
SPIRONOLACTONE, BUT NOT ENALAPRIL, POTENTIATES HYPOXIA-INDUCIBLE FACTOR-1α AND ETS-1 EXPRESSION IN NEWBORN RAT KIDNEY

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Hypoxia is regarded as an important physiological factor that controls nephrogenesis. We investigated whether the renin-angiotensin-aldosterone system (RAAS) affects hypoxia-related target genes in developing kidneys. Newborn rat pups were treated with enalapril (30 mg/kg/d) or spironolactone (200 mg/kg/d) for 7 days. Tissue hypoxia was assessed by the uptake of a hypoxyprobe-1, pimonidazole (200 mg/kg), and the expression of hypoxia-responsive genes. In the enalapril group, hypoxia-inducible factor (HIF)-1α, HIF-2α, and Ets-1 protein expression were not changed, compared to the control group. In the spironolactone group, HIF-1α and Ets-1 protein expression were significantly increased by immunoblots and immunohistochemistry, whereas HIF-2α protein expression was not changed, compared to the control group. In the enalapril group, the immunoactivity of pimonidazole was not significantly different from that of the controls. However, in the spironolactone group, pimonidazole staining demonstrated that the cortex and medulla underwent severe hypoxia. In summary, our data showed that aldosterone inhibition in the developing kidney augmented the hypoxic responses, and up-regulated the expression of key mediators of hypoxia including HIF-1α and Ets-1. Angiotensin II inhibition did not affect hypoxia-related alterations in the developing kidney. The components of RAAS may differentially modulate renal hypoxia and its related target genes in the developing rat kidney.

Key words: angiotensin, aldosterone, hypoxia, kidney development, hypoxia-inducible factor, angiotensin converting enzyme

INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) has been implicated as a potent mitogenic signal that regulates the genes involved in cell proliferation and growth of the developing kidney; in addition, it is a major contributor in the development of renal disease (1). Previously, we suggested the mitogen activated protein kinase family as one of the angiotensin II- and aldosterone-related intracellular signaling pathways of renal proliferation and apoptosis in the developing kidney (2, 3). It has been reported that the mitogen activated protein kinase reaction cascade is involved in hypoxia-induced gene expression (4), and that this interaction suggests a cooperation between hypoxic and growth factor signals.

Of note, hypoxia has been proposed as a major regulatory factor associated with nephrogenesis (5). In comparison to maternal blood oxygen tension, fetal blood pO₂ is low (6). During early development, the poorly developed vascular tree may contribute to inadequate local blood delivery; such regional hypoxia is believed to play a major regulatory role in tissue maturation in the developing kidney. Hypoxia markers have been activated in areas where cells are undergoing high rates of proliferation and in tissues with incomplete or developing vascular networks (7, 8). However, the underlying molecular mechanisms involved in these processes are incompletely understood (9).

A key mediator of the altered gene expression in hypoxic response is the hypoxia-inducible factor (HIF), which is a heterodimeric transcription factor consisting of α-(HIF-1α or HIF-2α) and β-subunit (HIF-1β). Under normoxic conditions, the prolines in the HIFα chains are hydroxylated by oxygen-dependent prolyl hydroxylases. The hydroxylated prolyl residues have been noted in the Von Hippel Lindau protein as components of an E3 ubiquitin ligase, which destroys HIFα during proteasomal degradation. Under hypoxic conditions, prolyl hydroxylation and HIFα degradation are avoided (10). Vascular abnormalities and embryonic lethality have been observed in mice with targeted disruption of the HIF-1α, HIF-2α, and HIF-1β (11-13).

Hypoxia induces transcription factor Ets-1 expression via the activity of HIF-1α in endothelial cells and renal tubular cells (14, 15). The Ets family is defined by a conserved DNA-binding Ets domain that forms a winged helix-turn-helix structure motif and is involved in cellular growth, migration, and differentiation (16). Ets-1 was the first member identified in the Ets family and mediates embryogenesis, angiogenesis, cell migration, and invasion (17). During morphogenesis, Ets-1 is expressed in vascular structures and branching tissues, including the kidneys (18).

The neonatal rat is a useful model for kidney developmental studies since rats are born with immature kidneys and there is considerable postnatal renal development. The developing rat kidney is dependent on an intact RAAS from gestational day 15 to postnatal day 13 (19). All components of the RAAS are crucially expressed in the developing kidney in a spatial and temporal pattern, and plasma aldosterone levels are highest in newborns (20).
The goal of this study was to determine whether hypoxia is present during renal development and whether the RAAS is involved in hypoxia-regulated cellular processes of the developing kidneys. To investigate this, we examined the expression pattern of hypoxia-related target genes in newborn rat kidneys and enalapril- or spironolactone-treated kidneys. To correlate the findings with the degree of hypoxia, we also evaluated the immunoreactivity of pimonidazole, a recognized marker of severe cell and tissue hypoxia (21).

MATERIALS AND METHODS

Animal preparation

Neonatal rat pups from six pregnant Sprague Dawley rats were breastfed by their own mother throughout the study and divided into two groups. Group 1 was given 30 mg/kg of enalapril (the enalapril-treated group, n=22) dissolved with water or the same amount of vehicle (the control group 1, n=16) via an orogastric tube. This dose of enalapril is known to block the effects on angiotensin II (22). Group 2 was treated with 200 mg/kg of spironolactone (Sigma Chemical Co., St. Louis, MO, USA) in olive oil (the spironolactone group, n=20) or olive oil (the control group 2, n=16). This dose of spironolactone is known to protect the cardiovascular system against the deleterious effects of hemodynamic overload (23). Because spironolactone was not dissolved in saline, we liquefied it in the olive oil. To identify the effects of the olive oil, we compared its effect with that of normal saline in control kidneys, and there were no differences observed (Western blots for HIF-1α, HIF-2α and Ets-1; data not shown).

The rats were sacrificed at eight days post partum (N8). Their kidneys were harvested and processed for the study. One whole kidney (right kidney) from each rat was used for light microscopy and immunohistochemistry, and the other whole kidney (left kidney) for Western blot analysis. The experimental protocol was approved by the Animal Care Committee of the Korea University Guro Hospital.

Protein extraction

The kidney tissues were frozen in liquid nitrogen and stored at -70°C. Total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, OH, USA) and homogenized by a homogenizer (Model 985-370, Biospec Products, Bartlesville, OK, USA). Then, 37% chloroform (200 μl/ml TRI REAGENT) was added to the homogenates. The homogenates were centrifuged at 12000 rpm for 15 min at 4°C, and separated into three layers, RNA, DNA, and protein. To precipitate the DNA, the interface and organic phases remaining after the RNA extraction, as described previously (2), were treated with 100% alcohol and centrifuged at 5000 rpm and 4°C for 5 min. Isopropanol was added to the residual phenol-ethanol upper layer, centrifuged, and the precipitate was treated with 0.3 M guanidine hydrochloride dissolved in 95% ethanol. This layer was centrifuged at 10000 rpm and 4°C for 10 min after incubation at room temperature for 15 min; this process was repeated three times. It was then washed with 0.3 M guanidine hydrochloride dissolved in 95% ethanol to obtain a protein pellet. The protein extract was dissolved in 1% sodium dodecyl sulfate (SDS) and stored at -20°C. The Bradford method was used to quantify the protein.

Immunoblot analysis

The extracted proteins were solubilized in 5×SDS loading buffer for 5 min at 95°C and separated by electrophoresis on 10% SDS-polyacrylamide gels under reducing conditions. Equal amounts of 5-15 μg of proteins were loaded per lane. Subsequently the proteins were transferred to nitrocellulose membranes (Amersham Life Science, Buckinghamshire, England). The nitrocellulose membranes were blocked in 5% skim milk with TBS-T (0.05% Tween 20 in 50 mM of Tris, 150 mM of NaCl, and 0.05% NaN3, (pH 7.4)) at room temperature for 1 h. The membranes were washed two times in TBS-T and incubated for 18 h at 4°C with rabbit polyclonal antiseraum HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:200), HIF-2α (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:200), and Ets-1 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:200). Thereafter, the membranes were washed two times with TBS-T and incubated for 40 min with an anti-rabbit IgG (Amersham Life Science) and an anti-mouse IgG (Amersham Life Science) at room temperature. After washing with TBS-T four times, the secondary antibody bound to the nitrocellulose was detected by incubation for 1 min with a detection reagent (Amersham Life Science) and then exposed to medical x-ray film (Agfa, Mortsel, Belgium) for 1 min. The film was developed with the FPM-3500 Fuji x-ray Film Processor (Fuji, Otawara, Japan). After that, for stripping and reprobing, the same membranes were submerged in stripping buffer (2% SDS in 62.5 mM of Tris-HCL and 0.1 M of [beta]-mercaptoethanol) and incubated at 50°C for 10 min with occasional shaking. To control for equal loading, α-tubulin (1:1000 dilution; Cell signalg technolgy, Beverly, MO, USA) and anti-mouse IgG conjugated horseradish peroxidase (1:1000 dilution; Amersham Life Science) were used as primary and secondary antibodies with the same method as described above. The developed x-rays were scanned using the Epson GT-9500 (Seiko Corp, Nagano, Japan) and quantified by a densitometer (Image PC alpha 9).

Reverse transcription and polymerase chain reaction (RT-PCR)

Oligo dT-primed first-strand DNA was synthesized from the 1 ul RNA template by using a cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN, USA). Synthetic cDNA was used to amplify HIF-1α, Ets-1 and glyceraldehye-3-phosphate dehydrogenase (GAPDH), a house-keeping gene. Using primers for GAPDH designed from DNA templates of rats (5'-AATGG AATGCTGCAACCCCAA-3', and 5'-GTAGCCATATCCTGAT CAT-3'), 515 base pairs (bp) of the PCR product were obtained (24). Products of 460 bp and 399 bp for each gene (HIF-1α and Ets-1) were obtained from primers of HIF-1α (5'-GCGCATCCCTGCTGCAAAAG-3', and 5'-TTGGCGCTTGAT CAT-3'), 515 base pairs (bp) of the PCR product were obtained (24). Products of 460 bp and 399 bp for each gene (HIF-1α and Ets-1) were obtained from primers of HIF-1α (5'-GCGCATCCCTGCTGCAAAAG-3', and 5'-TTGGCGCTTGAT CAT-3'), 515 base pairs (bp) of the PCR product were obtained (24).

Immunohistochemical staining

For assessing expression, five kidneys in each group were selected for representative immunohistochemical staining of HIF-1α and Ets-1, using an avidin-biotin immunoperoxidase method (Vectastain ABC kit, Burlingame, CA, USA). The harvested kidneys were treated in 10% formalin solution (Sigma Chemical
Co., St. Louis, USA.) and embedded in paraffin. The samples were then cut into 4 µm sections and dried onto siliconized slides (Muto-Glass, Japan). The paraffin sections were deparaffinized with xylene, followed by rehydration in a descending series of ethanol. Then, the endogenous peroxidase activity was quenched in 0.6 % hydrogen peroxide for 15 min. Antigen retrieval was performed with 0.1% citric acid (DAKO Co., CA, USA). After quenching and antigen retrieval, the sections were incubated with primary antibodies against HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:50) and Ets-1 (Santa Cruz Biotechnology; dilution 1:50). As negative controls, the primary antibody was substituted with PBS. The incubation time was overnight at 4°C. After incubation, the sections were washed twice in PBS for 5 min, and incubated for 30 min with secondary antibodies (peroxidase-conjugated anti-rabbit IgG (Vectastain ABC kit, Burlingame, CA, USA; dilution 1:200)). Then, the slides were washed in PBS, and incubated for 50 min with the Vectastain ABC reagent. The immunoreaction products were developed using 3, 3-diaminobenzidine as the chromogen, at standard development times. The sections were counterstained in 0.5% methyl green solution (Trevigen, Gaitisburg, MD, USA) for 5 min, dehydrated, and evaluated using light microscopy (×400).

**Detection of tissue hypoxia by pimonidazole adducts**

The newborn rats were injected intraperitoneally with pimonidazole (Hypoxyprobe-1; Natural Pharmacia International, Inc., Belmont, MA, USA) at a dose of 200 mg/kg, 1 hour prior to sacrifice. Pimonidazole, delivered in vivo, reliably binds to tissue at oxygen concentrations below 10 mmHg and forms immunogenic protein adducts in hypoxic cells (21). Formalin-fixed sections were processed by an avidin-biotin immunoperoxidase method (Vectastain ABC kit, Burlingame, CA, USA) followed by exposure, for 40 min, with 1:200 pimonidazole-adduct antibodies. The sections were then incubated for 10 min in rabbit anti-mouse IgG and streptavidin peroxidase, respectively. The immunoreaction products were developed in diaminobenzidine at standard development times. No counterstaining was employed.

**Identification of hypoxic signals in serial sections**

The immunohistochemical stains for pimonidazole, HIF-1α, and Ets-1 expression, in the serial sections from the same kidney of the spironolactone-treated group, were performed and the spatial changes were compared. All stained tubules in one serial section that could clearly be identified in a corresponding serial section, by local landmarks, were evaluated.

**Statistical analysis**

Data are presented as the mean±SEM. Differences between the groups were analyzed by the t test. Statistical significance was defined as a P<0.05. The sigmastat version 2.0 for Windows was used for the analysis.

**Fig. 1. Hypoxic cells in developing kidney. (A-C) control kidneys (D-F) enalapril-treated group (G-I) spironolactone-treated group. The arrows indicate strong positive pimonidazole staining in each group (A,D,G original magnification x 40; B,E,H x 100; C,F,I x 400).**
RESULTS

On day 1, the body weight was 7.43±0.34 g in the control group 1 and 7.47±0.19 g in the enalapril-treated group. On day 8, the body weight was 16.9±0.36 g in the control group 1 and 15.7±0.46 g in the enalapril-treated group, and the kidney weight was 0.128±0.005 g in the control group 1 and 0.129±0.002 g in the enalapril-treated group. The body and kidney weights of the enalapril-treated group were not different from those of the control group 1 (P>0.05).

On day 1, the body weight was 7.23±0.32 g in the control group 2 and 7.62±0.15 g in the spironolactone-treated group. On day 8, the body weight was 16.15±0.52 g in the control group 2 and 13.13±0.49 g in the spironolactone-treated group, and the kidney weight was 0.11±0.004 g in the control group 2 and 0.09±0.004 g in spironolactone-treated group. The body and kidney weights of the spironolactone-treated group were significantly lower than those of the control group 2 (P<0.05).

Detection of tissue hypoxia by pimonidazole adducts

In the control N8 kidney, hypoxic signals were observed in the cortex and medulla, and the expression was especially high in the medullary cells. Hypoxyprobe-1 binding was clearly

Fig. 2. HIF-1α, HIF-2α and Ets-1 protein expression in the enalapril-treated group. (A) HIF-1α (B) HIF-2α (C) Ets-1 (n=5 for each group) (CONT; control group, ENAL; enalapril-treated group).

Fig. 3. HIF-1α, HIF-2α and Ets-1 protein expression in the spironolactone-treated group. (A) HIF-1α (B) HIF-2α (C) Ets-1 (n=5 for each group) (CONT; control group, SP; spironolactone-treated group) (A,C; * P<0.05).
observed in the tubular and collecting duct cells of the renal medulla, whereas the renal cortex exhibited only moderate pimonidazole staining in some of the developing and maturing tubules (Fig. 1A-C).

**Enalapril and hypoxic signals**

In the enalapril-treated group, HIF-1α, HIF-2α, and Ets-1 protein expression were not changed, compared to the control group 1 (Fig. 2A-C). Pimonidazole staining was significant in the dilated cortical and medullary tubular cells in the enalapril-treated group; however, the overall intensity of the hypoxic signals was not much different from that of the control group (Fig. 1D-F). Immunohistochemistry and RT-PCR for HIF-1α, HIF-2α, and Ets-1 were not performed because there were no significant changes in western blotting between the two groups.

**Spironolactone and hypoxic signals**

1. **HIF-1α expression**

The immunoblots showed that HIF-1α/tubulin protein expression was significantly increased in the spironolactone-treated group compared to the control group 2 (P<0.05; Fig. 3A). There was no difference in HIF-1α/GAPDH mRNA expression between the two groups (Fig. 4A). The immunohistochemical staining showed that HIF-1α expression was easily detected within the glomeruli and the tubular epithelial cells throughout the cortex and medulla in the control N8 kidney (Fig. 5A and B).

![Image of immunohistochemstry and western blot](image-url)

**Fig. 4.** HIF-1α and Ets-1 mRNA expression in the spironolactone-treated group. (A) HIF-1α (B) Ets-1 (n=5 for each group) (CONT; control group, SP; spironolactone-treated group).

![Image of immunohistochemistry](image-url)

**Fig. 5.** Immunohistological examination of HIF-1α in the control and spironolactone-treated kidneys. (A) control kidneys (B) cortical expression within the glomeruli and tubules of the control group (arrows), (C) spironolactone-treated kidneys (D) cortical expression within the glomeruli and tubules of the spironolactone-treated group (arrows) (A,C original magnification x 100; B,D x 400).
In the spironolactone-treated group, HIF-1α was more intensely expressed in the medullary and cortical developing and maturing tubular cells, compared to the controls (Fig. 5C and D).

2. HIF-2α expression

The immunoblots showed that HIF-2α/tubulin protein expression was not different in comparisons between the spironolactone-treated group and the control group 2 (Fig. 3B). RT-PCR and immunohistochemical staining for HIF-2α were not performed because there was no change in western blotting between the two groups.

3. Ets-1 expression

Ets-1/tubulin protein expression was significantly increased in the spironolactone-treated group, compared to the control group 2 (P<0.05; Fig. 3C). RT-PCR also showed increased mRNA expression of Ets-1/GAPDH in the spironolactone-treated group (Fig. 4B). Immunohistochemically, Ets-1 expression was easily detected in the medullary and cortical tubular epithelial cells and glomeruli in the control group 2 (Fig. 6A and B). In the spironolactone-treated group, it was more strongly detected in almost all of the medullary and cortical tubular segments, compared to the control group 2 (Fig. 6C and D).

4. Hypoxyprobe-1 staining and serial sections

In the spironolactone-treated group, the hypoxic signals were more prominent than in the control group, and almost all tubular segments in the medulla and cortex showed strong positive pimonidazole staining. Severe hypoxia was confirmed in almost all tubular epithelial cells and glomeruli (Fig. 1G-I). Moreover, the distributional pattern of the hypoxyprobe-1 generally co-localized with the HIF-1α and Ets-1 in the serial sections (Fig. 7A-C).

DISCUSSION

The main finding of this study was that aldosterone blockade, in the newborn rat kidney, increased the hypoxic response and up-regulated the expression of key mediators of hypoxia including HIF-1α and Ets-1. There were no significant changes in HIF-1α, HIF-2α and Ets-1 expression after enalapril treatment. We also confirmed that hypoxia existed in the control rat kidney at eight days post partum. This finding was demonstrated by experiments with the hypoxia marker pimonidazole and the assessment of hypoxia-related target genes.

We showed that severe hypoxia occurred in the tubular and collecting duct cells of the renal medulla, whereas the renal cortex...
exhibited only moderate hypoxia in the kidneys of the newborn control rat, by immunostaining with pimonidazole; these findings are consistent with those reported by Freeburg et al. (7). Tubular and collecting duct cells of the renal medulla reacted strongly with the hypoxyprobe-1, whereas diffuse weak binding was observed in the nephrogenic outer cortex. Hypoxyprobe-1 forms immunogenic protein adducts only in severely hypoxic cells (with half-maximal labeling of cells at O2 concentrations as low as 0.1 to 0.3%) (25). Hypoxia might have already occurred at oxygen concentrations well above the Hypoxyprobe-1 binding range, and therefore relatively weak binding or a negative kidney stain can not rule out hypoxia completely.

Because the renal medulla is relatively 'underperfused' in contrast to the 'overperfused' renal cortex (26), the cells in this region of the kidney function normally under hypoxic conditions. The countercurrent oxygen diffusion between the descending and ascending vasa recta and the high metabolic ion transport activity of thick ascending limb of Henle also lead to low pO2 in the renal medulla (27). Although the precise mechanism mediating renal medullary adaptation to low pO2 remains poorly understood, many studies have suggested that the molecular regulation of gene expression is significantly involved including HIF (26). Bernhardt et al. (9) showed that both HIF isoforms were expressed at early postnatal stages and disappeared until day 14 when rat kidney development was completed. Prominent signals for HIF-1α were found mostly in the medulla, compatible with our findings.

In this study, Ets-1 was also detected in the developing rat kidney. Though Ets-1 expression has been shown in renal tissues during murine embryo development (18), and has been shown to contribute to kidney development (28), the histological evidence of its expression in the newborn rat kidney has been insufficient. Occurrence and tissue distribution of the Ets-1 protein was similar to the HIF-1α staining pattern observed. Our data also indicated, for the first time, that spironolactone treatment promotes the hypoxic response in the developing rat kidney. In accordance with enhanced expression of the hypoxyprobe-1, HIF-1α and Ets-1 expression were up-regulated in the spironolactone group. Bernhardt et al. (9) showed that under systemic hypoxia in newborn rats HIFα accumulation was further enhanced, but did not change the tissue expression patterns; these findings suggested the lack of oxygen supply as the major stimulus for HIFα expression. They suggested that growth factors and cytokines as well as oxygen are involved in HIF activation of the developing kidney. Thus, our results imply that aldosterone blockade plays some role in the process of tissue hypoxia in the developing rat kidney by activating HIF-1α and Ets-1 expression.

Aldosterone inhibition disturbs sodium reabsorption, which requires more energy consumption for compensation. Na+ and K+ homeostasis by aldosterone is maintained chiefly by regulation of electrogenic Na+ reabsorption in the kidney. Na+ is reabsorbed from tubular fluid via amiloride-sensitive epithelial Na+ channels (ENaC) which comprises the rate-limiting step for transepithelial Na+ transport (29). ENaC is located in the luminal membrane of distal renal tubule cells whereas the (Na+K+)-ATPase in the basolateral membrane. The activity of ENaC and thereby the electrogenic Na+ transport is controlled by aldosterone (30). Aldosterone inhibition affects Na+ balance and may increase the sodium load delivered to distal nephron segments for compensation. This leads to increase medullary sodium reabsorption and O2 consumption, thereby decreasing medullary oxygenation (31). It has been proposed that low pO2 in the renal medulla leads to an increase of HIF-1α levels and activates the transcription of many oxygen-sensitive genes as well as protective factors. These factors not only dilate the vasa recta, but also inhibit tubular ion transport activity, resulting in the reduction of sodium reabsorption (26). Spironolactone treatment increases renal water and sodium excretion and may thereby exaggerate this HIF-1α-mediated regulation of renal medullary oxygenation in developing rat kidneys.

Another possible explanation may be that aldosterone contributes directly to the cellular effects. Differential responses to enalapril versus spironolactone in the present study support this straight cellular action of aldosterone, even though angiotensin converting enzyme (ACE) inhibition should result in a consequent inhibition of downstream aldosterone too. Numerous studies have suggested that aldosterone contributes to the progression of renal injury; in many cases the effects cannot be explained by its hemodynamic activity (32, 33). Aldosterone has been shown to cause proximal tubular cell apoptosis through oxidative stress, and this effect of aldosterone is mediated by mineralocorticoid receptors (34). Spironolactone has been reported to suppress peritubular capillary loss in deoxycorticosterone acetate/salt-induced tubulointerstitial fibrosis (35). Phylogenetic evidence also supports that aldosterone had hypoxic functions in addition to the regulation of the salt balance in the distant past (36).

In this study, HIF-2α expression was not changed in the spironolactone-treated group. Freeburg et al. (7) showed that HIF-2α, similar to HIF-1α, was expressed in developing kidney organ cultures under normoxia, and were further stimulated by hypoxia. By contrast, many studies have shown that HIF-2α was undetectable in renal tubular epithelial cells during acute renal ischemia (37, 38). These results support that HIF-1α, but not HIF-2α, is the key mediator of hypoxic HIF signaling.

Although our results, from experiments on developing rat kidneys, are opposite to the findings obtained from studies using adult rat models of renal injury, it may be speculated that spironolactone is involved in the hypoxic cellular changes noted in developing rat kidneys through enhanced expression of HIF-1α and Ets-1. To estimate the significance of our findings, further assays that address functional consequences of HIF-1α up-regulation, such as changes in vascular patterning, vascular endothelial growth factor and/or vascular endothelial growth factor receptor expression, are required.

Much to the interest, the preservation of the natural hypoxic conditions was observed despite enalapril treatment. This finding suggests that angiotensin II inhibition do not influence the original hypoxic conditions of the developing kidney. Diverse factors might participate in the expression of HIF and Ets-1 associated with angiotensin II in the developing rat kidney. It is postulated that ACE-independent mechanism may be involved in angiotensin II generation. Utrata et al. (39) identified chymase, a serine protease, as the major angiotensin II-forming enzyme in the human heart that converts angiotensin 1 to angiotensin II. Chymase is expressed at very low levels in normal kidneys (40); however, chymase-dependent angiotensin II generation is activated under certain pathophysiological conditions and when ACE is inhibited (41). Another carboxypeptidase, ACE2, a key enzyme catalyzing the cleavage of both angiotensin I and angiotensin II, directly converts angiotensin II to angiotensin 1-7. The conversion of angiotensin II to angiotensin 1-7 appears to be the preferred pathway with a 500-fold greater efficiency than that for angiotensin 1. Accumulating data suggest that angiotensin 1-7 acts as an endogenous antagonist of angiotensin II-induced activity. ACE2 appears to control angiotensin II production by ACE, either by stimulating an alternative pathway for angiotensin I degradation or by promoting the conversion of angiotensin II to angiotensin 1-7 (42).

Furthermore, ACE not only converts angiotensin I into angiotensin II, but also degrades bradykinin into inactive peptides. Hence, the pharmacological effects of ACE inhibitors are in part mediated via both the reduction of angiotensin II and
the accumulation of bradykinin, an important endogenous vasodilator (43). It has been found that ACE has a higher affinity for bradykinin than for angiotensin I and that ACE inhibition significantly increases bradykinin concentrations (44, 45).

Thus, the ACE inhibition, in this study, may have activated other pathways to compensate for the consequent decrease in angiotensin II levels, resulting in the absence of change in the expression of HIF and Ets-1. We cannot exclude totally the possible angiotensin II participation in newborn rat renal hypoxia. However, the results of the present study do not provide enough data to confirm this. Further investigations involving the precise measurement of angiotensin II levels or the direct participation of hypoxia in newborn rat kidneys, we have yet to investigate which kinds of signal cascades exist between RAAS and hypoxic changes in developing kidney.

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