnetic antibodies and cell separation were performed according to the manufacturers’ instructions. Total lung cell suspensates were first depleted of CD45⁺ cells and then positively selected for CD31⁺ cells. Each cells’ subfraction was dissolved in TRIZOL buffer and RNA was isolated. qPCR with Ucp2 specific primers (5) and 18S primers (6) for as a reference gene was performed for each group of cells, such as CD45⁺, CD45⁻/CD31⁺, and CD45⁻/CD31⁻.

RVSP measurements.

Mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (5 mg/kg) by intraperitoneal injection. Mice were then intubated, placed on positive pressure ventilation and light anesthesia maintained by inhaled isofluorane. A 1.9 French transducer tipped catheter (Millar Inc., Houston, TX) was inserted into right jugular vein and then advanced into the right ventricle for measurement of RVSP, positive and negative dp/dt. Data were recorded on a PowerLab instrument (ADInstruments) and analyzed using Chart 5 Pro software (ADInstruments).

Cell culture.

Mouse Lung Endothelial Cells (MLEC) were isolated from C57Bl6 mice as we previously reported (7). Cells were maintained at 37°C/5% CO₂ in F15/DMEM containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 20% FBS. Chemical hypoxia “CoCl₂ IH” was used to simulate IH in vitro (8). Cellular intermittent hypoxia model consisted of 6 cycles of 5 min hypoxia with 500µM CoCl₂ followed by 20 min reoxygenation with regular cell media. After the 6th reoxygenation cells were harvested on ice. Room air control cells were subjected to similar media changes, but without CoCl₂.

For calcium chelator experiments cells were incubated with 1µM BAPTA-AM.

Pulmonary artery endothelial cells (PAEC) from four PH patients and four healthy donors were obtained from Dr. Serpil Erzurum (9, 10). Cells were grown in endothelial basal medium on 1mg/ml fibronectin-coated tissue culture dishes according to manufacturer’s instructions. Cells were harvested and lysates were used for protein immunolottings.
Ucp2 and Pink1 siRNA transfection. MLEC were plated into 6 well plates overnight to reach 60% confluence. The next morning cells were transfected using TransIT-TKO siRNA transfection reagent according to manufacturer protocol. Briefly, 250 mL of OptiMEM media was combined with 80pg of siRNA and 8 μL of TransIT-TKO reagent in sterile 1.5 mL tube, and incubated for 25 min. After the incubation, the mix was added to each well containing MLEC with 1.5 mL cellular media. Optimal transfection time was detected 48 hours later.

Apoptosis assessment.
Apoptosis assessments in MLEC were performed with FACS Calibur machine using FITC Annexin V Apoptosis Detection kit. Briefly, cells were harvested and labeled with FITC annexin V and propidium iodide antibodies for 15 min at room temperature. FACS analysis was performed using FL-1 and FL-2 channels on Calibur FACS machine (BioRad Laboratories, CA). Final data are presented as a % of Annexin V positive cells.

Apoptosis assessments in mouse lung were performed using in situ cell death detection kit according to manufacturer’s instructions with slight modifications. Briefly, paraffin-fixed lung sections were dewaxed and hydrated, and target retrieval was performed by microwaving samples in Citrate retrieval buffer (DAKO, Carpinteria, Ca, USA) on high power (750W) for 1 min. Slides were cooled down for 20 min and immersed in serum-free blocking solution (DAKO, Carpinteria, Ca, USA) for 30 min. Anti-vWF specific antibodies were diluted in the same blocking solution 1:500 and the mix was applied on slides, and incubated overnight in humidifying conditions at 4 C. Next day slides were washed twice with 1xPBS and TUNEL mix was applied for 1h at 37C in humidifying conditions. Secondary antibodies (Alexa Fluor 555) were diluted 1:300 in PBS and added to slides for another 1h of incubation at room temperature. Slides were washed twice with PBS, mounted with DAPI containing mounting media(Vector Laboratories, Inc, Burlingame, CA, USA) and observed under fluorescent microscope Eclipse Ti fluorescent microscope using a ×40 lens.

Mitochondrial mass, mitochondrial ROS and mitochondrial potential assessment in MLEC.
Mitochondrial mass was detected with MitoTracker Green FM kit using FACS Calibur machine at channel FL-1 according to manufacturer protocol.
Mitochondrial potential was assessed with MitoTracker® Red CMXRos kit using FACS Calibur machine at channel FL-4 according to manufacturer protocol. Final data are presented as a percent of WT MLEC mitochondrial potential readings.
Mitochondrial ROS were detected with MitoSox Red kit using FACS Calibur machine at channel FL-3 according to manufacturer protocol. Data are presented as a ratio to MLEC transfected with sc siRNA at room air.

Mitochondrial mass and mitochondrial potential assessment in endothelial cells isolated from fresh mouse lungs.
Lungs were perfused with PBS through right ventricle, removed from mice and preserved in cold RPMI media. Two large lobes from the right lung were chopped with a sterile razor in RPMI media on ice and single cell suspension was prepared. The suspension was filtered through 100μM filter and 70 μM filters, and centrifuged at 300g for 10 min at 4C. Cells were washed
once with 3ml of PBS with 1% BSA and counted on Beckman Coulter Cell Counter. One million cells were incubated in RPMI media with CD45-PE antibodies (1:1000 dilution), CD31-APC (1:500 dilution), MitoTracker Green and MitoTracker® Red CMXRos dyes (1:2000 dilution) for 30 min at 37C. After the incubation cells were washed once with 3ml of PBS with 1% BSA, resuspended in 500 μL of PBS with 1% BSA, and pallied for FACS separation on Calibur FACS machine. MitoTracker Green was detected at FL-1 channel, CD45-PE labeled cells were detected at FL-2 channel, MitoTracker® Red CMXRos was detected at FL-3, and CD31-APC labeled cells were at FL-4 channel. Unlabeled and single dye labeled cells from Flox lung were used to set up necessary compensations for all the channels. Mitochondrial mass and mitochondrial potential were detected on CD45 negative / CD31 positive cells. Data is presented as a percent of Flox cells.

Mitochondrial isolation.

Frozen small lung lobe was homogenized in 500 uL ice-cold homogenization buffer containing 100 mM sucrose, 180 mM KCl, 10 mM EDTA, 5 mM MgCl2, 50 mM Tris/HCl, pH 7.4, and protease inhibitor cocktail. The homogenate was centrifuged at 1000 \( g \) for 5 min at 4 °C. The supernatant was saved and centrifuged at 11000 \( g \) for 15 min at 4 °C. The pellet containing mitochondrial was dissolved in 60-80 uL RIPA buffer containing protease inhibitor cocktail.

Measurement of Autophagic Flux

Autophagic flux was assessed by transfection with ptfLC3 plasmid (11). MLEC were transfected with ptfLC3 plasmid as well as Ucp2 and/or Pink1 siRNA using FuGene6 transfection reagent and subjected to chemical hypoxia 48 hours later. Cells were imaged with a Nikon Eclipse Ti fluorescent microscope using a ×40 lens. The numbers of GFP and RFP positive cells were counted for each experimental condition.

Measurement of mitophagy flux.

Mitophagy was evaluated by transfection with MT-mKeima-Red plasmid (12, 13). MLEC were simultaneously transfected with MT-mKeima-Red plasmid, Ucp2 and/or Pink1 siRNA using program T-023 on Amaxa Nucleofector system (Lonza, Cologne, Germany) with Basic Endothelial Cells Nucleofector® Kit. 48 hours later cells were subjected to chemical hypoxia, media was replaced by phenol-free DMEM and fluorescence was read at 444nm excitation/612nm emission and 544nm excitation/612nm emission using XS Gemini. A 544nm/444nm wavelength ratio was used to evaluate mitophagy flux.

Construction of lentiviral vectors

Lentivirus miRNA vectors with a ubiquitin promoter have been described previously (14). To silence mouse Pink1 expression in vivo, target site 1283–1303 (GenBank accession AB053476.1) was used to design Pink1 miRNA. This target sequence is 5’-GTGCGGTAATTGACTACAGCA-3’. Invitrogen’s RNAi Designer acted as an online tool to design pre-miRNA sequences. Two oligos, 5’-TGCTGTGCTGTAGTCAATTACGCACGTTTTGGCCACTGACTGACGTGCGGTATGACTACAGCA-3’ and 5’-CCTGTGCTGTAGTCAATTACGCACGTTTTGGCCACTGACTGACGTGCGGTATGACTACAGCA-3’ were annealed and ligated into pcDNA6.2-GW/EGFP-miR to generate Pink1 miRNA according to the manufacturer’s instructions. The lentiviral construct of EGFP-Pink1 miRNA was constructed by first amplifying the fragments from pcDNA6.2-GW/EGFP-Pink1 miRNA with
primers: sense: 5'-AGGCGCGCCTGGCTAACTAGAAC-3' and antisense: 5'-GGAATTCTATCTGAGTGCGGC-3'. The EGFP-Pink1 miRNA was recovered from the PCR fragment by Ascl and EcoRI digestion and then inserted into the Ascl and EcoRI sites of FUW to generate lenti-Pink1 miRNA. Lenti- NC have been described previously (14). The endothelial-specific Pink1 miRNA lentiviral construct has been described previously (15).

Intranasal administration of Pink1 miRNA lentivirus.

Intranasal administration of either the lenti-Pink1 miRNA or lenti-control was performed on 10 weeks old Ucp2 KO mice and WT littermate mice using 1x10^8 TU/mouse as previously described (14). Mice received intranasal treatment 1 week before IH experiment.

Western blotting.

Lung protein analyses were performed according to Zhang et al. (14). Whole lung tissues and cell pellets were homogenized in RIPA lysis buffer containing protease inhibitors. The protein concentrations of the lysates were determined by BCA Protein Assay. Samples were electrophoresed in a 4-20% ready-made Tris-HCl gel and transferred onto a PVDF membrane using Trans Blot Turbo transfer system. Membranes then were incubated overnight at 4C with primary antibodies. HRP-conjugated secondary antibodies incubation followed by the detection of signal with a Super Signal Femto chemiluminescence detection kit was used.

Statistics.

Data were represented as mean ± SD. Comparison of two groups was by 2-tailed Student’s t-test. Multiple group comparisons were performed using ANOVA F-test with Tukey’s post hoc test of means. Mean values were considered different at p<0.05.

References


