Protective Roles of Endothelial AMP-Activated Protein Kinase Against Hypoxia-Induced Pulmonary Hypertension in Mice

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Rationale: Endothelial AMP-activated protein kinase (AMPK) plays an important role for vascular homeostasis, and its role is impaired by vascular inflammation. However, the role of endothelial AMPK in the pathogenesis of pulmonary arterial hypertension (PAH) remains to be elucidated.

Objective: To determine the role of endothelial AMPK in the development of PAH.

Methods and Results: Immunostaining showed that endothelial AMPK is downregulated in the pulmonary arteries of patients with PAH and hypoxia mouse model of pulmonary hypertension (PH). To elucidate the role of endothelial AMPK in PH, we used endothelial-specific AMPK-knockout mice (*eAMPK*$^{-/-}$), which were exposed to hypoxia. Under normoxic condition, *eAMPK*$^{-/-}$ mice showed the normal morphology of pulmonary arteries compared with littermate controls (*eAMPK*$^{flox/flox}$). In contrast, development of hypoxia-induced PH was accelerated in *eAMPK*$^{-/-}$ mice compared with controls. Furthermore, the exacerbation of PH in *eAMPK*$^{-/-}$ mice was accompanied by reduced endothelial function, upregulation of growth factors, and increased proliferation of pulmonary artery smooth muscle cells. Importantly, conditioned medium from endothelial cells promoted pulmonary artery smooth muscle cell proliferation, which was further enhanced by the treatment with AMPK inhibitor. Serum levels of inflammatory cytokines, including tumor necrosis factor-α and interferon-γ were significantly increased in patients with PAH compared with healthy controls. Consistently, endothelial AMPK and cell proliferation were significantly reduced by the treatment with serum from patients with PAH compared with controls. Importantly, long-term treatment with metformin, an AMPK activator, significantly attenuated hypoxia-induced PH in mice.

Conclusions: These results indicate that endothelial AMPK is a novel therapeutic target for the treatment of PAH. (*Circ Res*. 2016;119:197-209. DOI: 10.1161/CIRCRESAHA.115.308178.)

Key Words: cell proliferation ■ cytokines ■ inflammation ■ metformin ■ pulmonary hypertension

Cytokines/chemokines and growth factors regulate pulmonary endothelial function and influence the development of pulmonary arterial hypertension (PAH).[1] PAH is characterized by pulmonary vascular remodeling and perivascular inflammation, leading to right ventricular (RV) failure and premature death.[2–5] Endothelial dysfunction is a crucial pathogenic status that triggers a variety of vascular disorders, such as PAH.[6,7] Endothelial dysfunction is also considered a key underlying feature in most forms of clinical and experimental PAH, which is enhanced by inflammatory cytokines/

chemokines and growth factors.[1,8] Indeed, we experience rapid progression and worsening of PAH, especially when complicated with infectious diseases, such as pneumonia and catheter-related infection.[8] Pulmonary endothelial dysfunction in patients with PAH enhances pulmonary vascular remodeling through impaired release of vasodilators, such as nitric oxide (NO) and prostacyclin.[9–11]

AMP-activated protein kinase (AMPK) is a heterotrimeric complex consisting of a catalytic subunit α and 2 regulatory subunits β and γ, and it is expressed in various tissues and

Nonstandard Abbreviations and Acronyms	
ACC	acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
CM	conditioned medium
DMEM	Dulbecco modified Eagle's medium
eNOS	endothelial nitric oxide synthase
FGF-2	fibroblast growth factor-2
IL	interleukin
NO	nitric oxide
PAEC	pulmonary artery endothelial cell
PAH	pulmonary arterial hypertension
PASMC	pulmonary artery smooth muscle cell
PDGF-BB	platelet-derived growth factor-BB
PH	pulmonary hypertension
RVH	right ventricular hypertrophy
RVSP	right ventricular systolic pressure
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cells
WT	wild-type

subcellular locations.[12] AMPK is an evolutionary conserved serine/threonine kinase that functions as an important energy sensor[13] and is activated by inhibition of Rho-kinase,[14] which plays a crucial role for PAH.[15–17] AMPK has an antiapoptotic effect in endothelial cells[18] and a proapoptotic effect in vascular smooth muscle cells (VSMC),[19] which are critical for vascular remodeling. Endothelial cell dysfunction and interaction between pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs) play a crucial role for the development of PAH.[20,21] Both endothelial NO production and NO-mediated signaling in VSMC are targets and effectors of the AMPK signaling pathway.[13] In endothelial cells, AMPK positively regulates NO production. In VSMC, AMPK reduces intracellular signaling and secretion of many growth factors, promoting VSMC proliferation and vascular remodeling.[13] Recent evidence suggests that AMPK regulates many other stimuli that modulate vascular functions, including reactive oxygen species, and promotes VSMC proliferation by auto/paracrine growth mechanisms.[13] Previous studies have reported crucial roles of AMPK signaling in animal models of pulmonary hypertension (PH). For example, Ibe et al[22] demonstrated that $AMPK\alpha_1$ and $AMPK\alpha_2$ play differential roles in the survival of PASMCs during hypoxia and hypoxia-induced PH. In addition, Teng et al[23] reported that both activity and expression level of AMPK are decreased in PAECs with in utero PH and suggested that AMPK activation improves angiogenesis. Moreover, we have recently demonstrated that endothelial AMPK plays an important role in microvascular homeostasis and regulation of systemic arterial pressure in mice in vivo.[24] Several drugs (eg, statins and metformin) and molecules (eg, apelin) activate AMPK, all of which could be potentially protective against PAH.[25–29] However, the role of endothelial AMPK in the pathogenesis of PH remains to be elucidated. In this study, we thus tested our hypothesis that endothelial AMPK plays protective roles against hypoxia-induced PH in mice.

Methods

Animal Experiments
All animal experiments were performed in accordance with the protocols approved by the Tohoku University Animal Care and Use Committee (No. 2013-Kodo-009). Hypoxia-induced PH models were used to assess the development of PH in mice.[30] Eight-week-old male wild-type (WT) mice on a normal chow diet were exposed to normobaric hypoxia (10% O_2) or normoxia for 4 weeks as previously described.[25,31] Briefly, hypoxic mice were housed in an acrylic chamber with a nonrecirculating gas mixture of 10% O_2 and 90% N_2 by adsorption-type oxygen concentrator to utilized exhaust air (Teijin, Tokyo, Japan), whereas normoxic mice were housed in room air (21% O_2) under a 12-hour light and dark cycle. After 4 weeks of exposure to hypoxia (10% O_2) or normoxia, mice were anesthetized with isoflurane (1.0%). To examine the development of PH, we measured RV systolic pressure (RVSP), RV hypertrophy (RVH), and pulmonary vascular remodeling.[25,30,31] For right heart catheterization, a 1.2-F pressure catheter (SciSense Inc, Ontario, Canada) was inserted in the right jugular vein and advanced into the RV to measure RVSP.[15] All data were analyzed using the PowerLab data acquisition system (AD Instruments, Bella Vista, Australia) and averaged >10 sequential beats.[25,30,31]

Generation and Genotyping of eAMPK$^{-/-}$ Mice
AMPK-floxed mice were obtained from Dr Viollet at Cochin Institute (Paris, France).[32] We generated *eAMPK$^{-/-}$* mice by crossing AMPK-floxed mice with Tie2-Cre mice on a C57BL/6 background.[24,33] The genotype of mice was confirmed by polymerase chain reaction using primers specific for the $AMPK\alpha_1$ gene (5′-TATTGCTGCCATTAGG CTAC-3′ and 5′-GACCTGACAGAATAGGATATGCCCAACCTC-3′), the $AMPK\alpha_2$ gene (5′-GCTTAGCACGTTACCCTGGATGG-3′ and 5′-GTTATCAGCCCAACTAATTACAC-3′), and the Tie2-Cre transgene (5′-GCGGTCTGGCAGTAAAAACTATC-3′ and 5′-GTGAAACAGCA TTGCTGTCACTT-3′). All mice were genotyped by polymerase chain reaction amplification of tail-clip samples, and all experiments were performed with male mice using littermate as WT controls. Animals were housed under a 12-hour light and 12-hour dark regimen and placed on a normal chow diet as previously described.[24]

Histological Analysis
After hemodynamic measurements, the heart and lungs were perfused with cold phosphate–buffered saline (PBS) and fixed in 10% formaldehyde for 24 hours. The whole heart and lungs were embedded in paraffin, and cross sections (3 μm) were prepared. Paraffin sections were stained with Elastica–Masson (EM) or used for immunostaining. Antibodies used were as follows: α-smooth muscle actin (Sigma-Aldrich, 1:400), Ki67 (Abcam, 1:800). Pulmonary arteries adjacent to an airway distal to the respiratory bronchiole were evaluated as previously reported.[30] Briefly, arteries were considered fully muscularized if they had a distinct double-elastic lamina visible throughout the diameter of the vessel cross section. The arteries were considered partially muscularized if they had a distinct double-elastic lamina visible for at least half the diameter. The percentage of vessels with double-elastic lamina was calculated as the number of muscularized vessels per total number of vessels counted. In each section, a total of 60 to 80 vessels were examined by the use of a computer-assisted imaging system (BX51, Olympus, Tokyo, Japan). This analysis was performed in the small vessels with external diameters of 20 to 70 μm.

Assessment of RVH
Formaldehyde-fixed dry hearts were dissected, and the RV wall was removed from the left ventricle (LV) and septum. The ratio of the RV to the LV plus septum weight (RV/[LV+Sep]) was calculated to determine the extent of RVH.[30]

Human Lung Samples
All protocols using human specimens were approved by the Institutional Review Board of Tohoku University, Sendai, Japan (No. 2013-1-160). Lung tissues were obtained from patients with PAH at the time of lung transplantation (n=8) or from control

patients (n=6) at the time of thoracic surgery for lung cancer at a site far from the tumor margins as previously described.[30] We analyzed lung specimens from 8 patients with PAH (6 idiopathic PAH, 1 PAH with shunt disease, and 1 PAH with collagen disease) obtained during lung transplantation and those obtained from 6 controls during pneumonectomy procedure for lung cancer. The average age (mean±SEM) was 36.0±4.2 years in patients with PAH and 66.8±2.9 years in controls. In the PAH group, mean pulmonary artery pressure was 51.2±7.5 mm Hg (range, 38.0–80.0 mm Hg), mean pulmonary vascular resistance was 1160±490 dyne·s·cm^{-5} (range, 619–1637 dyne·s·cm^{-5}), and mean cardiac index was 2.5±0.6 L/min per m^2 (range, 1.7–3.2 L/min per m^2). All patients provided written consent for the use of their lung tissues for research. Small pulmonary arteries were also obtained at the time of lung transplantation from patients with PAH. PASMCs from patients with PAH were isolated from pulmonary arteries <1.5 mm in outer diameter.[15,30] Primary human PASMCs were commercially obtained from Lonza (Basel, Switzerland). PASMCs were cultured in Dulbecco modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. PASMCs of passages 4 to 7 at 70% to 80% confluence were used

for experiments. We isolated PAECs from explanted recipient lungs, using a modified elastase/collagenase digestion protocol as previously described.[34] Additional primary PAECs were commercially obtained from Lonza. PAECs were grown in EBM-2 basal medium supplemented with EGM-2 (Lonza). PAECs of passages 3 to 5 at 70% to 80% confluence were used for experiments.

Harvest of Mouse PASMCs and PAECs

Mouse PASMCs were cultured from each group of 23- to 26-g male mice and maintained in DMEM containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air as previously described.[15,30] PASMCs of passages 4 to 7 at 70% to 80% confluence were used for experiments. Mouse PAECs were isolated by digesting minced lung with collagenase type 2 (GIBCO) and gentle agitation for 45 minutes at 37°C. Using a 50-mL syringe attached firmly to a cannula, the suspension was triturated 12×, filtered through 40-μm cell strainers and then centrifuged at 400 g for 5 minutes at 4°C. Cells were resuspended in 2 mL of cold PBS plus 0.1% bovine serum albumin, and the cell suspension was incubated with CD31 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). PAEC were positively selected
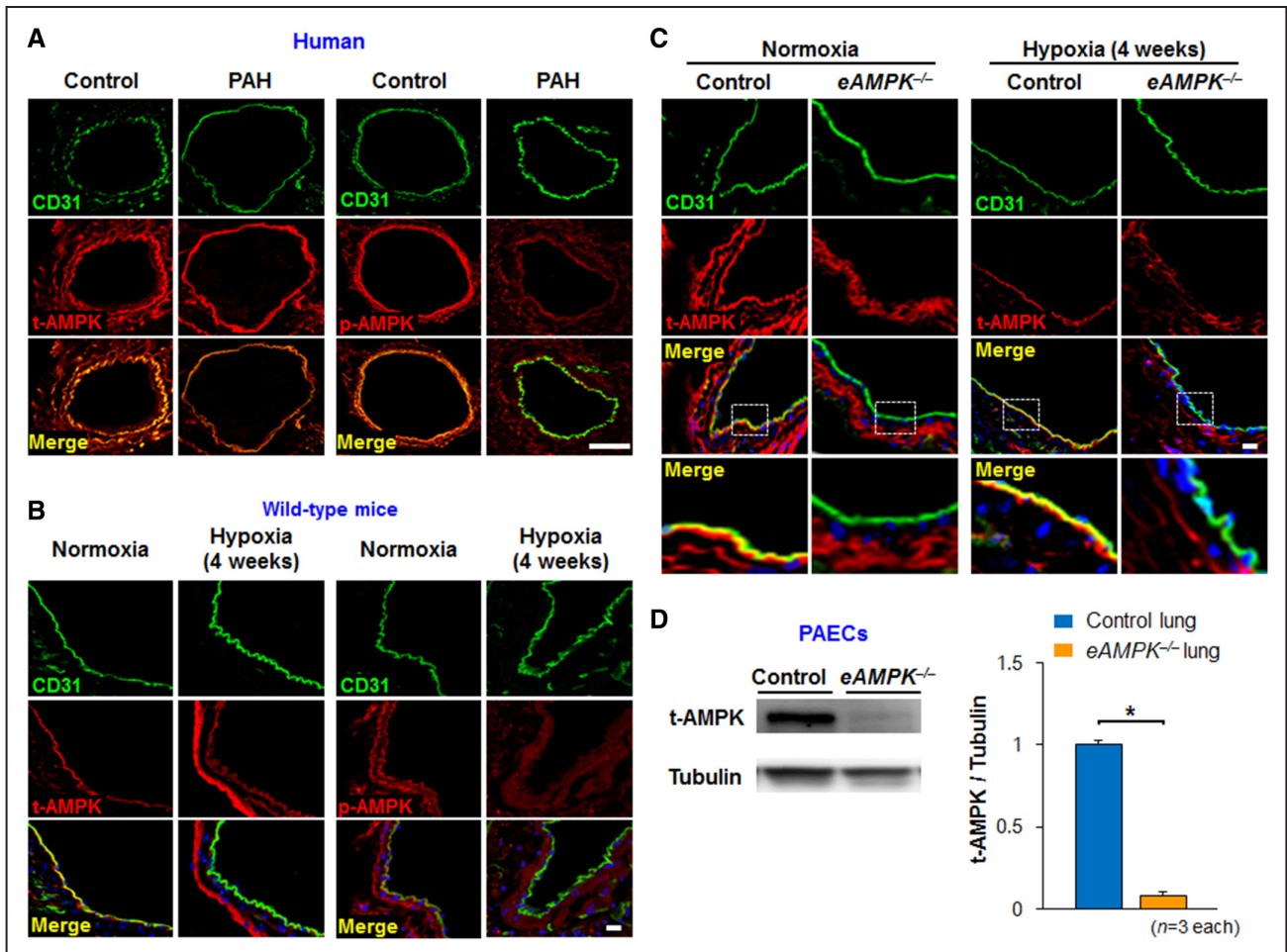
Figure 1. Downregulation of endothelial AMP-activated protein kinase (AMPK) in patients with pulmonary arterial hypertension (PAH). A, Representative immunostaining pictures of the distal pulmonary arteries from patients with PAH (n=8) and controls (n=6). The endothelium was visualized by CD31 (Alexa Fluor-488, green). Double immunostaining for total AMPK (t-AMPK, Alexa Fluor-563, red), phosphorylated-AMPK at Thr172 (p-AMPK, Alexa Fluor-563, red), and CD31 (green). Scale bars, 25 μm. **B,** Representative immunostaining pictures of the distal pulmonary arteries from wild-type mice in normoxia and hypoxia (10% O_2). The endothelium was visualized by CD31 (Alexa Fluor-488, green). Immunostaining for t-AMPK (Alexa Fluor-563, red), p-AMPK (Alexa Fluor-563, red), CD31 (green), and 4′,6-diamidino-2-phenylindole [DAPI], blue). Scale bars, 25 μm. **C,** Representative immunostaining pictures of the distal pulmonary arteries from normoxic and hypoxic (10% O_2) endothelial–specific AMPK-knockout mice (eAMPK$^{-/-}$) and control mice. Immunostaining for t-AMPK (Alexa Fluor-563, red), p-AMPK (Alexa Fluor-563, red), CD31 (green), and DAPI (blue). Scale bar, 25 μm. **D,** Western blot analysis of total AMPK protein in pulmonary artery endothelial cells (PAECs) harvested from eAMPK$^{-/-}$ mice and controls (n=3 each). *P<0.05.

for CD31 by the Magnetic Cell Sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. PAECs of passages 4 to 5 at 80% to 90% confluence were used for experiments.

Preparation of Conditioned Medium

For the preparation of conditioned medium (CM; ex vivo culture), fresh lung samples obtained from mice were maintained equal wet weight of the minced tissue in each well of 6-well plates with DMEM. We collected the CM 24 hours after incubation as previously described.[30] Human PAECs obtained from Lonza in 10-cm dishes were treated with compound C (5 µmol/L, Merck Millipore), an AMPK inhibitor, or vehicle for 24 hours. After the incubation period, the medium was collected as CM as we described previously.[30]

Measurement of Cytokines/Chemokines and Growth Factors With the Bioplex System

The tissue levels of cytokines/chemokines and growth factors in the whole lung were measured with the Bioplex system (Bio-Rad, Tokyo, Japan) according to the manufacturer's instructions. To analyze the levels of cytokines/chemokines in lung tissues, pulmonary arteries were perfused with PBS, and the circulating blood was

completely removed. Lung tissues were homogenized with Tissue Protein Extraction Reagent (Pierce, Rockford, IL) and centrifuged (4°C, 2500 g, 20 minutes), and thereafter, clear supernatants were standardized for total protein content using the BCA Protein Assay Kit (Pierce, Rockford, IL).[35]

Cell Proliferation Assay

WT mouse PASMCs and human PASMCs were seeded in 96-well plates (15 000 cells/well) in DMEM with 10% fetal bovine serum. The cells were allowed to adhere for 24 hours and then stimulated with DMEM as control or CM for 2 days. Proliferation was measured by the Cell-Titer 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI).[36] Human PAECs and human PASMCs were seeded in 96-well plates (15 000 cells/well). The cells were allowed to adhere for 24 hours and stimulated with 5% serum from patients with PAH or healthy volunteers for 3 days. Cell proliferation was measured by the Cell-Titer 96 AQueous One Solution Cell Proliferation Assay Kit.

Immunofluorescence Staining

Lung tissues were embedded in optimum cutting temperature compound and quickly frozen. The tissues were cut into 10-µm thick slices. Antibodies used were as follows: CD31 (BD Pharmingen, 1:500), AMPK (Abcam, 1:500), phosphorylated AMPK at Thr172
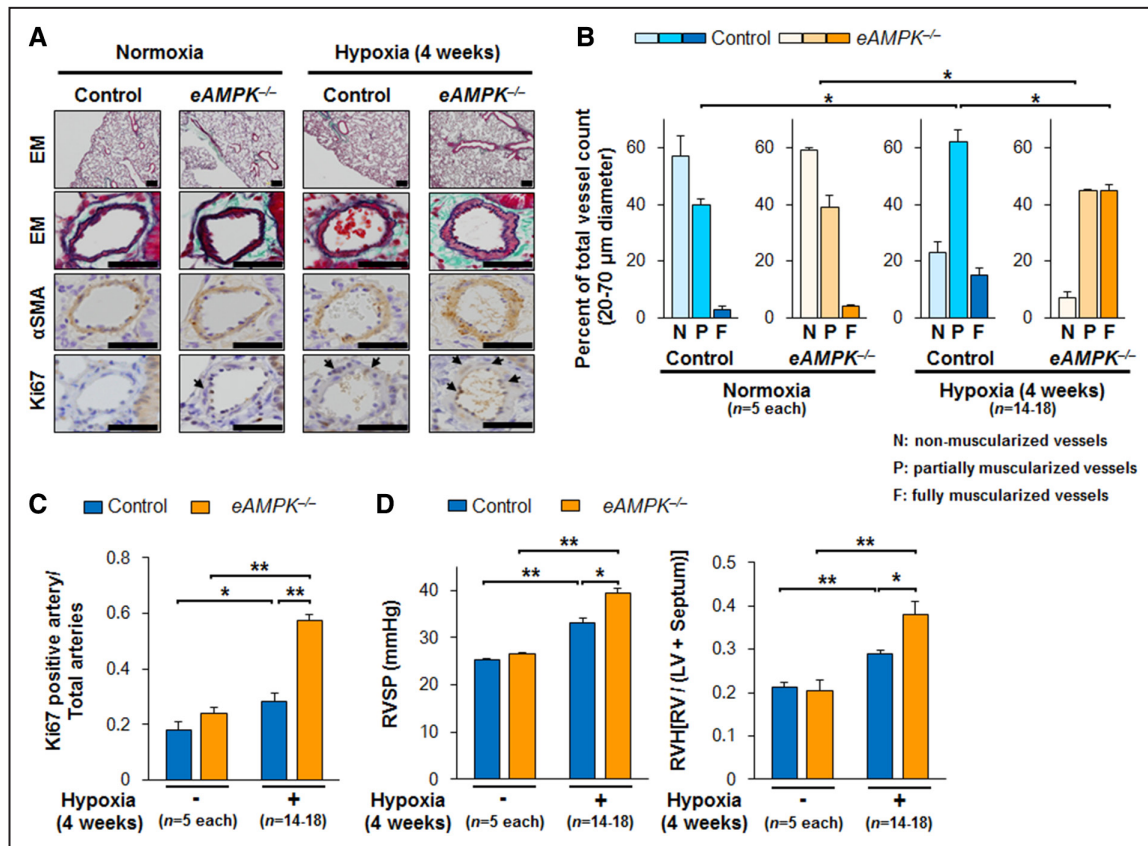
Figure 2. Endothelial AMP-activated protein kinase (AMPK) deletion accelerates hypoxia-induced pulmonary hypertension. A, Representative Elastica–Masson (EM) and immunostaining for α-smooth muscle actin (α-SMA) and Ki67 of the distal pulmonary arteries exposed to normoxia or hypoxia (10% O$_2$) for 4 wk. Arrows indicate Ki67-positive staining. Scale bars, 50 µm. **B**, Muscularization of the distal pulmonary arteries with a diameter of 20 to 70 µm. Endothelial-specific AMPK-knockout mice (*eAMPK*$^{-/-}$) and control mice in normoxia (n=5 each) vs *eAMPK*$^{-/-}$ (n=14) and control mice (n=18) after hypoxia for 4 wk. The arteries were considered fully muscularized (F) if they had a distinct double-elastic lamina visible throughout the diameter of the vessel cross section. The arteries were considered partially muscularized (P) if they had a distinct double-elastic lamina visible for at least half the diameter. The percentage of vessels with double-elastic lamina was calculated as the number of muscularized vessels per total number of vessels counted. Results are expressed as mean±SEM. *P<0.05. **C**, The numbers of Ki67-positive arteries in *eAMPK*$^{-/-}$ and control lungs. Results are expressed as mean±SEM. *P<0.05 and **P<0.01. **D**, Right ventricular systolic pressure (RVSP) and RV hypertrophy (RVH) assessed by the ratio of RV to left ventricle plus septum weight. Results are expressed as mean±SEM. *P<0.05 and **P<0.01. F indicates fully muscularized vessels; N, nonmuscularized vessels; and P, partially muscularized vessels.

(Cell Signaling, 1:500), platelet-derived growth factor-BB (PDGF-BB, R&D systems, 1:500), and fibroblast growth factor-2 (FGF-2, Santa Cruz, 1:500). Lung tissues obtained from patients with PAH at the time of lung transplantation (n=8) or from control patients (n=6) at the time of thoracic surgery for lung cancer at a site far from the tumor margins and murine lung tissues from littermate controls and *eAMPK*^{−/−} mice exposed to normoxia or hypoxia were analyzed. Slides were viewed with a fluorescence microscopy (LSM 780, Carl Zeiss, Oberkochen, Germany).

Western Blot Analysis

Human PAECs were seeded in 10-cm dishes in DMEM with 10% fetal bovine serum. PASMCs were allowed to adhere for 24 hours, washed 2×, and starved in serum-free medium for 24 hours. These quiescent cells were then stimulated with CM from PAECs treated with compound C or vehicle for 24 hours. PASMCs were washed with cold PBS and lysed with cell lysis buffer (Cell Signaling) and protease inhibitor cocktail (Sigma-Aldrich) after the incubation period. Total cell lysates from lung homogenates and human PASMCs were loaded on the SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (GE Healthcare) after blocking for 1 hour at room temperature in 5% bovine serum albumin in *tris*-buffered saline with tween 20.[16] The primary antibodies were against caspase-3 (Cell Signaling, 1:1000), proliferating cell nuclear antigen (Santa Cruz, 1:500), AMPK (Cell Signaling, 1:1000), phosphorylated AMPK at Thr172 (Cell Signaling, 1:500), acetyl-CoA carboxylase (ACC, Cell Signaling, 1:1000), phosphorylated ACC at Ser79 (Cell Signaling, 1:500), p53 (Santa Cruz, 1:500), endothelial nitric oxide synthase (eNOS, BD Biosciences, 1:1000), phosphorylated eNOS at Ser1177 (BD Transduction Laboratories, 1:500), and vascular cell adhesion molecule-1 (VCAM-1, Santa Cruz, 1:500). Proteins were visualized by the enhanced chemiluminescence system (ECL Western Blotting Detection Kit; GE Healthcare) as previously described.[16] Densitometric analysis was performed with Image J Software (NIH, Bethesda, MD).

In-Cell Western Blot Assay Using LI-COR System

In-cell Western assay is a rapid, reproducible alternative to traditional Western blotting. The in-cell Western assay is a quantitative immunofluorescence assay performed in microplates (96-well format).[37] We have performed analysis based on the recommendations of the manufacturer (https://www.licor.com/bio/applications/in-cell_western_assay/).
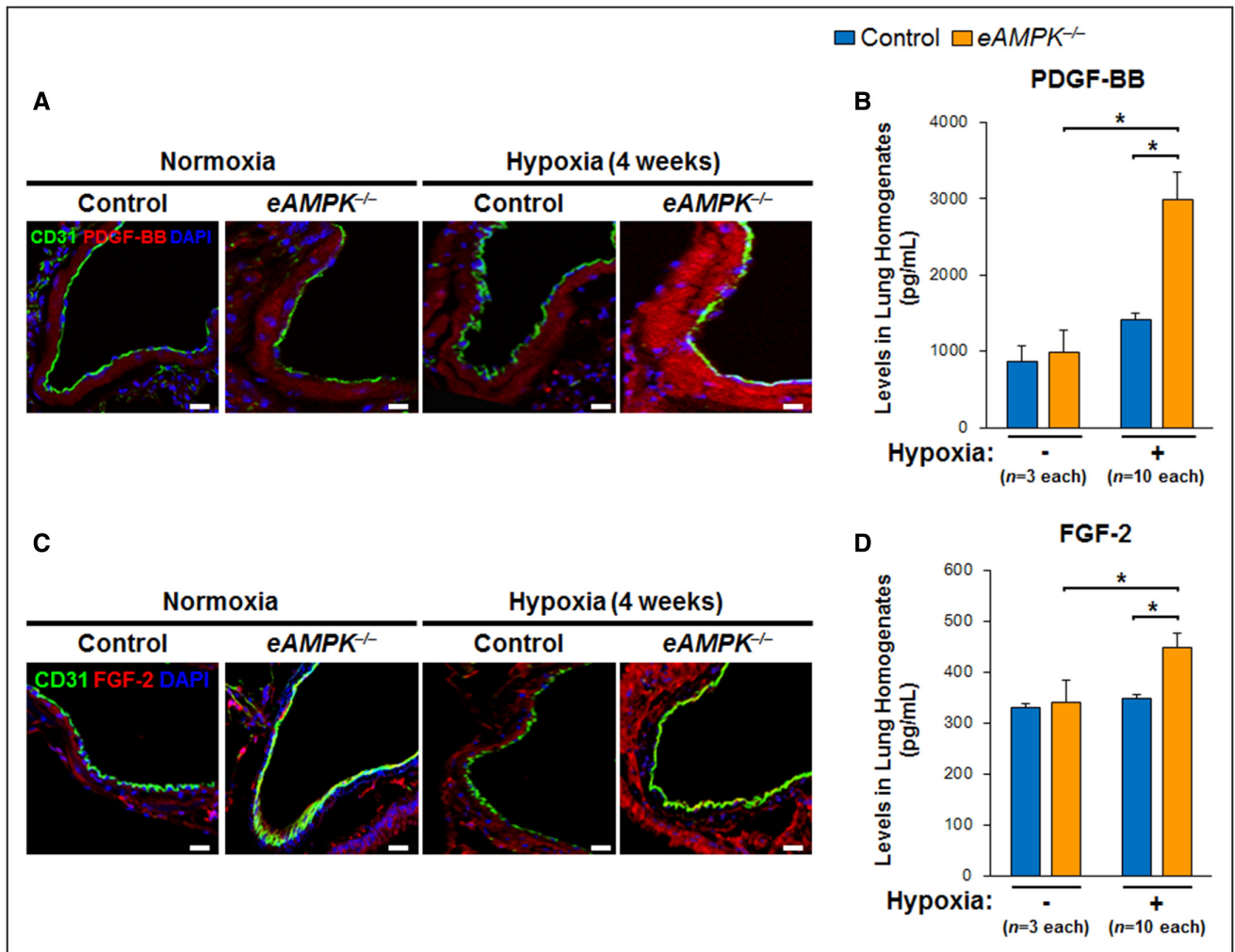
Figure 3. Endothelial-specific AMP-activated protein kinase (AMPK) deletion increases growth factors in the lung. A, Representative pictures of distal pulmonary arteries from normoxic and hypoxic (10% O₂) endothelial–specific AMPK-knockout (*eAMPK*^{−/−}) and control mice. Immunostaining for platelet-derived growth factor-BB (PDGF-BB, Alexa Fluor-563, red), CD31 (Alexa Fluor-488, green), and (4′,6-diamidino-2-phenylindole [DAPI], blue). Scale bars, 25 μm. **B**, Levels of PDGF-BB in lung homogenates after normoxia (n=3 each) and hypoxic exposure (n=10 each) for 4 wk in *eAMPK*^{−/−}and control mice. Results are expressed as mean±SEM. *P<0.05. **C**, Immunostaining for fibroblast growth factor-2 (FGF-2, red), CD31 (green), and DAPI (blue). Scale bars, 25 μm. **D**, Levels of FGF-2 in the lung after normoxia (n=3 each) and hypoxic exposure (n=10 each) after hypoxic exposure for 4 wk in *eAMPK*^{−/−} and control mice. Results are expressed as mean±SEM. *P<0.05.

Human PAECs and PASMCs were seeded in clear-bottomed 96-well plates (15 000 cells/well) and were allowed to adhere for 24 hours. The medium was removed, and the cells were washed with PBS before stimulating with human serum for 24 hours. The cells were then fixed in 3.7% formaldehyde and incubated at room temperature for 20 minutes. The cells were permeabilized with PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and blocked for 1.5 hours in PBS containing 0.1% Triton X-100 and 3% bovine serum albumin at room temperature with gentle rocking. The cells were incubated with primary antibodies overnight at 4°C. The primary antibodies were against ACC (Cell Signaling, 1:400), phosphorylated ACC at Ser79 (Cell Signaling, 1:400), VCAM-1 (Santa Cruz, 1:500), and α-tubulin (Sigma-Aldrich, 1:500). The next day, the cells were washed with PBS containing 0.1% Tween 20 and incubated with secondary antibodies. The secondary antibodies were as follows: IRDye 680RD Goat anti-Rabbit (LI-COR, 1:400) and IRDye 800CW Goat anti-Mouse (LI-COR, 1:800 dilution). After incubation, the cells were washed and analyzed with the Odyssey CLx (LI-COR, Bad Homburg, Germany).

Metformin Treatment
Eight-week-old male WT mice were randomized to be treated with either metformin (100 mg/kg per day) or vehicle in drinking water and were exposed to hypoxia (10% O_2) for 3 weeks. After 3 weeks of hypoxia, RVSP and RVH were measured as described above.[38,39]

Serum Samples From Patients With PAH and Healthy Controls
We collected serum samples from patients with symptoms or signs of PH who were referred to Tohoku University Hospital for right heart catheter examination from February 2009 to December 2011. Patients with PAH (n=30) were enrolled, and those with cancer were excluded. As healthy controls, we collected serum samples from healthy volunteers (n=15).

Statistical Analyses
All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test. Comparisons of mean responses associated with the 2 main effects of the different genotypes and the severity of pulmonary vascular remodeling were performed by 2-way ANOVA, followed by Tukey honestly significant difference multiple comparisons. All reported *P* values are 2-tailed, with a *P* value of <0.05 indicating statistical significance.[15,30] Statistical significance was evaluated with JMP 10 (SAS Institute Inc, Cary, NC).

Results

Knockdown of Endothelial AMPK Promotes the Development of Hypoxia-Induced PH
Immunostaining of the lung tissues from patients with PAH (n=8) showed reduced endothelial expression of phosphorylated AMPK in distal pulmonary arteries when compared with non-PAH controls (n=6; Figure 1A; Online Figures I and II). Moreover, we evaluated the expression of AMPK in the endothelium of the distal pulmonary arteries in experimental mouse model of PH: chronic hypoxia–induced PH model.[30] WT mice exposed to hypoxia for 4 weeks revealed decreased expression of phosphorylated AMPK in the endothelium of the distal pulmonary arteries when compared with those in normoxia (Figure 1B). Thus, we generated *eAMPK*$^{-/-}$ mice to examine the specific roles of endothelial AMPK in vivo. In the distal pulmonary arteries, we found costaining of the endothelial marker CD31 and AMPK in control mice, whereas there was no expression of endothelial AMPK in the pulmonary arteries of *eAMPK*$^{-/-}$ mice (Figure 1C). In addition, the protein
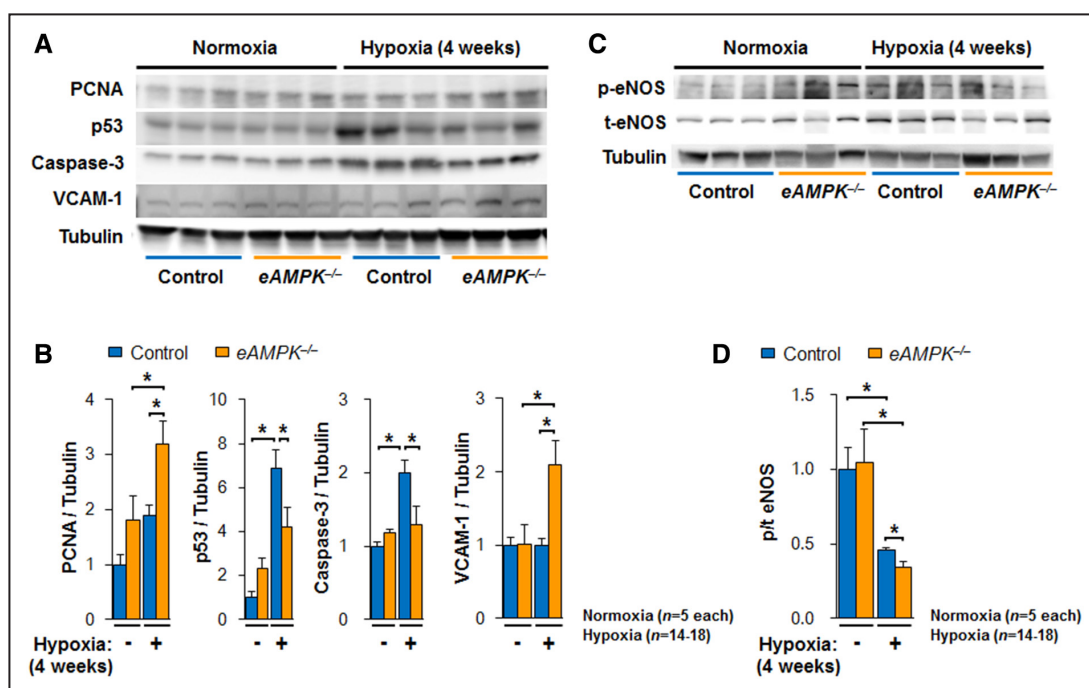

Figure 4. Endothelial-specific AMP-activated protein kinase (AMPK) deletion promotes proliferation in lung tissue. A, Representative Western blotting of proliferating cell nuclear antigen (PCNA), p53, caspase-3, and vascular cell adhesion molecule-1 (VCAM-1) in normoxic and hypoxic (10% O_2) endothelial–specific AMPK-knockout (*eAMPK*$^{-/-}$) and control mice. **B**, Densitometric analyses of Western blotting. Normoxic *eAMPK*$^{-/-}$ and control mice (n=5 each) vs hypoxic *eAMPK*$^{-/-}$ (n=14) and control mice (n=18). Results are expressed as mean±SEM. *P<0.05. **C**, Representative Western blotting of phosphorylated endothelial nitric oxide synthase (p-eNOS) at Ser1177 and total eNOS (t-eNOS). Normoxic *eAMPK*$^{-/-}$ and control mice (n=5 each) vs hypoxic *eAMPK*$^{-/-}$ (n=14) and control mice (n=18). **D**, Densitometric analyses of Western blotting. Results are expressed as mean±SEM. *P<0.05.

level of AMPK was significantly less in PAECs harvested from *eAMPK^−/−* lungs compared with those from controls (Figure 1D). The morphology of distal pulmonary arteries in normoxic *eAMPK^−/−* mice did not differ compared with control mice (Figure 2A). In contrast, mice exposed to hypoxia for 4 weeks exhibited a significant difference in the medial thickness of pulmonary arteries (Figure 2A). When compared with control mice, *eAMPK^−/−* mice exhibited significantly severe muscularization of distal pulmonary arteries after hypoxic exposure (Figure 2A and 2B). The number of Ki67-positive cells in the pulmonary arteries of normoxic *eAMPK^−/−* mice did not differ compared with control mice (Figure 2A and 2C). In contrast, the number of Ki67-positive cells in the pulmonary arteries of *eAMPK^−/−* mice was significantly increased compared with controls after hypoxic exposure. Consistent with these morphological changes, control mice exhibited increased RVSP after chronic hypoxia, which was exaggerated

in *eAMPK^−/−* mice (Figure 2D). The increased ratio of RV to LV plus septum weight (RV/[LV+Sep]) was also enhanced in *eAMPK^−/−* mice compared with controls (Figure 2D).

Knockdown of Endothelial AMPK Increases PDGF-BB and FGF-2 in Hypoxic Lung

Assessment of cytokines/chemokines and growth factor levels in *eAMPK^−/−* lungs compared with control lungs revealed significant increase in VEGF, but a decrease in RANTES (regulated on activation, normal T cell expressed and secreted) and interleukin-6 (IL-6) in *eAMPK^−/−* lungs after hypoxia (Online Figure III). PDGF-BB and FGF-2 were also significantly upregulated after hypoxic exposure in *eAMPK^−/−* lungs compared with control lungs (Figure 3A through 3D). Furthermore, immunostaining showed that the increased expressions of PDGF-BB and FGF-2 were especially strong in the smooth muscle layers of distal pulmonary
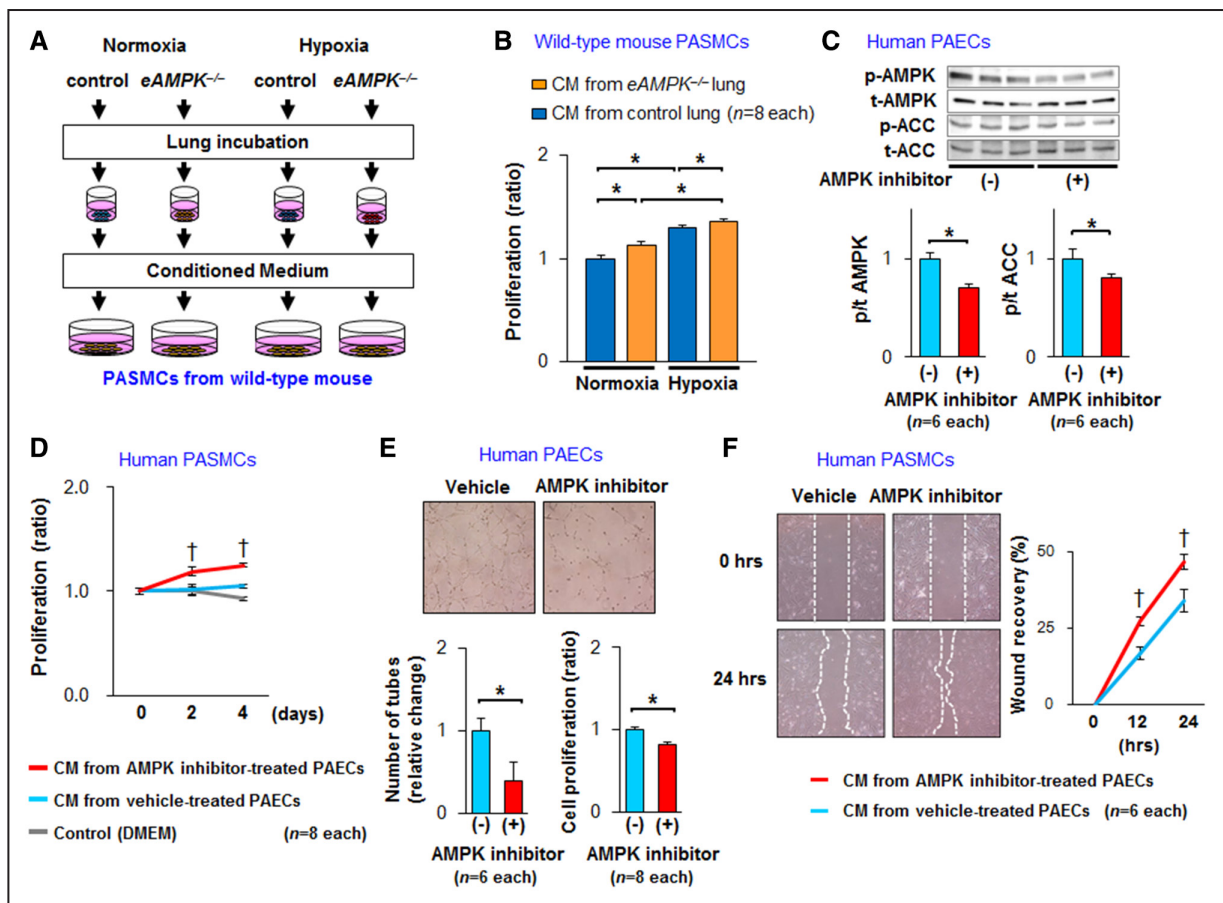
Figure 5. Endothelial AMP-activated protein kinase (AMPK) is crucial for pulmonary artery smooth muscle cell (PASMC) proliferation. A, Experimental design using PASMCs from wild-type mice, which were stimulated with conditioned medium (CM) prepared from normoxic and hypoxic (4 wk in vivo, 10% O₂) endothelial–specific AMPK-knockout (*eAMPK^−/−*) lungs or control lungs. **B**, Wild-type mouse PASMC proliferation by the treatment with CM prepared from normoxic and hypoxic *eAMPK^−/−* lungs or control lungs (n=8 each). Results are expressed as mean±SEM. *P<0.05. **C**, Representative Western blotting and densitometric analyses of phosphorylated AMPK at Thr17 (p-AMPK), total AMPK (t-AMPK), phosphorylated acetyl-CoA carboxylase (ACC) at Ser79 (p-ACC), and total ACC (t-ACC) in human pulmonary artery endothelial cell (PAEC) treated with AMPK inhibitor, compound C or vehicle. Results are expressed as mean±SEM (n=6 each). *P<0.05. **D**, Human PASMC proliferation by the treatment with CM from compound C–treated PAECs or vehicle-treated PAECs. Results are expressed as mean±SEM (n=8 each). †P<0.05 compared with CM from vehicle-treated PAECs. **E**, Representative pictures of tube formation assay in human PAECs treated with compound C or vehicle. Tube number was assessed 8 h after the treatment with compound C or vehicle. Results are expressed as mean±SEM (n=6 each). *P<0.05. Cell proliferation of human PAEC by the treatment with compound C or vehicle for 72 h. Results are expressed as mean±SEM (n=8 each). *P<0.05. **F**, Representative pictures from wound healing assay of PASMCs stimulated with CM prepared from compound C–treated PAEC or vehicle-treated PAEC. Results are expressed as mean±SEM (n=6 each). †P<0.05 compared with CM from vehicle-treated PAECs.

arteries (Figure 3A through 3C). In addition, Western blotting showed an increased proliferating cell nuclear antigen expression in *eAMPK*$^{-/-}$ lungs compared with control lungs (Figure 4A and 4B). In contrast, the expression of p53, which suppresses cell proliferation, was significantly downregulated in *eAMPK*$^{-/-}$ lungs compared with control lungs after hypoxic exposure (Figure 4A and 4B). Moreover, the expression of caspase-3 was downregulated in *eAMPK*$^{-/-}$ lungs compared with control lungs after hypoxia (Figure 4A and 4B). We further assessed eNOS activity in lung tissues and found significantly reduced phosphorylation of eNOS in *eAMPK*$^{-/-}$ lungs compared with control lungs after hypoxic exposure (Figure 4C and 4D). Finally, the expression of adhesion molecule, VCAM-1, was significantly upregulated in *eAMPK*$^{-/-}$ lungs compared with control lungs after hypoxia (Figure 4A and 4B).

Interaction Between Endothelial Cells and Smooth Muscle Cells in Pulmonary Arteries

We harvested PASMCs from WT mice and stimulated them with CM prepared from *eAMPK*$^{-/-}$ lungs or control lungs (Figure 5A). PASMC proliferation was significantly increased by the treatment with CM from hypoxic lungs (4 weeks in vivo) compared with normoxic lungs (Figure 5B). Moreover, PASMC proliferation was enhanced by the treatment with CM from *eAMPK*$^{-/-}$ lungs compared with control lungs (Figure 5B). We next assessed whether inhibition of endothelial AMPK could promote PASMC growth in a paracrine manner. The treatment of endothelial cells with an AMPK inhibitor, compound C (5 μmol/L, 24 hours), significantly downregulated AMPK and its downstream ACC (Figure 5C). Importantly, CM from compound C–treated endothelial cells significantly increased PASMC proliferation compared with vehicle-treated endothelial cells (Figure 5D). Consistently, compound C–treated human PAECs showed significantly reduced angiogenesis assessed by tube formation assay and cell proliferation (Figure 5E). Furthermore, to determine the interaction between PAECs and PASMCs, we used CM to stimulate human PASMCs. Interestingly, wound-healing assay demonstrated that CM from compound C–treated PAECs significantly promoted migration of PASMCs compared with CM from vehicle-treated PAECs (Figure 5F), which implicates the interactions between PAECs and PASMCs through endothelial AMPK signaling.

Circulating Inflammatory Cytokines Downregulate Pulmonary Endothelial AMPK

We performed analyses of the serum from patients with PAH by using the Bioplex cytokine array system (Table). We found a significant increase in serum levels of inflammatory cytokines, including interferon-γ and tumor necrosis factor-α, in patients with PAH (Online Figures IV and V). These results implicate the involvement of multiple signaling pathways in pulmonary vascular inflammation and remodeling in patients with PAH. Thus, we next examined the impact of increased serum levels of inflammatory cytokines on PAECs and PASMCs in vitro (Figure 6). Treatment with the serum from patients with PAH significantly downregulated AMPK signaling in healthy PAECs, as assessed by ACC phosphorylation compared with

Table. Baseline Characteristics of Patients With PAH

|	Healthy Volunteers (n=15)	PAH (n=30)
Age, y	36±3	44±3
Male/Female, n	10/5	10/20
Body mass index, kg/m²	20.5±1.2	23.6±0.7
WHO class		
I/II, %	100	82
III, %		12
IV, %		6
Mean RAP, mm Hg		6.4±0.7
Mean PAP, mm Hg		44.7±2.6
Mean PCWP, mm Hg		9.1±0.4
Cardiac index, L/min per m²		2.6±0.1
PVR, dyne·s·cm⁻⁵		685.7±74.5
SvO₂, %		69.0±1.1

Results are expressed as mean±SEM. PAP indicates pulmonary artery pressure; PAH, pulmonary arterial hypertension; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; RAP indicates right arterial pressure; and SvO₂, mixed venous oxygen saturation.

controls (Figure 6A). In addition, the serum from patients with PAH upregulated VCAM-1 in healthy PAECs compared with controls (Figure 6A). Consistently, treatment with the serum from patients with PAH significantly reduced PAEC proliferation compared with controls (Figure 6A). Similarly, the serum from patients with PAH significantly downregulated ACC phosphorylation and upregulated VCAM-1 in PASMCs compared with controls (Figure 6B). In contrast, the serum from patients with PAH increased healthy PASMC proliferation compared with controls (Figure 6B). We further performed analyses using PAECs and PASMCs harvested from patients with PAH (Figure 6C and 6D). Interestingly, similar responses were noted in PAH PAECs (Figure 6C) and PAH PASMCs (Figure 6D), except the findings in the augmented proliferation of PAH PAECs in response to the serum from patients with PAH (Figure 6C).

Metformin Ameliorates Hypoxia-Induced PH in Mice

Finally, we found that the treatment with metformin significantly ameliorates hypoxia-induced PH in mice. Metformin upregulated AMPK and its downstream ACC in WT lungs (Figure 7A). The expression of VCAM-1 was significantly downregulated in the metformin-treated lungs compared with controls (Figure 7A). In addition, the phosphorylation of eNOS was significantly upregulated in the metformin-treated mice compared with vehicle controls (Figure 7A; Online Figure VI). Importantly, under normoxia, mice treated with metformin alone showed no significant morphological or hemodynamic changes compared with vehicle-treated mice (Figure 7B and 7C). In contrast, metformin-treated mice exhibited fewer muscularized distal pulmonary arteries after hypoxic exposure compared with vehicle controls (Figure 7B). Consistent with these morphological changes, the development of hypoxia-induced PH, as assessed by RVSP and RVH,
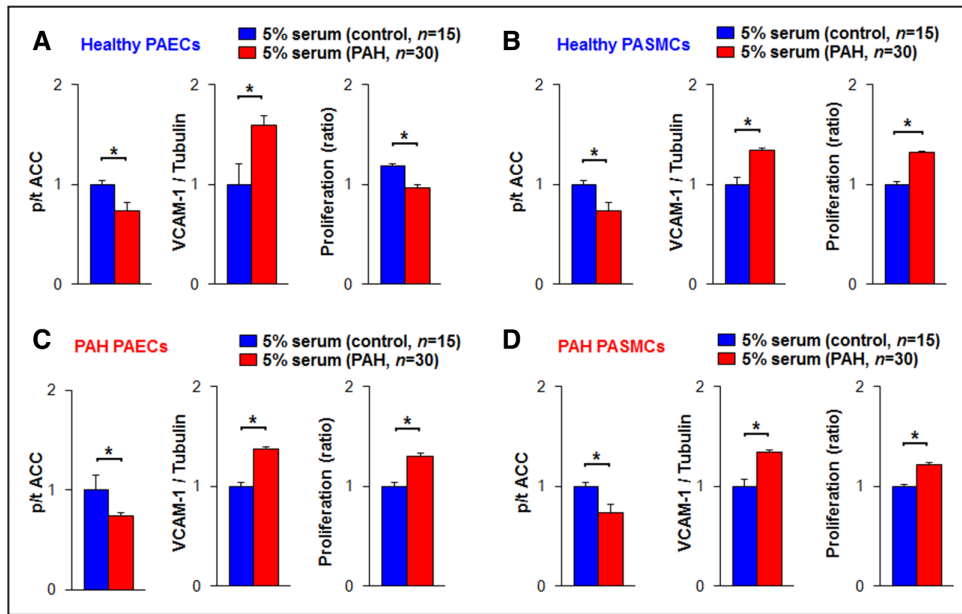
Figure 6. Circulating inflammatory cytokines downregulate endothelial AMP-activated protein kinase (AMPK) in pulmonary arterial hypertension (PAH). A, Quantitative analysis of the in-cell Western blot assay. Expression of phosphorylated acetyl-CoA carboxylase (ACC) at Ser79 (p-ACC), total ACC (t-ACC), and vascular cell adhesion molecule-1 (VCAM-1) in healthy pulmonary artery endothelial cells (PAECs) treated with the serum from patients with PAH (n=30) or healthy volunteers (n=15). Results are expressed as mean±SEM. *P<0.05. Cell proliferation of healthy PAECs treated with the serum (5%) from patients with PAH (n=30) or healthy volunteers (n=15) for 72 h. Results are expressed as mean±SEM. *P<0.05. **B**, Quantitative analysis of the in-cell Western blot assay. Expression of p-ACC, t-ACC, and VCAM-1 in healthy pulmonary artery smooth muscle cells (PASMCs) treated with the serum from patients with PAH (n=30) or healthy volunteers (n=15). Results are expressed as mean±SEM. *P<0.05. Cell proliferation of healthy PASMCs treated with the serum (5%) from patients with PAH (n=30) or healthy volunteers (n=15) for 72 h. Results are expressed as mean±SEM. *P<0.05. **C**, Quantitative analysis of the in-cell Western blot assay. Expression of p-ACC, t-ACC, and VCAM-1 in PAH PAECs treated with the serum from patients with PAH (n=30) or healthy volunteers (n=15). Results are expressed as mean±SEM. *P<0.05. Cell proliferation of PAH PAECs treated with the serum (5%) from patients with PAH (n=30) or healthy volunteers (n=15) for 72 h. Results are expressed as mean±SEM. *P<0.05. **D**, Quantitative analysis of the in-cell Western blot assay. Expression of p-ACC, t-ACC, and VCAM-1 in PAH PASMCs treated with the serum from patients with PAH (n=30) or healthy volunteers (n=15). Results are expressed as mean±SEM. *P<0.05. Cell proliferation of PAH PASMCs treated with the serum (5%) from patients with PAH (n=30) or healthy volunteers (n=15) for 72 h. Results are expressed as mean±SEM. *P<0.05.

was significantly ameliorated in the metformin-treated mice compared with vehicle controls (Figure 7C).

Discussion

The major findings of this study are that (1) endothelial AMPK activity was reduced in distal pulmonary arteries of patients with PAH and experimental mouse model of PH, (2) endothelial AMPK knockdown promoted the development of hypoxia-induced PH, (3) endothelial AMPK knockdown increased PDGF-BB and FGF-2 expression in PASMCs, (4) serum from patients with PAH reduced PAEC proliferation and increased PASMC proliferation, and (5) metformin ameliorated hypoxia-induced PH in mice. On the basis of these findings, we propose that endothelial AMPK protects against the development of hypoxia-induced PH (Figure 7D).

Endothelial AMPK Downregulation Increases PDGF-BB and FGF-2 and Promotes PASMC Proliferation

There are increasing reports that support the role of cross talk between PAECs and PASMCs in the development of PAH.[20,40] In this study, we aimed to further elucidate the key role of endothelial AMPK because it mediates the interaction between PAECs and PASMCs. Using hypoxia-induced PH model in eAMPK^{−/−} mice, we demonstrated a decreased phosphorylation

of eNOS in eAMPK^{−/−} mice after chronic hypoxia. These data are consistent with the previous study demonstrating that endothelial AMPK is essential for endothelial function and vascular homeostasis, especially in hypoxia.[41] Indeed, activation of eNOS catalyzes the production of NO, which is a crucial molecule for the maintenance of endothelial function and vascular homeostasis.[42] Indeed, Afolayan et al[43] demonstrated that phosphorylation of APMK and mitochondrial biogenesis are downregulated in fetal lambs with persistent PH of the newborn. In addition, eNOS phosphorylation in PAEC increased superoxide dismutase-2 levels and decreased the mitochondrial superoxide levels in this animal model of persistent PH of the newborn.[43] Thus, our study supports the crucial role of endothelial AMPK in the maintenance and homeostasis of pulmonary vasculature. We demonstrated that the expression of adhesion molecule, VCAM-1, was upregulated in eAMPK^{−/−} lungs after chronic hypoxia in addition to impaired endothelial function. This observation is consistent with the recent study demonstrating that VCAM-1 in PAEC is upregulated in patients with PAH and experimental PH models.[44] Next, we demonstrated that growth factors, such as PDGF-BB and FGF-2, both of which are upregulated in PAH,[45] were significantly increased in eAMPK^{−/−} lungs compared with control lungs. Although AMPK was specifically knockdown for endothelial cells in eAMPK^{−/−} mice, the enhanced expressions of PDGF-BB and FGF-2 were
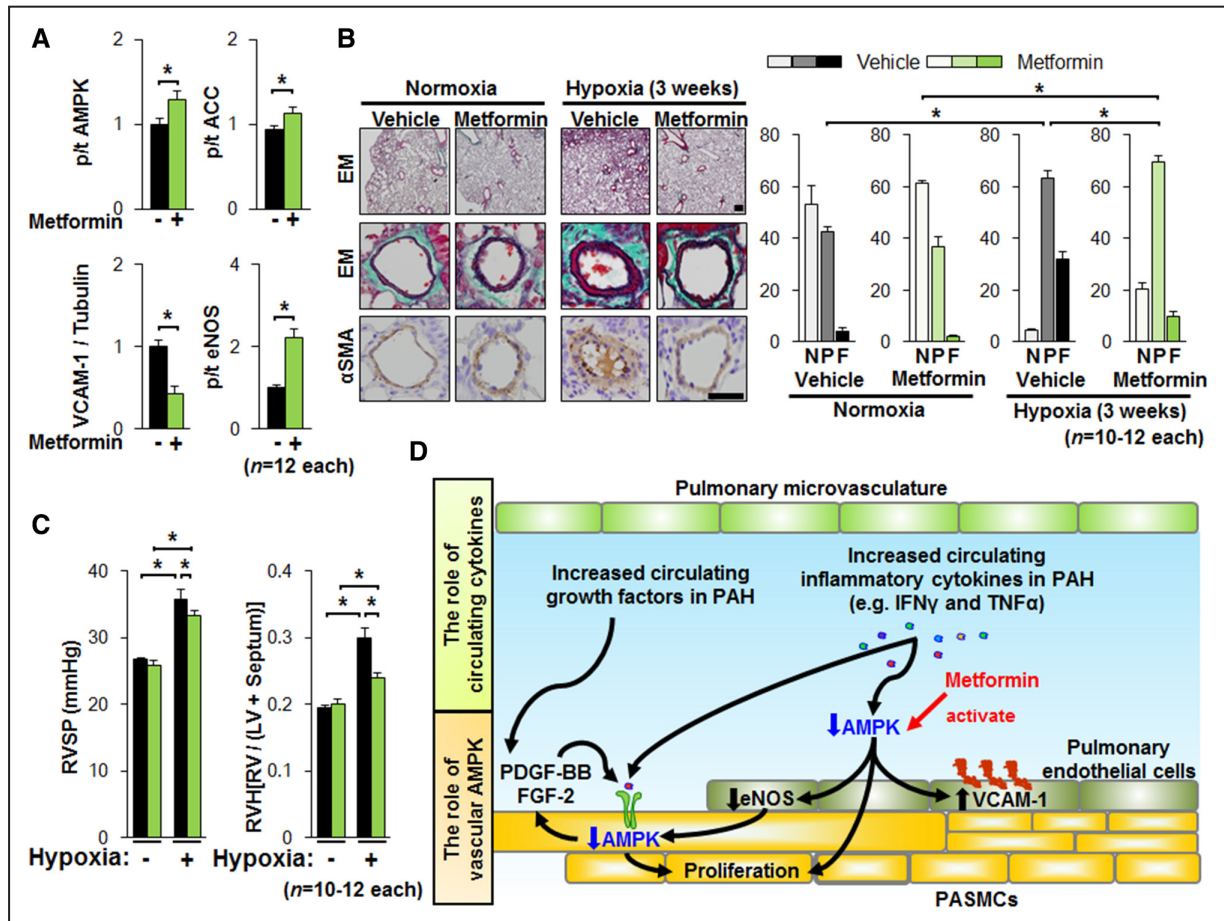
Figure 7. Metformin ameliorates hypoxia-induced pulmonary hypertension. A, Densitometric analyses of phosphorylated AMP-activated protein kinase (AMPK) at Thr17 (p-AMKP), total AMPK (t-AMPK), phosphorylated acetyl-CoA carboxylase (ACC) at Ser79 (p-ACC), total ACC (t-ACC), vascular cell adhesion molecule-1 (VCAM-1), phosphorylated endothelial nitric oxide synthase (eNOS) at Ser1177 (p-eNOS), and total eNOS (t-eNOS) in wild-type mice exposed to hypoxia (10% O$_2$) treated with or without an AMPK activator, metformin (100 mg/kg per d, PO), for 3 wk. Results are expressed as mean±SEM (n=12 each). *$P<0.05$. **B**, Representative Elastica–Masson (EM) and immunostaining for α-smooth muscle actin (α-SMA) of the distal pulmonary arteries exposed to normoxia or hypoxia (3 wk) and treated with metformin or vehicle. Scale bars, 50 μm. Muscularization of the distal pulmonary arteries with a diameter of 20 to 70 μm. The arteries were considered fully muscularized (F) if they had a distinct double-elastic lamina visible throughout the diameter of the vessel cross section. The arteries were considered partially muscularized (P) if they had a distinct double-elastic lamina visible for at least half the diameter. The percentage of vessels with double-elastic lamina was calculated as the number of muscularized vessels per total number of vessels counted. Results are expressed as mean±SEM (n=10–12 each). *$P<0.05$. **C**, Right ventricular systolic pressure (RVSP) and RV hypertrophy (RVH) assessed by the ratio of RV to left ventricle (LV) plus septum weight in wild-type mice treated with or without metformin. Results are expressed as mean±SEM (n=10–12 each). *$P<0.05$. **D**, Schematic representation of molecular mechanisms of endothelial AMPK–mediated pulmonary vascular remodeling. F indicates fully muscularized vessels; FGF-2, fibroblast growth factor-2; IFN-γ, interferon-γ; LV, N, nonmuscularized vessels; P, partially muscularized vessels; PAH, pulmonary arterial hypertension; PASMC, pulmonary artery smooth muscle cells; PDGF-BB, platelet-derived growth factor-BB; and TNF-α, tumor necrosis factor-α.

noted in the pulmonary artery medial layers of the mice, supporting the possible cross talk between PAECs and PASMCs in vivo.[20,46] Indeed, CM prepared from the *eAMPK*$^{-/-}$ lungs promoted PASMC proliferation compared with control lungs. Taken together, these data suggest that endothelial AMPK downregulation induces endothelial dysfunction and promotes adjacent PASMC proliferation in mice in vivo.

Increased Serum Levels of Inflammatory Cytokines Downregulate Endothelial AMPK

Increased inflammatory cytokines in patients with PAH promote the development of PAH.[47] Endothelial dysfunction is a trigger of the development of PAH.[6,48] Among the inflammatory cytokines, tumor necrosis factor-α directly induces endothelial dysfunction.[49] Indeed, we demonstrated that the serum

levels of tumor necrosis factor-α were significantly increased in patients with PAH, which is consistent with the previous report.[47] In addition, immunostaining showed reduced endothelial AMPK activity in pulmonary arteries of patients with PAH and experimental mouse model of PH. Moreover, treatment with the serum from patients with PAH significantly downregulated AMPK signaling and upregulated VCAM-1 expression in PAECs and PASMCs. These findings are consistent with the recent study demonstrating that inflammatory cytokines impair endothelial function and endothelial phenotype in PAH.[44]

Study Limitations

Several limitations should be mentioned for this study. First, in this study, we tried to prepare CM prepared from PAECs derived from explanted PAH lungs without success. For the preparation

of CM, we needed a large amount of pure PAECs. However, it was difficult to increase the amount of pure PAECs because of the contaminated nonendothelial cells that expanded after several passages. This is potentially because of the increased proliferative capacity of contaminated fibroblasts and PASMCs from patients with PAH. Thus, we were unable to prepare CM from pure PAECs derived from explanted PAH lungs. Second, although we used Tie2 as a driver for endothelial cell expression in this study, it is known that Tie2 is unique not only to endothelial cells but also to some subsets of monocytes and macrophages. Thus, we need to consider the possible involvement of hematopoietic subsets, including monocytes and macrophages, in the phenotype of Tie2-Cre–mediated AMPK-knockout mice. Third, several studies demonstrated that IL-6 plays a crucial role in the development of PH. Among them, Hashimoto-Kataoka et al[50] demonstrated the crucial role of IL-6 in the pathogenesis of PAH. In contrast, in this study, we found significantly less IL-6 levels in the eAMPK$^{-/-}$ lung homogenates under chronic hypoxia compared with control lung homogenates. The discrepancy between the study by Hashimoto-Kataoka et al[50] and our study could be explained, at least in part, by the difference in the experimental settings, as they used WT mice and we used endothelial-specific AMPK-knockout mice and chronic hypoxia. Indeed, IL-6 is known as a hormone that has both proinflammatory and anti-inflammatory actions. For example, IL-6 is produced in skeletal muscle in response to exercise associated with enhanced AMPK activity in several organs.[51] In addition, AMPK is ubiquitously expressed in vascular cells, inflammatory cells, perivascular adipocytes, and skeletal muscle cells, all of them will show different responses under different conditions (eg, hypoxia). Thus, AMPK-mediated regulation of IL-6 production may differ depending on the cell types in the lung (eg, PAECs, PASMCs, and migrating inflammatory cells), environmental circumstances (eg, hypoxia and duration), and conditions (eg, ages). Indeed, it was reported that exercise-induced increase in AMPK activity was reduced in muscle and adipose tissue of younger IL-6–knockout mice, whereas this increase was not found in older IL-6–knockout mice.[51] Thus, the role of AMPK would be different in each cell type and condition in vivo.

Clinical Implication and Conclusions

There are several medications and compounds to activate endothelial AMPK signaling in vivo, including salicylate and methotrexate.[52,53] Salicylate is an ancient drug, which is the major breakdown product of aspirin.[54] The low dose of aspirin exerts antiplatelet effects in patients with coronary artery disease, which contributes to the significant improvement of long-term survival in patients with coronary artery disease.[55] When we consider these backgrounds,[52,54] it could be possible that the efficacy of aspirin in patients with coronary artery disease is partially because of its stimulatory effect on endothelial AMPK signaling. In this study, the treatment with metformin significantly ameliorated hypoxia-induced PH in mice. This is consistent with the recent report in monocrotaline-induced PH in rats.[56] Our data are novel because we demonstrated the specific role of AMPK in mice in vivo. Importantly, we found that PASMC-specific AMPK-knockout mice were embryonic lethal. Thus, we consider that the predominant role of AMPK is in the endothelial cells in hypoxia-induced PH in mice. Our

results suggest the potential role of circulating inflammatory cytokines for inducing endothelial dysfunction in pulmonary circulation.[1,47] Thus, this study indicates that endothelial AMPK and circulating inflammatory cytokines may be therapeutic targets for the treatment of PAH. The human equivalent dose of metformin we used in this study was much lower than the maximum recommended human dose.[39] Taken together, the efficacy of metformin in improving AMPK signaling could be valuable in treating PAH and nonvascular diseases, including cancer, in which AMPK signaling is reduced.[54] Next, increased serum levels of cytokines in inflammatory status contribute to the acute progression and worsening of clinical status in patients with PAH.[8] Present findings suggest that AMPK is a key molecule at the crossroad of inflammation and pulmonary artery endothelial dysfunction in the pathogenesis of PAH. Thus, a strategy targeting endothelial AMPK may be promising for the development of novel therapy in patients with PAH.

In conclusion, this study demonstrates that endothelial AMPK plays protective roles against hypoxia-induced PH.

Disclosures

None.

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Novelty and Significance

What Is Known?

- Endothelial cell dysfunction and interaction between pulmonary artery endothelial cells and pulmonary artery smooth muscle cells play a crucial role for the development of pulmonary arterial hypertension (PAH).
- AMP-activated protein kinase (AMPK) plays an important role in microvascular homeostasis.

What New Information Does This Article Contribute?

- Endothelial AMPK activity is reduced in patients with PAH and an experimental mouse model of pulmonary hypertension.
- Serum from patients with PAH downregulates AMPK signaling with aberrant cell proliferation.
- Endothelial AMPK protects against the development of hypoxia-induced pulmonary hypertension.

To our knowledge, this is the first study demonstrating that AMPK is a crucial molecule at the crossroad of inflammation and aberrant cell proliferation in the pathogenesis of PAH. We showed that the endothelial-specific AMPK knockdown induced pulmonary artery endothelial cells dysfunction and promoted adjacent pulmonary artery smooth muscle cells proliferation. We also demonstrated that serum from patients with PAH, which contained increased inflammatory cytokines, significantly downregulated AMPK activity in pulmonary artery endothelial cells. Moreover, metformin, an AMPK activator, ameliorated hypoxia-induced pulmonary hypertension in mice. On the basis of these findings, we propose that the activation of AMPK signaling may be promising for the development of novel therapy in patients with PAH.

Circulation Research

Protective Roles of Endothelial AMP-Activated Protein Kinase Against Hypoxia-Induced Pulmonary Hypertension in Mice

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SUPPLEMENTAL MATERIAL

Protective Roles of Endothelial AMP-activated Protein Kinase Against Hypoxia-induced Pulmonary Hypertension in Mice

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Online Supplement Detailed Methods
Supplemental Figures I-VI
Supplemental Figure Legends I-VI

A detailed, expanded Methods section

Wound Healing Assay

Confluent human pulmonary artery smooth muscle cells (PASMCs) obtained from Lonza (Basel, Switzerland) were starved for 24 h in 60-mm dishes.   Then, a linear wound (600 μm in width) was made through the center of dish using a pipette tip and medium was replaced with condioned medium (CM) from compound C-treated pulmonary artery endothelial cells (PAECs) or vehicle-treated PAECs. 12 h and 24 h after incision, three different fields were chosen randomly in each dish and were photographed by a phase-contrast microscope.   The area of gap closure was measured using Image J Software (NIH, Bethesda, USA).

Immunohistochemical Analysis

Immunohistochemical analysis was performed with sections of lung tissues which were obtained from pulmonary arterial hypertension (PAH) patients at the time of lung transplantation (n=8) or from control patients (n=6) at the time of thoracic surgery for lung cancer at a site far from the tumor margins.   Antibodies used were as follows; CD31 (BD Pharmingen, 1:500), AMP-activated protein kinase (AMPK, Cell Signaling, 1:500), phosphorylated AMPK at Thr172 (Cell Signaling, 1:500). After subtraction of background noise, fluorescence intensity (mean optical density) of AMPK or phosphorylated AMPK at Thr172 within CD31-positive areas of distal pulmonary arteries was calculated in arbitrary units (AU) with image J Software.

Tube Formation Assay

Sterile 6-well plates coated with matrigel were incubated at 37°C for 0.5h to form gels.   After polymerization of the gels, human PAECs obtained from Lonza were seeded into each well (50,000 cells/well) and incubated with EBM-2 basal medium containing 1% FBS and compound C (5 μmol/L) for 6 h.   Six different fields were chosen randomly in each well, and were photographed by a phase-contrast microscope.   Number of the tubes was calculated with Image J Software.
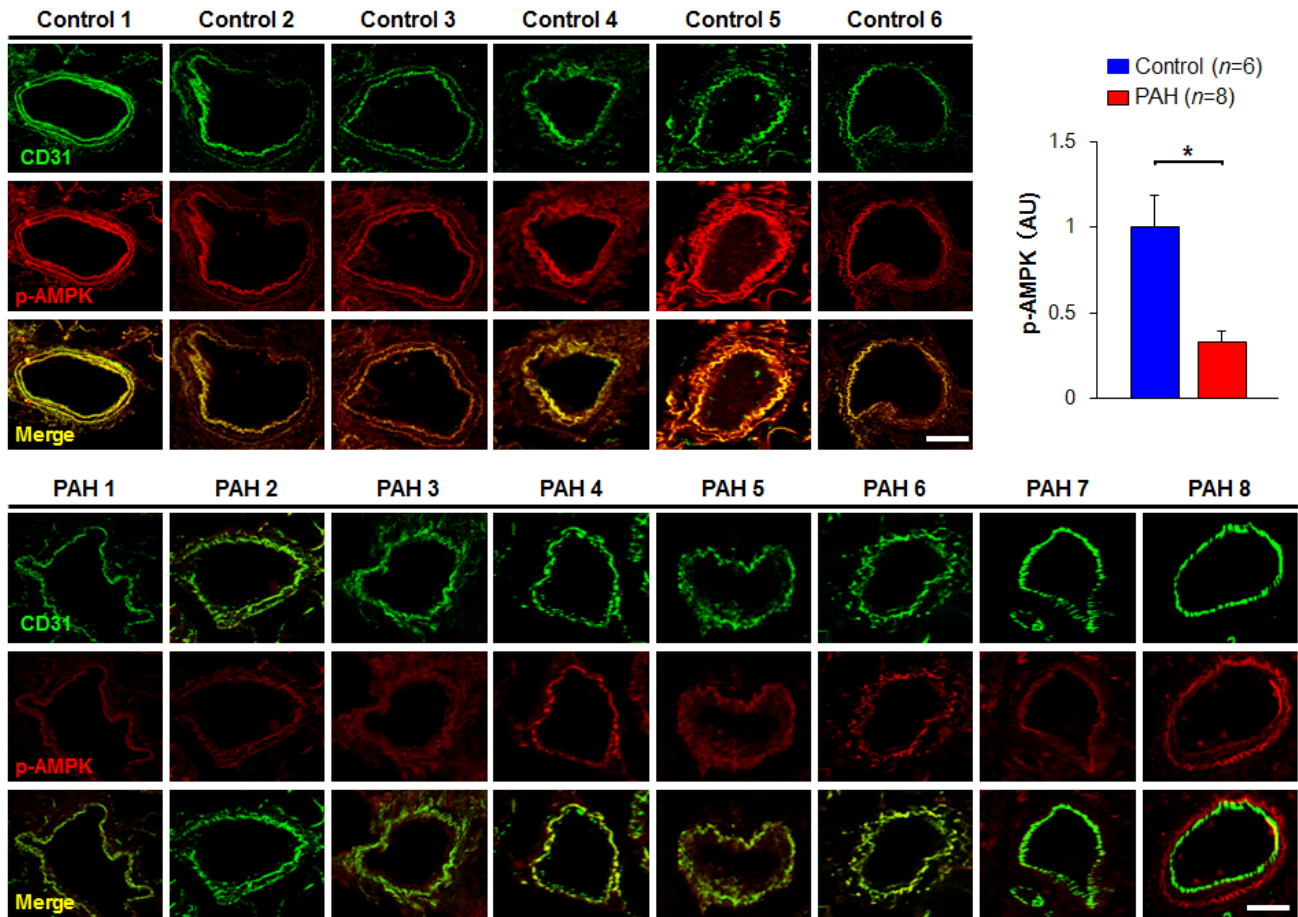
Supplementary Figure I
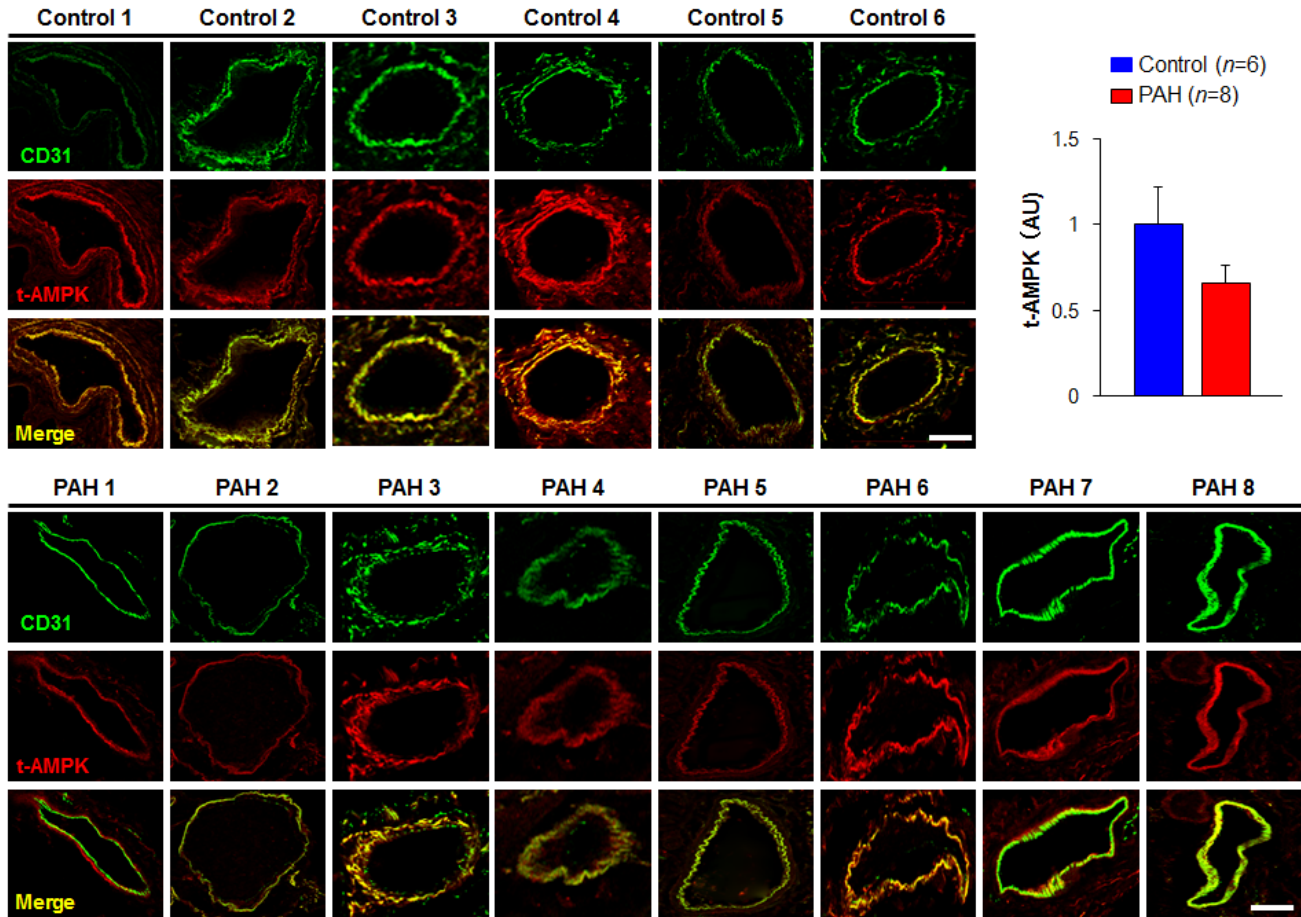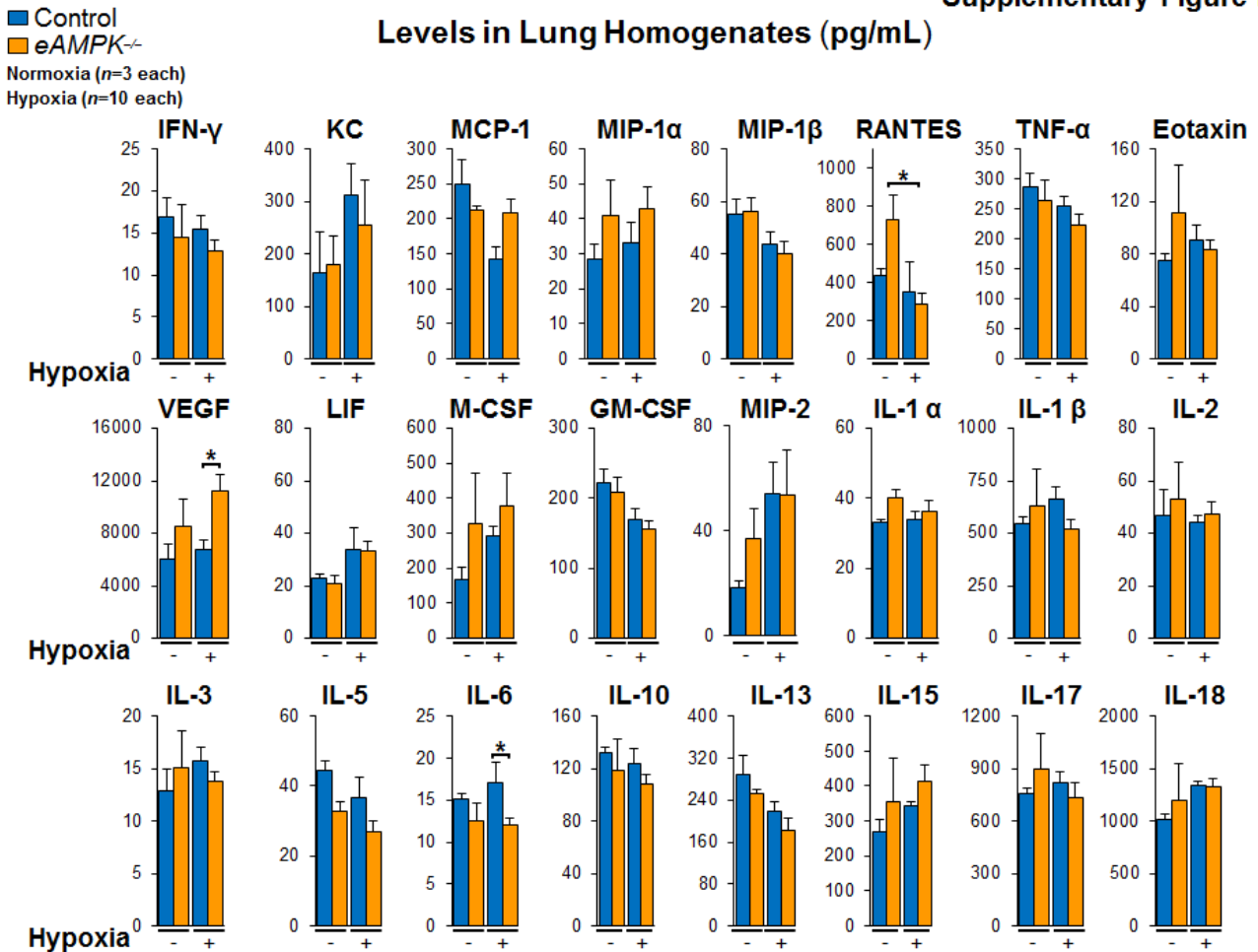

Supplementary Figure I.   Down-regulation of Endothelial Total AMPK in Patients with Pulmonary Arterial Hypertension (PAH)

Representative immunostaining pictures of the distal pulmonary arteries from PAH patients (n=8) and controls (n=6).   The endothelium was visualized by CD31 (Alexa Fluor-488, green). Double-immunostaining for total AMPK (t-AMPK, Alexa Fluor-563, red) and CD31 (green). Quantitative analysis of t-AMPK in CD31-positive area in the distal pulmonary arteries from PAH patients and controls.   Scale bars, 25 μm.

Supplementary Figure II. Down-regulation of Endothelial Phosphorylated-AMPK in Patients with Pulmonary Arterial Hypertension (PAH)

Representative immunostaining pictures of the distal pulmonary arteries from PAH patients (*n*=8) and controls (*n*=6). The endothelium was visualized by CD31 (Alexa Fluor-488, green). Double-immunostaining for phosphorylated-AMPK at Thr172 (p-AMPK, Alexa Fluor-563, red) and CD31 (green). Quantitative analysis of p-AMPK in CD31-positive area in the distal pulmonary arteries from PAH patients and controls. Scale bars, 25 μm.
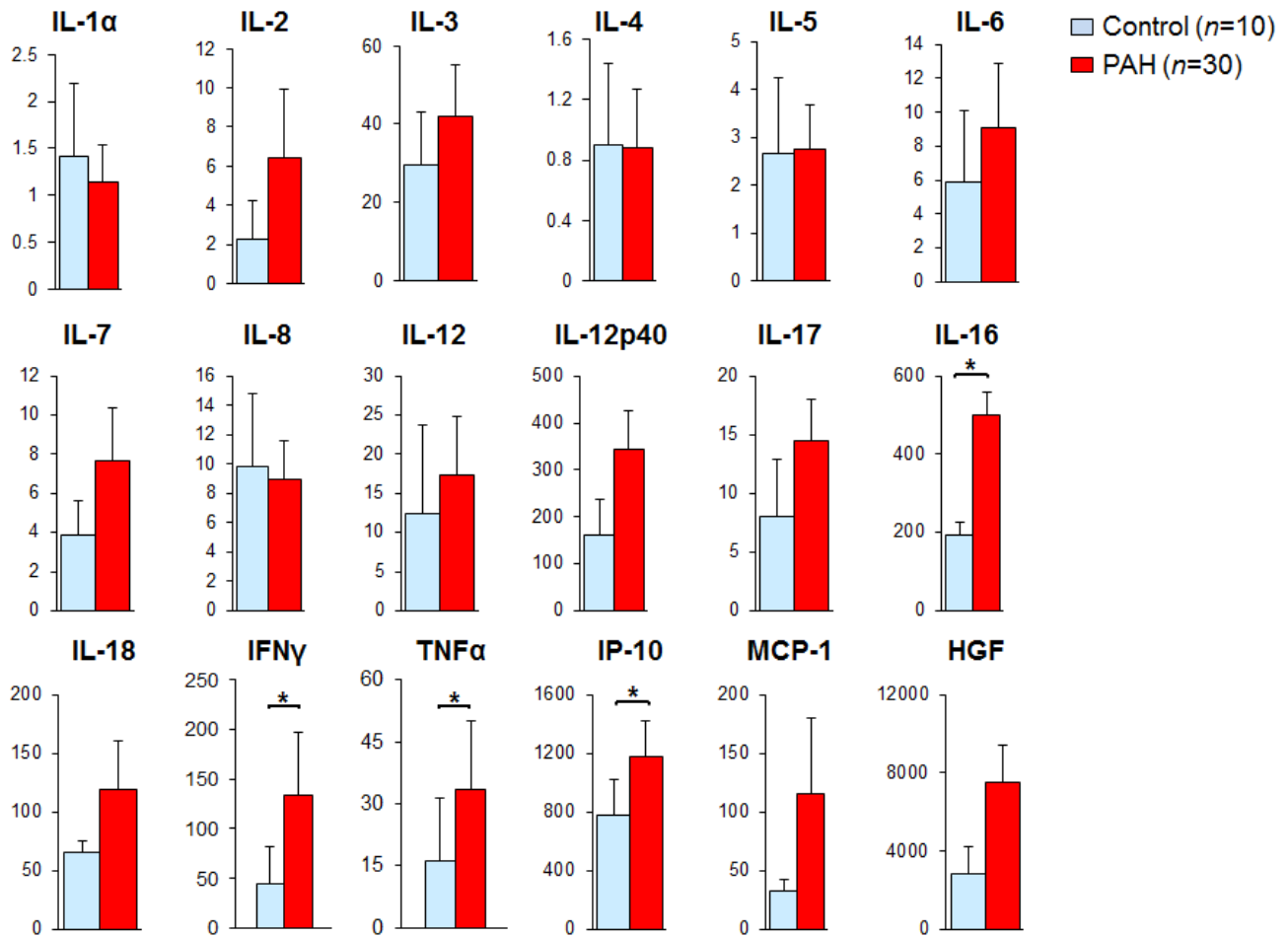
Supplementary Figure III. Cytokines/Chemokines and Growth Factors in Lung Homogenates

The levels of cytokines/chemokines and growth factors in lungs of *eAMPK*$^{-/-}$ or control mice after 4 weeks of normoxia (*n*=3 each) or hypoxia (10% O$_2$, *n*=10 each). Results are expressed as mean ± SEM. *P<0.05.

Supplementary Figure IV

Serum Levels of Cytokines/chemokines and Growth Factors (pg/mL)
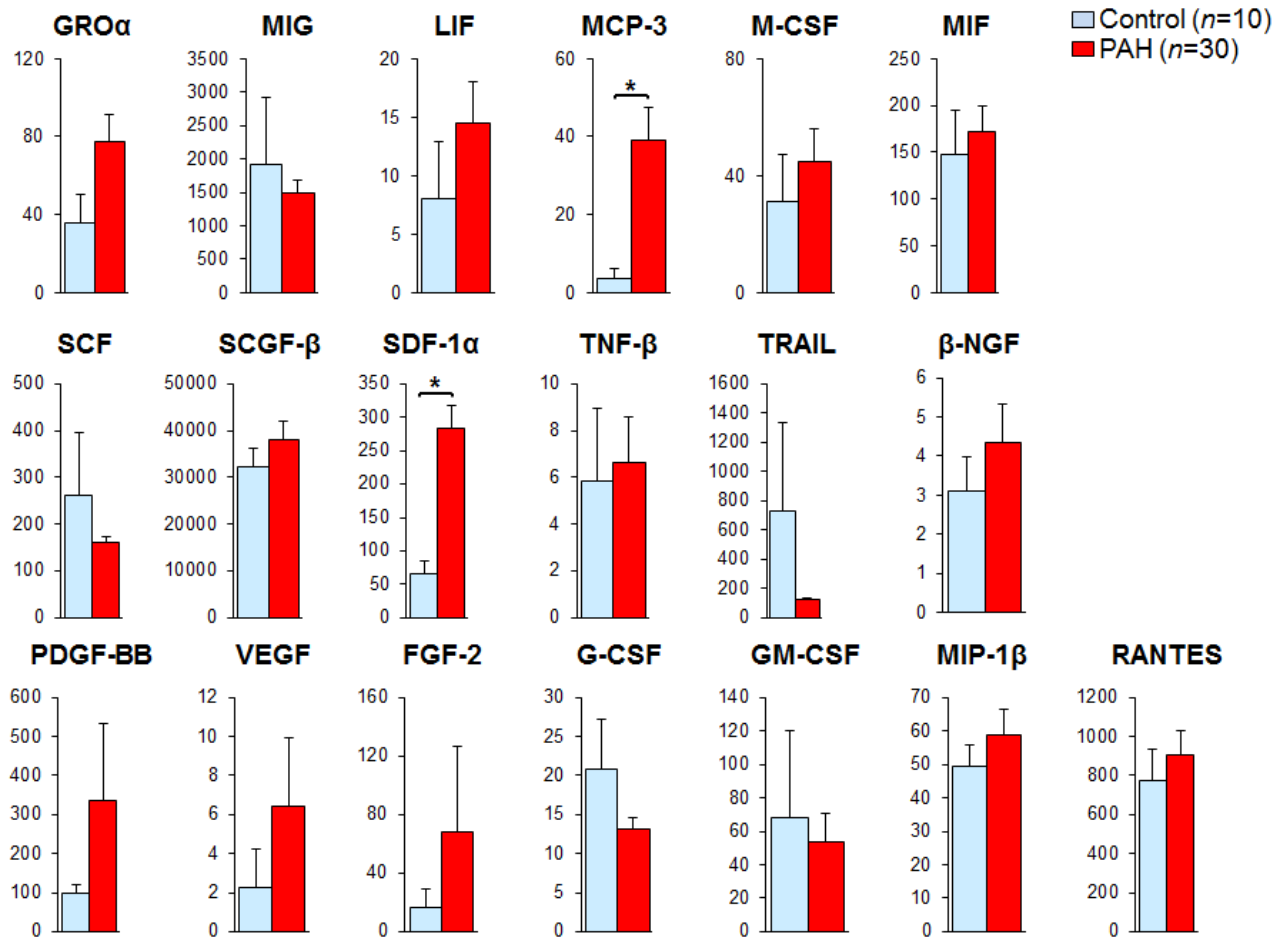

Supplementary Figure IV.   Serum Levels of Cytokines/Chemokines and Growth Factors

Serum levels of cytokines/chemokines and growth factors in patients with PAH (*n*=30) or control (*n*=10).   Results are expressed as mean ± SEM.   *P<0.05.
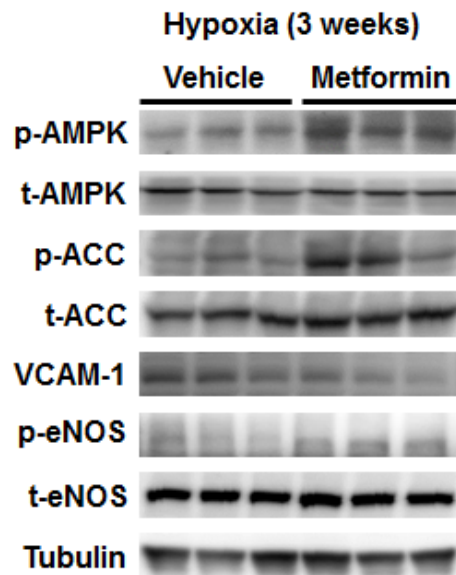
Supplementary Figure V.   Serum Levels of Cytokines/Chemokines and Growth Factors

Serum levels of cytokines/chemokines and growth factors in patients with PAH (*n*=30) or control (*n*=10).   Results are expressed as mean ± SEM.   *P<0.05.

Supplementary Figure VI

Supplementary Figure VI.   Metformin Ameliorates Hypoxia-induced Pulmonary Hypertension
Representative Western blotting of phosphorylated AMPK at Thr17 (p-AMKP), total AMPK
(t-AMPK), phosphorylated ACC at Ser79 (p-ACC), total ACC (t-ACC), vascular cell adhesion
molecule-1 (VCAM-1), phosphorylated eNOS at Ser1177 (p-eNOS), and total eNOS (t-eNOS) in
wild-type mice exposed to hypoxia (10% O_2) treated with or without an AMPK activator, metformin
(100 mg/kg/day, PO), for 3 weeks.   Results are expressed as mean ± SEM (*n*=12 each).