Angiotensin II receptor (type 1 and 2) expression peaks when placental growth is maximal in sheep

IRENE KOUKOULAS, TOMRIS MUSTAFA, REBECCA DOUGLAS-DENTON, AND E. MARELYN WINTOUR
Howard Florey Institute of Experimental Physiology and Medicine, The University of Melbourne, 3010 Victoria, Australia

Received 4 February 2002; accepted in final form 20 June 2002

Koukoulas, Irene, Tomris Mustafa, Rebecca Douglas-Denton, and E. Marelyn Wintour. Angiotensin II receptor (type 1 and 2) expression peaks when placental growth is maximal in sheep. Am J Physiol Regul Integr Comp Physiol 283: R972–R982, 2002; 10.1152/ajpregu.00070.2002.—In sheep, placental size is maximal by midgestation, but blood flow continues to increase until term. No nerves are present and ANG II is thought to be a major regulator of vascular tone. We hypothesized that angiotensin type 2 receptors (AT2) would predominate over type 1 (AT1) until late in gestation and be primarily expressed in the vasculature. Real-time PCR, hybridization histochemistry, and ligand-binding studies were performed on placenta and fetal membranes at 27, 45, 66, and 130 days of gestation (term ≈ 150 days) to determine quantitative changes and localization. The maximum level of AT1 expression occurred in the 45-day placenta and was located predominantly in the maternal stromal cells. AT1 receptors were expressed in the endothelial cells of the chorion in the first half of pregnancy, where later in gestation, both AT1 and AT2 receptors were predominant in blood vessels. These results suggest that ANG II, via the AT1 receptor, may have hitherto unsuspected important roles in the growth/function on the ovine placenta during the maximal growth phase.

AT1 receptor; AT2 receptor; placenta; pregnancy; real-time polymerase chain reaction

Optimal growth of a fetus depends on adequate provision of oxygen and substrates as well as the appropriate endocrine environment (33). Intrauterine growth retardation (IUGR), resulting in low birth weight for gestational age, is now known to be associated with an increased risk for the development of cardiovascular and metabolic disease in the adult (39). Successful placental function depends on the appropriate growth and adequate perfusion of both maternal and fetal components of the placenta (42). In sheep, maximal placental growth occurs in the first half of pregnancy, whereas blood flow and exchange capacity continue to increase until term, supporting the major growth of the fetus in the last third of gestation (42).

One system implicated in normal vascular placenta and blood flow control is the renin-angiotensin-system (RAS) and, in the human, a complete RAS is found in the placenta (41). Prorenin and angiotensinogen are produced by the decidua, a maternal tissue not found in many other species (27). Angiotensin-converting enzyme (ACE) mRNA is found in the human placenta (53), and ANG II receptors, predominantly of the AT1 type, are present in both the syncytiotrophoblasts and cytotrophoblasts (7, 28) and are reduced in IUGR. There is also evidence that abnormalities of AT1 expression are important in preeclampsia (14). An agonist antibody to AT1, which can stimulate the production of tissue factor, has been shown to be produced in preeclampsia (48). Recently, it has been shown that AT1 in the human placenta is upregulated at both the mRNA and protein level in preeclampsia (26).

Most studies of fetal physiology have been conducted in sheep, which is a nondeciduate species and in which there is no substantial evidence for the production of renin by the placenta. There have been extensive studies on the effects of ANG II on both the uterine artery and umbilical artery, although these have been done almost exclusively in the last third of pregnancy (8, 43). The AT2 receptor type has been found to predominate in the smooth muscle of the maternal uterine artery (34) and in the majority of fetal blood vessels with the notable exception of the external umbilical artery, in which AT1 predominates, at least in the second half of pregnancy (5). There has been, however, no systematic study of the proportions and location of AT1/AT2 expression throughout pregnancy in sheep.

The ovine placenta differs from that of the human in that it consists of 60–100 individual cotyledons, which are formed by the attachment of the fetal trophotroblast cells (which also form the chorion) at predetermined sites (caruncles) in the uterine wall (52). The fetal membranes consist of the chorion, which overlies both the amnion and the allantois; the fetus develops within the amniotic cavity. The allantoic cavity is a second fluid-filled sac that occupies the tip of the pregnant horn and all the non-pregnant horn in a singleton
pregnancy. Fetal urine enters the allantoic sac via the urachus, which arises in the bladder wall and travels in the center of the umbilical cord (51).

The hypothesis tested in the current study was that AT1/AT2 expression would be confined to blood vessels in the ovine placenta and fetal membranes and that AT2 expression would predominate until late in pregnancy. The unexpected finding was that AT1 was most highly expressed early in pregnancy and that the maternal stroma of the placenta was the major site of expression, which raises exciting new possibilities for the function of ANG II in early placental growth/function.

MATERIALS AND METHODS

Animals. Animals were killed by an overdose of pentobarbital sodium (Lethabarb, Arnolds, Boronia, Australia; 100 mg/kg body wt). Allantois, amnion, chorion, and cotyledon tissue samples were collected from four fetuses from the following age groups: 65–77, 96–104, and 140 days of gestation (term is 145–150 days) for real-time PCR studies. Cotyledon tissue samples were also collected from four fetuses at 27 days of gestation and six fetuses at 45 days of gestation. Intercarunculi uterine tissue was collected from four pregnant ewes at 27 days of gestation. Additional cotyledon samples were also collected at 41 and 51 days of gestation as well as 130 days of gestation for in situ hybridization histochemistry and receptor binding assays. Fresh tissues were rinsed in physiological saline (0.9%) and cleaned with gauze to remove excess blood before being placed in physiological saline (0.9%) and cleaned, tissue was immediately frozen in liquid nitrogen and stored at −80°C until further use or fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4 h at room temperature and routinely processed.

Isolation of total RNA. Total RNA was extracted from frozen tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method (6).

DNase treatment of total RNA. For each total RNA sample, 20 μg was DNase treated in a 100-μl reaction containing 10 mM DTT, 5 mM MgCl2, 40 mM Tris-HCl (pH 7.5), 0.2 U/μl of RNase inhibitor, and 0.03 U/μl of RNase-free DNase I. The reactions were incubated at 37°C for 15 min prior to a heat inactivation step at 65°C for 10 min followed by three phenol extractions and one chloroform extraction. The total RNA samples were ethanol precipitated to remove residual organic solvent contamination and resuspended in milli Q water (0.05% DEPC treated). The total RNA quality and content was established after obtaining absorbance readings at 260 and 280 nm. The integrity of the total RNA was examined after fractionating 1 μg onto a formaldehyde agarose gel. Samples were stored at −80°C until further use.

dDNA synthesis. Each cDNA synthesis reaction consisted of 1× TaqMan Universal Master Mix (including the passive reference ROX), 50 nM 18S TaqMan probe, 20 nM 18S forward primer, 80 nM reverse primer, 150 nM AT1, Taqman probe, 900 nM forward and reverse primers or 75 nM AT2 TaqMan probe, and 300 nM forward and reverse primers. cDNA (5 ng) and no RT preparations (no RT prep) were used to perform the quantitation of gene expression using the comparative C T method. Separate AT1 and AT2 reactions were set up with 18S was detected in both of these reactions, although the primers for 18S were limited. In pilot experiments, multiplex vs. non-multiplex Ct–values were compared, where for all genes studied, the Ct values were identical, suggesting that multiplex reactions did not affect Ct values. A validation experiment was also performed to test whether the comparative Ct method could be used for the relative quantitation of gene expression. Here, approximately equal efficiencies of AT1 and AT2 amplifications together with 18S were tested using different template concentrations and, in both sets of multiplex reactions, approximately equal PCR efficiencies were obtained.

PCR reactions were carried out in 25-μl volumes consisting of 1× TaqMan Universal Master Mix (including the passive reference ROX), 50 nM 18S TaqMan probe, 20 nM 18S forward primer, 80 nM reverse primer, 150 nM AT1, Taqman probe, 900 nM forward and reverse primers or 75 nM AT2 TaqMan probe, and 300 nM forward and reverse primers. cDNA (5 ng) and no RT preparations (no RT prep) were used to perform the quantitation of gene expression using the comparative Ct method. Separate AT1 and AT2 reactions were set up with 18S was detected in both of these reactions, although the primers for 18S were limited. In pilot experiments, multiplex vs. non-multiplex Ct–values were compared, where for all genes studied, the Ct values were identical, suggesting that multiplex reactions did not affect Ct values. A validation experiment was also performed to test whether the comparative Ct method could be used for the relative quantitation of gene expression. Here, approximately equal efficiencies of AT1 and AT2 amplifications together with 18S were tested using different template concentrations and, in both sets of multiplex reactions, approximately equal PCR efficiencies were obtained.

Real-time PCR. For the relative quantification of gene expression, real-time PCR was performed (16) using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reverse transcription reactions were performed in a GeneAmp PCR System 9600 (Applied Biosystems) with incubations at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. EDTA was added at a final concentration of 0.01 M to each reaction tube before storage at −80°C.

In vitro transcription of riboprobes. After the recombinant plasmids were linearized, both antisense and sense (negative control) riboprobes were prepared by in vitro transcription using the Promega riboprobe kit (Promega, Madison, WI), where [α-35S]UTP (100 Ci/mmol) was incorporated (Bresatec, Thebarton, Australia). The riboprobes were hydrolyzed (9),
precipitated, and resuspended in 10 mM DTT before hybridization histochemistry. Plasmid constructs consisting of the ovine AT₁ and AT₂ receptor sequences were kindly provided by Dr. Jean Robillard, Ann Arbor, MI.

In situ hybridization histochemistry. Paraffin sections (4 µm) were cut and mounted onto silanized slides and dried overnight at 37°C. The slides were subsequently dewaxed and rehydrated before sections were digested at 37°C for 10 min with Pronase E (Sigma) at a final concentration of 125 µg/ml. The sections were rinsed twice in 0.1 M phosphate buffer (pH 7.4) before post-fixing in 4% paraformaldehyde for 10 min at room temperature. Again, the sections were rinsed twice in 0.1 M phosphate buffer (pH 7.4) before dehydration and air drying. Hybridization was performed by applying ~60 µl of riboprobe, at a final concentration of 0.02 ng/µl, onto each section, which were subsequently covered with a coverslip. The hybridization buffer consisted of 10% of 10x salts (100 mM Na₂HPO₄, 3 M NaCl, pH 6.8, 100 mM Tris·HCl, pH 7.5, 50 mM EDTA, pH 8.0, 0.2% bovine serum albumin, 0.2% Ficoll 400, 0.2% polyvinyl pyrrolidone), 50% formamide, 0.7 mg/ml yeast tRNA, 10 mM DTT, and 10% dextran sulfate. Sections were hybridized overnight at 50°C in a sealed humidified chamber. On the following day, coverslips were removed after thorough washing in formamide (50% formamid, 10% 10x salts) at 50°C and three subsequent washes at 1 h each with gentle rocking. The sections were rinsed in RNase A buffer (0.5 M NaCl, 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, pH 8.0), followed by RNase A (150 µg/ml; Sigma-Aldrich) treatment at 37°C for 2 h with gentle rocking to remove free riboprobes. Once the digestion was complete, nonspecific binding was removed after three washes with 2x SSC at 65°C for 30 min each. The sections were then rinsed briefly in milli Q water to remove residual salt, dehydrated, air dried, and exposed to Fuji phosphorimaging plates (BASII) overnight to determine potential hybridizing sites after scanning on a Fujix BAS 2000 scanner. Slides were dipped in liquid emulsion (Ilford, Essex, UK) and exposed for 10 days at room temperature before developing in filtered Kodak D19 developer for 2 min and fixing in Ilford Hypam xer (1/5 dilution) for 2 min. Sections were finally stained with hematoxylin and eosin.

Image acquisition. For in situ hybridization histochemistry, light and dark images were captured on a Nikon Microphot microscope linked to a Sony 930P video camera (Sony, Australia). Digitized images were subsequently processed using microcomputer imaging device software (Image Research, St. Catherine's, Canada).

Membrane fraction preparation. Frozen placental tissues were moderately thawed, diced, and homogenized in ice-cold hypotonic buffer consisting of 50 mM Tris, 5 mM EDTA, pH 7.4. After centrifugation at 600 g for 5 min at 4°C to remove cellular debris, supernatants were centrifuged at 50000 g for 20 min at 4°C and the recovered membrane pellets were resuspended in isotonic binding buffer consisting of 10 mM Na₂HPO₄, 150 mM NaCl, 5 mM EDTA, and 0.02% NaN₃, pH 7.4. Protein content was subsequently determined using the Bradford protein assay (3).

Radioligand. The ANG II antagonist [125I]-[Sar¹,Ile⁸]ANG II was radioiodinated using the lactoperoxidase-glucose oxidase oxidase method and subsequently purified by HPLC on a C₁₈ reverse-phase column (32).

Receptor binding assays. Saturation binding studies were carried out by incubating 20 µg of freshly prepared placental membranes (45 or 130 day, n = 4 for each age) in the presence of increasing concentrations (1–12,000 pM) of [125I]-[Sar¹, Ile⁸]ANG II in binding buffer (10 mM Na₂HPO₄, 150 mM NaCl, 5 mM EDTA, 0.02% NaN₃, 0.2% BSA, 0.5 µg/ml bacitracin, 100 µM PMSF, pH 7.4) for 1 h at 22°C. Nonspecific binding was determined in the presence of 10 µM unlabeled ANG II. Free from bound radioligand was separated by vacuum filtration through a cell harvester (Brandel) using Whatman GF/B glass fiber filter paper (Whatman International) presoaked in 1% polyethyleneimine. Ice-cold wash buffer consisting of 10 mM Na₂HPO₄, 150 mM NaCl, 5 mM EDTA, 0.02% NaN₃, pH 7.4, was used to wash the filter paper before the retained radioactivity was measured using an automatic gamma counter (Packard, Meriden, CT). Tripli- cate points were performed where all eight samples were studied simultaneously and the raw data were averaged and analyzed using GraphPad Software, version 3.0 (San Diego, CA).

AT₁ and AT₂ levels were determined by incubating 30 µg of freshly prepared placental membranes with 0.5 µCi/ml of [125I]-[Sar¹, Ile⁸]ANG II in the presence of 1 µM PD 123319 (AT₂ receptor antagonist) or 1 µM candesartan (AT₁ receptor antagonist) for 1 h at 22°C. Nonspecific binding was determined in the presence of 1 µM cold ANG II. Free from bound radioactivity was then separated using the standard filtration method described above.

Statistical analyses. Data in different groups were measured by one-way ANOVA with all pairwise multiple comparison procedures (Tukey test). All data are reported as means ± SE, unless otherwise stated. The level of significance for all tests was set at P < 0.05.

RESULTS

Quantitation of AT₁ and AT₂ gene expression. Five different placental ages were studied (27, 45, 66 ± 1, 100 ± 4, and 140 days of gestation) as well as 27-day intercaruncular (uterus between cotyledons) and adult kidney samples. All of the samples studied were compared with the mean of one 140-day placenta sample assayed four times. A statistically significant increase in AT₁ gene expression was detected in the 45-day cotyledon (12.2 ± 1.1) compared with the 27 (2.7 ± 0.24), 66 ± 1 (4.6 ± 0.17), 100 ± 4 (1.9 ± 0.48), and 140 days of gestation (4.1 ± 0.27). AJP-Regul Integr Comp Physiol • VOL 283 • OCTOBER 2002 • www.ajpregu.org

![Fig. 1. AT₁ gene expression in the placenta at 27, 45, 66 ± 1, 100 ± 4, and 140 days of gestation (open bars). Intercotyledonal uterus at 27 days of gestation (filled bar) and adult kidney (hatched bar) are also presented. *Statistically significant difference (P < 0.05).](http://ajpregu.physiology.org/DownloadedFrom/)
(1.1 ± 0.066) day cotyledons (Fig. 1). AT₁ gene expression in the intercaruncular part of the uterus at 27 days of gestation (2.3 ± 0.67) was very similar to the 27-day cotyledon (2.7 ± 0.24). The adult kidney displayed a marked amount of AT₁ gene expression (8.5 ± 0.54) compared with most of the placenta samples studied (P < 0.05). Only the 45-day cotyledon had a greater amount of AT₁ gene expression (1.5-fold) compared with the adult kidney, although this comparison was not statistically significant.

The greatest amount of AT₂ gene expression was detected in the 45-day cotyledon (5.3 ± 1.6) compared with the 140-day cotyledon, although this was not significant (Fig. 2). Therefore, AT₂ gene expression followed a similar pattern to that of AT₁. Similar AT₂ mRNA levels were also observed in the 66 ± 1-day cotyledon (4.8 ± 0.93). Once again, at 100 ± 4 days of gestation (3.3 ± 0.45), AT₂ gene expression was not significantly lower compared with the 45- and 66 ± 1-day cotyledons. The lowest levels of AT₂ mRNA were observed in the 27 (0.36 ± 0.22)- and 140 (0.7 ± 0.29)-day cotyledons as well as the 27-day uterus (0.75 ± 0.39) and adult kidney (0.33 ± 0.19).

The fetal membranes, together with the placenta, at 66 ± 1, 100 ± 4, and 140 days of gestation were also studied for AT₁ and AT₂ gene expression (Fig. 3). The mean of 66 ± 1-day amnion samples from four different animals was used as a calibrator in these studies. In support of the above findings, no statistically significant difference in AT₁ mRNA content was observed between the 66 ± 1 (2.3 ± 0.35)-, 100 ± 4 (1.1 ± 0.29)-, and 140 (0.61 ± 0.15)-day cotyledons (Fig. 3). A statistically significant increase in AT₁ gene expression, however, was observed in the 66 ± 1-day chorion (3.23 ± 0.63) compared with the 140 (0.72 ± 0.37)-day chorion but not the 100 ± 4-day chorion (1.7 ± 0.36) (Fig. 3). No statistically significant change in AT₁ gene expression was observed between the 66 ± 1 (0.63 ± 0.17)-, 100 ± 4 (1.4 ± 0.55)-, and 140 (0.57 ± 0.24)-day allantois groups as well as the 66 ± 1 (1.0 ± 0.0)-, 100 ± 4 (0.67 ± 0.19)-, and 140 (0.35 ± 0.13)-day amnion groups (Fig. 3).

Once again, no statistically significant difference in AT₂ gene expression was observed between the 66 ± 1 (4.1 ± 1.1)-, 100 ± 4 (2.9 ± 0.31)-, and the 140 (1.8 ± 1.3)-day cotyledons (Fig. 3). No statistically significant difference in AT₂ gene expression was observed between the 66 ± 1 (5.4 ± 1.4)-, 100 ± 4 (3.3 ± 0.86)-, and 140 (2.1 ± 0.55)-day chorion groups (Fig. 3). This also applied to the allantois groups studied, where at 66 ± 1, 100 ± 4, and 140 days of gestation, the levels of AT₂ gene expression were 1.1 ± 0.18, 1.9 ± 0.87, and 1.8 ± 0.49, respectively (Fig. 3). No difference in AT₂ gene expression was observed between the 66 ± 1 (1.0 ± 0.0)-, 100 ± 4 (2.5 ± 1.1)-, and 140 (2.7 ± 1.1)-day amnion groups (Fig. 3). The 66 ± 1-day chorion dis-
played the greatest amount of AT2 gene expression (Fig. 3) and a statistically significant decrease (107-fold) was observed in the adult kidney (0.05 ± 0.025), where the lowest levels of AT2 mRNA were found (data not shown).

**Localization of AT1 and AT2 gene expression.** Abundant levels of AT1 gene expression were observed in the cotyledon at 41 and 51 days of gestation. AT1 mRNA localization was observed in the maternal component of the cotyledon surrounding the fetal villi (Fig. 4, A and C). AT1 mRNA was also localized in the blood vessels of the chorion at 140 days of gestation, where both the endothelial and smooth muscle cells demonstrated hybridization (see Fig. 6A). Earlier in gestation, little AT1 mRNA was present in the epithelial cells of the chorion (67 days) and none in the blood vessels (see Fig. 6E). Only sparse AT1 receptor gene expression was observed in the 27-, 66 ± 1-, 100 ± 4- (data not shown), and 140 (Fig. 4E)-day placenta, although still in the maternal component of the placenta.

AT2 mRNA localization was less prevalent in the placenta (Fig. 5). Gene expression was also confined to the maternal component of the cotyledon although primarily adjacent to the fetal villi (Fig. 5, A and C). This part of the placenta is the syncytium where fetal binucleate cells and uterine epithelial cells fuse, therefore establishing a fetomaternal hybrid tissue. AT2 mRNA is clearly absent, however, in the fetal trophoblast cells (Fig. 6A). Hybridization was also present in the blood vessels of the chorion, although, in contrast to the AT1, gene expression was only present in the smooth muscle cells and not the endothelial cells (Fig. 6C). AT2 mRNA was absent in the chorion early in gestation (65 days) (Fig. 6G) and this was also true for the 27-, 66 ± 1-, 100 ± 4- (data not shown), and 140 (Fig. 5E)-day placenta.

**Angiotensin receptor levels in the 45- compared with the 130-day placenta.** Scatchard plots generated from saturation binding studies indicated significantly higher levels of total ANG II binding sites in the 45
maximal binding ($B_{\text{max}}$) = 1 fmol/µg of protein]- compared with the 130 ($B_{\text{max}}$ = 0.252 fmol/µg of protein)-day sheep placenta (Fig. 7). $^{125}$I-[Sar$^1$,Ile$^8$]ANG II bound with similar affinity to both 45- and 130-day placental membranes with $K_d$ values of 0.5 and 0.6 nmol, respectively. These results clearly suggest a change in total ANG II binding sites in the 45-day placental tissue rather than a change in affinity for the radioligand. Incubation of 45- and 130-day placental membranes in the presence of specific AT$_1$ and AT$_2$ antagonists indicated significantly higher AT$_1$ levels in the 45-day placental tissue than AT$_2$ (Fig. 8), where a 1.8-fold difference was observed. In the 130-day placenta, AT$_1$ and AT$_2$ levels were approximately equal (Fig. 8). Again, ANG II binding was greater in the 45- compared with the 130-day placenta, where a statistically significant increase was observed in the total and AT$_1$ and AT$_2$ binding samples, where 4.5-, 5.8-, and 3.4-fold differences were observed, respectively (Fig. 8). Nonspecific binding was also low for both placental ages (Fig. 8).

**DISCUSSION**

**Summary of results.** In this study, using the sensitive real-time PCR technique, gene expression for both AT$_1$ and AT$_2$ receptors was detected in all the ovine fetal membrane and placental samples studied. A statistically significant increase in AT$_1$ gene expression was observed in the 45-day placenta compared with 27, 66 ± 1, 100 ± 4, and 140 days of gestation as well as the 66 ± 1-day chorion compared with 100 ± 4 and 140 days of gestation. By in situ hybridization histochemistry, AT$_1$ and AT$_2$ mRNA was localized in the blood vessels of the chorion later in gestation, whereas earlier in gestation, AT$_1$ mRNA only was localized to the epithelial cells. AT$_1$ and AT$_2$ mRNA was observed early in the placenta (41–51 days of gestation), primarily in the maternal stromal cells rather than in the blood vessels. Receptor ligand binding studies supported the above finding where greater levels of angiotensin receptors were observed earlier in gestation than late and that the predominant receptor was of the AT$_1$
Because the receptor ligand binding studies represented intracellular membranes also, and recent findings have identified intracellular ANG II (47, 56), the role of intracellular angiotensin receptor function in the placenta cannot be excluded.

**Source of ligand.** AT1 and AT2 receptors are responsible for mediating the effects of ANG II; however, adequate amounts of renin, angiotensinogen, and ACE are required for ANG II to be produced. All three of these components are made in early ovine fetuses, where Wintour and coworkers (49) demonstrated that by 41 days of gestation, the lung, brain, and liver tissues contained angiotensinogen and ACE gene expression. The kidney also, constituting the meso- and metanephros, contained renin and angiotensinogen mRNA, as well as protein (49). In the pregnant ewe, plasma renin concentrations have been observed to increase to 60 days of gestation (50); however, it is only until mid-pregnancy that this increased renin activity is observed. No renin mRNA can be detected in ovine placental and fetal membranes (unpublished results), which is not surprising because the main intrauterine

---

**Fig. 6.** Dark-field (A, C, E, and G) and bright-field (B, D, F, and H) photomicrographs of tissue hybridized with AT1 (A, B, E, and F) and AT2 (C, D, G, and H) 35S-labeled riboprobes. Photomicrographs represent chorion samples at 140 (A-D; magnification, ×200), 67 (E and F; magnification, ×100), and 65 (G and H, magnification, ×100) days of gestation. AT1 mRNA was localized in the primary placental arteries of the chorion, where both endothelial and smooth muscle cells displayed hybridization (A and B). In photomicrographs C and D, AT2 mRNA was localized in the smooth muscle cell layer of the primary placental arteries only. AT1 gene expression was observed in the chorion early in gestation, primarily in the epithelial cells and not the blood vessels (E and F). AT2 gene expression, however, was very low in the 65-day chorion (G and H). bv, Blood vessels.
source of prorenin in deciduate species (e.g., humans) is the maternal decidua and the sheep is nondeciduate. ANG-(1–7) is also increased in the fetus compared with the ewe and could potentially affect the placenta, because renin, AT_1, and AT_2 receptor gene expression levels increase in the fetal kidneys with ANG-(1–7) administration (37).

Studies on the AT_1 receptor in the sheep. In the ovine placenta, the AT_1 gene appeared to be expressed largely at 45 days of gestation and decreased thereafter to term, whereas at 27 days of gestation, levels were similar to late gestation. Zheng and coworkers (55) also studied AT_1 expression in the sheep placenta, although only at 130 days of gestation. By immunohistochemistry, AT_1 binding was observed in the caruncular and intercaruncular components of the uterus, as well as the fetal component of the cotyledons. Microvessels in these components stained positive for AT_1 receptors, which consisted of both smooth muscle and endothelial cells. In the fetal component of the cotyledon, staining was predominantly in the trophoblast and binucleate cells lining the fetal villi. AT_1 staining was also localized in nonvascular cells of the placenta and uterus, which included the epithelium, stroma, and smooth muscle cells of the myometrium (55). The distribution of AT_1 in the fetal component of the sheep placenta differs from that found in this study, where AT_1 gene expression was strictly localized to the maternal component of the placenta in the stromal cells throughout gestation, even at 140 days of gestation where little expression was observed.

AT_1 and AT_2 receptor species differences in the placenta. AT_1 mRNA, as well as protein, has previously been identified in the human placenta throughout pregnancy (7, 20, 28, 40). RT-PCR was employed by Cooper and coworkers (7), and AT_1 gene expression was demonstrated in the first, second, and third trimesters of human pregnancy, although these results were not quantitative. This group also observed greater AT_1 immunoreactivity in the first and second trimesters of human pregnancy compared with term (7). Again in the human, AT_1 receptors have been
The differences in AT\textsubscript{1} and AT\textsubscript{2} receptor distribution between the bovine and ovine placentae is interesting because both sheep and cows are ruminants, and the sheep placenta resembles the human in that predominantly AT\textsubscript{1} receptors exist, although in all three species, the level of AT\textsubscript{1} receptors do predominate early in gestation, unlike in the pig. Therefore, the distribution of AT\textsubscript{1} and AT\textsubscript{2} receptor subtypes varies among different species, suggesting that the RAS may perform different functions in placentae of different animals (Table 1).

**AT\textsubscript{1} and AT\textsubscript{2} receptors in fetal membranes.** In the human, significant amounts of AT\textsubscript{1} receptors have been noted in the amnion and predominantly AT\textsubscript{2} receptors in the chorion (21). Previously, few ANG II receptors were reported in both the human amnion and chorion by others (15). In the cow, AT\textsubscript{2} receptors are predominant in fetal membranes, similar to the mRNA findings in this study compared with AT\textsubscript{1} receptor mRNA levels, where they have been localized in the mesenchyme cells adjacent to the trophoblast cell layer and surrounding arteries (45). Fewer AT\textsubscript{1} ligand binding sites were localized in these regions (45). AT\textsubscript{2} ligand binding was most dominant in the allantochorionic membrane of the cotyledon and also at the fetal side on the mesenchymal cells (45). No AT\textsubscript{1} or AT\textsubscript{2} ligand binding was detected on the allantoic endoderm and trophoblast cells or the binucleate and trinucleate cells in the trophoblast cell layer and maternal uterine epithelium (45).

**Uterus.** Little AT\textsubscript{1} and AT\textsubscript{2} gene expression was detected in the 27-day uterus by real-time PCR. AT\textsubscript{1} and AT\textsubscript{2} ligand binding studies were previously performed in late gestation ovine uteri, where only AT\textsubscript{1} receptors were identified in the myometrium (35). Contrary to this, AT\textsubscript{2} receptors were found to be more abundant in the myometrium in nonpregnant sheep, where lower levels of both AT\textsubscript{1} and AT\textsubscript{2} receptors were detected in the endometrium (35). In the human, however, AT\textsubscript{2} receptors were found to decrease in the myometrium as pregnancy proceeded (31); therefore, few are found near term such as in the sheep. Most of the angiotensin receptors in the human endometrium have also been classified as the AT\textsubscript{2} type during pregnancy (2). AT\textsubscript{1} receptors have only been localized in the glandular and vascular epithelial cells of the human endometrium (44). Overall, differences exist in the distribution of the AT\textsubscript{1} and AT\textsubscript{2} receptors in uteri.

Table 1. AT\textsubscript{1} and AT\textsubscript{2} receptors in the placenta of different mammals during early and late pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Amount of AT\textsubscript{1} vs. AT\textsubscript{2} Receptors</th>
<th>Early vs. Late Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>AT\textsubscript{1} ( \text{R} &gt; \text{AT}_{2\text{R}} )</td>
<td>Early ( &gt; ) Late</td>
</tr>
<tr>
<td>Human</td>
<td>AT\textsubscript{1} ( \text{R} \gg \gg \text{AT}_{2\text{R}} )</td>
<td>Early ( &gt; ) Late</td>
</tr>
<tr>
<td>Cow</td>
<td>AT\textsubscript{2} ( \text{R} &gt; \text{AT}_{1\text{R}} )</td>
<td>Early ( &gt; ) Late</td>
</tr>
<tr>
<td>Pig</td>
<td>AT\textsubscript{2} ( \text{R} \gg \gg \text{AT}_{1\text{R}} )</td>
<td>Early ( = ) Late</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>AT\textsubscript{1} ( \text{R} \gg \gg \text{AT}_{2\text{R}} )</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>AT\textsubscript{2} ( \text{R} \gg \gg \text{AT}_{1\text{R}} )</td>
<td></td>
</tr>
</tbody>
</table>

R, receptor.
during pregnancy among the different mammals studied to date.

Mediating effects of angiotensin receptors on growth. In this study, a significant increase in AT1 mRNA at 45 days of gestation coincided with the period of maximal placental growth. Therefore, the AT1 receptor may play a key role in ovine placental growth/differentiation and/or function. It is generally accepted that ANG II, via the AT1 receptor, promotes cell growth, whereas the AT2 receptor mediates antiproliferation and apoptosis as determined in rat coronary endothelial, rat PC12W, and mouse R3T3 cells (46, 54). This AT1 receptor mitogenic effect has been demonstrated in cell types such as rat vascular smooth muscle cells and chick cardiac myocytes (1, 11). Other studies in mice also supported the opposing effects of the two receptor subtypes and that a functional interaction may exist (17). A cross-talk mechanism was subsequently suggested to exist between these two receptor subtypes, as determined in rat catecholaminergic neurons (12). Recently, however, growth-promoting effects of the AT2 receptor have been suggested from rat optic nerve studies (29).

Perspectives

The major original finding in the current study is that AT1 receptors are most highly expressed in the sheep placenta in the first third of pregnancy. It is now appreciated that ACE inhibitors are contraindicated in pregnancy, but the main concern has been to avoid them in the second and third trimesters of pregnancy (24). This is largely attributed to the effects on the fetal kidney, in which ANG II is essential to maintain normal perfusion and urine flow (30) and the consequent anuria results in oligohydramnios and lung hypoplasia. However, more recent reports on the use of specific AT1 antagonists from conception have indicated that these may also be associated with more serious toxic effects on the fetus (4). In the case reported by Briggs and Nageotte (4), intrauterine death occurred at 33 wk when the drug valsartan was stopped at 24 wk of gestation. Interestingly, the placenta was extremely small in weight (48% of the 10th percentile for gestational age). RAS is also important early in gestation, particularly because elevated levels of prorenin are found in gestational sac fluid (18) and the placenta (10) in humans. These findings that the AT1 receptor is highly expressed early in pregnancy in the maternal stroma of the ovine placenta, when maximal growth of the placenta occurs, suggest that ANG II, via the AT1 receptor, may play a vital role in the growth/function of the placenta in this species.

The authors thank M. Goga for excellent technical assistance with the receptor ligand binding studies and K. Johnson for technical advice during the generation of the in situ hybridization histochemistry photomicrographs. Dr. J. Burrell and Prof. E. Lumbers are also thanked for technical advice with the receptor ligand binding studies.

The purchase of the ABI PRISM 7700 Sequence Detection System was possible due to funding from the following foundations: the Clive and Vera Ramaciotti Foundation, the Harold and Cora Brennen Benevolent Trust, the Philip Bushell Foundation, and the Sylvia and Charles Viertel Foundation.

REFERENCES


