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Expression and actions of HIF prolyl-4-hydroxylase in the rat kidneys

Ningjun Li, Fan Yi, Christina M. Sundy, Li Chen, Molly L. Hilliker, Dustin K. Donley, Daniel B. Muldoon, and Pin-Lan Li

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia

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Li N, Yi F, Sundy CM, Chen L, Hilliker ML, Donley DK, Muldoon DB, Li P-L. Expression and actions of HIF prolyl-4-hydroxylase in the rat kidneys. *Am J Physiol Renal Physiol* 292: F207–F216, 2007. First published August 1, 2006; doi:10.1152/ajprenal.00457.2005.—Hypoxia inducible factor (HIF) prolyl-4-hydroxylase domain-containing proteins (PHDs) promote the degradation of HIF-1α. Because HIF-1α is highly expressed in the renal medulla and HIF-1α-targeted genes such as nitric oxide synthase, cyclooxygenase, and heme oxygenase are important in the regulation of renal medullary function, we hypothesized that PHD regulates HIF-1α levels in the renal medulla and, thereby, participates in the control of renal Na^+ excretion. Using real-time RT-PCR, Western blot, and immunohistochemical analyses, we have demonstrated that all three isoforms of PHD, PHD1, PHD2, and PHD3, are expressed in the kidneys and that PHD2 is the most abundant isoform. Regionally, all PHDs exhibited much higher levels in renal medulla than cortex. A furosemide-induced increase in renal medullary tissue P_{O_2} significantly decreased PHD levels in renal medulla, whereas hypoxia significantly increased mRNA levels of PHDs in cultured renal medullary interstitial cells, indicating that O_2 regulates PHDs. Functionally, the PHD inhibitor L-mimosine (L-Mim, 50 $mg \cdot kg^{-1} \cdot day^{-1}$ ip for 2 wk) substantially upregulated HIF-1α expression in the kidneys, especially in the renal medulla, and remarkably enhanced (by >80%) the natriuretic response to renal perfusion pressure in Sprague-Dawley rats. Inhibition of HIF transcriptional activity by renal medullary transfection of HIF-1α decoy oligodeoxynucleotides attenuated L-Mim-induced enhancement of pressure natriuresis, which confirmed that HIF-1α mediated the effect of L-Mim. These results indicate that highly expressed PHDs in the renal medulla make an important contribution to the control of renal Na^+ excretion through regulation of HIF-1α and its targeted genes.

fluid homeostasis; anoxia; natriuretic factor; gene transcription; renal tubules; renal hemodynamics

IT IS WELL KNOWN that P_{O_2} is much lower in the renal medulla than in the renal cortex because of relative "underperfusion," the countercurrent O_2 diffusion between descending and ascending vasa recta, and the high metabolic ion transport activity of the thick ascending limb of Henle in the renal medullary region (6, 10). To adapt to this low-P_{O_2} milieu, the renal medulla has naturally developed various mechanisms to protect the cells from ischemic and hypoxic injury (5, 6, 15) and to ensure that the cells in this kidney region function normally in a hypoxic environment (6, 10). Increase in tissue blood perfusion to facilitate the O_2 supply and inhibition of tubular metabolic activity to decrease O_2 demands (5, 6, 15, 53) are the common outcomes of various adaptive actions induced by different renal medullary factors such as nitric oxide (NO), prostaglandins, and heme oxygenase (HO) products. Hypoxia inducible factor (HIF)-1α, the master regulator of adaptation to hypoxia, has been shown to activate gene transcription of many O_2-sensitive genes such as NO synthase (NOS), cyclooxygenase (COX), HO, and vascular endothelial growth factor (21, 25, 26, 32, 38, 45, 50, 53). The participation of the products controlled by these genes is important in the adaptation of the renal medulla to hypoxic challenges (53).

We recently reported that HIF-1α is abundantly expressed in renal medullary tissue (54). However, it remains unknown how the renal medullary HIF-1α levels are regulated in response to low P_{O_2}. Recent studies have indicated that HIF prolyl-4-hydroxylase activities initiate the degradation of HIF-1α by promoting the O_2-dependent proline hydroxylation of HIF-1α (7, 23, 24). Under normoxic conditions and with O_2 as a cofactor, HIF prolyl-4-hydroxylase catalyzes site-specific proline hydroxylation of HIF-1α, which allows recognition by the von Hippel-Lindau tumor suppressor protein, a component of an E3 ubiquitin ligase complex, which targets these subunits for degradation by the ubiquitin-proteasome pathway. Under hypoxic conditions, this hydroxylation is inhibited, allowing HIF-1α to escape von Hippel-Lindau tumor suppressor protein-mediated recognition and, consequently, proteasome degradation. Three HIF prolyl-4-hydroxylase domain-containing (PHD) proteins including PHD1, PHD2 and PHD3 have recently been identified (7, 14, 23), and they exert important regulatory actions on tissue levels of HIF-1α and its target genes (37, 49). Little is known regarding expressions and actions of PHDs in the kidneys. Whether this prolyl hydroxylation of HIF-1α participates in the control of renal function and arterial blood pressure is also unknown.

Because HIF-1α transcriptionally regulates several enzymes such as NOS, COX-2, and HO-1 (21, 25, 26, 32, 38, 50), which produce antihypertensive factors in the kidneys, the regulation of HIF-1α by PHD may contribute to the control of renal function and blood pressure. To test this hypothesis, the present study was designed, first, to characterize the expression of PHDs in the kidneys with the use of real-time RT-PCR, Western blot analyses, and immunohistochemistry and, then, to determine the regulation of HIF-1α by PHDs with use of the inhibitors of PHD. We also examined the effect of inhibition of PHD activities on pressure natriuresis. Our results provide evidence that PHDs are highly expressed in the renal medulla and that these medullary PHDs exert a tonic regulatory action on the HIF-1α levels, thereby participating in the control of pressure natriuresis and renal function.

Address for reprint requests and other correspondence: N. Li, Dept. of Pharmacology & Toxicology, Medical College of Virginia Campus, Virginia Commonwealth Univ., PO Box 980613, Richmond, VA 23298 (e-mail: nli@vcu.edu).

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MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Harlan Sprague Dawley, Madison, WI; 250–300 g body wt) were maintained on a standard pellet diet (Purina Mills) with free access to water in the Animal Resource Center of Virginia Commonwealth University. All animal procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

RNA extraction and quantitative RT-PCR analysis of the mRNA levels of PHD and HO-1. Total RNA from renal cortical and medullary tissues was extracted using TRIzol solution (Life Technologies, Rockville, MD) and then reverse transcribed with a cDNA synthesis kit (Bio-Rad, Hercules, CA). The RT products were amplified using real-time quantitative PCR kits. For the analyses of PHD mRNA levels, a SYBR green real-time quantitative PCR kit (Bio-Rad) was used. A primer design computer program (Beacon Designer, Bio-Rad) based on sequences of rat PHD mRNA and 18S rRNA was used to design primers: GCTGCTGCGTTGGTTAC (sense) and GCCTCCTGGTTCTCTTG (antisense) for PHD1 (GenBank accession no. NM_001004083), CTGGGACGCCAAGGTGA (sense) and CAATGTCAGCAAACTGG (antisense) for PHD2 (GenBank accession no. NM_178334), GTTCAGCCCTCCTATGC (sense) and ACCACCGTCAGTCTTTA (antisense) for PHD3 (GenBank accession no. NM_019371), and 5′-CGCCGCTAGAGGTGAAATTC-3′ (sense) and 5′-TCTTGGCAAATGCTTTCGC-3′ (antisense) for 18S rRNA (GenBank accession no. M11188). Real-time quantitative PCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad) according to the manufacturer's manual. Post-PCR melting curves verified the specificity of single-target amplification, which was also confirmed by separating the PCR product by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Equal amplification efficiencies of 18S rRNA and target genes were tested, and optimization of primers and cDNA concentrations was performed in preliminary experiments. Data were gathered and analyzed by the same real-time PCR detection system. The cycle threshold (C_t) values were exported into a Microsoft Excel worksheet for calculation of gene expression in accordance with the $\Delta\Delta C_t$ method. The C_t values were first normalized with respect to 18S rRNA levels to obtain ΔC_t values. The ΔC_t value for PHD1 from the renal cortex of control animals was used as a reference to calculate $\Delta\Delta C_t$ values for all other samples. Relative mRNA levels are expressed as $2^{-\Delta\Delta C_t}$ (Tn).

For analysis of HO-1 mRNA levels, a TaqMan gene expression assay kit (Applied Biosystems) was used. This kit contains HO-1 primers and FAM dye-labeled probes and has been tested and optimized for the analysis of rat HO-1 mRNA expression by the manufacturer. A TaqMan gene expression assays kit for detecting the levels of 18S rRNA was used as an endogenous control. Real-time quantitative PCR was performed using the same real-time PCR detection system, and the data were analyzed in accordance with the $\Delta\Delta C_t$ method (see above).

Preparation of homogenates from renal tissues and Western blot analysis of PHD protein levels. Tissue homogenate was prepared as we described previously (52). The dissected cortical, outer medullary (OM), and inner medullary (IM) tissues were homogenized with a glass homogenizer in ice-cold HEPES buffer containing (in mmol/l) 25 Na-HEPES, 1 EDTA, and 0.1 PMSF. After centrifugation of the homogenate at 6,000 *g* for 5 min at 4°C, the supernatant containing membrane and cytosolic components, termed homogenate, was divided into aliquots, frozen in liquid nitrogen, and stored at −80°C. Western blot analyses were performed as described previously (54). Briefly, protein samples (20 μg) were subjected to 10% SDS-PAGE gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were probed with antibodies (1:1,000 dilution) against PHD1, PHD2, and PHD3 (catalog nos. NB-100–137, NB-139, and NB-310, respectively, Novus Biologicals, Littleton, CO) overnight in a cold (4°C) room. The membrane was washed and then incubated for 1 h with horseradish peroxidase-labeled donkey anti-rabbit IgG (1:3,000 dilution). Then enhanced chemiluminescence detection solution (Pierce) was added directly to the blots on the surface carrying proteins, and the membrane was wrapped in plastic wrap and exposed to Kodak Omat film. The intensity of the blots was determined using a microcomputer imaging system (MCID, Imaging Research). Using recombinant rat FLAG-His-PHD1, PHD2, and PHD3 or in vitro transcription/translation products of full-length rat PHD1, PHD2, and PHD3 cDNA, the manufacturer has confirmed the specificities of the primary antibodies, rabbit polyclonal antibodies that are affinity-purified IgG from rabbit sera. It has been shown that there is no cross-reaction among these antibodies (http://www.novusbio.com).

In vitro transcription/translation was performed using a IVTT kit (Promega) to generate radiolabeled proteins using [^{35}S]methionine (Amersham) and plasmids encoding full-length PHD1, PHD2, and PHD3 cDNAs (generous gifts from Dr. Frank S. Lee, University of Pennsylvania). IVTT proteins and renal tissue homogenates were subjected to Western blot analysis using antibodies against PHD1, PHD2, and PHD3, and the radioactive signals of the blot were determined by autoradiography. The autoradiographic intensities of IVTT proteins from full-length PHD1, PHD2, and PHD3 cDNA were normalized with that of a serially diluted IVTT reaction solution with known radioactivity adjusted according to the number of methionine residues in PHD proteins. Densitometries of Western blot bands corresponding to PHDs from renal tissues were normalized to those of IVTT proteins, which were calibrated by autoradiography. Therefore, the relative protein levels of PHD1, PHD2, and PHD3 in the kidney tissues were corrected with the affinities of the antibodies.

Preparation of renal nuclear extracts, Western blot analyses for HIF-1α protein levels, and HIF-1α binding assay. Renal nuclear protein was prepared as we described previously (54). Briefly, dissected cortical, OM, and IM tissues were minced and washed with PBS by centrifugation at 2,000 rpm and then homogenized with a glass homogenizer in ice-cold HEPES buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 10% Nonidet P-40]. After centrifugation of the homogenate at 2,000 rpm for 5 min at 4°C, the pellets containing cell nuclei were collected and incubated with ice-cold HEPES buffer containing 5 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 26% glycerol for 30 min to release nuclear proteins. The reaction mixtures were centrifuged at 23,000 rpm for 30 min, and the supernatant was collected and snap frozen in liquid nitrogen until use as nuclear extracts. HIF-1α levels were determined by Western blot analysis (see above) using antibody against HIF-1α.

The anti-HIF-1α antibody was prepared and validated as described previously (54). A 13-residue peptide from rat HIF-1α consisting of NH_2-CIHVYDTSSNQPQ-COOH (amino acids 199–210 plus a cysteine end) was synthesized, conjugated to keyhole limpet hemocyanin, and then used to immunize rabbits. The serum titer was detected even at a dilution of 1:100,000, as measured by a standard ELISA. When the synthetic antigen peptide was used to prereact with this serum, the antigen-antibody reaction in ELISA was completely blocked, suggesting that the serum is specific to this peptide. To further determine the specificity of anti-HIF-1α antibody, a positive nuclear extract from HeLa cells was used for Western blot analyses and peptide block experiments. In this HeLa cell nuclear extract, a 96-kDa immunoreactive band was detected and substantially blocked by preincubation of the antibody with the synthetic peptide. The size of this 96-kDa protein was predicted by rat and human HIF-1α cDNAs.

HIF-1α binding activities in the nuclear extracts were detected using an ELISA-based TransBinding HIF assay kit (Panomics) following the manufacturer's instructions. Briefly, activated HIF-1α from nuclear extracts specifically binds to oligodeoxynucleotides (ODNs) containing an HIF consensus-binding site. The ODNs are then immobilized on a 96-well plate. The complex bound to the ODNs is detected by an antibody against the HIF-1α, followed by addition of

a horseradish peroxidase-conjugated secondary antibody and colorimetric reagent tetramethyl benzidine, which is quantified by spectrophotometry. The ELISA-based HIF binding kit provides a fast, sensitive, and specific measurement of HIF-1α binding activities (27).

Immunohistochemical analysis of PHDs in rat kidney. The kidneys were removed, cut longitudinally, and fixed in 10% neutral buffered formalin. The kidneys were then embedded in paraffin, and 4-μm sections were cut, deparaffinized, and stained. The slides were incubated in 0.3% H_2O_2 in 100% MeOH for 30 min to block endogenous peroxidase activity. The sections were incubated at room temperature for 30 min in PBS containing 1% milk and 3% goat serum to block nonspecific binding and then incubated overnight at 4°C in a humidified chamber with an antibody against PHD1, PHD2, or PHD3 (rabbit polyclonal IgG, Novus Biologicals) diluted 1:50 in PBS containing 1.5% goat serum and 0.5% milk. Then the slides were incubated for 60 min at room temperature in a humidified chamber with a biotinylated goat anti-rabbit IgG diluted 1:200 in PBS as a secondary antibody. The slides were subsequently placed in 3% H_2O_2 for 10 min before incubation with streptavidin-horseradish peroxidase diluted 1:50 in PBS for 30 min at room temperature in a humidified chamber. Then the slides were incubated with 50 μl of diaminobenzadine (BioGenex, San Ramon, CA) as a substrate, counterstained with hematoxylin (Sigma), dehydrated, and fixed with Permount histological mounting medium (Fisher Scientific). Normal rabbit serum, instead of the primary antibodies, was used for negative controls. The negative controls showed no immunoreactivity.

Treatment of rats with furosemide. To examine the effect of alteration in renal medullary oxygenation on PHD expressions, male Sprague-Dawley rats received a single intraperitoneal injection of vehicle ($NaCO_3$ solution, pH 8.5) or furosemide in $NaCO_3$ solution at 10 mg/kg, a dose that was found in previous studies (5, 54) to acutely increase (by 10–30 mmHg) renal medullary P_{O_2}. At 24 h after injection of furosemide, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and the kidneys were harvested immediately. The renal cortex, OM, and IM (~100 mg) were dissected, collected, and transferred to microcentrifuge tubes containing 450 μl of TRIzol reagent. Total RNA was extracted, and real-time RT-PCR analysis was performed to determine the effects of furosemide on the mRNA levels of PHDs (see above).

Isolation and culture of rat renal medullary interstitial cells. Renal medullary interstitial cells (RMICs) were isolated and cultured as described previously (54). Briefly, inbred male Sprague-Dawley rats (300–350 g body wt; Harlan Sprague Dawley) were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). The left kidney was removed, and the renal IM was dissected, finely minced, and resuspended in 3 ml of basic medium Eagle's. This tissue suspension was injected subcutaneously into two to four vertical tracks on the abdominal wall of a recipient rat (from the same litter). At 4 days after injection, the firm yellow nodules at the injection sites were dissected, removed, minced, and treated with 0.05% trypsin-EDTA solution at 37°C for 20–30 min and then washed and centrifuged to yield a cell pellet. The cell suspension was transferred to plastic tissue culture flasks and then incubated with basic medium Eagle's containing fetal bovine serum (10%, vol/vol), amino acid mixtures (10%, vol/vol), lactalbumin hydrolysate (0.25%, wt/vol), yeast extracts (0.05%, wt/vol), and antibiotics [penicillin (100 U/ml) and streptomycin (100 μg/ml)] in a 37°C incubator in 95% air-5% CO_2. The culture medium was replaced with fresh medium on *day 5* and then changed every 3 days. These cells, which formed a confluent monolayer in 18–21 days, were treated with trypsin and subsequently replanted in flasks. The cells of *passages 7–8* were used for experiments. The identity of these cells was confirmed by oil red O- and Sudan black B-stained rich lipid granules within the cells, as described previously (54).

Cell hypoxia or changes in osmolarity of the culture medium. For induction of hypoxia, the RMICs were cultured in plastic culture plates until a monolayer of these cells formed. The plates were then moved to a humidified modular chamber and flushed with 95% N_2-5% CO_2 for 2 h. Previous reports showed that P_{O_2} in the culture medium was maintained at 5–10 mmHg and that 2 h of hypoxia produced maximal changes in the expression of HIF-1α (46, 54). After hypoxia, the culture medium was rapidly replaced by RNA extraction solution (TRIzol), and then total RNA was extracted according to the protocol of the manufacturer. The osmolarity in the culture medium was changed by addition of NaCl + urea to the culture medium, which adjusted the osmolarity from 300 to 1,200 mosmol/l. Use of NaCl + urea was based on previous studies showing that NaCl or urea at high concentrations individually exhibited toxic effects on rat RMICs and mouse IM collecting duct cells, whereas NaCl + urea increased (>1,000 mosmol/l) osmolarity without causing cell death (44, 54).

Determination of prolyl hydroxylase activity. HIF-1α peptide-specific conversion of 2-oxoglutarate to succinate provides a hydroxyl group for prolyl hydroxylation of HIF-1α. This reaction has been widely used for the determination of PHD activity (11, 43). Briefly, dissected renal cortical and medullary tissues were homogenized on ice using a Dounce homogenizer in 6 vol of buffer containing 0.15 mM $MgCl_2$, 10 mM KCl, and 10 mM Tris·HCl (pH 6.7). Dextrose was added to a final concentration of 0.25 M, and the homogenate was centrifuged at 1,500 *g* for 15 min at 4°C to remove nuclei. The mitochondrial fraction was separated by centrifugation at 6,500 *g* for 10 min at 4°C. The hydroxylase reaction was carried out using 30 μg of protein in the reaction buffer containing 40 mM Tris·HCl (pH 7.5), 50 μM $FeSO_4$, 0.1 mM L-[α-5-^{14}C]ketoglutaric acid (Moravek Biochemicals, Brea, CA), 0.25 mM ascorbate, 0.4 mg/ml catalase, 0.5 mM DTT, and 200 μM O_2-dependent degradation domain peptide of HIF-1α (amino acids 556–575 of rat HIF-1α; Advanced ChemTech, Louisville, KY). Samples were incubated for 1 h at 37°C in a final volume of 50 μl. After incubation, 25 μl of a mixture of 20 mM succinate and 2-oxoglutarate and 25 μl of 0.16 M 2,4-dinitrophenylhydrazine in 30% $HClO_4$ were added. After the addition of 50 μl of 1 M 2-oxoglutarate, the samples were left for 30 min at room temperature. Supernatants were separated by spinning at 3,000 *g* for 5 min, and radioactivity was measured using a liquid scintillation counter. Each set of experiments included control reactions without addition of O_2-dependent degradation domain peptide of HIF-1α to calculate HIF-1α-dependent conversion of 2-oxoglutarate to succinate, which represents specific HIF prolyl hydroxylase activity.

Effect of inhibition of PHD activity on renal function and pressure natriuresis. Animals were treated with daily intraperitoneal injections of vehicle or L-mimosine (L-Mim) (Sigma), an inhibitor of PHD activity (35, 48), for 2 wk at 50 mg/kg to inhibit this enzyme activity. On the day of the acute experiment, rats were prepared for the study of pressure natriuresis, as previously described (13). Briefly, after they were anesthetized with ketamine (30 mg/kg im) and thiobutabarbital (Inactin; 50 mg/kg ip), the rats were placed on a thermostatically controlled warming table to maintain body temperature at 37°C. A catheter was placed in the left femoral vein for intravenous infusion and in the femoral artery for monitoring of renal perfusion pressure (RPP). For renal blood flow (RBF) measurement, a transonic flow probe (2 mm) was placed around the left renal artery to measure RBF with a flowmeter (Transonic Systems), as described previously (51). A plastic catheter was inserted into the ureters for urine collection.

After surgery, the animals received a continuous infusion of 0.9% NaCl solution containing 2% albumin at 100 $\mu l \cdot min^{-1} \cdot 100$ g body wt^{-1} throughout the experiment. For pressure natriuresis, vasopressin (52 pg/min), aldosterone (20 ng/min), norepinephrine (100 ng/min), and hydrocortisol (20 μg/min) were included in the intravenous infusion solution to fix the circulating levels of these hormones, as previously described (13). After 1 h of equilibration, the celiac and mesenteric arteries were tied off to acutely increase RPP, which was set to 160, 120, and 80 mmHg, respectively, by an adjustable clamp placed on the aorta above the renal arteries. At each RPP, after 10 min of equilibration, urine and blood samples (100 μl) were collected during a 20-min clearance period. To measure glomerular filtration rate (GFR), a 0.5-ml bolus of FITC-inulin (8.0 mg/ml) was given, and

a steady intravenous infusion of FITC-inulin (4.0 mg/ml) continued throughout the experiment (30, 47, 51). After each experiment, FITC-inulin fluorescence in blood and urine samples was measured with excitation and emission wavelengths of 480 and 530 nm, respectively, using an automatic microplate reader (model KC4, Bio-Tek Instruments, Winooski, VT). The urine flow rate was determined gravimetrically, and Na^+ concentrations of urine samples were measured using a flame photometer (Buck Scientific, East Norwalk, CT). GFR, urine flow, and urinary Na^+ excretion were factored per gram kidney weight. At the end of the experiment, the kidneys were removed, weighed, dissected into cortical and medullary tissues, and rapidly frozen in liquid nitrogen for later measurement of PHD activity and Western blot analysis of HIF-1α expression (see above). The animals were then euthanized with an excess dose of pentobarbital sodium (150 mg/kg iv).

Renal medullary transfection of HIF-1α decoy ODN. HIF-1α hypoxia-responsive element containing ODNs were synthesized on the basis of the sequence reported elsewhere (46, 50). After rats were anesthetized with ketamine (80 mg/kg ip) and xylazine (6 mg/kg), an interstitial catheter (5 mm) was placed into the left renal medulla and anchored in the kidney surface with Vetbond tissue adhesive (3M). A mixture of 40 nmol of HIF-1α decoy ODNs or scrambled ODNs in 0.6 ml of 50% microbubble (Optison, GE HealthCare) in saline was infused into the renal medulla at a rate of 10 μl/min. After the infusion, the catheter was cut and blocked by a piece of fat tissue with Vetbond tissue adhesive. An ultrasound transducer (Ultax UX-301, Celcom Medico) was directly applied to the kidney with a continuous-wave output of 1-MHz ultrasound at 5% power output, for a total of 60 s at 30-s intervals (28) in the middle and at the end of the infusion. This ultrasound-microbubble technique has been shown to effectively (>90% transfection) deliver plasmid to cells in the kidneys (28). Our preliminary study also demonstrated >90% transfection of FITC-labeled ODNs into medullary tubular cells. Three groups of animals (6 in each group) were studied: *1*) the control group was transfected with scrambled ODNs and intraperitoneally injected with vehicle daily, *2*) the L-Mim group was transfected with scrambled ODNs and intraperitoneally injected with L-Mim daily, and *3*) the decoy group was transfected with HIF-1α decoy ODNs and intraperitoneally injected with L-Mim daily. After 7 days, pressure natriuresis was studied in these animals (see above), and the kidneys were dissected into cortical and medullary tissues, snap frozen in liquid nitrogen, and stored at −80°C for analysis of HIF banding activity and HO-1 mRNA levels (see above).

Statistics. Values are means ± SE. The significance of differences in mean values within and between multiple groups was evaluated using ANOVA followed by Duncan's multiple range test. Student's *t*-test was used to evaluate statistical significance of differences between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of PHDs in renal cortex, OM, and IM. Real-time quantitative RT-PCR analysis of mRNA expression of PHDs is presented in Fig. 1. A typical gel document from real-time RT-PCR reactions (Fig. 1*A*) shows a single band for each product, which confirms the specificity of target gene amplification in this analysis. The relative quantitation of PHD mRNA expressions in different kidney regions is shown in Fig. 1*B*. PHD1, PHD2, and PHD3 mRNAs were detected in three different kidney regions. The expressions of mRNA of three PHD isoforms were all much higher in the medullary than in the cortical area, and PHD2 was the most abundant isoform expressed in these kidney regions.

The results obtained from Western blot analyses of PHDs proteins are presented in Fig. 2. Typical gel documents of the immunoreactive blots on the nitrocellulose membranes carrying IVTT proteins and renal cortical and medullary proteins probed with specific antibodies against PHD1, PHD2, or PHD3 are shown in Fig. 2*A*, and the results of these protein analyses are summarized in Fig. 2*B*. Similar to the mRNA expression pattern, the blot intensities of PHD proteins were significantly higher in the OM and IM than in the renal cortex. Protein expression of the PHDs was much higher in the renal medulla, and PHD2 was the most abundance protein.

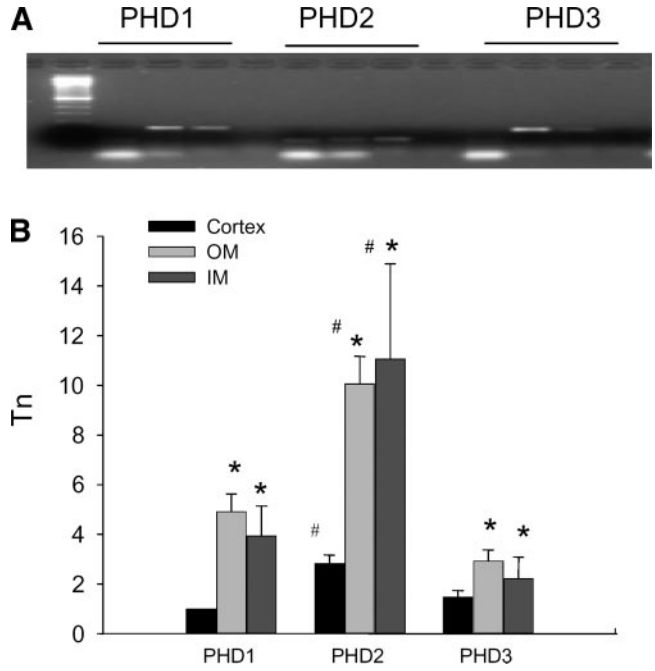

Fig. 1. Prolyl-4-hydroxylase domain-containing protein (PHD) mRNA expression in kidneys. *A*: typical agarose gel electrophoresis of real-time RT-PCR products of PHDs visualized by ethidium bromide staining. *B*: relative quantitation (Tn) of mRNA levels in PHDs by real-time RT-PCR analysis in renal cortex, outer medulla (OM), and inner medulla (IM). PHD1 from renal cortex was used as a reference for calculation of $\Delta\Delta C_t$. Values are means ± SE ($n = 6$). $^*P < 0.05$ vs. cortex. $^\#P < 0.05$ vs. PHD1 and PHD3.

To further define the protein expression of PHDs in the kidney, immunohistochemistry was performed. As shown in Fig. 3, PHD1, PHD2, and PHD3 were detected in all kidney regions, including the cortex, OM, and IM, mainly in distal tubules and collecting ducts. Strong signals for PHD2 were also found in the proximal straight tubules in the cortex and OM, and weak staining for PHD1 was observed in the OM. The immunostaining patterns were similar to the results from Western blot analyses, in which PHD2 signals were stronger than the others, and the OM and IM exhibited more staining than the cortex in all PHD isoforms. The PHD immunostaining was visualized mainly in the cytoplasm of renal tubular cells.

Effects of furosemide on mRNA expression of PHDs in the kidneys. Figure 4 shows the effect of furosemide on PHD mRNA levels in three different kidney regions. Furosemide was used to increase tissue P_{O_2} in the renal medulla (~10–15 mmHg) by inhibiting tubular transport activity. The value of PHD1 in the cortical tissue of vehicle-treated animals was used as the reference for calculation of the relative mRNA levels of all other samples, including PHD2 and PHD3. Furosemide treatment remarkably reduced the mRNA levels of all three isoforms in the medullary area but had no significant effect on the mRNA levels of PHDs in the renal cortex (Fig. 4).

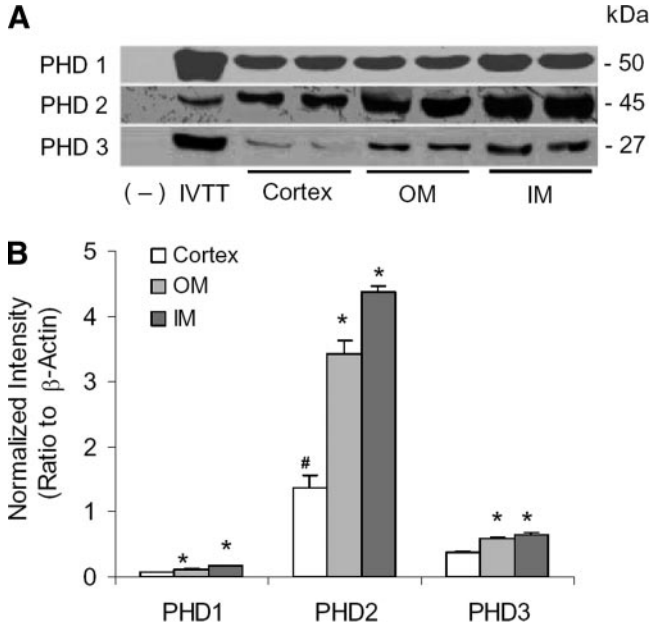

Fig. 2. Western blot analysis of PHD protein expression in kidneys. *A*: representative enhanced chemiluminescence gel documents of Western blot analyses depicting protein levels of PHDs in kidneys. *B*: intensities of PHD blots normalized to β-actin in renal cortex, OM, and IM. In vitro transcription/translation (IVTT) proteins from full-length PHD1, PHD2, and PHD3 cDNAs were used to calibrate immunoblots and estimate relative abundance of PHD proteins in tissues. Products from IVTT reactions using empty vectors were loaded as negative controls. Values are means ± SE ($n = 6$). $^{*}P < 0.05$ vs. cortex. $^{\#}P < 0.05$ vs. PHD1 and PHD3.

Effects of hypoxia on mRNA expression of PHDs in RMICs. To further explore the mechanism that increases renal medullary PHDs expression, we determined the effect of hypoxia on the mRNA levels in cultured RMICs (Fig. 5*A*). Incubation of RMICs in a hypoxia chamber for 2 h significantly increased the mRNA levels of PHD1 and PHD2, but not PHD3, compared with control cells. In hypoxic cells, mRNA levels of PHD1 and PHD2 increased twofold, the mRNA level of PHD3 did not change significantly.

Effects of elevated osmolarity in the culture medium on mRNA expression of PHDs in RMICs. Because high osmolarity and furosemide-induced changes osmolarity in the renal medulla in vivo may also influence the gene expression in renal medullary cells, additional experiments were conducted to examine the effects of changes in extracellular osmolarity on the expression of PHDs in RMICs (Fig. 5*B*). Elevations of the osmolarity in the culture medium from 300 to 1,200 mosmol/l induced by addition of NaCl + urea had no effect on the mRNA levels of PHDs, indicating that the mRNA levels of PHDs in these renal medullary cells are not associated with the changes in extracellular osmolarity.

Effects of L-Mim on PHD activity and HIF-1α expression in rat kidneys. L-Mim is a PHD inhibitor that binds to the active site of PHDs and interferes with the reconstitution of the active enzyme (35). It has been reported that this inhibitor of PHD activity upregulates the HIF-1α levels (11). The PHD activities in renal tissue homogenates, presented as the HIF-1α peptide-dependent conversion rate of [^{14}C]-2-oxoglutarate to succinate, were higher in the renal medulla than in the renal cortex, which is consistent with higher PHD expression levels in the renal medulla. Pretreatment of animals with L-Mim for 2 wk significantly reduced PHD activity in the rat kidneys. This inhibitory effect was greater in the renal medulla than in the renal cortex (data not shown; see online version of this article for supplemental information). Western blot analysis was performed to detect the HIF-1α levels in the kidneys from control and L-Mim-treated rats. These data are shown in Fig. 6. Typical gel documents of the immunoreactive blots, demonstrating significant increases in the protein levels of HIF-1α in the kidneys from L-Mim-treated animals compared with vehicle-treated animals are shown in Fig. 6*A*, and the data from these experiments are summarized in Fig. 6*B*. Pretreatment of animals with L-Mim substantially increased the HIF-1α levels in the renal cortex and medulla, but the effect was much greater in the medulla than in the cortex.

Effects of inhibition of PHD activity and HIF-1α decoy on HIF-1α transcriptional activities in the renal medulla. HIF-1α transcriptional activities were examined by detection of its

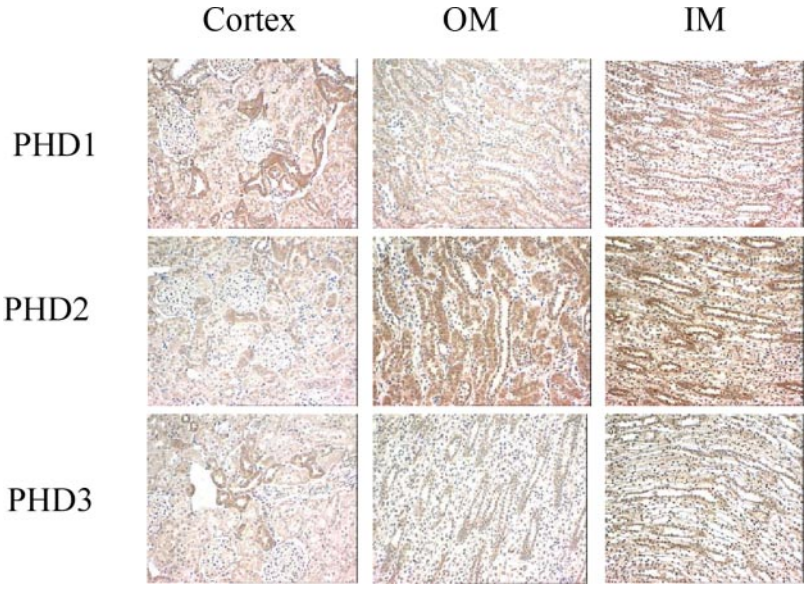

Fig. 3. Immunohistochemical staining of PHD1, PHD2, and PHD3 proteins in rat kidney. Results are representative of staining of PHDs in renal cortex, OM, and IM in kidneys from 5 rats.

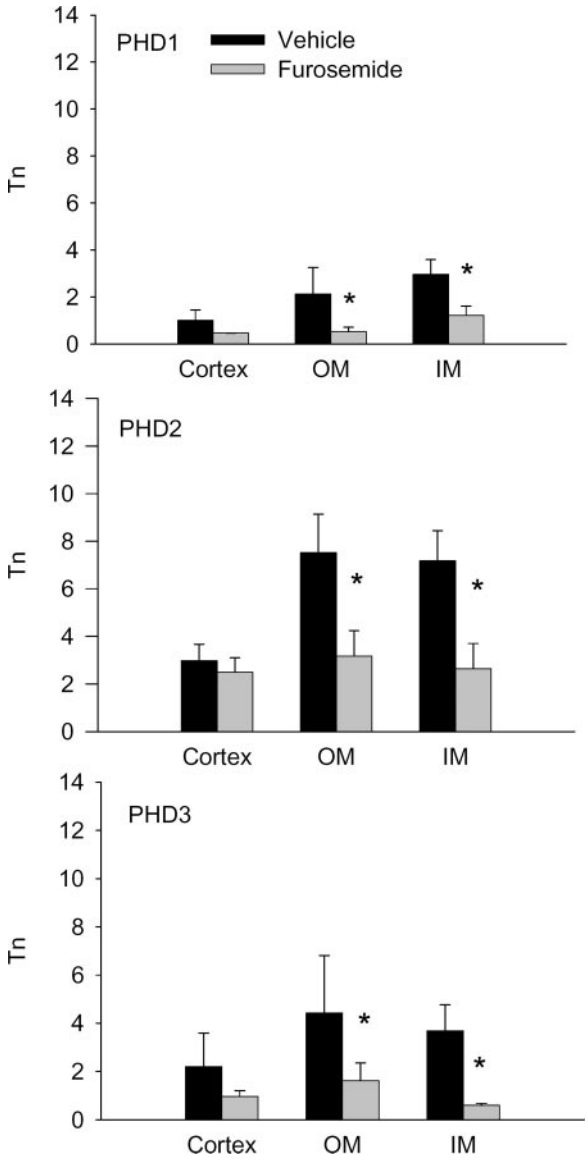

Fig. 4. Effects of furosemide on mRNA levels of PHDs in renal cortex, OM, and IM. Values are means ± SE ($n = 5$). *$P < 0.05$ vs. vehicle.

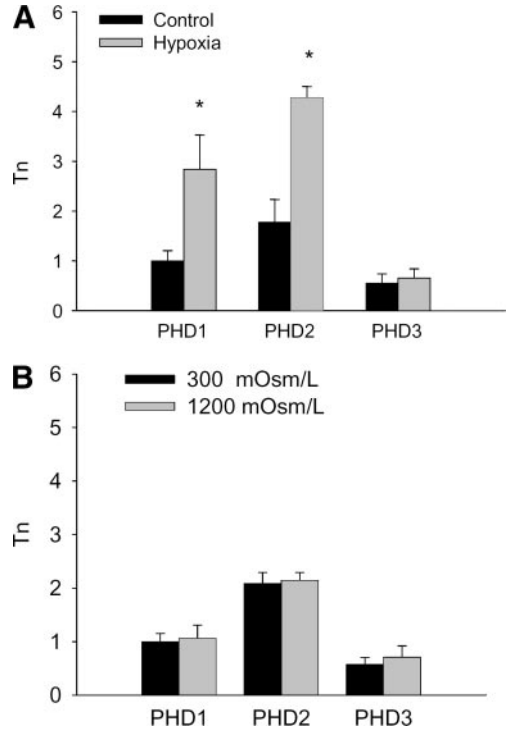

Fig. 5. Effects of hypoxia (*A*) and extracellular osmolarity (*B*) on mRNA levels of PHDs in renal medullary interstitial cells (RMICs). NaCl + urea was used to increase osmolarity from 300 to 1,200 mosmol/l. Values are means ± SE ($n = 8$). *$P < 0.05$ vs. control.

binding activities and the transcription of its target gene. Consistent with the increased HIF-1α levels induced by L-Mim, HIF-1α binding activities in the renal medulla were significantly increased in L-Mim-treated animals (Fig. 7*A*). Meanwhile, renal medullary mRNA levels of HO-1, a prototype HIF-1α target gene, were substantially upregulated in L-Mim-treated rats compared with control animals (Fig. 7*B*). These L-Mim-induced increases in HIF-1α binding activities and HO-1 mRNA levels were abolished by the decoy of HIF-1α in the renal medulla (Fig. 7).

Effects of inhibition of PHD activity and HIF-1α decoy on renal function and pressure natriuresis. To explore the physiological significance of the PHD regulation of HIF-1α, we determined pressure natriuresis in animals treated with L-Mim, an inhibitor of PHDs. The effects of inhibition of PHD activity on pressure natriuresis are presented in Fig. 8. In vehicle-treated rats, urine flow and Na^+ excretion were increased >10- and 15-fold, respectively, as RPP was increased from 80 to 157 mmHg. In rats chronically treated with L-Mim, the diuretic and natriuretic responses to elevations in RPP were remarkably enhanced, and >20-fold diuretic and 30-fold natriuretic responses were observed when RPP increased from 80 to 155

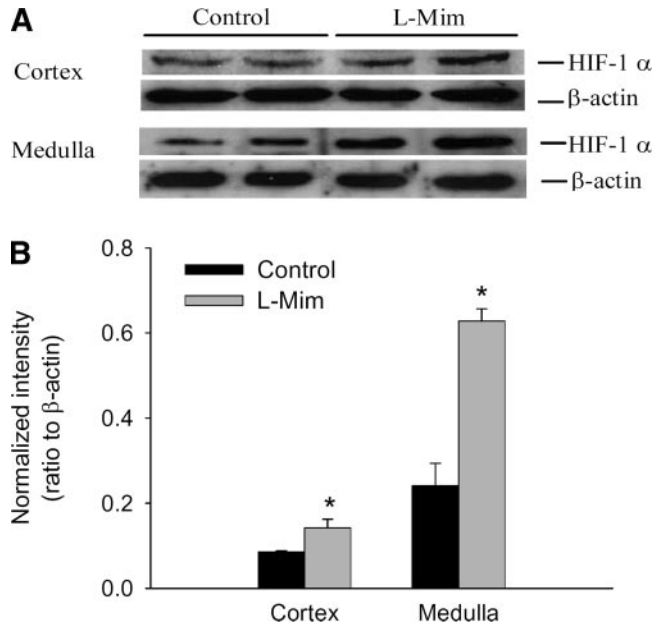

Fig. 6. Effects of inhibition of PHD activity on protein expression of hypoxia inducible factor (HIF)-1α in rat kidneys. *A*: typical enhanced chemiluminescence gel documents of HIF-1α protein expression in renal cortical and medullary regions. *B*: intensity of immunoreactive band of HIF-1α protein normalized with β-actin. L-Mim, L-mimosine. Values are means ± SE ($n = 6$). *$P < 0.05$ vs. control.

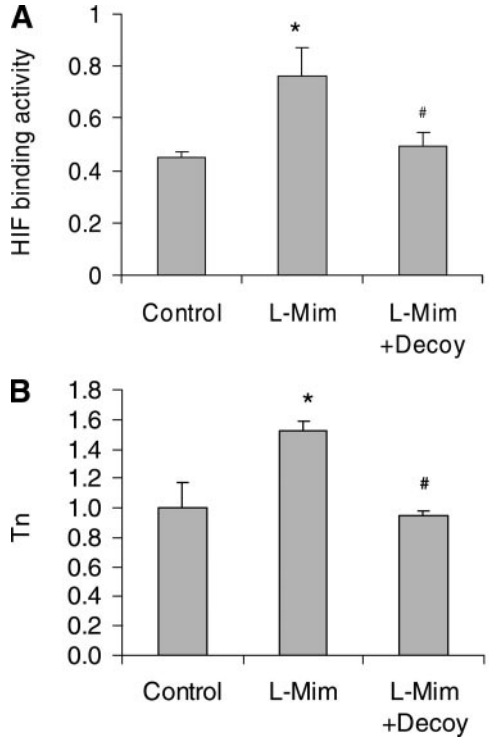

Fig. 7. Effects of inhibition of PHD activity and HIF-1α decoy on HIF binding activities (*A*) and heme oxygenase (HO)-1 mRNA levels (*B*) in renal medulla. Values are means ± SE ($n = 6$). *$P < 0.05$ vs. control. #$P < 0.05$ vs. L-Mim.

mmHg. However, this L-Mim-induced enhancement in diuretic and natriuretic responses to elevated RPP was significantly attenuated in the animals transfected with HIF-1α decoy ODNs in the renal medulla (Fig. 8, *A* and *B*). There was no significant difference in the GFR and RBF in response to the elevations of RPP among the vehicle-, L-Mim-, and HIF-1α decoy ODN-treated rats when RPP was elevated (Fig. 8, *C* and *D*).

DISCUSSION

In the present study, all three isoforms of PHD were detected in the renal cortex and medulla, and PHD2 was the most abundant isoform. The mRNA and protein levels of these isoforms of PHD were much higher in medullary than in cortical regions, which might be associated with the low O_2 levels in the renal medulla. Inhibition of PHD activity increased HIF-1α levels in the kidneys and enhanced pressure natriuresis in rats.

The present study, for the first time, characterized the expressions of three isoforms of PHD and their distributions in different kidney regions. Real-time RT-PCR, Western blot, and immunohistochemical analysis demonstrated that mRNAs and proteins of all three PHDs were much higher in the renal medulla than in the renal cortex and that PHD2 was the most predominant isoform in the rat kidney. Immunohistochemistry revealed that PHDs were mainly present in the distal tubules and collecting ducts, and PHD2 was also detected in proximal straight tubules in the cortex and OM. The precise intracellular localization of PHDs in the renal tubular cells requires further investigation. In this regard, in a study using human osteosarcoma cells, PHD1 was exclusively present in the nucleus, PHD2 was located in the cytoplasm, and PHD3 was homogeneously distributed in the cytoplasm and the nucleus (36), whereas another study showed little staining of PHDs in the nucleus in human prostate cancer cells (4).

Strong immunostaining of PHDs in the distal tubules and collecting ducts was also detected in the renal cortex, despite the high Po_2 in this area. A similar finding has also been observed in the immunostaining of HIF-1α in a previous report of predominantly hypoxic activation of HIF-1α in the connect-

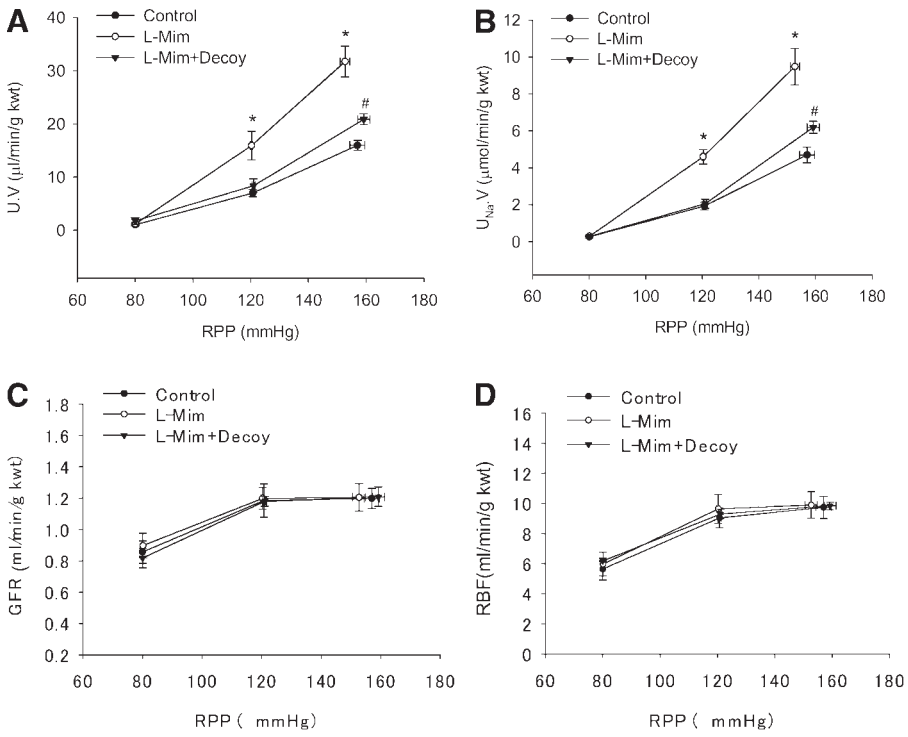

Fig. 8. Effects of chronic treatment of rats with L-Mim and HIF-1α decoy on diuretic and natriuretic responses to elevations of renal perfusion pressure (RPP): urinary volume (UV, *A*), urinary Na^+ excretion ($U_{Na}V$, *B*), glomerular filtration rate (GFR, *C*), and renal blood flow (RBF, *D*). Values are means ± SE ($n = 6$). *$P < 0.05$ vs. corresponding values from other two groups. #$P < 0.05$ vs. control.

ing tubules and collecting ducts, irrespective of known differences in local P_{O_2} in the cortex and medulla (42). It seems that the expression of PHDs in some cells may not be closely associated with tissue O_2 levels. Indeed, there is increasing evidence of O_2-independent regulation of HIF-1 and PHD expression (8, 31).

Because P_{O_2} is lower in renal medullary than in renal cortical tissue (5, 15, 54) and hypoxic upregulation of the expression of PHDs has been reported in other tissues or cells (9, 11, 14, 29, 34), the increased levels of PHDs in the renal medulla may be associated with a low-P_{O_2} milieu in this kidney region. To further determine whether the levels of PHDs are associated with tissue oxygenation in the renal medulla, we examined changes in the mRNA levels of PHDs when tissue P_{O_2} was increased in the renal medulla. It has been reported that a classic loop diuretic, furosemide, inhibits active ion transport activity in the thick ascending limb of Henle and significantly increases renal medullary tissue P_{O_2} but does not affect cortical P_{O_2} in rat kidneys (5, 54). We therefore treated the animals with furosemide and found that the mRNA levels of PHDs were significantly decreased in the renal medulla, but not in the renal cortex. This is consistent with our previous studies demonstrating that HIF-1α, an O_2-sensitive gene, was highly expressed in the renal medulla and that administration of furosemide decreased the mRNA levels of HIF-1α in the renal medulla (54). These results support the idea that the high levels of PHDs may be due to the low P_{O_2} in the renal medulla. The consistency of increased expressions in mRNA and protein of PHDs in the renal medulla suggests that the regulation of PHD by O_2 levels may be at the transcriptional level.

Since the in vivo experiments with furosemide could not exclude the effects of other factors such as osmolarity, hemodynamic changes, or shear stress on the expressions of PHDs in this kidney region, we determined the effects of hypoxia on the expression of PHDs in cultured renal medullary cells to confirm that high levels of PHDs in the renal medullary area are related to the low P_{O_2}. With respect to the possible effects of osmolarity, in vivo furosemide treatment could also decrease renal medullary osmolarity and, thereby, influence PHD expression; therefore, experiments are needed to exclude the effects of osmolarity. As documented in the literature, it is not possible to alter renal medullary oxygenation in vivo without changing tissue osmolarity in this kidney region. Also, factors that change renal osmolarity, such as water diuresis, water restriction, and others, clearly influence renal medullary oxygenation (16, 17, 39, 40). For these reasons, we performed in vitro experiments using cultured RMICs as prototype cells of renal medullary cells to dissect the effects of low P_{O_2} and high osmolarity on renal medullary PHD levels. Our data demonstrated that, in RMICs, mRNA expressions of PHD1 and PHD2 were markedly increased in response to hypoxia, providing direct evidence that hypoxia upregulates PHDs in these renal medullary cells. However, increasing extracellular osmolarity from 300 to 1,200 mosmol/l had no effect on the mRNA levels in any of the isoforms of PHD, suggesting that high levels of osmolarity are not associated with increases in mRNA levels of PHDs in the renal medulla. From these results, it can be further concluded that hypoxia, per se, is a primary stimulus for increased expression of PHDs in renal medullary cells or tissue.

The present study showed that PHD2 was the dominant isoform in kidney tissues and cultured RMICs. The cell-specific expression and response to hypoxia of different PHD isoforms have been observed in some other tissues and cells. For example, all PHD genes were expressed to a similar level in the heart, whereas PHD3 mRNA was expressed at a low level in smooth muscle cells; PHD1 was the most abundant mRNA in human aortic endothelial cells, and PHD2 was the most abundant in skeletal muscle (3, 9). In addition, in RMICs, PHD3 expression was not increased by hypoxia, a finding that is not consistent with the results from the in vivo experiments. This discrepancy in PHD3 expression levels may be related to the lack of a response of PHD3 to hypoxia in RMICs, but in vivo data may include some responses from other types of cells in this kidney region. In response to hypoxia, tissue- or cell-specific changes in different PHDs have also been observed. In the HeLa cell line, transcripts for PHD2 and PHD3, but not PHD1, are induced by hypoxia (14). In cardiac myocytes, smooth muscle cells, and endothelial cells, hypoxia results in significant time-dependent increases in PHD3 mRNA levels, but not PHD1 or PHD2 mRNA levels, which are correlated with an increase in HIF-1α protein expressions (9, 29). All PHD isoforms have been reported to regulate the expression of HIF (1, 9, 22, 29), but different PHD isoforms are responsible for the regulatory effect in a specific cell type (1, 14). Tissue- or cell-specific response of the expressions of different PHD isoforms to hypoxia seems to be a common phenomenon in the regulation of levels of this enzyme. This phenomenon may indicate the development of specific pharmacological intervention of PHD isoforms for selective modulation of the HIF-1α response in different cells (1).

It has been documented that the major function of PHDs is to promote the degradation of HIF-1α (7, 23, 24). Thus the upregulation of PHDs and HIF-1α during hypoxia seems paradoxical. However, recent studies have demonstrated that hypoxic upregulations of PHDs may represent a feedback-adaptive mechanism in response to high levels of HIF-1α, which limit an accumulation of this transcription factor to prevent the overexpression of its target genes by accelerated degradation of HIF-1α (11, 34). In this regard, the mechanism by which hypoxia induces PHDs has been proposed to be HIF-1α dependent; that is, under hypoxic conditions, HIF-1α levels are increased by decreased PHD activity, which enhances the transcription of PHDs to promote the degradation of this transcription factor, playing a feedback-regulatory role (2, 12, 34). Furthermore, activities of PHDs are mainly dependent on tissue P_{O_2}, rather than the expression levels of PHDs (11). The opposite dual actions of hypoxia on PHDs, inhibiting the activities and activating the expressions of PHDs, may allow a precise and rapid regulation of HIF-1α and its target genes. There is a concern regarding the role of higher medullary PHD levels in the hypoxic upregulation of HIF-1α in the kidneys. Rosenberger et al. (42) showed that HIF-1α expression in response to hypoxia was higher in the renal medulla than in the cortex, which corresponds to an established gradient of hypoxia sensitivity. Because the major function of PHDs is promotion of the HIF-1α degradation, the high levels of PHDs in the renal medulla shown in the present study seem not to be responsible for the stronger hypoxic HIF stabilization in this kidney region. It is possible that a high level of PHD expression in this kidney region is related to a feedback induction by a high level of HIF-1α expression (see above).

In the present study, we also determined the role of PHD activity in the control of renal function, which addresses the

physiological relevance of this hydroxylase. As discussed above, PHDs have been shown to regulate several target genes of HIF-1α (37, 49) such as NOS (25, 26, 38), COX-2 (21, 32), and HO-1 (26, 50). Because of the importance of the products of these genes in the regulation of renal medullary function or Na^+ excretion, we wondered whether PHD activities are involved in the control of Na^+ excretion and blood pressure through the actions of HIF-1α-targeted gene products. To test this hypothesis, we performed a series of in vivo experiments to test the effects of treatment of animals with an inhibitor of PHD activity on urinary Na^+ excretion in response to the elevations of RPP, namely, pressure natriuresis. L-Mim was chosen, because it was reported to more effectively induce HIF-1α in the kidneys in vivo than other inhibitors (48). Consistent with the previous study (48), administration of L-Mim significantly decreased PHD enzyme activity and, thereby, upregulated HIF-1α levels in the kidneys, especially in medullary areas. We further confirmed that L-Mim increased HIF-1α binding and the transcription of HO-1, a prototype HIF-1α target gene, in the renal medulla. This inhibition of PHD activity and consequent increases in HIF-1α expression and transcriptional activity markedly enhanced pressure natriuresis, as shown by a significant left-upward shift of the pressure-natriuresis curve. Since pressure natriuresis is a key mechanism in the long-term control of arterial blood pressure (19, 20), these results suggest that renal PHD activity, in particular in the renal medulla, may make an important contribution to the control of normal arterial blood pressure.

The present study did not attempt to further explore which pathway regulated by PHD/HIF-1α is important in the regulation of pressure natriuresis and Na^+ excretion. The products of several HIF-1α-targeted genes, such as NOS, COX-2, and NO-1, have been reported as crucial regulators of renal medullary function and Na^+ excretion (18, 33, 41, 52). Therefore, modification of HIF-1α by alteration of PHD activity or expression would be expected to regulate the levels of these products of HIF-1α-targeted genes, thereby changing Na^+ excretion and participating in the control of arterial blood pressure. The regulation of HIF-1α by PHDs in the renal medulla may represent a novel and important mechanism responsible for the regulation of renal water and Na^+ excretion and arterial blood pressure. Since the effects of L-Mim on PHD activities and HIF-1α expressions were observed in cortical and medullary tissues, although the effects were more predominant in the renal medulla, the PHD/HIF pathway in nephron segments located in the renal cortex may also be involved in resetting the pressure-natriuresis curve after administration of L-Mim.

Although L-Mim induced increases in HIF-1α levels and its transcriptional activities, whether the effects of L-Mim on renal function are mediated by increased HIF-1α could not be firmly concluded. To address this issue, we examined the effect of inhibition of HIF binding activity on the L-Mim-induced shift of the pressure-natriuresis curve. Decoy of HIF-1α in the renal medulla eliminated the effects of L-Mim on HIF binding and HO-1 expression in this kidney region. Consequently, inhibition of HIF transcriptional activity diminished the L-Mim-induced enhancement of pressure natriuresis. These results support the notion that effects of inhibition in PHD activity on renal function are dependent on induction of HIF-1α levels. Although the decoy of HIF-1α abolished the L-Mim-induced increases in HIF binding activities and HO-1 expression in renal medullary tissues, inhibition of HIF binding activities in the renal medulla by decoy only partially attenuated L-Mim-induced enhancement of pressure natriuresis. L-Mim also affected PHD activities and HIF-1 levels in the cortex (see above); however, these effects were small. Medullary transfection of HIF-1α decoy ODNs in the present study may not block the effect of L-Mim in the renal cortex, which also participates in the regulation of renal function.

In summary, the present study demonstrated that all three isoforms of PHD are present in the kidneys and that their levels are much higher in the renal medulla. PHD2 was the most abundant isoform in all kidney regions. Moreover, we found that high levels of PHDs in the renal medulla were associated with the low O_2 levels in this region. Increases in HIF-1α levels and transcriptional activities by inhibition of PHD activity enhanced the pressure-natriuresis response, suggesting that PHDs may serve as O_2 sensors to make an important contribution to the normal control of renal function and Na^+ excretion. Thus renal prolyl hydroxylase, especially in the renal medulla, may be one of the most important enzymes that controls expression and function of O_2-sensitive genes in this kidney region, thereby participating in the regulation of blood pressure.

GRANTS

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