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Role of HIF-1α in the regulation ACE and ACE2 expression in hypoxic human pulmonary artery smooth muscle cells

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Zhang R, Wu Y, Zhao M, Liu C, Zhou L, Shen S, Liao S, Yang K, Li Q, Wan H. Role of HIF-1α in the regulation ACE and ACE2 expression in hypoxic human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 297: L631–L640, 2009. First published July 10, 2009; doi:10.1152/ajplung.90415.2008.—Angiotensin-converting enzyme (ACE) enhances the proliferation and migration of pulmonary artery smooth muscle cells (PASMCs), which contribute to the pathogenesis of hypoxic pulmonary hypertension (HPH). Previous reports have demonstrated that hypoxia upregulates ACE expression, but the underlying mechanism is unknown. Here, we found that ACE is persistently upregulated in PASMCs on the transcriptional level during hypoxia. Hypoxia-inducible factor 1α (HIF-1α), a key transcription factor activated during hypoxia, was able to upregulate ACE protein expression under normoxia, whereas knockdown of HIF-1α expression in PASMCs inhibited hypoxia-induced ACE upregulation. Furthermore, HIF-1α can bind and transactivate the ACE promoter directly. Therefore, we report that ACE is a novel target of HIF-1α. Recently, a homolog of ACE, ACE2, was reported to counterbalance the function of ACE. In contrast to ACE, we found that ACE2 mRNA and protein levels increased during the early stages of hypoxia and decreased to near-baseline levels at the later stages after HIF-1α accumulation. Thus HIF-1α inhibited ACE2 expression, and the accumulated ANG II catalyzed by ACE is a key mediator in the downregulation of ACE2 by HIF-1α. Moreover, a reduction of ACE2 expression in PASMCs by RNA interference was accompanied by significantly enhanced proliferation and migration during hypoxia. We conclude that ACE is directly regulated by HIF-1α, whereas ACE2 is regulated in a bidirectional way during hypoxia and may play a protective role during the development of HPH. In sum, these findings contribute to the understanding of the pathogenesis of HPH.

hypoxia-inducible factor 1α; angiotensin-converting enzyme 2; angiotensin II; hypoxia

EXPOSURE TO CHRONIC HYPOXIA leads to hypoxic pulmonary hypertension (HPH), which is associated with the structural remodeling of pulmonary vessels (13). Many pulmonary disorders are associated with chronic hypoxia, and most of these pulmonary disorders are accompanied by pulmonary hypertension and fatal right heart failure resulting from pulmonary vascular remodeling (17). Vascular remodeling, which occurs in all layers of the vessel wall, is the rearrangement of the vascular architecture characterized by changes in smooth muscle cell proliferation, intimal thickening, and the extension of smooth muscle into previously nonmuscularized arterioles. The key pathological findings of pulmonary vascular remodeling are the increased thickening of pulmonary vessel walls and the muscularization of small arteries (2). Interestingly, decreases in ambient oxygen exposure cause similar pathological changes, including pulmonary smooth muscle hypertrophy and proliferation, in laboratory animals (39). Furthermore, several in vitro studies have also shown that exposure to hypoxia stimulates pulmonary arterial smooth muscle cell (PASMC) proliferation and migration, which may recapitulate an important mechanism underlying pulmonary vessel remodeling associated with HPH in vivo (5, 27).

Chronic hypoxia-induced distal muscularization of small pulmonary arteries is associated with the activation of the renin-angiotensin system (RAS) (4), which has a role in the autocrine and paracrine regulation of vascular function. The RAS, an endocrine cascade, results in the conversion of the inactive prohormone ANG I to the active peptide hormone ANG II by angiotensin-converting enzyme (ACE). ANG II, acting through the ANG II type 1 (AT_1) receptor, is a potent vasoconstrictor and has been demonstrated to stimulate cell proliferation and migration (23). Previous studies have asserted that ANG II contributes to the development of HPH via its vasoconstrictive action or through AT_1 receptor-mediated effects on the migration and growth of vascular smooth muscle cells (1, 21). Consistent with these findings, ACE expression is enriched in the walls of newly muscularized small pulmonary arteries (20). Additionally, in patients and experimental models, HPH was attenuated by a specific AT_1 receptor antagonist and by an ACE inhibitor (ACEI) (12, 22). These results indicate that ACE mediates an important role in the occurrence of HPH because of the formation of ANG II. As a recently reported homolog of ACE, ACE2 is a new component of the updated RAS (7). ACE2 has been shown to be critical in the conversion of the hyperplastic, hypertrophic, and profibrotic hormone ANG II directly to ANG-(1–7), which has the opposite function of ANG II: it induces vasodilatation and is antiproliferative. ACE2 appears to counterbalance the ANG II hypertension-promoting effect of ACE and plays a protective role in many diseases (18, 26, 32).

Activation of the inducible transcription factor hypoxia-inducible factor 1α (HIF-1α) is a critical hypoxia-driven signaling pathway in the lung vasculature (33, 35). HIF-1 is a heterodimeric transcription factor composed of a regulatory α-subunit and a constitutive β-subunit (HIF-1β). HIF-1α is selectively stabilized under hypoxia, whereupon it translocates into the nucleus, binds to hypoxia-responsive elements (HREs), and activates the expression of genes that promote vascular development, glycolytic metabolism, and cell cycle control (28). Although previous reports have shown that hypoxia can regulate

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ACE expression in vivo and in vitro (15, 19), the expression of ACE2 during hypoxia has not been examined. HIF-1α is one of the principal transcription factors regulated by hypoxia, but whether HIF-1α can regulate ACE and ACE2 remains unknown. Hence, we attempted to investigate the potential relationship between HIF-1α and ACE, as well as its homolog ACE2. Our results demonstrate that hypoxia can elicit changes in the expression profiles of ACE and ACE2 in human PASMCs (hPASMCs). HIF-1α can upregulate ACE directly, and ACE was identified as a novel target gene of HIF-1α. However, HIF-1α could also inhibit ACE2 protein expression in an indirect way. In addition, we demonstrated a reduction of ACE2 expression in hPASMCs by RNA interference that was accompanied by significantly enhanced levels of proliferation and migration under hypoxia. Importantly, these findings may contribute to the elucidation of the mechanism underlying vessel remodeling in HPH.

MATERIALS AND METHODS

Materials. DMEM, penicillin-streptomycin, amphotericin B (Fungizone), and trypsin (0.05% in 0.53 mM EDTA) were purchased from Invitrogen (Carlsbad, CA); FBS from Hyclone (Logan, UT); antibodies to ACE and ACE2 from Santa Cruz Biotechnology (Santa Cruz, CA); antibody for HIF-1α from BD Biosciences (San Diego, CA); β-actin antibody from Merck (Darmstadt, Germany); horseradish peroxidase-linked secondary antibodies from Cell Signaling Technology (Beverly, MA); the ELISA kit for detection of ANG II from R & D (Minneapolis, MN); the ACEI captopril, the specific ANG II type 2 (AT_2) receptor antagonist PD-123319, and ANG II from Sigma (St. Louis, MO); and the specific AT_1 receptor antagonist telmisartan from Boehringer Ingelheim (Ingelheim, Germany).

hPASMCs. hPASMCs were obtained commercially from Cascade Biologics (Portland, OR). Cells were maintained in smooth muscle growth medium (medium 231, Cascade Biologics) supplemented with smooth muscle growth supplement (Cascade Biologics), which had been optimized for the growth of human vascular smooth muscle cells. Cells were incubated in a CO_2 incubator (Heraeus Instruments) set at 37°C in a 5% CO_2 atmosphere. Cells used in all experiments were between *passage 4* and *passage 10*. Confluent cells were removed from the culture flasks with use of trypsin-EDTA, centrifuged at 650 rpm for 5 min, resuspended, and counted for use in subsequent experiments. Before exposure to hypoxia or treatment with different agents, cells were made quiescent by incubation in serum-free medium for 24 h. Media were changed every 2–3 days.

Exposure of hPASMCs to hypoxia. Cells were exposed to hypoxia as described in previous studies (10, 24, 29). Briefly, hPASMCs were cultured in a hypoxia incubator (Thermo Electron, Forma, MA) under hypoxic conditions (2% O_2-93% N_2-5% CO_2) with temperature maintained at 37°C. The incubator chamber was sealed and purged with 2% O_2-5% CO_2-balance N_2 for 60 min. Cells were exposed to hypoxia for 0, 6, 12, 24, and 48 h.

RT-PCR. Total RNAs were extracted from cells by TRIzol reagent (GIBCO BRL, Gaithersburg, MD). RNA was treated with DNase (Promega, Madison, WI), and cDNA was synthesized using the cDNA synthesis kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Fluorescence real-time RT-PCR was performed with the double-stranded DNA dye SYBR Green PCR core reagents (PE Biosystems, Warrington, UK) using an ABI PRISM 7300 system (Perkin-Elmer, Torrance, CA). The reaction of SYBR Green assay contained 1 μl of 10× SYBR Green PCR buffer, 0.8 μl of dNTP mixture, 0.1 μl of AmpErase UNG (1 U/μl), 0.05 μl of AmpliTaq Gold DNA polymerase (5 U/μl), 1.2 μl of $MgCl_2$ (25 mM), 0.1 μl of forward and reverse primer (20 μM), 1 μl of cDNA, and 5.65 μl of double-distilled H_2O. The following specific oligonucleotide primers were used: 5′-CGC TGA AAC CGC TGT ACG A-3′ (sense) and 5′-TGG GGG AGT TGT ACC AGG AG-3′ (antisense) for ACE and 5′-AAC TGC TGC TCA GTC CAC C-3′ (sense) and 5′-AAA AGG CAG ACC ATT TGT CCC-3′ (antisense) for ACE2; β-actin [5′-CAT CCT CAC CCT GAA GTA CCC-3′ (sense) and 5′-AGC CTG GAT AGC AAC GTA CAT G-3′ (antisense)] was used as the internal control. PCR was carried out as follows: one cycle of 50°C for 2 min (UNG incubation) and 10 min at 95°C (hot-start PCR) followed by 45 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. After PCR amplification cycles, a dissociation (melting) curve was constructed in the range of 65°C to 95°C. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical adhesive covers (Applied Biosystems). PCR was performed in triplicate, and standard deviations representing experimental errors were calculated. All data were analyzed using ABI PRISM SDS 2.0 software (Perkin-Elmer), which is coupled to the instrument and allows determination of the threshold cycle (C_t), which represents the number of the cycle where the fluorescence intensity is significantly above the background fluorescence intensity. With use of the ΔC_t method, β-actin was coamplified to normalize the amount of RNA added to the reaction, and the data were subjected to cycling threshold analysis as described previously (41). For semiquantitative RT-PCR, the primers for ACE, ACE2, and β-actin described above were used. PCR products were analyzed on a 1% agarose gel. All RT-PCR were repeated at least three times.

Western blot analysis. Cells were scraped from culture dishes, and protein lysates were mixed with an equal volume of 2× Laemmli buffer [62.5 mM Tris·HCl (pH 6.8), 2% SDS, 50 mM DTT, 10% glycerol, and 0.01% bromphenol blue], boiled for 10 min at 100°C, and then resolved by SDS-PAGE on an 8% gel using a Mini-Gel apparatus (Bio-Rad). Subsequently, the proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with a 5% nonfat dry milk solution in TBS with 0.1% Tween 20 (TBS-T, pH 7.6) for 1 h at room temperature and then incubated in primary antibody dissolved in the blocking solution at 4°C overnight. The ACE protein was detected by a mouse anti-ACE monoclonal antibody, ACE2 was detected by a goat anti-ACE2 polyclonal antibody, and HIF-1α was detected by a mouse anti-HIF-1α monoclonal antibody; a mouse anti-β-actin monoclonal antibody was used to confirm equal loading. After the membranes were washed three times for 5 min in TBS-T, they were incubated with HRP-conjugated secondary antibody corresponding to the primary antibody in the blocking buffer for 1 h at room temperature. After three washes, the proteins were detected by luminol detection reagent (Santa Cruz Biotechnology) and developed on Kodak X-ray films.

Immunofluorescence. hPASMCs were grown on chamber slides and used for experiments when they reached 75% confluence. Subsequently, cells were washed in PBS and fixed immediately in acetone-methanol (1:1). Fixed cells were incubated overnight at 4°C with PBS containing an ACE or ACE2 antibody diluted 1:50. Indirect immunofluorescence was observed after incubation with an FITC-conjugated IgG antibody (DAKO, Glostrup, Denmark) diluted 1:100 in PBS.

Overexpression of HIF-1α in hPASMCs. pEF-BOS is a powerful mammalian expression vector. The expression vector of HIF-1α (pEF-BOS-HIF-1α) and pEF-BOS empty plasmid were kindly provided by Dr. Y. Fujii-Kuriyama (University of Tsukuba, Tsukuba, Japan) (8). At 1 day before transfection, the hPASMCs were collected and seeded into six-well plates at 2×10^5 cells per well. The cells were transfected with 400 ng of pEF-BOS-HIF-1α plasmid by use of Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The transfection mixture was removed 12 h after transfection, and fresh medium was added. The transfection efficiency in the cells was monitored by the cotransfection of 50 ng of pEGFP plasmid (Clontech, Palo Alto, CA), which expressed green fluorescence protein (GFP). pEF-BOS plasmid was transfected as a negative control. Transfected cells were analyzed 36 h after transfection.

Small interfering RNA knockdown of HIF-1α, ACE, and ACE2 expression. Small interfering RNA (siRNA) was designed and transfected into cells as described previously with some modifications (11). The sequence for the siRNA against HIF-1α was as follows: 5′-UGU GAG UUC GCA UCU UGA U dTdT-3′ (forward) and 5′-AUC AAG AUG CGA ACU CAC A dTdT-3′ (reverse; JieKai, Shanghai, China). As a control for HIF-1α siRNA, we used a corresponding random siRNA sequence (5′-UAC ACC GUU AGC AGA CAC C dTdT-3′). The sequence for siRNA targeting of ACE2 was as follows: 5′-CCA UCU ACA GUA CUG GAA A dTdT-3′ (forward) and 5′-UUU CCA GUA CUG UAG AUG G dTdT-3′ (reverse; JieKai). As a control for ACE2 siRNA, we used a corresponding random siRNA sequence [5′-UUC UCC GAA CGU GUC ACG U dtdt-3′ (forward) and 5′-ACG UGA CAC GUU CGG AGA A dtdt-3′ (reverse)]. The sequence for siRNA targeting of ACE was as follows: 5′-CUA UCA AGC GGA UCA UAA A dTdT-3′ (forward) and 5′-UUU AUG AUC CGC UUG AUA G dTdT-3′ (reverse; JieKai). As a control for ACE siRNA, we used a corresponding random siRNA sequence [5′-UUC UCC GAA CGU GUC ACG U dtdt-3′ (forward) and 5′-ACG UGA CAC GUU CGG AGA A dtdt-3′(reverse)]. hPASMCs were treated with siRNAs targeting HIF-1α (100 nM), ACE2 (100 nM), or ACE (100 nM) and then incubated under normoxia and hypoxia. siRNA transfection was performed with Lipofectamine 2000 (Invitrogen).

Expression constructs and site-directed mutagenesis. Human ACE promoter luciferase constructs include the following sequences: *1*) pGL3-ACE, which contains 2,659 nt of the 5′-flanking region from +1743 to −916 bp of the ACE gene; *2*) pGL3-ACE-1, which contains 599 nt of the 5′-flanking region from +1743 to +1144 bp of the ACE gene; and *3*) pGL3-ACE-2, which contains 2,078 nt of the 5′-flanking region from +1162 to −916 bp of the ACE gene. In addition, human pGL3-ACE2 contains 3,332 nt of the 5′-flanking region from +1990 to −1342 bp of the ACE2 gene. All the fragments from the ACE and ACE2 promoters were subcloned into the *Mlu* and *Xho* sites of the pGL3-basic luciferase reporter plasmid. A mutant HRE binding site (ACGTG to AAATG) was introduced into the pGL3-ACE-1 construct by using a site-specific mutagenesis kit (Stratagene, La Jolla, CA). The pGL3-basic plasmid and the pSV40-*Renilla* plasmid were purchased from Promega.

Luciferase assay. Cos-7 cells were seeded at 1×10^5 per well in 12-well plates and incubated overnight. Cells were cotransfected with 50 ng of pGL3-ACE or pGL3-ACE2, 1 ng of pSV40-*Renilla*, and 100 ng of pEF-BOS-HIF-1β, which was combined with 0, 50, 100, or 200 ng of the pEF-BOS-HIF-1α expression plasmid. The cells were also cotransfected with 50 ng of each of the reporter constructs (pGL3-ACE, pGL3-ACE-1, pGL3-ACE-2, and the specific site-directed mutant construct), 1 ng of pSV40-*Renilla*, and 100 ng of pEF-BOS-HIF-1β combined with 0 or 200 ng of the pEF-BOS-HIF-1α expression plasmid. For each transfection, the total plasmid concentration was 750 ng and was supplemented with the pEF-BOS empty vector as necessary. Plasmids were transfected into Cos-7 cells using the Polyfect transfection reagent (Qiagen, Valencia, CA). Transfected cells were harvested after 36 h, and luciferase activity was measured using the dual-luciferase assay system (Promega) according to the manufacturer's instructions.

Chromatin immunoprecipitation assay. As described previously (38), cells were exposed to normoxia or hypoxia for 24 h, treated with 1% formaldehyde for 10 min and 0.125 M glycine for 5 min, and analyzed using the chromatin immunoprecipitation assay (Upstate Biotechnology) with anti-HIF-1α and rabbit IgG (Santa Cruz Biotechnology). PCR was performed using primers to amplify DNA sequences from the ACE gene. PCR primer pairs for amplification of the ACE promoter region (−1345 to −1130 bp) were as follows: 5′-TGC AAC ACG TCA TCT GAC AAG T-3′ (sense) and 5′-GGG ACA GCT GCT CCC AAG A-3′ (antisense).

ANG II concentration detection in culture medium. hPASMCs were plated at 1×10^5 cells per well in 12-well plates. When confluent, cells were incubated with 1 ml of fresh medium and then cultured under hypoxia or normoxia. After specified intervals, the cell culture supernatants were aspirated and frozen at −80°C until assayed. ANG II concentrations in the culture medium were detected by ELISA kits according to the manufacturer's instructions.

Cell growth assay. Cell growth was assessed by cell counting (Cell Counting Kit-8, Dojindo, Kumamoto, Japan). hPASMCs (2×10^3), transfected with ACE- or ACE2-targeting and -nontargeting siRNA, were plated in 96-well microplates. Cells were incubated in serum-free medium under hypoxia and normoxia for 24 h. After addition of 10 μl of the Cell Counting Kit-8 reagent and 3 h of incubation, the plates were read at 450 nm in a spectrophotometer (Molecular Devices, Sunnyvale, CA). Experiments were carried out in triplicate and repeated at least three times.

hPASMC migration assay. hPASMC migration was assayed in 24-well Millicell hanging cell culture inserts (Millipore, Boston, MA) with 8-μm pores. At 24 h after transfection with ACE- or ACE2-targeting and -nontargeting siRNA, cells were harvested and seeded in serum-free medium on top of the culture insert at 2×10^4 cells per insert in a 0.2-ml volume. The lower chamber contained 1.25 ml of complete medium. Migration was allowed to proceed for 6 h under hypoxia and normoxia. Cells remaining on the upper surface of the filters were mechanically removed, and then filters were fixed in 3% glutaraldehyde and stained with Giemsa. The number of cells that had migrated to the lower surface was determined by counting under high-power microscopy (×40 objective). Three inserts were counted for each treatment in each experiment, and experiments were carried out in triplicate.

Statistical analysis. Values are means ± SE. Statistical comparisons were performed using Student's *t*-test or ANOVA, as appropriate. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of hypoxia on ACE and ACE2 expression in hPASMCs. Cultured hPASMCs were incubated under hypoxic conditions (2% O_2) for 0, 6, 12, 24, and 48 h. Total RNA was isolated from the cells, and ACE and ACE2 mRNA levels were quantified by real-time RT-PCR. The results were normalized to β-actin, which served as an unchanged control, and expressed as a ratio. ACE mRNA was markedly increased in hPASMCs during hypoxia, showing time-dependent regulation of the gene by hypoxia (Fig. 1, *A* and *B*). ACE2 mRNA was also increased during hypoxia but peaked at 12 h and then began to decline (Fig. 1, *A* and *B*). The upregulation of ACE mRNA by hypoxia also increased the concentration of ACE protein. The immunoreactivity of ACE protein was increased after incubation under hypoxia as determined by Western blotting and immunofluorescence assays (Fig. 1, *C–E*). ACE2 protein was also increased during the early stages of hypoxia; it peaked at 24 h and then declined (Fig. 1, *C–E*). In addition, HIF-1α protein also accumulated during hypoxia (Fig. 1*C*).

ACE was upregulated and ACE2 was downregulated by HIF-1α. HIF-1α is a key transcription factor that regulates many genes at the transcriptional level during hypoxia (34). To determine whether HIF-1α could regulate the expression of ACE and ACE2, hPASMCs were transfected with the pEF-BOS-HIF-1α plasmid or the pEF-BOS empty vector as a negative control. Examination of the transfection efficiency revealed that ~50% of cells showed green fluorescence (Fig. 2*A*). These results demonstrated that overexpression of HIF-1α could upregulate ACE protein expression, but downregulate ACE2, in the absence of hypoxia (Fig. 2*B*). RNA interference for HIF-1α was also accomplished by transfection of hPASMCs with an siRNA sequence for HIF-1α (HIF-1α-siRNA). As expected, we ob-

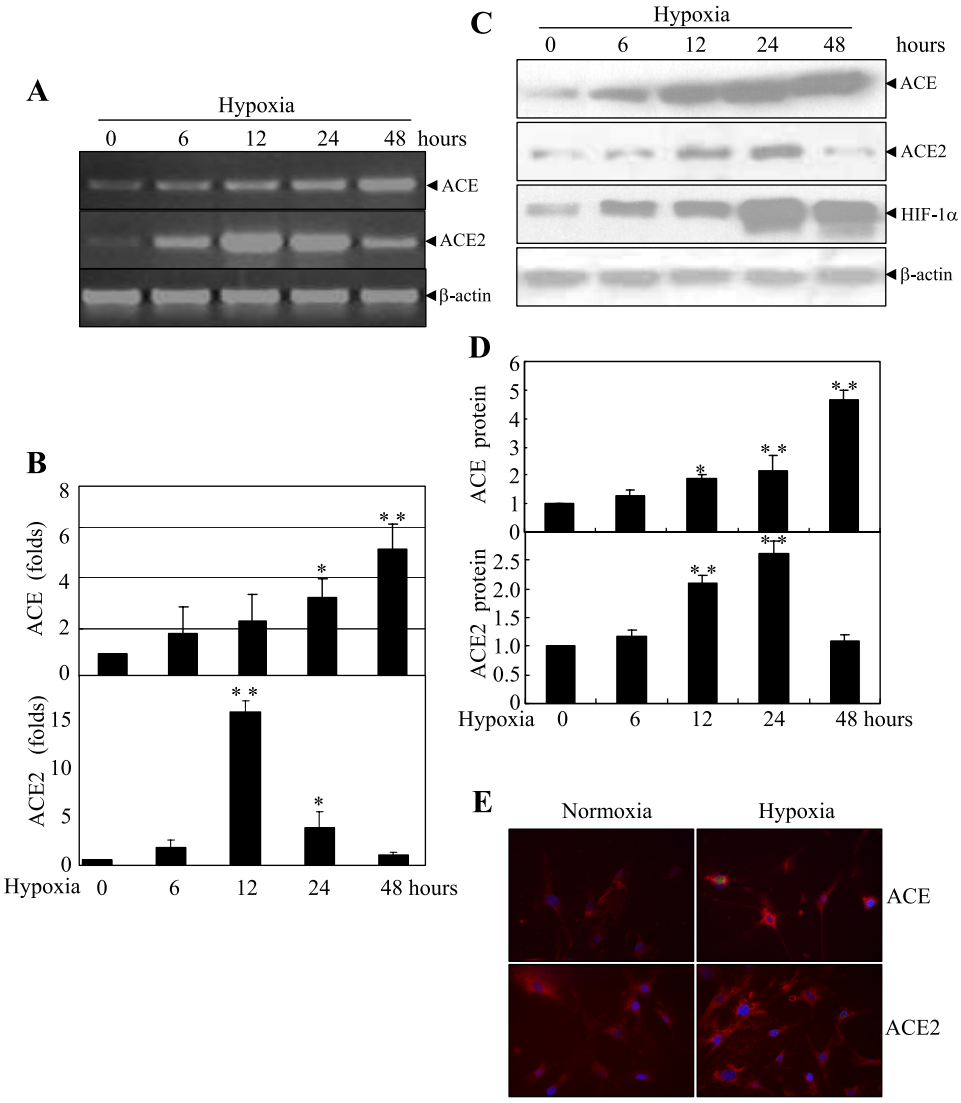

Fig. 1. Effects of hypoxia on angiotensin-converting enzyme (ACE) and ACE2 expression in human pulmonary artery smooth muscle cells (hPASMCs) exposed to 2% O_2 for 0, 6, 12, 24, and 48 h. *A* and *B*: semiquantitative RT-PCR and real-time quantitative RT-PCR assessment of mRNA levels of ACE and ACE2, with β-actin used as an internal control. *C*: Western blot detection of ACE, ACE2, and hypoxia inducible factor 1α (HIF-1α) proteins, with β-actin used as a loading control. *D*: densitometric ratios of ACE and ACE2 proteins to β-actin. *E*: immunofluorescence detection of ACE and ACE2 after 24 h of hypoxia or normoxia. Values in *B* and *D* (means ± SE) are fold increases, with 0 h taken as 1. All experiments were repeated in triplicate with similar results. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. 0 h.

served the typical upregulation of ACE during hypoxia, but not in HIF-1α-siRNA-treated cells. In contrast, the ACE2 protein level was markedly increased in HIF-1α-siRNA-treated cells compared with controls during hypoxia (Fig. 2*C*). This study demonstrates that HIF-1α can upregulate ACE but it can downregulate ACE2.

HIF-1α enhances the transcriptional activity of ACE protein. Because HIF-1α-dependent target genes are controlled via the HREs of their respective promoters, bioinformatics analysis was performed with an ~3-kb region (upstream 2,000 bp to downstream 1,000 bp to the transcription starting site) of the ACE and ACE2 genes. The results showed three typical HREs (A/GCGTG) in the putative promoter region of ACE that were located at −1239 to −1235 bp, −921 to −917 bp, and +187 to +191 bp of the gene, and five typical HREs in the promoter region of ACE2, which were located at −1541 to −1537 bp, −870 to −866 bp, +149 to +153 bp, +487 to +491 bp, and +543 to +547 bp of the gene. Fragments of the human ACE promoter (−1743 to +916 bp) and ACE2 promoter (−1990 to +1342 bp) were subcloned into the luciferase reporter plasmid pGL3-basic to form pGL3-ACE and pGL3-ACE2, respectively. We measured the promoter activities using a dual-luciferase reporter assay. The HIF-1α expression vector pEF-BOS-HIF-1α and the HIF-1β expression vector pEF-BOS-HIF-1β were cotransfected along with the pGL3-ACE or pGL3-ACE2 plasmid into cells. The results demonstrated that the ACE promoter activity of luciferase was significantly increased in a dose-dependent manner when HIF-1α was overexpressed in the cells (Fig. 3*A*). In contrast, the ACE2 promoter activity of luciferase was unchanged compared with the control (Fig. 3*B*).

To further investigate which HRE site was involved in HIF-1α-mediated regulation, the whole ACE promoter region was divided into two segments, and two luciferase reporter plasmids were constructed (pGL3-ACE-1 and pGL3-ACE-2). As shown in Fig. 3*C*, the pGL3-ACE-1 promoter construct containing one of the three HREs increased luciferase reporter activity to ~10-fold above the comparable levels of pGL3-ACE when pEF-BOS-HIF-1α was cotransfected. However, the pGL3-ACE-2 promoter construct containing two of the three HREs could not elevate luciferase reporter activity compared with the control. Furthermore, after introduction of a mutation into the HRE site in pGL3-ACE-1, the reporter gene induction by HIF-1α was substantially reduced. This result indicated that

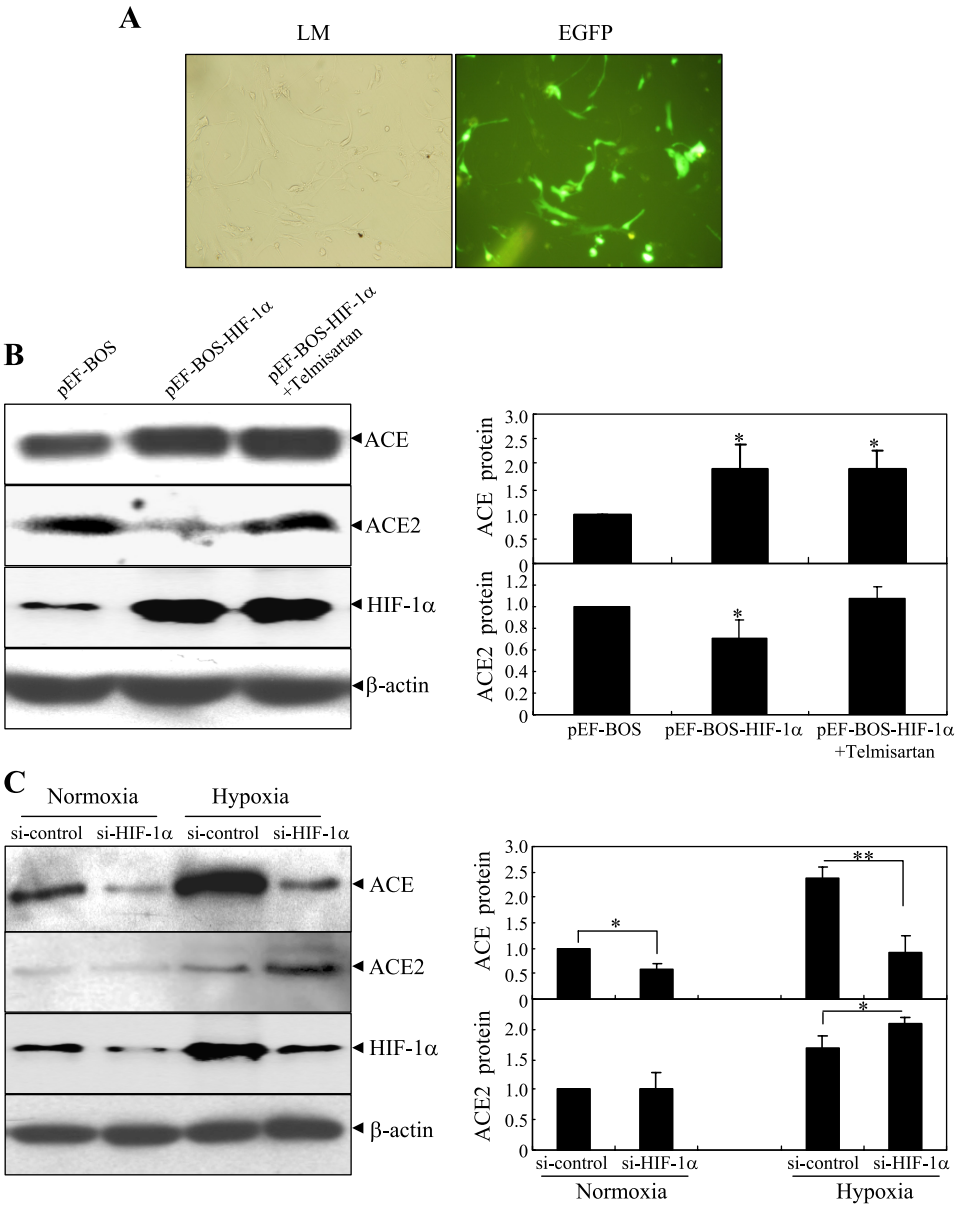

Fig. 2. ACE was upregulated and ACE2 was downregulated by HIF-1α. *A*: light-microscopic (LM) and enhanced green fluorescence protein (EGFP) images of hPASMCs. To assess transfection efficiency, we cotransfected cells with the pEGFP and pEF-BOS plasmids at the same time, which expressed green fluorescence protein. *B*: Western blot detection of ACE, ACE2, and HIF-1α proteins, with β-actin used as a loading control. hPASMCs were transiently transfected with pEF-BOS or pEF-BOS-HIF-1α during normoxia, and telmisartan (5 μM) was also added to pEF-BOS-HIF-1α-transfected cells. Values (means ± SE) are densitometric ratios of ACE and ACE2 proteins to β-actin, with pEF-BOS taken as 1. All experiments were repeated in triplicate with similar results. $^*P < 0.05$ vs. pEF-BOS. *C*: Western blot detection of ACE, ACE2, and HIF-1α proteins, with β-actin used as a loading control. Cells were transiently transfected with small interfering RNA (siRNA) targeting HIF-1α during normoxia and hypoxia; siRandom was used as a control siRNA (si-control) for siHIF-1α. Values (means ± SE) are densitometric ratios of ACE and ACE2 proteins to β-actin, with si-control under normoxia taken as 1. All experiments were repeated in triplicate with similar results. $^*P < 0.05$. $^{**}P < 0.01$.

this site was indeed required for HIF-1α-mediated regulation. To identify whether hypoxia could enhance the binding of HIF-1α to the ACE promoter in live cells, we performed a chromatin immunoprecipitation assay using the primers covering the suggested HRE binding site. As shown in Fig. 3*D*, hypoxia did augment the binding of HIF-1α to the ACE promoter.

Accumulated ANG II was a key mediator in the downregulation of ACE2 by HIF-1α. Previous studies showed that ANG II could inhibit the synthesis of ACE2 at mRNA and protein levels (9, 14). In the present study, we found that the ANG II concentration in cell culture medium is higher under hypoxia than normoxia at the same time point (Fig. 4*A*). Subsequently, cultured hPASMCs were incubated with synthetic ANG II. In addition, EDTA (0.5 mmol/l) was added to the culture medium to prevent the degradation of ANG II by blocking the activity of metalloproteinases, such as neprilysin and ACE2. As previously reported (9, 14), ANG II downregulated the expression of ACE2 protein, and the downregulation of ACE2 by the peptide was dose dependent (Fig. 4*B*). ANG II downregulated ACE2 protein expression at 1 μmol/l, and the inhibitory effect reached a maximal level at 10 μmol/l.

To confirm whether ANG II is involved in the downregulation of ACE2 in hPASMCs during hypoxia, an ACEI was used. This agent suppresses ANG II generation by inhibiting its conversion from ANG I and does not affect ACE2 activity (30). As shown in Fig. 4*C*, captopril could upregulate ACE2 expression during hypoxia. This demonstrates that ANG II contributes to the downregulation of ACE2 in hPASMCs during hypoxia.

Specific angiotensin receptor antagonists were also used to identify the receptor subtype that mediated the downregulation of ACE2 protein by ANG II. The addition of the specific AT_1 receptor antagonist telmisartan effectively upregulated ACE2 protein expression during hypoxia (Fig. 4*D*). In contrast, the AT_2 receptor antagonist PD-123319 had no effect on the regulation of ACE2 protein expression during hypoxia (Fig.

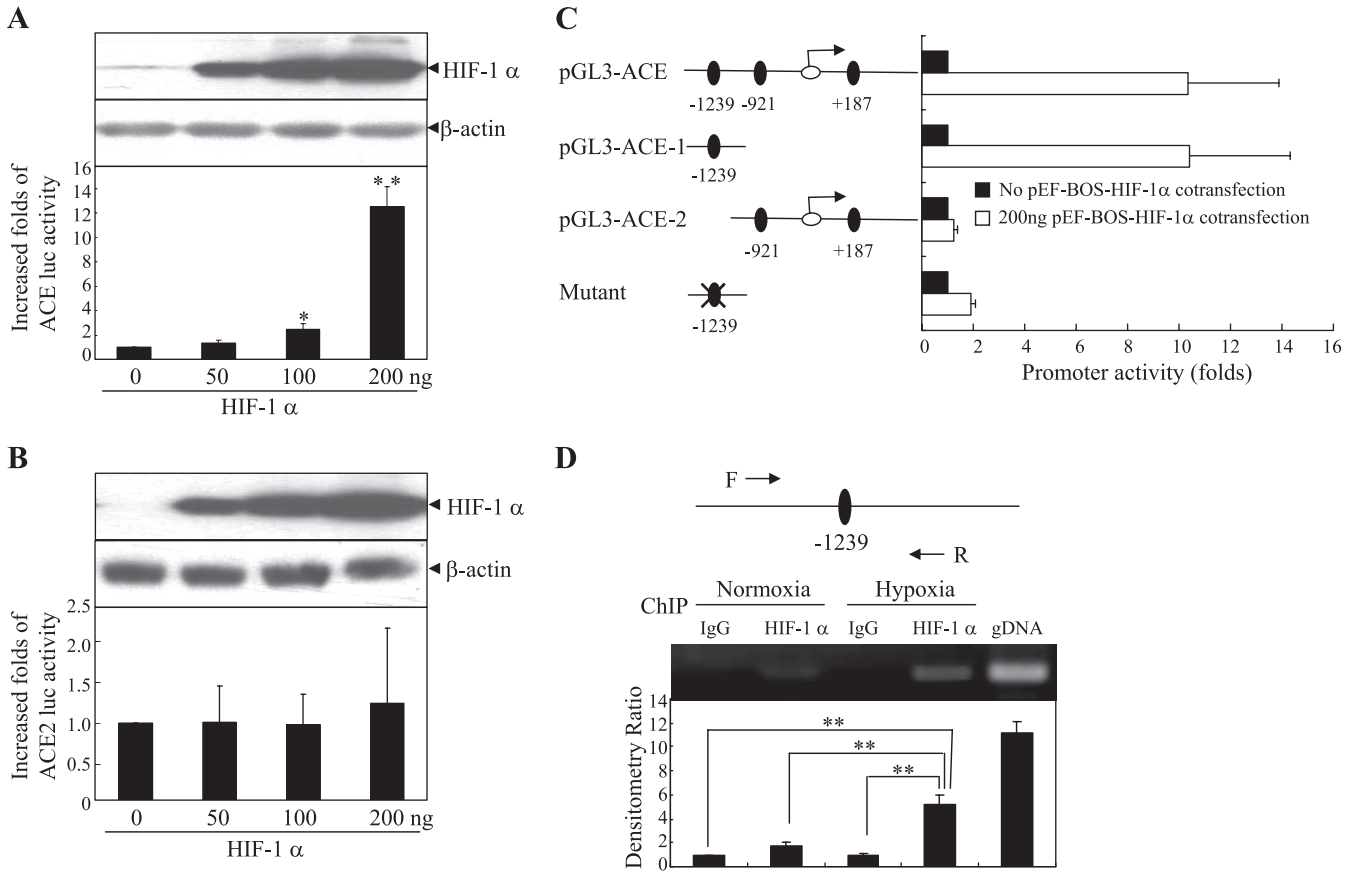

Fig. 3. HIF-1α transactivates the ACE promoter. *A* and *B*: pGL3-ACE and pGL3-ACE2 reporter plasmids were transiently cotransfected with pEF-BOS-HIF-1α, pEF-BOS-HIF-1β, and pSV40-*Renilla* into Cos-7 cells. *C*: pGL3-ACE, pGL3-ACE-1, pGL3-ACE-2, and site-directed mutant constructs were transfected into cells. Total amount of introduced DNAs was supplemented to 750 ng by pEF-BOS empty vector. Promoter activity was measured as the ratio of luciferase (luc) activity 36 h after transfection. Promoter activity in the presence of 0 ng of pEF-BOS-HIF-1α plasmid was defined as 1, and promoter activity in the presence of different concentrations of pEF-BOS-HIF-1α expression vectors was defined relative to that value. Values are means ± SE of 3 independent samples with the same treatment. $*P < 0.05$ and $**P < 0.01$ vs. 0 ng of pEF-BOS-HIF-1α. HIF-1α protein was detected by Western blot, with β-actin as a loading control. ●, Hypoxia-responsive elements (HREs); ✕, mutant forms; ⇀, transcription start site. *D*: chromatin immunoprecipitation assay of HIF-1α binding in hypoxia-exposed hPASMCs. Chromatin from cells exposed to normoxia or hypoxia was immunoprecipitated with IgG or anti-HIF-1α and analyzed by PCR using primers spanning the candidate HIF-1α-binding site. Genomic DNA (gDNA) was a positive control in which PCR was performed using total human gDNA, rather than immunoprecipitated chromatin. F, forward; R, reverse. Values (means ± SE) are densitometric ratios of PCR products, with IgG under normoxia taken as 1. All experiments were repeated in triplicate with similar results. $**P < 0.01$.

4*E*). Additional experiments were performed to assess whether the downregulation of ACE2 by HIF-1α was ANG II dependent. We added telmisartan to hPASMCs overexpressing HIF-1α, and the results demonstrated that the HIF-1α-mediated downregulation of ACE2 is completely blocked by the addition of telmisartan (Fig. 2*B*). These results indicate that accumulated ANG II catalyzed by ACE is a key mediator in the downregulation of ACE2 protein by HIF-1α. Hence, the downregulation of ACE2 by ANG II is an AT_1 receptor-mediated process.

RNA interference-mediated reductions in ACE2 and ACE expression resulted in changes in cell proliferation and migration abilities. RNA interference was effective in reducing ACE2 and ACE protein expression in hPASMCs (Fig. 5, *A* and *D*). Cell counting (Cell Counting Kit-8) and Millicell inserts were used to assess the effects of ACE2 and ACE knockdown on cell proliferation and motility, respectively. In the cell proliferation assay, we found that hPASMCs that were transfected with ACE2-targeted siRNA exhibited significantly higher WST-8 cleavage levels than nontargeted controls under hypoxia (Fig. 5*B*). However, hPASMCs transfected with ACE-targeting siRNA showed lower WST-8 cleavage levels than controls during hypoxia (Fig. 5*E*). In the case of the migration assay, cell numbers translocating across the microporous membranes after treatment with nontargeting or ACE2-targeting siRNA were obtained. As shown in Fig. 5*C*, the number of cells translocating across the membranes during hypoxia was increased in cells that were knockdowns of ACE2. In contrast, the number of cells translocating across the membranes during hypoxia was decreased in the cell knockdowns of ACE (Fig. 5*F*). This study demonstrated that ACE2 could inhibit the proliferation and migration of hPASMCs during hypoxia. On the contrary, ACE could promote the proliferation and migration of hPASMCs during hypoxia.

DISCUSSION

In the present study, the expression profiles of ACE at the mRNA and protein levels were upregulated during hypoxia. However, ACE2 was increased at an early stage and decreased to near baseline levels at the later stages of hypoxia. Our results

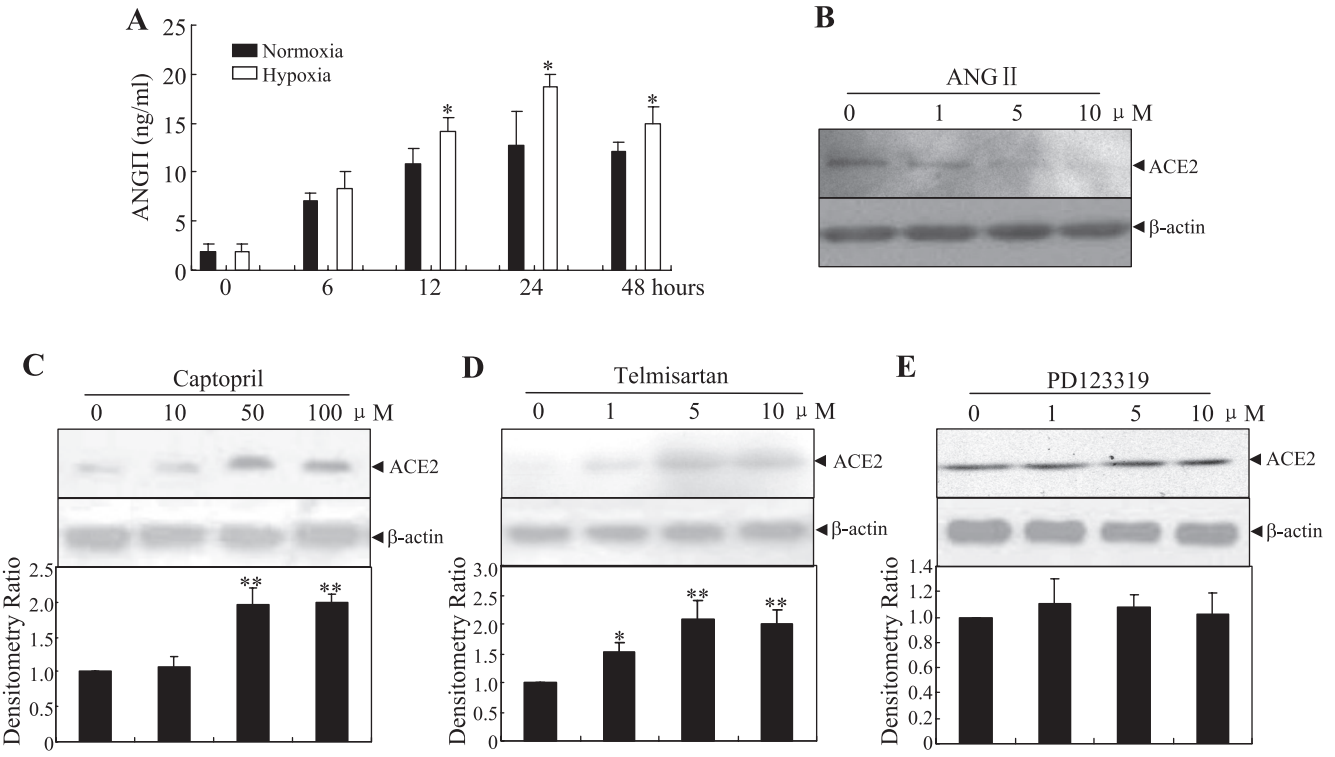

Fig. 4. ANG II downregulated ACE2 in hPASMCs. *A*: ELISA of ANG II concentration in supernatants of hPASMCs exposed to normoxia and hypoxia for 0, 6, 12, 24, and 48 h. Values are means ± SE of 3 independent samples with the same treatment. All experiments were repeated in triplicate with similar results. **P* < 0.05 vs. normoxia at the same time point. *B*: Western blot of ACE2 protein in hPASMCs incubated with 0, 1, 5, or 10 μM ANG II in serum-free medium containing 0.5 mmol/l EDTA for 24 h. *C*: Western blot of ACE2 protein in hPASMCs incubated with 0, 10, 50, and 100 μM captopril for 48 h under 2% O_2. *D* and *E*: Western blot of ACE2 protein in hPASMCs incubated with 0, 1, 5, and 10 μM telmisartan and PD-123319 for 48 h under 2% O_2. Values (means ± SE) in *C–E* represent densitometric ratios of ACE2 protein to β-actin (loading control), with 0 concentration taken as 1. All experiments were repeated in triplicate with similar results. **P* < 0.05 and ***P* < 0.01 vs. 0 concentration.

also indicate that the increase in ACE2 protein is maximal at 24 h. However, the ACE2 mRNA at 24 h is markedly reduced compared with 12 h. The changes in mRNA and protein levels are not entirely in accordance with one another. Nevertheless, perhaps the mRNA level changes more rapidly than the protein level. Thus the peak mRNA levels emerged earlier than the peak protein levels in the present study.

These experiments strongly suggest that hypoxia regulates ACE and ACE2 expression in very different ways. Although the factors controlling the transcriptional regulation of ACE and ACE2 are poorly understood, we examined the putative promoter regions of the two genes that contain potential binding sites for a variety of transcription factors, including HIF-1α. Previous reports have demonstrated numerous pulmonary responses to hypoxia that are regulated by this transcription factor, including decreased K^+ channel activity, PASMC hypertrophy, alterations in Ca^{2+} homeostasis, and pulmonary vascular remodeling (39). In the present study, we used hPASMCs that were transfected for the overexpression or knockdown of HIF-1α to elucidate the role of HIF-1α in the regulation ACE and ACE2. We found that HIF-1α upregulated ACE, yet it downregulated ACE2. Furthermore, overexpression of HIF-1α could transactivate the activity of the ACE promoter, but not ACE2. In addition, the HRE site (as tested in pGL3-ACE-1) appeared to exhibit HIF-1α-mediated regulation. Our results indicate that HIF-1α upregulates ACE expression directly, and ACE is a novel target gene of HIF-1α.

ANG II concentrations are increased in the cell culture medium under hypoxic conditions. Consistent with previous reports (9, 14), we found that ANG II could significantly inhibit ACE2 protein expression in hPASMCs. ACE2 was downregulated by ANG II in a dose-dependent manner, and this response was mediated by the AT_1 receptor. In contrast, ACEI and AT_1 receptor antagonists increased ACE2 protein expression in hPASMCs under hypoxia. Accordingly, these two agents have been shown to attenuate the actions of ANG II or reduce its production and are effective for HPH therapy. Moreover, they could upregulate ACE2, which could increase the formation of the vasodilator antiproliferative peptide ANG-(1–7) and, consequently, reduce the concentration of ANG II (42). Therefore, we propose that ANG II contributes to the downregulation of ACE2 in hPASMCs during hypoxia. The downregulation of ACE2 by ANG II may serve as a mechanism within the lung artery to favor ANG II-mediated responses during HPH. The protective effects of ACEI and AT_1 receptor blockers may be due, at least in part, to increases in ACE2, which shift the angiotensin peptide balance to favor the metabolism of ANG II and lead to the production of ANG-(1–7).

ANG II is mainly derived from ANG I catalyzed by ACE, which was upregulated by HIF-1α in our study. We have demonstrated that HIF-1α can also downregulate ACE2 protein expression. Thus it was necessary to determine whether the downregulation of ACE2 protein expression by HIF-1α was ANG II dependent. To address this issue, we used telm-

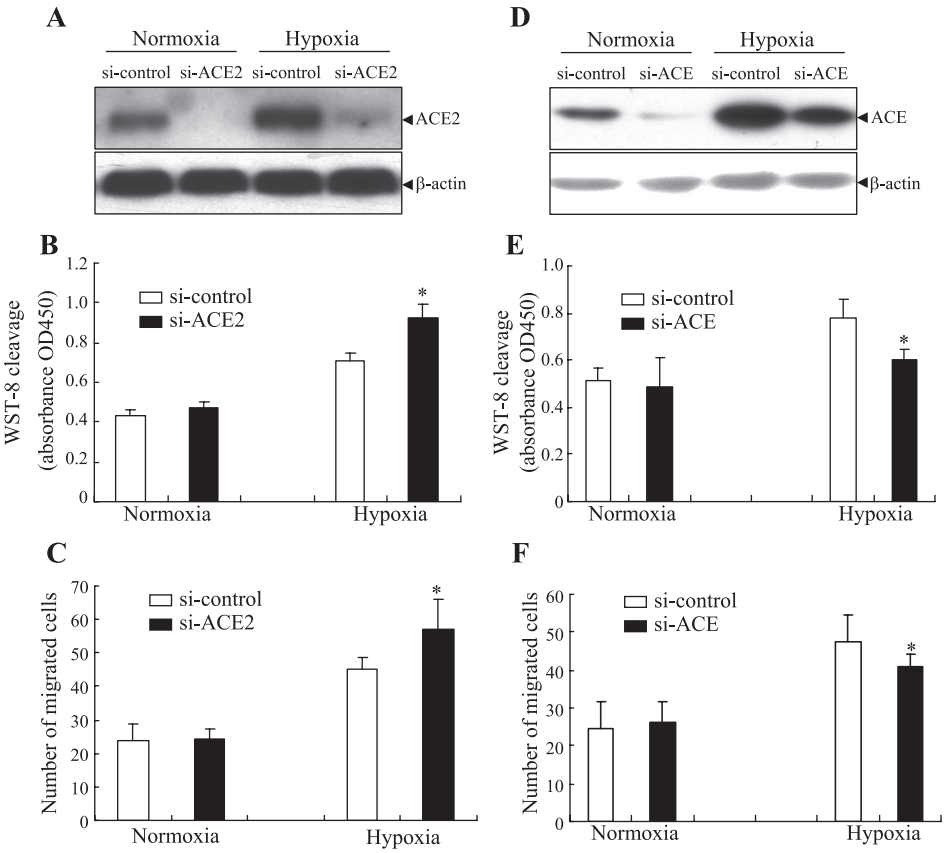

Fig. 5. RNA interference-mediated reduction in ACE and ACE2 expression resulted in altered ability of the cells to proliferate and migrate. hPASMCs were transiently transfected with siRNA targeting ACE2 (*A*) or ACE (*D*). Random siRNA was used as a control siRNA. ACE2 and ACE proteins were detected by Western blot, with β-actin as a loading control. *B* and *E*: effects of ACE2 and ACE knockdown on proliferation of hPASMCs. Cells transfected with ACE2 or ACE targeting siRNA were cultured under hypoxia and normoxia, and WST-8 cleavage in each group was measured as absorbance at 450 nm [optical density at 450 nm (OD_{450})]. Cells transfected with ACE2 (*C*) or ACE (*F*) targeting siRNA were trypsinized and plated into a Millicell insert at 2×10^4 cells per 24-well plate. Number of cells that migrated to the lower membrane surface was counted after 6 h. Values are means ± SE of 3 independent tests. $^*P < 0.05$ vs. hypoxia control.

isartan to block the action of ANG II in HIF-1α-overexpressing cells. Thus we could asses the ability of HIF-1α to alter ACE2 expression under normoxia. We found that overexpression of HIF-1α could not inhibit ACE2 protein expression when an AT_1 receptor antagonist was used. Thus the inhibition of ACE2 protein expression by HIF-1α was completely blocked by telmisartan. These results indicate that HIF-1α downregulation of ACE2 was an indirect effect mediated by ANG II.

Taken together, our data provide evidence that ACE and ACE2 are regulated in two different ways during hypoxia (Fig. 6). Hypoxia initiates cellular responses via HIF-1α or HREs that transactivate an autocrine loop of ACE upregulation, ANG II formation, and subsequent AT_1 receptor activation on hPASMCs to reduce ACE2 protein. Hypoxia can also stimulate ACE2 expression via HIF-1α-independent pathways at the same time. Thus ACE2 is regulated in a bidirectional way during hypoxia, and although hypoxia can upregulate ACE2 expression, it remains unclear which factors contribute to this progress. Hypoxia is a complex physiological phenomenon, and many transcriptional regulators are activated during hypoxia (16, 37). Perhaps, some of these factors are involved in the regulation of ACE2. On the other hand, the regulation of ACE2 mRNA may not be at the level of transcription, and the effect may be due to alterations in mRNA stability under hypoxia. ACE2 decreased to near-baseline levels at the later stages of hypoxia after HIF-1α accumulation, but the upregulation of ACE2 protein by hypoxia may counterbalance the effect of increased ACE during the early stage of hypoxia exposure. ACEI and AT_1 receptor antagonist can upregulate ACE2 under hypoxic conditions by blocking the formation and action of ANG II. These two drugs have been used for HPH patients for many years, but their underlying mechanisms of action are not fully understood. The present results should be helpful in further elucidating the mechanism of action for these HPH treatments.

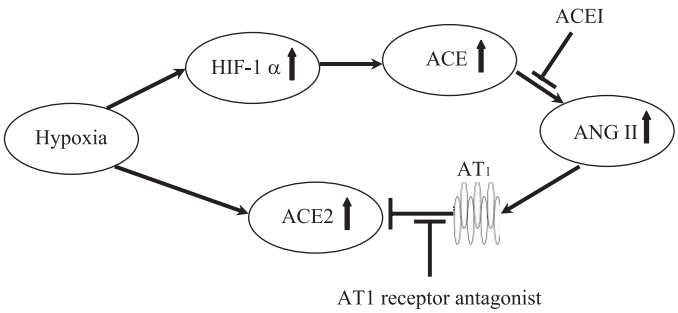

Fig. 6. Scheme of hypoxia-driven regulation of ACE and ACE2. Hypoxia responses via HIF-1α transactivate ACE and lead to ANG II formation. In parallel, hypoxia upregulates ACE2 levels via an HIF-1α-independent pathway. ANG II activates the ANG II type 1 (AT_1) receptor on hPASMCs to reduce ACE2 protein synthesis. ACE inhibitor (ACEI) and AT_1 receptor antagonists can upregulate ACE2 during hypoxia by blocking formation and action of ANG II, respectively.

To further study the function of ACE2 in the pathogenesis of HPH, ACE2 expression was reduced in hPASMCs by RNA interference. We observed that the reduction of ACE2 expression was accompanied by a significantly enhanced ability of cells to proliferate and migrate during hypoxia. Consistent with previous reports (25, 40), we found that a reduction of ACE expression resulted in reduced ability of cells to proliferate and migrate during hypoxia. These results suggest that the function

of ACE2 is the opposite of the function of ACE. Therefore, ACE2 can inhibit the proliferation and migration of hPASMCs during hypoxia and may play a protective role in HPH by blocking the formation of lung vessel remodeling. However, it should be emphasized that HPH is a complicated condition in which many factors are involved. So, further experiments, ideally using animal models, are also required to formally determine whether ACE2 is important in the process of vessel remodeling.

It is also worthy to note that the ratio of ACE to ACE2 is a potential prognostic indicator. Recent research has shown that an imbalance of this ratio leads to many disease states (3, 31). Mice with a targeted disruption of ACE2 develop abnormal heart function that progresses with age (6). In another study, mice lacking ACE2 developed pulmonary congestion and an increased incidence of cardiac death compared with wild-type mice (36). In our study, we found that ACE is persistently upregulated in PASMCs during hypoxia. Although ACE2 increased during the early stages of hypoxia, it decreased to near-baseline levels during the later stages, which led to an imbalance of the ACE-to-ACE2 ratio. Therefore, we presume that long-term hypoxia would also lead to an imbalance of the ACE-to-ACE2 ratio, which may be involved in the pathogenesis of HPH.

In summary, the present study is the first to demonstrate that HIF-1α upregulates ACE directly and ACE is a novel target gene of HIF-1α. We also found that ACE2 is regulated in a bidirectional way during hypoxia. In addition, we observed that a reduction of ACE2 expression by RNA interference could promote the proliferation and migration of hPASMCs during hypoxia. We speculate that ACE2 plays a protective role during the development of HPH, but further studies are necessary to substantiate this conclusion.

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GRANTS

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