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Hypertension 2010, 55:1129-1136: originally published online March 22, 2010
doi: 10.1161/HYPERTENSIONAHA.109.145896
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Hypoxia-Inducible Factor Prolyl-Hydroxylase 2 Senses High-Salt Intake to Increase Hypoxia Inducible Factor 1α Levels in the Renal Medulla

Zhengchao Wang, Qing Zhu, Min Xia, Pin-Lan Li, Shante J. Hinton, Ningjun Li

Abstract—High salt induces the expression of transcription factor hypoxia-inducible factor (HIF) 1α and its target genes in the renal medulla, which is an important renal adaptive mechanism to high-salt intake. HIF prolyl-hydroxylase domain-containing proteins (PHDs) have been identified as major enzymes to promote the degradation of HIF-1α. PHD2 is the predominant isoform of PHDs in the kidney and is primarily expressed in the renal medulla. The present study tested the hypothesis that PHD2 responds to high salt and mediates high-salt–induced increase in HIF-1α levels in the renal medulla. In normotensive rats, high-salt intake (4% NaCl, 10 days) significantly inhibited PHD2 expressions and enzyme activities in the renal medulla. Renal medullary overexpression of the PHD2 transgene significantly decreased HIF-1α levels. PHD2 transgene also blocked high-salt–induced activation of HIF-1α target genes heme oxygenase 1 and NO synthase 2 in the renal medulla. In Dahl salt-sensitive hypertensive rats, however, high-salt intake did not inhibit the expression and activities of PHD2 in the renal medulla. Correspondingly, renal medullary HIF-1α levels were not upregulated by high-salt intake in these rats. After transfection of PHD2 small hairpin RNA, HIF-1α and its target genes were significantly upregulated by high-salt intake in Dahl salt-sensitive rats. Overexpression of PHD2 transgene in the renal medulla impaired renal sodium excretion after salt loading. These data suggest that high-salt intake inhibits PHD2 in the renal medulla, thereby upregulating the HIF-1α expression. The lack of PHD-mediated response to high salt may represent a pathogenic mechanism producing salt-sensitive hypertension.

Key Words: hypertension ■ transcription factor ■ gene transfection ■ Dahl salt-sensitive rats ■ small interfering RNA

The transcription factor hypoxia inducible factor (HIF) 1α and some of its target genes, such as NO synthase (NOS), cyclooxygenase 2 (COX-2), and hemeoxygenase 1 (HO-1), are highly expressed in the renal medulla.1–6 The products of these HIF-1α target genes play critical roles in regulating renal medullary blood flow and tubular activity, thereby maintaining the constancy of body fluid volume and arterial blood pressure. These genes in the renal medulla are upregulated in response to high-salt intake,2,4–7 and inhibition of these genes and/or the enzymes encoded by these genes within the renal medulla reduces sodium excretion and increases salt sensitivity of arterial blood pressure.2–4,6,8–10 We have shown previously that high-salt intake increases HIF-1α levels in the renal medulla.11 Inhibition of HIF-1α blocks the activation of its target genes in the renal medulla in response to high-salt intake and consequently promotes sodium retention, inducing salt-sensitive hypertension.11 It is suggested that HIF-1α–mediated gene regulation in the renal medulla represents an important molecular adaptive mechanism in response to high-salt intake and plays a crucial role in the maintenance of sodium balance. However, it remains unclear how high-salt intake induces increases in the HIF-1α level in the renal medulla.

It has been demonstrated recently that HIF prolyl-hydroxylases are the major enzymes to promote the degradation of HIF-1α.12–14 HIF prolyl-hydroxylases catalyze site-specific proline hydroxylation of HIF-1α using oxygen as a cofactor, and prolyl-hydroxylated HIF-1α is recognized and targeted for degradation by the ubiquitin-proteasome pathway. Although HIF prolyl-hydroxylases work as oxygen sensors to regulate the destruction of HIF-1α,12–14 recent evidence has clearly shown that the activities and expressions of HIF prolyl-hydroxylases are also regulated independent of oxygen levels by a variety of factors.15–19

Three isoforms of HIF prolyl-hydroxylase, including prolyl-hydroxylase domain-containing proteins (PHDs) 1, 2, and 3, have been identified.12–13,20 It has been demonstrated that PHDs are present in the kidneys, with PHD2 as the predominant isoform of PHDs,21–25 and PHD2 is most abundantly expressed in the renal medulla.21–25 We have shown that PHDs participate in the regulation of renal medullary function.21 Given the important role of PHDs in the regulation
of HIF-1α levels and renal function, we hypothesize that PHD2 responds to high-salt intake and mediates the high-salt–induced increase of HIF-1α in the renal medulla. We examined the effect of high-salt intake on the expression of PHD2 and determined the role of PHD2 in high-salt–induced activation of HIF-1α by transfection of PHD2 transgenes into the renal medulla. We also revealed that there was an impaired response of HIF-1α to high salt, which was associated with PHD2, in the renal medulla from Dahl salt-sensitive rats by transfection of PHD2 small hairpin RNA (shRNA). Our results demonstrate that PHD2 is the mediator for the adaptive activation of renal medullary HIF-1α and its target genes in response to a high-salt challenge.

Materials and Methods

Animal
Experiments were performed in male Sprague-Dawley rats (Harlan, Madison, WI), as well as Dahl salt-sensitive and SS13BN rats (Charles River, Wilmington, MA), weighing 250 to 350 g. Animals were kept on a low-salt diet, and some of them were fed with a high-salt diet (4% NaCl) during experiments, as indicated in the Results section. All of the animal procedures were approved by the institutional animal care and use committee of the Virginia Commonwealth University.

Plasmids Expressing Rat Full-Length PHD2 cDNA and Rat PHD2 shRNA
Plasmids encoding rat full-length PHD2 cDNA were generous gifts from Dr Frank S. Lee (University of Pennsylvania). The expression and function of rat PHD2 protein by the plasmids have been validated by Dr Lee and colleagues26–27 and in our previous study.21 Rat PHD2 small interfering RNA sequences (sense: GUG UGA CAU GUA UAU AUU A; antisense: UAA UAU AUA CAU GUC ACA C) were designed and synthesized by QIAGEN. The target sequence is ATG TGT GAC ATG TAT ATA TTA (accession No.: NM_178334). After the confirmation of effective knocking down of PHD2 genes by these small interfering RNAs, the sequences were constructed into a pRNA-CMV3.2 vector (Genscript) to produce shRNA. The effective gene silencing of renal PHD2 by shRNA in vivo was also verified in preliminary experiments.

Transfection of DNA into the Renal Medulla
Rats were uninephrectomized 1 week before, and the remaining left kidney was transfected with designated plasmids (50 μg) into the renal medulla using in vivo-jetPEI (Polyplus-Transfection), a polyethylenimine derivative, in combination with ultrasound radiation. Previous studies have shown that DNA was successfully delivered into the renal medulla using in vivo-jetPEI,28 and that combination of ultrasound significantly enhanced the DNA transfection with different transfection reagents,11,29,30 including polyethylenimine nanoparticles.31 For the details of this and the following methods, see the expanded Materials and Methods section in the online Data Supplement, available at http://hyper.ahajournals.org. Our previous studies using a similar technique for DNA delivery into the cells in the renal medulla showed that >90% of cells were transfected with no cell-type selectivity.11 We also showed that the expression of transgene in the kidney peaked on approximately days 5 to 7 and gradually decreased thereafter, whereas the mRNA levels in transfected animals remained 4.5 times higher than that in control animals 4 weeks after transfection.12 The in vivo expression time period of the transgene in our studies is consistent with reports by others using nonviral vectors and different DNA delivery methods, which have shown that in vivo overexpression of transgenes lasts for ≥2 or 4 weeks.33–38

RNA Extraction and Quantitative RT-PCR
Analysis of PHD2, HO-1, NOS-2, and COX-2 mRNA
The relative mRNA levels were measured by real-time RT-PCR using TaqMan Gene Expression Assays kits (Applied Biosystems) with an iCycler iQ Real-Time PCR Detection System (Bio-Rad).

Preparation of Tissue Homogenate and Nuclear Extracts and Western Blot Analyses for Protein Levels of HIF-1α and PHD2
Renal tissue homogenates and nuclear protein were prepared, and Western blot analyses were performed as described previously.21 Primary antibodies used in the present study included anti-rat HIF-1α (monoclonal, Novus Biologicals, 1:300 dilution) and PHD2 (rabbit polyclonal, Novus Biologicals, 1:300). The intensities of the blots were determined using an imaging analysis program (ImageJ, free download from http://rsweb.nih.gov/ij/).

Determination of HIF Prolyl-Hydroxylase Activity
HIF-1α peptide-specific conversion of 2-oxoglutarate into succinate provides a hydroxyl group for HIF-1α to be prolyl-hydroxylated. This reaction has been widely used for the determination of PHD activity by measuring an HIF-1α–dependent conversion rate of 2-oxoglutarate into succinate.36,37

Measurement of Daily Sodium Balance
Additional groups of Sprague-Dawley rats were uninephrectomized and transfected with control or PHD2 plasmids into the renal medulla of the remaining kidney and then housed in metabolic cages 8 days after PHD2 transfection. Daily indices of sodium balance were computed by subtracting urinary sodium excretion from total sodium intake. After 2-day control measurements, the animals were switched from tap water to 2% NaCl water, and sodium balance measurements were continued for 4 additional days.11,38,39 At the end of experiment, PHD2 mRNA levels in the renal medulla were measured by real-time RT-PCR.

Statistics
Data are presented as mean±SE. The significance of differences in mean values within and between multiple groups was evaluated using an ANOVA test followed by a Duncan multiple range test. Student t test was used to evaluate statistical significance of differences between 2 groups. P<0.05 was considered statistically significant.

Results

Effect of High-Salt Intake on PHD2 Levels in the Renal Medulla in Sprague-Dawley Rats
As summarized in Figure 1, after a 10-day high-salt challenge, both mRNA and protein levels of renal medullary PHD2 were remarkably decreased in both control and PHD2 plasmid-transfected animals. Overexpression of PHD2 transgene produced a large increase in PHD2 expression compared with that in control animals. Although a high-salt diet also inhibited PHD2 levels in rats transfected with PHD2 plasmids, the PHD2 expression remained at the level shown in control rats on a low-salt diet. The HIF-PHD activity in the renal medulla, as measured by the conversion of 2-OG (2-oxoglutarate) into succinate, significantly increased in PHD2 plasmid-transfected rats on a low-salt diet (Figure 1D), suggesting that PHD activities were well correlated with PHD2 levels in the renal medulla.
Effect of PHD2 Transgene Overexpression on High-Salt–Induced Increase in HIF-1α Levels and Transcription of Its Target Genes in the Renal Medulla in Sprague-Dawley Rats

As shown in Figure 2, high-salt intake significantly increased the HIF-1α protein level, which is consistent with our previous finding.11 In PHD2-transfected rats, HIF-1α levels were remarkably decreased. Notably, high-salt–induced increases in HIF-1α levels were blocked in PHD2-transfected rats to a level similar of that in control rats on a low-salt diet.

The mRNA levels of 2 important HIF-1α target genes, HO-1 and NOS-2, in the renal medulla were shown in Table 1. Similar to the patterns of HIF-1α protein levels, high-salt–induced activation of both HO-1 and NOS-2 transcriptions was remarkably inhibited in PHD2-transfected rats, and the mRNA levels of HO-1 and NOS-2 in these rats fed with a high-salt diet were decreased to a level similar as that in control rats on a low-salt diet.

Comparison of High-Salt–Induced Changes in HIF-1α and PHD2 Levels in the Renal Medulla Between Dahl Salt-Sensitive and SS13BN Rats

SS13BN rats exhibit minimal differences in genotype from Dahl salt-sensitive rats and are considered as the best controls for hypertension studies in Dahl salt-sensitive rats.40–42 As such, SS13BN rats were used in the present study as the control for Dahl salt-sensitive rats. As shown in Figure 3, high-salt intake increased HIF-1α levels in SS13BN rats as in Sprague-Dawley rats presented above. However, the renal medullary HIF-1α level was significantly lower in Dahl salt-sensitive rats, and high-salt–induced increases in HIF-1α levels were absent in this rat strain compared with that in SS13BN rats. Interestingly, the PHD2 levels were signifi-
significantly higher, and high-salt–induced inhibition in PHD2 levels in the renal medulla was not observed in Dahl salt-sensitive rats (Figure 4A and 4B). Biochemical analysis showed that changes in PHD activities in response to a high-salt diet were different between these 2 rat strains, which followed the similar patterns observed in PHD2 expression (Figure 4C).

Comparison of High-Salt–Induced Increases in mRNA Levels of HIF-1α Target Genes in the Renal Medulla Between Dahl Salt-Sensitive and SS13BN Rats

The mRNA expressions of HIF-1α target genes HO-1, NOS-2, and COX-2, which play important roles in regulating renal medullary functions, were evaluated between Dahl salt-sensitive and SS13BN rats. As shown in Figure 5, high salt induced dramatic increases in mRNA levels of all 3 of the HIF-1α target genes in SS13BN rats. However, these high-salt–induced increases in the expressions of HO-1, NOS-2, and COX-2 genes were almost abolished in Dahl salt-sensitive rats.

Effect of PHD2 Gene Silencing on High-Salt–Induced Changes in HIF-1α Levels and Transcriptions of Its Target Genes in the Renal Medulla From Dahl Salt-Sensitive Rats

Intrarenal medullary transfection of PHD2 shRNA plasmids knocked down the PHD2 protein expression by 78% in Dahl salt-sensitive rats, as shown in Figure 6A and 6B. This PHD2 gene knocking down was accompanied by a significant upregulation of HIF-1α in the renal medulla in response to high-salt intake (Figure 6C and 6D). Meanwhile, 3 important HIF-1α target genes, HO-1, NOS-2, and COX-2, were significantly increased in PHD2 shRNA-transfected rats (Table 2).

Effects of Renal Medullary PHD2 Transgene Overexpression on Salt Balance

High-salt intake produced a positive daily sodium balance. The positive salt balances were progressively increased in the first 2 days and started to decrease on the third day of high-salt intake. The high-salt–induced positive salt balance was significantly greater in PHD2-transfected rats than that in control rats (Figure 7A). The mRNA levels were increased by 3-fold in PHD2-transfected rats (Figure 7B), which confirmed the overexpression of the PHD2 transgene in these rats.

Discussion

The present study showed that high-salt intake decreased PHD2 levels in the renal medulla and that overexpression of the PHD2 transgene blocked high-salt–induced activation in HIF-1α and its target genes, suggesting that high salt increases the HIF-1α level and thereby enhances expression of its target genes through inhibition of PHD2. However, the
activation of renal medullary HIF-1α and its target genes in response to a high-salt diet was not observed in Dahl salt-sensitive rats, which was associated with an absence of high-salt–induced inhibition in PHD2. Gene silencing of PHD2 restored high-salt–induced activation of the HIF-1α and its target genes in the renal medulla from Dahl salt-sensitive rats. Moreover, overexpression of the PHD2 transgene in the renal medulla impaired renal sodium excretion after salt loading. These results demonstrate that high salt inhibits PHD2 and thereby activates HIF-1α and its target genes in the renal medulla and that an impairment of this molecular adaptive response to high-salt intake may mediate sodium retention in salt-sensitive individuals.

HIF-1α target genes, such as NOS, COX-2, and HO-1, have been reported as crucial regulators in renal medullary function and sodium excretion, as well as pressure natriuresis. Activation of HIF-1α and its target genes by high-salt intake has been proposed to promote the excretion of extra sodium loading by regulating the renal medullary functions and thereby regulating the sodium balance in the body and salt sensitivity of blood pressure. The present study found that high-salt–induced activation of HIF-1α and its target genes was associated with PHD2 inhibition and that

### Table 2. Effect of PHD2 Transgene on HIF-1α Target Genes HO-1, NOS-2, and COX-2 mRNA Levels in the Renal Medulla in Dahl Salt-Sensitive Rats

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ctrl + LS</th>
<th>Ctrl + HS</th>
<th>PHD2shRNA + HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>1.01 ± 0.15</td>
<td>1.41 ± 0.16</td>
<td>7.28 ± 1.71*</td>
</tr>
<tr>
<td>NOS-2</td>
<td>0.99 ± 0.13</td>
<td>1.46 ± 0.14*</td>
<td>5.81 ± 1.15*</td>
</tr>
<tr>
<td>COX-2</td>
<td>1.00 ± 0.13</td>
<td>1.52 ± 0.18*</td>
<td>9.28 ± 2.28*</td>
</tr>
</tbody>
</table>

Ctrl indicates control plasmids; PHD2, PHD2 plasmids; LS, low-salt intake; HS, high-salt intake. Data were normalized to Ctrl + LS.

*P < 0.05 vs others (n = 6).
overexpression of PHD2 transgenes blocked the activation of HIF-1α and its target genes after high-salt challenge. These results indicate that PHD2 functions as an upstream signal that initiates HIF-1α-mediated gene activations in the renal medulla in response to high salt. Given the critical roles of the products of HIF-1α target genes HO-1, NOS-2, and COX-2 in the regulation of renal adaptation to high-salt challenge and the maintenance of sodium homeostasis, it is suggested that PHD regulation of HIF-1α in response to high salt in the renal medulla may reveal a novel mechanism involved in renal salt handling.

The above information suggests that the PHD/HIF-1α pathway is an important molecular mediator in renal salt adaptation under normal conditions. We wondered whether this PHD/HIF-1α pathway was involved in the pathogenic mechanism of abnormal renal sodium management in salt-sensitive hypertensive individuals. It has been reported that there is a defect in NOS-2, one of the HIF-1α target genes,18,53,54 whereas high-salt intake has been shown to inhibit PHDs in hypoxia.15 One of the possible mechanisms mediating downregulation of PHD2 by a high-salt diet may be that high-salt intake initially increases renal tubular activities35 and decreases renal medullary oxygen levels,32 which inhibits PHD2 activity and activates HIF-1α-mediated adaptive genes. The proteins encoded by these genes produce mediullary protective factors, including NO, which, in turn, inhibit PHD2. In addition, several inflammatory factors have been implicated in the regulation of PHD2 expression and/or HIF-1α,18,53,54 whereas high-salt intake has been shown to cause inflammation in the kidneys.55,56 Therefore, high-salt–induced inflammatory factors may be involved in the inhibition of PHD2. The interesting finding that the expression of the PHD2 transgene was decreased by high-salt intake in the present study indicated that reduced mRNA stability might be associated with high-salt–induced PHD2 inhibition. The exact mechanisms for high salt to inhibit PHD2 require further exploration.

Regarding the mechanism causing impaired PHD2 response to high salt in Dahl rats, increased oxidant stress57,58 might be one of the mechanisms. It has been shown that inhibition of NAD(P)H oxidase restores the diminished activation of NOS-2 in response to high-salt intake in the renal medulla in Dahl salt-sensitive rats.50 However, it is not yet clear how inhibition of NAD(P)H oxidase restores NOS-2 level in the kidneys in this rat strain. Because superoxide has been demonstrated to stimulate PHDs and thereby inhibit mediated gene activations in these renal medullary protective factors are impaired in this rat strain. In parallel to these results, a higher level of PHD2 and failed inhibition of PHD2 in response to high-salt intake in the renal medulla from Dahl salt-sensitive rats were observed. In addition, reduction in PHD2 levels by shRNA restored the levels of HIF-1α and its target genes HO-1, NOS-2, and COX-2 in the renal medulla in Dahl salt-sensitive rats. It is suggested that diminished HIF-1α in Dahl salt-sensitive rats is caused by abnormal PHD2 response to a high-salt diet. These data further support the view that PHD2 responds to high-salt intake and, thus, controls HIF-1α-mediated gene activation, consequently maintaining sodium balance. Our results further suggest that deficient PHD/HIF-1α-mediated molecular adaptation in response to high-salt intake in the renal medulla may represent a pathogenic mechanism producing salt-sensitive hypertension.

To further elucidate the role of renal medullary PHD2 in the regulation of sodium excretion, we compared the sodium balance between control and PHD2-transfected rats after salt loading. It was demonstrated that renal medullary overexpression of the PHD2 transgene remarkably impaired the capability of the kidneys to remove extra sodium load, which resulted in sodium retention. These data additionally suggest that renal medullary PHD2 is a crucial mediator in the signaling pathway sensing sodium intake and thereby importantly participates in the regulation of sodium excretion.

The present study did not attempt to explore the mechanisms by which high-salt challenge inhibits PHD2 in the renal medulla and what causes the abnormal response of PHD2 in Dahl salt-sensitive rats. In this regard, several pathways might be accountable for it. For example, NO has been shown to inhibit PHDs in hypoxia.15 One of the possible mechanisms mediating downregulation of PHD2 by a high-salt diet may be that high-salt intake initially increases renal tubular activities35 and decreases renal medullary oxygen levels,32 which inhibits PHD2 activity and activates HIF-1α-mediated adaptive genes. The proteins encoded by these genes produce medullary protective factors, including NO, which, in turn, inhibit PHD2. In addition, several inflammatory factors have been implicated in the regulation of PHD2 expression and/or HIF-1α,18,53,54 whereas high-salt intake has been shown to cause inflammation in the kidneys.55,56 Therefore, high-salt–induced inflammatory factors may be involved in the inhibition of PHD2. The interesting finding that the expression of the PHD2 transgene was decreased by high-salt intake in the present study indicated that reduced mRNA stability might be associated with high-salt–induced PHD2 inhibition. The exact mechanisms for high salt to inhibit PHD2 require further exploration.

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HIF-1α, it is possible that high-salt–induced oxidative stress induces PHD2 and thereby reduces HIF-1α levels in the renal medulla in Dahl salt-sensitive rats. Detailed mechanisms need to be clarified in future investigations.

Perspectives

Our results showed that PHD2-mediated activation of HIF-1α and its target genes in the renal medulla was an important molecular adaptive mechanism in response to high-salt intake, which augments the enzymes producing natriuretic factors. It was also demonstrated that there was a defect in this salt adaptive pathway in Dahl salt-sensitive rats, and impairment in this adaptive pathway caused sodium retention. It is concluded that high-salt–induced inhibition of PHD2 and subsequent HIF-1α–mediated gene activation may play important roles in the maintenance of sodium homeostasis under various sodium loadings. Therefore, modification of the PHD/HIF-1α pathway in the renal medulla may serve as a therapeutic approach for the management of salt-sensitive hypertension.

Sources of Funding

This work was supported by National Institutes of Health grant HL-89563.

Disclosures

None.

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HIF Prolyl-Hydroxylases Sense High Salt Intake to Increase Hypoxia Inducible Factor-1α Levels in the Renal Medulla

Zhengchao Wang, Qing Zhu, Min Xia, Pin-Lan Li, Shante J. Hinton, Ningjun Li

Department of Pharmacology & Toxicology, Medical College of Virginia,
Virginia Commonwealth University, Richmond, VA 23298

Supplement methods

**Transfection DNA into renal medulla.** Rats were uninephrectomized one week before and the remaining left kidney was transfected with designated plasmids into renal medulla. To deliver the plasmids into renal medulla, the anesthetized rats were placed on a thermostatic table to maintain a body temperature of 37°C, and then the left kidney was exposed by a flank incision (1-1.5 cm) and placed in a cup to stabilize the organ for implanting a medullary interstitial catheter (tapped tip, 4-5mm) into the kidney. The catheter was anchored into place on the kidney surface with Vetbond Tissue Adhesive (3M). A mixture containing 50 μg of DNA and 8 μl of *in vivo-jetPEI™* (Polyplus-transfection, New York, NY), a polyethylenimine derivative, in 500 μl 10% glucose was infused into renal medulla at a speed of 20 μl/min. After infusion, the catheter was cut and blocked by a piece of fat tissue with Vetbond Tissue Adhesive. An ultrasound transducer (Sonitron 2000, Rich-Mar) was directly applied onto the kidneys with a 1-MHz ultrasound at 10% power output, for a total of 60 s with 30-s intervals on each side of the kidney in the middle and at the end of the infusion. A previous study evidenced that shRNA expression plasmids were successfully delivered into renal medulla using *in vivo-jetPEI™*. It has been shown in our previous studies and others that combination of ultrasound significantly enhances the DNA transfection with different transfection reagents including polyethylenimine and polystyrene nanoparticles. In the present study, combination of ultrasound with *vivo-jetPEI* produced a high efficient gene delivery into the renal medulla as evidenced by a 4-fold increase in overexpression of transgene and 78% decrease in the protein expression of shRNA target gene.

**RNA extraction and quantitative RT-PCR analysis of PHD2, heme oxygenase (HO)-1, nitric oxide synthase (NOS)-2 and cyclooxygenase (COX)-2 mRNA.** Total RNA from renal medullary tissues was extracted using TRIzol solution and then reverse-transcribed (RT) (cDNA Synthesis Kit, Bio-Rad). The RT products were amplified using TaqMan® Gene Expression Assays kits (Applied Biosystems) with an iCycler iQ Real-Time PCR Detection System (Bio-Rad). The levels of 18S ribosomal RNA (rRNA) were used as an endogenous control. The relative gene expressions were calculated using cycle threshold (Ct) values in accordance with the ΔΔCt method. The Ct values were first normalized with respect to 18S rRNA levels to obtain ΔCt values. The averaged ΔCt value from the control group was used as a reference to calculate ΔΔCt values for all samples. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$. 

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Preparation of tissue homogenate and nuclear extracts, and Western blot analyses for protein levels of HIF-1α and PHD2. Nuclear protein was prepared as we described previously. Briefly, renal medullary tissue was homogenized with a glass homogenizer in ice-cold HEPES buffer (A) containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10% Nonidet P-40. After centrifugation of the homogenate at 1,000 x g for 5 min at 4°C, the supernatants and pellets were collected separately. The supernatants were centrifuged again at 6,000 x g for 10 min and resulting supernatants were used for Western blot analyses of PHD2.

For nuclear fraction isolation, the pellets from the first centrifugation, which contains cell nuclei, were washed with buffer A and then incubated with ice-cold HEPES buffer (B) containing 5 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 300 mM NaCl, 400 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 26% glycerol for 30 min to release nuclear proteins. Next, the reaction mixtures were centrifuged at 23,000 rpm for 30 min, and the supernatant was collected and frozen in liquid nitrogen until use as nuclear extracts for Western blot analyses of HIF-1α levels.

Western blot analyses were performed as described previously. Primary antibodies used in the present study included anti-rat HIF-1α (monoclonal, Novus Biologicals, 1:300 dilution) and PHD2 (rabbit polyclonal, Novus Biologicals, 1:300). The intensity of the blots was determined using an imaging analysis program (ImageJ, free download from http://rsbweb.nih.gov/ij/).

Determination of prolyl hydroxylase activity. Peptide-specific conversion of 2-oxoglutarate into succinate provides a hydroxyl group for HIF-1α to be prolyl hydroxylated. This reaction has been widely used for determination of PHD activity. Briefly, dissected renal cortical and medullary tissues were homogenized on ice using Dounce homogenizer in 6 volumes of buffer containing 0.15 mM MgCl₂, 10 mM KCl, 10 mM Tris-HCl, pH 6.7. Dextrose was added to a final concentration of 0.25 M, and the homogenate was centrifuged at 1500 x g for 15 min at 4 °C to remove nuclei. The mitochondrial fraction was separated by the second centrifugation at 6500 x 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 FeSO₄, 0.1 mM L-[5-¹⁴C]α-ketoglutaric acid (Moravek Biochemicals, Brea, CA), 200 μM ODD peptide (amino acids 556–575 of rat HIF-1α) (Advanced ChemTech, Louisville, KY), 0.25 mM ascorbate, 0.4 mg/ml catalase and 0.5 mM dithiothreitol. Samples were incubated for 1 h at 37 °C in a final volume of 50 μl. After incubation, 25 μl of mixture of 20 mM succinate and 2-oxoglutarate was added, followed by the addition of 25 μl of 0.16 M 2,4-dinitrophenylhydrazine in 30% HClO₄. The samples were allowed to sit for 30 min at room temperature following the addition of 50 μl of 1 M of 2-oxoglutarate. Supernatants were separated by spinning at 3000 x g for 5 min, and radioactivity measured using a liquid scintillation counter. Each set of experiments included control reactions without
addition of peptide of HIF-1α to calculate HIF-1α–dependent conversion of 2-oxoglutarate into succinate, which represents specific HIF prolyl-hydroxylase activity.

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