

Fig. 5. Protein expression of MAP kinase (p-ERK1/2/ERK 1/2) 14 days after stenting.

A, Photographs of immunoblots of tissues from neointima (neo) and media plus adventitia (Med + A) from normal coronary artery sites (NCA), bare metal stent sites, and imatinib-NP-eluting stent sites.

B, Densitometric analysis of protein expression ($n=4$ each). * $p < 0.05$ versus bare metal stent site.

extracellular space as PLGA is hydrolyzed, resulting in prolonged delivery of imatinib into the stented coronary artery. In this regard, we recently reported that this bioabsorbable polymeric NP-eluting stent system has unique aspects in vascular compatibility and an efficient drug delivery system (stable delivery of NP into the neointima and medial layers until day 28 after deployment of a NP-eluting stent), compared to a dip-coated polymer-eluting stent¹⁹.

In contrast to our present findings, prior studies failed to demonstrate the inhibitory effect of imatinib on in-stent neointima formation in rabbits (oral administration at 10 mg/kg per day for 6 weeks)¹⁶, pigs (600 μ g/stent)¹⁵, and patients (oral administration at 600 mg/body per day for 10 days)¹⁷. The estimated dose of imatinib loaded on our NP-eluting

stent was 21 ± 8 μ g/stent, which is markedly lower than the doses used in these prior studies; therefore, it is likely that the inhibition of in-stent neointima formation is mediated by slower release and longer retention of imatinib at the imatinib-NP-eluting stent site in this porcine coronary artery model. To confirm this hypothesis, we tried to measure local tissue concentrations of imatinib immediately after and 6 hours after deployment of a imatinib-NP-eluting stent by the HPLC system as a preliminary experiment, which was under the limit of detection (1 ng/mL). Local concentrations of imatinib after deployment of imatinib-NP-eluting stent are unclear; however, our present data (Fig. 5) demonstrated that the attenuation of in-stent neointima formation by an imatinib-NP-eluting stent was associated with inhibition of the downstream signal of PDGF receptor (ERK) *in vivo*. Therefore, our present data provide evidence that PDGF receptor signaling blockade by an imatinib-NP-eluting stent may be a promising means for preventing in-stent neointima formation *in vivo*.

An impaired arterial healing process has been demonstrated to be a major histopathological feature in arteries exposed to currently marketed DES in experimental animals^{29, 30} and in humans⁴⁻⁶. In this study, neither FITC- nor imatinib-NP-eluting stents had apparent effects on inflammation, injury, and re-endothelialization in porcine coronary arteries *in vivo*, suggesting that this NP-eluting stent system may not impair the healing process and endothelial regeneration in this model. Collectively, these data on vascular compatibility support the notion that this bioabsorbable PLGA NP-eluting stent system could be applied to human subjects. One limitation of this interpretation is that we did not compare delayed endothelial healing effects between our NP-eluting stent and current DES devices. In this respect, we do not know whether this approach may have an advantage over currently marketed first-generation DES devices. Future studies are needed to prove this point. Another limitation is that this study was performed in normal pigs without pre-existing atherosclerotic coronary lesions, although this porcine coronary artery model is regarded as an appropriate and standard pre-clinical study model³¹. A long-term efficacy study is also needed.

We and others have reported that monocyte-mediated inflammation induced by monocyte chemoattractant protein-1 (MCP-1) plays a central role in the pathogenesis of neointima formation^{24, 32-36} and in atherogenesis^{37, 38}. If imatinib and anti-MCP treatment exert their effects through different pathways, it would be interesting to examine whether combined

blockade of PDGF-R and MCP-1 would have additive inhibitory effects on in-stent stenosis.

In conclusion, blockade of PDGF signaling by imatinib-NP inhibited the proliferation of VSMC with no adverse effects on endothelial cells *in vitro*, and an imatinib-NP-eluting stent attenuated in-stent neointimal formation in porcine coronary arteries *in vivo*. This molecular-targeting NP-eluting stent system may be an innovative platform for delivering agents that target future diagnosis and treatment of atherosclerotic vascular disease.

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Disclosures

Dr. Egashira hold a patent on the results reported in the present study. The remaining authors report no conflicts.

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Renin-Angiotensin System

Inhibition of Prolyl Hydroxylase Domain-Containing Protein Downregulates Vascular Angiotensin II Type 1 Receptor

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See Editorial Commentary, pp 354–355

Abstract—Inhibition of prolyl hydroxylase domain-containing protein (PHD) by hypoxia stabilizes hypoxia-inducible factor 1 and increases the expression of target genes, such as vascular endothelial growth factor. Although the systemic renin-angiotensin system is activated by hypoxia, the role of PHD in the regulation of the renin-angiotensin system remains unknown. We examined the effect of PHD inhibition on the expression of angiotensin II type 1 receptor (AT₁R). Hypoxia, cobalt chloride, and dimethyloxalylglycine, all known to inhibit PHD, reduced AT₁R expression in vascular smooth muscle cells. Knockdown of PHD2, a major isoform of PHDs, by RNA interference also reduced AT₁R expression. Cobalt chloride diminished angiotensin II–induced extracellular signal–regulated kinase phosphorylation. Cobalt chloride decreased AT₁R mRNA through transcriptional and posttranscriptional mechanisms. Oral administration of cobalt chloride (14 mg/kg per day) to C57BL/6J mice receiving angiotensin II infusion (490 ng/kg per minute) for 4 weeks significantly attenuated perivascular fibrosis of the coronary arteries without affecting blood pressure level. These data suggest that PHD inhibition may be beneficial for the treatment of cardiovascular diseases by inhibiting renin-angiotensin system via AT₁R downregulation. (*Hypertension*. 2011;58:386–393.) • **Online Data Supplement**

Key Words: angiotensin II type 1 receptor ■ renin angiotensin system
■ prolyl hydroxylase domain-containing protein ■ vascular remodeling

Renin-angiotensin system (RAS) physiologically and pathophysiologically plays a pivotal role in the cardiovascular system. RAS modulates blood pressure, fluid and electrolyte homeostasis, and neuronal function.¹ RAS is also critical for the pathogenesis of cardiovascular diseases, such as hypertension, atherosclerosis, ischemic heart disease, and congestive heart failure.² Angiotensin II (Ang II), the primary active circulating component of the RAS, is a multifunctional hormone responsible for many cellular processes, such as inflammation, fibrosis, migration, proliferation, hypertrophy, and apoptosis, resulting in the cardiovascular remodeling.³ The effects of Ang II are mediated by Ang II receptors, and 2 distinct isoforms of 7-transmembrane, G protein-coupled receptors have ever been cloned, Ang II type 1 receptor (AT₁R)⁴ and Ang II type 2 receptor.⁵ It is generally accepted that AT₁R mainly contributes to the progression of cardiovascular diseases. Indeed, many large-scale randomized clinical trials showed the beneficial effects of AT₁R antagonists in the treatment of cardiovascular diseases.⁶

Cardiovascular diseases are intimately related to the reduced oxygen concentration state (hypoxia). Cardiomyocytes in ischemic heart disease, peripheral organs in heart failure,

ischemic limb in arteriosclerosis obliterans, and the brain in cerebral infarction are subject to hypoxia. Recently, it was reported that hypoxia activates both circulating and local RAS.^{7,8}

Hypoxia-inducible factor 1 (HIF-1) is a key transcription factor that plays a crucial role in the cellular adaptive response to hypoxia, such as erythrocytosis, glycolysis, and angiogenesis.⁹ HIF-1 is a heterodimeric transcription factor composed of an O₂-regulated subunit HIF-1 α and a constitutively expressed subunit HIF-1 β .¹⁰

Expression of HIF-1 α is negatively regulated by prolyl hydroxylase domain-containing protein (PHD). PHD mediates O₂-dependent hydroxylation of proline residue of HIF-1 α , which triggers subsequent ubiquitination and proteasomal degradation of HIF-1 α .¹¹ The PHD subfamily of 2-oxoglutarate/Fe²⁺-dependent dioxygenases includes 3 isoforms (PHD1, PHD2, and PHD3). PHD2 is expressed more abundantly than other isoforms and is responsible for hypoxic response.¹² Hypoxia inhibits PHD activity, which stabilizes HIF-1 α , resulting in the accumulation of target genes in the nucleus and activation of expression of target genes such as vascular endothelial growth factor (VEGF) and phosphoglycerate kinase 1. In addition to

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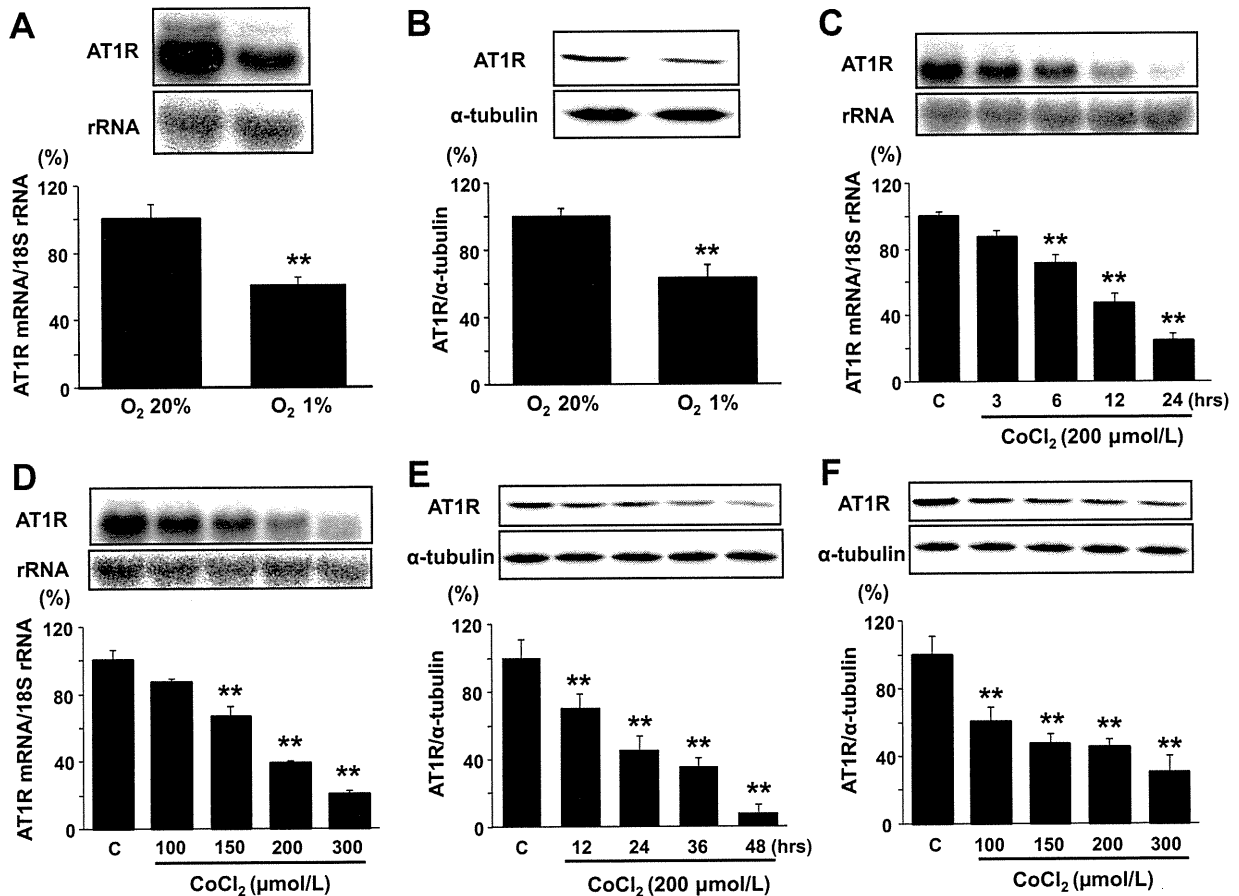


Figure 1. Hypoxia and cobalt chloride (CoCl₂) suppressed angiotensin II type 1 receptor (AT₁R) expression. **A** and **B**, Vascular smooth muscle cells (VSMCs) were incubated for 24 hours under normoxia (O₂ 20%) or hypoxia (O₂ 1%; n=4). **C**, and **E**, VSMCs were incubated with CoCl₂ (200 μmol/L) for varying periods as indicated in the figure (n=4). **D** and **F**, VSMCs were incubated with CoCl₂ at varying concentrations as indicated in the figure for 24 (for mRNA), and 48 hours (for protein), respectively (n=4). **A**, **C**, and **D**, Total RNA was isolated, and the expression of AT₁R mRNA and 18S rRNA was determined by Northern blot analysis. **B**, **E**, and **F**, Expression of AT₁R and α-tubulin protein was detected by Western blot analysis. The ratio of AT₁R/18S rRNA and the ratio of AT₁R/α-tubulin are shown in the bar graph. Values (mean±SEM) are expressed as a percentage of culture at 20% O₂ concentration (100%) or control culture (C). *P<0.05, **P<0.01 vs O₂ 20% or control.

hypoxia, cobalt chloride (CoCl₂) and dimethylxalylglycine (DMOG) also inhibit PHD and stabilize HIF-1. Therefore, they are often referred to hypoxia mimetics.¹³

Although systemic RAS is activated by hypoxia,^{7,8} the role of PHD in the regulation of RAS remains uncertain. In the present study, we examined whether PHD inhibition affects AT₁R expression and the Ang II signaling pathway in the vascular smooth muscle cell (VSMC) and vascular remodeling process.

Materials and Methods

To clarify the effect of PHD inhibition on vascular AT₁R expression, VSMCs from the thoracic aorta of Sprague-Dawley rats, which exclusively express AT₁R, were exposed to hypoxia, incubated with various PHD inhibitors (CoCl₂ and DMOG), or transfected with PHD2-specific small interfering RNA (siRNA). Expression of AT₁R, VEGF, and phosphoglycerate kinase 1 mRNA was examined by Northern blot analysis or quantitative RT-PCR. Expression of AT₁R, PHD2, and HIF-1α protein and extracellular signal-regulated kinase (ERK) phosphorylation was examined by Western blot analysis. Promoter activity was examined by luciferase assay. Cell viability was evaluated by trypan blue assay.

Nine-week-old male C57BL/6J mice were allocated into the following 4 groups: (1) control; (2) Ang II; (3) CoCl₂; and (4) Ang II+CoCl₂. In the Ang II group, 490 ng/kg per minute of Ang II was infused IP. CoCl₂ dissolved in water at 0.01% was administered ad libitum. Body weight, heart rate, and systolic blood pressure were measured. After 4 weeks, the perivascular fibrosis area of the small coronary arteries and the expression of AT₁R protein in the aorta were examined. Renin mRNA of the kidney and angiotensin-converting enzyme and Ang II type 2 receptor mRNA of the aorta were examined by real-time quantitative PCR. Serum angiotensinogen was examined by ELISA.

Statistical analysis was performed using a Student *t* test for the comparison of 2 groups. One-way ANOVA with Fishers post hoc test was used for multiple comparisons. The experiment indicated in Figure 3C was statistically analyzed by a 2-way ANOVA. Detailed information of materials and methods used in this study is available in the online Data Supplement (please see <http://hyper.ahajournals.org>).

Results

PHD Inhibition Suppressed AT₁R Expression in Rat VSMCs

Hypoxia (O₂ 1%; 24 hours) reduced AT₁R mRNA and protein expression (Figure 1A and 1B). Hypoxia increased

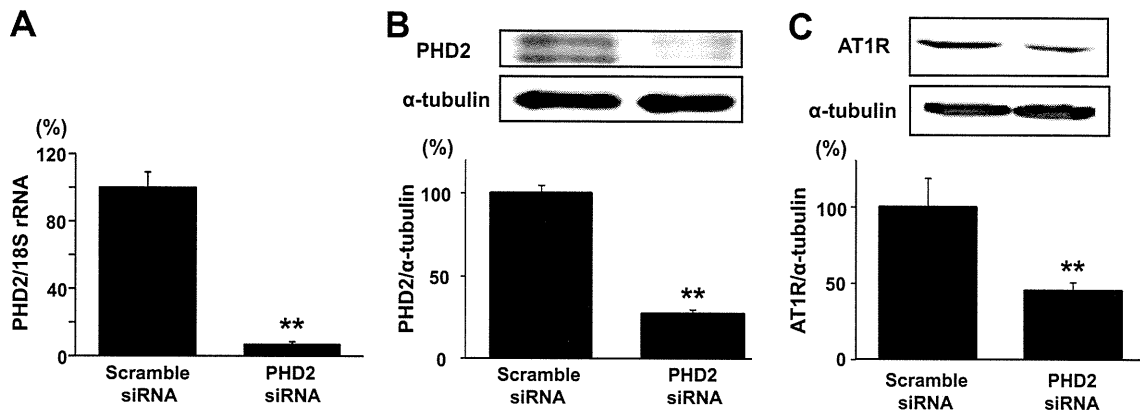


Figure 2. Knockdown of prolyl hydroxylase domain-containing protein (PHD) 2 suppressed angiotensin II type 1 receptor (AT₁R) expression. **A** through **C**, Vascular smooth muscle cells (VSMCs) were transfected with scramble small interfering RNA (siRNA) or PHD2-specific siRNA (n=3). After 72 hours, expression of PHD2 and 18S rRNA mRNA and expression of PHD2, AT₁R, and α-tubulin protein were determined by real-time quantitative PCR and Western blot analysis, respectively. **A**, The ratio of PHD2 mRNA/18S rRNA is shown in the bar graph. **B** and **C**, The ratio of PHD2 or AT₁R to α-tubulin is shown in the bar graph. Values (mean±SEM) are expressed as a percentage of scramble siRNA (100%). **P<0.01 vs scramble siRNA.

expression of nuclear HIF-1α protein and mRNA expression of VEGF, its target gene, was also increased (Figure S1A and S2A, available in the online Data Supplement). Hypoxia (O₂ 1%) increased hypoxia response element (HRE)-driven promoter activity measured by luciferase assay to 208.8±41.1% (Figure S3A). We then examined the effect of CoCl₂, one of the hypoxia mimetics, on AT₁R expression. Incubation of VSMCs with CoCl₂ (200 μmol/L) for varying time periods significantly reduced the expression level of AT₁R mRNA and significantly increased the expression level of VEGF mRNA in a time-dependent manner (Figures 1C and S2B). Incubation of VSMCs with varying concentrations of CoCl₂ resulted in dose-dependent downregulation of AT₁R mRNA and upregulation of VEGF mRNA (Figures 1D and S2C). CoCl₂ also increased nuclear HIF-1α expression and dose-dependently increased HRE-driven promoter activity (Figures S1A and S3B). These data indicate that CoCl₂ increased HIF-1. CoCl₂ induced AT₁R protein downregulation in a time- and dose-dependent manner (Figure 1E and 1F). DMOG, another PHD inhibitor, also downregulated AT₁R protein expression in a time- and dose-dependent manner (Figure S4A and S4B). Treatment of cells with control vehicle did not affect the expression of AT₁R protein at each time point (Figure S5A and S5B). To exclude a possible toxic effect of hypoxia, CoCl₂, and DMOG on VSMCs, we assessed the cell viability by trypan blue staining. Incubation of VSMCs under hypoxia (O₂ 1%), with CoCl₂ (200 μmol/L) or with DMOG (1.0 mmol/L), did not affect the viability of VSMCs (percentage of viable cells: control 90.9±1.1%, hypoxia 93.1±0.1%, CoCl₂ 92.7±1.0%, DMOG 92.5±1.8%; P value not significant; n=4).

To examine whether the suppressive effects of hypoxia, CoCl₂, and DMOG on vascular AT₁R expression are indeed mediated by PHD inhibition, *Phd* gene expression was knocked down by siRNA introduction. There are at least 3 PHD isoforms. PHD2, however, is believed to play a pivotal role, because PHD2 is broadly expressed in essentially all of the tissues examined,^{14–16} and PHD2 knockout results in embryonic lethality.¹⁷ Knockdown of PHD2 (Figure 2A

and 2B) downregulated AT₁R protein expression (Figure 2C), indicating that at least PHD2 is involved in PHD inhibition-induced AT₁R downregulation. PHD2 knockdown by siRNA increased nuclear HIF-1α expression (Figure S1B) and mRNA expression of VEGF and phosphoglycerate kinase 1 (Figure S6), of which expression depends on HIF-1, indicating that downregulation of PHD2 by siRNA inhibited PHD2 activity and activated HIF-1. Because hypoxia mimetics are useful to study dose- and time-dependent effects of PHD inhibition on AT₁R expression and can be used for in vivo study, we used hypoxia mimetics in the following experiments.

CoCl₂ Inhibited AT₁R mRNA Expression Through Transcriptional and Posttranscriptional Mechanisms

The effect of CoCl₂ on AT₁R gene promoter activity was examined. CoCl₂ suppressed the AT₁R gene promoter activity in a dose-dependent manner (Figure 3A). The data suggest that CoCl₂ suppresses AT₁R gene expression at the transcriptional level.

Next, the deletion mutants of AT₁R gene promoter/luciferase fusion DNA were used to locate the specific DNA element responsible for CoCl₂-induced AT₁R suppression. The luciferase activity was suppressed in all of the mutants, suggesting that the most proximal region of the AT₁R gene promoter is critical for downregulation by CoCl₂ (Figure 3B).

The effect of CoCl₂ on AT₁R mRNA stability was examined. CoCl₂ enhanced AT₁R mRNA degradation (Figure 3C). The expression level of AT₁R mRNA was reduced to 42.7±2.8% after 12 hours in vehicle-treated cells. In contrast, CoCl₂ increased the AT₁R mRNA degradation rate (28.4±4.4% at 12 hours). The data suggest that CoCl₂ downregulates AT₁R gene expression through the posttranscriptional mechanism.

Next, we examined whether CoCl₂-induced AT₁R mRNA downregulation requires de novo protein synthesis. Incubation with cycloheximide alone for 24 hours upregulated the

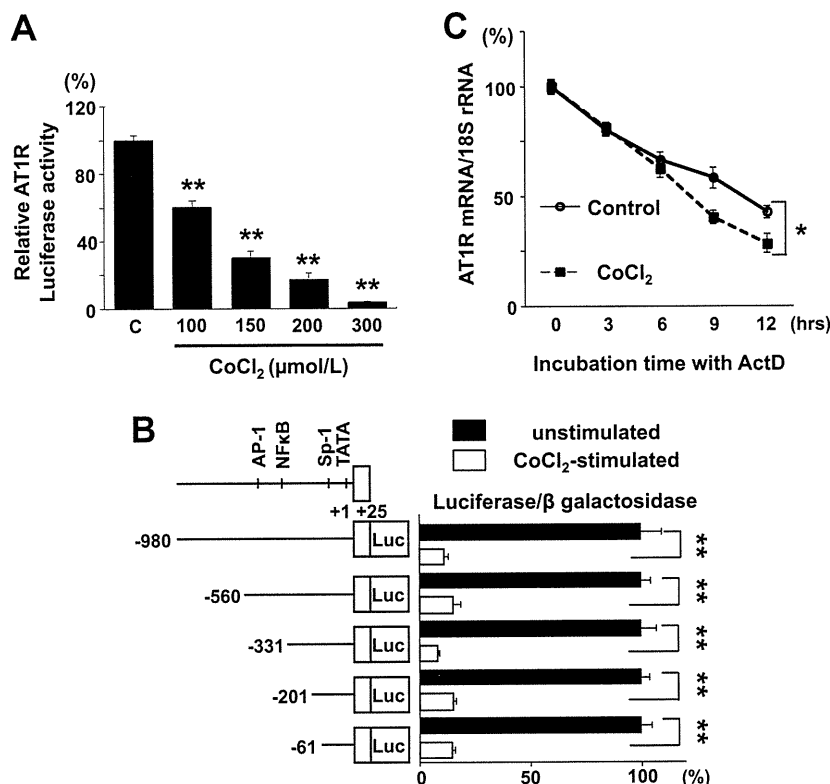


Figure 3. Effect of cobalt chloride (CoCl₂) on angiotensin II type 1 receptor (AT₁R) gene promoter activity and mRNA stability in vascular smooth muscle cells (VSMCs). **A** and **B**, AT₁R gene promoter/luciferase DNA construct and LacZ gene were introduced into VSMCs. **A**, VSMCs were stimulated with CoCl₂ for 24 hours at varying concentrations as indicated in the Figure. Luciferase activity was normalized by β -galactosidase activity. Relative luciferase activity of control (C) was set as 100%. Data are shown as mean \pm SEM (n=3). ***P*<0.01 vs C. **B**, The scheme of deletion mutants of AT₁R gene promoter/luciferase fusion DNA construct is shown in the left panel. Relative AT₁R luciferase activity normalized by β -galactosidase activity corresponding to the left luciferase DNA construct is indicated by the bar graph in the right panel. Relative luciferase activity of unstimulated VSMCs in each construct was set as 100%. ■ and □ indicate the relative luciferase activity of unstimulated and CoCl₂-stimulated VSMCs, respectively. Values (mean \pm SEM) are expressed as a percentage of control culture (unstimulated); n=6; ***P*<0.01. **C**, VSMCs were stimulated with CoCl₂ (200 μ mol/L) for 3 hours. Then actinomycin D (ActD; 5 μ g/mL) was added and incubated for the indicated periods. In the control experiment, only ActD was added to the medium. Northern blot analyses were performed as described in the legend to Figure 1. The ratio of AT₁R mRNA/18S rRNA before the ActD addition in each group was designated 100%. Results are expressed as mean \pm SEM (n=4). **P*<0.05 vs control (2-way ANOVA).

AT₁R mRNA expression. In the presence of cycloheximide, CoCl₂ still significantly suppressed the AT₁R mRNA expression (Figure S7). The data suggest that de novo protein synthesis is not required for CoCl₂-induced AT₁R mRNA downregulation.

CoCl₂ and PHD2 Knockdown Reduced Cellular Response to Ang II

Ang II is known to induce phosphorylation of ERK in VSMCs through AT₁R.¹⁸ VSMCs were pretreated with CoCl₂ (200 μ mol/L) for 30 minutes, 24 hours, and 48 hours and then stimulated with Ang II (100 nmol/L) for 5 minutes. Ang II-induced ERK phosphorylation was not affected by 30 minutes of preincubation with CoCl₂. ERK phosphorylation was significantly reduced after 24 or 48 hours of preincubation with CoCl₂ (Figure 4A). The data suggest that CoCl₂-induced AT₁R downregulation results in the attenuation of the cellular response to Ang II. The lack of suppression of Ang II-induced ERK activation after preincubation with CoCl₂ for 30 minutes suggests that the direct inhibition of

AT₁R signaling by CoCl₂ is unlikely. Phorbol-12-myristate-13-acetate-induced ERK phosphorylation was not affected by preincubation with CoCl₂, confirming that the ERK activation pathway is not inhibited by CoCl₂ (Figure S8). PHD2 knockdown by siRNA also significantly attenuated Ang II-induced ERK phosphorylation (Figure 4B).

PHD Inhibition-Induced AT₁R mRNA Downregulation Was Independent of HIF-1 α

To examine whether HIF-1 α is involved in PHD inhibition-induced AT₁R mRNA downregulation, the effect of the overexpression of constitutively active HIF-1 α on AT₁R mRNA expression was examined. The constitutively active HIF-1 α vector was introduced into VSMCs with either the HRE/luciferase construct or the AT₁R gene promoter/luciferase construct. As a control, an empty vector was used. HRE-driven promoter activity was increased significantly by introduction of constitutively active HIF-1 α , but AT₁R gene promoter activity was not affected (Figure 5). These data

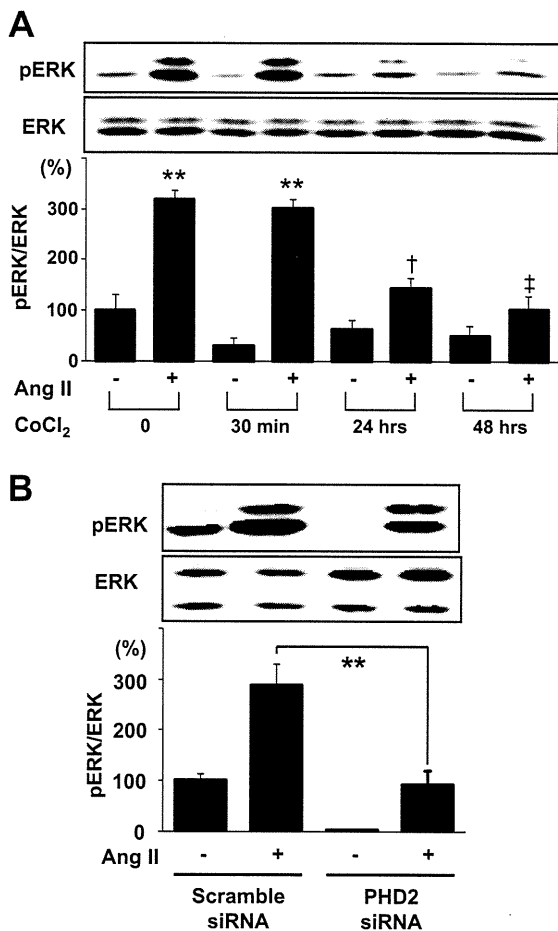


Figure 4. Cobalt chloride (CoCl₂) and prolyl hydroxylase domain-containing protein (PHD) 2 knockdown attenuated angiotensin (Ang) II-induced extracellular signal-regulated kinase (ERK) phosphorylation. **A**, Vascular smooth muscle cells (VSMCs) were pretreated with CoCl₂ (200 μ mol/L) for 30 minutes, 24 hours, and 48 hours and then stimulated with Ang II (100 nmol/L) for 5 minutes (n=4). **B**, VSMCs were transfected with scramble small interfering RNA (siRNA) or PHD2-specific siRNA for 72 hours and then stimulated with Ang II (100 nmol/L) for 5 minutes (n=3). Phospho (p)ERK and ERK protein were detected by Western blot analysis. The ratio of pERK/ERK is shown in the bar graph. Values (mean \pm SEM) are expressed as a percentage of control culture or scramble siRNA (100%). ** P <0.01 vs Ang II (-) at time 0 or scramble siRNA and Ang II (+). † P <0.05, ‡ P <0.01 vs Ang II (+) and CoCl₂ (-) at time 0.

suggest that HIF-1 α is not involved in PHD inhibition-induced AT₁R mRNA downregulation.

CoCl₂ Attenuated the AT₁R Expression in the Aorta and Perivascular Fibrosis of the Coronary Arteries

We examined whether CoCl₂ affects the expression of AT₁R in vivo. Systolic blood pressure was elevated in mice receiving Ang II, and CoCl₂ did not affect systolic blood pressure level (Table). The expression level of AT₁R protein in the aorta was significantly decreased in mice receiving CoCl₂ (Figure 6A). However, expression of renin in the kidney and expression of angiotensin-converting enzyme and Ang II type 2 receptor in the aorta were not affected. Serum concentration

of angiotensinogen was not elevated either (Figure S9). Perivascular fibrosis of the small coronary arteries of the heart was increased by Ang II infusion, which was inhibited by CoCl₂ treatment (Figure 6B and 6C). In contrast, interstitial fibrosis of the kidney was not reduced by CoCl₂ (Figure S10). Medial thickness of the coronary arteries was slightly increased by Ang II. However, the increase was not statistically significant, and we did not observe a significant effect of CoCl₂ (data not shown).

Discussion

In the present study, we demonstrated that hypoxia, CoCl₂, and DMOG suppressed AT₁R expression in cultured VSMCs. PHD2 knockdown by RNA interference also reduced AT₁R expression in VSMCs. The AT₁R expression was specifically suppressed because VEGF mRNA was upregulated. CoCl₂ suppressed AT₁R expression through transcriptional and posttranscriptional mechanisms. CoCl₂ and PHD2 knockdown suppressed Ang II-induced ERK phosphorylation in vitro, and CoCl₂ attenuated Ang II-induced perivascular fibrosis of the small coronary arteries in vivo.

Cobalt has been reported to attenuate the lesion formation in many experimental animal models, including renal ischemic injury,¹⁹ myocardial ischemia-reperfusion injury,²⁰ and cerebral ischemia,²¹ in which activation of RAS is involved in the development of these lesions. Because AT₁R expression was downregulated by CoCl₂, we suppose that suppression of Ang II-induced perivascular fibrosis of the coronary arteries in this study and attenuation of ischemic injury in previous studies by CoCl₂^{19–21} might be ascribed, at least in part, to inhibition of AT₁R expression. It is interesting that Ang II-induced renal interstitial fibrosis was not affected by CoCl₂. This may suggest that renal interstitial fibrosis is more dependent on blood pressure that was not affected by CoCl₂.

We showed a direct inhibitory effect of CoCl₂ on AT₁R expression in VSMCs. However, our study could not exclude the possible indirect effects of CoCl₂ on the Ang II signaling in vivo. Recent studies showed that inhibition of PHD, using different PHD inhibitors, lowered the levels of proinflammatory cytokines, such as tumor necrosis factor- α , in different models.^{22–24} Tumor necrosis factor- α and interleukin 1 β have been reported to transcriptionally enhance the AT₁R gene expression in cardiac fibroblasts.^{25–27} Therefore, reduction of Ang II-induced cytokine production may also be responsible for CoCl₂-induced AT₁R downregulation in vivo.

It is interesting that Ang II alone did not affect AT₁R expression in the aorta, although Ang II is known to suppress AT₁R in VSMCs,²⁸ which is designated homologous downregulation. The lack of homologous downregulation may also be explained by compensation by Ang II-induced cytokines that upregulate AT₁R as described above.^{25–27}

CoCl₂ is known to inhibit PHD activity and increase HIF-1 level.¹³ Several mechanisms for PHD inhibition by CoCl₂ have been suggested.²⁹ The most likely mechanism is that Co²⁺ substitutes for Fe²⁺, the cofactor directly required for PHD catalytic activities.³⁰ Another proposed mechanism is that cobalt produces reactive oxygen species, which inactivate the PHD either directly or indirectly by depleting

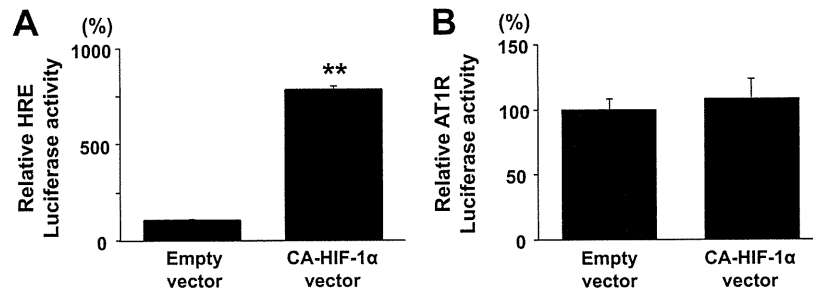


Figure 5. Effect of overexpression of constitutively active (CA) Hypoxia-inducible factor 1 α (HIF-1 α) on angiotensin II type 1 receptor (AT₁R) mRNA expression in vascular smooth muscle cells (VSMCs). **A** and **B**, VSMCs were transfected with CA-HIF-1 α vector and either the hypoxia response element (HRE)/luciferase construct or the AT₁R gene promoter/luciferase construct. HRE-driven promoter and AT₁R gene promoter activities were measured after 48 hours of the transfection, as described in the legend to Figure 3. As a control, an empty vector was used instead of CA-HIF-1 α . Values (mean \pm SEM) are expressed as a percentage of control culture (empty vector, n=3 each). ** P <0.01 vs empty vector.

ascorbate, which is essential for the regeneration of Fe²⁺ from Fe³⁺ after a hydroxylation reaction.³¹

Because PHD is a negative regulator for HIF-1 expression, we expected that increased HIF-1 may be responsible for the AT₁R downregulation. In this study, however, overexpression of the constitutively active HIF-1 α did not affect the AT₁R gene promoter activity, although it strongly activated HRE-dependent transcription as expected. HRE does not exist in the AT₁R gene promoter region, which also suggests that PHD inhibition-induced AT₁R downregulation is independent of HIF-1. Therefore, PHD inhibition may induce a transcription repressor or inhibit the transcription factor that activates AT₁R gene expression. Further study is needed to clarify this point.

The circulating RAS has been reported to be activated by hypoxia.⁷ It was also reported that AT₁R mRNA in VSMCs was upregulated under hypoxia (3% O₂, 24 hours).³² However, in the present study, both AT₁R mRNA and protein were downregulated under hypoxia (1% O₂, 24 hours). Although it is possible that the different oxygen concentration may affect the result, the reason for this discrepancy between the present study and the previous one is not clear at this time. We showed that several hypoxia mimetics and PHD2 downregulation suppressed AT₁R expression. Therefore, it is plausible to assume that PHD inhibition, possibly including hypoxia, negatively regulates AT₁R expression. Our results may also indicate that AT₁R downregulation by PHD inhibition under hypoxia antagonizes the circulating RAS activation.

Table. Body Weight, Heart Rate, and Systolic Blood Pressure of Mice

Groups	BW, g	HR, bpm	SBP, mm Hg
Control	24.9 \pm 0.4	553 \pm 21	102 \pm 1
Ang II	25.8 \pm 0.2	600 \pm 23	128 \pm 3*
CoCl ₂	25.0 \pm 0.4	535 \pm 13	100 \pm 3
Ang II+CoCl ₂	25.5 \pm 0.3	582 \pm 21	123 \pm 2*

BW indicates body weight; HR, heart rate; SBP, systolic blood pressure; CoCl₂, cobalt chloride; Ang II, angiotensin II. BW, HR, and SBP were recorded in mice 4 wk after administration of CoCl₂ and infusion of Ang II. Data are expressed as mean \pm SEM.

* P <0.05 vs control, n=5 to 6 in each group.

The limitation of this study is that we used chemical inhibitor CoCl₂ to inhibit PHD. We cannot completely exclude the possible nonspecific effects. However, CoCl₂ increased nuclear HIF-1 α expression and VEGF mRNA, suggesting that it inhibited PHD and stabilized HIF-1. In addition, hypoxia and DMOG also suppressed AT₁R expression (Figures 1A, 1B, S4A, and S4B). Knockdown of PHD2 also resulted in AT₁R downregulation (Figure 2). Therefore, we suppose that PHD inhibitors suppress AT₁R expression, at least in part, via PHD2 inhibition. However, it is not clear how PHD regulates AT₁R gene expression. Sp1 consensus sequence and TATA box are located in the proximal promoter region of the AT₁R gene. So far, it has never been reported that activity of Sp1 or TATA box binding protein is regulated by hydroxylation. Further study is needed to determine the PHD regulation of AT₁R gene expression.

In the present study, CoCl₂ reduced AT₁R in aorta and diminished Ang II-induced perivascular fibrosis but it did not affect Ang II-induced hypertension. Although the mechanism remains uncertain, it is known that the nondepressor dose of angiotensin receptor blocker is effective for the inhibition of perivascular fibrosis or cardiac hypertrophy induced by pressure-overload.³³ These data suggest that cardioprotection by angiotensin receptor blocker or AT₁R downregulation might be independent of the blood pressure-lowering effect, at least in terms of Ang II-induced fibrosis.

Perspectives

In summary, we demonstrated in the present study that PHD inhibition downregulates AT₁R expression, reduces the cellular response to Ang II, and attenuates the profibrotic effect of Ang II on the coronary arteries. Although further studies are required to determine the detailed molecular mechanism for AT₁R downregulation, PHD inhibition may be beneficial for the treatment of cardiovascular diseases, in which activation of RAS plays a critical role.

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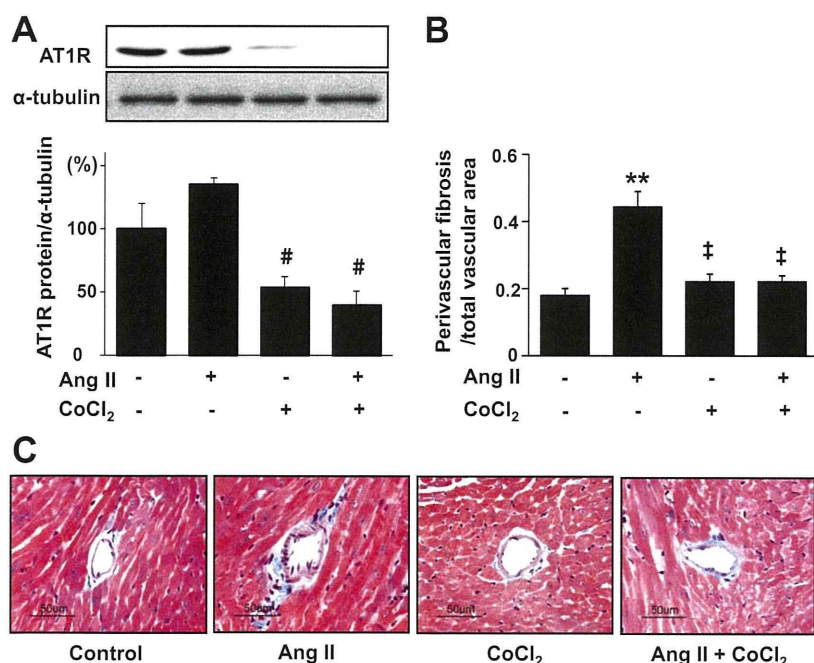


Figure 6. Cobalt chloride (CoCl₂) decreased aortic angiotensin II type 1 receptor (AT₁R) expression and attenuated perivascular fibrosis of the coronary arteries in mice. Angiotensin II (Ang II) was intraperitoneally infused through an osmotic minipump. CoCl₂ was administered ad libitum in drinking water (0.01%). **A**, After 4 weeks of administration, AT₁R and α-tubulin expression in the aorta was determined by Western blot analysis. The ratio of AT₁R/α-tubulin is shown in the bar graph (n=5 to 6, each group). *P*<0.05 vs Ang II (-) and CoCl₂ (-). **B**, Perivascular fibrosis of the coronary arteries was examined. Ang II significantly increased perivascular fibrosis of the coronary arteries. CoCl₂ inhibited the Ang II-induced perivascular fibrosis (n=5 to 6, each group). ***P*<0.01 vs Ang II (-) and CoCl₂ (-). *‡P*<0.01 vs Ang II (+) and CoCl₂ (-). **C**, Representative microphotographs of Masson trichrome-stained mice coronary arteries from each group are shown (n=5 to 6, each group).

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Disclosures

None.

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ONLINE SUPPLEMENT

Inhibition of Prolyl Hydroxylase Domain-containing Protein Downregulates Vascular Angiotensin II Type 1 Receptor

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Supplemental Materials and Methods

Materials

Dulbecco's Modified Eagle Medium was purchased from GIBCO-BRL, Invitrogen Co. (Carlsbad, CA, U.S.A.). Fetal bovine serum was purchased from SAFC Biosciences Inc. (Lenexa, KS, U.S.A.). Bovine serum albumin, cobalt chloride (CoCl₂), phorbol-12-myristate-13-acetate (PMA), actinomycin D (ActD), cycloheximide (CHX), and a monoclonal anti- α -tubulin antibody were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Dimethylxallylglycine (DMOG) was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). [α -³²P] dCTP was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA, U.S.A.). Luciferase assay system was purchased from Promega Co. (Madison, WI, U.S.A.). **Complete Protease Inhibitor Cocktail solution was purchased from Roche Applied Science (Indianapolis, IN, U.S.A.).** A polyclonal antibody against AT1R was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). A monoclonal antibody against phosphorylated ERK (pERK) and a polyclonal antibody against ERK were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). **A polyclonal antibody against HIF-1 α and a monoclonal antibody against PHD2 were purchased from Novus Biologicals, Inc. (Littleton, CO, U.S.A.).** Horseradish peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.). Angiotensin (Ang) II was purchased from Peptide Institute Inc. (Osaka, Japan). Lipofectamine RNAiMAX was purchased from Invitrogen Co. (Carlsbad, CA, U.S.A.). Other chemical reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise stated.

Cell Culture

Vascular smooth muscle cells (VSMCs) were prepared, maintained, and serum-starved as described previously¹ and stimulated as indicated in the text. Our VSMCs do not express detectable level of Ang II type 2 receptor.

Northern Blot Analysis

Northern blot analysis was performed as described previously.¹ **An Eco RI fragment of the third exon of rat AT1a gene cDNA were labeled with ³²P by a Prime It kit (Stratagene/Agilent Technologies, Inc., Santa Clara, CA, U.S.A.) and used as an AT1R cDNA probe.**² The nucleotides from 1363 to 1659 bp of *Vegfa* were used as a vascular endothelial growth factor (VEGF) cDNA probe. The expression level of AT1R or VEGF mRNA was indicated as a ratio of AT1R or VEGF mRNA to 18S rRNA. To analyze the stability of AT1R mRNA, Act D (5 μ g/mL) was added after 3 hours of

stimulation with CoCl_2 (200 $\mu\text{mol/L}$) and cells were harvested at 3, 6, 9, and 12 hours after addition of ActD. The level of AT1R mRNA and 18S rRNA was examined by Northern blot analysis. In a control experiment, only ActD was added. To determine whether CoCl_2 -induced AT1R mRNA downregulation requires de novo protein synthesis, VSMCs were pretreated with or without CHX (10 $\mu\text{g/mL}$) for 30 minutes and incubated in the presence or absence of CoCl_2 (200 $\mu\text{mol/L}$) for 24 hours. Then the level of AT1R mRNA and 18S rRNA was determined by Northern blot analysis.

Measurement of Cell Viability

Confluent VSMCs were serum-deprived for 48 hours and then exposed to hypoxia (O_2 1%) or treated with CoCl_2 (200 $\mu\text{mol/L}$), or DMOG (1mM). Total cell numbers seeded were equivalent and the experiments were performed under the same conditions. After 24 hours of incubation, the attached cells were harvested with trypsin-EDTA solution. Cells in the medium were collected by centrifugation. These cells were stained with 0.4% trypan blue. The number of total and dead cells was counted with a hemocytometer.

Western Blot Analysis

Western blot analysis was performed as described previously.¹ To prepare nuclear protein, cells were suspended in a buffer containing 10 mmol/L HEPES-KOH (pH 7.9), 1.5mmol/L MgCl_2 , 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L CoCl_2 , 1x complete Protease Inhibitor Cocktail solution (Roche Applied Science) and 0.6% Nonidet P-40. After centrifugation (1000 g), nuclear proteins were extracted with a buffer containing 20 mmol/L HEPES-KOH (pH 7.9), 1.5mmol/L MgCl_2 , 420 mmol/L NaCl, 0.5 mmol/L DTT, 0.2 mmol/L CoCl_2 , 25% glycerol, and 1x complete Protease Inhibitor Cocktail solution (Roche Molecular Biochemicals). Nuclear or total cell extracts were cleared by centrifugation and used for Western blotting. Expression level of AT1R, PHD2 and HIF-1 α was indicated as a ratio of AT1R, PHD2 and HIF-1 α to α -tubulin. Activation of ERK was expressed as a ratio of pERK to ERK (which recognizes both phosphorylated and nonphosphorylated forms).

siRNA Transfection and Analysis

VSMCs were plated at subconfluent density and scramble or PHD2-specific siRNAs complexed with Lipofectamine RNAiMAX was transfected into cells on the following day. The concentration of siRNA used was 50 nmol/L. After 72 hours, the expression of PHD2 mRNA and AT1R protein was evaluated by Real-time Quantitative RT-PCR and Western blot analysis, respectively. Rat PHD2 small interfering RNA (sense:

GUG UGA CAU GUA UAU AUU A; antisense: UAA UAU AUA CAU GUC ACA C) was synthesized by Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). The target sequence is GTG TGA CAT GTA TAT ATT A (accession No. : NM_178334). To verify specificity of the observed effect, one set composed of 4 pooled scramble siRNAs (catalog no. D-001206-14-05, Thermo Fisher Scientific Inc.) was used.

Measurement of Transcriptional Activity of AT1R Gene Promoter and hypoxia response element (HRE)-Driven Promoter

AT1a gene promoter fused with luciferase gene was described previously.³ A luciferase construct with 7 copies of HRE in the promoter region was a generous gift from Dr. Masaomi Nangaku (University of Tokyo, Japan).⁴ An expression vector of constitutively active (CA) form of HIF-1 α (CA-HIF-1 α) was kindly provided by Dr. Kiichi Hirota (Kyoto University, Japan). Since CA-HIF-1 α lacks a part of an oxygen-dependent degradation domain, it is not degraded under normoxia.⁵ Two μ g of each luciferase fusion DNA construct and 2 μ g of LacZ gene driven by simian virus 40 promoter-enhancer sequence were introduced into VSMCs by the DEAE-dextran method according to the manufacturer's protocol (Promega Co.). After transfection, the cells were cultured in DMEM with 10% FBS for 24 hours, washed twice with phosphate-buffered saline (PBS), serum-starved and stimulated with CoCl₂ (0–200 μ mol/L) or incubated under normoxia (O₂ 20%) or hypoxia (O₂ 1%) for 24 hours. Then the cells were lysed in 200 μ L of Reporter Lysis Buffer (Promega Co.). One hundred μ L of the lysate was used for luciferase activity assay in a Lumat luminometer (LB9501, Berthold Technologies GmbH & Co., KG, Germany). The assay was started by adding 100 μ L of Luciferase Assay Reagent (E1501, Promega Co.) to cell lysate, and integrated peak luminescence was determined over 57-second window after a 3-second delay. The β -galactosidase activity in the same sample was measured spectrophotometrically and used to normalize the luciferase activity¹.

Animal Experiment

All procedures were approved by Animal Care and Use Committee, Kyushu University and conducted in accordance with the institutional guidelines. Nine-week-old male C57BL/6J mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and fed a normal chow. CoCl₂ was dissolved in water at 0.01% and administered *ad libitum*. The estimated dose of orally-ingested CoCl₂ was 14mg/kg/day. In Ang II group, 490 ng/kg/min of Ang II was infused intraperitoneally via an ALZET osmotic mini-pump (Durect Co., Cupertino, CA, U.S.A.). The following 4 groups were examined: (1) Control, (2) Ang II, (3) CoCl₂, and (4) Ang II + CoCl₂. Heart rate (HR) and systolic blood pressure (SBP) were measured using tail-cuff

method (BP-98A, Softron Co., Tokyo, Japan).

Histological Analysis

After 4 weeks, mice were euthanized under pentobarbital anesthesia. The hearts and kidneys were removed and fixed in 10% neutral buffered formalin solution, embedded in paraffin, and then cut in the microtome. The tissue slices were stained with Masson-trichrome solutions and hematoxylin-eosin, respectively. The sections were scanned using a light microscope (BX50, Olympus Co., Tokyo, Japan). The perivascular fibrosis area of the small coronary arteries and the total vascular area were quantified by an image processing software (ImageJ). The extent of perivascular fibrosis was evaluated by the ratio of the perivascular fibrosis area to the total vascular area.⁶ In each heart, 10 coronary arteries were examined. The interstitial fibrosis of the kidneys were quantified by an image processing software (ImageJ). At least 10 randomly selected cortical tubulointerstitial areas from each sample were evaluated. The fibrotic area or positive staining area of the kidneys was measured as a percentage of the total area of the each image.

Real-time Quantitative RT-PCR (qPCR) Analysis

The aortas were removed, minced into small pieces, and homogenized in ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) on ice, and total RNA was extracted according to the manufacturer's protocol. One µg of total RNA of the aorta or cultured VSMCs in vitro study was reverse-transcribed with ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan). Real-time qPCR analysis was performed using the Power SYBR Green PCR Master Mix (Life Technologies Co., Carlsbad, CA, U.S.A.) and the Applied Biosystems 7500 Real-Time PCR System (Life Technologies Co.) according to the manufacturer's protocol. Expression of AT1R, VEGF, PGK1, Renin, ACE, and AT2R was presented as the relative mRNA level to that of 18S rRNA. The following primers were used: for *Agtr1a*, 5'-GGACACTGCCATGCCATAAC-3' and 5'-TGAGTGCGACTTGGCCTTTG-3'; for *Vegf-a*, 5'-GCACATAGGAGAGATGAGCTTCC-3' and 5'-CTCCGCTCTGAACAAGGCT-3'; for *Pgk1*, 5'-TGGATGAGGTGGTCAAAGCC-3' and 5'-GCACAGCAAGTGGCAGTGTC-3'; for *Ren1*, 5'-CTCCTGGCAGATCACGATGAAG-3' and 5'-GGAGCTCGTAGGAGCCGAGATA-3'; for *Ace*, 5'-TTCCTGCGAACGTGCCATAC-3' and 3'-TTACTGTAGCCCAGCTTCATGG-5'; for *Agtr2*, 5'-CTTGGATGCTCTGACCTGGATG-3' and 3'-AAGCGGTTTCCAACGAAACAATAC-5'; for *18S rRNA*, 5'-ACTCAACACGGGAAACCTCA-3' and 5'-AACCAGACAAATCGCTCCAC-3'.

ELISA for Angiotensinogen

Mouse Total Angiotensinogen Assay Kit-IBL (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) was used. An equal amount of mice serum was added to the 96-well plate, followed by incubation with anti-angiotensinogen antibody for 1 hour, and then samples were incubated with HRP-conjugated anti-IgG antibody for 1 hour. Then samples were subjected to colorimetric reaction and absorbance at 450 nm was read by Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany).

Statistical Analysis

Statistical analysis was performed using a Student's t test for the comparison of two groups. One-way analysis of variance (ANOVA) with Fisher's post hoc test was used for multiple comparisons. The experiment indicated in Figure 3C was statistically analyzed by a two-way ANOVA. Data are shown as mean±SEM. A value of P < 0.05 was considered to be statistically significant.

Supplemental References

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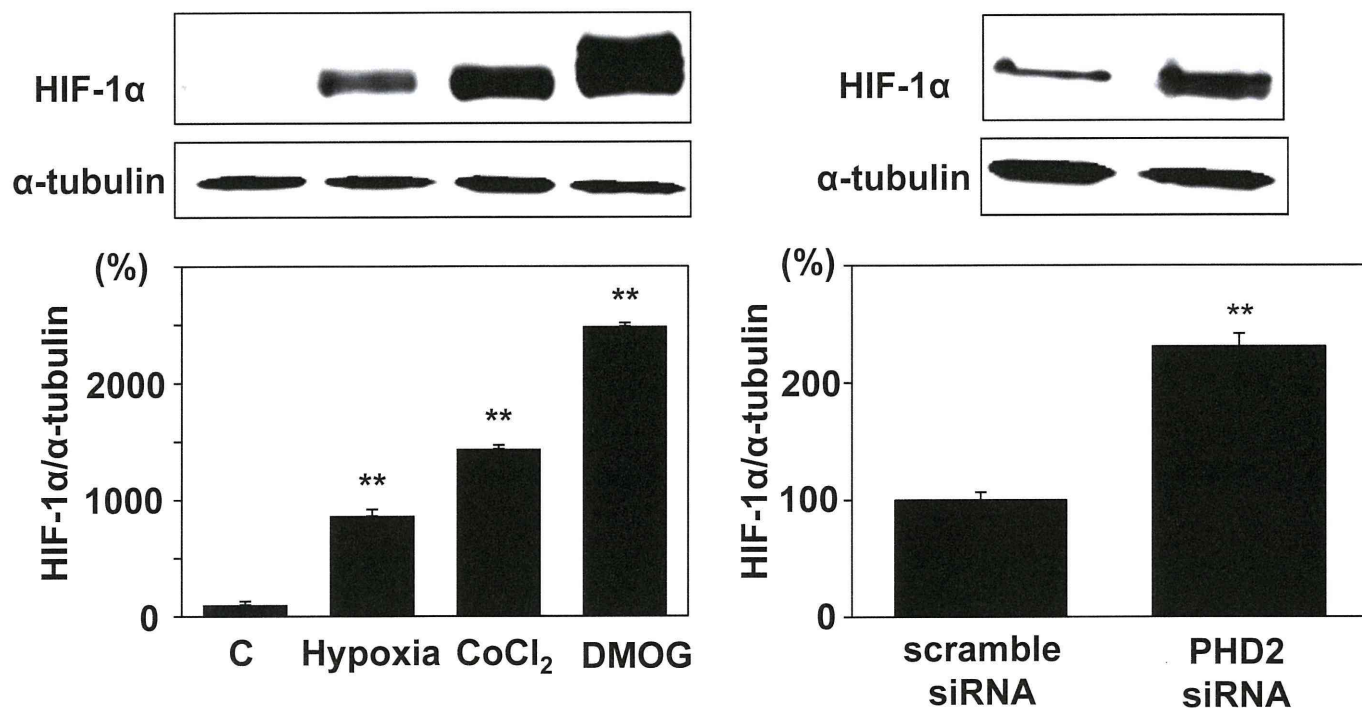


Figure S1 PHD inhibition increased nuclear HIF-1α expression.

(A) VSMCs were incubated under hypoxia (O₂ 1%), with CoCl₂ (200 μmol/L), or DMOG (1.0 mmol/L) for 24 hours (n=4). (B) VSMCs were transfected with scramble siRNA or PHD2-specific siRNA for 72 hours (n=3). The ratio of HIF-1α to α-tubulin is shown in the bar graph. Values (mean±SEM) are expressed as a percentage of control culture (C) or scramble siRNA (100%). **P<0.01 vs control or scramble siRNA.

