3,3'-Diiodothyronine Sulfate Excretion in Maternal Urine Reflects Fetal Thyroid Function in Sheep

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ABSTRACT

We have shown that there is significant fetal-to-maternal transfer of sulfated metabolites of thyroid hormone after fetal infusion of a pharmacologic amount of 3,3',5-triiodothyronine (T_3) or sulfated T_3 in late pregnancy in sheep (Am J Physiol 277:E915, 1999). The transferred iodothyronine sulfoconjugate, i.e. 3,3'-diiodothyronine sulfate (T_2S), of fetal origin appears in maternal sheep urine. The present study was carried out to assess the contribution of T_2S of fetal origin to the urinary pool in ewes. Eighteen date-bred ewes (mean gestational age of 115 d) and their twin fetuses were divided into four groups. In group I (control, $n = 5$), both ewes (M) and their fetuses (F) were sham operated for thyroidectomy (Tx). In group II, the ewes (MTx, $n = 4$) and, in group III, the fetuses (FTx, $n = 4$) were subjected to Tx. In group IV (MTx · FTx, $n = 5$), both the ewe and fetus had Tx. After 10–12 d, fetal and/or maternal hypothyroidism were confirmed by serum thyroxine (<15 nmol/L) measurements. In addition, we infused radioactive T_3 without disturbing the T_3 pool in three singleton near-term fetuses and assessed the amount of radioactive iodothyronine that appeared in maternal urine (MU). After infusing [^{125}I -3'],3,5- T_3 via fetal vein to the near-term normal fetuses, radioactive T_2S was identified as the major metabolite in MU by HPLC and T_2S -specific antibody. MU T_2S excretion (pmol/mmol creatinine) was significantly

reduced by FTx and MTx · FTx but not by MTx. In addition, positive correlations ($p < 0.01$) were found between MU T_2S excretion and fetal serum thyroxine and T_3 concentrations but not with maternal serum thyroxine or T_3 levels. T_2S of fetal origin contributes significantly to the MU pool. (*Pediatr Res* 50: 358–364, 2001)

Abbreviations

TH, thyroid hormone
 T_2S , 3,3'-diiodothyronine sulfate
Tx, thyroidectomy
 T_4 , thyroxine
 T_3 , 3,3',5-triiodothyronine
MU, maternal urinary
D1, type-I iodothyronine deiodinating activity
D3, type-III deiodinating activity
 rT_3 , 3,3',5'-triiodothyronine (reverse T_3)
 T_4S , sulfated thyroxine
 T_3S , sulfated T_3
 rT_3S , sulfated rT_3
 T_2 , 3,3'-diiodothyronine
cr, creatinine
IST, iodothyronine sulfotransferase

In developing mammals, D1 is low (1) whereas D3 is relatively high in fetal tissues, placenta, and uterus (2, 3). As a result, the circulating fetal levels of T_3 are reduced and rT_3 are elevated (1). In addition, in the mammalian fetus, we observed significantly high concentrations of T_4S , T_3S , rT_3S , and T_2S (1,

4–8). These sulfated iodothyronines are known to be deiodinated mainly by D1 (9), and we have shown that infusion of the ovine fetus with pharmacologic amounts of T_3 (10) or T_3S (11) resulted in significant fetal-to-maternal transfer of T_3S and T_2S . High concentrations of a T_2S -like material (Compound W) also has been demonstrated in the serum and urine of pregnant women, and the levels of Compound W increase with the progression of pregnancy and peak shortly before parturition (12). Further evidence suggests that Compound W is derived from thyroid hormones of fetal origin (13, 14), and we have suggested that it may serve as a marker for fetal thyroid function.

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There is evidence that the diiodothyronines (3,3'-T₂ and 3,5-T₂) may be biologically active, stimulating mitochondrial thermogenesis (15, 16). These data suggest that sulfoconjugation of T₂ and other iodothyronines may serve the following functions: 1) to accelerate degradation and excretion of TH and their derivatives; 2) to provide a reservoir for TH such as T₃ (13, 17, 18) and metabolites including rT₃ (19) and T₂ (15, 16) that can be recovered from these respective sulfated derivatives by sulfatase in selected tissues; 3) to provide via further accelerated deiodination a source of iodine for new hormone synthesis; and 4) to facilitate fetal-to-maternal transfer of T₃S metabolites, which may serve to maintain the low serum T₃ levels in late gestation in precocial species. Additionally, T₂S of fetal origin may serve as a noninvasive marker in maternal serum and urine for fetal thyroid function *in utero*. To further document this possibility, we conducted the present study to measure the contribution of T₂S of fetal origin to the urinary pool in ewes and to assess the correlation between fetal TH concentration and the amount of T₂S excreted in maternal urine.

METHODS

Animal preparation. Western mixed-breed time-dated pregnant ewes with twin pregnancies were obtained from the Nebeker Ranch (Lancaster, CA, U.S.A.) and acclimated to our laboratory conditions and food. Animals were studied in four groups. In group I ($n = 5$), both the ewes and fetuses were sham-operated for Tx. In group II ($n = 4$), the ewes and, in group III ($n = 4$), the fetuses were thyroidectomized. In group IV, both ewes and fetuses were thyroidectomized.

The ewes (113–121 d gestation, term = 150 d) were sedated (1.2 mg atropine and 700 mg ketamine intramuscularly), and a continuous infusion of ketamine (100 mg/h) was started via a jugular venous catheter. After local anesthesia of the abdominal wall (2% lidocaine), a midline incision was followed by palpation of the uterus and fetal parts and identification of both fetal heads. A hysterotomy was performed over the fetal necks, and fetal membranes were marsupialized to the skin of the fetal neck, avoiding loss of amniotic fluid. The necks of the twin fetuses were infiltrated with 1% lidocaine, followed by dissection and complete removal of the thyroid gland (Tx) in groups III and IV. The neck incisions were closed. Sham-operated twin fetuses (groups I and II) were handled in a similar manner except that a sham operation was conducted and the thyroid gland was left intact. The maternal thyroid glands were removed after neck dissection in groups II and IV. In the sham-operated group I and III ewes, the maternal thyroid was left intact after neck dissection. In each group, the ewes were treated for 3 d postoperatively with oxacillin (2 g) and gentamicin (80 mg) given intramuscularly in divided doses. Ten to 12 d after the initial operation, the ewes were killed. The 10–12-d interval from Tx to death was chosen based on the serum half-life of T₄ (24 h) in the ovine fetus (8). All experiments were approved by the Harbor-UCLA Medical Center Animal Use Committee. Maternal urinary samples (~50 mL) were obtained through catheter at the end of experiments. Fetal

and maternal serum samples were collected at the time of initial operation (sham or Tx) and at the end of experiments.

In a separate experiment involving three pregnant sheep with singleton fetuses, hysterotomy was performed (131 ± 1 d of gestation), and a catheter was inserted in the fetal femoral vein. One week later, outer-ring labeled ¹²⁵I-T₃ (250 μCi, sp act 1300 μCi/μg) diluted in saline was given in a single bolus via the catheter. Maternal serum and urine samples were collected hourly for 4 h.

Preparation of test serum and urine. Samples were extracted with 2 vol of 95% ethanol before assay. Preliminary experiments showed that the extraction efficiency for T₂S and T₃S in serum and urine was 91 to 96%. Final values of T₂S and T₃S concentration were not corrected for recovery efficiency. The preliminary experiments demonstrated that the immuno-reactive T₂S and T₃S in ethanol extracts of fetal and MU cochromatographed with the synthetic compounds by HPLC.

T₂, T₃, T₂S, and T₃S RIA. T₂, T₂S, and T₃S levels in serum and urine were measured by specific and sensitive RIA methods (4, 10, 20). Serum and urine samples were extracted with 2 vol of 95% ethanol (final ethanol concentration, 63%) as described previously (4, 10, 20). The lower limit of detection for T₂ was 2 ng/dL. T₂S RIA has a lower limit of detection of 2 ng/dL (33 pmol/L). Of various TH analogs studied and known to exist in sheep serum or urine, only T₃S, rT₃S, and T₄S cross-react significantly (3.2, 1.4, and 0.02%, respectively) in the T₂S RIA; T₄, T₃, rT₃, and T₂ cross-reacted <0.0001%. The T₂S concentrations in serum and urine were corrected for the cross-reactivity of T₃S. The T₃S RIA has a lower limit of detection of 2 ng/dL (27 pmol/L). Analog cross-reactivities in the T₃S RIA are T₄, <0.001; rT₃, <0.001; T₃, <0.001; rT₃S, <0.007; and T₄S, 3.3%. Serum T₃ and T₄ were assayed using commercial kits purchased from Diagnostic System Laboratory (Webster, TX, U.S.A.) and Nichols Institute Diagnostics (chemiluminescence assay, San Juan Capistrano, CA, U.S.A.), respectively. The lower limit of detection for T₄ was 0.2 μg/dL (2.6 nmol/L) and for T₃, 5 ng/dL (77 pmol/L).

Identification of [¹²⁵I]-3,3'-T₂S in maternal sheep urine. Radioactive metabolites were identified in MU extracts of sheep whose fetuses were infused via the fetal femoral vein with [¹²⁵I]-T₃. Maternal urine and serum samples were extracted with 2 vol of 95% ethanol and subsequently lyophilized. The dried extracts were dissolved in 1 mL of H₂O and purified using an LH-20 column as previously described (10). After application to an HPLC uBondapak C₁₈ column, the serum extract was eluted isocratically with a mixture of acetonitrile and 0.02 M ammonium acetate, pH 4.0 (22:78 vol/vol), at a flow rate of 2 mL/min. Aliquots of eluent in 1-mL fractions were collected, and 100 μL of these aliquots with significant radioactivities were subjected to immunoprecipitation with T₂S-specific antibody.

Source of materials. 3,3'-T₂, T₃, and T₄ were purchased from Henning-Berlin Co. (Berlin, Germany). [¹²⁵I]-3,3'-T₂ was prepared by radioiodination using the method described previously (21). T₃S and T₂S were synthesized by the method described previously (22, 23). Chlorosulfonic acid, 99%, was purchased from Aldrich Chemical Co. (Milwaukee, WI,

U.S.A.). [^{125}I]- T_3 was purchased from Dupont NEN (Boston, MA, U.S.A.).

Statistical analysis. ANOVA was used for multigroup comparisons. If significant differences were detected, the Dunnett multiple comparison test was used to compare the control or baseline mean and the mean values of other groups (24). Unpaired t test was used only to compare differences between the two groups. Significance was defined as $p < 0.05$. Results are reported as the mean $\pm$ SEM.

RESULTS

Effect of fetal or/and maternal Tx on the amount of T_2S , T_3S , T_2 , and T_3 in MU. As shown in Table 1, maternal or/and fetal Tx resulted in significant decreases in serum T_4 levels in ewes or/and fetuses, respectively. A significant reduction in serum T_3 concentrations occurred in ewes but not in lambs after respective Tx.

MU T_2S excretion (pmol/mmol cr) was significantly reduced by fetal Tx and by combined maternal and fetal Tx (489 ± 31 and 340 ± 39 , respectively, *versus* sham control, 778 ± 52 , $p < 0.05$) but not by maternal Tx (742 ± 40 , $p > 0.05$) (Table 2). In addition, positive correlations were found between MU T_2S excretion and fetal serum T_4 and T_3 ($R = 0.796$, $p < 0.001$ and $R = 0.547$, $p < 0.01$, respectively) concentrations but not maternal serum T_4 or T_3 concentrations (Fig. 1). A significant decrease in urinary T_3S excretion was observed after either maternal or fetal Tx or both, and there was a positive correlation ($R = 0.596$, $p < 0.01$) between the amount of maternal T_3S excretion (pmol/mmol cr) and T_4 levels in maternal serum but weakly positive with T_3 and T_4 levels in fetal serum ($0.025 < p < 0.05$) (Fig. 2). A significant reduction in T_3 excretion in MU was observed in ewes after Tx, but this was not the case after fetal Tx (group III) (Table 2).

Effect of fetal or/and maternal Tx on the maternal and fetal T_2S and T_3S concentrations in serum. Fetal serum T_2S levels were reduced after fetal Tx as well as maternal Tx (Table 3). However, the T_2S levels in group II were not significantly different between prematernal (1835 ± 165 pmol/L) and post-maternal Tx (Table 3). On the other hand, fetal T_3S concentrations were not significantly altered by fetal hypothyroidism (group III in Table 3). There were significant decreases in fetal serum T_3S levels after maternal Tx (group II) and after combined maternal and fetal Tx (group IV). Maternal serum T_2S and T_3S concentrations were reduced only in group IV in which both the ewe and fetuses were thyroidectomized.

Recovery of radioactivity in MU and serum after fetal infusion of [^{125}I]- T_3 . After fetal bolus i.v. infusion (at 138 ± 1 d gestation) with [^{125}I]- T_3 , hourly MU and serum samples were collected. After 4 h, the cumulative radioactivity recovered in MU represented $19 \pm 1.5\%$ ($N = 3$) of the infusion dose. As shown in Figure 3, a mean of $43.6 \pm 5.3\%$ of the total radioactivity recovered in MU was T_2S during the first hour. Thereafter, the proportion of urinary radioactivity as T_2S gradually decreased to a mean of $20.9 \pm 0.3\%$ during the fourth hour. Confirmation of the identity of the MU T_2S peak was determined by both its HPLC retention time (identical to that of known [^{125}I]-3,3'- T_2S standard) and by immunoprecipitation with T_2S -specific antibody. In addition, immunoreactive T_2S in ethanol extracts of MU cochromatographed with the synthetic compound by HPLC (results are not shown). One minor HPLC peak at 15 min cochromatographed with synthetic T_3S . There was another minor HPLC peak at 18 min in which total radioactivity increased between the first and fourth hours (Fig. 3). Other minor peaks at 31/2 and 10 min contained radioactivities that remained essentially constant. Radioactivity in the free iodine peak increased from a mean of $39.1 \pm 5.6\%$ in the first hour to $57.6 \pm 3.2\%$ at the fourth hour.

DISCUSSION

In recent reported studies, we demonstrated that there is significant fetal-to-maternal transfer of sulfated metabolites of TH after fetal infusion of relatively large amounts of T_3 or T_3S in late pregnancy in sheep (10, 11). The transferred iodothyronine sulfoconjugates (*i.e.* T_2S and T_3S) of fetal origin appear in MU. The significant amount of T_3S of fetal origin found in MU was apparently a result of infusing a pharmacologic dose of T_3 that was more than 20 times the fetal daily production rate of T_3 (10). In the present study, we infused radioactive T_3 into the fetal circulation without altering the T_3 pool and demonstrated T_2S but not T_3S as the major sulfoconjugate of fetal origin in MU. This would represent a more physiologic state in assessing fetal-to-maternal transfer. In addition, maternal excretion of T_2S was reduced after fetal but not maternal Tx. Furthermore, strong positive correlations were found between MU T_2S excretion (pmol/mmol cr) and fetal serum T_3 and T_4 concentrations but not with maternal serum T_3 or T_4 levels. We could not explain that maternal serum T_2S levels are the same in either maternal (group II) or fetal (group III) Tx and not significantly reduced compared with the sham-operation control (group I). It is speculated that rapid renal

Table 1. Maternal and fetal serum T_4 and T_3 concentrations before and at end of study

		Gestational age (d)		Serum T ₄ (nmol/L)					Serum T ₃ (nmol/L)			
		Maternal		Maternal		n	Fetal		Maternal		Fetal	
Group	n	Surgery	End	Start	End		Start	End	Start	End	Start	End
I	5	114 (0.5)	125 (0.2)	82 (12)	109 (14)	10	117 (4)	96 (8)	1.5 (0.3)	1.0 (0.3)	0.23 (0.03)	0.19 (0.02)
II	4	115 (0.3)	126 (0.3)	82 (8)	6.5 (0.9)*	8	125 (8)	147 (5)	0.9 (0.2)	0.3 (0.2)*	0.25 (0.03)	0.14 (0.02)
III	4	114 (0.3)	125 (0.3)	103 (8)	124 (8)	8	117 (5)	13 (2.0)*	1.2 (0.2)	1.1 (0.1)	0.20 (0.05)	0.10 (0.02)
IV	5	117 (1.2)	129 (2.0)	80 (8)	7.8 (0.5)*	10	142 (6)	6.1 (0.8)*	1.1 (0.2)	0.3 (0.1)*	0.18 (0.05)	0.10 (0.02)

Group I: both mothers and fetuses were sham operated; group II: maternal Tx only; group III: fetal Tx only; group IV: both mothers and fetuses had Tx. Data are presented as means (SE).

* $p < 0.05$ (*vs* starting baseline value).

Table 2. Effect of fetal (F) or/and maternal (M) Tx on the amount of T₂S, T₃S, T₂ and T₃ in MU

Group	n	Surgery	T ₂ S	T ₃ S (pmol/mmol creatinine)	T ₂	T ₃
I	5	Sham	778 (52)	435 (38)	358 (74)	512 (41)
II	4	MTx	742 (40)	264 (22)**	255 (61)	138 (29)**
III	4	FTx	489 (31)*	260 (48)**	207 (57)	440 (46)
IV	5	MTx + FTx	340 (39)**	102 (18)**	174 (69)	85 (38)**

* $p < 0.05$, ** $p < 0.01$ vs group I, sham-operated control group. Data are presented as means (SE).

MU samples were collected 11–12 d after sham or Tx surgery.

Figure 1. Correlation between MU T₂S excretion (pmol/mmol cr) and the corresponding (A) fetal serum T₄ concentrations (nmol/L), (B) fetal serum T₃ concentrations (pmol/L), (C) maternal serum T₄, and (D) maternal serum T₃ concentrations. ■ represents group I, control; ●, group II, maternal Tx; ◇, group III, fetal Tx; and ▲ group IV, maternal and fetal Tx.

clearance of T₂S may play a role. Although fetal serum T₂S levels were significantly decreased in group II compared with group I, the decreases were not significantly different from the baseline values. On the other hand, MU T₃S excretion was reduced after either maternal or fetal Tx. Using 2 × 2 factorial

analysis in SPSS with fetal Tx and maternal Tx as the two factors (25), we assessed effects of fetal Tx (group I and II versus III and IV) and of maternal Tx (group I and III versus II and IV). An additive effect of fetal Tx was found to be significant in MU T₂S and T₃S excretion as well as fetal serum

Figure 2. Correlation between MU T₃S excretion (pmol/mmol cr) and the corresponding (A) fetal serum T₄ concentrations, (B) fetal serum T₃ concentrations, (C) maternal serum T₄ concentrations, and (D) maternal serum T₃ concentrations. The respective units of concentration and symbols are shown in Figure 1.

Table 3. Effect of fetal or/and MTx on the T₂S and T₃S concentrations in maternal and fetal sera

Group	n	T ₂ S (pmol/L)						T ₃ S (pmol/L)					
		Maternal			Fetal			Maternal			Fetal		
		Start	End	n	Start	End	n	Start	End	n	Start	End	n
I	5	413 (45)	496 (83)	10	1967 (132)	2661 (116)	5	356 (70)	301 (60)	10	793 (81)	862 (178)	10
II	4	446 (55)	463 (40)	8	1835 (165)	1901 (264)*	4	342 (64)	333 (37)	8	917 (114)	465 (67)*	8
III	4	380 (36)	463 (73)	8	1504 (116)	1190 (149)*	4	328 (41)	328 (137)	8	917 (75)	725 (130)	8
IV	5	430 (26)	264 (35)*	10	1686 (116)	446 (66)*	5	328 (66)	52 (14)**	10	889 (66)	98 (18)**	10

Group I, II, III, and IV: see Table I; Data are presented as means (SE).
* *p* < 0.05, ** *p* < 0.01 vs group I sham-operated control.

T₂S. On the other hand, the additive effect of maternal Tx was found to be significant in maternal serum T₃ and T₄ as well as MU T₃ and T₃S but not T₂S. Thus, it appears that the major determinant of the amount of MU T₂S excretion is the fetal thyroid status.

In humans, a precocial species similar to sheep, we found high levels of T₂S-like material (Compound W) in maternal serum (12) and urine (14). Levels increase with the progression of pregnancy, peaking near term at which time a 20-fold increase in serum levels of this T₂S-like material is

Figure 3. Identification of [^{125}I]-3,3'- T_2S by specific antibody and by cochromatographing with synthetic-labeled T_2S in urine extract of maternal ewes eluted from HPLC (C_{18} column) isocratically with a mixture of acetonitrile and 0.02 M ammonium acetate, pH 4.0 (22:78, vol/vol), at a flow rate of 2 mL/min.

found compared with nonpregnant women. Serum levels return to nonpregnant values by 7–10 d postpartum (12). Further clinical studies suggest that Compound W is derived from TH of fetal origin, analogous to the present studies in sheep, and can be a practical marker for fetal thyroid function (13).

The mechanism and site(s) of fetal-to-maternal transfer of T_2S are unclear. 3,3'- T_2 is a metabolite derived from both inner-ring deiodination of T_3 and outer-ring deiodination of rT_3 . In addition, T_2 is a preferred substrate for IST in rat liver (26) and uterus (27). T_2S can also be formed at an accelerated rate from further deiodination of T_3S and rT_3S , both of which are abundant in the fetal circulation and biologic fluids in lambs late in gestation (6, 8, 28, 29). It is likely that there is an increasing production of T_2S in precocial species fetuses in late gestation as the hypothalamic-pituitary-thyroid axis matures. In addition to T_2S formation, an active mechanism to convert fetal T_4 to rT_3 , T_2 , T_4S , rT_3S , T_3S , and T_2S may be important to normal fetal development, especially in late gestation. T_2 has been shown to stimulate mitochondrial respiration in various rat tissues (15, 16), and it is possible that a tight regulation of T_2 concentration by sulfation and fetal-to-maternal transfer would have physiologic value. Because sulfation is a reversible process, tissue sulfatase enzyme(s) can desulfate T_2S , converting it back to an active iodothyronine. Thus, the fetal-to-maternal transfer may protect the fetus from excessive mitochondrial thermogenesis stimulated by high fetal concentration of T_2 . T_2S species (*i.e.* 3',5'- T_2S and 3,5- T_2S) other than 3,3'- T_2S have not been measured.

More recently, a placental IST was detected in the cytosol of sheep cotyledon (Wu SY, unpublished data). Again, T_2 was shown to be the preferred substrate for the placental IST in sheep, and we observed significant D3 activity in the cotyledon homogenates (Wu SY, Polk DH, Fisher DA, unpublished observation). The observation that D3 metab-

olites, namely T_2 and rT_3 , are the preferred metabolites for the placental IST suggests that D3 may be linked to IST to facilitate fetal-to-maternal transfer of T_2S . In perfusing the fetal side of guinea pig with TH, Castro *et al.* (30) found T_3 is much more rapidly inner-ring deiodinated than T_4 . In addition, Rudolph and Heymann (31) have shown in sheep that the placenta received 40 to 50% of the fetal cardiac output. Furthermore, Sack *et al.* (32) showed that umbilical cord cutting triggers hypertriiodothyroninemia in the newborn lamb and that the postnatal T_3 peak can be delayed until well after the TSH peak by delaying umbilical cord cutting. Recently, Santini *et al.* (33) found that the placenta may play an important role in maintaining the low serum T_3 in fetuses late in gestation. These findings suggest the importance of the placenta in fetal T_3 metabolism, and it is possible that fetal-to-maternal transfer of the T_3S metabolite (presumably via placenta) can be a further novel mechanism involved in reducing serum T_3 concentrations in the fetus.

The present study also revealed another interesting aspect of fetal-maternal TH homeostasis in sheep. In agreement with our previous study, fetal serum levels of T_3S were not reduced by fetal hypothyroidism (8). The reason is unclear; the fetal serum T_3S level does not correlate with fetal serum T_3 or T_4 but weakly correlates with maternal serum T_4 . In contrast, fetal serum T_2S levels decreased significantly after fetal Tx and showed a significant correlation with the low fetal serum T_4 concentrations.

In conclusion, we have demonstrated significant fetal-to-maternal transfer of T_2S in late gestation in sheep. The present study also suggests that the amount of T_2S excretion in maternal sheep urine reflects fetal thyroid status.

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