Ontogeny of Expression of Organic Anion Transporters 1 and 3 in Ovine Fetal and Neonatal Kidney

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Organic ions are excreted into the urine via the action of organic anion transporters (OATs). In adult kidney, both OAT1 and OAT3, both multispecific transporters, are abundant; OAT1 is a known transporter of para-aminohippurate (PAH) and OAT3 is a known transporter of sulfoconjugated estrogens. The present study was designed to test the hypotheses that the expression of both OAT1 and OAT3 are developmentally regulated and that the expression increases in late gestation. Fetal kidneys were collected at sacrifice of fetal sheep at 80, 100, 120, 130, and 145 days of gestation, as well as 1 day and 1 week after birth ($n = 4$–5 per group). Renal tissue was separated into cortex and medulla and snap-frozen in liquid nitrogen for later extraction of mRNA. The expression levels of OAT1 and OAT3 were measured using real-time reverse transcriptase polymerase chain reaction (RT-PCR), with specific probes and primers designed in our laboratory. Cellular distribution of protein expression was identified using immunohistochemistry with commercially available antisera. The OAT1 and OAT3 mRNA in renal cortex was increased in the more mature animals. At 145 days of gestation, OAT1 mRNA abundance was increased and remained elevated postnatally. Compared with prenatal ages, OAT3 mRNA was increased postnatally. The expression of both transporters was not significantly changed as a function of development in the renal medulla. The protein expression of OAT1 and OAT3 was identified in tubular epithelium in renal cortex, although the immunoreactivity for OAT1 was greater than for OAT3. We conclude that there is a developmental pattern of expression of both OAT1 and OAT3 in ovine renal cortex, and that the pattern of expression suggests that the function of both transporters is likely to be greater starting in late gestation. Exp Biol Med 230:668–673, 2005

Key words: OAT; fetal sheep; fetal kidney; qPCR

Introduction

In adult animals, renal organic anion transporters remove anionic compounds from the blood to the lumen of the proximal tubules (1,2). Members of the organic anion transporter (OAT) family are found in tubular epithelial cells, mainly on the basolateral surface. The most abundant OATs found in the kidney are OAT1 and OAT3 (3,4). The first OAT to be cloned and localized to the basolateral membrane of the renal proximal tubule was OAT1 (5). Organic anion transporter-3 is found in proximal tubules, the loop of Henle and collecting duct, and is also found in the basolateral surface (6). These transporters have substrate specificities that are distinct, yet overlapping. Dantzler and Wright reported that rabbit OAT1 readily transports para-aminohippurate (PAH) and only inefficiently transports estrogen sulfate (ES); rabbit OAT3 avidly transports ES but does not transport PAH (3). Renal transport of ES is reduced in OAT3-knockout mice (7). Sulfoconjugated estrogens circulate in high concentrations in ovine fetal plasma (8, 9). Although the clearance of the sulfoconjugated estrogens from fetal plasma is not completely understood, it is possible that, in the fetal kidney, OAT3 might facilitate excretion of sulfoconjugated estrogen into the fetal urine.

Although the transport of organic anions in the adult kidney has been a subject of intense investigation, relatively little is known about the prenatal development of these transporters. In the fetal sheep, renal transport mechanisms are relatively immature (10). Studied between 120 and 140 days of gestation, net secretion of PAH by the ovine fetal kidney could not be found, leading Elbourne and coworkers to suggest that the excretion of organic anions develops late, perhaps even after birth (10). In the rat, a species in which the newborn is quite immature compared with the human being or sheep, OAT1 expression in the renal cortex was low prenatally, then increased dramatically postnatally (11). There have been no reports of renal handling of known substrates of OAT3, even though this is now recognized as a key transport system in the adult kidney (12). The aim of this study was to identify the expression of both OAT1 and OAT3 in renal cortex and medulla of the ovine fetus, to

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identify ontogenetic changes in the level of expression, and to confirm that the cellular sites of expression of both OAT1 and OAT3 are consistent with known sites of expression in the adult kidney.

Materials and Methods

Tissues used in this study were obtained from newborn ($n = 4$) and 1-week-old ($n = 4$) lambs and from 24 fetal sheep of the following ages: 80 ($n = 5$), 100 ($n = 4$), 120 ($n = 4$), 130 ($n = 4$), and 145 ($n = 4$) days of gestation. The animals were humanely euthanized using an intravenous overdose of sodium pentobarbital. The kidneys were rapidly isolated, dissected into cortex and medulla, snap-frozen in liquid nitrogen, and stored at −80°C until studied. These experiments were approved by the University of Florida Institutional Animal Care and Use Committee.

Messenger RNA (mRNA) was isolated using Trizol (Gibco, Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. After isolation, mRNA was stored in RNA Secure (Ambion Corp., Austin, TX) at −80°C until use. Total mRNA in each sample (4 μg) was converted to cDNA using a High Capacity cDNA Archive kit using methodology recommended by the kit manufacturer (Applied Biosystems, Foster City, CA). The newly synthesized cDNA, stored at −20°C, was used for assay of mRNA for OAT1 and OAT3 by real-time polymerase chain reaction (PCR). Real-time PCR reactions were run using AmpliTaq Gold DNA Polymerase (Applied Biosystems) and primers and probes (Geno-Mechanix, Alachua, FL) were specifically designed using Primer Express software (Applied Biosystems). Probes for OAT1 and OAT3 were labeled with 6-carboxyfluoresceine (6-FAM) in the 5′ position and carboxytetramethyl rhodamine (TAMRA) in the 3′ position. Sequences of primers and probes are reported in Table 1. In each sample, 18S ribosomal RNA was also measured using real-time reverse transcriptase (RT)-PCR methodology, with probe, primers, and reagents purchased from Applied Biosystems. All mRNA abundances for OAT1 and OAT3 were normalized to the abundance of 18S rRNA, using the relative cycle threshold (ΔCt) method. All reactions were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems), using 100 ng of cDNA, 100 n*M* primers, and 200 n*M* probe. Reactions were amplified using the following conditions: 48°C for 30 mins and 95°C for 10 mins, followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. For both assays, we ran appropriate "no-RT" (reaction using mRNA alone as the substrate) and "no template" (reaction using water alone as the substrate) controls. In both cases, omitting cDNA from the reaction resulted in a lack of product generation.

Ovine OAT1 was partially sequenced from a 200 base pair PCR product derived from ovine kidney cDNA. The primers (forward, 5′-TCCATGCTGTGGTTTGCCA-3′ reverse, 5′-TGATGAGGATGCAGATGCCTG-3′) were designed from bovine OAT1 mRNA sequence (accession number AJ549816). The PCR reaction was performed in an ABI Prism 7000 Sequence System (Applied Biosystems), using 100 ng of cDNA, 900 n*M* primers, and 2× SYBR Green Master mix from Applied Biosystems. The reaction was performed under the following conditions: 95°C for 10 mins followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. The PCR product was purified with the Wizard PCR Preps DNA Purification System (Promega, Madison, WI), and was sequenced using the Big Dye sequencing protocol in the DNA sequencing core laboratory of Center for Mammalian Genetics, University of Florida. The partial ovine OAT1 sequence was aligned with human and bovine OAT1 mRNA sequences, and is reported in Table 2. Using Primer Express software from Applied Biosystems, the ovine OAT1 primers and probe for the real-time PCR were designed from this newly defined partial ovine OAT1 mRNA sequence. OAT3 probe and primers were designed from bovine OAT3 mRNA sequence (accession number AJ627254).

The expression of OAT1 and OAT3 mRNA were normalized to 18S mRNA and reported graphically as the change relative to the mean concentration at 80 days of gestation. The calculation of relative expression was performed using the ΔΔCt method, as described previously (13). Statistical analysis, on the other hand, was performed on the values of ΔCt, because these values are normally distributed (13). Values of ΔCt (for mRNA) and relative optical density (for protein) were analyzed using one-way analysis of variance (Sigma Stat; SPSS, Chicago, IL), and pairwise comparisons of group means were performed using Duncan's multiple range test (14). The null hypothesis (i.e., all groups are similar) was rejected when $P < 0.05$.

The cellular distribution of immunoreactive OAT1 and OAT3 protein was measured using immunohistochemistry. Renal cortex and renal medulla samples were collected at necropsy from adult nonpregnant sheep ($n = 19$) and from 3-day-old postnatal lambs ($n = 3$). Tissue samples were collected immediately after euthanasia induced by intravenous injection of pentobarbital. Tissue samples were immersion-fixed in 4% buffered paraformaldehyde over-

Table 1. Probe and Primer Sequences for Real-Time RT-PCR Analysis of OAT1 and OAT3 mRNA

Gene of interest	Forward primer	Reverse primer	Probe
oOAT1	CATCTACCTAATCCAGGTGATCTTTG	TGTTGATGACAAGGAAGCTCACA	TGCTGTGGACCTGCCTGCCAAG
bOAT3	CTGTGTGGCTTCGGCATCT	GGACACCCACTCGACATTCAA	AGGCATTACCCTGAGCACCGTCA

Table 2. Partial Sequence of Ovine OAT1[a]

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Human	CCTGCAGGGCTTTGGAGTCAGCATCTACCTAATCCAGGTGATCTTTGGTGCTGTGGACCTGCCT
Bovine	CCTTCAGGGTTTTGGGGTCAGCATCTACCTAATCCAGGTGATCTTTGGTGCTGTGGACCTGCCT
Ovine	CCTGCAGGGTTTTGGGGTCAGCATCTACCTAATCCAGGTGATCTTTGGTGCTGTGGACCTGCCT
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Human	GCCAAGCTTGTGGGCTTCCTTGTCATCAACTCCCTGGGTCGCCGGCCTGCCCAGA
Bovine	GCCAAGCTTGTGAGCTTCCTTGTCATCAACAATGTGGGCCGCCGGCCTGCCCAGA
Ovine	GCCAAGCTTGTGAGCTTCCTTGTCATCAACAATGTGGGCCGCCGGCCTGCCCAGA

[a] +, designated for the homology between three species; ., not homologous with human but homologous with bovine.

night, and then transferred to 70% reagent alcohol. Samples were subsequently transferred to graded increases in alcohol concentration, then cleared in xylene and embedded in paraffin. Specimens were cut in 5-μm–thick sections on a Zeiss rotary microtome and mounted on poly-L-lysine–coated glass slides. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol in water. Immunohistochemistry was performed using commercial antibodies for OAT1 and OAT3 (cat #OAT11-S and OAT311-S, respectively; Alpha Diagnostics, San Antonio, TX). Primary antibodies were diluted 1:500 in 10% normal goat serum in phosphate buffered saline. Visualization was accomplished using biotinylated anti-rabbit and anti-mouse IgG and streptavidin-conjugated horseradish peroxidase from Zymed, Inc. (South San Francisco, CA), and metal-enhanced diaminobenzidine (DAB) from Pierce Co. (Rockford, IL). Sections were counterstained using hematoxylin (Fisher Scientific, Fairlawn, NJ). For both transporters, immunoreactivity was eliminated when the primary antibody was omitted.

Results

The expression of OAT1 and OAT3 were significantly related to fetal gestational age in the renal cortex (Fig. 1B and E). The expression of OAT1, classically thought of as the PAH transporter in the renal proximal tubule, was significantly decreased at 100, 120, and 130 days of gestation compared with 80 days of gestation (Fig. 1B). The expression of OAT1 subsequently was significantly increased in 145-day fetal, and 1- and 7-day postnatal lambs compared with values in the younger fetuses (Fig. 1B). The general pattern of expression of OAT3 as a function of developmental age was generally similar. The expression of OAT3 was increased significantly only in postnatal ages (1- and 7-day lambs) when compared with expression levels in fetuses (Fig. 1E).

The expression of OAT1 and OAT3 in the renal medulla was more variable. Although the data visually seemed to follow a pattern of increasing expression in more mature animals, the apparent changes were not statistically significant (Fig. 1C and F). The levels of expression in the renal medulla were far more variable than in the cortex; the lack of statistical significance is likely a reflection of that increased variability.

Immunohistochemical analysis of adult and neonatal kidneys revealed a pattern of immunoreactivity for both OAT1 and OAT3 that is consistent with localization in tubular epithelium. Representative immunoreactivity in adult renal cortex is shown in Figure 1A and D. Although the present study was not designed to distinguish proximal from distal convoluted tubules, the pattern of immunoreactivity is consistent with localization in the proximal tubule. The general pattern of immunoreactivity for OAT3 was similar to that of OAT1; however, the intensity of staining for OAT3 was consistently less than that of OAT1.

Discussion

This is the first study to quantify the transcriptional expression of OAT1 and OAT3 as a function of fetal development in sheep. Although there have been several studies published that report the expression of both of these transporters in prenatal and postnatal rodent kidneys (11, 15), this is the first study to investigate the development of these transporters in sheep, a species whose maturity at birth is more similar to human than to rodent species.

The kidney of the developing fetus plays an important role with regard to fetal fluid balance and blood pressure (16). Amniotic fluid is produced mainly by the fetal kidney and lungs; the volume of amniotic fluid is adjusted by changes in the rate of urine and lung liquid formation and the rate of reabsorption via the intramembranous pathway (17). The role of the fetal kidneys in the regulation of fetal blood pressure is not strictly parallel to that of the kidneys in the adult animal. Nevertheless, the secretion of renin from the juxtaglomerular cells of the fetus is an important determinant of fetal blood pressure and fluid balance between fetal and maternal vascular space and amniotic fluid space (18).

Organic anion transporters are likely to play important roles in the maintenance of fetal homeostasis and transition from fetal to neonatal life. After birth, the major route of excretion of potentially toxic organic anions is via the urine

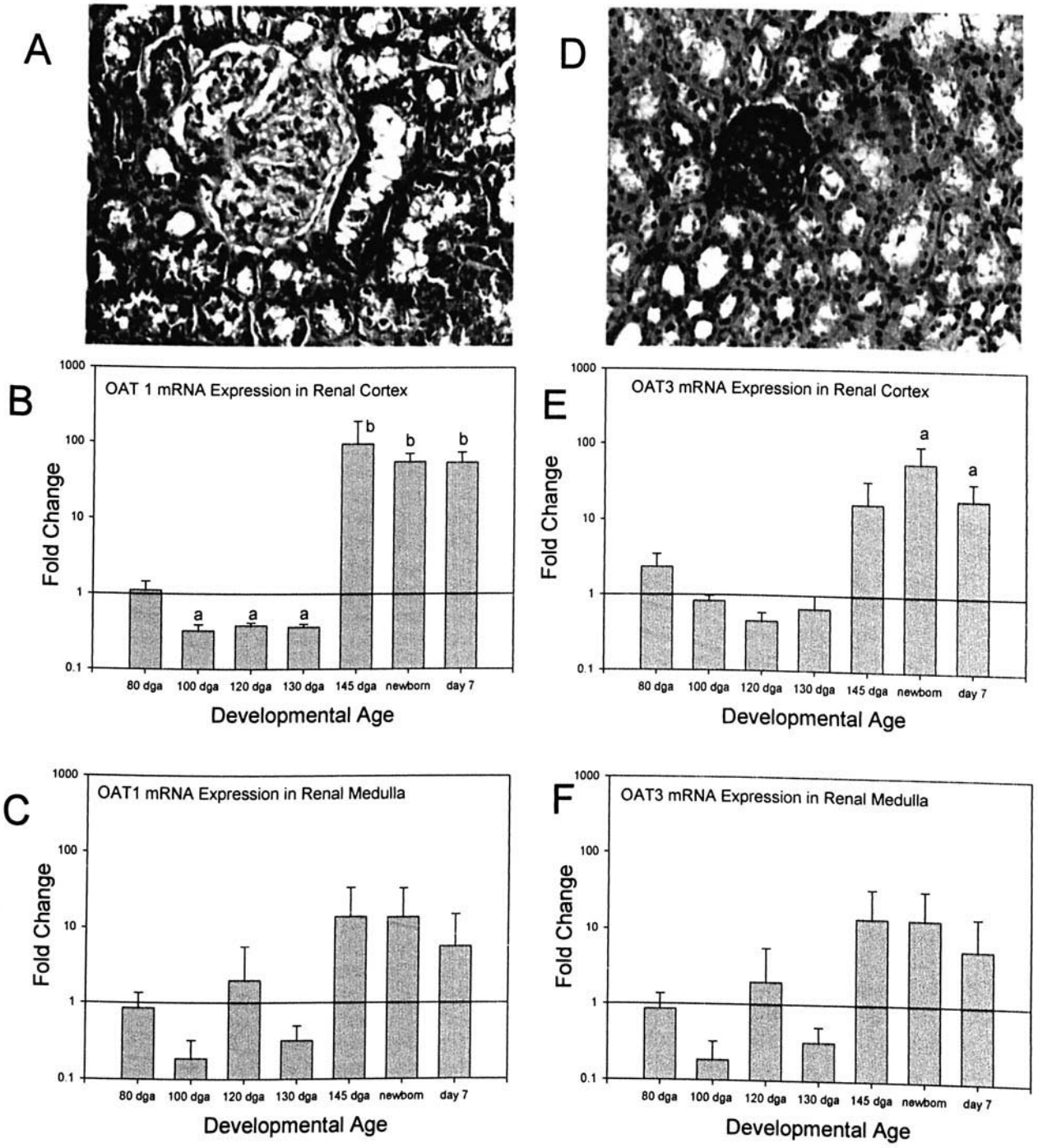

Figure 1. (A) Immunohistochemical localization of OAT1 protein to tubular epithelium in adult renal cortex. Immunoreactivity is brown (DAB); section was counterstained with hematoxylin (blue). (B) Ontogeny of expression of OAT1 in renal cortex in fetal and neonatal sheep. Values are represented as mean values ± 1 SEM. "a" represents statistically significant decrease relative to values at 80 days, and "b" represents statistically significant increase relative to values at 80 days. (C) Ontogeny of expression of OAT1 in renal medulla in fetal and neonatal sheep. Values are represented as mean values ± 1 SEM. (D) Immunohistochemical localization of OAT3 protein to tubular epithelium in adult renal cortex. Immunoreactivity (DAB, brown) was much less intense for OAT3; sections are counterstained with hematoxylin (blue). (E) Ontogeny of expression of OAT3 in renal cortex in fetal and neonatal sheep. Values are represented as mean values ± 1 SEM. "a" represents statistically significant increase relative to values at 80 days. (F) Ontogeny of expression of OAT3 in renal medulla in fetal and neonatal sheep. Values are represented as mean values ± 1 SEM.

(2). Before birth, the major route of excretion is via transplacental transfer from fetal to maternal blood, then excretion into the maternal urine. Relatively little is known about the expression of organic anion transporters in placenta, except that one or more members of a related family of transporters (OATp) are expressed in the placenta of the rat (19), and that OAT4 is expressed in the syncitiotrophoblast layer of the human placenta (20). Physiologic data from chronically catheterized fetal sheep between approximately 0.8 and 0.95 gestation have

demonstrated that there is no net secretion of PAH acid into the urine in late-gestation fetal sheep (10). The mRNA expression pattern reported in this study is consistent with the physiologic data. Expression of OAT1, the major PAH transporter in the kidney, is low during fetal life and increases only in the last several days before spontaneous parturition.

The function of OAT3 in the sheep has not been specifically tested, although in rabbit, human, rat, and mouse, this transporter is a multifunctional transporter that has an affinity for organic anions, such as PAH, but also for sulfoconjugated estrogens (3). We and others have reported that fetal plasma contains a relatively high concentration of estrone-3-sulfate and estradiol-3-sulfate (9, 21). These hydrophilic steroids are present in high abundance. For example, the concentration of estradiol-3-sulfate in fetal plasma is approximately 40 times the concentration of unconjugated estradiol (9). Soodvilai and coworkers have proposed that OAT1 and OAT3, expressed in the renal cortex, work in concert with each other to provide the capacity for transport of a variety of substances into the urine (3). The present data demonstrate expression of both genes in the renal cortex of the developing sheep fetus and lamb. The OAT3 data in renal cortex suggest that the majority of expression of this transporter is postnatal (Fig. 1E). We interpret these data to suggest that the expression of OAT1 and OAT3 are similar and analogous to each other. We believe that it is unlikely that the ovine fetal kidney effectively excretes sulfoconjugated estrogens into the urine. If so, the low level of transport would allow relatively high concentrations of the steroids to remain in fetal plasma, and at the same time keep the concentration of sulfoconjugated estrogen in amniotic fluid relatively low.

The ontogenetic pattern of expression of OAT1 and OAT3 in the renal cortex suggests the possibility that the expression of either or both of these proteins might be increased by the preparturient rise in fetal plasma cortisol concentration. Bahn and colleagues reported that treatment of kidney slices *in vitro* with dexamethasone increased the transcription of OAT1 (22). The possible roles of androgen and estrogen, also known to increase in fetal plasma at the end of gestation, are somewhat more complex. In adult rats, orchidectomy of males decreased OAT1 expression and ovariectomy of females moderately increased OAT1 expression (23). Treatment with estrogen decreased OAT1 expression (23). The involvement, if any, of glucocorticoids, androgens, and estrogens in the upregulation of OAT1 is not known; all increase to differing degrees at the end of gestation in sheep. It is possible that the mechanism of the maturational effect on OAT3 expression is similar to that of OAT1. Although little is presently known about possible steroid hormone regulation of OAT3 expression, sulfoconjugated estrogens might be involved. Eraly and coworkers have reported that OAT1 and OAT3 can be classified as a tightly linked pair, based on their phylogenetic footprints (24). On the basis of their analysis, these investigators predicted a similar pattern of expression of these transporters. Our results are consistent with this predicted similarity of expression (Fig. 1).

We conclude that the expression of OAT1 and OAT3 in fetal ovine renal cortex is immature relative to postnatal expression. The expression of both transporters is greater in renal cortex than in medulla, and the level of expression increases very late in fetal life. We propose that the postnatal expression of both transporters coincides with the increased importance of the kidney as the primary route for excretion of organic anions.

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