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Differential Gene Expression and Regulation of Renal Angiotensin II Receptor Subtypes (AT_1 and AT_2) during Fetal Life in Sheep

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ABSTRACT

Previous studies have shown that angiotensin II subtype 2 (AT_2) receptors appear early during renal embryonic development. Factors involved in the regulation of AT_2 receptors during renal development, however, have not been investigated. The present study was designed *1*) to characterize the ontogeny of renal AT_2 gene expression during the last half of gestation in fetal sheep and newborn lambs, *2*) to compare changes in AT_1 and AT_2 gene expression during renal development, *3*) to determine the influence of AII in modulating renal AT_1 and AT_2 gene expression during fetal life, and *4*) to characterize the role of cortisol in modulating renal AT_2 gene expression during the last trimester of gestation in fetal sheep. To perform these studies, we first isolated and cloned a polymerase chain reaction product that has 92 and 90% homology with the cDNA encoding the human and rat AT_2 receptors, respectively. Using this sheep AT_2 cDNA probe, we demonstrated that the sheep AT_2 gene was encoded in a single locus. In addition, we showed that renal AT_2 mRNA expression was high early during fetal life (60–90-d gestation) and decreased rapidly thereafter. In contrast, the expression of renal AT_1 receptor gene was low at 60-d gestation and increased during the last trimester of gestation. We found that a continuous i.v. infusion (1 mL/h) of AII (9.5 nM/h) for 24 h, which raised plasma AII levels from 84 $\pm$ 9 pg/mL to 210 $\pm$ 21 pg/mL, decreased the expression of both renal AT_1 and AT_2 genes in third trimester fetal sheep. On the other hand, we observed that cortisol, known to decrease AT_1 gene expression in the fetus, had no effect on AT_2 gene expression. In summary, this study demonstrates that AII, but not glucocorticoids, contributes to the regulation of renal AT_2 gene expression during development and that there is differential regulation of AT_1 and AT_2 receptors. **(*Pediatr Res* 38: 896–904, 1995)**

Abbreviations

AII, angiotensin II
AT_1, angiotensin II subtype 1 receptor
AT_2, angiotensin II subtype 2 receptor
PRA, plasma renin activity
PCR, polymerase chain reaction
rRNA, ribosomal RNA

Compared with adult blood values, levels of various components of the renin-angiotensin system are elevated during fetal life and in the newborn period (1). Circulating renin and plasma AII levels are high in fetal and newborn animals (2), and the expression of renin, angiotensinogen, and angiotensin-converting enzyme genes appear to be developmentally regulated (3–6).

It has been suggested that AII is implicated in the regulation of renal function (1) and renal growth (7) during development. The biologic effects of AII are mediated by two distinct specific receptors (AT_1 and AT_2) located in the plasma membrane of different tissues (8). Studies in rats (9, 10) and sheep (11) have shown that the expression of kidney AT_1 receptor mRNA is developmentally regulated. In the sheep, renal AT_1 mRNA expression is elevated during the last trimester of gestation and decreases during the second postnatal week (11). In the rat, the expression of renal AT_1 receptor mRNA is also higher in immature than in adult animals (12). Discrete expression of AT_1 receptor has been observed as early as 2 d of postnatal age in rat immature glomeruli (10).

Both *in situ* hybridization and autoradiographic studies have also shown that AT_2 receptors are present in the fetal mesenchyme (13), in the mesonephros before its involution (14), in the nephrogenic zone of the metanephric renal cortex (10, 14), and in immature glomeruli (10, 15, 16). Factors involved in the regulation of AT_2 receptor expression during renal development, however, have not been investigated.

To further understand the ontogeny of AT_2 receptor during fetal renal development, we isolated and characterized a por-

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tion of the sheep AT_2 receptor cDNA sequence. Studies were then performed *1*) to characterize the ontogeny of renal AT_2 gene expression during the last half of gestation in fetal sheep and in newborn lambs, *2*) to compare the changes in AT_1 and AT_2 mRNA expression during renal development, *3*) to characterize the role of cortisol in modulating renal AT_2 gene expression during the last trimester of gestation, and *4*) to determine the influence of AII in modulating AT_1 and AT_2 gene expression during fetal life in sheep.

METHODS

Animals. Studies were performed in fetal sheep and newborn lambs of Dorset and Suffolk mixed breeding, obtained from a local source. The gestational ages of the fetuses were based on the induced ovulation technique as previously described (17).

Surgical preparation and experimental protocols. Anesthesia and surgery of the ewe and fetus were performed as previously described (18). Briefly, the ewe was fasted for 24 h before surgery and anesthetized using a mixture of halothane (1%), oxygen (33%), and nitrous oxide (66%).

In a first series of studies, we determined the ontogeny of the kidney AT_2 receptor during the fetal and neonatal periods in sheep. For these studies, pregnant ewes were anesthetized as described, and the uterus was exteriorized to gain access to the fetus. Samples of fetal kidney cortex were obtained at 60, 90, 120, and 140 d of gestation, term 145 d: four different fetuses were studied at 60-, 90-, and 120-d gestation and five fetuses were studied at 140-d gestation. Similar procedures were performed in anesthetized newborn lambs at 1 (n = 5) and 10 d (n = 6) of age. After being removed, the kidney cortex was snap frozen in liquid nitrogen and stored at −70°C.

In a second series of studies, we investigated the effects of cortisol on the renal expression of both AT_1 and AT_2 gene expression in 10 pairs of chronically instrumented twin fetal sheep at 130-d gestation. For these studies, surgery was performed under sterile conditions. Briefly, the uterus was opened over the fetal hindlimbs, and polyethylene catheters were placed into the fetal femoral arteries and veins of both twin fetuses as previously described (11). Additional catheters were secured in the peritoneal cavity of each twin. At the end of surgery, the fetal incisions were closed, and the fetus was returned to the uterus. Uterine and maternal abdominal muscles and maternal skin were closed in separate layers. All catheters were exteriorized through a s.c. tunnel and placed in a cloth pouch on the ewe's flank. Ampicillin sodium (Wyeth Laboratories, Philadelphia, Pa) was administered to the ewe intramuscularly before surgery (2 g) and infused into the amniotic cavity after surgery (2 g). Pregnant ewes were returned to individual pens and allowed free access to food and water.

After a 72-h recovery period from surgery, one of each set of twins was given a continuous intraperitoneal infusion of cortisol (MW 484.52) for exactly 48 h at a rate of 3 mg/h (6.2 μmol/h) (1 mL/h). The other twin served as control. The infusion was carried out with a portable peristaltic infusion pump (Cormed, Inc., Middleport, NY) secured on the back of the ewe in a specially designed jacket that allowed the animals to move freely during the infusion. Fetal arterial blood pressure, heart rate, and amniotic pressure were monitored in each twin fetus for 30 min before and at the end of the 48-h intraperitoneal infusion of cortisol. Fetal arterial blood from each twin fetus was taken before and 48 h after starting the cortisol infusion for determination of arterial pH, P_{CO_2}, P_{O_2}, hematocrit, and plasma cortisol and AII concentrations and PRA. At the end of the 48-h infusion period, the ewes were anesthetized as described, the uterine cavity was opened, and kidneys from both fetuses were removed, snap frozen in liquid nitrogen, and stored at −70°C.

In a third series of studies, we investigated the effects of AII on the renal expression of AT_1, AT_2, and renin genes in nine pairs of chronically instrumented twin fetal sheep at 129 ± 2 d of gestation (3.18 ± 0.11 kg). The surgical preparation was identical to what has been described for the second series of studies. Briefly, after a 72-h recovery from surgery, one of each set of twins was administered a continuous i.v. infusion of AII (MW 1046) at a rate of 10 μg/h (9.5 nM/h) (3.17 ± 0.11 $\mu g \cdot kg^{-1} \cdot min^{-1}$) (1 mL/h) for exactly 24 h, as described for the second series of studies. Fetal arterial blood pressure, heart rate, and amniotic pressure were monitored in each twin fetus for 30 min before and at the end of the 24 h intraperitoneal infusion of AII. Fetal arterial blood from each twin fetus was taken before and 24 h after starting the AII infusion for determination of arterial pH, P_{CO_2}, P_{O_2}, hematocrit, PRA, and plasma AII concentrations. At the end of the 24-h infusion period, the kidneys were removed from both fetuses as described for the second series of studies, snap frozen in liquid nitrogen, and stored at −70°C.

All procedures were performed within the regulations of the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the University of Iowa Animal Care and Use Committee.

Analytical procedures. Arterial blood for pH, P_{CO_2}, and P_{O_2} was collected anaerobically in heparinized glass syringes, and measurements were immediately determined with appropriate pH, P_{CO_2}, and P_{O_2} electrodes at 39°C using an IL-1303 pH blood gas analyzer (Laboratory Instruments, Schaumburg IL). Hematocrit was determined in duplicate using a meter caliper. RIA, previously established in our laboratory, were used to measure plasma cortisol (19) and plasma AII (20) concentrations and PRA (20, 21).

Isolation of RNA. Total renal cortical cellular RNA was isolated using TRI-REAGENT (Molecular Research Center, Cincinnati OH). RNA was quantified spectrophotometrically by absorbance at 260 nm. RNA samples were stored as an ethanol precipitate at −70°C until further analysis.

Isolation, characterization, and sequencing of AT_2 partial cDNA from sheep kidney. A partial AT_2 cDNA clone was amplified by PCR from approximately 1 ng of cDNA after reverse transcription. Reverse transcription was performed with 1 μg of total RNA from sheep kidney using an avian myeloblastoma virus reverse transcriptase (Boehringer-Mannheim, Indianapolis IN).

PCR was performed using two specific primers prepared by a model 391 DNA synthesizer (Applied Biosystems, Foster

City, CA) and derived from the first and sixth transmembrane domains of the rat AT_2 receptor (22, 23). The sequence of primer 1 (forward) was 5′-GCA GAT AAG CTT TTG GAA GCA ATT CCT G-3′, which was defined by bp 118–145 (22, 23). The AT_2 primer 2 (reverse) encompassed bp 947–920 (22, 23), and the sequence was 5′-AAG GAA TTC ACA CAG CTG TTG GTG AAT C-3′. The primer sequences differed from the corresponding rat AT_2 sequence (22, 23) by 1 nucleotide in primer 1 and 2 nucleotides in primer 2. Primer 1 had a T substituted for the A at site 128, and primer 2 had an A substituted for the G at site 943 and a C substituted for the A at site 939. These sequence alterations created a *Hin*dIII restriction site at the 5′ end of the PCR product and an *Eco*RI restriction site at the 3′ end. The conditions for PCR were 94°C for 1 min, 53°C for 1 min and 72°C for 1 min for 35 cycles; the first cycle was held at 94°C for 3 min instead of 1 min, and the final cycle had 5 min of primer extension time at 72°C.

The 830-bp PCR product was gel-purified (Qiaex; Qiagen, Chatsworth, CA) and cloned into the plasmid vector pT7/T318U (Ambion). Before ligation, both the PCR product and the plasmid vector were digested with *Hin*dIII and *Eco*RI. DNA sequencing was performed at the University of Iowa DNA Core Facility (Dave Moser, Director) on a model 373A automated DNA sequencer (Applied Biosystems) using vector- and sequence-specific primers, Taq DNA polymerase and fluorescent dye-labeled terminators. DNA and protein sequences were aligned using the program Bestfit from the Sequence Analysis Software Package of the Genetics Computer Group (24).

Preparation of AT_2 RNA probe. The plasmid pAT_2 was treated with the restriction enzyme *Hin*dIII and then purified by phenol chloroform extraction and ethanol precipitation. The resultant linear plasmid was used to generate a labeled antisense RNA probe using T7 RNA polymerase (United States Biochem Corp., Cleveland, OH) and [d-^{32}P]UTP (Amersham Corp, Arlington Heights, IL).

Preparation of AT_1 and renin RNA probes. A sheep AT_1 receptor partial cDNA sequence from nucleotide 114 to 783 was isolated as previously described (11). The plasmid pAT_1 was then treated with the restriction enzyme *Nde*I and purified by phenol extraction and ethanol precipitation. The resultant linear plasmid was used to generate a labeled antisense RNA probe (11).

A clone containing a 0.7-kb fragment of rat renin cDNA was also prepared from a full-length 1.43-kb rat renin cDNA (25) obtained from Dr. K. R. Lynch (University of Virginia, Charlottesville). This cDNA yields antisense RNA when transcribed from the T7 promoter after linearizing with *Eco*RI.

Preparation of 18S rRNA probe. An 18S rRNA probe was used to confirm equal loading and transfer of RNA. The 18S rRNA probe was prepared from an 18S cDNA clone corresponding to a 82-bp fragment of a highly conserved region of human 18S rRNA obtained from Ambion.

Northern blot hybridization. Aliquots of 5 μg of RNA were fractionated by formaldehyde-agarose gel electrophoresis (26). After electrophoresis, RNA was transferred to a 0.45-μm Nytran filter. The filters were prehybridized for 1 h at 60°C in a solution of 50% deionized formamide, 5 × SSPE, 5 × Denhardt's reagent, 0.5% SDS, and 200 μg/mL denaturated sheared salmon sperm DNA. Hybridization of filters was carried out with fresh hybridization buffer solution containing 2×10^6 counts·min^{-1}·mL^{-1} of the radiolabeled probe. The hybridization reaction was carried at 60°C for 12–18 h. Filters were then sequentially washed according to the manufacturer's specifications. This included three low stringency washes (1 × SSPE, 0.5% SDS) at 68°C and a high stringency wash (0.1 × SSPE, 0.5% SDS) at 65°C. Hybridization signals were detected and quantitated using an AMBIS 4000 Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA) as previously described (11). Background counts above each lane were determined and subtracted from total counts generated in each region of interest to yield a net count value. In addition, the washed filters were exposed to Kodak XAR film at −70°C.

Southern blot hybridization. To test whether the sheep AT_2 gene was encoded in a single locus, genomic sheep DNA was isolated from sheep blood. Ten micrograms of DNA was digested with each of the following restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I according to the manufacturer (New England Biolabs, Inc., Berverly, MA). The digested DNA was separated on a 0.7% agarose gel and visualized on a UV transilluminator after staining with ethidium bromide. The DNA was transferred to a nylon membrane (Zeta Probe; Bio-Rad Laboratories, Hercules, CA) by capillary action and immobilized on the membrane by UV cross-linking (Stratalinker; Stratagene, LaJolla, CA). The partial cDNA probe of the sheep AT_2 gene was labeled with [α-^{32}P]CTP by PCR using gene-specific primers (27). Hybridization and detection were performed as suggested by the manufacturer (Bio-Rad).

Data analysis. For quantitation of mRNA abundance, all samples were analyzed together on a single Northern blot hybridization to control for day-to-day variations in hybridization efficiency. Northern blots were done in triplicate. Abundance of AT_1, AT_2, and renin mRNAs was expressed as total net radioactive counts, after subtracting background counts for each sample.

Comparisons among the different groups of animals were performed using one-way analysis of variance. When the analysis of variance indicated significant differences among groups, as calculated by the F statistic, the Duncan multiple comparison procedure was performed to determine significant differences between each group (28). Paired and unpaired t tests were also used to compare means between two groups. Statistical significance is defined as $p < 0.05$, and the results are presented as mean ± SEM.

RESULTS

Isolation, characterization, and sequencing of AT_2 receptor partial cDNA from sheep kidney. The sheep AT_2 partial cDNA sequence from nucleotide 142 to 921 and corresponding AT_2 cDNA sequences from other species are presented in Figure 1. The deduced amino acid sequence from the sheep AT_2 partial cDNA and comparison between the sheep sequence and sequences from other species are presented in Figure 2. The percent homology between the sheep AT_2 partial

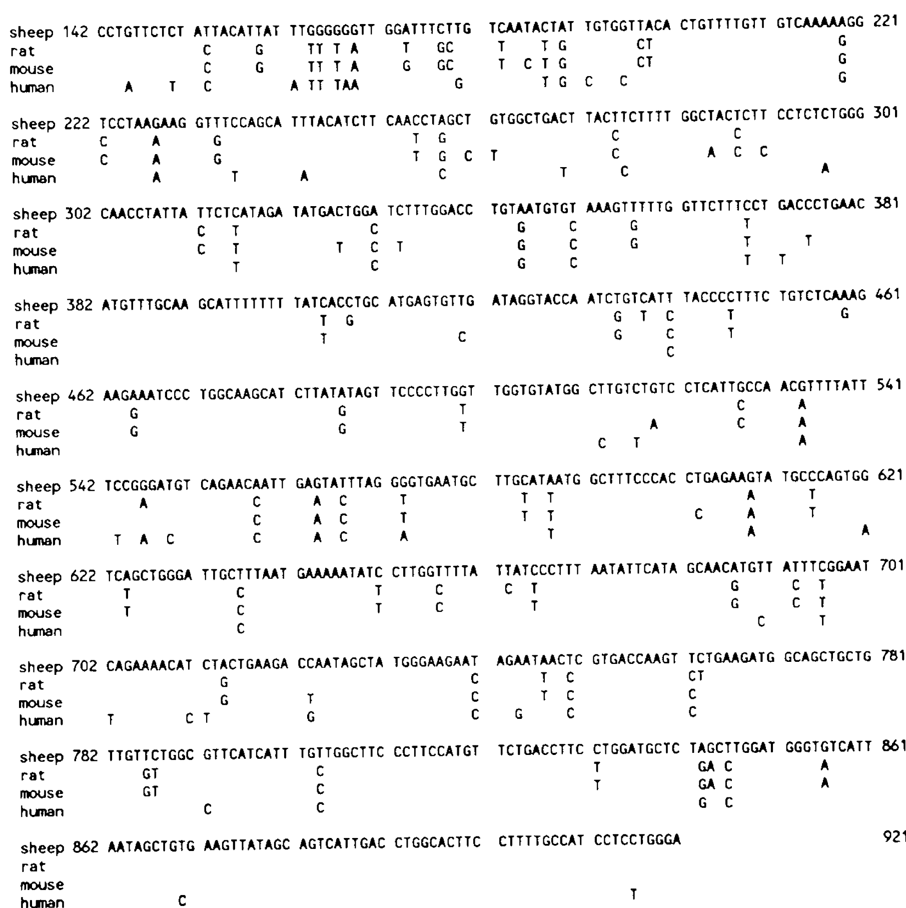

Figure 1. Sheep AT_2 partial cDNA sequence from nucleotide 142 to 921 and interspecies comparisons. The percent homology between AT_2 cDNA sequence from sheep kidney and corresponding AT_2 cDNA sequences from other species is 90% with the rat (22, 23) and the mouse (30), and 92% with the human (29).

cDNA sequence and corresponding AT_2 cDNA sequences from other species is 92% with the human (29) and 90% with both the rat (22, 23) and the mouse (30). The percent homology between the sheep AT_2 partial amino acid sequence and corresponding AT_2 amino acid sequences from other species is 96% with the human (29) and 95% with both the rat (22, 23) and the mouse (30).

To test whether the sheep AT_2 gene was encoded in a single locus, Southern blot analysis was performed on genomic sheep DNA using a partial cDNA probe from the sheep AT_2 gene (Fig. 3). Several different restriction enzymes were tested and a single DNA fragment was detected suggesting the presence of a single AT_2 locus in the sheep.

Renal AT_2 mRNA expression in fetal and newborn sheep. Renal AT_2 mRNA expression was higher early during fetal life (60- and 90-d fetal sheep) and decreased rapidly thereafter (Fig. 4*A*); the expression of the renal AT_2 gene decreased by 75% during the last trimester of gestation. After the first week of life, renal AT_2 gene expression was only 12% of the expression observed at 60-d gestation. Because 18S ribosomal mRNA levels (Fig. 4*A*, *upper panel*) tended to be lower at 90-d gestation than at any other gestational age, one may suggest that renal AT_2 mRNA levels in 90-d fetuses may even be higher when factored for 18S ribosomal mRNA levels.

In contrast to the high level of AT_2 mRNA expression early during fetal life, the expression of renal AT_1 receptor gene (Fig. 4*B*) was low at 60-d gestation, increased during the last trimester of gestation (from 90–140-d gestation, term 145 d) and decreased rapidly after birth. Some of these results have been published previously (11).

Effects of cortisol infusion on kidney AT_2 mRNA expression in third trimester fetal sheep. Previous studies by our group (11) have shown that cortisol produced a significant decrease in renal AT_1 mRNA abundance in fetal sheep during the last trimester of gestation. Using total RNA from kidneys used previously to investigate the role of cortisol on renal AT_1 mRNA expression (11), we characterized the effect of cortisol on renal AT_2 gene expression in 10 pairs of chronically instrumented fetal sheep studied at 130 ± 1 d of gestation (term, 145 d in sheep). One of each set of twins received a continuous intraperitoneal infusion (1 mL/h) of cortisol for 48 h (3 mg/h) (6.2 μmol/h). Plasma cortisol concentrations increased from 32 ± 7.1 nmol/L to 1126 ± 231 nmol/L in cortisol treated fetuses ($p > 0.001$) and changed only slightly in control fetuses from 22.7 ± 3.6 nmol/L to 45.8 ± 8.2 nmol/L. Cortisol infusion had no significant effect on fetal arterial pH, Po_2, and Pco_2 and produced no significant changes in fetal plasma angiotensin II levels, as demonstrated previously (11).

A rise in fetal plasma cortisol levels had no significant effect on renal AT_2 mRNA levels (Fig. 5). Hybridization of 18S rRNA probe showed no significant differences in renal 18S

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                            I                                 II
sheep   48   PVLYYIIWGV GFLVNTIVVT LFCCQKGPKK VSSIYIFNLA VADLLLLATL   97
rat               M FVI    A  IV   S
mouse             M FVI    A  IV   S                        L
human         I     FVI       IV

                                                    III
sheep   98   PLWATYYSHR YDWIFGPVMC KVFGSFLTLN MFASIFFITC MSVDRYQSVI   147
rat                  Y     L
mouse                Y     L
human                Y     L       L

                                    IV
sheep  148   YPFLSQRRNP WQASYIVPLG WCMACLSSLP TFYFRDVRTI EYLGVNACIM   197
rat                          V   V
mouse                        V   V
human                            V

                                     V
sheep  198   AFPPEKYAQW SAGIALMKNI LGFIIPLIFI ATCYFGIRKH LLKTNSYGKN   247
rat
mouse
human

                                    VI
sheep  248   RITRDQVLKM AAAVVLAFII CWLPFHVLTF LDALAWMGVI NSCEVIAVID   297
rat                                               T   I
mouse                                             T   I
human

                     VII
sheep  298   LALPFAILLG                                               307
rat
mouse
human
```

Figure 2. Sheep AT_2 partial amino acid sequence and interspecies comparisons. The percent homology between sheep kidney AT_2 partial amino acid sequence and corresponding AT_2 amino acid sequences from other species is 95% with the rat (22, 23), and the mouse (30), and 96% with the human (29).

rRNA levels between control (1330 ± 50 net counts) and cortisol-treated fetuses (1324 ± 59 net counts).

Effects of AII infusion on kidney AT_1, AT_2, and renin mRNA levels in third trimester fetal sheep. This series of studies was designed to characterize the effects of AII in regulating the expression of kidney AT_1, AT_2, and renin mRNAs during the last trimester of gestation in fetal sheep. Nine pairs of chronically instrumented fetal sheep (129 ± 2 d of gestation) were studied. One of each set of twins received a continuous i.v. infusion (1 mL/h) of AII (10 μg/h or 9.5 nM/h) for 24 h. The effects of AII infusion on fetal arterial blood values, mean arterial blood pressure, and heart rate in both control and AII-treated fetuses are presented in Table 1. No significant changes in arterial pH, Pco_2, Po_2, and hematocrit were observed in both control and AII-treated fetuses. AII infusion, however, produced a significant increase in plasma AII concentration in AII-treated fetuses (from 84 ± 9 to 210 ± 21 pg/mL), but had no effect in twin-match control fetuses. Significant decreases in PRA (from 4.68 ± 1.49 to 2.05 ± 0.67 ng $AI \cdot mL^{-1} \cdot h^{-1}$) were also observed in AII-treated fetuses. AII infusion increased arterial blood pressure from 54 ± 2 to 76 ± 5 mm Hg in AII-treated fetuses, but had no effects in control twin fetuses.

The effects of i.v. AII infusion on renal AT_1, AT_2, and renin mRNA levels are presented in Figures 6-8. Northern blot hybridization demonstrated significant ($p < 0.05$) decreases in both renal AT_1 (Fig. 6) and AT_2 (Fig. 7) mRNA levels in AII-treated fetuses when compared with twin-match control fetuses. Infusion of AII produced significant decreases in renal AT_1 (−27.4 ± 11.2%) and AT_2 (−31.1 ± 6.7%) mRNA levels. Twenty-four hours after starting the AII infusion, renal AT_1 and AT_2 mRNA levels expressed as ^{32}P were, respectively, 548 ± 97 and 386 ± 63 net counts in twin-match control fetuses and 375 ± 57 and 253 ± 38 net counts in AII-treated fetuses. AII infusion produced also a significant decrease ($p < 0.01$) in renal renin mRNA expression (Fig. 8). No significant differences were observed in renal 18S rRNA levels between AII-treated and twin-matched control fetuses, indicating similar RNA loading.

DISCUSSION

The present study suggests that the AT_2 gene is present in a single locus in the sheep, and demonstrates a high level of homology between the sheep AT_2 partial cDNA sequence and corresponding AT_2 cDNA sequences from other species (22, 23, 29, 30). In addition, we have shown that the expression of AT_2 receptor mRNA in fetal sheep kidney is high during the second trimester of gestation and decreases rapidly thereafter. In contrast, renal AT_1 mRNA expression is low early during fetal life (60-d gestation), increases during the last trimester of gestation, and decreases after birth, confirming previous results

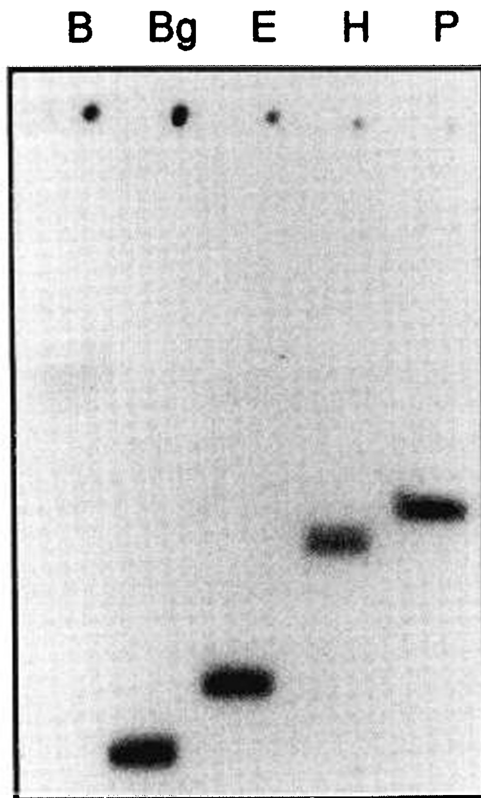

Figure 3. Southern blot analysis of genomic sheep DNA hybridized with a partial cDNA probe from the sheep AT_2 gene. Each lane represents 10 μg of sheep genomic DNA digested with the following restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; and P, *Pst*I.

(11). This study also demonstrates that a rise in fetal plasma AII levels produces significant decreases in the expression of both renal AT_1 and AT_2 genes and in renal renin mRNA levels. Finally, we observed that cortisol, which is known to decrease renal AT_1 mRNA expression (11), had no significant effects on renal AT_2 expression in near-term fetuses.

Previous studies using autoradiographic techniques have demonstrated that AT_2 receptors predominate in 15–17-wk gestation human fetal kidneys and are localized in both cortex and medulla (31, 32). Similarly in the rat, AT_2 receptor binding sites appear first in immature renal structures (S-shaped glomeruli) (15), whereas AT_1 receptor sites are observed when kidney development reaches more advanced developmental stages (10, 15). Using *in situ* hybridization, investigators have shown that the expression of both AT_1 and AT_2 mRNAs appear simultaneously in embryonic fetal mouse kidney (14-d gestation) (14). However, it appears that, during fetal life in the rat, the pattern of distribution of AT_1 and AT_2 receptors differs (16). Interestingly, the intensity of AT_2 mRNA's signal is greater than the AT_1 mRNA's signal in several nephrogenic areas that undergo apoptosis after nephrogenesis (14).

In agreement with previous fetal studies using autoradiographic techniques (10, 15, 31, 32) and *in situ* hybridization (14, 16, 33), we found that both AT_1 and AT_2 receptors are developmentally regulated in the fetal sheep kidney. We have shown that the expression of AT_2 receptors is elevated early during fetal life (60-d gestation) and decreases rapidly during

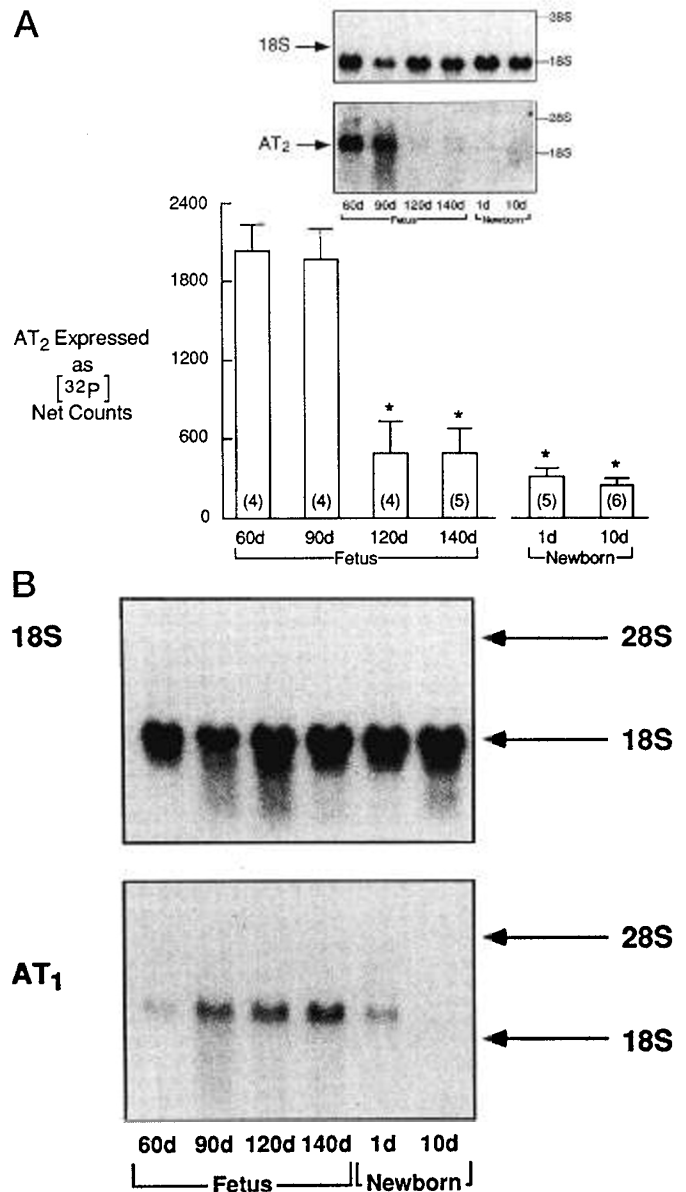

Figure 4. (*A*) *Upper panel*: representative autoradiogram of Northern blots of total renal cortical RNA hybridized with a human 18S rRNA probe and with a sheep AT_2 RNA probe labeled with ^{32}P. *Lower panel*: abundance of renal AT_2 mRNA is expressed as total net counts of ^{32}P in the six age groups. Numbers in parentheses refer to the number of animals in each group. $*p < 0.05$ when compared with levels from 90-d fetus. (*B*) Representative autoradiograms of Northern blots of total renal cortical RNA hybridized with a human 18S rRNA probe and with a sheep AT_1 RNA probe labeled with ^{32}P.

the third trimester of gestation, whereas AT_1 mRNA levels increase rapidly before birth, as previously described (11). Adding to these previous results (11), we are now showing that the renal expression of AT_1 gene is low during the second trimester of gestation when renal AT_2 gene expression is elevated.

Factors regulating these changes is of intense current interest. It has been suggested that the differential regulation of AT_1 and AT_2 receptors during embryonic development is related to the different cell types in which these receptors are expressed (14). For example, the cellular location of AT_2 expression in the embryonic kidney overlaps with cells undergoing apoptosis

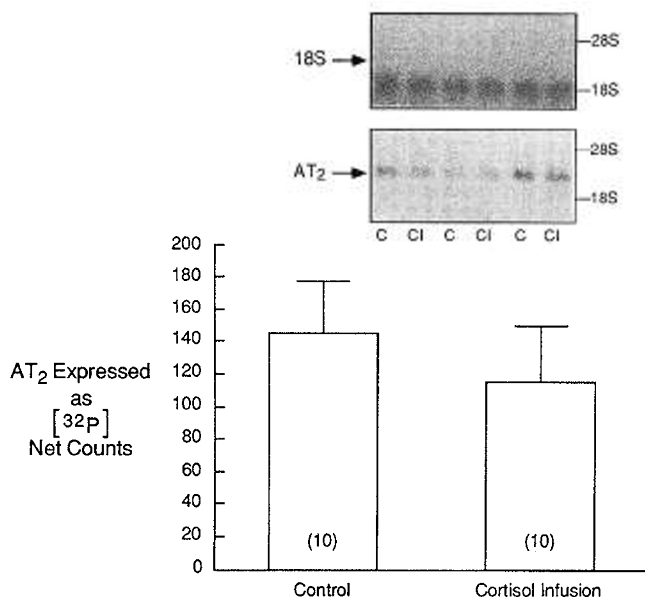

Figure 5. *Upper panel*: representative autoradiogram of Northern blots of total renal cortical RNA hybridized with a human 18S rRNA probe and with a sheep AT_2 RNA probe labeled with ^{32}P. Total renal cortical RNA was extracted from control and cortisol-infused twin fetal sheep at 130-d gestation. Each pair of control and cortisol-infused blots represents twin fetuses studied simultaneously. Numbers in parentheses refer to number of nimals in each group; *C*, control; *CI*, cortisol infusion. *Lower panel*: abundance of renal AT_2 mRNA is expressed as total net counts of ^{32}P. Ten pairs of twin fetuses were studied. Values are means ± SEM. Numbers in parentheses refer to the number of animals in each group; *C*, control; *AII*, angiotensin II infusion.

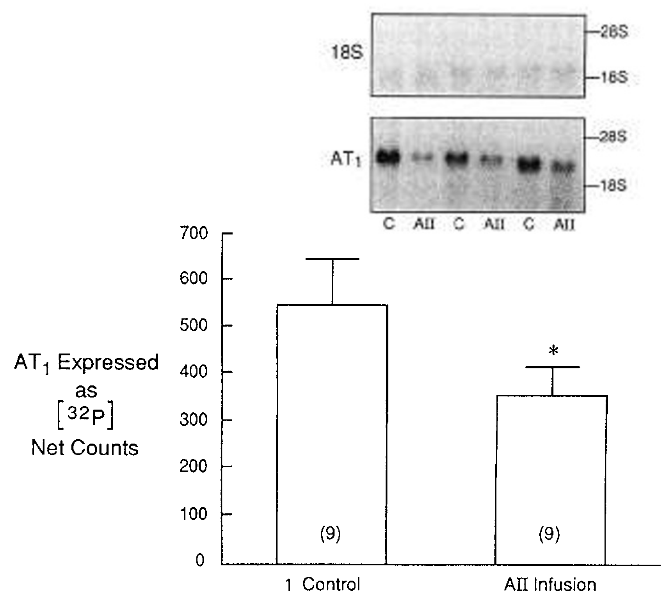

Figure 6. *Upper panel*: representative autoradiogram of Northern blots of total renal cortical RNA hybridized with a human 18S rRNA probe and with a sheep AT_1 RNA probe labeled with ^{32}P. Total renal cortical RNA was extracted from control (*C*) and AII-infused (*AII*). *Lower panel*: Abundance of renal AT_1 mRNA is expressed as total net counts of ^{32}P. Nine pairs of twin fetuses were studied. Values are means ± SEM. Numbers in parentheses refer to the number of animals in each group; *C*, control; *AII*, angiotensin II infusion.

Table 1. *Arterial blood values, mean arterial blood pressure, and heart rate in both control and AII-treated fetal sheep*

	Control		AII-treated	
	Pre	24 h	Pre	24 h
pH	7.31 ± 0.02	7.30 ± 0.01	7.31 ± 0.02	7.29 ± 0.01
Pco_2 (mm Hg)	44 ± 1	45 ± 1	44 ± 1	45 ± 2
Po_2 (mm Hg)	20 ± 1	21 ± 2	20 ± 1	22 ± 1
Hematocrit (%)	26 ± 1	27 ± 1	27 ± 1	27 ± 2
PRA (ng AI· mL^{-1}·h^{-1})	4.36 ± 1.12	3.05 ± 1.13†	4.68 ± 1.49	2.05 ± 0.67*
AII (pg/mL)	99 ± 15	95 ± 9†	84 ± 9	210 ± 21*
HR (beats/min)	173 ± 5	170 ± 5	168 ± 4	166 ± 7
MABP (mm Hg)	52 ± 2	54 ± 3†	54 ± 2	76 ± 5*

Pre, before starting the i.v. infusion (1 ml/h) of AII (9.5 nM/h); 24 h, 24 h after starting the intravenous infusion of AII; PRA, plasma renin activity; AII, angiotensin II; HR, heart rate; MABP, mean arterial blood pressure. Conversion factor for SI units: 1 mm Hg = 133.3 Pa, 1 mol of AI=1296 g, 1 mol AII-1046 g. Values are means ± SEM.

* $p < 0.05$ when compared with Pre values.

† $p < 0.05$ when compared with corresponding 24-h AII-treated values.

after nephrogenesis (14). This suggests that programmed death of embryonic cells may contribute to the decreased expression in AT_2 receptors later during development. In addition, the modulatory effects of several growth factors may be also involved in regulating the expression of both AT_1 and AT_2 receptors during development (34, 35). For example, platelet-derived growth factor-BB reduces AT_2 and increases AT_1 binding sites in culture of rat aorta smooth muscle cells (35). Other growth factors such as epidermal growth factor and endothelin-1 have a suppressive effect on AT_2 receptors (35).

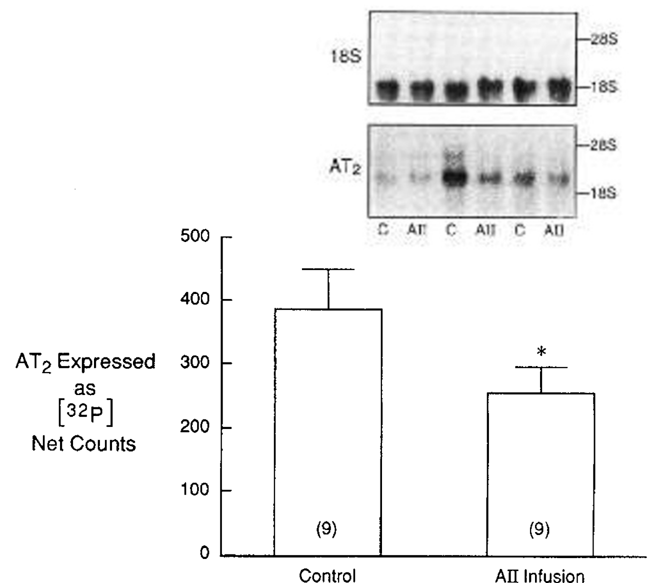

Figure 7. *Upper panel*: representative autoradiogram of Northern blots of total renal cortical RNA hybridized with a human 18S rRNA probe and with a sheep AT_2 RNA probe labeled with ^{32}P. Total renal cortical RNA was extracted from control (*C*) and AII-infused (*AII*) twin fetal sheep at 130-d gestation. Each pair of control and AII-infused blots represents twin fetuses studied simultaneously. *Lower panel*: abundance of renal AT_2 mRNA is expressed as total net counts of ^{32}P. Nine pairs of twin fetuses were studied. Values are means ± SEM.

The present results demonstrate that a rise in circulating levels of AII produces a significant decrease in AT_1 receptor mRNA levels in fetal sheep kidney. These results differ from previous studies in adults (36, 37) that found no change in glomerular and mesangial AT_1 mRNA levels after exposure to

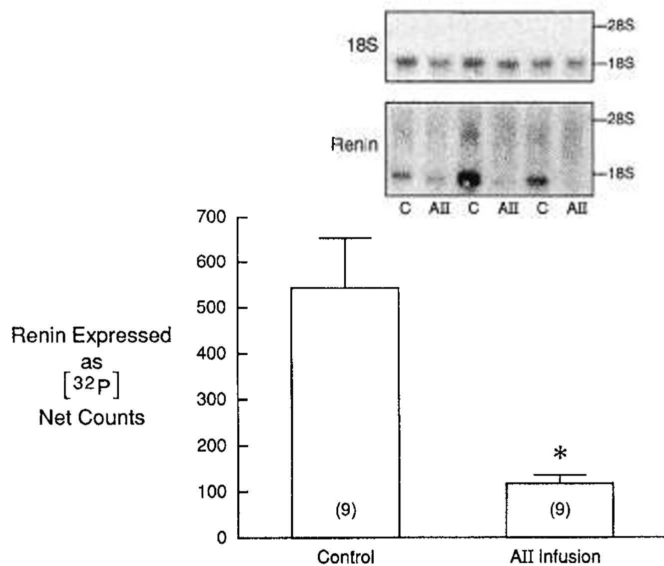

Figure 8. *Upper panel*: representative autoradiogram of Northern blots of total renal cortical RNA hybridized with a human 18S rRNA probe and with a rat renin RNA probe labeled with ^{32}P. Each pair of control and AII-infused blots represents twin fetuses studied simultaneously. Total renal cortical RNA was extracted from control (*C*) and AII-infused (*AII*) twin fetal sheep at 130-d gestation. *Lower panel*: abundance of renal renin mRNA is expressed as total net counts of ^{32}P. Nine pairs of twin fetuses were studied. Values are means ± SEM.

AII. Similarly, sodium depletion, a condition associated with high plasma AII levels, is not associated with a decrease in AT_1 receptor mRNA levels in adult rat kidneys (14). Sodium depletion, however, produces a down-regulation of AII receptor density (38–40). On the other hand, other investigators have shown that AII reduces AT_{1A} receptor mRNA expression in cultured vascular smooth muscle cells (41) and mesangial cells (42). Recent studies have also shown that agents that increase intracellular cAMP may induce a decrease in AT_1 receptor mRNA levels in glomerular mesangial cells (42). Finally, it has been suggested that activation of protein kinases C and A can up-regulate both AT_1 receptor mRNA levels (43) and binding sites (44). Further studies are needed to determine the role of these factors in the regulation of transduction and translation of AT_1 receptors during renal development.

As for the AT_1 receptors, AT_2 receptors located in the kidney are down-regulated by AII during the last trimester of gestation in fetal sheep. This finding represents the first demonstration of a role of AII in modulating AT_2 receptor expression in the developing kidney. Because we observed, however, that AII produced an increase in fetal arterial blood pressure, one may suggest that blood pressure itself may participate in the down-regulation of AT_2 gene expression. In addition, because the present studies were performed in third trimester fetuses where the expression of AT_2 receptor gene had already started to decrease, it remains unclear if AII can decrease the expression of AT_2 receptors during the second trimester of gestation, when the expression of the AT_2 gene is elevated. Further studies are needed to determine the exact role of these receptors as well as the role of cGMP and phosphotyrosine phosphatase (45) in modulating the action, distribution, and regulation of AT_2 binding sites and gene expression during fetal renal development.

The present study also demonstrates that chronic infusion of AII decreases fetal PRA and renal renin mRNA levels. Previous studies have shown that a rise in plasma AII levels decreases PRA in the fetus (20). To our knowledge, it is the first demonstration that AII participates in the regulation of renal renin gene expression during fetal life. The present results, however, do not allow us to determine whether this decline in renal renin gene expression during a rise in plasma AII levels is secondary either to a direct action of AII on the juxtaglomerular cells or to an increase in fetal renal perfusion pressure or both.

Another interesting observation is that glucocorticoids, which decrease renal AT_1 gene expression during the last trimester of gestation in fetal sheep (11), do not alter AT_2 mRNA levels. These results are somewhat different from previous studies in adult pancreatic acinar cells which demonstrate that glucocorticoids down-regulate AT_2 receptors (46). It is not clear, however, if glucocorticoids would have an effect of AT_2 receptor gene expression at an earlier time during gestation when the expression of these receptors is higher. Additional studies are needed to clarify these issues.

In summary, renal AT_2 gene expression is high during the second trimester of gestation and decreases rapidly thereafter, whereas the expression of AT_1 receptor gene increases during the third trimester of gestation to decrease after birth. AII produces similar decrease in the expression of both renal AT_1 and AT_2 genes during the third trimester of gestation in sheep. Glucocorticoids, which are known to decrease renal AT_1 gene expression in the fetus (11), do not significantly alter the expression of renal AT_2 receptor gene during the last trimester of gestation.

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