Hypoxia-inducible factor-1α contributes to the profibrotic action of angiotensin II in renal medullary interstitial cells

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To examine whether hypoxia-inducible factor (HIF)-1α mediates the profibrotic effects of angiotensin II, we treated cultured renal medullary interstitial cells with angiotensin II and found that it increased HIF-1α levels. This was accompanied by a significant upregulation of collagen I/III, the tissue inhibitor of metalloproteinase-1, elevation of the proliferation marker proliferating cell nuclear antigen, and a transdifferentiation marker vimentin. All these effects of angiotensin II were completely blocked by siRNA for HIF-1α but not HIF-2α. Overexpression of a prolyl-hydroxylase domain-containing protein 2 (PHD2) transgene, the predominant renal HIF prolyl-hydroxylase, attenuated the effects of angiotensin II and its gene silencing enhanced the effects of angiotensin II. Removal of hydrogen peroxide eliminated angiotensin II-induced profibrotic effects. A 2-week infusion of rats with angiotensin II increased the expression of HIF-1α and α-smooth muscle actin, another marker of transdifferentiation, in renal medullary interstitial cells in vivo. Thus, our study suggests that HIF-1α mediates angiotensin II-induced profibrotic effects through activation of cell transdifferentiation. We propose that redox regulation of prolyl-PHD2 plays a critical role in angiotensin II-induced activation of HIF-1α in renal cells.

Interstitial fibrosis is correlated with the progression of chronic renal diseases and has been proposed as a final common pathway to end-stage renal diseases.[1–3] Hypoxia-inducible factor (HIF)-1α has been recently associated with the progression of chronic renal injuries.[2,4–6] Although upregulation of HIF-1α has been shown to be protective in acute ischemic injury,[7–9] long-term activation of HIF-1α in chronic renal diseases is implicated to be pathogenic.[2,3,5,6,8,10–12] HIF-1α has been reported to be upregulated in chronic renal diseases.[2,4,5,8,13] It has also been demonstrated that HIF-1α stimulates collagen accumulation by activation of fibrogenic factors, such as plasminogen activator inhibitor and tissue inhibitor of metalloproteinase (TIMP).[13–16] Angiotensin II (ANG II) is a major pathogenic factor producing renal fibrosis in chronic renal injury.[3,4,17–19] Meanwhile, it has been shown that ANG II stimulates HIF-1α accumulation.[20,21] However, the contribution of HIF-1α to ANG II-induced profibrotic action has not been evidenced. In addition, the role of HIF prolyl-hydroxylases, the enzymes that promote the degradation of HIF-1α,[22–24] in the regulation of fibrogenesis has not been investigated. A recent study has shown that ANG II inhibits HIF prolyl-hydroxylase activity and increases HIF-1α level,[25] indicating a possible role of HIF prolyl-hydroxylases in ANG II-induced profibrotic action. HIF prolyl-hydroxylases are present in the kidneys and regulate HIF-1α levels.[7,26–28] Three HIF prolyl-hydroxylases, including prolyl-hydroxylase domain-containing proteins 1, 2, and 3 (PHD 1, 2, and 3), have recently been identified,[22,23,29] and PHD2 is the primary PHD in the kidneys.[7,26–28] This study was designed to test the hypothesis that HIF-1α accumulation by PHD inhibition is a critical mediator in the profibrotic action of ANG II using renal interstitial cells, one of the important cell types involved in progression of chronic renal diseases.[30–32]

We first utilized HIF-1α small interference RNA (siRNA) to silence the gene expression of HIF-1α and evaluated the contributing role of HIF-1α in ANG II-induced increases in collagen I/III and TIMP-1 in cultured renal medullary interstitial cells (RMICs). We then transfected the vectors expressing rat full-length PHD2 or rat PHD2 siRNA into the cells to determine whether PHD2 was involved in

ANG II-induced profibrotic action. To our knowledge, this study provides the first evidence suggesting that PHD/HIF-1α-mediated gene regulation importantly participates in ANG II-induced profibrotic effects in renal cells.

RESULTS
HIF-1α siRNA blocked ANG II-induced increases in collagen I/III, TIMP-1, proliferating cell nuclear antigen (PCNA), and vimentin

Our result showed that in RMICs, ANG II induced the accumulation in collagen I/III and TIMP-1 protein levels, which is consistent with previous reports.[18,33,34] To determine the role of HIF-1α in ANG II-induced increases in these fibrogenesis-associated factors, we examined whether gene silencing of HIF-1α would block these profibrotic effects of ANG II. As shown in Figure 1, in the cells transfected with HIF-1α siRNA, ANG II-induced increases in collagen I/III and TIMP-1 were abolished, suggesting that ANG II-induced stimulatory effects on collagen I/III and TIMP-1 are through the activation of HIF-1α. Figure 1d and e confirmed the accumulation of HIF-1α induced by ANG II, which was abolished by HIF-1α siRNA. The concentration of ANG II (10^{-6} M) used in this study was a concentration that induced the maximal activation of HIF-α based on the preliminary experiments (Supplementary Figure S2 online). This high concentration of ANG II allowed us to determine the inhibitory effect of HIF-1α siRNA on ANG II-induced activation of fibrogenic factors under the maximal stimulation.

Because both hypoxia and activation of ANG II have been implicated in chronic renal injury,[4] we determined whether ANG II and hypoxia synergistically stabilized HIF-1α. Our results demonstrated that hypoxia alone exhibited a stronger effect on HIF-1α levels than ANG II alone. However, ANG II + hypoxia did not show significantly further effects on HIF-1α accumulation compared with hypoxia alone (Supplementary Figure S3 online). These data indicate that ANG II and hypoxia may share the same pathway, probably by inhibition of PHD activity, in stabilizing HIF-1α.

In addition, cell proliferation and transdifferentiation have been reported to participate in ANG II-induced renal tubulointerstitial injury.[35,36] Therefore, we further determined the role of HIF-1α in ANG II-induced cell proliferation and transdifferentiation. Our results showed that gene silencing of HIF-1α blocked ANG II-induced increases in the transcriptions of PCNA, a marker of cell proliferation,[37] and vimentin, a marker of cell transdifferentiation[38] (Figure 2a and b). As ANG II has been reported to induce epithelial-to-mesenchymal transdifferentiation/transition,[39,40] we also detected PCNA and vimentin mRNA levels in rat renal tubular cells (NRK-52E; ATCC, Manassas, VA) and demonstrated that HIF-1α siRNA similarly blocked ANG II-induced increases in PCNA and vimentin in renal epithelial cells (Figure 2c and d). These results suggest that activation of HIF-1α mediates ANG II-induced cell proliferation and transdifferentiation.
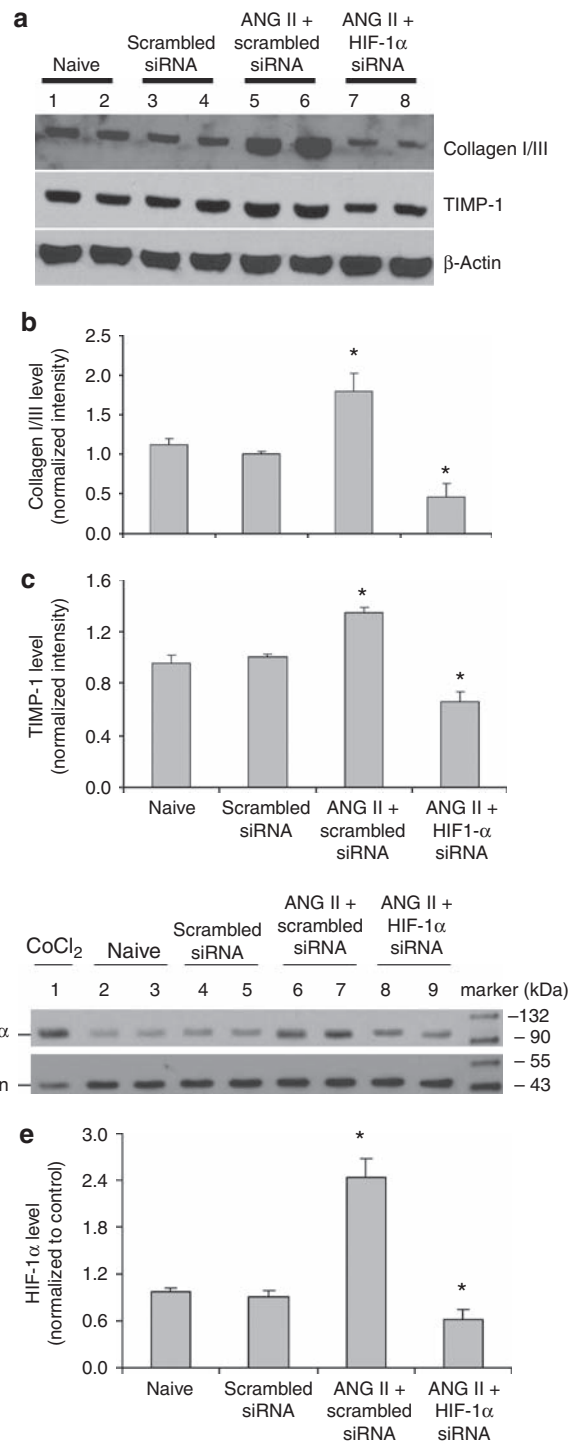

Figure 1 | Effect of hypoxia-inducible factor (HIF)-1α small interference RNA (siRNA) on angiotensin II (ANG II)-induced increases in collagen I/III and tissue inhibitor of metalloproteinase-1 (TIMP-1) in renal medullary interstitial cells (RMICs) by western blot analysis. (**a, d**) Representative enhanced chemiluminescence (ECL) gel documents of western blot analyses depicting the protein levels of collagen I/III, TIMP-1, and HIF-1α. (**b, c, e**) Summarized intensities of collagen I/III, TIMP-1, and HIF-1α blots normalized to control. *$P < 0.05$ vs all other groups ($n = 6$). In (**d**), the sample from CoCl$_2$-treated cells was used as a positive control. A blot image containing full-size markers for (**d**) is presented in Supplementary Figure S1 online to further illustrate the location of HIF-1α blots.
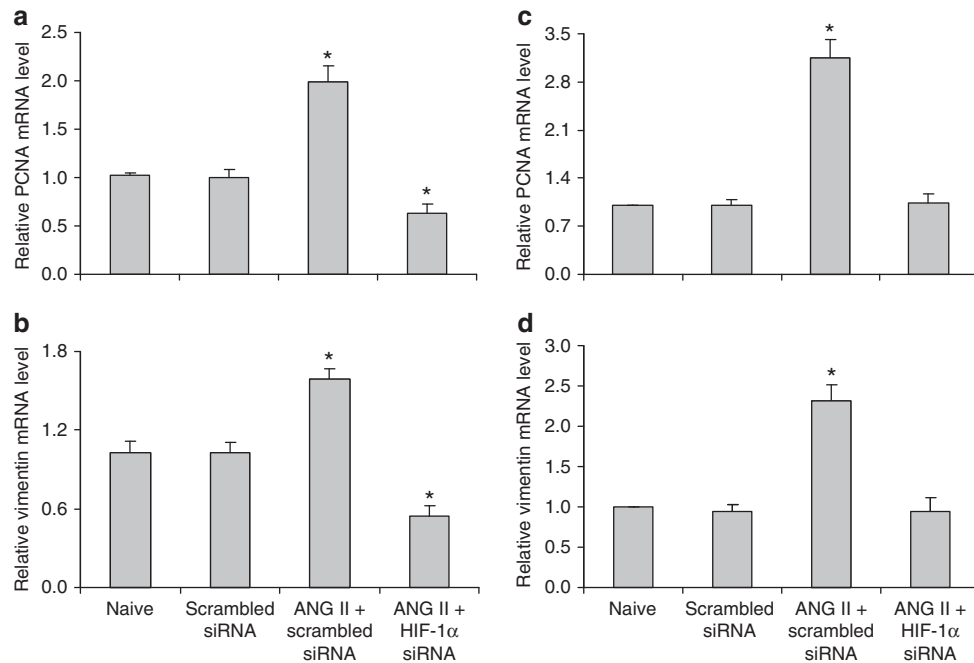
Figure 2 | **Effect of hypoxia-inducible factor (HIF)-1α small interference RNA (siRNA) on angiotensin II (ANG II)-induced increases in proliferating cell nuclear antigen (PCNA) and vimentin mRNA levels by real-time reverse transcriptase (RT)-PCR analysis. (a, b)** In renal medullary interstitial cells (RMICs) and (**c, d**) in tubular cells. *$P < 0.05$ vs all other groups ($n = 6$).

HIF-2α siRNA did not affect ANG II-induced increases in collagen I/III and TIMP-1 in RMICs

It has been shown that HIF-2α is expressed in renal interstitial cells.[41,42] We determined the contribution of HIF-2α to the effects of ANG II on collagen I/III and TIMP-1 in RMICs. Our results showed that the mRNA levels of HIF-2α was about 25 times less than that of HIF-1α, and that although HIF-2α siRNA decreased HIF-2α levels by 76%, it did not affect ANG II-induced changes in collagen I/III and TIMP-1 (Figure 3). These data suggest that HIF-2α is not the primary isoform of HIF in these cells and considerably low level of HIF-2α does not significantly contribute to the profibrotic action of ANG II in RMICs in this study.

PHD2 regulated HIF-1α, collagen I/III, and TIMP-1 levels

It has been well characterized that PHDs promote the degradation of HIF-1α.[22] Although the results above showed that HIF-1α was involved in ANG II-induced profibrotic action, the role of PHDs in this process remained to be proven. To address this issue, we first verified the over-expression or silencing of *PHD2* gene by transfection of PHD2-expressing plasmid or PHD2 siRNA vectors (Figure 4a). We also confirmed that expression of PHD2 transgene decreased HIF-1α protein level and silencing of *PHD2* gene increased the HIF-1α levels in our experiments (Figure 4b and c). Interestingly, overexpression of PHD2 transgene decreased collagen I/III and TIMP-1, and silencing of *PHD2* gene increased collagen I/III and TIMP-1 protein levels (Figure 5). These results suggest that PHD2 regulates fibrogenesis-associated factors in RMICs. PHD2 was chosen because it is the predominant PHD in the kidneys[7,27,28,43] and

is also quantitatively predominant in the cells used in this study (Supplementary Figure S4 online).

Manipulation of *PHD2* gene altered ANG II-induced increases in collagen I/III and TIMP-1

The above data proved that PHD2 regulated fibrogenesis-associated factors. However, it was not clear whether PHDs participated in the physiological/pathological processes associated with these fibrogenesis-associated factors. To determine the role of PHD in the regulation of fibrogenic factors in response to pathogenic stimulation, we examined the effect of gene silencing or gene transfection of PHD2 on ANG II-induced profibrotic action. Figure 6 shows that in RMICs transfected with PHD2 plasmids, ANG II-induced increases in collagen I/III and TIMP-1 were significantly attenuated. In contrast, these ANG II-induced increases in collagen I/III and TIMP-1 were significantly enhanced when *PHD2* gene was silenced. These results demonstrated that PHD2 mediated the effects of ANG II on collagen I/III and TIMP-1. Our data showed that ANG II increased the levels of PHD2, indicating that the stimulatory effects of ANG II on HIF-1α, collagen I/III, and TIMP-1 were not via the downregulation of PHD2 expression.

As PHD1 and PHD3 are present in renal cortical interstitial cells,[44] and HIF-1α in turn regulates the levels of PHDs,[44-47] we also evaluated the effects of ANG II and PHD2 siRNA on PHD1 and PHD3 levels. It was found that ANG II and PHD2 siRNA did not affect PHD3 levels, whereas PHD1 was marginally increased by ANG II (Supplementary Figure S4 online). It is known that the expression, regulation, and action of three PHDs are different in specific tissues or cells.[29,44-47]

Figure 4 | Effect of prolyl-hydroxylase domain-containing protein 2 (PHD2) or PHD2 small interference RNA (siRNA) expression vectors on the levels of PHD2 and hypoxia-inducible factor (HIF)-1α. (a) The relative mRNA levels of PHD2 by real-time reverse transcriptase (RT)-PCR analysis. (b) Representative enhanced chemiluminescence (ECL) gel documents of western blot analyses depicting the protein levels of HIF-1α. (c) Summarized intensities of HIF-1α blots normalized to control. *$P < 0.05$ vs all other groups ($n = 6$).

These data suggest that PHD2, but not PHD1 and PHD3, is the predominant enzyme in the cells used in this study.

ANG II inhibited PHD activity and increased collagen I/III and TIMP-1 via stimulation of H_2O_2 production

The next question was that by what mechanism ANG II inhibited the action of PHD2. As ANG II did not down-regulate the levels of PHD2, as shown in Figure 6, we investigated the effects of ANG II on PHD activities and consequent changes in collagen I/III and TIMP-1. PHDs catalyze site-specific proline hydroxylation of HIF-1α, which allows recognition by the von Hippel–Lindau tumor-suppressor protein that targets hydroxylated HIF-1α (HIF-1α-OH) for its degradation by the ubiquitin–proteasome pathway. Therefore, the levels of HIF-1α-OH represent the activity of PHDs. As illustrated in Figure 7, ANG II
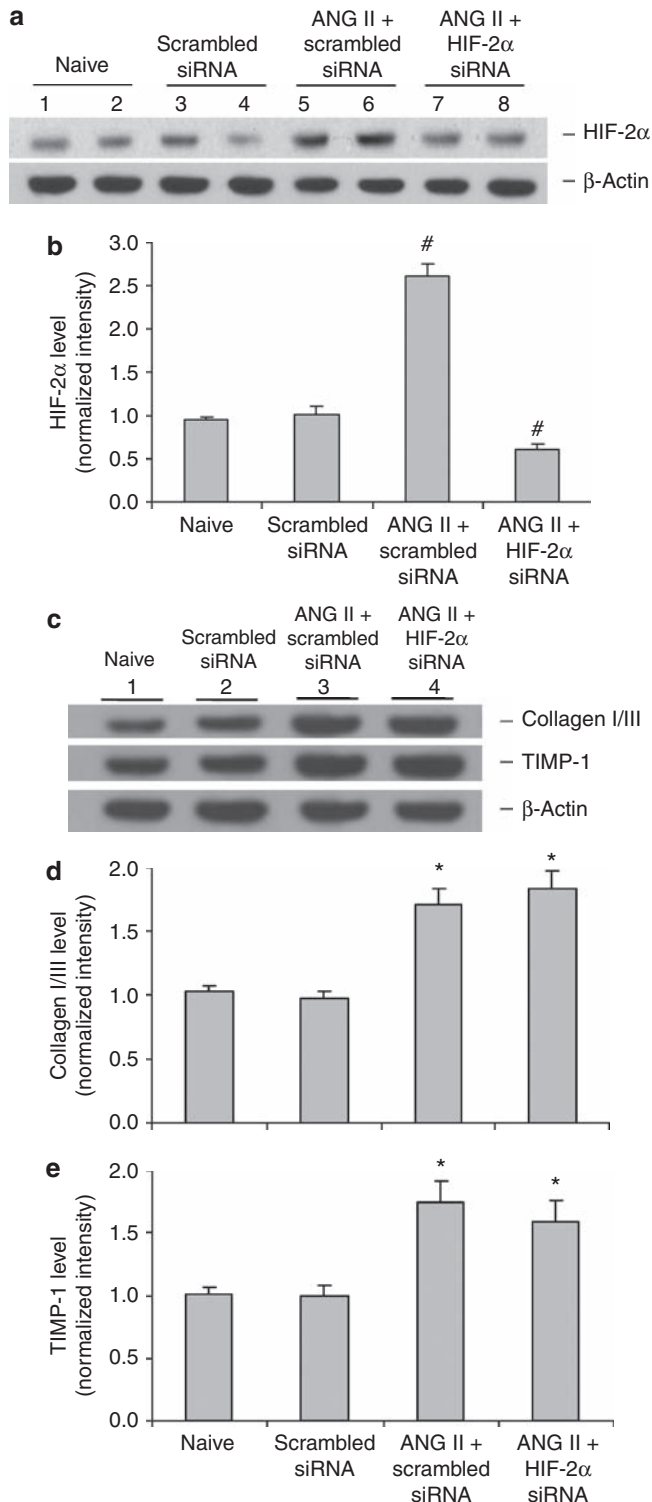
Figure 3 | Effect of hypoxia-inducible factor (HIF)-2α small interference RNA (siRNA) on angiotensin II (ANG II)-induced increases in collagen I/III and tissue inhibitor of metalloproteinase-1 (TIMP-1) in renal medullary interstitial cells (RMICs) by western blot analysis. (a, c) Representative enhanced chemiluminescence (ECL) gel documents of western blot analyses depicting the protein levels of HIF-2α, collagen I/III, and TIMP-1. (b, d, e) Summarized intensities of HIF-2α, collagen I/III, and TIMP-1 blots normalized to control. #$P < 0.05$ vs all other groups. *$P < 0.05$ vs control groups. ($n = 6$).
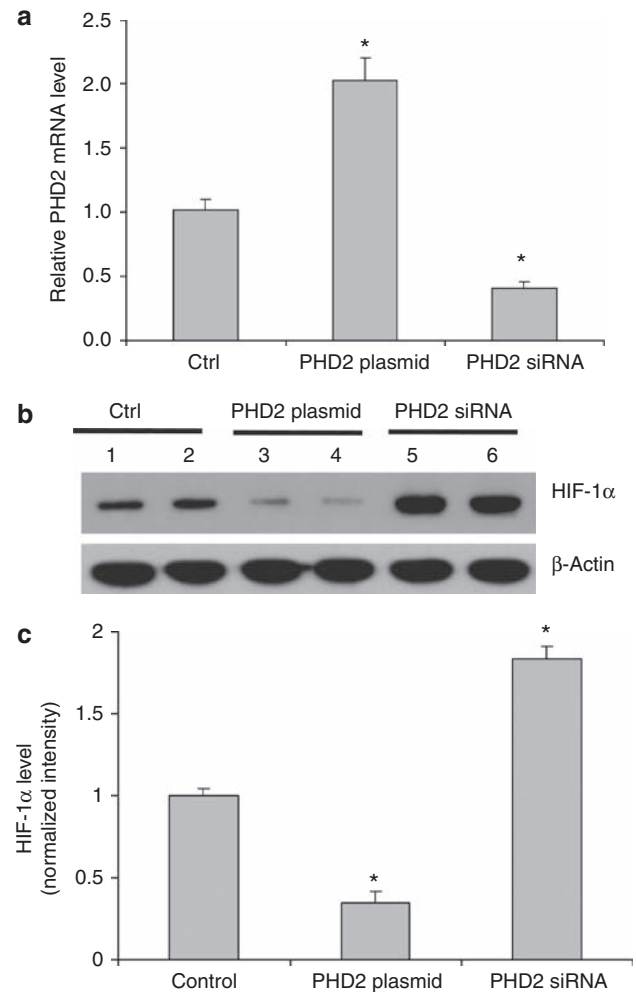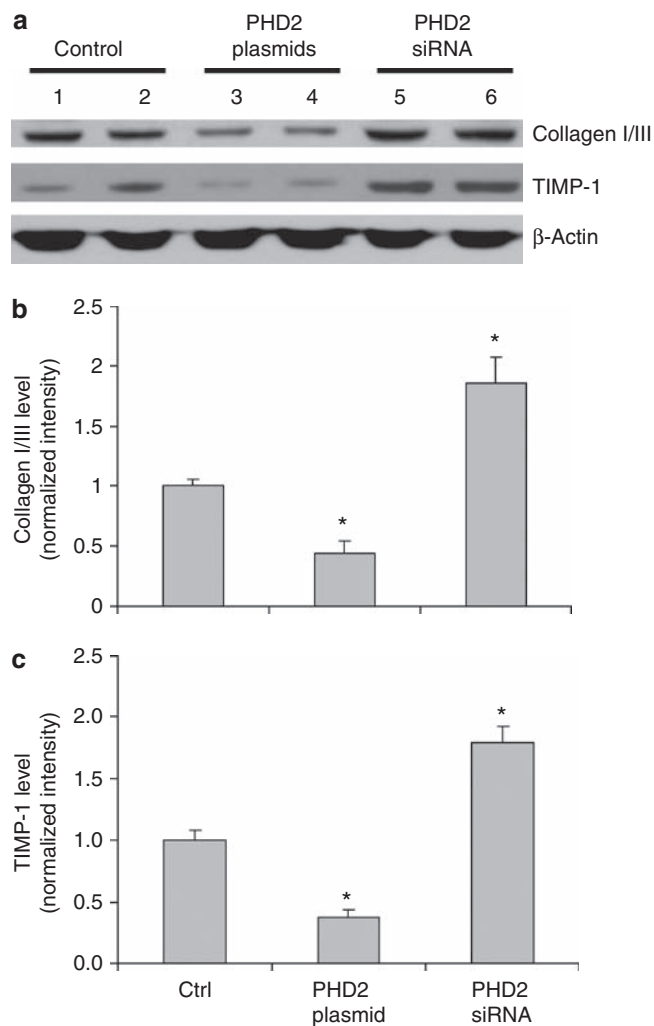
Figure 5 | Effect of prolyl-hydroxylase domain-containing protein 2 (PHD2) or PHD2 small interference RNA (siRNA) expression vectors on the levels of collagen I/III and tissue inhibitor of metalloproteinase-1 (TIMP-1) in renal medullary interstitial cells (RMICs) by western blot analysis.
(**a**) Representative enhanced chemiluminescence (ECL) gel documents of western blot analyses depicting the protein levels of collagen I/III and TIMP-1. (**b, c**) Summarized intensities of collagen I/III and TIMP-1 blots normalized to control. *$P < 0.05$ vs all other groups ($n = 6$).

significantly reduced the HIF-1α-OH levels (Figure 7a, lines 3 and 4), suggesting that ANG II inhibits the hydroxylylating activities of PHDs. Reduced levels of HIF-1α-OH were accompanied by increases in collagen I/III and TIMP-1, indicating that ANG II-induced profibrotic effects are through inhibition of PHD activities in RMICs.

As ANG II was shown to increase H_2O_2,[48,49] and H_2O_2 to inhibit PHD2 activity,[25] we further elucidated the role of H_2O_2-induced PHD2 inhibition in the profibrotic action of ANG II in RMICs. These data are presented in Figure 7. In the cells treated with ascorbate or catalase to eliminate H_2O_2 effects or remove H_2O_2, ANG II-induced decreases in HIF-1α-OH were returned to the control levels (Figure 7a, lines

5–8). In contrast to the changes in HIF-1α-OH, ANG II-induced increases in collagen I/III and TIMP-1 were decreased to normal levels by these antioxidative treatments. These results demonstrated that increased H_2O_2 production by ANG II inhibited PHD activities and consequently promoted the activation of fibrogenic factors. In additional experiments, we also tested the effects of exogenous H_2O_2 and found that exogenous H_2O_2 exhibited similar inhibitory effects on HIF-1α-OH levels and stimulatory effects on collagen I/III and TIMP-1 as ANG II did (Figure 7a, lines 9 and 10). These results further indicated that the actions of ANG II were through stimulation of H_2O_2 production. ANG II-induced oxidative stress was confirmed by detecting the increases in superoxide production (Figure 8a and b) and H_2O_2 levels (Figure 8c) in ANG II-treated cells. Overall, these data showed that ANG II increased TIMP-1 and induced the accumulation of collagen I/III via inhibition of PHD activity by stimulating the production of H_2O_2.

Infusion of ANG II stimulated the expression of HIF-1α and α-smooth muscle actin (SMA) in RMICs *in vivo* in the kidneys
Infusion of ANG II (150 ng/kg/min) for 2 weeks significantly increased the number of HIF-1α- and SMA-positive RMICs in the kidneys by immunohistochemical analysis (Figure 9). SMA is also a molecular hallmark of fibroblast activation during fibroblast to myofibroblast transition.[50–52] These data demonstrate that ANG II-stimulated activation of HIF-1α and transdifferentiation in RMICs also occurs in the kidneys *in vivo*.

DISCUSSION
This study showed that gene silencing of HIF-1α blocked ANG II-induced increases in TIMP-1 and collagen I/III, as well as PCNA and vimentin. It was also demonstrated that PHD2 mediated the effects of ANG II on HIF-1α, collagen I/III, and TIMP-1, and that ANG II induced collagen I/III and TIMP-1 via stimulating the production of H_2O_2 that inhibited PHD activity. These results suggest that ANG II-induced profibrotic action may be mediated by activation of HIF-1α because of redox inhibition of PHD activity.

In this study, we used the increases of TIMP-1 and collagen as indicators of ANG II-induced profibrotic action. To our knowledge, the findings from this study provide the first evidence that ANG II-induced activation of fibrogenesis-associated genes is mediated by activation of HIF-1α. This study did not show the significant contribution of HIF-2α in ANG II-induced profibrotic action in RMICs. The reason for a very low level of HIF-2α is probably that HIF-2α-expressing cells are mainly located in the cortex and outer strip of outer medulla,[41,42] whereas the RMICs used in this study are isolated from the inner medulla. The cells used in this study seem not to be characterized similar to the interstitial cells located in the cortex with respect to HIF-2α expression. However, it has been reported that the medullary interstitial fibrotic injuries are similar to or more profound than cortical interstitial fibrotic injuries in chronic renal diseases.[53,54]
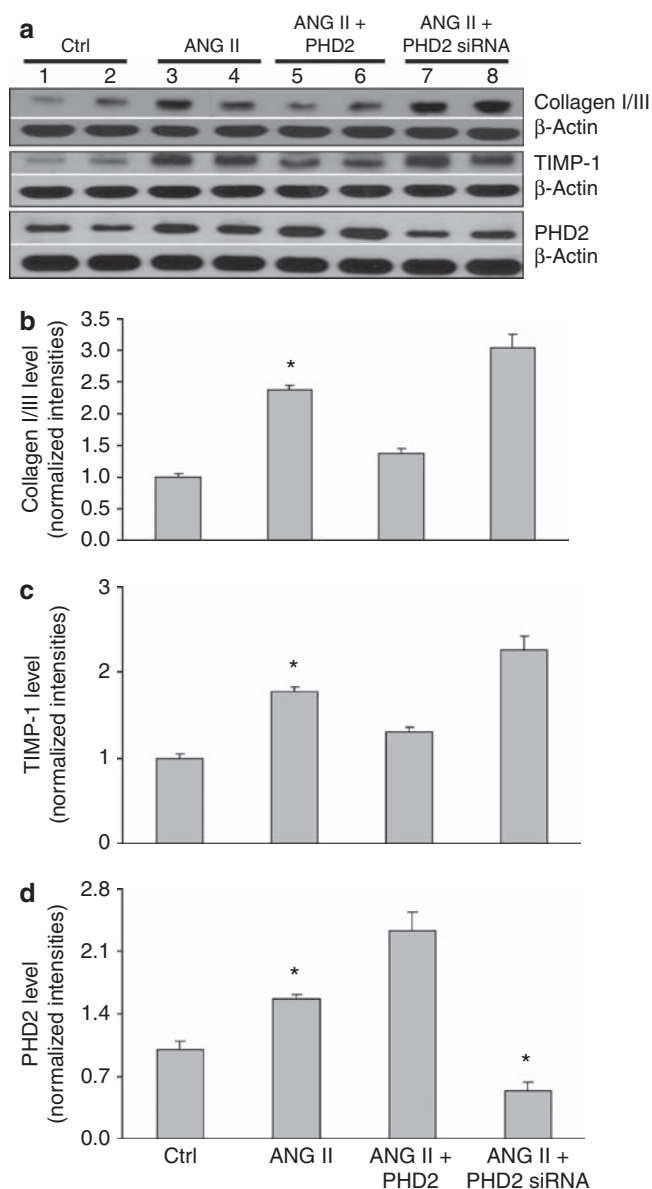
Figure 6 | Effect of prolyl-hydroxylase domain-containing protein 2 (PHD2) or PHD2 small interference RNA (siRNA) expression vectors on the levels of collagen I/III, tissue inhibitor of metalloproteinase-1 (TIMP-1), and PHD2 in the presence of angiotensin II (ANG II) in renal medullary interstitial cells (RMICs) by western blot analysis. (a) Representative enhanced chemiluminescence (ECL) gel documents of western blot analyses depicting the protein levels of collagen I/III, TIMP-1, and PHD2. (**b–d**) Summarized intensities of collagen I/III, TIMP-1, and PHD2 blots normalized to control. *$P < 0.05$ vs all other groups ($n = 6$).

Therefore, the medullary interstitial cells used in this study may present a model of cells involved in renal medullary interstitial damage.

Another interesting finding in this study is that HIF-1α may also mediate ANG II-induced cell transdifferentiation. Cell transdifferentiation, including epithelial–mesenchymal transition, importantly contributes to the development of renal fibrosis in chronic renal diseases.[55–58] During

fibrogenesis, renal resident fibroblasts are activated to transform/transdifferentiate into myofibroblasts, which are primary cell resources to produce extracellular matrix.[59–61] Increased expression of vimentin has been shown to be the indicator of transition from fibroblasts to myofibroblasts.[31,52] Although both HIF-1α[5,16,62] and ANG II[39,40] have been shown to promote cell transdifferentiation, the interaction between HIF-1α and ANG II in the process of cell trans-differentiation remains unclear. Our results demonstrate that HIF-1α may participate in ANG II-induced transdifferentiation of renal cells. These data suggest that HIF-1α pathway may be involved in the relatively early stage of ANG II-induced profibrotic actions, which may represent a novel mechanism linking HIF-1α-regulated gene transcription to ANG II-induced profibrotic action.

HIF-1α regulates many target genes and thereby has an important role in many physiological processes. Therefore, there may be a concern that blockade of HIF-1α-mediated gene expression may also prevent some important physio-logical responses and impair normal cell functions. Because there is an overactivation of HIF-1α after ANG II treatment or in chronic renal diseases, inhibition of HIF-1α under these pathological conditions should aim at counteracting excessive HIF-1α activity and eliminating its pathological impact, thereby restoring normal physiological regulation. Therefore, inhibition of HIF-1α could be a useful strategy to reduce the fibrogenesis by resetting such excessive HIF-1α activity to the normal level in a variety of pathological conditions.

PHDs have been reported to regulate the target genes of HIF-1α via their actions degrading HIF-1α.[63–66] Given the critical role of HIF-1α in fibrogenesis, PHDs may also be regulators of fibrogenesis. Indeed, our results demonstrated that PHD2 regulates the levels of TIMP-1 and collagen in renal cells, which may reveal a novel pathway that modulates the fibrotic process. Although PHDs work as oxygen sensors to regulate the destruction of HIF-1α by promoting the oxygen-dependent proline hydroxylation of HIF-1α,[22–24] recent evidences have clearly shown the oxygen-independent regulation of HIF-1α and PHDs.[67–70] Most importantly, ANG II has also been shown to inhibit PHD activity and upregulate HIF-1α levels.[25] This study proved the hypothesis that PHD participates in the regulation of fibrogenic factors and is involved in ANG II-induced profibrotic action. Overexpression of PHD2 transgenes overcame ANG II-induced profibrotic effects, suggesting that PHD may be used as an anti-fibrotic factor under different pathological situations, such as activation of ANG II in chronic renal diseases. In this respect, targeting PHD to regulate HIF-1α and its target genes is emerging as a novel therapeutic strategy in a variety of disease conditions such as tumor[64] and postischemic organ injuries.[63,71–73] The findings from this study that PHDs participate in the regulation of fibrogenic factors under control condition and after ANG II treatment may stimulate the development of intervention associated with PHD/HIF pathway to retard the progression of chronic renal diseases.
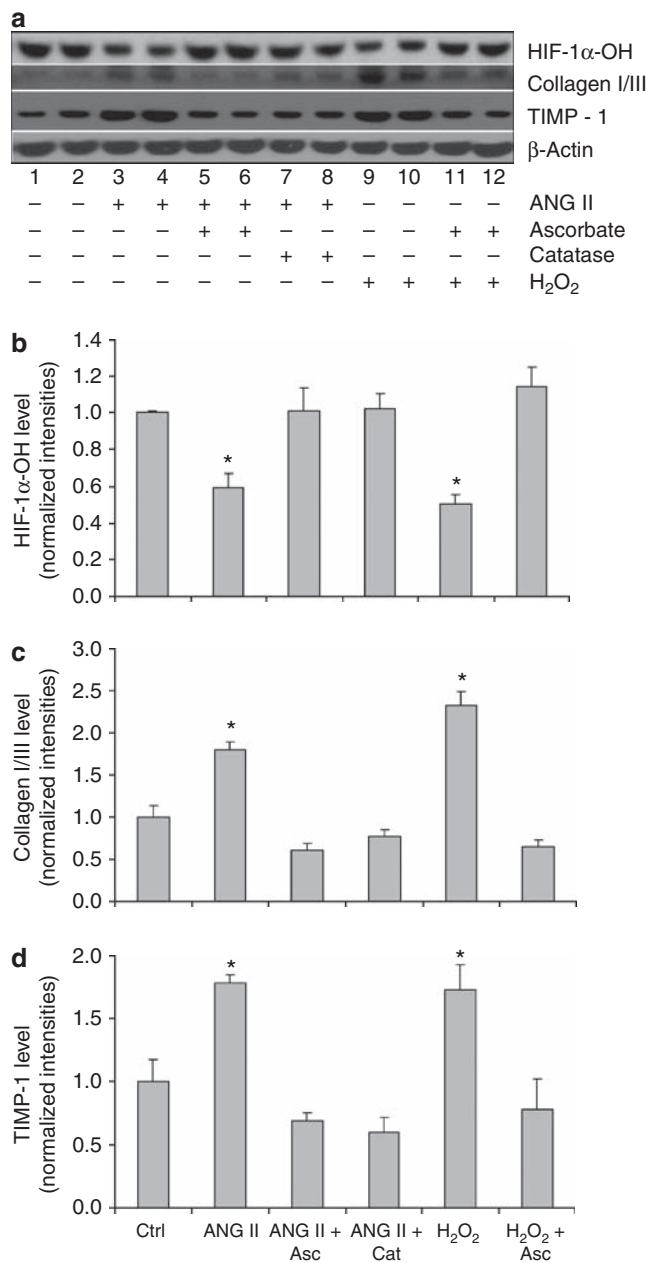
Figure 7 | Effect of angiotensin II (ANG II) or H_2O_2 on the levels of hydroxylated hypoxia-inducible factor-1α (HIF-1α-OH), collagen I/III, and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the presence or absence of catalase or ascorbate by western blot analysis. (a) Typical enhanced chemiluminescence (ECL) gel documents of HIF-1α-OH, collagen I/III, and TIMP-1 protein levels. (b–d) Summarized intensities of HIF-1α-OH, collagen I/III, and TIMP-1 blots normalized to control. *$P < 0.05$ vs all other groups except the group also marked with * ($n = 6$).

Notably, our results showed that ANG II increased PHD2 levels. This ANG II-induced increase in PHD2 levels is probably caused by a feedback mechanism because of increase in HIF-1α level, because PHD2 is one of HIF-1α target genes and activation of HIF-1α upregulates PHD2 levels.[74] This study found that ANG II stimulated HIF-1α activation and increases in collagen I/III and TIMP-1 through

inhibition of PHD activity rather than downregulation of the PHD expression. In addition, we showed that ANG II inhibited PHD activity via stimulating H_2O_2 production. These data demonstrate, for the first time, that ANG II stimulates fibrogenic factors via activation of H_2O_2 production and that H_2O_2 promotes fibrotic process via inhibition of PHD activity. Although redox signaling has been indicated in chronic renal injury, detailed mechanisms remain to be clarified. Our data suggest that redox regulation of PHD activity and thereafter manipulation of fibrogenic factors may represent an important mechanism mediating chronic renal injury by oxidant stress.

This study focused on the role of HIF-1α/PHD pathway in ANG II-induced activation of fibrogenic factors using cultured RMICs. Our finding that ANG II stimulated HIF-1α and SMA in RMICs in vivo further suggests that HIF-1α/ PHD pathway may be involved in ANG II-induced chronic renal injury. The exact significance of HIF-1α/PHD pathway in chronic renal injury needs to be further clarified using chronic renal disease models associated with ANG II activation. In this regard, there were controversial reports on the role of HIF-1α in different chronic renal disease models. A recent study demonstrated that stable expression of HIF-1α in tubular epithelial cells promoted interstitial fibrosis in 5/6 nephrectomy mice,[12] whereas a previous report showed that inhibition of PHD to upregulate HIF-1α protected the kidneys from damage in 5/6 nephrectomy rats.[75] In addition, it was reported that genetic ablation of renal epithelial HIF-1α inhibited the development of renal tubulointerstitial fibrosis in unilateral ureteral obstruction rats,[16] whereas upregulation of HIF-1α by cobalt, an inhibitor of PHD activity, was shown to ameliorate renal injury in an obese, hypertensive type 2 diabetes rat model.[76] These discrepancies might be attributed to the differences in disease models, disease stages, and approaches manipulating HIF-1α. Apparently, more detailed investigations are required regarding the role of HIF-1α/PHD pathway in the chronic renal diseases.

In summary, this study demonstrated that ANG II stimulated H_2O_2 production, which inhibited PHD activity and thereby upregulated HIF-1α levels, and consequently activated TIMP-1, resulting in collagen I/III accumulation in RIMC cells. It is concluded that PHD2 as a novel redox-sensitive enzyme is critical to the regulation of HIF-1α levels when renal interstitial cells were exposed to ANG II. Such PHD-mediated regulation of HIF-1α level and activity could be one of the important early mechanisms inducing transdifferentiation and promoting the upregulation of fibrogenic genes in renal cells under profibrotic stimulations.

MATERIALS AND METHODS
Animals
Experiments were performed on male Sprague-Dawley rats (Harlan, Madison, WI), weighing 250–300 g. All animal procedures were approved by the institutional animal care and use committee of the Virginia Commonwealth University.
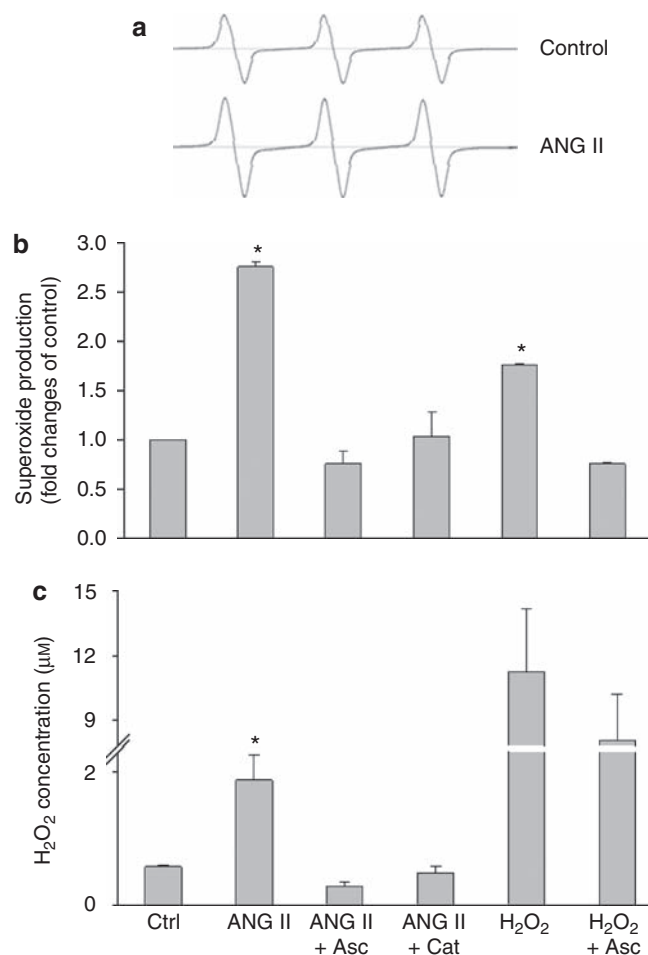
Figure 8 | Effects of angiotensin II (ANG II), catalase, and ascorbate on the levels of superoxide (O$_2^{\bullet-}$) and H$_2$O$_2$. (a) Representative electronic spin resonance (ESR) spectrograph of O$_2^{\bullet-}$ trapped by 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine in control and ANG II-treated cells. (b) Summarized data showing O$_2^{\bullet-}$ production in cells treated with different reagents. (c) Summarized data showing H$_2$O$_2$ levels in culture media from cells treated with different reagents. *$P < 0.05$ vs all other groups. ($n = 6$). Asc, ascorbate; Cat, catalase.

Isolation and culture of rat RMICs

RMICs were isolated and cultured as described previously.[43] Briefly, the renal inner medulla from inbred male Sprague-Dawley rats was finely minced, and the tissue suspension was injected subcutaneously into a recipient rat (from the same litter). At 4 days after injection, the firm yellow nodules located at the site of injections were dissected, removed, minced, and trypsinized, and then washed and centrifuged to obtain a cell pellet. The cell suspension was transferred to plastic tissue culture flasks and then incubated with modified culture medium. These cells formed a confluent monolayer in 18–21 days. The cells of seventh and eighth passage were used for experiments. The identity of these cells was confirmed by rich lipid granules within the cells stained with Sudan Black B and Oil Red O (Figure 10), which are characteristics of these cells.[77–79]

Transfection of HIF-1α and HIF-2α siRNA

Transfection of siRNA was performed using the siLentFect lipid reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. For a 10 cm dish, 200 pmoles of siRNA was used. After 5 h incubation

in transfection reagent, the cells were then switched to normal medium for additional 16 h and were ready for experiment. The sequence of HIF-1α siRNA was: sense, 5′-GGAAAGAGACUCAUAGAAA-3′ antisense, 5′-UUUCUAUGACUCUCUUUCC-3′ (Sigma-Aldrich, St Louis, MO). The sequence of HIF-2α siRNA was: sense, 5′-GCAGAUGGA UAACUUGUAC-3′; antisense, 5′-GUACAAGUUAUCCAUCUGC-3′ (Applied Biosystems, Carlsbad, CA). A scrambled small RNA (QIAGEN, Valencia, CA), which was confirmed as non-silencing double-stranded RNA, was used as control for siRNA experiments.

Transfection of plasmids expressing rat PHD2 or rat PHD2 siRNA into the cells

Plasmid transfections were performed using lipids (DOTAP/DOPE; Avanti Polar Lipids, Alabaster, AL) according to the manufacturer's instructions. In brief, 5 μg of DNA was mixed with lipid solution in a ratio of 1:10 (DNA/lipid, w/w) in serum-free culture medium (5 ml for a 10 cm dish). Cells were incubated with this transfection medium for 5 h and switched to normal medium for another 16 h. The cells were then ready for experiment. In preliminary experiments, almost all cells were positive after transfection with luciferase plasmids when detected by bioluminescent imaging (IVIS200; Caliper Life Sciences, Hopkinton, MA), demonstrating a high transfection efficiency (data not shown). Sequences used for producing rat PHD2 siRNA were: sense, 5′-GTGTGACATGTATATATTA-3′; antisense, 5′-TAATATATA CATGTCACAC-3′(QIAGEN). The DNA sequence was constructed into pRNAT-CMV3.2 vector (GenScript, Piscataway, NJ) to generate plasmids that express rat PHD2 siRNA. Plasmids encoding full-length rat PHD2 were generous gifts from Dr Frank S. Lee (University of Pennsylvania). The expression and function of rat PHD2 protein by this plasmid has been validated by Dr Lee[80,81] and by us.[43] Our preliminary data showed effective gene knockdown or gene overexpression of PHD2 by these plasmids in cultured RMICs. Luciferase plasmids (Promega, Madison, WI) were used as control for PHD2 and PHD2 siRNA expression vector transfection experiments.

Cell treatment and experimental groups

After siRNA or plasmid transfections, the cells were switched to serum-free medium containing 10^{-6} M of ANG II. After ANG II treatment for 20 h, the cells were harvested for protein and RNA isolation as described below. Some of the cells were treated with H$_2$O$_2$ (5×10^{-5} M), ascorbate (10^{-4} M), or catalase (1000 U/ml) as indicated in the experimental groups in results. The concentrations of H$_2$O$_2$ and ANG II used in this study did not cause detectable cell damage as measured by lactate dehydrogenase activity (Assay Kit; Cayman Chemical, Ann Arbor, MI) (Supplementary Figure S5 online).

Preparation of nuclear extracts and cytosolic protein, western blot analyses for protein levels of HIF-1α, HIF-2α, TIMP-1, collagen I/III, and PHD2

Nuclear protein was prepared as we described previously.[43] Cytosolic protein and nuclear protein were collected separately. The cytosolic protein was used for western blot analyses of TIMP-1, collagen I/III, and PHD2. The reason for detecting collagen I/III is that the subtype of collagen is tissue/cell specific and collagen I/III is the one expressed in renal interstitial cells. The nuclear fraction was used for western blot analyses of HIF-1α and HIF-2α. Western blot analyses were performed as described previously.[43] The intensity of the blots was determined using an imaging analysis program (ImageJ, free download from http://rsbweb.nih.gov/ij/). The primary antibodies used in this study included anti-rat HIF-1α and HIF-2α
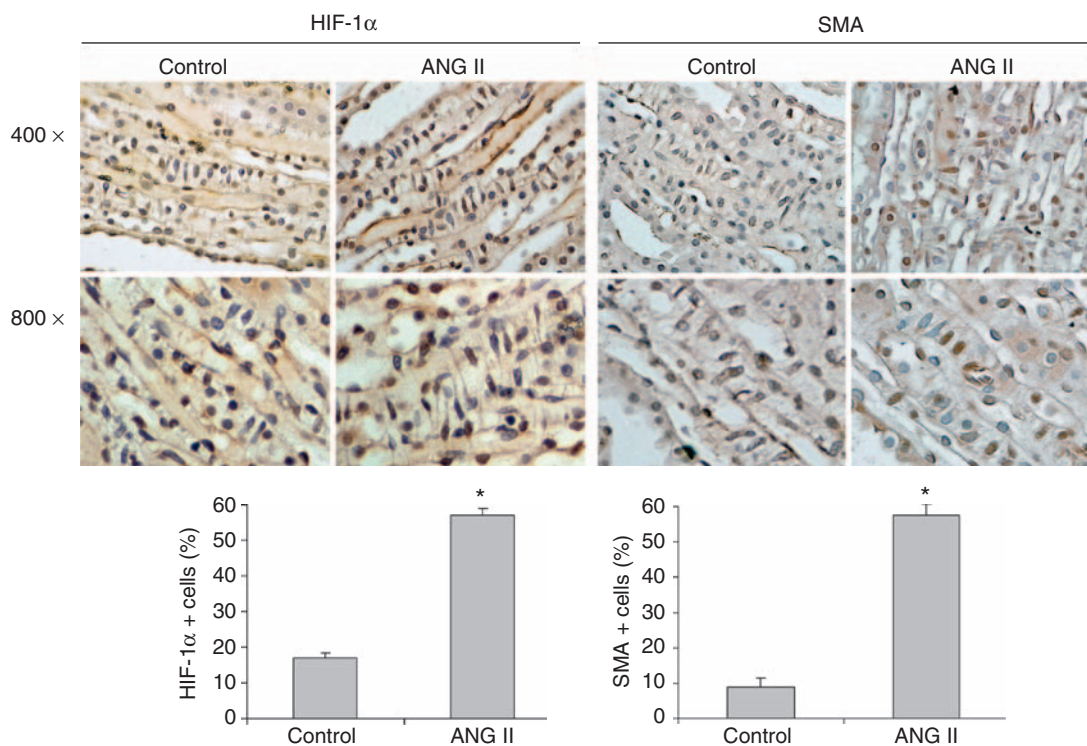
Figure 9 | Immunohistochemical staining of hypoxia-inducible factor (HIF)-1α and α-smooth muscle actin (SMA) in the renal inner medulla. (Top panels) Representative photomicrographs. Brown color indicates positive staining. (Bottom panels) Percentage quantitation of positive cells. *$P < 0.05$ vs control ($n = 5$). Renal medullary interstitial cells (RMICs) were identified by their unique morphological features of a ladder-like arrangement with the long axis of the cells perpendicular to the long axis of the papilla.
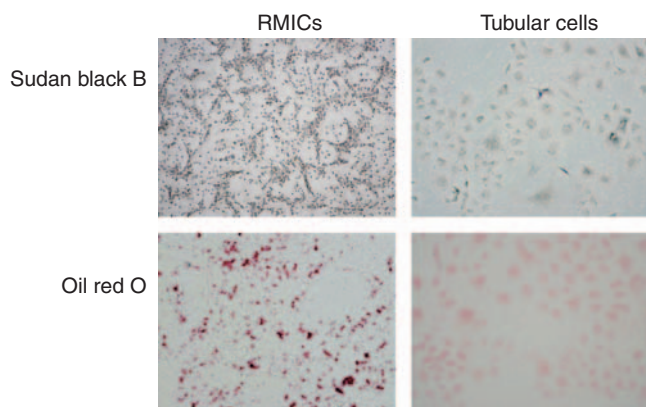

Figure 10 | Sudan Black B and Oil Red O staining in renal medullary interstitial cells (RMICs) and tubular cells. The cell shapes were apparently different between these two types of cells. There were numerous positive-staining vesicles in RMICs. In contrast, there were few such positive-staining vesicles observed in tubular cells (original magnification × 200).

(monoclonal, 1:300 dilution; Novus Biologicals, Littleton, CO), HIF-1α-OH (rabbit polyclonal, 1:500; Novus Biologicals), PHD2 (rabbit polyclonal, 1:300; Novus Biologicals), TIMP-1 (monoclonal, 1:1000; R&D Systems, Minneapolis, MN), and collagen I/III (rabbit polyclonal, 1:300; Calbiochem, San Diego, CA). For the details of this and the following methods, see the Expanded Materials and Methods section in online Supplement.

RNA extraction and quantitative reverse transcriptase-PCR analysis of the mRNA levels of PNCA, vimentin, and PHD

Total RNA was extracted using TRIzol solution (Life Technologies, Rockville, MD) and then reverse-transcribed (cDNA Synthesis Kit; Bio-Rad). The reverse-transcribed products were amplified using a TaqMan Gene Expression Assays kit (Applied Biosystems). A kit for detecting the levels of 18S ribosomal RNA was used as an endogenous control. The relative gene expressions were calculated in accordance with the $\Delta\Delta$Ct method. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

Superoxide ($O_2^{\cdot-}$) detection by electronic spin resonance

The measurement of $O_2^{\cdot-}$ by electronic spin resonance was performed according to the methods in our previous studies.[82,83]

Fluorescence spectrometric assay of H_2O_2 concentrations

Amplex red is a fluorogenic substrate with very low background fluorescence; it reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin.[84] Fluorescence spectrometric assay of H_2O_2 levels in culture medium was performed using an Amplex red kit (Molecular Probes, Eugene, OR) as we described previously.[85]

Infusion of ANG II and immunohistochemical analysis of HIF-1α and α-SMA expression in RMICs in the kidneys

Rats were infused with ANG II (150 ng/kg/min; Sigma-Aldrich) for 2 weeks using ALZET mini-osmotic pumps (Model 2002) implanted intraperitoneally. At the end of infusion, kidneys were removed, cut

longitudinally, fixed in 10% neutral buffered formalin, and then processed for immunostaining as we described before[43] using antibody against rat HIF-1α 1:200 and SMA (rabbit polyclonal, 1:200; Abcam, Cambridge, MA). Expressions of HIF-1α and SMA in RMICs were evaluated by counting RMICs in 10 microscopic fields (×400, around 100 cells) and then the percentages of positive-staining RMICs were calculated. RMICs were identified by their unique morphological features, that is, ladder-like arrangement with the long axis of the cells perpendicular to the long axis of the papilla.[86,87]

Statistics

Data are presented as means ± s.e. The significance of differences in mean values within and between multiple groups was evaluated using an analysis of variance 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.

SUPPLEMENTARY MATERIAL

Figure S1. Representative ECL gel documents of western blot analysis with full-size markers depicting the location of HIF-1α blots.
Figure S2. Effect of ANG II on HIF-1α levels at different concentrations in RMICs by western blot analysis.
Figure S3. HIF1α protein level in the RMICs treated with Ang II and/or hypoxia (1% O_2 for 4 h).
Figure S4. Effect of ANG II and HIF-1α siRNA on the mRNA levels of PHD1, 2, and 3 in RMICs by real-time RT-PCR analysis.
Figure S5. Effect of ANG II and H_2O_2 on lactate dehydrogenase (LDH) activity (assay kit; Cayman).
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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