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Changes of Renal AT_1/AT_2 Receptors and Structures in Ovine Fetuses following Exposure to Long-Term Hypoxia

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Key Words
Hypoxia · Angiotensin II receptors · Ovine fetus

Abstract
Background/Aims: The present study tested the hypothesis that chronic hypoxia adversely affects renal development in the ovine fetus. ***Methods:*** Kidneys were collected from near-term fetuses of pregnant ewes maintained at sea level or high altitude (3,801 m, PaO_2: approx. 60 mm Hg) for 110 days (n = 6 for each group). ***Results:*** Long-term high altitude hypoxia reduced the fetal kidney/body weight ratio. Histological analysis showed a significant enlargement in the Bowman's space and swelling of tubule epithelial cells in the kidney of the hypoxic fetus. The histological alterations were limited to the cortical, but not medullary, zone. These alterations were associated with an increase in serum creatinine and a decrease in the BUN-to-creatinine ratio in hypoxic fetuses. Angiotensin II receptors (AT_1R and AT_2R) were detected in the glomerular and tubular regions of the kidney. Chronic hypoxia caused a significant increase in AT_1R and a decrease in AT_2R protein and mRNA abundance, resulting in a large increase in the AT_1R/AT_2R ratio in the fetal kidney. ***Conclusion:*** The results demonstrate an adverse effect of chronic hypoxia on renal AT_1R and AT_2R expression and functions in the fetus, suggesting a possible role of fetal hypoxia in the programming of renal diseases in fetal origins.

Introduction

Abnormal maternal anatomy in the reproductive system, such as obstructive uropathy, or adverse environmental factors, including malnutrition during pregnancy, may cause impaired fetal developmental and pathological changes in the kidney [1–5]. Retarded growth and restricted organ development in fetuses may be linked to a poor supply of nutrients, including oxygen. Notably, a large number of studies have demonstrated that alterations in fetal development may lead to diseases after birth in adults, including hypertension and metabolic diseases [6, 7]. However, there is limited information on how fetal kidney development is affected by hypoxia. The present study used the ovine fetal model to test whether, and to what extent, low levels of oxygen affect fetal kidney development inside healthy maternal sheep with intact reproductive systems (i.e. no obstructive anatomy).

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Previous studies have demonstrated that fetuses exposed to malnutrition or other adverse environmental factors have smaller kidneys with a reduction of the number of nephrons [2, 4]. However, specific information on the details of the changes in the nephron has not been elucidated. In addition, the influence of hypoxia on the fetal renal units, such as the glomerular and tubule systems, is largely unknown.

Among several mechanisms which produce developmental problems in fetal organs, we focused on the renin-angiotensin system and its receptor subtypes such as the angiotensin II receptor subtype 1 (AT_1R) and subtype 2 (AT_2R). It is known that AT_1R and AT_2R play an important role in cell growth, differentiation and apoptosis during development [8–12]. Abnormal expression of AT_1R and AT_2R in the renal system or in the body has also been shown to be related to kidney and cardiovascular diseases [13–16]. Therefore, expression of AT_1R and AT_2R, as well as their ratio (AT_1R/AT_2R), at both the mRNA and protein level, as well as with fetal renal functions, were determined in the present study. Our data provide novel information for the study of hypoxia-mediated mechanisms in renal development and for disease development in fetal origins.

Methods

Experimental Animals

As previously described [17, 18], time-dated pregnant sheep were obtained from the Nebeker Ranch (Lancaster, Calif., USA; altitude: approx. 300 m; arterial P_aO_2: 102 ± 2 mm Hg) and served as the sea-level normoxic control (n = 6). For chronic hypoxic treatment (n = 6), pregnant ewes (30 days of gestation) were transported to the Barcroft Laboratory, White Mountain Research Station (Bishop, Calif., USA; altitude: 3,801 m; maternal P_aO_2: 60 ± 2 mm Hg), and maintained there for approximately 110 days. As previously reported, fetal P_aO_2 in the hypoxic group was 19.3 ± 0.8 mm Hg and 23.3 ± 0.5 mm Hg in the normoxic control [18]. The animals were transported to the laboratory immediately before the studies. Ewes were anesthetized with thiamylal (10 mg/kg) administered via the left external jugular vein. The animals were then intubated and anesthesia was maintained on 1.5–2.0% halothane in oxygen throughout surgery. An incision was made in the abdomen to expose the fetus. Maternal blood samples were collected from the maternal jugular vein and fetal blood samples were collected from the umbilical cord (vein) immediately after opening the uterus. Following the collection of blood samples, fetuses were removed and weighed, and fetal kidneys were collected and weighed. Blood samples were centrifuged and serum was measured for the determination of electrolytes (Na^+, K^+ and Ca^{2+}), albumin (ALB), amylase (AMY), total bilirubin (Tbil), creatinine (CRE) and blood urea nitrogen (BUN) using Vetscan (Abaxis, Union City, Calif., USA). ALB, AMY and Tbil were measured to determine fetal metabolic status related to liver function under the hypoxic condition. All procedures and protocols used in the present study were approved by the IACUC of Loma Linda University and followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Histological Analysis

Fetal kidney tissue was used. Deparaffinized sections (4 μm) were treated with 2 changes of xylene, 10 min each, and rehydrated in descending alcohol (absolute alcohol for 5 min, 95% alcohol for 1 min, 85% alcohol for 1 min and 75% alcohol for 1 min, sequentially). The sections were washed briefly in distilled water and then stained in Mayer's hematoxylin solution for 5 min. Counterstaining was done in eosin-phloxine B solution for 2 min. The sections were dehydrated through alcohol solutions, and cleared in phenol xylene mounted with xylene-based mounting medium. The slices were viewed, analyzed and diagnosed with a Nikon microscope by experienced pathologists in Soochow University's Pathology Lab in a blinded manner. Images were captured with an attached SPOT digital camera imaging system.

Immunoblotting

Protein levels of renal AT_1R and AT_2R were determined with Western blot analysis as reported previously [16]. Briefly, fetal renal tissue was homogenized in lysis buffer containing 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 2 g/ml aprotinin, pH 7.4. Homogenates were centrifuged at 4°C for 10 min at 12,000 *g*, and supernatants were collected. Samples with equal protein (80 μg) were loaded on 7.5% polyacrylamide gel and separated by electrophoresis at 100 V for 90 min. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding was blocked in TBST containing 5% dry milk for 50 min at room temperature. The membranes were incubated with rabbit AT_1R and AT_2R polyclonal antibody (1:300; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) overnight at 4°C. The membranes were then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2,000). Protein bands were visualized with enhanced chemiluminescence reagents, and the blots were exposed to Hyperfilm. For comparison of the levels of AT_1R and AT_2R protein (relative density) between the 2 groups, bands were normalized to β-actin, which was used as a control. Results were quantified by the Kodak electrophoresis documentation and analysis system with Kodak ID image analysis software as reported [16].

Real-Time RT-PCR

RNA was extracted from fetal renal tissue using TRIzol reagents (Invitrogen, Carlsbad, Calif., USA). PCR was performed in triplicate. mRNA abundance of AT_1R and AT_2R was determined by real-time RT-PCR using an Icycler Thermal Cycler (Bio-Rad, Hercules, Calif., USA). The primer sequences for AT_1R were forward 5′-CGGCCTTCGGATAACATGA-3′ and reverse 5′-CCTGTCACTCCACCTCAAAACA-3′. The primer sequences for AT_2R were forward 5′-CAATCTGGCTGTGGCTGACTT-3′ and reverse 5′-TGCACATCACAGGTCCAAAGA-3′. Real-time RT-PCR was performed in a final volume of 25 μl. Each PCR reaction mixture consisted of 600 nM of primers, 33 units of M-MLV re-

verse transcriptase (Promega, Madison, Wisc., USA) and iQ SYBR Green Supermix (Bio-Rad) containing 0.625 units of Taq polymerase; 400 μM each of dATP, dCTP, dGTP, and dTTP; 100 mM KCl; 16.6 mM ammonium sulfate; 40 mM Tris-HCl; 6 mM $MgSO_4$; SYBR Green I; 20 nM fluorescein; and stabilizers. RT-PCR was performed under the following conditions: 42°C for 30 min and 95°C for 15 min, followed by 45 cycles of 95°C for 20 s and 52°C for 1 min. GAPDH was used as an internal reference and serial dilutions of the positive control were performed on each plate to create a standard curve. The amount of target gene was normalized to the reference GAPDH to obtain the relative threshold cycle.

Immunohistochemistry

Fetal kidneys were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin. Ten-micrometer sections were cut through the fetal kidney on a cryostat. Immunohistochemical detection of AT_1R and AT_2R was performed using the avidin-biotin-peroxidase technique, as described previously [16]. Tissue sections were incubated with primary antibodies against AT_1R or AT_2R (1:1,000) overnight at 4°C. After rinsing the sections in 0.01 M phosphate-buffered saline for 5 min (3 times), they were incubated with biotinylated goat anti-rabbit Ig (1:500) for 60 min at room temperature. The sections were then treated with 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma; 0.02% hydrogen peroxide). The negative control of immunostaining was performed in the absence of the primary antibody. The slices were viewed with a Nikon microscope, and images were captured with an attached SPOT digital camera imaging system.

Data Analysis

Results from all experiments described above were expressed as means ± SEM obtained from the number of animals given (n = 6 for each group). Differences were evaluated for statistical significance ($p < 0.05$) by 2-way ANOVA followed by the Newman-Keuls post-hoc test or t test, where appropriate.

Results

Blood Values

There was no difference in maternal ALB, AMY, Tbil, CRE or BUN/CRE between the normoxic and hypoxic groups (fig. 1; table 1). In the fetuses, serum ALB was significantly less in the hypoxic group than in the control group. Fetal serum CRE was significantly higher and the ratio of BUN/CRE was significantly lower in the hypoxic fetuses than in the normoxic controls (fig. 1). However, fetal serum AMY and Tbil levels were the same between the control and hypoxic animals (table 1). Although serum Na^+ and Ca^{2+} levels in the maternal and fetal sheep were the same between the control and hypoxic groups, serum K^+ concentrations in both maternal and fetal sheep were significantly lower in the hypoxic group than in the normoxic group (table 1).

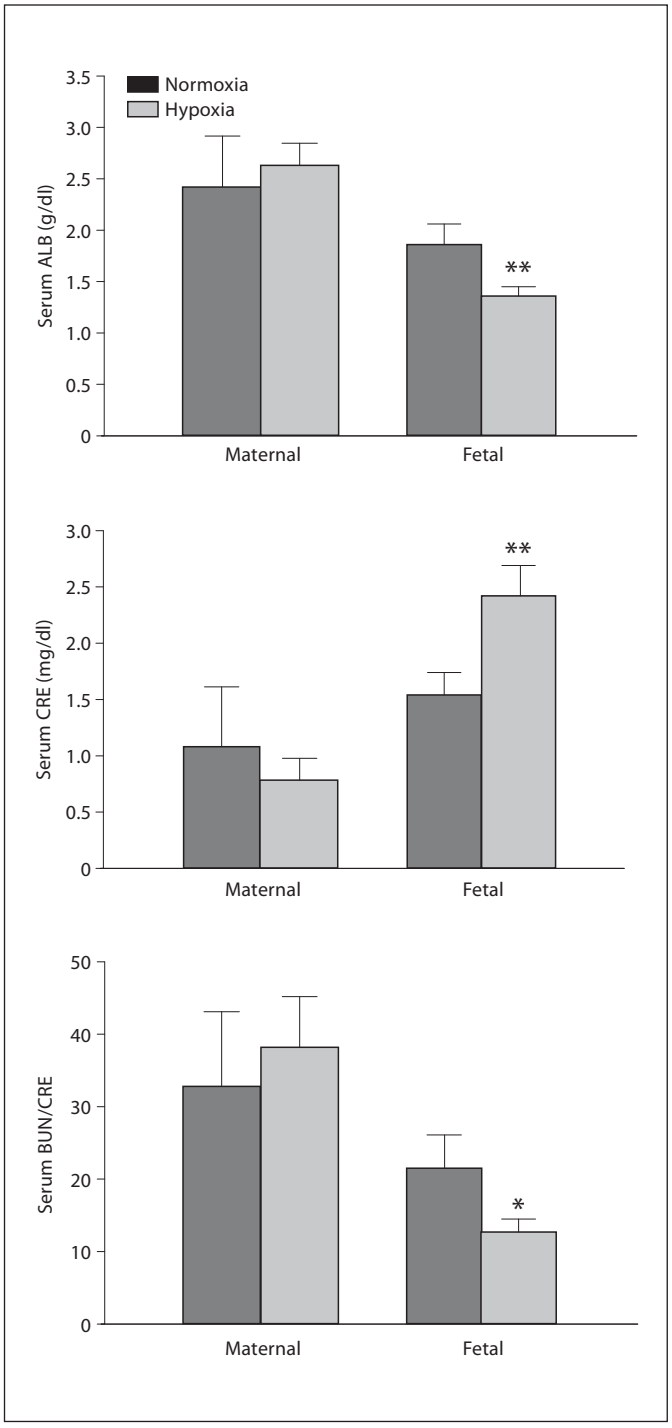

Fig. 1. Maternal and fetal serum values following chronic hypoxia. Normoxia represents the control, hypoxia represents exposure to hypoxia. * $p < 0.05$; ** $p < 0.01$.

Table 1. Maternal and fetal serum values

	Adult		Fetus	
	normoxia	hypoxia	normoxia	hypoxia
AMY, U/l	14.60 ± 5.93	10.00 ± 2.34	5.20 ± 1.50	4.00 ± 0.00
Tbil, mg/dl	0.56 ± 0.05	0.58 ± 0.07	0.72 ± 0.08	0.77 ± 0.07
Na^+, mM/l	143.00 ± 3.19	143.5 ± 1.29	142.2 ± 1.03	142.71 ± 1.34
K^+, mM/l	5.38 ± 0.06	4.37 ± 0.10*	5.50 ± 0.35	4.81 ± 0.13*
Ca^{2+}, mM/l	9.74 ± 0.94	9.28 ± 0.27	11.76 ± 0.95	12.78 ± 0.32

Values are expressed as means ± SEM. Normoxia represents the control, hypoxia represents exposure to hypoxia. * $p < 0.05$.

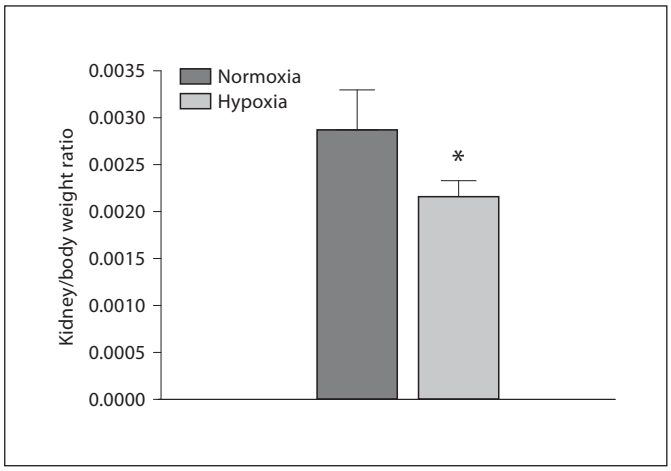

Fig. 2. Fetal kidney/body weight ratio following exposure to hypoxia. Normoxia represents the control, hypoxia represents exposure to hypoxia. * $p < 0.05$.

Ratio of Fetal Kidney/Body Weight

There was no significant difference in fetal body weight between the control and the hypoxic groups (3,760 ± 69 vs. 3,972 ± 43 g, $p > 0.05$). When the normoxic and the hypoxic groups were compared at the same gestational age, statistical analysis showed that the ratio of fetal kidney/body weight was significantly lowered in the hypoxic animals (fig. 2).

Histological Changes

Compared with the control group, the histological changes in the fetal kidney of the hypoxic group were mainly located in the cortical zone of the kidney, including the glomeruli and renal tubule. No significant histological changes were observed in the medullary zone of the kidneys in either group. The most notable histological change in the glomeruli was that the Bowman's space was greatly enlarged, while the sizes of the solid parts of the glomeruli were reduced, accompanied by an increased red-stained renal matrix in the glomeruli of the fetuses exposed to hypoxia (fig. 3). It also appeared that the number of parenchymal cells in glomeruli was decreased in the hypoxic group compared to that in the control fetuses (fig. 3). The major histological change in the renal tubule was cellular swelling or hydropic degeneration in the epithelium of the proximal convoluted renal tubule, where epithelial cells were enlarged and cytoplasm was translucent and loosened (fig. 3). No obvious histological changes were observed in other sections of the renal tubule, including the loop of Henle, the distal convoluted tubule and the collecting tubule.

AT_1R and AT_2R Protein Levels

Western blot analysis showed that the total amount of AT_1R protein demonstrated by the relative density of AT_1R in the fetal kidney was significantly increased following chronic exposure to hypoxia during pregnancy, while levels of renal AT_2R protein were significantly decreased in the hypoxic fetuses (fig. 4).

AT_1R and AT_2R mRNA Levels

Real-time PCR analysis showed that renal levels of AT_1R mRNA were also significantly increased in the fetuses exposed to hypoxia in comparison to the control animals. In addition, renal AT_2R mRNA was significantly lower ($t = 5.963$, $p < 0.05$, $n = 6$) in the fetal kidney of the hypoxic group than in the normoxic group (fig. 5). Furthermore, the ratio of renal AT_1R/AT_2R mRNA levels was significantly increased (approx. 4 fold) in the hypoxic fetus (fig. 5).

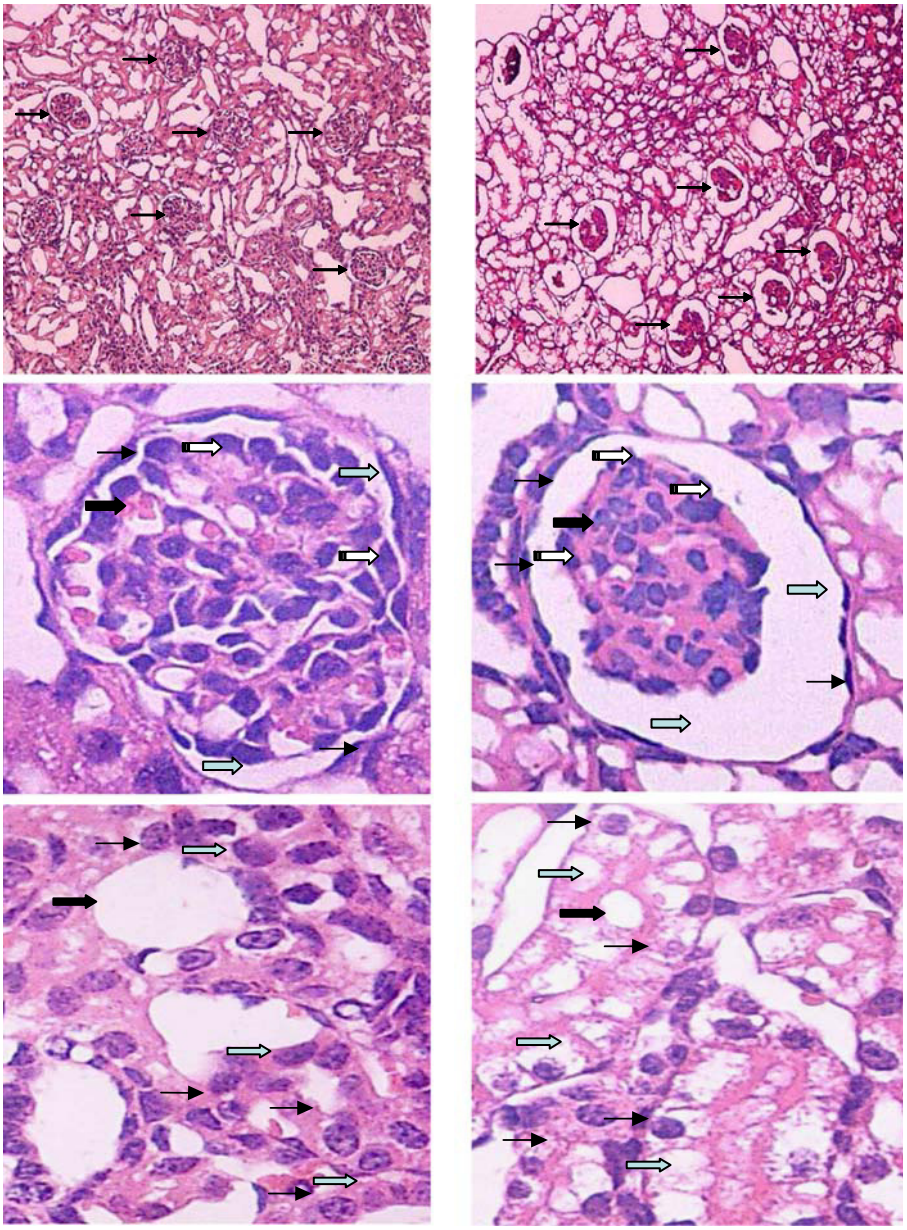

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Fig. 3. Histological changes in the fetal glomeruli (top and middle panels, 10× and 40×) and the renal tubule (bottom panel, 40×). The left panels show tissue from a normoxic control fetus, the right panels show tissue from a hypoxic fetus. The Bowman's space was enlarged, while the size of the solid core of the glomeruli was reduced, accompanied by an increased red-stained renal matrix in the fetuses exposed to hypoxia (top and middle panels). The number of parenchymal cells of partial glomeruli was decreased in the right panels (thick solid arrows show glomeruli, thin solid arrows show parietal cells, gray arrows show the Bowman's space and white arrows show podocytes in middle panels). Cellular swelling was observed in the epithelium of the proximal convoluted renal tubule, where epithelial cells were enlarged, and the cytoplasm was translucent and loosened in the bottom right panel (thick solid arrows show renal tubule, thin solid arrows show cytoplasm of tubular epithelium and gray arrows show epithelial cells of the renal tubule in the bottom panels).

AT_1R and AT_2R Immunostaining

In fetal glomeruli and the proximal convoluted renal tubule, histological analysis showed AT_1R immunostaining in podocytes, parietal cells and epithelia in kidneys of both the control and hypoxic groups. The AT_1R immunosignal in the glomeruli of the fetuses exposed to hypoxia was stronger than that in the control group (fig. 6). Immunohistochemistry also showed similar AT_2R immunostaining in podocytes and the epithelia in fetal kidneys of both the control and hypoxic groups (fig. 7).

Discussion

It has been demonstrated that anatomical problems like uropathy and environmental insults such as malnutrition [1–5] may cause renal developmental changes in fetuses. Although mechanisms for obstructive uropathy-induced developmental problems in fetuses include a deficient supply of oxygen in utero, few studies examined the effect of hypoxia on fetal renal development during pregnancy. The present study demonstrated that chronic

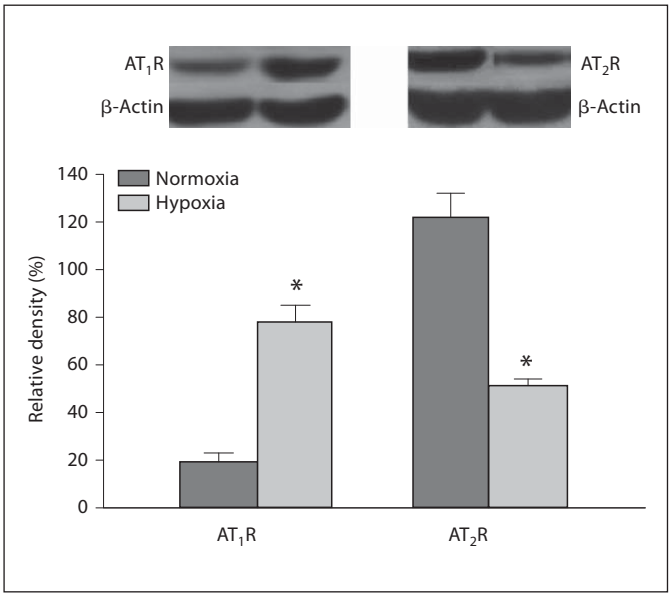

Fig. 4. Fetal renal angiotensin II receptor protein following exposure to hypoxia. Normoxia represents the control, hypoxia represents exposure to hypoxia. * $p < 0.05$.

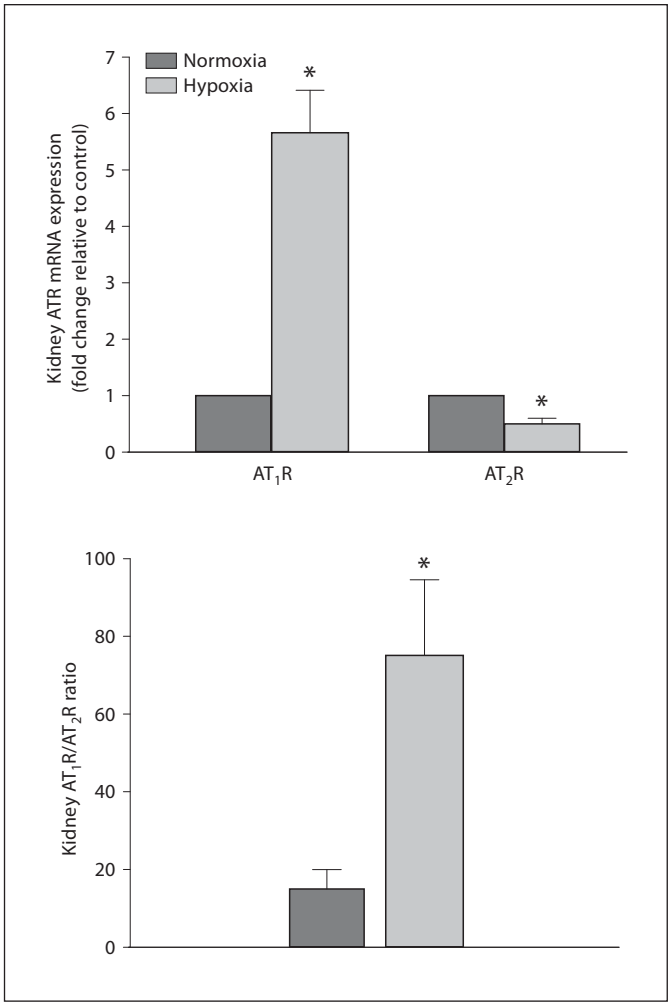

Fig. 5. Fetal renal angiotensin II receptor mRNA (top panel) and the AT_1R/AT_2R mRNA ratio (bottom panel) following exposure to hypoxia. Normoxia represents the control, hypoxia represents exposure to hypoxia. * $p < 0.05$ (n = 5–6). ATR = Angiotensin II receptor.

in utero hypoxia caused alternations in kidney glomeruli and the renal renin-angiotensin system in near-term fetuses.

Previous studies have indicated that the development of the fetal kidney is affected by malnutrition and other adverse environmental factors [2, 7]. In the present study, we demonstrated a significant decrease in the kidney/body weight ratio in ovine fetuses exposed to long-term high altitude hypoxia, indicating impairment in fetal renal development. The findings of an enlarged Bowman's space and reduced size of the glomeruli accompanied by an increased red-stained renal matrix in hypoxic fetuses suggest that chronic hypoxia adversely affects glomeruli and tubular systems in the fetal kidney. These changes in histology show that hypoxia mainly affects the glomeruli and renal tubule in the cortical zone, not the medullary zone, of the fetal kidney. Previous studies showed that multiple factors could reduce nephron number in the fetus [3–5, 7, 19]. Given that maternal food intake and body weight at high altitude were not significantly decreased, the effects observed in the present study are likely caused by hypoxia. The finding of the enlarged Bowman's space and shrunken core of glomeruli caused by in utero hypoxia is intriguing and suggests a likelihood of increased risk of renal disease in the postnatal development. Whether and to what extent the histological changes in the glomeruli in the fetus are reversible after birth remains an interesting area for further investigation.

We also measured fetal serum CRE and other chemicals for a preliminary assessment of renal functions. CRE is a break-down product of creatine phosphate and is chiefly filtered by the kidneys. If renal filtering is deficient, serum CRE rises. As a result, CRE levels in blood may reflect the glomerular filtration rate and renal functions. Measuring serum CRE is the most commonly used indicator of renal functions, and a rise in blood CRE levels is often indicative of marked damage to functioning nephrons [20–24]. In the present study, fetal serum CRE

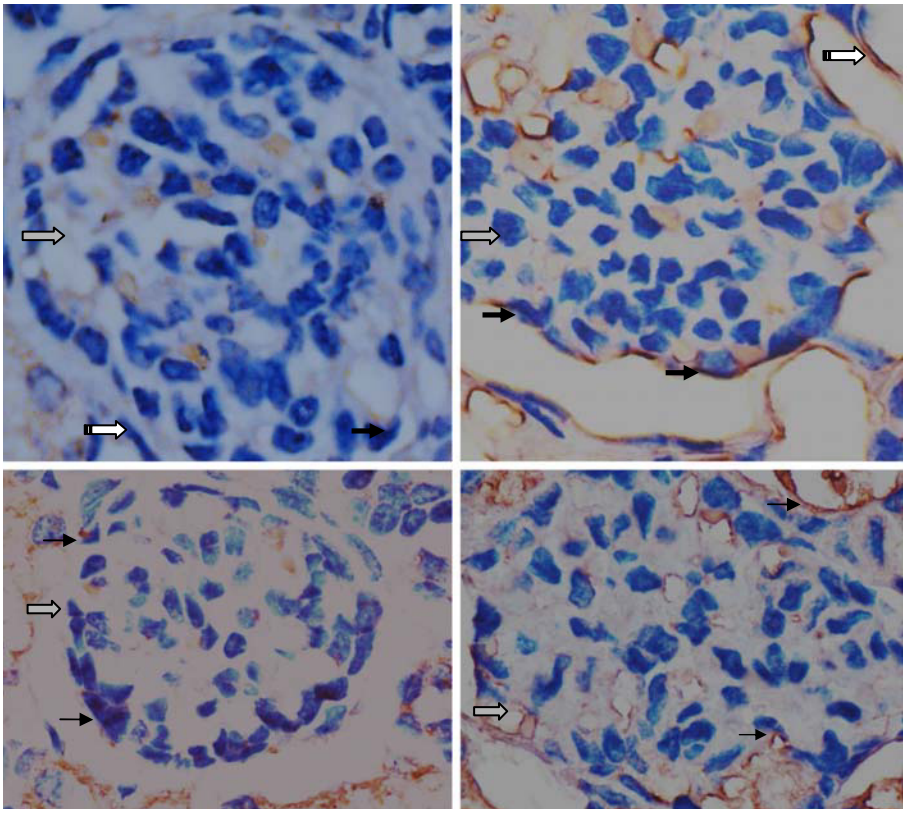

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Fig. 6. Immunohistochemical staining showing AT_1R (top panel) and AT_2R (bottom panel) expression patterns in fetal glomerular podocytes and parietal cells of the Bowman's space. AT_1R staining was relatively stronger in the top right panel, while AT_2R staining appeared similar between the 2 groups. The left panels show the control, the right panels show the treated hypoxia. Gray arrows show glomeruli, solid arrows show AT_1R or AT_2R on podocytes, and white arrows show parietal cells of the Bowman's space. 40×.

levels were significantly increased following exposure to hypoxia, while maternal CRE remained unchanged. This demonstrated that the mature kidney in the mother could still handle CRE when exposed to hypoxia, but the immature fetal kidney failed to remove CRE sufficiently, suggesting that the capability of fetal renal filtering was damaged. The BUN-to-CRE ratio was also reduced (<10) in the fetuses exposed to hypoxia, further indicating that the problems were intrinsic in the kidney. Serum AMY and Tbil, which can reflect both maternal and fetal liver function, were not changed between the normoxia and hypoxia groups in the present study. Maternal and fetal blood sodium and calcium levels were also unchanged by hypoxia. However, fetal serum ALB and potassium were significantly lowered in the fetuses exposed to hypoxia. Low ALB levels can reflect excess excretion by the kidney as seen in nephrotic syndrome [25]. Low potassium also can be caused by renal tubular problems. Many factors can cause low blood ALB and potassium, thus renal influence is not the only possible interpretation of the data. However, due to the evidence of a higher fetal serum CRE associated with the histological changes in renal filtering units and tubular structures, low serum ALB and potassium in the fetus could be suspected as being consequences of renal structural changes following exposure to hypoxia.

We were also interested in the mechanisms involved in hypoxia-induced renal histological and functional alterations in the fetus. Among many possibilities, we focused on renal angiotensin II receptors because several lines of studies have shown that activation of these receptors makes important contributions to cell growth, differentiation and apoptosis during development [22–33]. We found that both fetal renal AT_1R protein and mRNA levels were significantly increased, while AT_2R protein and its mRNA levels were decreased in the ovine fetuses exposed to hypoxia. In addition, the renal AT_1R/AT_2R mRNA ratio was significantly increased (approx. 4-fold) in the hypoxia-treated fetuses compared to that of the control. Immunostaining also showed both AT_1R and AT_2R on glomerular podocytes, parietal cells of Bowman's space and the epithelia of the proximal convoluted renal tubule in fetal kidneys. Notably, AT_1R immunostaining was more intense in the fetal glomeruli and renal

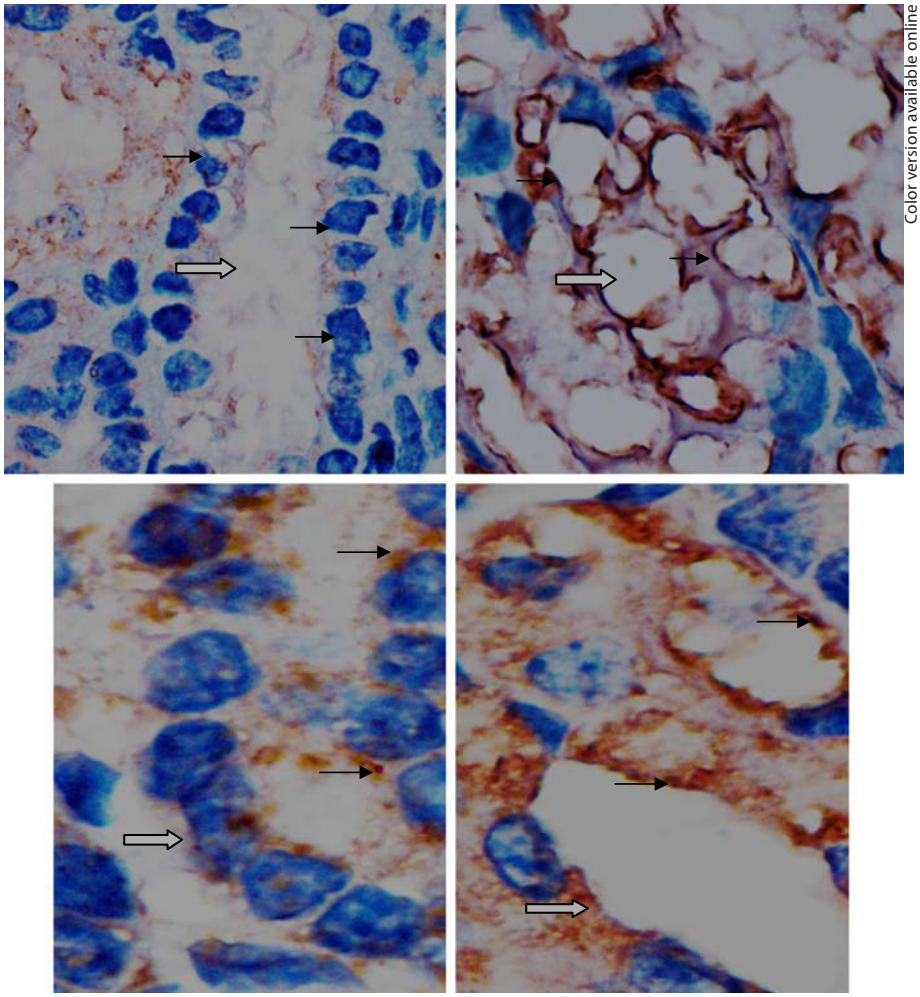

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Fig. 7. Immunohistochemical staining showing AT_1R (top panels, 40×) and AT_2R (bottom panels, 100×) expression patterns in the epithelia of the proximal convoluted renal tubule. AT_1R staining was relatively stronger in the top right panel, while AT_2R staining appeared similar between the 2 groups. The left panels show the control, the right panels show the treated hypoxia. Gray arrows show the renal tubule and solid arrows show AT_1R or AT_2R on the epithelia of the proximal convoluted tubule.

tubule following hypoxia than in the control. Angiotensin II receptor subtypes play a critical role in renal development, including glomerular apoptosis [17, 34–36]. Both AT_1R and AT_2R have been suggested to be involved in cellular development, including cellular proliferation and differentiation [8–12, 36]. In light of this, at least one explanation was considered for the data gained in the present study: an increase of fetal renal AT_1R and a decrease of AT_2R following hypoxia may contribute to cell growth, differentiation, migration and apoptosis [37, 38] in the cortical zone of the fetal kidney and may be involved in alterations in glomerular and tubular histology. Although future studies are needed for further clarification, the present study was the first to demonstrate that the fetal renal AT_1R/AT_2R ratio was significantly increased following hypoxia in association with glomerular and tubular alterations in utero.

In conclusion, the present study on hypoxia-mediated alterations in the fetal renal development demonstrated that hypoxia during pregnancy specifically affected the development of the cortical zone, but not the medullary zone, of the fetal kidney at near-term. Hypoxia altered fetal renal histological structures mainly located at the Bowman's space, the core of glumeruli and the renal tubule in the cortical zone. Notably, these alterations were associated with the changes in fetal serum CRE and the BUN-to-CRE ratio that may reflect impaired renal functions. Importantly, both the mRNA and protein levels of AT_1R and AT_2R, as well as the AT_1R/AT_2R mRNA ratio, in the fetal kidney were significantly altered by hypoxia, indicating a possible mechanism of hypoxia-mediated poor development of fetal kidneys in utero. The findings in the present study also generated new questions and ideas for further and future investigation. These include

(1) how to test and clarify the hypothesis that the renal renin-angiotensin system plays an important role in the fetal glomerular and tubular development, (2) whether prenatal environmental insult-induced alterations in fetal renal structures are reversible or permanent following birth or after removing hypoxia stimulation, and (3) whether the altered gene and protein expression of renal AT_1R and AT_2R at the fetal stage would last in the offspring. Answering these questions will assist in further understanding the development, prevention and treatment of diseases in fetal renal origins.

Acknowledgments

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