

Renal renin-angiotensin system dysregulation caused by partial bladder outlet obstruction in fetal sheep

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Background. To determine whether fetal renal obstruction activates the renal renin-angiotensin system (RAS), an important mediator in normal kidney development and obstructive nephropathy, we used a model of fetal partial bladder outlet obstruction (PBOO).

Methods. Total RNA and protein was extracted from kidney of sheep fetuses with partial bladder outlet obstruction created at 95 days gestation, after 2 ($N = 6$) and 5 weeks of obstruction (term; $N = 6$), and from normal fetal sheep at various time points between 60 and 135 days of gestation (total $N = 19$). Relative levels of mRNA for renin, angiotensinogen, type 1 and 2 angiotensin II (Ang II) receptors (AT-1 and AT-2), and transforming growth factor- β 1 (TGF- β 1) were assessed by semiquantitative reverse transcription-polymerase chain reaction. Expression levels of AT-2 receptor protein were measured by Western blot analysis.

Results. Renin mRNA expression was increased (250%) after two weeks of obstruction. In normal fetuses, AT-1 expression was low at 60 to 75 days of gestation and increased toward the end of gestation, whereas AT-2 expression showed a reversed pattern. At 109 days, PBOO caused an increased expression of AT-2 mRNA compared with normals (400%). Correspondingly, AT-2 receptor protein was more abundant in obstructed kidneys. TGF- β 1 mRNA expression was significantly increased in obstructed kidneys at 109 days gestation.

Conclusions. These observations confirm the reciprocal developmental regulation of AT-1 and AT-2 receptors' expression, suggesting their functional role in renal development. Partial bladder outlet obstruction produces specific alterations: increased renin expression and altered balance of receptor subtypes, which may induce altered functional and vascular regulation of the obstructed fetal kidney. TGF- β 1, a mediator of Ang II-induced fibrosis, may play a role in inducing and propagating interstitial fibrosis.

Key words: RAS, bladder, obstructive nephropathy, kidney development, type 1 angiotensin II receptor.

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Partial bladder outlet obstruction during fetal life causes significant kidney injury. Posterior urethral valves are a leading cause of end-stage renal failure before the age of two years and a significant cause of chronic renal failure throughout childhood and adolescence [1]. Renal interstitial fibrosis is thought to be one of the important features of prenatal obstructive nephropathy. In many postnatal kidney diseases, the renal renin-angiotensin system (RAS) has been shown to play an important role in the induction of renal interstitial fibrosis, possibly mediated through transforming growth factor- β 1 (TGF- β 1) [2]. Pharmacological manipulation of the RAS has been shown to be beneficial in slowing the progression of renal fibrosis in animal models [3, 4] as well as human diseases [reviewed in 5].

The RAS has well-established physiological functions. Angiotensin II (Ang II) regulates blood pressure and fluid and electrolyte balances, and it is also thought to mediate renal growth and differentiation. The renal RAS plays a crucial role during fetal kidney development. We investigated the effects of fetal partial bladder outlet obstruction on mRNA expression of critical elements of the renal RAS. These elements include substrate, activating enzyme, and receptors for Ang II, angiotensinogen, renin and the two major receptor subtypes, Ang II type 1 (AT-1) and Ang II type 2 (AT-2), respectively. TGF- β 1 expression was investigated as a possible downstream mediator of Ang II-induced fibrosis, with the hypothesis that up-regulation of the renal RAS occurs with fetal obstruction and may contribute to the initiation and propagation of renal fibrosis, as well as to alterations in renal growth and differentiation [6, 7].

METHODS

Surgical procedure

Bladder outlet obstruction was created in fetal sheep at 95 days of gestation (term 140 days). Time-dated pregnant ewes underwent open hysterotomy under general

anesthesia (halothane/oxygen after induction with ketamine) in accordance with institutional animal care and use guidelines. Surgical and anesthetic details have been published previously [8]. The fetal hind end was exposed, and the urachus was identified and occluded by a surgical clip through a small incision at the base of the umbilical cord. Additionally, in male sheep, the perineal urethra was mobilized, and an occluding gold jewelry ring (internal diameter of 2 mm) was placed around it. In female sheep, the bladder neck was exposed via a suprapubic midline incision, and the occluding gold jewelry ring was placed around the proximal urethra. All animals were returned to the uterus, and pregnancy was allowed to continue. At 109 ($N = 6$) and 135 days ($N = 6$) of gestation, animals were delivered via Cesarean section and were sacrificed. Normal, unmanipulated, and age and sex-matched animals (total $N = 8$) served as controls: usually twins of the obstructed animals. Additional normal sheep fetuses were sacrificed at 60, 75, 90 and 135 days of gestation (total $N = 11$ animals). Gross changes in urinary tracts were assessed, and kidneys were rapidly retrieved, drained, and weighed. Representative whole kidney samples were snap frozen in liquid nitrogen and stored at -80 to 76°C .

Reverse transcription-polymerase chain reaction

Total RNA extraction was performed using Tri-Reagent[®] (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Reverse transcription (RT) was performed using MMLV-RT (Ambion, Austin, TX, USA) with oligo-dT (GIBCO, Grand Island, NY, USA) as the first-strand primer. cDNA was precipitated with linear acrylamide, ammonium acetate, and ethanol and was redissolved in sterile water. Primers were selected from the previously published sheep angiotensinogen [9], sheep renin [10], sheep AT-1 and AT-2 receptor [11], sheep TGF- β 1 [12] and human β -actin [13], all found through GeneBank database search. An approximate 350 bp β -actin product was amplified using the following primer pair: sense 5'-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3' (383 to 413) and antisense 5'-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3' (703 to 733). An approximate 201 bp angiotensinogen product was amplified using the following primer pair: sense 5'-ACT TGT CCA CGG ACC CAA ATC-3' (717 to 737) and antisense 5'-TGG TGT TGT CCA CCC AGA ACT C-3' (917 to 896). An approximate 370 bp renin product was amplified using the following primer pair: sense 5'-CTG GCA GAT CAG AAT GAA AGG G-3' (824 to 845) and antisense 5'-GAA CTT GCG GAT AAA GGT GGC-3' (1193 to 1173). An approximate 442 bp AT-1 product was amplified using the following primer pair: sense 5'-TGG TGG GGA TAT TTG GAA ACA G-3' (6 to 27) and antisense 5'-TGG GAT TCG TAA TGG

AAA GCG-3' (447 to 427). An approximate 523 bp AT-2 product was amplified using the following primer pair: sense 5'-TTC AAC CTA GCT GTG GCT GAC TTA C-3' (109 to 133) and antisense 5'-CCA TCT TCA GAA CTT GGT CAC GAG-3' (631 to 608). An approximate 349 bp TGF- β 1 product was amplified using the following primer pair: sense 5'-GGC GAC CCA CAG AGA GGA AAT AG-3' (620 to 643) and antisense 5'-AGG CAG AAA TTG GCG TGG TAG C-3' (968 to 947). Polymerase chain reactions (PCR) were performed in a total volume of 25 μl , containing 22 μl of PCR SuperMix[®] (GIBCO), 0.5 μl each of sense and antisense primer (20 pmol/ μl), 0.1 μl of ^{32}P dCTP (3000 Ci/mmol; Amersham, Arlington Heights, IL, USA) and 2 μl of cDNA. PCR amplification was performed for 30 cycles at 94°C (denature), 58°C (anneal), and 72°C (extend) for 40 seconds each. PCR products were subjected to size separation by polyamide gel electrophoresis. Additional confirmation of PCR products was made by sequence-specific restriction enzyme digest. All samples were normalized to β -actin mRNA levels, and a limiting dilution method was used to make semiquantitative comparisons. Band intensity was measured by scanning densitometry according to manufacturer's instructions using a IS-1000 Digital Imaging System (version 2.00; Alpha Innotech, San Leandro, CA).

Western blot analysis

Whole kidney samples were homogenized in lysate solution with a polytron device. The lysate solution contains Triton X-100 (5 ml/100 ml), 4-(2-aminoethyl)-bensenesulfonyl fluoride (0.125 mM), aprotinin (0.01 mg/ml), leupeptin (0.01 mg/ml), ethylenediaminetetraacetic acid (EDTA; 2 mM), and dithiothreitol (DTT; 5 mM) in $1 \times$ phosphate-buffered saline (PBS; all purchased from Sigma Chemicals, St. Louis, MO, USA). Temperature was maintained at 4°C throughout the entire procedure. The concentration of protein in each sample was determined using the Bio-Rad D C Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA). Equal amounts of protein from each sample were then incubated with 10 μl of anti-AT-2 receptor goat antibody (C-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for one hour and precipitated with 40 μl of Protein A/G Plus-Agarose (Santa Cruz Biotechnology) overnight. As a negative control, 10 μl of anti-AT-2 antibody was suspended in PBS and precipitated in the same manner as previously described.

The immunoprecipitates were then collected by centrifugation at 2500 r.p.m. for five minutes. After washing with PBS/1 M NaCl, the precipitates were resuspended in electrophoresis buffer, which contains Tris-chloride, pH 6.8 (50 mM), 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, DTT (67 mM), and 10% glycerol (all purchased from Sigma Chemicals). Ten microliters of the

immunoprecipitates were subjected to size separation by 12% polyacrylamide gel electrophoresis in the presence of SDS. The separated protein samples were then electrotransferred to Immobilon P transfer membrane (Millipore Corporation, Bedford, MA, USA). Subsequently, the membrane was washed, blocked with 10% Blotto solution, and probed with a 1:1000 dilution of anti-AT-2 receptor antibody. The membrane was then incubated with a secondary antibody, an anti-goat IgG-horseradish peroxidase conjugate at a 1:40,000 dilution. Finally, the bound secondary antibody was detected using the enhanced chemiluminescence (ECL) system (Amersham). The experiments were repeated using the antibody preincubated with blocking peptide (BP) to confirm the specificity of the AT-2 receptor antibody.

Statistical analysis

Numerical results between experimental groups were analyzed for statistical significance within the same RT-PCR run, using the two-tailed alternate Welch *t*-test, which does not assume equal variances. All other data were analyzed using Student's *t*-test, with a *P* value <0.05 indicating statistical significance.

RESULTS

At the time of sacrifice, all obstructed kidneys were hydronephrotic and larger than normals. Kidney weight was increased at 109 days gestation in obstructed animals to a mean of 9.6 g/kidney (SD = 1.51), significantly (*P* < 0.005) greater than 6.8 g/kidney (SD = 0.63) in normal 109-day-old fetuses. At term, kidney weight was significantly increased to a mean of 21.7 g/kidney (SD = 15.3) in obstructed animals, whereas normals at term had a mean weight of 9.1 g/kidney (SD = 1.68, *P* = 0.0007). Bladder weights at 109 days were increased in obstructed animals to a mean of 4.1 g/bladder (SD = 4.16). Normal animals had a mean bladder weight of 1.5 g/bladder (SD = 0.29; NS *P* = 0.39). At term, the mean bladder weight was 13.5 g/bladder (SD = 12.7) in obstructed animals and 2.9 g/bladder in normals (SD = 0.65, *P* < 0.001). Mean animal weights were equal in both groups at 109 days as well as at term.

At 109 days gestation, after two weeks of partial bladder outlet obstruction, renin mRNA expression was significantly increased in obstructed kidneys (230% of normal, *P* = 0.009; Fig. 1). At this time point, angiotensinogen mRNA expression was detectable in all kidneys but was unchanged by partial bladder outlet obstruction. Renin mRNA expression in kidneys at term was similar in normal and obstructed animals (data not shown).

In normal fetuses, AT-1 mRNA expression was low at 60 to 75 days of gestation and increased toward the end of gestation. In contrast, AT-2 mRNA expression was highly expressed at earlier time points and decreased

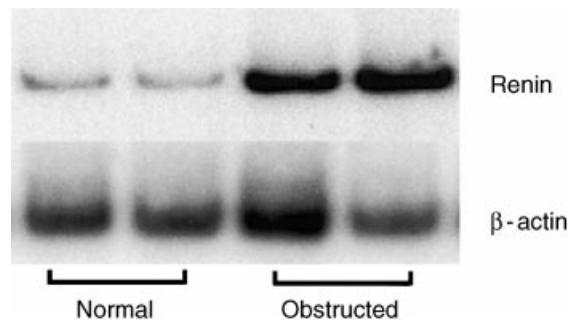


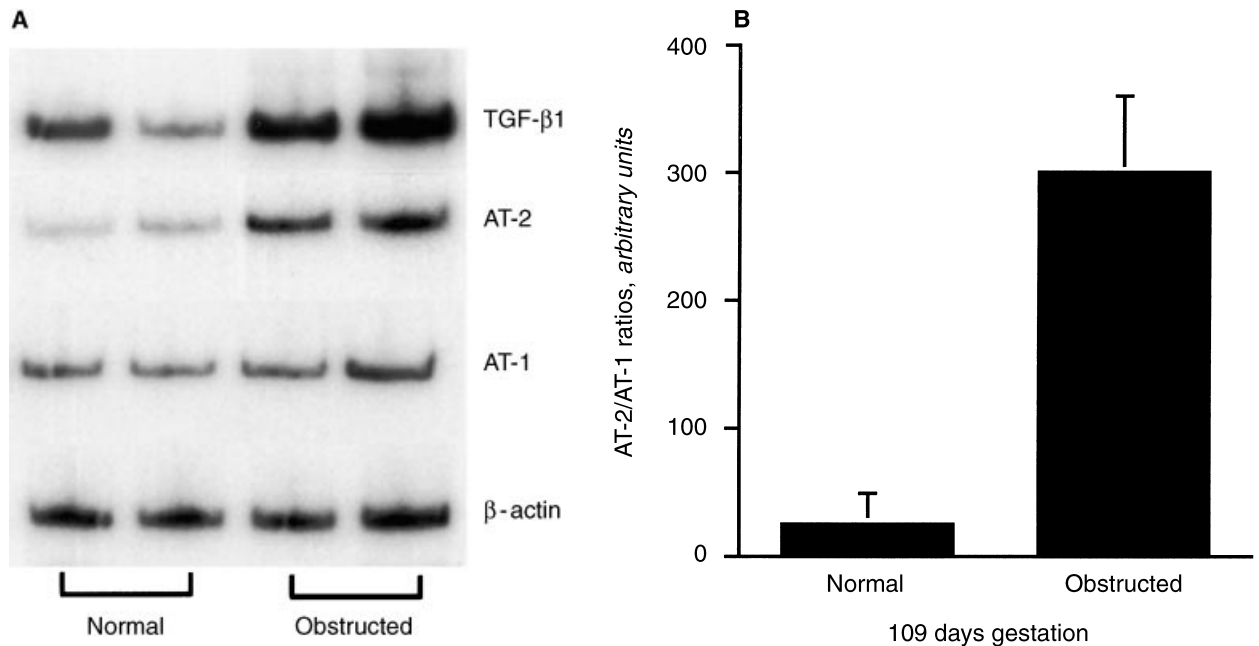
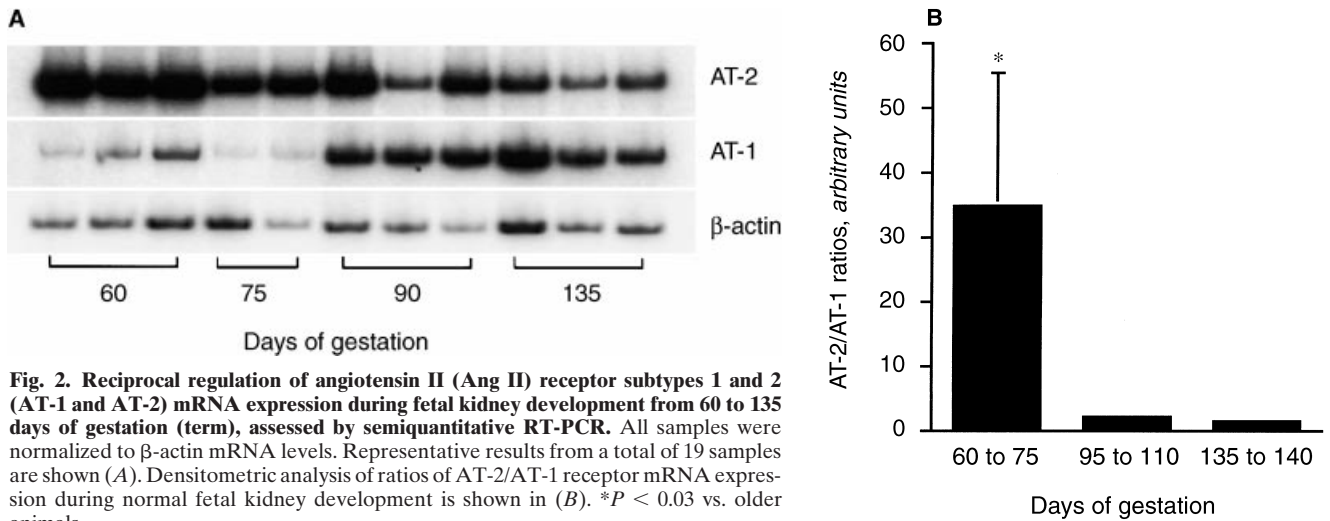
Fig. 1. Increased renin mRNA expression in two-weeks obstructed versus normal kidneys of fetal sheep at 109 days of gestation, assessed by semiquantitative RT-PCR. All samples were normalized to β -actin mRNA levels. The representative gel from two independently performed experiments is shown.

toward the end of gestation (Fig. 2A). AT-2/AT-1 ratios were therefore high at 60 to 75 days of gestation and declined thereafter (Fig. 2B). Partial bladder outlet obstruction caused persistent high expression of AT-2 mRNA in kidneys at 109 days gestation (400% of normal, *P* = 0.007), whereas AT-1 mRNA expression levels were unchanged in obstructed animals compared with controls (Fig. 3A). The mean AT-2/AT-1 ratios at 109 days were therefore significantly increased compared with normal kidneys (*P* = 0.03; Fig. 3B). Similarly, AT-2 receptor protein levels were increased in obstructed kidneys at 109 days (Fig. 4). At term, the mean AT-2/AT-1 ratios were not statistically significantly different (data not shown).

Transforming growth factor- β 1 (TGF- β 1) mRNA expression was increased in obstructed kidneys at 109 days gestation, but not at term (Fig. 3A).

DISCUSSION

Fetal partial bladder outlet obstruction causes significant changes in gene expression of several components of the renal RAS. Using semiquantitative RT-PCR, this study showed increased mRNA levels for renin, the activating enzyme of the RAS, after two weeks of obstruction. Persistently elevated levels of angiotensin receptor type 2 mRNA in two-week obstructed fetal kidneys are a novel finding in the context of obstructive nephropathy. The precise function of AT-2 receptor is not known, but it is well established that AT-2 is expressed in areas of mesenchymal differentiation in many fetal tissues [14]. Although AT-2 gene disruption studies have shown that AT-2 is not required for embryonic development [15, 16], Ang II and AT-2 receptor are linked to growth and differentiation processes as well as blood pressure regulation [reviewed in 17]. The increase in AT-2 gene expression correlated with its increased protein levels in this study, indicating the potential functional significance of this effect.



In this study, using RT-PCR, the expression of mRNA for all components of the renal RAS was found in the developing sheep kidney. This is in agreement with several other reports that describe the fetal RAS in rat [18], sheep [11, 19], and human [20] kidneys. In a previous study using Northern blot analysis, no detectable renal angiotensinogen expression during late gestation in fetal sheep was found, but angiotensinogen was highly expressed in fetal liver [21]. The increased sensitivity of

RT-PCR analysis may have permitted angiotensinogen mRNA detection. The second possibility—that mRNA for angiotensinogen was amplified from an origin other than renal—is not likely, as others found renal angiotensinogen expressed with a peak around birth in rat [22], as well as sheep [23], fetuses using nonisotopic hybridization histochemistry. In our report, mRNA levels for angiotensinogen were unchanged by partial bladder outlet obstruction, possibly because of an abundance of this

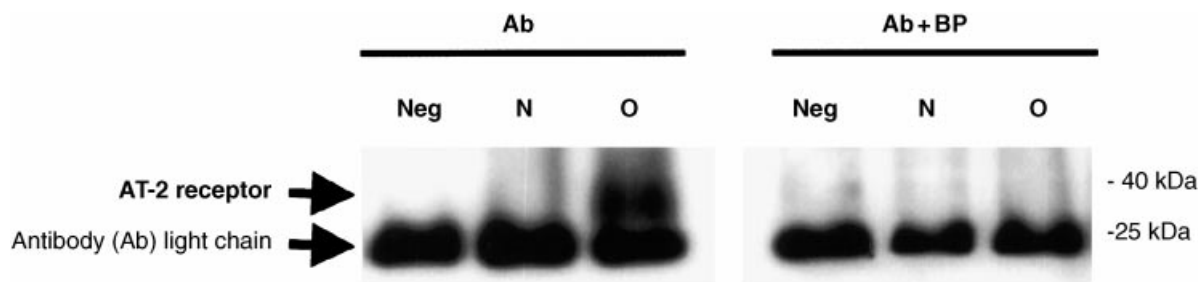


Fig. 4. Western blot analysis of angiotensin II receptor subtype 2 in normal and two-weeks obstructed kidneys at 109 days of gestation. The representative gel of three independently performed experiments is shown ($N = 4$ each gel). Ab, Increased AT-2 receptor protein expression in two-weeks obstructed (O) kidney versus normal (N) kidney of fetal sheep at 109 days gestation assessed by Western blot analysis after immunoprecipitation. Negative control (Neg). Ab + BP, specificity of AT-2 receptor antibody was confirmed by preincubating the Ab with blocking peptide (BP). Band intensity of light chains from the antibody used for immunoprecipitation indicate equal protein loading.

substrate for the more tightly regulated expression of renin.

The critical role of the renin-angiotensin system in kidney development and pathology has been demonstrated in a variety of investigations [reviewed in 24, 25]. Renin is present in the renal vasculature during fetal kidney development and changes to its mature, juxtaglomerular cell localization during postnatal life [26]. This pattern suggests an important role of the RAS in renal vascular development. Fetal partial bladder outlet obstruction induced increased levels of renal renin mRNA after two weeks of obstruction, which might suggest adaptive vascular development. In neonatal rat kidneys, renin was identified not only in the juxtaglomerular region but along the afferent arteriole in both obstructed and in the contralateral kidney exposed to unilateral ureteral obstruction, again in a pattern resembling that of earlier stages in development [27].

Further evidence for a functional role of the RAS in normal kidney development has been provided by pharmacological manipulations and targeted disruptions of RAS genes causing morphological as well as functional abnormalities of the kidneys. Inactivation of angiotensinogen [28] and angiotensin-converting enzyme (ACE) produced renal abnormalities in mice including hyperplastic arterial walls, papillary atrophy, tubular dilation, and interstitial fibrosis [29], similar to changes seen in the knockouts of both AT-1 receptor subtypes. Renal functional studies in mice lacking ACE revealed elevated urinary output, decreased urine osmolality, and slightly elevated serum creatinine levels [30]. Pharmacological manipulation of the RAS in the developing kidney in weanling rats with ACE inhibitors or AT-1 blockers results in similar histological changes, as described in the ACE knockout mice. Further studies described additional, striking vascular changes induced by Losartan (AT-1 blocker) not readily detectable on conventional histology, such as fewer distorted afferent arterioles [31]. Functional studies in these animals showed

normal glomerular filtration rates but altered tubular function [32]. Although the precise mechanisms of RAS-mediated regulation of renal development remain to be defined, its critical role in kidney development is evident from these studies.

Changes in activity of the angiotensin receptors have been shown to contribute to alterations in renal development. Nullizygote mice for both AT-1 receptor subtypes AT-1a and AT-1b showed normal survival *in utero* but decreased survival postnatally. Animals were growth impaired and hypotensive and showed abnormal kidney morphology with delayed glomerular growth and maturation, hypoplastic papillae, and renal artery hypertrophy [33]. In other studies, however, disruption of the murine Ang-II receptors AT-1a [34, 35], AT-1b [36], and AT-2 genes did not induce apparent renal pathology (abstract; Matsusaka et al, *J Am Soc Nephrol* 7:1602, 1996). AT-2 knockout mice did, however, have significant anomalies of the upper urinary tract, including the ureter and renal pelvis [25].

Gene expression of both Ang II receptor subtypes (AT-1 and AT-2) in the fetal sheep was present with reciprocal developmental regulation. This finding confirmed previous reports of developmental regulation of renal AT-1 and AT-2 receptor gene expression [11, 19], with increasing AT-1 and decreasing AT-2 message levels. Using *in situ* hybridization, the authors localized AT-1 mRNA to presumptive mesangial cells of glomeruli in 40-day gestation fetuses with continued expression in lambs up to two days old. AT-1 mRNA was also found to be expressed in medullary interstitial cells and in the cortical medullary rays. AT-2 mRNA was found in epithelial cells of the macula densa during nephrogenesis and in interstitial cells surrounding developing glomeruli, but not in the nephrogenic mesenchyme [19]. This finding would suggest that the AT-1 receptor may mediate early differentiation events, whereas the AT-2 receptor may regulate positioning of the macula densa at the vascular pole of its glomerulus. In fetal and postnatal weanling rats, AT-2

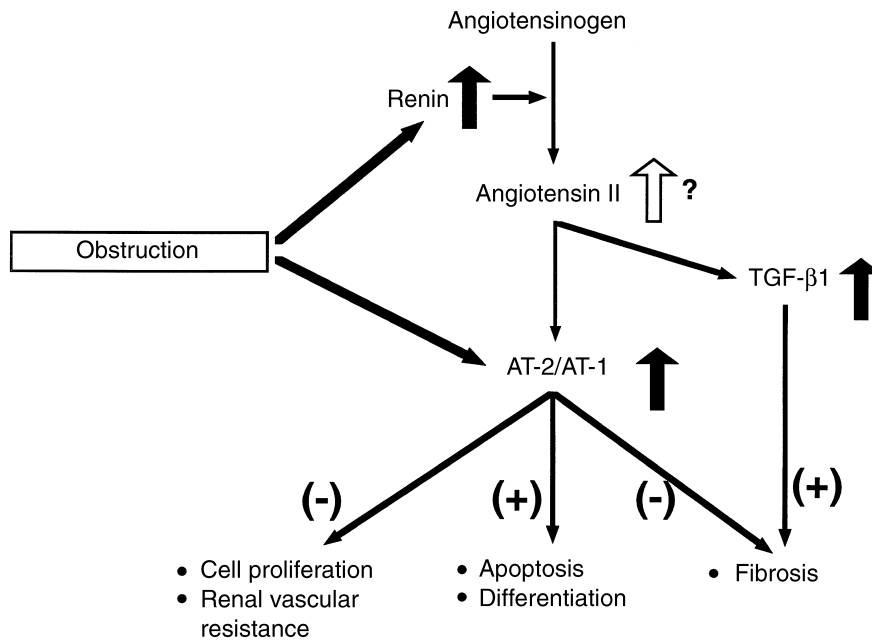


Fig. 5. A hypothesis of the effects of partial bladder outlet obstruction on the renal RAS, and its effects on fetal renal growth, differentiation, and regulation of extracellular matrix deposition.

receptors in the nephrogenic zone of the metanephric renal cortex and the advancing tubules and ampullae of the ureteric bud were described [37, 38], indicating its possible role in renal morphogenesis by modulating cell proliferation, and inducing apoptosis and early differentiation of the nephron.

This concept of the AT-2 receptor as a regulator of renal differentiation is supported by several *in vitro* cell proliferation and differentiation assays. Ang II inhibits basic fibroblast growth factor-induced proliferation in the R3T3 mouse embryonic fibroblast line that expresses only the AT-2 receptor [39], and the density of membrane AT-2 receptor in R3T3 cells responds to a variety of mitogenic and antiproliferative stimuli [40, 41]. Cell differentiation processes have been attributed to Ang II stimulation via AT-2 receptor in neuronal cell lines [42]. Ang II has been shown to induce apoptosis, mediated by the AT-2 receptor [43] in mouse embryonic fibroblasts and in several other cell lines [44, 45]. In contrast, the pattern of AT-1 expression suggests a role in final differentiation of the nephron and in mediating the classic hemodynamic effects of Ang II [reviewed in 24]. This body of evidence supports the view of the angiotensin receptors acting as a regulatory system for Ang II activity in early renal development and in response to obstruction. In general, the AT-1 receptor subtypes act in a growth-promoting manner, whereas AT-2 appears to act to promote differentiation.

Obstruction induces increased TGF- β 1 expression, as shown in kidneys exposed to two weeks of obstruction. Ang II is known to be a potent activator of fibrogenesis

through stimulation of TGF- β 1 expression [reviewed in 2]. This mechanism would explain the previously described renal interstitial fibrosis in prenatal obstructive uropathy [46]. Figure 5 depicts our findings and summarizes the known and discussed effects of RAS and the possible interaction with TGF- β 1 in the developing kidney exposed to obstruction.

We have shown that partial bladder outlet obstruction causes the immature pattern of angiotensin receptor gene expression of early gestation (60 to 75 days) to persist into the last trimester of gestation. This finding raises the possibility that the factors produced by obstruction (increased pressure, dilation, tubular stretch) are similar to the driving forces during early development, causing renal growth and early differentiation of nephrons. Persistence of the immature pattern of AT-2/AT-1 expression may be a compensatory response geared to adapt the renal architecture to its altered functional demands in the setting of obstruction. Renal fibrosis induced by obstruction is exaggerated in the AT-2 knockout mouse [47]. This suggests another possible compensatory response to the obstructive condition, whereby elevated AT-2 expression is maintained as a counterbalance to fibrosis and collagen deposition induced by increased TGF- β 1 activity. The developmentally regulated counterbalance of the activities of the AT-1 and AT-2 receptors and its dysregulation by obstruction appears therefore to be a potentially important factor in the pathophysiology of congenital obstructive nephropathy and associated growth alterations, interstitial fibrosis, and hemodynamic changes.

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