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Suppression of endothelial PGC-1α is associated with hypoxia-induced endothelial dysfunction and provides a new therapeutic target in pulmonary arterial hypertension

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Ye JX, Wang SS, Ge M, Wang DJ. Suppression of endothelial PGC-1α is associated with hypoxia-induced endothelial dysfunction and provides a new therapeutic target in pulmonary arterial hypertension. * Am J Physiol Lung Cell Mol Physiol 310: L1233–L1242, 2016.—Endothelial dysfunction plays a principal role in the pathogenesis of pulmonary arterial hypertension (PAH), which is a fatal disease with limited effective clinical treatments. Mitochondrial dysregulation and oxidative stress are involved in endothelial dysfunction. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a key regulator of cellular energy metabolism and a master regulator of mitochondrial biogenesis. However, the roles of PGC-1α in hypoxia-induced endothelial dysfunction are not completely understood. We hypothesized that hypoxia reduces PGC-1α expression and leads to endothelial dysfunction in hypoxia-induced PAH. We confirmed that hypoxia has a negative impact on endothelial PGC-1α in experimental PAH in vitro and in vivo. Hypoxia-induced PGC-1α inhibited the oxidative metabolism and mitochondrial function, whereas sustained PGC-1α decreased reactive oxygen species (ROS) formation, mitochondrial swelling, and NF-κB activation and increased ATP formation and endothelial nitric oxide synthase (eNOS) phosphorylation. Furthermore, hypoxia-induced changes in the mean pulmonary arterial pressure and right heart hypertrophy were nearly normal after intervention. These results suggest that PGC-1α is associated with endothelial function in hypoxia-induced PAH and that improved mitochondrial function is associated with improved mitochondrial respiration, reduced inflammation and oxygen stress, and increased PGC-1α expression. Taken together, these findings indicate that PGC-1α may be a new therapeutic target in PAH.

hypoxia; pulmonary arterial hypertension; endothelial dysfunction; proliferator-activated receptor-γ coactivator-1α; mitochondria

PULMONARY ARTERIAL HYPERTENSION (PAH) is a cardiopulmonary disease with a poor prognosis that is characterized by elevated pulmonary arterial pressure and leads to progressive right heart failure and ultimately death (57). Pharmacological agents moderately improve the patients’ symptoms and the hemodynamic parameters of severe PAH, but none significantly reduces mortality (4, 42). There is a pressing need to identify novel targets to treat PAH patients and to improve patient management.

Endothelial dysfunction, as exhibited by reduced nitric oxide (NO) production and/or utilization (14, 15, 22), plays a central role during the pathogenesis and progression of PAH (42) and is triggered by hypoxia, reactive oxygen species (ROS), and inflammation (36, 56). One of the early manifestations of endothelial dysfunction is dysregulated mitochondrial biogenesis, which changes cell metabolism with impaired NO bioavailability (44) and increases oxidative stress under pathological conditions. Mitochondria are a major source and target of vascular oxidative stress when dysregulated (20), leading to a vicious circle. Mitochondria, metabolism, and oxygen are intricably associated. Excessive mitochondrial ROS (5, 41, 43) not only reduces bioavailable NO but also leads to endothelial nitric oxide synthase (eNOS) uncoupling and the further production of ROS (40); the increased ROS then aggravates mitochondrial dysregulation (6). The factors that regulate mitochondrial biogenesis are potential therapeutic targets to ameliorate hypoxia-induced endothelial dysfunction in PAH (31, 60). Peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1α (PGC-1α) exerts numerous effects on cellular metabolism and is a key determining regulator of triglyceride accumulation and mitochondrial biogenesis (51).

PGC-1α, which is a powerful versatile transcriptional coactivator, belongs to a small family composed of PGC-1α, PGC-1β, and PGC-1-related coactivators (51). PGC-1α regulates metabolism in different organs and in various physiological and pathological states (49). In hepatocytes, PGC-1α activates gluconeogenesis (1). In brown adipose tissues, PGC-1α responds to cold exposure and drives thermogenesis (17, 37). In the heart, PGC-1α powerfully activates a broad program of mitochondrial biogenesis and fatty acid oxidation (32, 34, 46). Additionally, PGC-1α also works as an effective defender against oxidative stress in cells (45). Upregulating PGC-1α significantly attenuates oxidative damage (53). Moreover, PGC-1α is a broad and powerful regulator of mitochondrial biogenesis (16). Downregulation of PGC-1α directly induces mitochondrial dysfunction (30). Mitochondria are the main cellular producers of ROS, and a low oxygen level reduces the efficiency of the respiratory chain and leads to ROS...
production (27). Taken together, PGC-1α is a broad and powerful regulator of ROS production and extinction.

However, the PGC-1α function in the endothelium remains poorly understood. PGC-1α is involved in the regulation of antioxidant defense, antiapoptosis (48, 58), anti-inflammatory properties (29), and mitochondrial biogenesis (3, 55) in endothelial cells. Furthermore, overexpressing PGC-1α improves endothelium-dependent relaxation and preserves eNOS coupling (62). Studies of endothelial PGC-1α in intact animals are still limited. In the present study, we aimed to establish whether the suppression of PGC-1α expression is associated with hypoxia-induced endothelial dysfunction. We initially determined whether hypoxia downregulated endothelial PGC-1α in vitro and in vivo and then studied the effects of reduced PGC-1α on endothelial function in hypoxia-induced PAH. Furthermore, PGC-1α could be upregulated via the activation of PPAR receptors (rosiglitazone and bezafibrate), and its activity could be modulated via AMP-activated protein kinase (AMPK activation) [amino-imidazolecarboxamide ribonucleotide (AICAR), metformin, and resveratrol] (54). Thereafter, we addressed the role of upregulated PGC-1α in the improvement of hypoxia-induced endothelial dysfunction. Finally, we propose that PGC-1α is a potential therapeutic target for PAH in the hypoxia-induced PAH model.

METHODS

Ethics Statement

The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Institutional Animal Care and Use Committee of the Affiliated Drum Tower Hospital. Animal deaths used the approved method of euthanasia as stipulated in the American Veterinary Medical Association’s Guidelines on Euthanasia.

Cell Study

Cell culture. Human pulmonary artery endothelial cells (PAECs) (ScienCell, San Diego, CA) were resuspended and then cultured in endothelial cell medium (ScienCell, Carlsbad, CA) supplemented with 16.6% M199 and 1% fetal bovine serum. Human PAECs were washed twice with cold PBS, lysed with 0.5 M perchloric acid, and briefly sonicated (5 to 10 times with a 1-s burst) until cells were clearly disrupted. The samples were then neutralized with 2 M potassium hydroxide and centrifuged to remove the precipitate. The adenosine triphosphate (ATP) levels were measured by using the Enhanced ATP Assay Kit (Beyotime) according to the manufacturer’s instructions. The mitochondrial membrane potential (Δψm) was determined by flow cytometry using the Δψm-dependent fluorescent dye JC-1 (MitoProbe JC-1 assay kit for flow cytometry, Molecular Probes). The cells were rinsed with PBS and incubated in 2 μM JC-1 (final concentration) at 37°C for 20 min. The cells were then rinsed with PBS and analyzed by flow cytometry (FACScanto).

Cellular Western blot analysis. Cells were homogenized in ice-cold RIPA lysis buffer containing protease inhibitor cocktail and phosphoSTOP (Roche). Proteins were quantified by using a BCA protein assay kit (Pierce), separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore). Membranes were blocked with 2.5% phosphoBlocker Blocking Reagent (Cell Bioslabs) in Tris-buffered saline-Tween 20 and probed with the following antibodies: rabbit anti-AMPKα Thr172, anti-AMPKα, and anti-GAPDH (all 1:1,000, Cell Signaling Technology) or mouse anti-PGC-1α (1:200, Abcam). After washing, the membranes were incubated for 2 h at room temperature with secondary antibodies (goat anti-rabbit IgG 1:10,000 and goat anti-mouse IgG 1:2,500, Abkibine) and then washed again. Finally, chemiluminescence was detected with a mini-LAS4000 Fujifilm device (Fujifilm). Densitometry quantification was performed with Image Gauge software (Fujifilm) and normalized against GAPDH.

Animal Study

Animal model of chronic hypoxia-induced pulmonary arterial hypertension. Male Sprague-Dawley rats (6–7 wk old; weight 180–220 g) were used throughout the study and were provided by the Experimental Animal Center of Nanjing Medical University (license number: SYXX 2015-0015). All protocols and surgical procedures were approved by the local animal care committee. For the induction of PAH due to chronic hypoxia, rats were maintained in a normobaric hypoxic chamber (FIO2 10% O2) for up to 28 days.

Animal experimental groups. The rats were randomly assigned to the following four groups (15 rats per group): normoxia (Normoxia), normoxia + metformin (Metformin), hypoxia + vehicle (Hypoxia), and hypoxia + metformin (Hypoxia + Metformin). The dose of metformin (180 mg·kg⁻¹·day⁻¹) corresponded to a best measurement of the intracellular adenosine triphosphate levels and assessment of the mitochondrial membrane potential. Human PAECs were washed twice with cold PBS, lysed with 0.5 M perchloric acid, and briefly sonicated (5 to 10 times with a 1-s burst) until cells were clearly disrupted. The samples were then neutralized with 2 M potassium hydroxide and centrifuged to remove the precipitate. The adenosine triphosphate (ATP) levels were measured by using the Enhanced ATP Assay Kit (Beyotime) according to the manufacturer’s instructions. The mitochondrial membrane potential (Δψm) was determined by flow cytometry using the Δψm-dependent fluorescent dye JC-1 (MitoProbe JC-1 assay kit for flow cytometry, Molecular Probes). The cells were rinsed with PBS and incubated in 2 μM JC-1 (final concentration) at 37°C for 20 min. The cells were then rinsed with PBS and analyzed by flow cytometry (FACScanto).

Table 1. Primers in the cellular study

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<td>GTTCAACACCCAAAATGCTTAT</td>
<td>qPCR</td>
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<tr>
<td>PGC-1α (reverse)</td>
<td>ATCTACCTCGCTGAGACCCTT</td>
<td>qPCR</td>
</tr>
<tr>
<td>GAPDH (forward)</td>
<td>CGACTCCTCCAGCTTTGAC</td>
<td>qPCR</td>
</tr>
<tr>
<td>GAPDH (reverse)</td>
<td>ACCCTTGTGTTGTTGGGCA</td>
<td>qPCR</td>
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PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; qPCR, quantitative polymerase chain reaction.
daily dose of metformin in humans (2,000 mg/day) (21) (based on the body surface area transfer reduction formula between humans and rats). Metformin was administered starting either on the first (Hypoxia+Metformin) or 14th day of the 28 days of hypoxia (Hypoxia+Metformin 1/2). The endothelial cells were prepared as previously described (50). In brief, the lungs were pumped with medium 199 containing 0.25% collagenase (Worthington CLS2, pH 7.4) and 4 g% of bovine serum albumin (BSA). After digestion stopped, the cells were collected by centrifuging for 10 min at 1,000 rpm. The cells were resuspended and cultured in endothelial cell medium (ECM, ScienCell) at 37°C in an incubator with a humidified atmosphere of 5% CO₂.

Detection of blood glucose, serum TNF-α, and IL-6 levels. Blood was collected in tubes that were pretreated with heparin sodium and centrifuged at 3,500 rpm for 10 min at 4°C to obtain plasma. Blood glucose was detected by use of a Hitachi 7020 automatic analyzer (Hitachi). The serum TNF-α and IL-6 concentrations were detected by using commercial ELISA kits (GenStar) according to the manufacturer’s protocols.

Animal quantitative real-time PCR. The endothelial PGC-1α and brain natriuretic peptide (BNP) mRNA levels of the cardiomyocyte were quantified by real-time PCR. RNA was isolated from rat pulmonary artery endothelium with Trizol (Thermo Fisher). The primers that were used in this study are listed in Table 2. Steps were performed as described in the cellular part. Data were normalized to GAPDH, and the relative gene expression was calculated by the 2⁻ΔΔCt method.

Animal Western blot analysis. For Western blot analyses, rat pulmonary artery endothelium was prepared. The primary antibodies that were used included rabbit anti-eNOS (1:1,000, Abcam), anti-phospho-eNOS (1:500, Abcam), and anti-PGC-1α (1:15,000, Abcam). The protein expression and phosphorylation levels were normalized to GAPDH and to baseline expression.

NF-κB activity assay. An ELISA-based assay was used to measure the NF-κB activity as previously described (19). Rat pulmonary artery endothelium was immediately frozen in liquid nitrogen, diced and the NF-κB activity as previously described (19). Rat pulmonary artery endothelium was prepared, and RIPA containing a protease inhibitor cocktail (Roche) was added. The samples were quantified with BCA reagent (Pierce), the NF-κB activity was then detected with a TransAM NF-κB Chemi Kit (Active Motif) according to the manufacturer’s protocol.

Measurement of intracellular adenosine triphosphate levels. Rat pulmonary artery endothelium was prepared, and RIPA containing a protease inhibitor cocktail (Roche) was added. The samples were homogenized. After incubation on ice for 30 min, the lysate was centrifuged for 5 min at 4°C 12,000 g, and the supernatant was collected. The ATP levels were measured with the Enhanced ATP Assay Kit (Beyotime) according to the manufacturer’s instructions.

Electron transmission microscopy. Rat pulmonary artery endothelium was cut into 1-mm³ cubes and fixed with 2% glutaraldehyde in 0.1 mol/l sodium phosphate buffer, pH 7.4, overnight. The fixed samples were then postfixed with 1% OsO4. Copper grids were stained with 2% uranyl acetate, followed by dehydration with a series of ethanol. Samples were embedded with epoxy resin and polymerized at 60°C. The images were acquired via electron transmission microscopy (JEOL JEM-1400, Tokyo, Japan).

Measurement of hemodynamic and right ventricular hypertrophy. Rats were anesthetized via an intraperitoneal injection of ketamine and xylazine. Hemodynamic parameters were measured via a venous catheter that was inserted in the right jugular vein. The catheter was introduced into the right atrium, the right ventricle (RV), and the pulmonary artery. The mean pulmonary artery pressure (mPAP) was measured (Hewlett-Packard, M1106B). The heart was harvested and washed. RV hypertrophy was measured by using the ratio of the RV weight to the left ventricle (LV) plus interventricular septum weight (S) [RV/(LV+S)].

Statistical Analysis

The results are presented as means ± SD. SPSS 17.0 (Chicago, IL) was used for statistical analysis. Significance was calculated by an unpaired Student’s t-test or ANOVA with Dunnett’s posttest as appropriate. Significance was accepted at P < 0.05.

RESULTS

In vitro hypoxia effects on endothelial PGC-1α. Human PAECs were cultured under hypoxic (95% N₂, 5% CO₂) conditions for 12 h in glucose-containing culture medium, resulting in increased oxidative stress as measured in DCF fluorescence (Fig. 1A). Hypoxia did not reduce cell viability, as measured by FITC-Annexin V Apoptosis Detection Kits. We further examined the effects of 12-h hypoxia on PGC-1α mRNA and protein expression, which both decreased significantly by half (Fig. 1B). Then, we extended hypoxia to 24 h and observed a different response of PGC-1α mRNA expression, which nearly returned to the normoxic level (Fig. 1C). Further incubation of cells in hypoxia beyond 24 h resulted in widespread death. In our biocytoculture study, cells were initially maintained in 15 mM glucose, and fuel diminution induced ischemia-like conditions, which may upregulate PGC-1α expression directly via AMP-activated protein (AMPK) activation (25, 26, 47). To test this possibility, hypoxia experiments were repeated. Pretreatment with compound C, an AMPK blocker, completely abolished this effect, implicating AMPK activation in this process. Furthermore, we fed cells again in the middle of 24-h hypoxia with glucose-containing culture medium, which completely abolished this effect (Fig. 1C). Changes in AMPK were confirmed by Western blotting (Fig. 1D). In brief, hypoxia reduced the expression level of endothelial PGC-1α.

Hypoxia effects on endothelial mitochondria and upregulated PGC-1α restored mitochondrial dysfunction. Human PAECs were treated with or without metformin in vitro. We used independent parameters of ATP production and ΔΨm to evaluate mitochondrial function. The 12-h hypoxia induced a collapse of the ΔΨm and decreased ATP production in human PAECs (Fig. 2A). Upreregulated endothelial PGC-1α with metformin overcame the hypoxia-induced oxidative stress and mitigated hypoxia-induced mitochondrial dysfunction (Fig. 2, A–C). In addition, the Western blotting results showed that the p-AMPK/t-AMPK levels also increased in response to metformin. In all, upregulated endothelial PGC-1α could preserve endothelial function under hypoxic conditions.

Chronic hypoxia effects on endothelial PGC-1α in an in vivo chronic hypoxia-induced pulmonary arterial hypertension model. The mRNA and protein expression levels of PGC-1α were determined in rats. Compared with the

Table 2. Primers in the animal study

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<tr>
<td>GAPDH (forward)</td>
<td>GGGCTCTCTTCGCTCCCTTGT</td>
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<td>GAPDH (reverse)</td>
<td>CAGGCCTCTCGTACGCACAA</td>
<td>qPCR</td>
</tr>
<tr>
<td>BNP (forward)</td>
<td>CCGCAAAAGGACGTTGAACTA</td>
<td>qPCR</td>
</tr>
<tr>
<td>BNP (reverse)</td>
<td>GGGCCGAAGCAGGTTGAACTA</td>
<td>qPCR</td>
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BNP, brain natriuretic peptide.
Fig. 1. Effects of hypoxia on pulmonary artery endothelial cells and endothelial PGC-1α. A: oxidative stress during endothelial hypoxia was assayed with 2,7’-dichlorodihydrofluorescein (DCF) fluorescence (green). Scale bar = 100 μm. Quantitative analysis of DCF fluorescence intensity by flow cytometry. #P < 0.05 vs. Normoxia. B: quantitative (q) PCR and Western blot analysis of PGC-1α expression after hypoxia for 12 h. #P < 0.05 vs. Normoxia; n = 6. C: qPCR of PGC-1α expression after hypoxia for 12 or 24 h (left); qPCR of PGC-1α expression in pulmonary artery endothelial cells with 24 h of hypoxia and 40 μM compound C (middle); qPCR of PGC-1α expression in the normoxic group or in cells that were exposed to 24 h of hypoxia with or without repeat feeding with glucose-containing (15 mM) culture medium after 12 h of hypoxia or in the normoxic group. The results were normalized to total AMPK expression (left). Phosphorylated AMPKα (Thr172) protein expression was assayed by Western blot analysis of pulmonary artery endothelial cells that were exposed to 12 or 24 h of hypoxia or in the normoxic group. The results were normalized to total AMPK expression (left). Phosphorylated AMPKα (Thr172) protein expression in normoxic pulmonary artery endothelial cells or in cells that were exposed to 24 h of hypoxia with or without repeat feeding with glucose-containing (15 mM) culture medium after 12 h of hypoxia (right). #P < 0.05 vs. Normoxia; &P < 0.05 vs. Hypoxia-24 h; n = 6. PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; AMPK, AMP-activated protein kinase.
moxia groups, rats under hypoxia had significantly lower PGC-1α expression levels. Metformin significantly increased the PGC-1α mRNA level under both normoxic and hypoxic conditions (Fig. 3A), but the PGC-1α protein expression level was elevated only in the hypoxic group (Fig. 3A).

**Effects on hemodynamics and right ventricular hypertrophy.** Rats under chronic hypoxia for 4 wk displayed an increased hematocrit (Hypoxia vs. Hypoxia + Metformin vs. Normoxia or Metformin: 70 ± 1 vs. 71 ± 2 vs. 44 ± 2 or 43 ± 3%, n = 15, P < 0.01), whereas weight (Hypoxia vs. Hypoxia + Metformin vs. Normoxia vs. Metformin: 453 ± 4 vs. 451 ± 5 vs. 451 ± 3 vs. 449 ± 5 g), fasting blood glucose (Hypoxia vs. Hypoxia + Metformin vs. Normoxia vs. Metformin 4.13 ± 0.43 vs. 4.3 ± 0.19 vs. 4.3 ± 0.19 vs. 4.23 ± 0.15 mM), and postprandial blood glucose (Hypoxia vs. Hypoxia + Metformin vs. Normoxia vs. Metformin: 10.56 ± 1.24 vs. 11.13 ± 1.31 vs. 11.11 ± 1.21 vs. 11.07 ± 1.1 mM) were unchanged compared with the untreated rats (n = 15, all P > 0.05). This experiment administered metformin to rats for 4 wk. No differences in body weight or blood glucose were observed between the metformin groups and controls.

The rats that were exposed to chronic hypoxia to establish PAH were characterized by an increased mPAP and right ventricular remodeling, which was demonstrated as a significant increase in RV/(LV+S) (Fig. 3B). Metformin treatment throughout the hypoxia duration decreased both the mPAP and the RV/(LV+S) ratio to nearly normal levels (Fig. 3B). To further define the effects of metformin in the
rat chronic-hypoxia PAH model, we reassessed the effects of metformin treatment administered during the last 2 wk of the 4-wk hypoxia. Both mPAP and the RV/(LV+S) ratio were significantly reduced (Fig. 3B). To determine the effects on cardiac function, we examined the BNP mRNA level, which markedly decreased in the rats that were treated with metformin throughout the duration, indicating an improvement of cardiac function (Fig. 3D).

Fig. 3. Chronic hypoxia decreased endothelial PGC-1α expression and upregulated PGC-1α in a chronic hypoxia-induced pulmonary artery hypertension model. A: effects of chronic hypoxia on endothelial PGC-1α in a chronic hypoxia-induced pulmonary arterial hypertension model. Quantitative (q) PCR and Western blot analysis of PGC-1α was performed in normoxia+vehicle (Normoxia), normoxia+metformin (Metformin), rats that were exposed to hypoxia for 4 wk (Hypoxia) and rats that were exposed to hypoxia and treated with metformin for 4 wk (180 mg·kg⁻¹·day⁻¹) (Hypoxia+Metformin). #P < 0.05 vs. Normoxia; n = 15. B: effects on hemodynamic and right ventricular hypertrophy. The mPAP and [RV/(LV+S)] ratio were determined in the Normoxia, Metformin, Hypoxia, and Hypoxia+Metformin groups. C: mPAP and [RV/(LV+S)] ratio in the Normoxia, Metformin, Hypoxia, and rats that were exposed to hypoxia and treated with metformin for the final 2 wk of the 4 wk (180 mg·kg⁻¹·day⁻¹) (Hypoxia+Metformin 1/2). #P < 0.05 vs. Normoxia; *P < 0.05 vs. Hypoxia, n = 15. D: BNP mRNA level as assessed by qPCR. #P < 0.05 vs. Normoxia; *P < 0.05 vs. Hypoxia, n = 15.

PAH, pulmonary arterial hypertension; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; mPAP, mean pulmonary arterial pressure; [RV/(LV+S)], RV hypertrophy as measured using the ratio of RV weight to left ventricle (LV) plus interventricular septum weight (S); BNP, brain natriuretic peptide.
Upregulated PGC-1α protects against chronic hypoxia-induced mitochondrial dysfunction. Mitochondria, metabolism, and oxygen are inextricably intertwined. Mitochondria are energy units in every cell of the body and convert energy into ATP to power cellular functions. Mitochondria dysfunction is always accompanied by structural changes. The rats that were exposed to chronic hypoxia displayed massive swelling of the mitochondria and loss of cristae, as determined by electron transmission microscopy (Fig. 4A). In contrast, the rats that were treated with metformin throughout the duration had neat, regularly organized mitochondria (Fig. 4A).

Furthermore, chronic hypoxia decreased ATP production in the endothelium, but this treatment rescued mitochondrial function (Fig. 4, A and B).

Effects on NO bioavailability and NF-κB activation. A decrease in eNOS activation may be a major mechanism underlying endothelial dysfunction. Initially, metformin did not affect eNOS phosphorylation at Ser1177 under normoxic conditions (Fig. 5A). However, the phosphorylation levels of eNOS significantly decreased after chronic hypoxia exposure (Fig. 5A). In contrast, coinubation with metformin enhanced the reduced activation even past control levels (Fig. 5A).
Inflammation is the key factor that drives the progression of simple endothelial dysfunction to PAH. NF-κB activation is also associated with endothelial dysfunction, and NF-κB serves as a key transcription factor of major proinflammatory cytokines and adhesion molecules. Thus we examined inflammatory signaling through NF-κB and quantified the serum levels of IL-6 and TNF-α. Compared with the activation observed under normoxia in rats with or without metformin treatment, the activation of NF-κB significantly increased under chronic hypoxia (Fig. 5B). A similar trend was also observed in the serum levels of IL-6 and TNF-α. Upon treatment with metformin under chronic hypoxia, NF-κB activation significantly decreased (Fig. 5B). Together, these results suggest that metformin decreases inflammatory responses in the endothelium while improving eNOS activity.

DISCUSSION

PAH is characterized by a progressive increase of pulmonary vascular resistance and pulmonary arterial pressure, leading to right ventricle hypertrophy and death (23). Different forms of PAH share common pathogenic mechanisms involving inflammation, endothelial dysfunction (35, 39), oxidative stress, and mitochondrial dysfunction (12). Arguably, PGC-1α is a transcriptional coactivator and also acts as an important precursor for mitochondrial biosynthesis, which also controls inflammation (7) and decreases oxidative stress by enhancing the expression of antioxidant genes (60). In this study, we showed how hypoxia affects the expression of endothelial PGC-1α. Our results indicate that PGC-1α expression is associated with O2 content (Fig. 1C), in agreement with hypoxia exposure decreasing the PGC-1α levels in hepatocytes (38). In contrast, the ischemic condition corresponding to our 24-h hypoxia increased PGC-1α expression (Fig. 1D), which could be abrogated by blocking AMPK (compound C) or replenishing fuel (Fig. 1D). Ischemia likely induced PGC-1α expression via direct AMPK-mediated activation, and additional studies are needed to explore the mechanisms.

Hypoxia is a predominant factor of PAH, and mitochondria are the major source and target of oxidative stress in cells. Hypoxia generates ROS, and because we detected an increase in ROS, we hypothesized that metformin might decrease ROS as part of its protective effect in endothelial cells. The hypoxia-induced downregulation of endothelial PGC-1α could lead to endothelial dysregulation, resulting in endothelial dysfunctions. This result was associated with a decrease in membrane potential, decreased ATP production, and increased ROS levels (Fig. 2, A and B). Treatment with metformin sustained PGC-1α expression in PAECs, as indicated by increased mitochondrial integrity and ATP production and reduced ROS (Fig. 2, A–C). This result supports the hypothesis that the loss of PGC-1α disrupts energy metabolism and ATP production by dysregulated mitochondrial biogenesis and respiration. The improved endothelial function was associated with improved cellular mitochondrial respiration and ATP synthesis and increased PGC-1α expression in PAECs. Using a chronic hypoxia-induced PAH rat model, we further showed that chronic hypoxia without ischemia results in decreased PGC-1α expression (Fig. 3). These results agree with the decreased PGC-1α levels observed under chronic hypoxia in respiratory muscles (18) and cardiomyocytes (47). As previously mentioned, our findings support the notion that chronic hypoxia can reduce PGC-1α expression separate from fuel deprivation.

We thus propose that PGC-1α is associated with endothelial function in hypoxia-induced PAH and that PGC-1α could be a new therapeutic target for preventing the development of PAH. Furthermore, the improvement of endothelial function was associated with improved cellular mitochondrial respiration, reduced inflammation and oxygen stress, and increased PGC-1α expression (Figs. 3A, 4, and 5). We also observed that the hemodynamic right ventricular hypertrophy and cardiac BNP mRNA level as measurements of cardiac function in chronic hypoxia-induced PAH were attenuated after treatment (Fig. 3, B and C).

One of the determinate factors of vascular health is mitochondrial biogenesis in the endothelium. Hypoxia can decrease PGC-1α expression and cause mitochondrial dysfunction, along with a significant reduction of oxidative phosphorylation efficiency, resulting in membrane potential breakdown, ROS production, ATP depletion, and uncoupled oxidative phosphorylation (Figs. 1 and 2). PGC-1α functions as a key regulator of mitochondrial biogenesis. Here, we reconfirmed that upregulated PGC-1α expression could restore mitochondrial function, as demonstrated by increased ATP production and near, regular mitochondrial structure without swelling, rupture, or loss of cristae (Fig. 4).

Our results also suggest that PGC-1α could protect the endothelium through the inhibition of NF-κB activity (Fig. 5). The nuclear factor NF-κB is a central regulator of inflammatory processes. NF-κB activation is associated with inflammatory stimuli in endothelial cells (52). The change in NF-κB was in contrast to the change of PGC-1α, which agrees with the previous report that PGC-1α exerts many of its protective effects via the inhibition of NF-κB activity (13). As an inflammatory marker, NF-κB can also stimulate ROS production (24). Thus we reasoned that NF-κB inhibition might improve endothelium-dependent function by reducing oxidative stress and inflammation.

In our study, improvement of endothelial function was associated with increased eNOS phosphorylation and PGC-1α expression (Fig. 5). Improved eNOS activity is thought to increase NO bioavailability and facilitate angiogenesis (11). NO is one of the most important regulators of endothelial functions; thus increased NO produces a number of protective effects on the endothelium. NO could upregulate PGC-1α (8) and the eNOS-PGC-1α regulatory pathway, which preserves endothelial mitochondrial function. PGC-1α-mediated protection plays a role in preconditioning strategies; this protection is multifactorial and not limited to mitochondrial biogenesis (28). Several lines of evidence support the protective properties of metformin in PAH. Three studies have reported that metformin suppresses pulmonary arterial smooth muscle cell proliferation and may negatively modulate pulmonary vascular remodeling in PAH (2, 33, 59). Metformin can also increase eNOS phosphorylation, decrease Rho kinase activity, and improve vasodilation (4, 9, 10). Our data demonstrate the effects of metformin on endothelial function and mitochondria through PGC-1α, further supporting its protective effect in experimental models of PAH. Metformin significantly increased the PGC-1α mRNA level, but only the PGC-1α protein level was elevated in the hypoxia group (Fig. 3A). The reasons for this discrepancy are difficult to determine. Other factors may regulate this
process and require further study. PGC-1α may function as one of the key mediators in the metformin-mediated protective effect on PAH. One study reported that PGC-1 mediates the critical regulation of metformin in muscle irisin expression and function (61).

In summary, we have demonstrated the effect of hypoxia on endothelial PGC-1α, and our results suggest that PGC-1α protects endothelial cells by reducing oxidative stress, improving mitochondrial respiratory function, and inhibiting inflammation, which together ameliorate endothelial dysfunction. PGC-1α could be an ideal target to control or limit the damage associated with the defective mitochondrial function in PAH; therefore, it may be a new potent therapeutic target for PAH. Additionally, metformin is currently widely used in clinical practice, and its use as a new therapy for PAH might be plausible, practical, and safe.

There are some limitations in clinically translating the observations of this study. These limitations include the use of the hypoxia-induced PAH, which does not entirely recapitulate human PAH. Although this study mainly focuses on the effects of PGC-1α, other members of this family may affect other PAH-related signaling pathways. Thus one cannot exclude the possibility that metformin influences the development of PAH via additional mechanisms. The changes that we observed are limited to endothelial cells and do not extend to other cell types in PAH.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


