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ORIGINAL ARTICLE

AMP-activated Protein Kinase Phosphorylation of Angiotensin-Converting Enzyme 2 in Endothelium Mitigates Pulmonary Hypertension

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Abstract

Rationale: Endothelial dysfunction plays an integral role in pulmonary hypertension (PH). AMPK (AMP-activated protein kinase) and ACE2 (angiotensin-converting enzyme 2) are crucial in endothelial homeostasis. The mechanism by which AMPK regulates ACE2 in the pulmonary endothelium and its protective role in PH remain elusive.

Objectives: We investigated the role of AMPK phosphorylation of ACE2 Ser680 in ACE2 stability and deciphered the functional consequences of this post-translational modification of ACE2 in endothelial homeostasis and PH.

Methods: Bioinformatics prediction, kinase assay, and antibody against phospho-ACE2 Ser680 (p-ACE2 S680) were used to investigate AMPK phosphorylation of ACE2 Ser680 in endothelial cells. Using CRISPR-Cas9 genomic editing, we created gain-of-function *ACE2* S680D knock-in and loss-of-function *ACE2* knockout ($ACE2^{-/-}$) mouse lines to address the involvement of p-ACE2 S680 and ACE2 in PH. The AMPK–p-ACE2 S680 axis was also validated in lung tissue from humans with idiopathic pulmonary arterial hypertension.

Measurements and Main Results: Phosphorylation of ACE2 by AMPK enhanced the stability of ACE2, which increased Ang (angiotensin) 1–7 and endothelial nitric oxide synthase–derived NO bioavailability. *ACE2* S680D knock-in mice were resistant to PH as compared with wild-type littermates. In contrast, *ACE2*-knockout mice exacerbated PH, a similar phenotype found in mice with endothelial cell–specific deletion of *AMPKα2*. Consistently, the concentrations of phosphorylated AMPK, p-ACE2 S680, and ACE2 were decreased in human lungs with idiopathic pulmonary arterial hypertension.

Conclusions: Impaired phosphorylation of ACE2 Ser680 by AMPK in pulmonary endothelium leads to a labile ACE2 and hence is associated with the pathogenesis of PH. Thus, AMPK regulation of the vasoprotective ACE2 is a potential target for PH treatment.

Keywords: angiotensin-converting enzyme 2; AMP-activated protein kinase; pulmonary hypertension; vascular endothelium

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At a Glance Commentary

Scientific Knowledge on the Subject: Endothelial dysfunction is a pathophysiologic event involved in pulmonary hypertension (PH). ACE2 (angiotensin-converting enzyme 2) plays an important role in functional endothelium and has been implicated in PH amelioration. However, the molecular mechanism that regulates ACE2 in the endothelium in the context of PH is unknown. Furthermore, animal models harboring genetically manipulated ACE2 for PH study are scant.

What This Study Adds to the Field: We have identified that phosphorylation of ACE2 at Ser680 is critical for its stability via inhibiting the ubiquitination-related degradation. AMPK (AMP-activated protein kinase) phosphorylation of ACE2 Ser680, with the resultant increase in ACE2 expression in endothelium, is PH protective. Such a thesis involving the AMPK–phospho-ACE2 axis is demonstrated by enhanced PH in *ACE2*$^{-/-}$ and EC-*AMPKα2*$^{-/-}$ mice but decreased PH in *ACE2* S680D knock-in mice. Importantly, the impaired AMPK–phospho-ACE2 axis was validated in specimens from patients with idiopathic pulmonary arterial hypertension. In light of these findings, changes of ACE2 stability through modulating Ser680 phosphorylation have translational relevance in PH treatment.

Pulmonary hypertension (PH) is characterized by increased pulmonary arterial pressure (PAP) and progressive remodeling of the pulmonary vasculature, leading to right ventricular heart failure (1, 2). Although endothelial proliferation and apoptosis have been implicated in PH (3), the mechanism of endothelial dysfunction underlying this disease remains elusive.

Sharing approximately 42% homology with ACE (angiotensin-converting enzyme), ACE2 is a type I transmembrane metalloprotease. ACE2 cleaves Ang II (angiotensin II) to generate Ang 1–7 (angiotensin 1–7) that binds to Mas (4, 5). The ACE2–Ang 1–7–Mas pathway is the vasoprotective axis in the renin–angiotensin system (RAS). Highly expressed in the endothelium, ACE2 plays key roles in vasodilation, antifibrosis, and antihypertrophy (6, 7). Serum concentrations of ACE2 are lower in individuals with pulmonary arterial hypertension (PAH), representing group 1 of the World Health Organization clinical classification, than in those without it. Furthermore, serum concentrations of ACE2 have been found to be inversely correlated with mean PAP in humans (8). In a rodent model, namely monocrotaline (MCT)-treated rats, the synthetic activator of ACE2 attenuated PH progression (9). Administration of recombinant human ACE2 (rhACE2) to mouse models with PH could normalize pulmonary pressure (10). Although these previous studies have demonstrated that ACE2 can alleviate PH, there is a lack of mechanistic insight revealing the upstream molecular events regulating ACE2 stability. In addition, there is a need for PH study with animal models harboring genetically manipulated ACE2.

AMPK (AMP-activated protein kinase) is a metabolic master regulator in multiple tissues. AMPK is activated by the low ratio of ATP to AMP. Owing to the decreased concentration of cellular ATP under hypoxia, AMPK is activated as an adaptive response (11, 12). In endothelium, AMPK phosphorylates proteins that modulate vasodilation, antiinflammation, antioxidative stress, and mitochondrial biogenesis (e.g., eNOS [endothelial nitric oxide synthase] and peroxisome proliferator-activated receptor gamma coactivator 1-α) (13–17). With respect to PH, the antidiabetic drug metformin that activates AMPK *in vitro* and *in vivo* (18), is currently used in PAH clinical trials (www.clinicaltrials.gov identifier NCT01884051). Furthermore, hypoxia-induced PH is accelerated in mice with AMPK ablation in the endothelium (19).

In this paper, we show that ACE2 Ser680 is phosphorylated by AMPK to enhance the stability of ACE2, thereby leading to increased Ang 1–7 and eNOS-derived NO bioavailability. By using genetically manipulated mouse models and human idiopathic pulmonary arterial hypertension (IPAH) specimens, we elucidate that AMPK positively regulates ACE2–Ang 1–7 in cultured endothelial cells (ECs) *in vitro* and pulmonary endothelium *in vivo*. Our data demonstrate that the PH pathophysiology is closely related to perturbed AMPK phosphorylation of ACE2. Hence, this phosphorylation event while increasing ACE2 stability suggests a potential therapeutic target to treat this incurable disease.

Methods

Animal Models

Animal experiments were approved by the institutional animal care and use committee of the University of California, San Diego, and the institutional animal ethics committee of Xi'an Jiaotong University. All rodent models were kept on a 12-hour/12-hour light/dark cycle and fed *ad libitum* with chow diet.

EC-*AMPKα2*$^{-/-}$ Mice

To generate mice with EC-specific deletion of *AMPKα2* (EC-*AMPKα2*$^{-/-}$), mice homozygous for the floxed *AMPKα2* allele (The Jackson Laboratory) were crossed with Tie2-cre mice (The Jackson Laboratory). The resulting offspring were *AMPKα2*$^{fl/fl}$-Tie2-cre mice (EC-*AMPKα2*$^{-/-}$) and their control littermates *AMPKα2*$^{fl/fl}$. Eight- to 12-week-old male EC-*AMPKα2*$^{-/-}$ mice and their age-matched wild-type (WT) littermates were used for experiments.

ACE2 S680D Knock-in and *ACE2*-Knockout Mice

ACE2 S680D knock-in mice were developed with use of the CRISPR-Cas9 system. Three targeting guide RNAs (gRNAs) were subcloned into the px330 vector for *in vitro* validation of the targeting specificity in the mouse N2A cell line. On the basis of genomic sequencing results, one gRNA (AAGAAGTAGAAGGAGACTCT) was used for embryo injection together with Cas9 mRNA and a single-stranded DNA oligo with a TCC codon (coding Ser680) mutated to GAC (coding Asp) as the repair template. CRISPR also generated a frameshift mutation resulting in an *ACE2*-knockout (*ACE2*$^{-/-}$) mouse line. The genotypes of *ACE2* S680D and *ACE2*$^{-/-}$ mice were verified by genomic DNA sequencing. Knockout of *ACE2* was also confirmed by Western blot analysis. The generated mice were backcrossed with C57BL/6 mice for more than five generations.

SU5416/Hypoxia + Reoxygenation–induced PH Model

Male mice (8 to 12 wk old) were housed under hypoxic conditions (10% O_2) and given subcutaneous injections with the VEGF (vascular endothelial growth factor) receptor 2 inhibitor SU5416 (Sigma-Aldrich) at 20 mg/kg. The frequency of SU5416 treatment was once per week for 3 consecutive weeks. Animals receiving DMSO for the same duration were used as negative controls. Mice were then exposed to normoxia for an additional 2 weeks (reoxygenation). For the metformin treatment group, EC-*AMPKα2*$^{-/-}$ mice and their WT littermates were given intraperitoneal injections with metformin or saline (150 mg/kg/d) for 2 weeks after reoxygenation. Mice kept in the same housing condition served as normoxia controls.

MCT-induced Rat PH Model

Male Sprague-Dawley rats (200–250 g body weight) purchased from Charles River Laboratories were given intraperitoneal injections with 60 mg/kg body weight MCT (Sigma-Aldrich) or saline. Hemodynamics and right ventricular hypertrophy were assessed 3 weeks after MCT injection. Rats were then killed, and their tissues were collected for analysis.

Rodent Hemodynamics and PH Study

Rodent hemodynamics were measured as described previously (20). For the assessment of right ventricular hypertrophy, the right ventricle (RV) was dissected from the left ventricle (LV) and interventricular septum (S) and weighed. We assessed the ratio of RV weight to LV plus S weight [RV/(LV + S); i.e., Fulton index]. MICROFIL MV-122, MV-Diluent, and MV-Curing Agent (Flow Tech) were used for pulmonary angiography according to the manufacturer's instructions.

Human Samples

Normal control tissue was obtained from human organ donors whose lungs were deemed unsuitable for transplant. Per the donors' next of kin, no donor had a history of PAH, and none was found to have the physical sequelae of this disease (right ventricular hypertrophy and main pulmonary artery enlargement) at the time of organ harvest. IPAH lung tissue was obtained from individuals undergoing lung transplant for end-stage PAH. Procedures for measuring human PAP and for lung biopsy were as described previously (20). The patient demographics and clinical data are shown in Table E2 in the online supplement.

Most of our non-PAH donor tissue came from brain dead donors in the San Diego area. Because the organ donor pool in San Diego is principally young males, our control tissues were harvested from males, whereas our IPAH lung tissue patients were principally females. So, bias based on sex may have been possible. All individuals had given consent for lung biopsy. The study was approved by the institutional review board of the University of California, San Diego, and experiments were performed within relevant guidelines and regulations.

Statistics

Data are expressed as mean ± SEM. For normally distributed data, Student's *t* test was used to compare two groups. Analysis of variance followed by the Bonferroni *post hoc* test for equal sample sizes or the Tukey-Kramer test for unequal sample sizes was used to compare multiple groups. For nonnormally distributed data, the Kruskal-Wallis test for multiple groups or the Mann-Whitney *U* test was used to analyze data between two groups. Statistical significance was considered if the *P* value was less than 0.05. Statistical analyses were carried out using Prism 6 software (GraphPad Software).

Results

AMPK Phosphorylates ACE2 Ser680

On the basis of AMPK phosphorylation consensus sequence (21), we predicted that ACE2 Ser680 is an AMPK putative phosphorylation target that is conserved in diverse mammalian species (Figure 1A). By using an *in vitro* kinase assay, we verified that AMPKα2 phosphorylated an ACE2 oligopeptide containing Ser680 to a concentration like that of SAMS peptide, the canonical AMPK substrate, which was not seen in the dephosphorylated S680A mutant peptide (Figure 1B). In cultured human umbilical vein endothelial cells (HUVECs), AMPK activation by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) led to substantially increased phosphorylation of ACE2 Ser680 as well as expression of total ACE2 (Figure 1C). Consistently, AICAR treatment increased the phosphorylation of AMPK Thr172 and AMPK targeted acetyl coenzyme A carboxylase Ser79. Overexpression of the constitutively active form of AMPKα2 but not the dominant-negative form also increased the phosphorylated ACE2 Ser680 and total ACE2 protein concentrations in HUVECs (Figure 1D). Metformin, a vasoprotective antidiabetic drug, increased ACE2 phosphorylation and expression in HUVECs, which were attenuated by AMPKα siRNA knockdown or treatment with compound C, an AMPK inhibitor (18) (Figures 1E and 1F). In comparing lung ECs from EC-*AMPKα2*$^{-/-}$ mice or their EC-*AMPKα2*$^{+/+}$ littermates, *AMPKα2* ablation reduced ACE2 phosphorylation, even with metformin stimulation (*see* Figure E1A). AMPK phosphorylation of ACE2 Ser680 was further validated in human pulmonary artery endothelial cells (PAECs) (*see* Figure E1B).

Phosphorylation of ACE2 Ser680 Enhances ACE2 Stability and NO Bioavailability

Because increased ACE2 phosphorylation was associated with increased total ACE2 expression, we investigated whether AMPK phosphorylation of ACE2 Ser680 contributes to ACE2 protein stability. A phosphomimetic (S680D) or the complementary dephosphomimetic (S680A) mutant of ACE2 was transfected into human embryonic kidney 293 (HEK293T) cells that express minimal endogenous ACE2 (22). Cycloheximide chase experiments revealed a higher concentration of ACE2 in cells transfected with S680D than in those with S680A (Figure 2A), indicating augmented protein stability of the phosphomimetic S680D mutant. Similar results were seen in cycloheximide chase experiments with or without metformin treatment (*see* Figure E2A). To identify possible degradation pathways of nonphosphorylated ACE2, we overexpressed ACE2 S680A or S680D mutants together with ubiquitin in HEK293T cells. K48 ubiquitination and total ubiquitination were markedly lower in cells cotransfected with ACE2 S680D than ACE2 S680A (Figure 2B; *see also* Figure E2B). Furthermore, MG132 inhibition of the proteasome rectified the ACE2 concentration in cells transfected with

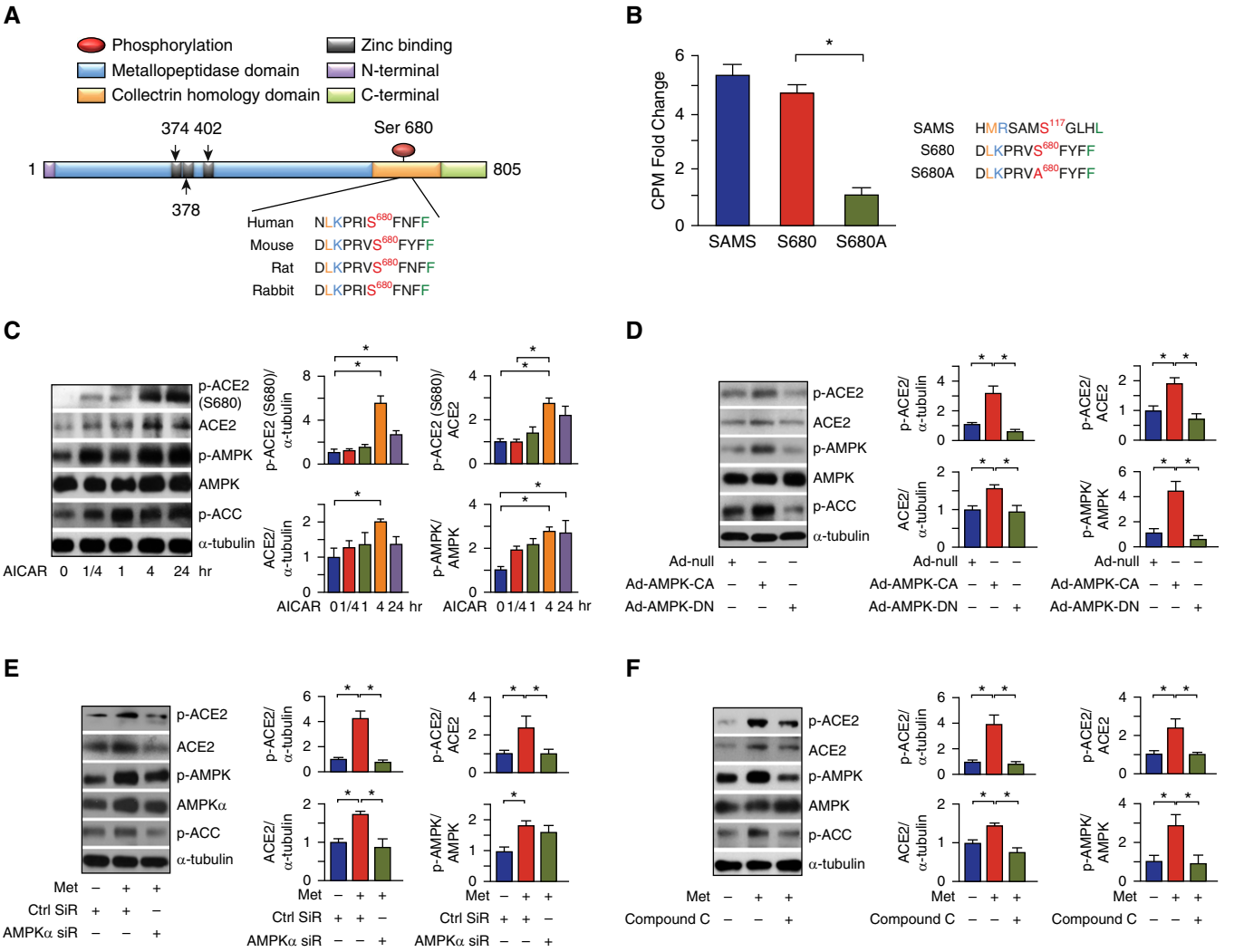

Figure 1. AMPK (AMP-activated protein kinase) phosphorylation of ACE2 (angiotensin-converting enzyme 2) Ser680 in human umbilical vein endothelial cells (HUVECs). (*A*) Schematic illustrating the functional domains of ACE2 that include a metallopeptidase catalytic domain (blue) and a collectrin homology domain (orange). The active zinc ion binding sites are at H374, H378, and E402 (gray). The AMPK phosphorylation consensus sequence is βΦβXXXS/TXXXΦ (hydrophobic, Φ = M, L, I, F, or V; basic, β = R, K, or H; X = any amino acid; S/T = phosphorylation site), which predicted ACE2 Ser680 phosphorylation (indicated by red oval) with conservation in human, mouse, rat, and rabbit. (*B*) AMPK activity assays were performed with ACE2 peptides spanning Ser680 or the dephosphomimetic with Ser replaced with Ala (S680A). SAMS peptide was used as a positive control. (*C–F*) Western blot analysis of indicated proteins in HUVECs treated with 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; 1 mmol/L) at increasing time points (*C*); HUVECs infected with adenosine (Ad)-null, constitutively active AMPK (Ad-AMPK-CA) or Ad-null, dominant-negative AMPK (Ad-AMPK-DN) at 10 multiplicities of infection for 48 hours (*D*); HUVECs transfected with control siRNA or AMPKα1/2 siRNA (20 nmol/L) for 48 hours before metformin (Met; 1 mmol/L) treatment for an additional 4 hours (*E*); and HUVECs pretreated with compound C (15 μmol/L) for 30 minutes followed by Met (1 mmol/L) for 4 hours (*F*). Data are mean ± SEM derived from three independent experiments. $^*P < 0.05$ between the indicated groups was considered statistically significant. ACC = acetyl coenzyme A carboxylase; CPM = scintillation counts per minute; Ctrl = control; p = phosphorylated; SiR = siRNA.

S680A (Figure 2C), which suggests that ACE2 Ser680 phosphorylation enhanced ACE2 stability by inhibiting ubiquitination and proteasomal degradation.

The conversion of Ang II to Ang 1–7 by ACE2 mediates eNOS-derived NO bioavailability (23). Bovine aortic ECs with exogenously expressed ACE2 S680D showed increased concentrations of Ang 1–7 and NO in the conditioned media (Figures 2D and 2E), as well as increased eNOS phosphorylation (Figure 2F) and intracellular NO production (Figure 2G). Metformin treatment also increased the phosphorylation of exogenously expressed eNOS in HEK293T cells cotransfected with ACE2 WT, similar to HEK293T cells transfected with ACE2 S680D, even in the absence of metformin treatment (*see* Figure E2C). In contrast, metformin activation of eNOS was not seen in cells transfected with ACE2 S680A (*see* Figure E2C).

PH Amelioration in *ACE2* S680D Mice

With the established AMPK–p-ACE2 Ser680 axis in cultured ECs, we then sought its translational implication in cardiovascular diseases. Using CRISPR-Cas9 genomic editing, we created an *ACE2* S680D knock-in mouse line to

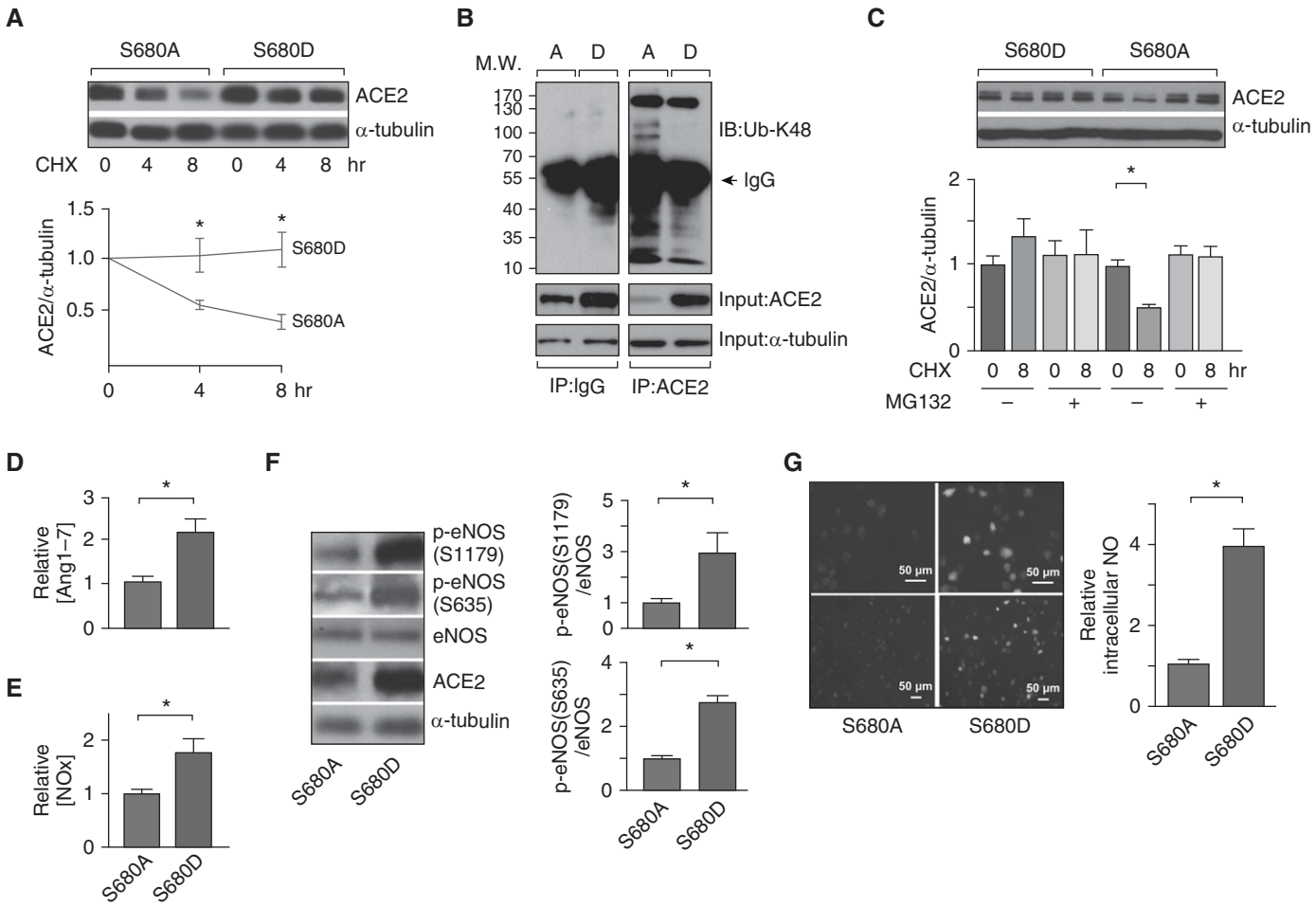

Figure 2. ACE2 (angiotensin-converting enzyme 2) Ser680 phosphorylation enhances ACE2 stability and NO bioavailability. (*A*) Western blot analysis of ACE2 and α-tubulin concentrations in human embryonic kidney 293 (HEK293T) cells transfected with ACE2 S680A or S680D plasmids (1 μg/ml) for 48 hours, then treated with cycloheximide (CHX; 100 μg/ml) for up to 8 hours. (*B*) HEK293T cells were cotransfected with ACE2 S680A (A) or S680D (D) plasmids together with ubiquitin plasmids (1 μg/ml) for 38 hours, then treated with MG132 (20 μmol/L) for an additional 10 hours. ACE2 was immunoprecipitated from cell lysates and then immunoblotted with anti–Ub-K48 (K48 ubiquitin). (*C*) Western blot analysis of ACE2 and α-tubulin protein concentrations in HEK293T cells transfected with ACE2 S680D or S680A mutant plasmids for 40 hours, then treated with CHX with or without MG132 for 8 hours. (*D*) Quantification of secreted Ang 1–7 (angiotensin 1–7) in bovine aortic endothelial cells transfected with ACE2 S680A or S680D plasmids for 48 hours by ELISA. (*E*) NO was detected as accumulated nitrite/nitrate in culture media by a nitrite/nitrate fluorometric assay. (*F*) Concentrations of indicated proteins were detected by Western blot analysis. (*G*) NO production revealed by fluorescence intensity evaluated in bovine aortic endothelial cells transfected with ACE2 S680A or S680D mutant plasmids for 48 hours and then loaded with Fluo-4 acetoxymethyl ester (5 μmol/L) for 30 minutes. Data are mean ± SEM derived from three independent experiments. *$P < 0.05$ between the indicated groups considered statistically significant. IB = immunoblot; IP = immunoprecipitation; M.W. = molecular weight; p-eNOS = phosphorylated endothelial nitric oxide synthase.

address the role of ACE2 Ser680 phosphorylation *in vivo*. Genotyping (shown in Figure 3A) confirmed that the TCC codon (encoding S680 in the WT) was replaced by GAC (encoding D680) in *ACE2* S680D knock-in mice. Flow cytometry revealed higher *ACE2* expression in lung ECs from ACE2 S680D knock-in mice than in WT littermates (*see* Figure E3). Consistently, these ECs showed increased NO bioavailability (i.e., phosphorylation of eNOS Ser1177 and Ser633) (Figure 3B).

AMPK and ACE2 have been shown to play a role in PH (9, 10, 19). However, mechanistic studies using endogenously altered ACE2 in the onset of PH *in vivo* are lacking. To investigate the functional role of the AMPK–p-ACE2 axis in the context of PH, we subjected *ACE2* S680D knock-in mice and their WT littermates to SU5416/hypoxia–reoxygenation (SU5416 administration once per week with 10% oxygen for 3 wk, followed by 2 wk of reoxygenation in normoxia) or kept them under the normoxia condition. Right ventricular systolic pressure (RVSP) and the Fulton index [RV/(LV + S) weight] were greater in WT mice than in *ACE2* S680D knock-in littermates under SU5416/hypoxia–reoxygenation or the two mouse lines under normoxia (Figures 3C and 3D). However, systolic blood pressure did not differ between the two groups (Figure 3E). As compared with WT littermates, *ACE2* S680D knock-in mice showed largely abrogated pulmonary artery muscularization and luminal narrowing (Figure 3F) as well as intact

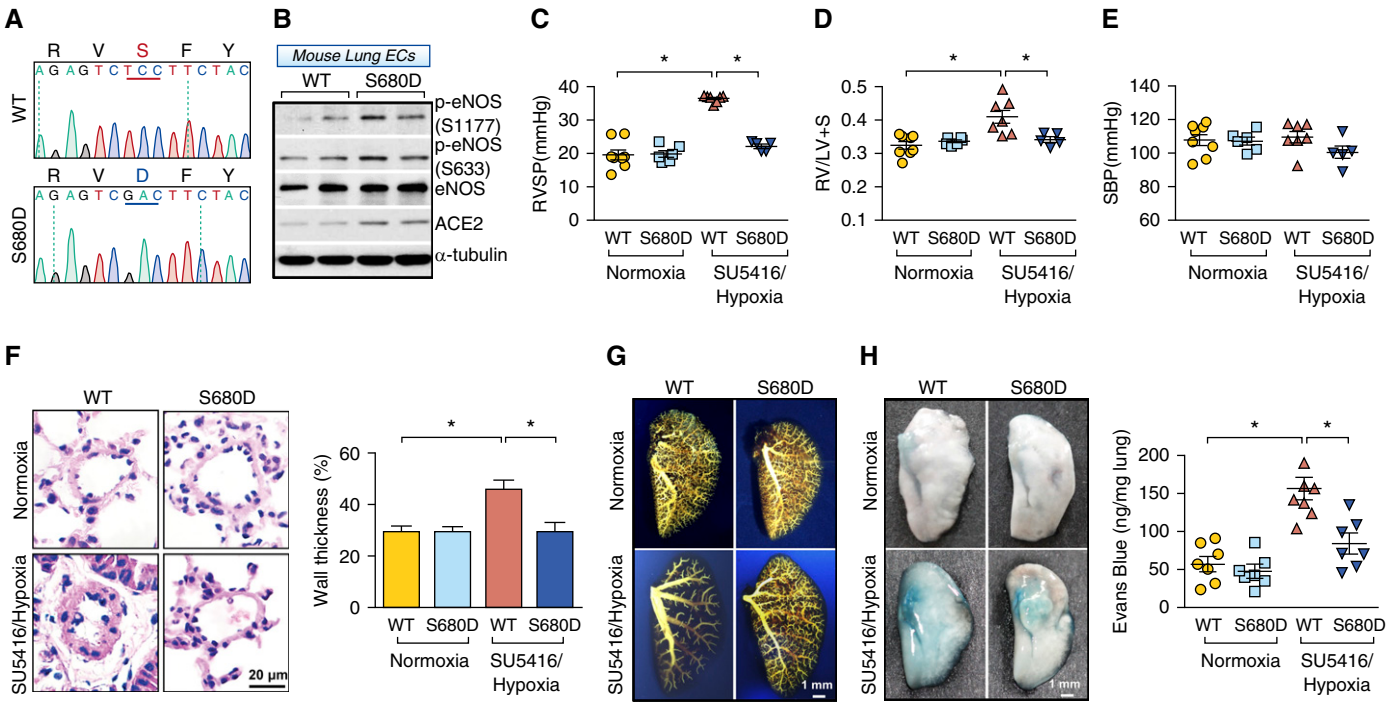

Figure 3. ACE2 (angiotensin-converting enzyme 2) Ser680 phosphorylation attenuates pulmonary hypertension susceptibility in mice. (*A*) Genotype confirmation of the Ser-to-Asp codon replacement in S680D knock-in mice. (*B*) Western blot analysis of indicated proteins in lung endothelial cells (ECs) from wild-type (WT) or *ACE2* S680D mice. (*C–H*) *ACE2* S680D and WT littermates were exposed to hypoxia (10% O_2) with subcutaneous injection of SU5416 at 20 mg/kg once weekly for 3 consecutive weeks, followed by reoxygenation (under normoxia) for 2 weeks. (*C–E*) Right ventricular systolic pressure (RVSP) (*C*), Fulton index [RV/(LV + S) weight] (*D*), and systolic blood pressure (*E*) were measured. (*F* and *G*) Vascularization was revealed by hematoxylin and eosin staining of pulmonary arteries and percentage of vessel wall thickness in reference to the outer diameter of the vessels (*F*), and angiography of pulmonary vasculature (*G*). (*H*) Vascular integrity was evaluated by intravenous injection of Evans blue dye. Scale bars are 20 μm in *F* and 1 mm in *G* and *H*. Data are mean ± SEM derived from five to seven mice per group. *$P < 0.05$ between the indicated groups was considered statistically significant. eNOS = endothelial nitric oxide synthase; LV = left ventricle; p = phosphorylated; RV = right ventricle; S = septum; SBP = systolic blood pressure.

vascularization (Figure 3G). To further address the role of ACE2 phosphorylation in endothelial function *in vivo*, we performed Evans blue staining to test the endothelial permeability of rodent pulmonary vessels. Compared with the lungs from WT littermates, those from *ACE2* S680D knock-in mice exhibited lower amount of Evans blue staining, indicating preserved vascular integrity under SU5416/hypoxia–reoxygenation (Figure 3H). Collectively, these results suggest that PH in the SU5416/hypoxia–reoxygenation mouse model could be reversed with a single gain-of-function mutation at ACE2 Ser680.

PH Augmentation in *ACE2*$^{-/-}$ Mice

To further address the role of ACE2 in PH, we used an *ACE2*$^{-/-}$ mouse line for loss-of-function approaches. *ACE2*$^{-/-}$ mice with SU5416/hypoxia treatment showed aggravated PH as compared with their WT littermates, characterized by elevated RVSP (Figure 4A), increased Fulton index (Figure 4B), vascular blushing of small pulmonary arteries (Figure 4C), and aggravated vasculature (Figure 4D). These phenotypic outcomes resulting from ACE2 ablation were recapitulated in control experiments using EC-*AMPKα2*$^{-/-}$ mice (*see* Figures E4A–E4D) and were also similar to findings in previously reported EC-*AMPKα1/α2* double-knockout mice (19). In addition, metformin administration decreased RVSP and the Fulton index in EC-*AMPKα2*$^{+/+}$ mice with PH onset but not in their EC-*AMPKα2*$^{-/-}$ littermates (*see* Figures E5A–E5C). Downstream eNOS phosphorylation was greatly reduced in the lungs of *ACE2*$^{-/-}$ mice during PH onset (Figure 4E). In a rescue experiment, we isolated lung ECs from EC-*AMPKα2*$^{-/-}$ mice and transfected them with ACE2 S680D or ACE2 S680A. Exogenously expressed ACE2 S680D but not ACE2 S680A ratified eNOS phosphorylation (Figure 4F). To further address the role of the AMPK–p-ACE2 axis in PH, we compared the expression profile of genes associated with PH (e.g., *EDN1* [endothelin-1], *NOTCH1*, *BMPR2* [bone morphogenetic protein receptor type II], and *VEGF*) (24–28) in lung ECs of EC-*AMPKα2*$^{-/-}$ mice with ACE2 S680D or S680A overexpression. ACE2 S680D reduced the mRNA concentration of *EDN1* and *NOTCH1* but increased that of *BMPR2* and *VEGF* as compared with ACE2 S680A (Figure 4G). Collectively, data from these loss-of-function approaches with *ACE2*$^{-/-}$ mice suggest that an impaired AMPK–ACE2 axis aggravated the onset of PH.

AMPK–p-ACE2 Axis Augments EC Function in Rat PH Model

Because ACE2 Ser680 is conserved among mice, rats, and humans (Figure 1A), we further examined the AMPK–p-ACE2 axis in a rat model with MCT-induced PH. Lungs of rats receiving MCT showed significantly decreased phosphorylation of ACE2 and AMPK as well as decreased

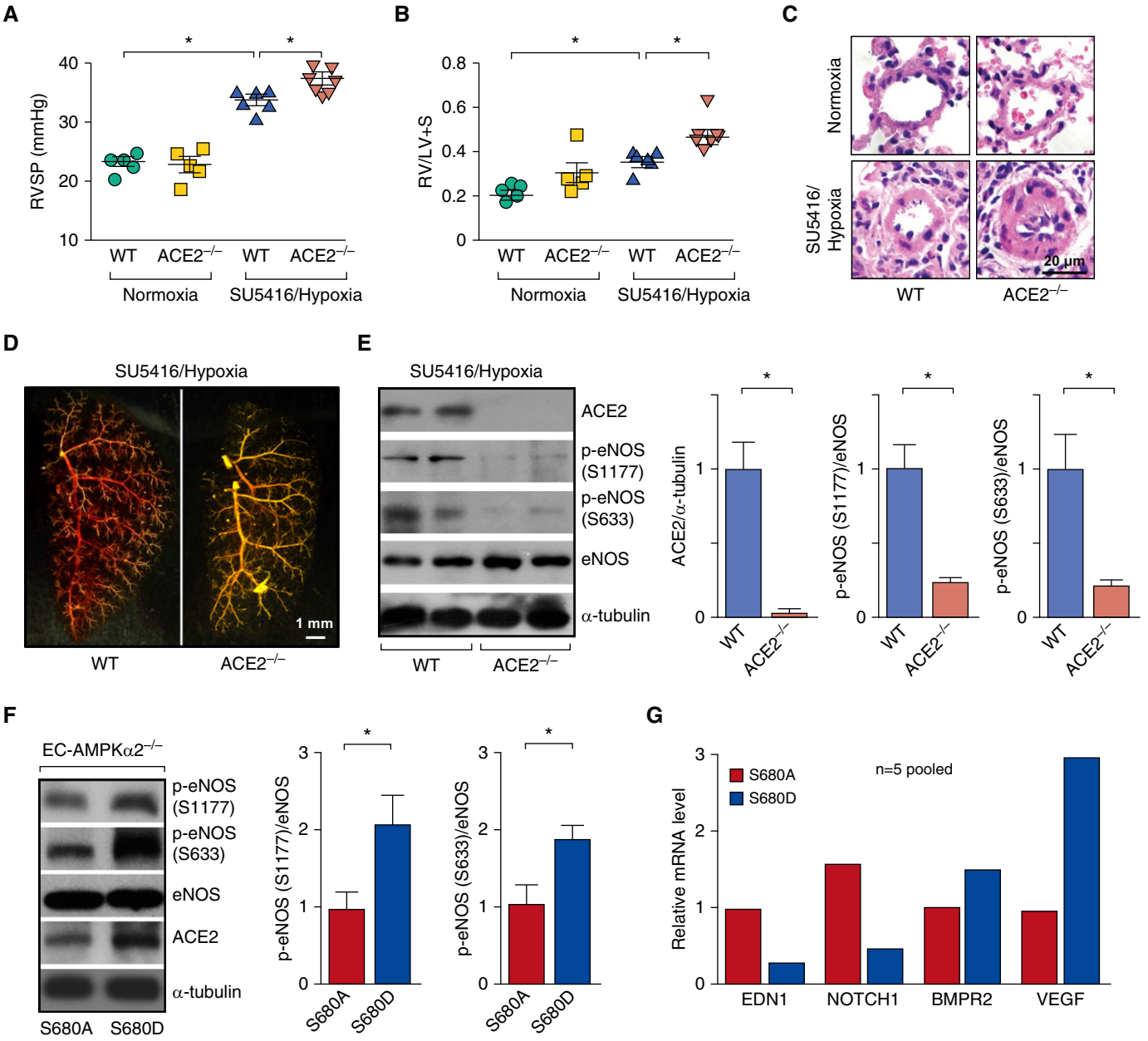

Figure 4. Pulmonary hypertension is aggravated by the ablation of the AMPK (AMP-activated protein kinase)–phosphorylated ACE2 (angiotensin-converting enzyme 2) axis. (*A–E*) *ACE2*$^{-/-}$ mice underwent SU5416/hypoxia followed by reoxygenation, as described in Figure 3. (*A* and *B*) Right ventricular systolic pressure (*A*) and RV/(LV + S) weight (*B*) were measured. (*C* and *D*) Vascularization was revealed by hematoxylin and eosin staining of pulmonary arteries (*C*) and angiography of pulmonary vasculature in the indicated groups (*D*). Scale bars are 20 μm in *C* and 1 mm in *D*. (*E*) Western blot analysis of indicated proteins in lung tissue of *ACE2*$^{-/-}$ mice and wild-type littermates. (*F* and *G*) Lung endothelial cells (ECs) isolated from EC-*AMPKα2*$^{-/-}$ mice were infected with ACE2 S680A or S680D adenovirus for 48 hours. (*F*) Western blot analysis of indicated proteins. (*G*) Quantitative PCR quantification of mRNA concentrations of *EDN1* (endothelin 1), *NOTCH1*, *BMPR2* (bone morphogenetic protein receptor type II), and *VEGF* (vascular endothelial growth factor). Data are mean ± SEM derived from five to seven mice per group. *$P < 0.05$ between the indicated groups was considered statistically significant. eNOS = endothelial nitric oxide synthase; LV = left ventricle; p-eNOS = phosphorylated endothelial nitric oxide synthase; RV = right ventricle; RVSP = right ventricular systolic pressure; S = septum; WT = wild type.

expression of ACE2 (Figures 5A and E6). In a rescue experiment, we isolated lung ECs from these MCT-treated rats and infected them with adenovirus overexpressing ACE2 S680A or S680D. eNOS phosphorylation was greater with ectopic expression of ACE2 S680D than S680A (Figure 5B). Furthermore, ACE2 S680D overexpression, though it reduced the mRNA concentrations of *EDN1* and *NOTCH1*, increased those of *BMPR2* and *VEGF* (Figure 5C). Taken together, the results shown in Figures 3, 4, and 5 suggest that an integral AMPK–p-ACE2 axis in the lung endothelium attenuated PH susceptibility in rodents.

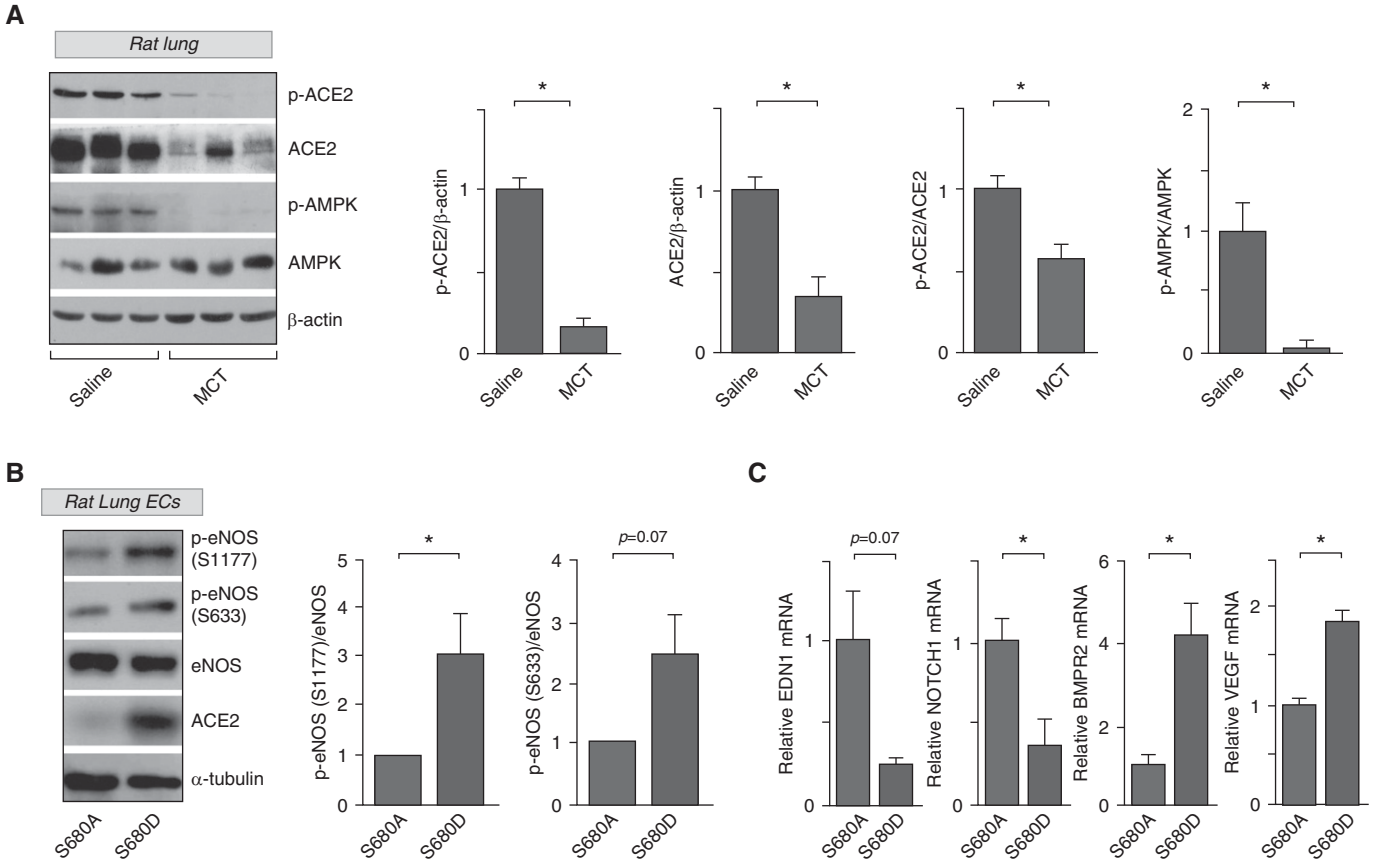

Figure 5. ACE2 (angiotensin-converting enzyme 2) S680D mitigates endothelial cell (EC) dysfunction in rat pulmonary hypertension model. Sprague-Dawley rats received subcutaneous injections with monocrotaline (60 mg/kg body weight) or an equal volume of saline for 3 weeks. (*A*) Lung tissues were isolated. (*B* and *C*) Lung ECs were isolated, then infected with ACE2 S680A or S680D adenovirus (10 multiplicities of infection) for 48 hours. (*A* and *B*) Western blot analysis of indicated protein concentrations. (*C*) Quantitative PCR of mRNA concentrations of *EDN1* (endothelin 1), *NOTCH1*, *BMPR2* (bone morphogenetic protein receptor type II), and *VEGF* (vascular endothelial growth factor). Data are mean ± SEM derived from three to five rats per group. $^*P < 0.05$ between the indicated groups was considered statistically significant. AMPK = AMP-activated protein kinase; eNOS = endothelial nitric oxide synthase; MCT = monocrotaline; p-ACE2 = phosphorylated ACE2; p-AMPK = phosphorylated AMP-activated protein kinase; p-eNOS = phosphorylated endothelial nitric oxide synthase.

AMPK–p-ACE2 Axis Is Impaired in Lungs of Humans with IPAH

Having found that AMPK phosphorylation of ACE2 was impaired in four rodent models of PH (i.e., *ACE2* S680D knock-in, *ACE2*$^{-/-}$, EC-*AMPKα2*$^{-/-}$, and MCT-treated rat), we then compared the AMPK–p-ACE2 axis in lung biopsies from humans with and without IPAH. As compared with non-IPAH control lungs, IPAH lungs showed reduced concentrations of p-AMPK, p-ACE2, and ACE2 (Figure 6A). In addition, decreased concentrations of p-AMPK, p-ACE2, and ACE2 were found in PAECs from patients with IPAH compared with non-IPAH PAECs (*see* Figure E7A). The dysregulated AMPK–p-ACE2 axis in small arteries of IPAH lungs was confirmed by immunostaining (Figure 6B). To reinforce that phosphorylation of ACE2 Ser680 is critical for eNOS-derived NO bioavailability in human pulmonary ECs, PAECs from patients with IPAH were infected with adenovirus overexpressing ACE2 S680A or S680D. As compared with ACE2 S680A, ACE2 S680D overexpression augmented eNOS phosphorylation at Ser1177 and Ser633 (Figure 6C). In addition, ACE2 S680D overexpression reduced the mRNA concentrations of *EDN1* and *NOTCH1* and increased those of *BMPR2* and *VEGF*. Functionally, ACE2 S680D overexpression attenuated IPAH PAEC proliferation when compared with these cells overexpressing ACE2 S680A (*see* Figure E7B). Taken together, the data shown in Figure 6 suggest a perturbed AMPK–p-ACE2 axis in lung ECs from patients with IPAH.

Discussion

Stemming from *in silico* analysis, the present study involving *in vitro* and *in vivo* rodent experiments as well as IPAH patient samples shows that the phosphorylation of ACE2 Ser680 is imperative for ACE2 stability, resulting in enhanced NO production in ECs. In terms of cardiovascular disease, AMPK phosphorylation of ACE2 Ser680, namely the AMPK–p-ACE2 axis, in the endothelium was perturbed in rodent models and human patients with IPAH.

We established that ACE2 Ser680 phosphorylation is indispensable for the protein stability of ACE2 and hence the expression level of ACE2 (Figure 2). Therapeutic use of rhACE2 has been tested in rodent models with PH (10). Because

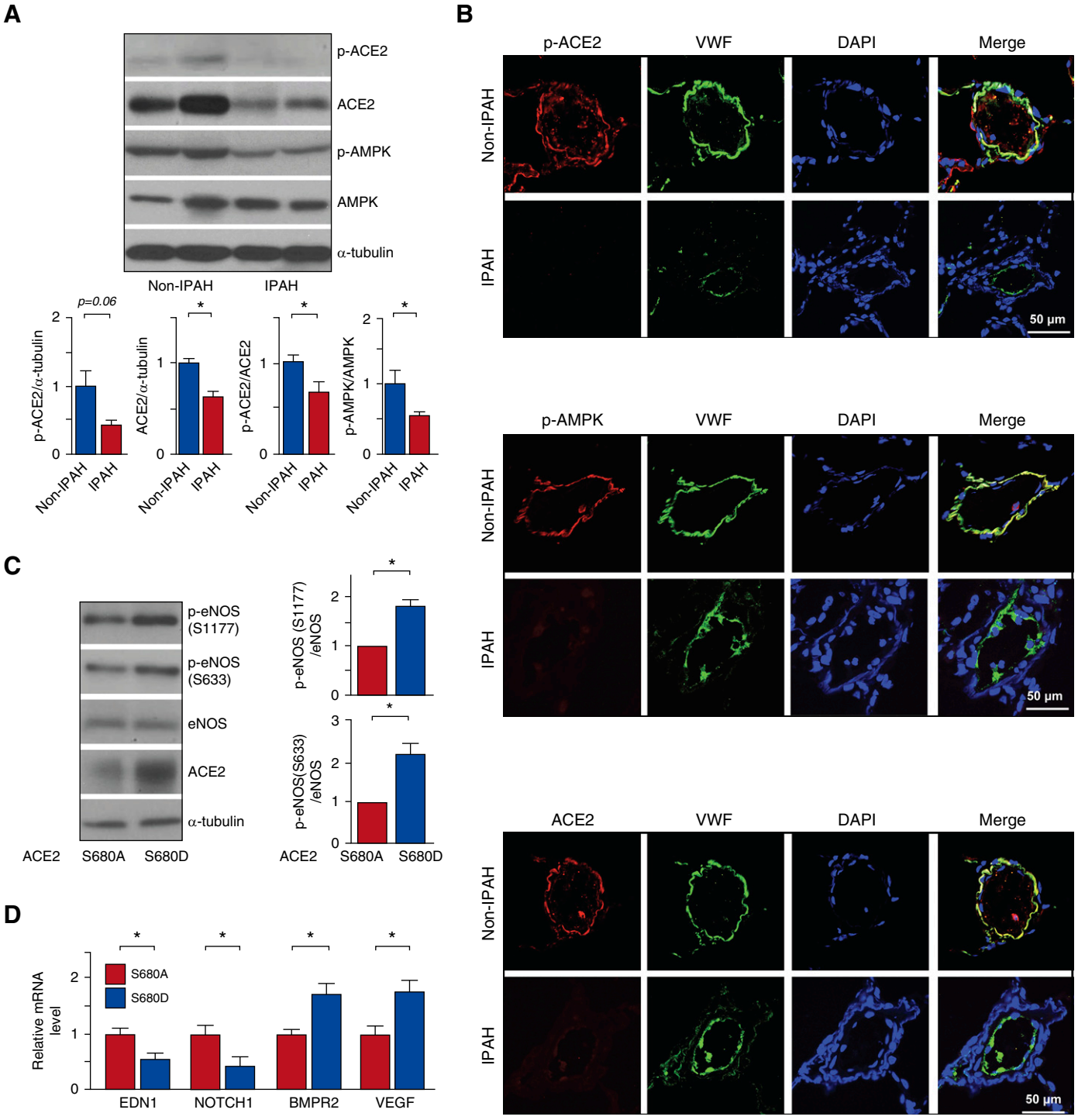

Figure 6. The AMPK (AMP-activated protein kinase)–phosphorylated ACE2 (angiotensin-converting enzyme 2)–axis is impaired in human idiopathic pulmonary arterial hypertension (IPAH). (*A* and *B*) Lung tissue was collected from patients with IPAH ($n = 6$) or control individuals without IPAH ($n = 6$). The indicated proteins were detected by (*A*) Western blot analysis and (*B*) immunostaining (scale bar, 50 μm). (*C* and *D*) Western blot analysis of indicated proteins (*C*) and quantitative PCR of mRNA concentrations of *EDN1* (endothelin 1), *NOTCH1*, *BMPR2* (bone morphogenetic protein receptor type II), and *VEGF* (vascular endothelial growth factor) (*D*) in human pulmonary artery endothelial cells isolated from patients with IPAH and then infected with ACE2 S680A or S680D adenovirus. Data are mean ± SEM derived from three independent experiments. *$P < 0.05$ between the indicated groups considered statistically significant. eNOS = endothelial nitric oxide synthase; p-eNOS = phosphorylated endothelial nitric oxide synthase; p-AMPK = phosphorylated AMP-activated protein kinase; VWF = Von Willebrand factor.

recombinant protein therapy depends on the duration of efficacy, the manipulation of Ser680 to enhance ACE2 stability would be a new therapeutic strategy of rhACE2. Interestingly, the region encoding Ser680 of ACE2 is conserved among mammalian species, and the human Ser680 coding region has no reported single-nucleotide polymorphism. The evolutionarily conserved ACE2 Ser680 suggests that phosphorylation at this site may play a crucial role in EC homeostasis, given that dephosphomimetic S680A mutant resulted in decreased eNOS-derived NO bioavailability (Figures 2E–2G). Ser680 of ACE2 is located at the extracellular domain (spanning amino acids 18 to 739), so how Ser680 is phosphorylated by cytoplasmic AMPK is an enigma. We performed immunostaining experiments to demonstrate that ACE2 is also localized at the cytoplasm when treated with metformin or AICAR (*see* Figure E8), which is consistent with previous reports (29).

In exploring the mechanism by which Ser680 phosphorylation affects ACE2 protein stability, we found that the S680D mutant showed less K48 ubiquitination than the S680A mutant (Figure 2B) and that MG132 stabilized the expression of ACE2 S680A (Figure 2C). These findings indicate that ACE2 Ser680 phosphorylation likely enhances ACE2 stability by inhibiting ubiquitin-related proteasomal degradation. Ang II–induced ubiquitination of ACE2 has been shown to increase ACE2 degradation (30). Cross-talk between phosphorylation and ubiquitination is prevalent in gene regulation at the post-translational level (31). Moreover, substrate phosphorylation can inhibit E3 ligase recognition; one example is S2 phosphorylation of c-Mos kinase preventing its ubiquitination by an E3 ligase (32). Using UbPred software (33), we predicted that Lys788 is a putative ubiquitination site of ACE2. Although the catalytic domain of ACE2 has been crystallized (34), the structural information for the region spanning Ser680 is unavailable. Because the catalytic site of ACE2 is deeply recessed and shielded from the surface of ACE2 (34), AMPKα2 phosphorylation of ACE2 may not directly facilitate substrate (i.e., Ang II) binding for proteolytic cleavage. Rather, AMPK phosphorylation of Ser680 may confer structural changes of ACE2, which are resistant to ubiquitination. If correct, future studies of the cross-talk of post-translational modifications and the involved E3 ligase are warranted.

Regarding downstream effectors of the AMPK–p-ACE2 axis, Ser680 phosphorylation affected eNOS-derived NO bioavailability, possibly through the Ang 1–7 receptor, Mas. The activation of Mas has been shown to activate Akt, which in turn phosphorylates eNOS (23). Furthermore, phosphorylation of ACE2 Ser680 mediated the expression of several PH-related genes, such as *EDN1*, *NOTCH1*, *BMPR2*, and *VEGF* (Figures 4G, 5C, and D6D). Accumulating evidence indicates that NO bioavailability is important for regulating EDN1, NOTCH1, BMPR2, and VEGF. NO inhibits EDN1 transcription, receptor binding, and release from the endothelium via cGMP-dependent pathways (35). NO can also inhibit NOTCH1 through nitration of the NOTCH1 intracellular domain to suppress downstream target genes (36). In contrast, NO can mediate the transcriptional expression of BMPR2 by *S*-nitrosylation of cysteine residues of *N*-ethylmaleimide–sensitive factor (37) as well as that of VEGF by enhancing hypoxia-inducible factor 1 and heme oxygenase 1 activity (38). Reciprocally, alterations in expression of these PH-related genes can regulate eNOS–NO homoeostasis (35, 38–41). Thus, the protective effect of the AMPK–p-ACE2 axis on PH would involve a comprehensive regulation of eNOS and these PH-related genes in the endothelium. Presumably, activation of the ACE2–Ang 1–7–Mas axis by AMPK would also counteract the ACE–Ang II–Ang II type 1 receptor arm of the RAS. Because the involvement of ACE, Ang II, and ACE–Ang II–Ang II type 1 receptor in PH is well documented (42), the beneficial role of AMPK phosphorylation of ACE2 in PH is mediated at least in part by a paradigm shift of the RAS.

In line with the notion that the AMPK–p-ACE2 axis is protective against PH, several AMPK activators, including metformin and AICAR, have shown therapeutic benefit in PH (43, 44). Such pharmacologically activated AMPK seems to be paradoxical to the pathophysiologically dampened AMPK. During PH pathogenesis, the endothelium would be in an energy-deficient state (e.g., hypoxia and mitochondrial damage), thereby activating AMPK to restore the ATP/AMP ratio (16, 45). In the endothelium, AMPK might be induced by an acute insult as part of a physiological or energy compensatory response. However, a prolonged PH milieu is associated with abrogated AMPK activity (46), resulting in impaired phosphorylation of ACE2 Ser680. This finding is line with the increased PH severity we observed in both EC-$AMPK\alpha2^{-/-}$ and $ACE2^{-/-}$ mice (Figure 4; *see also* Figures E4 and E5). Regarding the catalytic specificity of the AMPKα subunit (i.e., AMPKα1 vs. AMPKα2), AMPKα2 seemed to preferentially phosphorylate ACE2 Ser680 (*see* Figure E9). Such discrepancy is reminiscent of the selective AMPKα2 phosphorylation of eNOS and acetyl coenzyme A carboxylase in ECs (14). Thus, although AMPKα1 is more abundant in the endothelium (47), AMPKα2 has a catalytic preference toward a panel of molecules important for EC homeostasis (14, 48, 49). In the pulmonary vessels, endothelial function largely affects the homeostasis of the pulmonary artery smooth muscle cells through ECs releasing various bioactive molecules such as NO, EDN1, and angiotensin (50, 51). Our results show that phosphorylation of ACE2 Ser680 enhanced NO and Ang 1–7 while inhibiting EDN1 in ECs, which would exert a protective effect on pulmonary artery smooth muscle cells.

ACE2 has recently been shown to improve cardiomyopathy through activation of AMPK (52), suggesting a positive feedback loop regulation of AMPK-ACE2 to cause ACE2 phosphorylation/stabilization. To test whether such a feedback loop was present in our system, Western blot analysis was performed to assess the concentrations of p-AMPK and total AMPK in lung tissue from *ACE2* S680D knock-in and $ACE2^{-/-}$ mice. We found little change in p-AMPK/AMPK in the lung tissue from both *ACE2* S680D knock-in and $ACE2^{-/-}$ mice (data not shown). Thus, the putative feedback loop regulation of AMPK-ACE2 reported by Ma and colleagues was not apparent in our experimental system (53).

In summary, the present study demonstrates that phosphorylation of ACE2

at Ser680 is a crucial mediator of PAEC function. The core mechanism builds on the cross-talk between phosphorylation and ubiquitination to enhance the stability of ACE2. Abrogation of this post-translational modification–dependent AMPK–p-ACE2 axis aggravates PH. Therefore, preferential activation of this pathway in the pulmonary endothelium has potential as a new therapeutic strategy for PH. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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