

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.). Dimethylxalylglycine (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'-TGGATGAGGTGGTGAAGCC-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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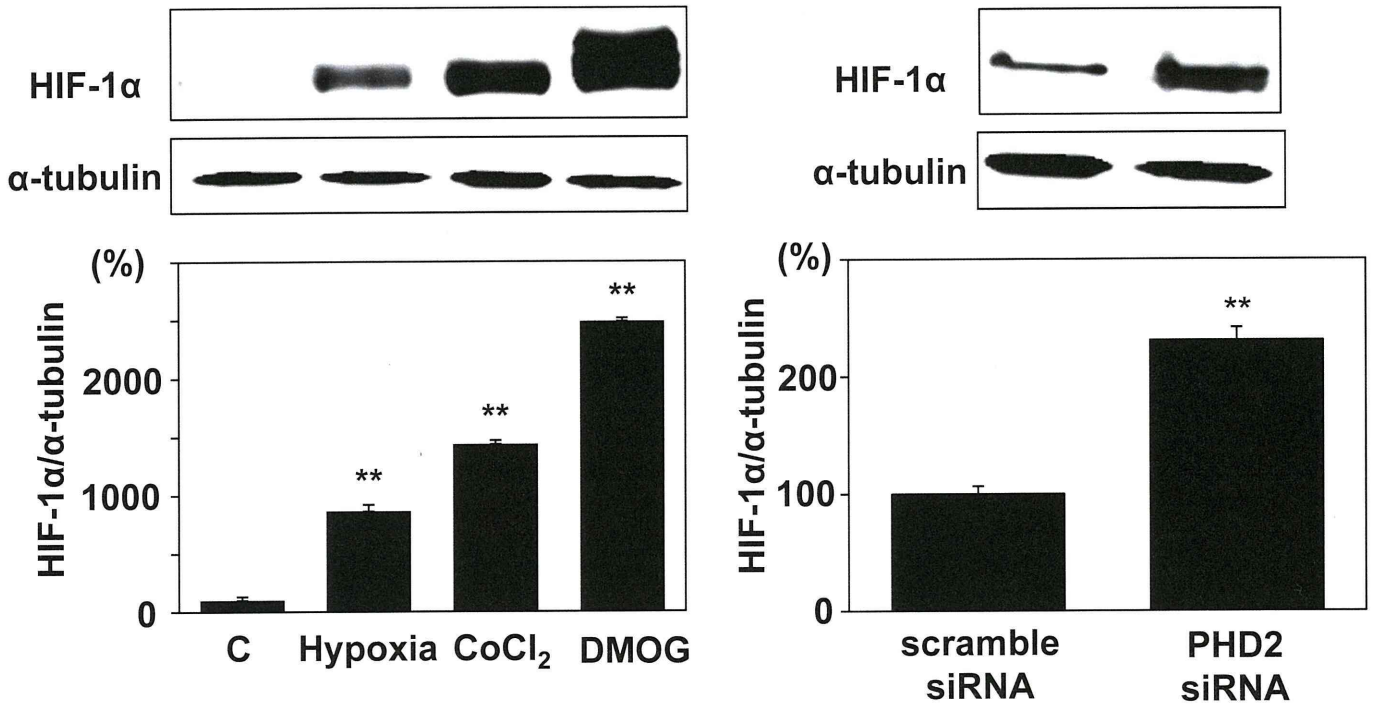
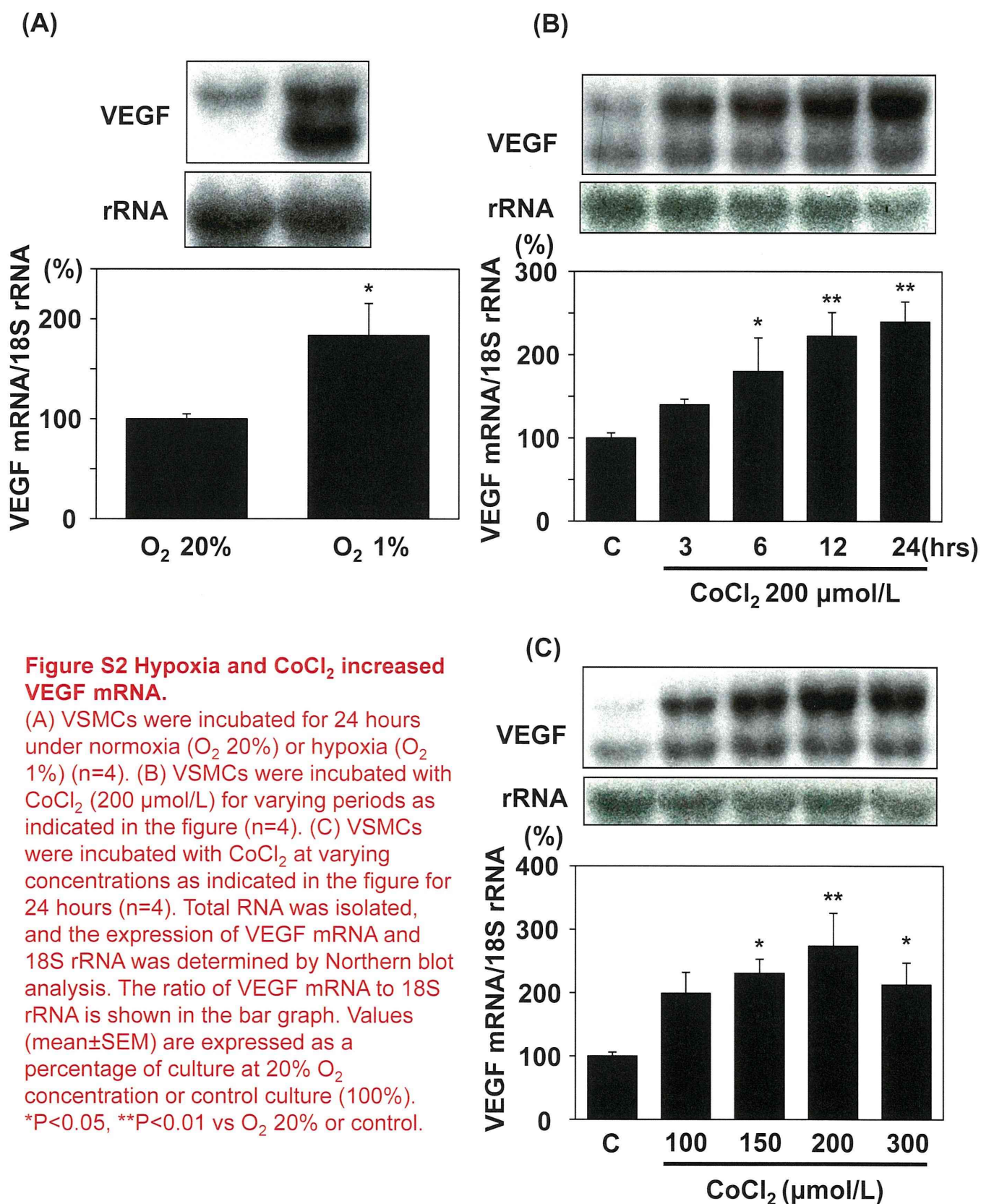


Figure S1 PHD inhibition increased nuclear HIF-1α expression.

(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.



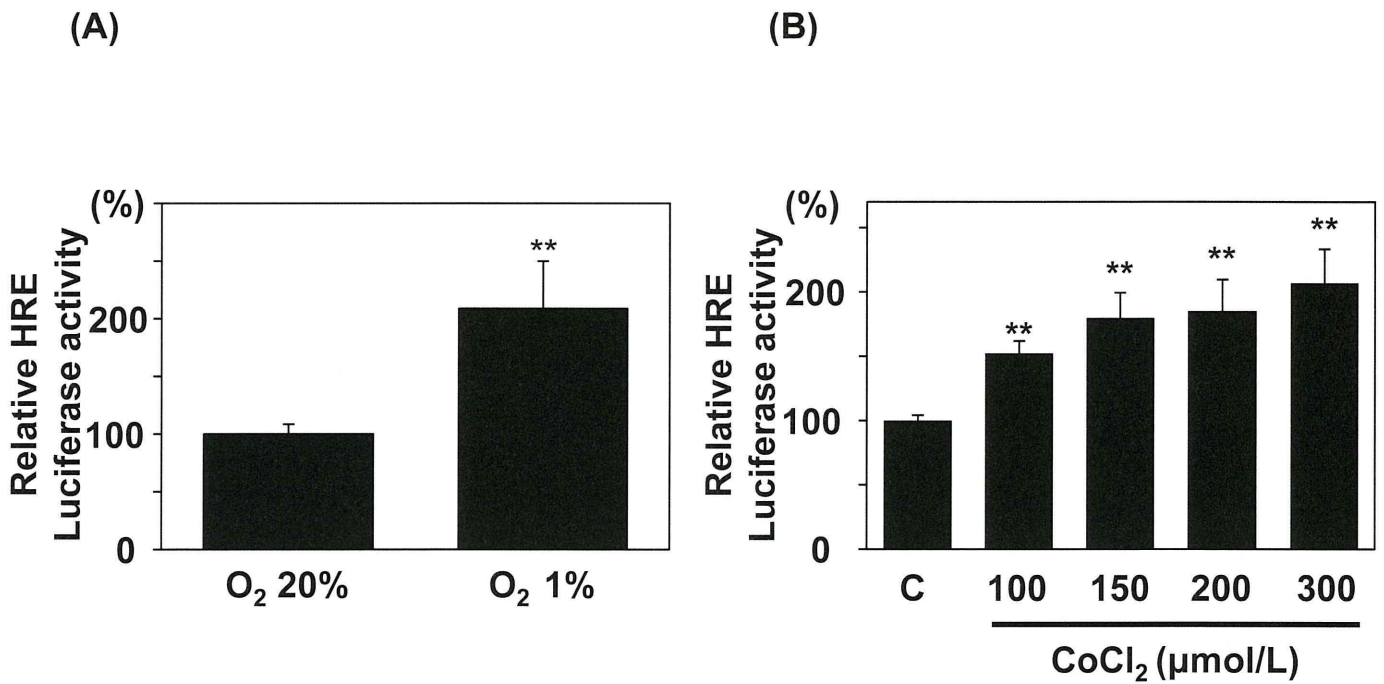


Figure S3. Hypoxia and CoCl₂ increased HRE-driven promoter activity.

HRE luciferase DNA construct and LacZ gene were introduced into VSMCs. VSMCs were incubated for 24 hours (A) under hypoxia (O₂ 1%) or (B) with varying concentrations of CoCl₂ as indicated in the figure. Luciferase activity was normalized by β-galactosidase activity. Relative luciferase activity obtained in VSMC culture at 20% O₂ concentration or control culture (C) was set as 100%. Data are shown as mean±SEM (n=7, n=3, respectively). **P<0.01 vs O₂ 20% or C.

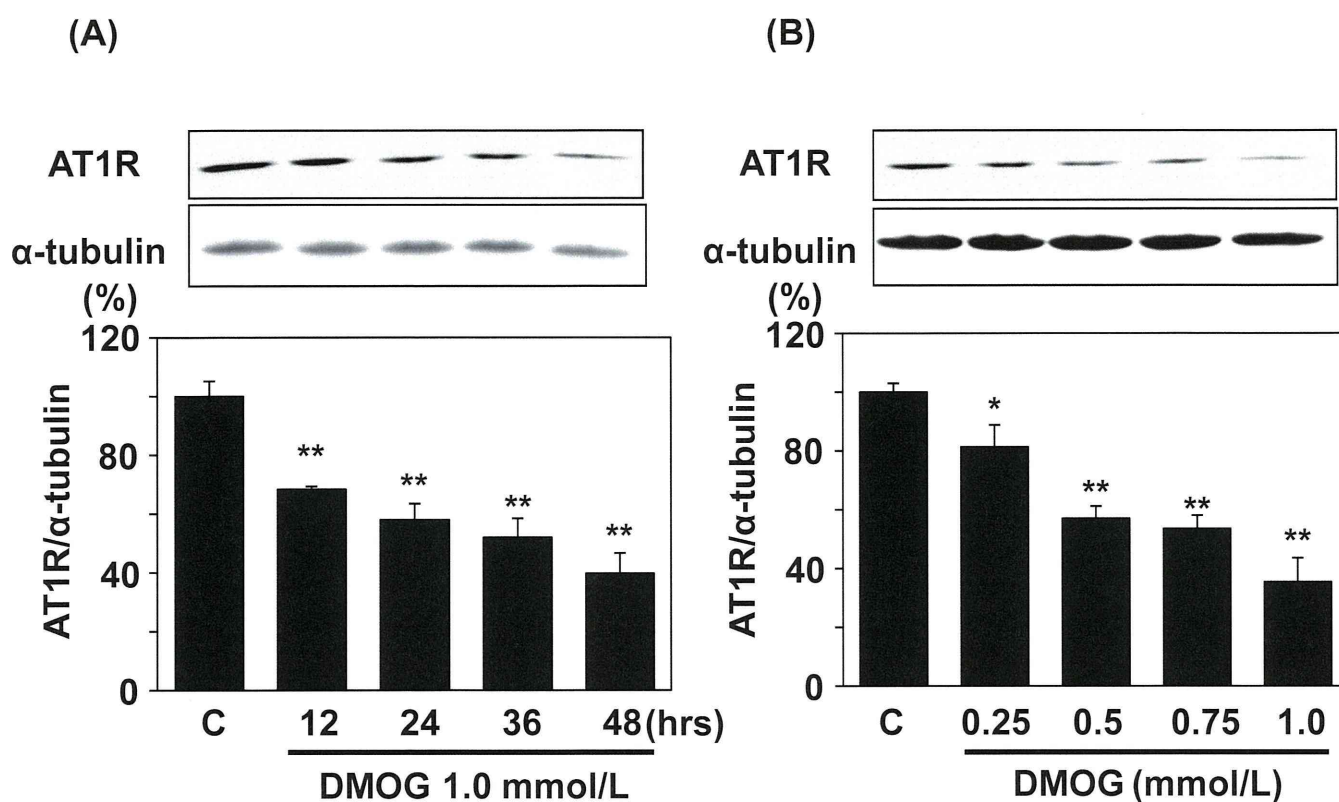


Figure S4. DMOG, another PHD inhibitor, also suppressed AT1R expression.

(A) VSMCs were incubated with DMOG (1.0 mmol/L) for varying periods as indicated in the figure (n=4). (B) VSMCs were incubated with DMOG at varying concentrations as indicated in the figure for 48 hours (n=4). (A), (B) Expression of AT1R and α -tubulin protein was detected by Western blot analysis. The ratio of AT1R to α -tubulin is shown in the bar graph. Values (mean \pm SEM) are expressed as a percentage of control culture (100%). *P<0.05, **P<0.01 vs control (C).

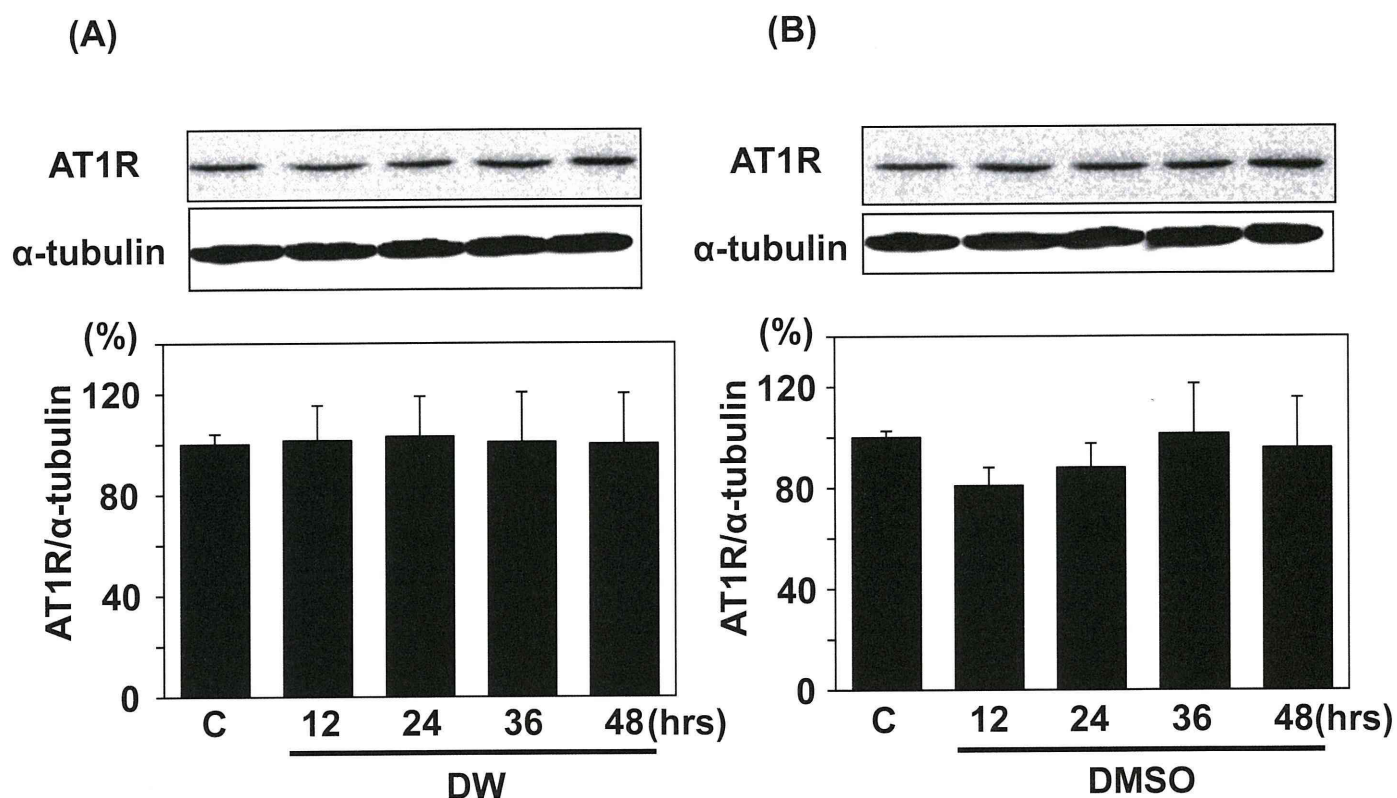


Figure S5. Control vehicle did not affect expression of AT1R protein.

(A) and (B) The same volume of vehicle (distilled water (DW) for CoCl_2 and dimethyl sulfoxide (DMSO) for DMOG) used to dissolve the chemical reagents at maximal doses was added to culture medium and VSMCs were incubated for varying periods as indicated in the figure ($n=4$). Expression of AT1R and α -tubulin protein was detected by Western blot analysis. The ratio of AT1R to α -tubulin is shown in the bar graph. Values (mean \pm SEM) are expressed as a percentage of control culture (100%).

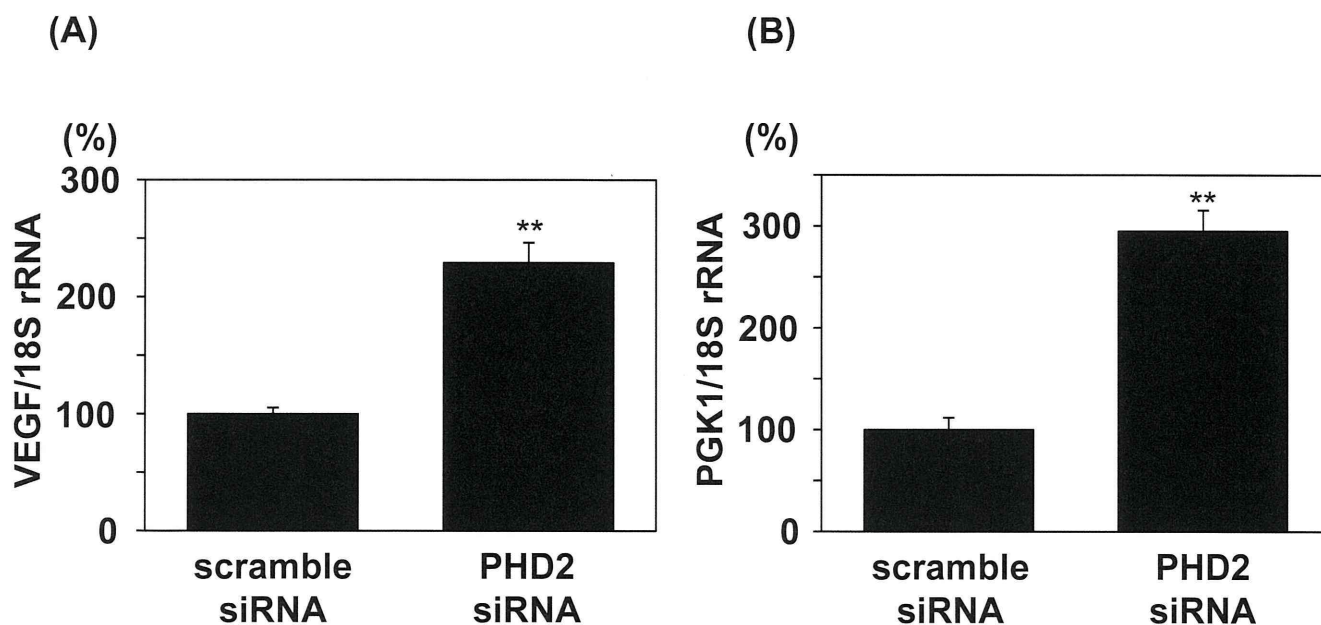


Figure S6. Knockdown of PHD2 mRNA upregulated VEGF and PGK1 mRNA expression. VSMCs were transfected with scramble siRNA or PHD2-specific siRNA. After 72 hours, the expression of VEGF (A) and PGK1 (B) was evaluated by Real-time Quantitative RT-PCR. The ratio of VEGF or PGK1 mRNA to 18S rRNA is shown in the bar graph. (n=3) Values (mean±SEM) are expressed as a percentage of scramble siRNA (100%). **P<0.01 vs scramble siRNA.

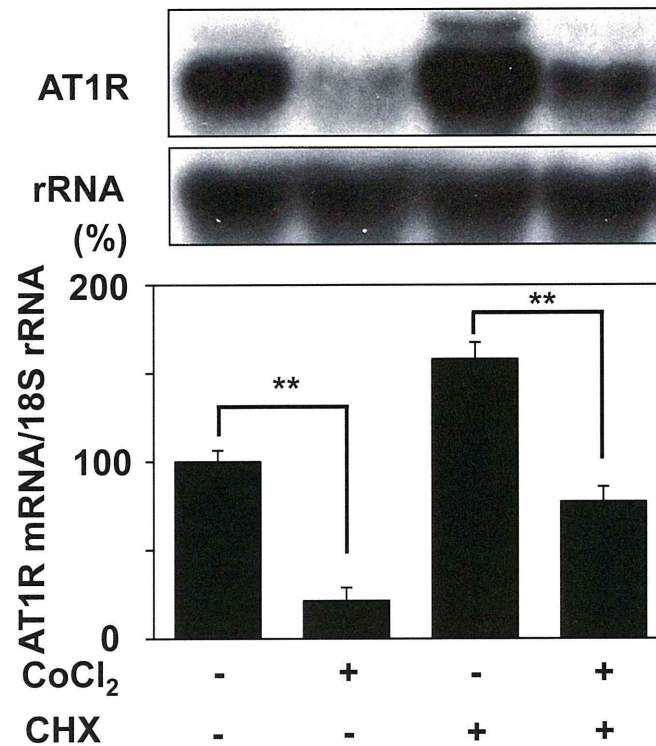


Figure S7. Effect of CHX on CoCl₂-Induced AT1R mRNA downregulation.

VSMCs were preincubated with or without CHX (10 µg/mL) for 30 minutes and then incubated in the presence or absence of CoCl₂ (200 µmol/L) for 24 hours. Expression of AT1R mRNA and 18S rRNA was determined by Northern blot analysis as described in the legend to Figure 1. Values (mean±SEM) are expressed as a percentage of control culture (100%) n=4. **P<0.01.

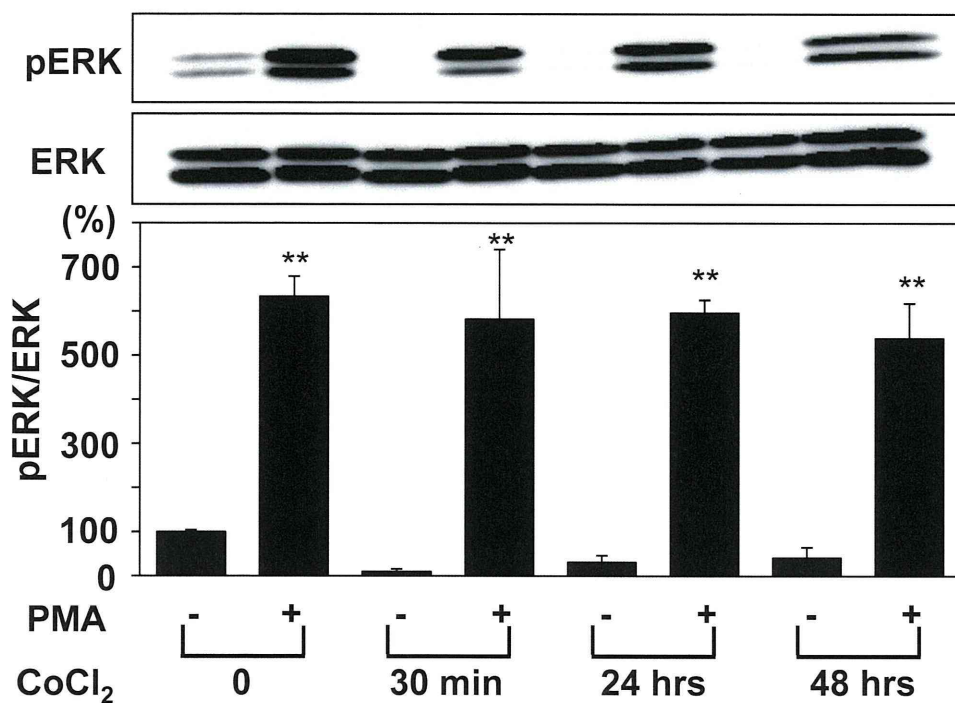


Figure S8. PMA-induced ERK phosphorylation was not affected by CoCl₂.

VSMCs were pretreated with CoCl₂ (200 μmol/L) for 30 minutes, 24 hours, and 48 hours, and then stimulated with or without PMA (100 nmol/L) for 5 minutes. pERK and ERK protein were detected by Western blot analysis. The ratio of pERK to ERK is shown in the bar graph.

Values (mean±SEM) are expressed as a percentage of control culture (100%) n=4. **P<0.01 vs PMA (-) at each time points.

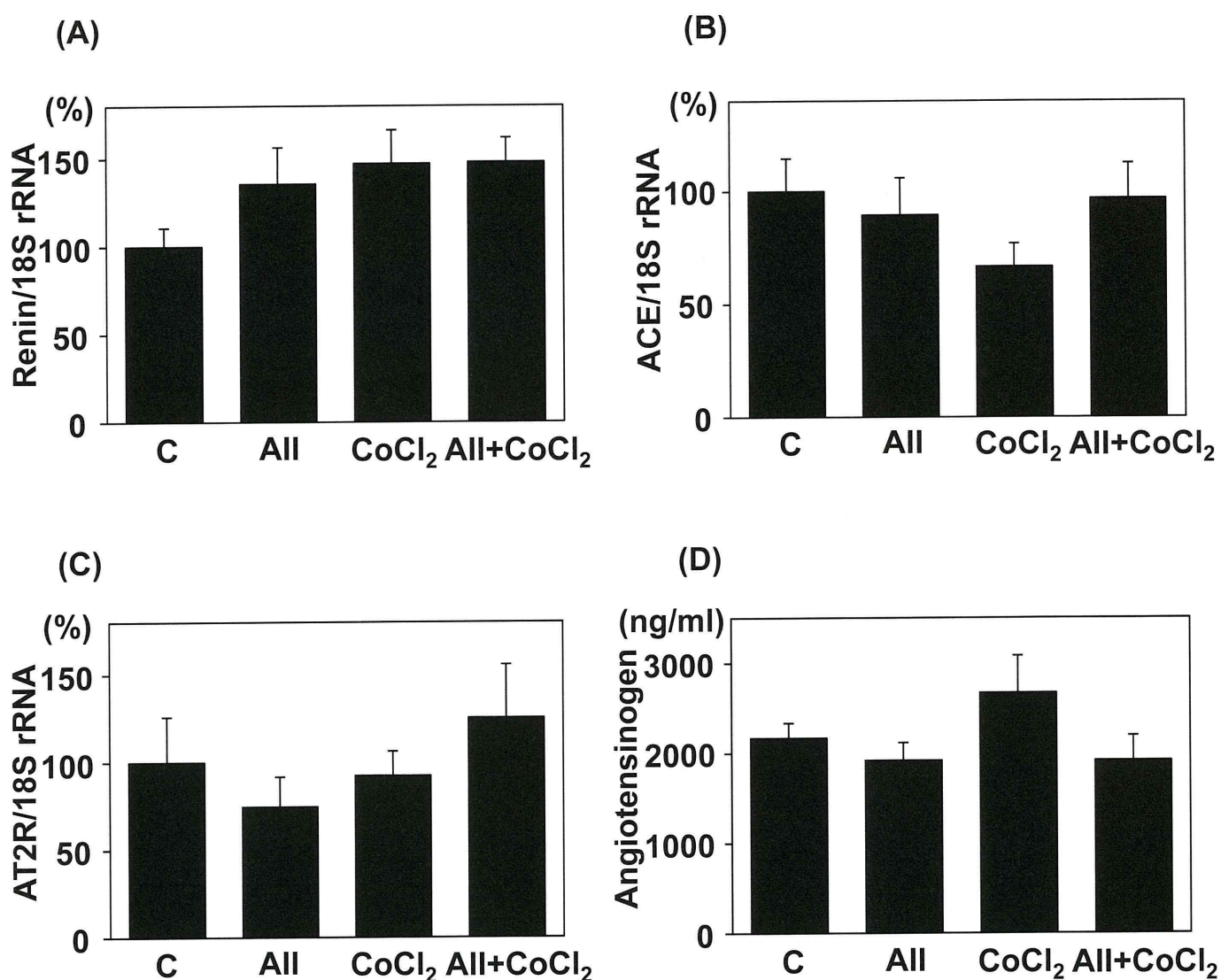
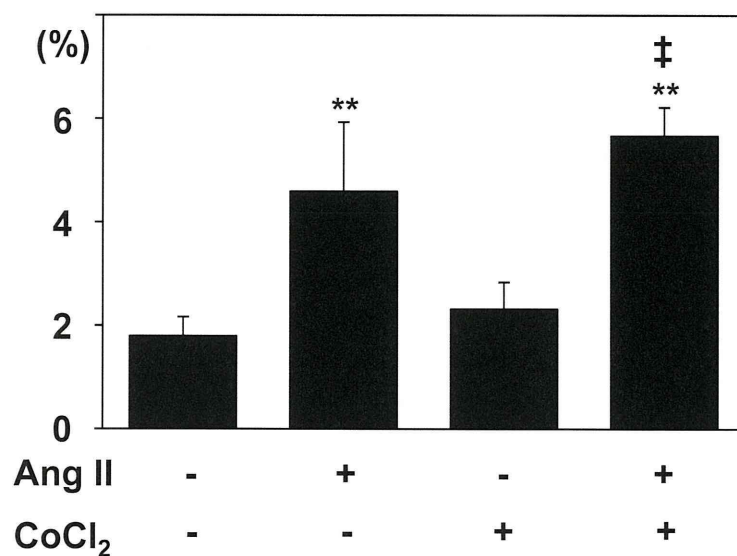


Figure S9. Renin, ACE, and AT2R mRNA of the mice aorta and serum Angiotensinogen. (A), (B) and (C) Expression of Renin mRNA from kidneys and ACE and AT2R mRNA from aortas of mice in each group of the animal experiment was examined by real time quantitative RT PCR. The ratio of Renin, ACE, and AT2R mRNA to 18S is shown in the bar graph. (D) Serum concentration of Angiotensinogen in each group of the animal experiment was examined by ELISA. Values (mean±SEM) are expressed as a percentage of control mice (100%).

(A)



(B)

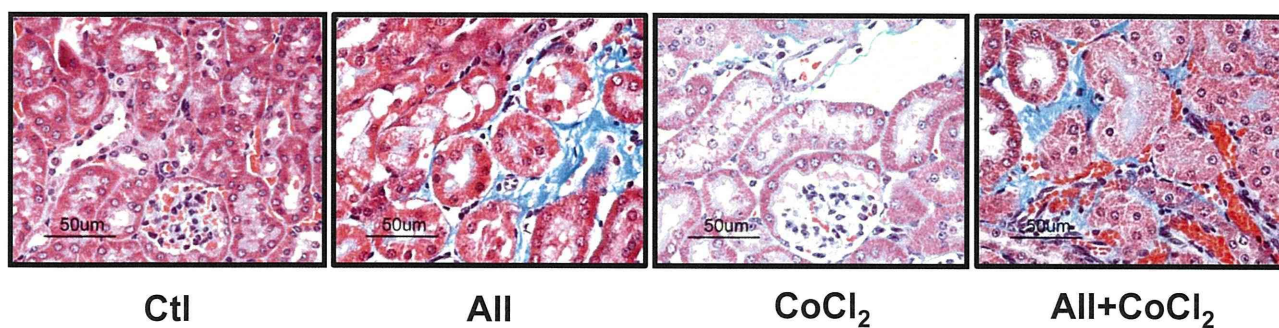


Figure S10. CoCl₂ did not affect All-induced interstitial fibrosis of the kidney in mice. Interstitial fibrosis of the kidneys was examined. Ang II significantly increased interstitial fibrosis of the kidneys. CoCl₂ did not inhibit the Ang II-induced interstitial fibrosis of the kidneys. (n=5–6, each group). **P<0.01 vs Ang II (-) and CoCl₂ (-). ‡P<0.01 vs Ang II (-) and CoCl₂ (+). (B) Representative microphotographs of Masson's Trichrome-stained mice kidneys from each group are shown. (n=5–6, each group).

Brain AT₁ Receptor Activates the Sympathetic Nervous System Through Toll-like Receptor 4 in Mice With Heart Failure

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Abstract: The activation of angiotensin II type 1 receptor (AT₁R) in the brain plays a pivotal role in enhanced sympathetic drive in heart failure (HF). Activation of the AT₁R in the brain produces oxidative stress and inflammation. Toll-like receptor 4 (TLR4) signaling in the brain induces the inflammatory cascade. We hypothesized that sympathoexcitation is mediated by the AT₁R-activated TLR4 in the brainstem in HF. As a model of HF, the left coronary artery was ligated to induce a large myocardial infarction and subsequent chronic heart failure (CHF) in Institute of Cancer Research mice. On day 10 after the surgery, we started intracerebroventricular infusion of losartan (CHF-Los) or vehicle (CHF-Veh) via osmotic minipumps for 14 days. Expression level of the TLR4 in the brainstem was significantly higher in HF mice than in sham mice and significantly lower in CHF-Los mice than in CHF-Veh mice. Urinary norepinephrine excretion was significantly higher in HF mice than in sham mice and was significantly lower in CHF-Los than in CHF-Veh. Chronic intracerebroventricular infusion of angiotensin II increased the expression level of the second messenger of the TLR4. These results suggest that activation of the TLR4 via AT₁R in the brainstem contributes to the sympathoexcitation probably due to the inflammation in the brain of the myocardial infarction-induced HF.

Key Words: heart failure, toll-like receptor, angiotensin II, brain inflammation

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INTRODUCTION

Heart failure (HF) is characterized by the activation of sympathetic nervous system (SNS),^{1,2} and the activation of the SNS contributes to the increased mortality and/or left ventricular (LV) dysfunction.^{2–5} Previous studies have shown that the

activation of the renin–angiotensin system (RAS) in the brain, in particular, the cardiovascular center in the brainstem, evokes the activation of the SNS in the HF and that the increase in oxidative stress is responsible for the brain angiotensin II type 1 receptor (AT₁R)–induced activation of the SNS in the HF.^{6–12} However, the downstream of the AT₁R and oxidative stress in the brain have not been fully determined.

Recent studies have suggested that the inflammation cascade in the brain is one of the important pathways in the activation of the SNS in the cardiovascular diseases.^{13–17} Central inflammation activates the SNS via nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase-dependent mitogen-activated protein kinase (MAPK) signaling.¹⁷ In the brain, after the myocardial infarction (MI), proinflammatory cytokines stimulates the activity of the SNS by inducing cyclooxygenase 2 activity and prostaglandin E₂ production in perivascular macrophages of the blood–brain barrier.¹⁴ Furthermore, the RAS in the brain is a major mechanism for sympathetic hyperactivity, LV remodeling, and LV dysfunction after the MI.¹¹ Previously, we demonstrated that the AT₁R and NAD(P)H oxidase—induced apoptotic pathway through the MAPK in the cardiovascular center of the brainstem contributes to the activation of the SNS.¹⁸ These results suggested that the RAS and inflammatory pathway in the brainstem cause the sympathoexcitation in the MI-induced HF. In the inflammatory cascade, toll-like receptors (TLRs) signaling plays an important role in immune response to pathogens.¹⁹ After the stimulation with an appropriate ligand, the TLRs relay a signal via myeloid differentiation primary response protein 88 (MyD88), a common signal adaptor molecule, and trigger the downstream stimulation of the nuclear factor- κ B (NF- κ B) and the induction of genes that encode proinflammatory cytokines.¹⁹ The inflammatory response is usually associated with the activation of innate immunity, specifically with the TLRs.¹⁹ Among the TLRs, the role of the TLR4 in the brain damage of stroke, neurodegenerative diseases, and alcohol ingestion has been suggested.^{20–23} Furthermore, several previous reports have suggested that the locally produced angiotensin II causes the inflammation via TLR4 pathway.^{24,25} However, the role of the TLR4 in the brain in the regulation of the SNS has not been determined.

Therefore, we hypothesized that the AT₁R in the brain may activate the SNS through in part the inflammation mediated by the TLR4 in the MI-induced HF, and the aim of the present study was to determine whether the inhibition of the

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activation of SNS via blockade of the AT₁R in the brain is through the TLR4 or not. For this purpose, we performed the chronic intracerebroventricular (ICV) infusion of the AT₁R blocker, losartan, to the mice with the MI-induced HF and examined the effects of the AT₁R blocker on the activation of the SNS and LV remodeling with the expression levels of the TLR4, MyD88, and NF- κ B in the brainstem.

MATERIALS AND METHODS

Animal Model and General Procedures

This study was reviewed and approved by the committee on ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male Institute of Cancer Research (ICR) mice (7–8 weeks old) were obtained from SLC Japan (Hamamatsu, Japan). Mice were fed a standard diet, and each strain was divided into 4 groups, sham-operated (Sham), chronic heart failure (CHF), CHF treated with vehicle (CHF-Veh), and CHF treated with losartan (CHF-Los).

Induction of CHF

We experimentally induced MI and subsequent CHF in ICR mice. Under anesthesia with pentobarbital and under mechanical ventilation, the thorax was opened at left intercostals space, and the left coronary artery (LCA) was ligated at 2 to 3 mm from its origin with 8-0 silk. Sham control mice underwent the same surgical procedure without ligation. After the experimental protocols, mice were euthanized with pentobarbital. The brain was removed and immediately frozen on dry ice. Moreover, the lung and heart were removed immediately and weighed.

Chronic ICV Infusion of Losartan or Vehicle to the CHF Mice

At day 10 after the interventions (LCA occlusion and sham operation), mice were instrumented with ICV cannula for chronic infusion of the losartan (10 μ g/h; Sigma-Aldrich, St Louis, MO)²⁶ or artificial cerebrospinal fluid (aCSF) into the lateral ventricles in the CHF-Los and CHF-Veh groups. Under light anesthesia of pentobarbital, mice were placed in a stereotaxic apparatus. The head of the mice were aligned so the lambda–bregma plane was horizontal, and a hole was made 0.4 mm posterior and 1.0 mm lateral to the bregma. The 30-gauge L-shaped cannula was inserted 0 mm below the skull surface and fixed with adhesive. The ICV cannula was connected, via a PE50 catheter, to a mini-osmotic pump implanted subcutaneously (Alzet; model 1002).

Echocardiographic Analysis

Echocardiographic studies were performed under light anesthesia of pentobarbital. An echocardiography system with dynamically focused 7.5-MHz linear array transducer was used. Two-dimensional parasternal short-axis view of the LV was obtained, and LV dimensions were measured.

Evaluation of the Activity of the SNS

As described previously, we measured the 24-hour urinary norepinephrine excretion at 4 weeks after the ligation of the LCA as a parameter of the activity of the SNS.^{16,18,27–29}

Expression Levels of the TLR4 and MyD88 in the Brainstem

Western blot analysis was performed to determine the expression levels of the TLR4 (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) and MyD88 (1:500; Cell Signaling Technology) of the brainstem as described previously.^{16,18,27–29}

Electrophoretic Mobility Shift Assay

The NF- κ B binding activity in the brainstem was measured by electrophoretic mobility shift assay. The brainstem tissue (30–40 mg) was suspended in hypotonic Tris buffer [400 μ L; 10 mM Tris–HCl (pH 7.8), 5 mM MgCl₂, 10 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.3 M sucrose, and 1 mM phenylmethylsulfonyl fluoride] and was homogenated. After the solution had been kept on ice for 15 minutes, 10% Nonidet P40 (20 μ L) was added followed by centrifugation at 8000g for 1 minute. The pellet was suspended in high-salt Tris buffer [100 μ L; 20 mM Tris–HCl (pH 7.8), 5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 320 mM KCl] on ice for 15 minutes. After centrifugation at 13500g for 15 minutes, the supernatant liquid, containing nuclear protein, was collected. Probe for NF- κ B was synthesized as double-stranded oligomers: 5'-AGTTGAGGGGACTTTCCAGGC-3'. This probe was end-labeled with [γ -³²P]-ATP by the activity of T4 polynucleotide kinase (Promega, Madison, WI). For this analysis, 2 μ g of nuclear extract was preincubated for 10 minutes with 2 μ g of poly-(di-dC) and bovine serum albumin in 4 μ L of binding buffer [100 mM Tris–HCl (pH 7.5), 10 mM EDTA, 40% glycerol, 1 M NaCl, and 100 mM DTT]. The probe was then added to the reaction mixture, which was incubated for 20 minutes. Subsequently, each sample was subjected to 5% polyacrylamide gel electrophoresis in buffer. Autoradiography was performed on the dried gel.

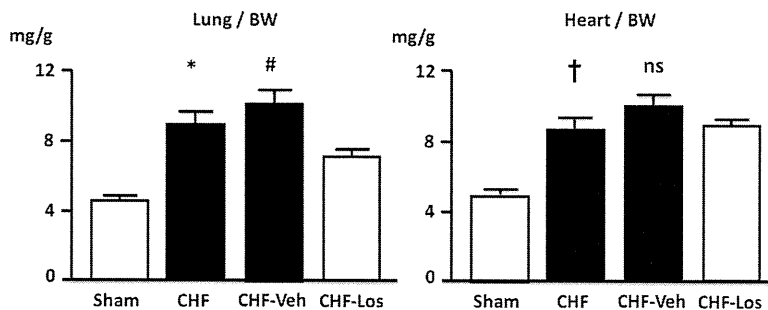
Chronic ICV Infusion of Exogenous Angiotensin II or Vehicle to the Normal Control Mice

To determine whether the brain AT₁R stimulation exogenously activate TLR4 in the brain, thereby increasing the central sympathetic outflow in normal control mice, we performed the chronic ICV infusion of exogenous angiotensin II (Ang II group) or aCSF (Veh group) to the normal male ICR mice without MI. Mice were instrumented with ICV cannula for the chronic infusion of angiotensin II (600 ng·kg⁻¹·min⁻¹; Sigma-Aldrich) or aCSF for 14 days. The dose of angiotensin II was determined according to the previous report in which the ICV of angiotensin II causes the sympathoexcitation in mice.³⁰

Statistical Analysis

All values are expressed as mean \pm standard error of the mean. Comparisons between any 2 mean values were

FIGURE 1. Organ weight of post-MI and sham-operated mice. Left, lung weight/body weight (BW) (**P* < 0.05 vs. Sham group, #*P* < 0.05 vs. CHF-Los group, n = 6 for each group). Right, heart weight/BW (†*P* < 0.05 vs. Sham group, n = 6 for each group). Data are shown as mean ± standard error of the mean.



performed using Bonferroni correction for multiple comparisons. Analysis of variance was used to compare all the parameters in the CHF, Sham, CHF-Veh, and CHF-Los groups. Differences were considered to be statistically significant at a *P* value of <0.05.

RESULTS

Measurement of Body Weight, Organ Weight, and Echocardiographic Data in CHF Mice

Mice developed CHF within 4 weeks after the MI. The weights of lung and heart were significantly higher in the CHF group than in the Sham group (Fig. 1). However, lung weights were significantly lower in the CHF-Los group than in the CHF-Veh group (Fig. 1). The percent fractional shortening was significantly lower, and LV diastolic diameter and LV systolic diameter were significantly higher in the CHF group than in the Sham group (Table 1). However, %FS was significantly higher, and LV diastolic diameter and LV systolic diameter were significantly lower in the CHF-Los group than in the CHF-Veh group (Table 1).

Urinary Norepinephrine Excretion as an Indicator of the Activation of the SNS

Urinary norepinephrine excretion was significantly higher in the CHF group than in the Sham group (Fig. 2). However, urinary norepinephrine excretion was significantly

lower in the CHF-Los group than in the CHF-Veh group (Fig. 2).

Expression Levels of the TLR4 and MyD88 in the Brainstem

The expression levels of the TLR4 and MyD88 in the brainstem were significantly higher in the CHF group than in the Sham group and significantly lower in the CHF-Los group than in the CHF-Veh group (Fig. 3).

NF-κB Binding Activity

Binding activity of the transcription factor NF-κB was significantly higher in the CHF group than in the Sham group and significantly lower in the CHF-Los group than in the CHF-Veh group (Fig. 4).

Chronic ICV Infusion of Exogenous Angiotensin II to the Normal Control Mice

Chronic ICV infusion of exogenous angiotensin II caused the sympathoexcitation (Fig. 5) as described in the previous study.³⁰ The expression level of the TLR4 in the brainstem was not different between the Ang II group and the Veh group (Fig. 6). However, the expression level of the MyD88 in the brainstem was significantly higher in the Ang II group than in the Veh group (Fig. 6).

TABLE 1. Echocardiographic Measurements

	LVDD (mm)	LVDs (mm)	%FS	Anterior Wall (mm)	Posterior Wall (mm)
Sham	3.43 ± 0.19	1.58 ± 0.16	53.7 ± 3.2	0.98 ± 0.07	1.02 ± 0.06
CHF	5.63 ± 0.12*	4.67 ± 0.11*	16.3 ± 0.6*	0.65 ± 0.04*	1.30 ± 0.04*
CHF-Veh	5.85 ± 0.07#	4.82 ± 0.06#	17.7 ± 0.7#	0.55 ± 0.07	1.35 ± 0.03
CHF-Los	5.43 ± 0.06	3.80 ± 0.07	30.8 ± 1.2	0.63 ± 0.05	1.32 ± 0.05

Data are shown as mean ± standard error of the mean.

**P* < 0.05 versus Sham group.

#*P* < 0.05 versus CHF-Los group, n = 6 for each group.

CHF, chronic heart failure induced by ligation of left coronary artery; Los, losartan; LVDD, left ventricular diastolic diameter; LVDs, left ventricular systolic diameter; Sham, sham-operated mice; Veh, vehicle (aCSF); %FS, percent fractional shortening.

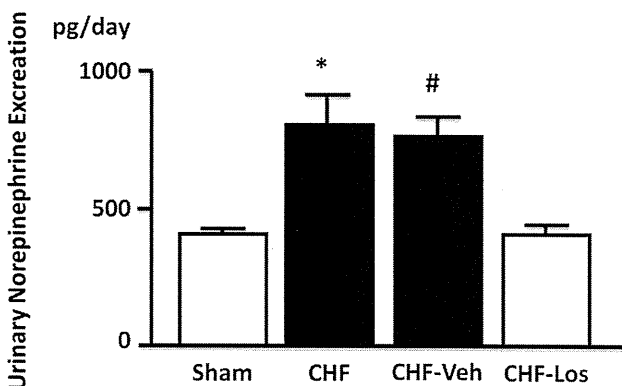


FIGURE 2. Twenty-four-hour urinary norepinephrine excretion as an indicator of activity of the SNS (**P* < 0.05 vs. Sham group; #*P* < 0.05 vs. CHF-Los group, n = 4 for each group).

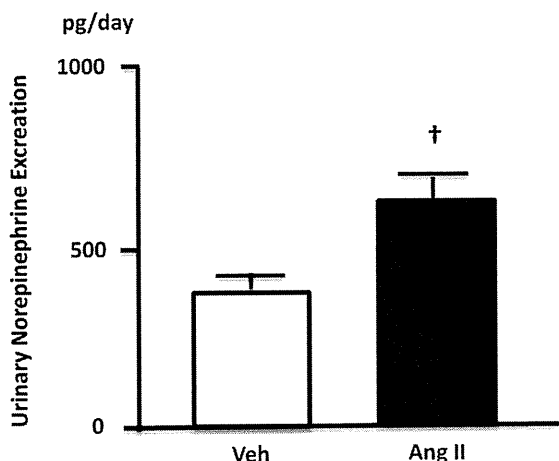


FIGURE 3. A, Western blots demonstrating the expression levels of the TLR4 and MyD88 in the brainstem in CHF group and Sham group (**P* < 0.05 vs. Sham group, *n* = 5, for each group). B, Western blots demonstrating the expression levels of the TLR4 and MyD88 in the brainstem in the CHF-Veh group and CHF-Los group (#*P* < 0.05 vs. CHF-Los group, *n* = 5, for each group).

DISCUSSION

The present study demonstrated that the expression levels of the TLR4 and MyD88, the second messenger of the TLR4, in the brainstem are enhanced in mice with the MI-induced HF and that the ICV infusion of the AT₁R blocker reduces the enhanced activity of the SNS associated with the inhibition of the expression levels of the TLR4, MyD88, and NF-κB in the brainstem. Furthermore, chronic exogenous

infusion of angiotensin II into the brain activates the SNS with the increase in the expression level of the MyD88. Our findings provide a novel insight, suggesting that the AT₁R in the brainstem activates the SNS through in part the inflammation mediated by the TLR4 and MyD88 in mice with the MI-induced HF (Fig. 7).

Previous studies have suggested that the central inflammation activates the SNS¹⁷ and that the RAS in the brain evokes the sympathetic hyperactivity, LV remodeling, and LV dysfunction in the MI-induced HF.^{11,14} These findings suggest that the RAS and inflammation have a close relationship in the brain of the MI-induced HF mice. It has been shown that angiotensin II activates the TLR4^{24,25} and that the AT₁R blocker inhibits the TLR4 in the mesangial and smooth muscle cells.³¹⁻³⁴ In the present study, we demonstrated that the blockade of AT₁R in the brainstem decreases the expression levels of the NF-κB, TLR4, and MyD88 in the MI-induced HF and that the ICV infusion of angiotensin II increases the expression levels of the NF-κB and MyD88. Exogenous angiotensin II infusion does not increase the expression level of the TLR4. However, the expression levels of the MyD88, the downstream of the TLR4, were increased by the exogenous infusion of angiotensin II. These findings suggest that the AT₁R stimulation in the brain by angiotensin II infusion elicits the TLR4–MyD88 activation because the increase in the expression level of the MyD88 strongly indicates the activation of the AT₁R and TLR4. We cannot exclude the possibility that other factors might also contribute to the activation of the TLR4–MyD88 pathway in the MI-induced HF. Another possibility is that exogenous angiotensin II infusion into the brain for 2 weeks might be insufficient for increasing the expression level of the TLR4. However, our findings support the concept that the AT₁R-induced

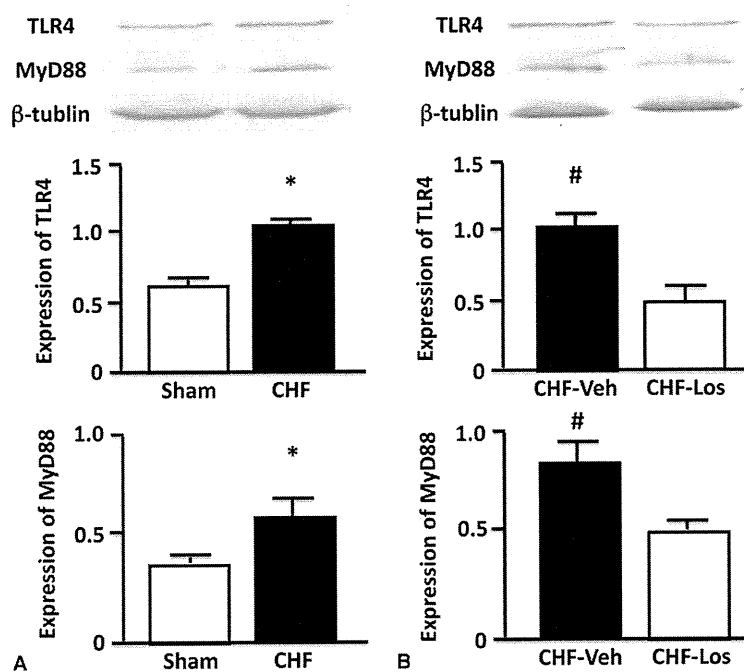


FIGURE 4. Electrophoretic mobility shift assay demonstrating the NF-κB binding activity in the brainstem in each group.