Thyroid hormone replacement normalizes renal renin and angiotensin receptor expression in thyroidectomized fetal sheep

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Submitted 5 April 2007; accepted in final form 3 June 2007

Chen K, Carey LC, Valego NK, Rose JC. Thyroid hormone replacement normalizes renal renin and angiotensin receptor expression in thyroidectomized fetal sheep. Am J Physiol Regul Integr Comp Physiol 293: R701–R706, 2007.—Previous studies have suggested that thyroid hormone influences maturation of the renin-angiotensin system (RAS) and cardiovascular function in the late-gestation fetal sheep. To further examine the importance of thyroid hormone in this regard, we used the technique of thyroidectomy (TX) to remove endogenous thyroid hormone from the circulation and then replaced it with physiological amounts of exogenous thyroxine. We hypothesized that the previously observed changes in RAS activity and cardiovascular function associated with TX would be normalized. TX was performed at 120 dGA, and control fetuses were sham operated. After 3 days of recovery, TX fetuses were continuously intravenously infused with thyroxine until delivery by cesarean section to term (138 dGA). Immediately before necropsy, TX fetuses were infused with isoproterenol, and the hemodynamic responses were noted. Thyroid hormone replacement normalized not only plasma triiodothyronine (T3) and thyroxine (T4) levels but also the TX-induced decreases in renal renin mRNA and renal renin content. Renal ANG II subtype receptor expression levels were also normalized for both mRNA and protein. Decreased basal heat rate and systolic blood pressure associated with TX returned to normal following replacement; however, changes in mean blood pressure and isoproterenol-induced changes in mean blood pressure were not altered. These findings demonstrate that replacement of thyroid hormone in hypothyroid sheep fetuses can restore renal ANG II receptor and renin expression and secretion to normal.

COMPONENTS OF THE RENIN ANGIOTENSIN SYSTEM (RAS) undergo maturational changes during fetal life. These changes appear to be important for regulating aspects of fetal development. Although the precise mechanisms mediating the changes in RAS expression are not completely understood, thyroid hormone, the levels of which increase dramatically close to term, is thought to be important in this regard. Findings from our recent studies lend support to this idea (6). We noted that fetal sheep thyroidectomized (TX) at 120 dGA did not show the expected increases in renin renin mRNA, renal active renin levels, and kidney ANG II receptor (AT1 and AT2) mRNA and protein expression close to term (6, 8).

Thyroid hormone is also thought to influence development of the cardiovascular system, particularly that pertaining to cardiac contractility and output, arterial relaxation, and systemic vascular resistance (14). Unsurprisingly, we found that TX sheep fetuses presented with altered cardiovascular function, namely decreased basal heart rate and blood pressure, and responsiveness to the beta adrenergic agonist isoproterenol (6).

Studies in the present investigation were designed to confirm the involvement of thyroid hormone in RAS and cardiovascular development. To achieve this, we used the technique of TX in conjunction with physiological thyroid hormone replacement (TX+R) in fetal sheep and hypothesized that the RAS and cardiovascular changes associated with TX alone would be normalized.

MATERIALS AND METHODS

Animals. Cross-bred pregnant ewes with known insemination dates were obtained from a local supplier. Ewes were housed in individual pens with food and water provided. After 5 days of acclimation, surgery was performed. After surgery, ewes were returned to their pens where they remained until fetuses were delivered. All procedures were approved by the Wake Forest University Animal Care and Use Committee. A total of 12 fetuses were used, 6 in each experimental group.

Surgical procedures. TX was performed similarly to the procedure described by Hopkins and Thorburn (9) at ~120 dGA. Briefly, the fetal neck was exposed and stabilized on a sterile tray, and the skin was opened at the midline. The fetal thyroid gland was exposed and removed. Control fetuses were sham operated, in which the thyroid was exposed but not touched. Catheters filled with sterile saline were placed in fetal carotid arteries and jugular veins. Additional catheters were inserted into the femoral arteries and veins, and they were advanced to the descending aorta and inferior vena cava. Catheters were also placed in the amniotic sac to measure intratherine pressure, as well as in the maternal femoral artery and vein. Gentamicin and ampicillin were administered to the ewe at the time of surgery and for the next 3 days through the maternal venous catheter. After 3 days of recovery, TX fetuses received a continuous intravenous infusion of thyroxine (T4) (~50 μg·kg−1·day−1; Sigma-Aldrich, St. Louis, MO) until delivery by cesarean section at ~138 dGA, hereafter referred to as TX+R. Control fetuses were infused with isotonic saline. Fetal plasma samples were collected following 3 days of postsurgery recovery, and just before necropsy for measurement of plasma triiodothyronine (T3), T4, active and prorenin, and arterial blood gases (O2 and CO2). Fetuses were delivered by cesarean section between 135 and 139 dGA (term in our flock is ~145 dGA), and the kidney cortex was collected and stored at ~80°C for later renal renin content and mRNA analysis, as well as ANG II receptor subtype mRNA and protein analysis.

Blood pressure and heart rate recording. Basal fetal hemodynamic and plasma active renin and prorenin levels and their responses to beta-adrenergic stimulation were studied in both TX+R and control...
was then centrifuged at 2,100 g and the supernatant was frozen at 0 °C until assay. The supernatant was homogenized on ice for 45 s in 4 ml of saline; the homogenate of isoproterenol (Abbott Laboratories, North Chicago, IL) infusion was collected. An aliquot was taken for protein determination and the remainder was frozen at −80 °C until assay. For the assay, samples were diluted with saline containing 5.2 mM BAL (2,3-dimercaptopropanol), 0.59 mM 8-hydroxyquinoline, and 10 mM disodium EDTA. ARC was determined as for plasma and is expressed as nanograms per milligram of protein per hour of incubation.

Prorenin concentration measurement. Prorenin concentration was determined by measuring active renin before and after treatment of plasma or kidney cortex homogenate with bovine pancreatic trypsin at a concentration designed to yield maximum renin activation. Each dose of trypsin was tested by constructing a dose-response curve with pooled plasma or kidney homogenate. Once the optimal dose of trypsin was established for each, this dose was used for subsequent assays. Trypsin activation was at 4°C and pH 7.3 for 30 min. The activation was stopped by the addition of trypsin inhibitor at room temperature for 15 min. The total renin concentration represented the sum of active renin and prorenin.

**RNA extraction and synthesis of antisense RNA probes.** Total tissue RNA was extracted using standard procedures described previously (6). The probe used for sheep renin mRNA is a partial sheep renin cDNA from coordinates 117–983 cloned into pGEM-T easy (Promega, Madison, WI) and cut with the restriction enzyme EcoRI to linearize the plasmid in preparation for in vitro transcription. The probe used for sheep AT1 mRNA is a partial sheep AT1 cDNA from coordinates 114–783 cloned into pGEM-T easy (Promega, Madison, WI) and cut with the restriction enzyme SpeI to linearize the plasmid in preparation for in vitro transcription. The probe used for sheep AT2 mRNA is a partial sheep AT2 cDNA from coordinates 142–921 cloned into pT7/T3U18 (Ambion, Austin, TX) and cut with the restriction enzyme HindIII to linearize the plasmid in preparation for in vitro transcription, as described previously (6).

**RNase Protection Assay and Western blot analysis.** Renin, AT1 and AT2 mRNA were quantified by RNase protection assay (RPA; RPA kit III; Ambion), and AT1 and AT2 protein were analyzed by Western blot analysis, as in a previous study (6).

**Densitometry.** Films were scanned and analyzed using DayOne software (PDI Imageware Systems, San Diego, CA). Sense RNA standards were used to calibrate the system for RPA data. The data were converted from optical density readings to pg mRNA/μg total RNA for RPA data. Western blot analysis data are reported in optical density units.

**Statistical analysis.** All data are expressed as means ± SE. The data for blood pressure, heart rate, and hormone levels were analyzed using two-way ANOVA, while differences for the remaining variables were determined by two-tailed t-test. A P < 0.05 was considered to be statistically significant.

**RESULTS**

**Confirmation of TX and fetal health.** The completeness of TX in each fetus was confirmed visually. Plasma T3 and T4 levels, measured immediately before the commencement of replacement, were significantly lower in TX fetuses (Fig. 1).

![Fig. 1. Plasma T3 (A) and T4 (B) levels during development in control and TX+R fetal sheep. Measurements were made just after surgery [120–125 days of gestational age (dGA)] and before death (135–139 dGA). Values are means ± SE; n = 6 for both groups. *Significant difference between the 120–125 and 135–139 dGA groups (P < 0.001). **Significant difference between TX+R and control fetuses (P < 0.001).](http://ajpregu.physiology.org/)

**Rens renin mRNA expression in control and TX+R fetal sheep.** Values are means ± SE; n = 6 for both groups. Inset: representative gel for these results.
Infusion of thyroxin normalized both T3 and T4 concentrations (Fig. 1). Fetal health, as assessed by arterial blood gas and pH measurements, was normal throughout the duration of the studies in both TX/H11001R and control fetuses.

**Effect of TX/H11001R on renal and plasma renin.** There were no differences in renal renin mRNA expression between TX/H11001R and control fetuses (Fig. 2). Thyroxine replacement also normalized total and active renal renin concentrations in TX+R fetuses (Fig. 3).

Plasma active renin concentrations were similar in both control and TX+R fetuses at both 120–125 and 135–139 dGA (Fig. 4). However, there was an age effect ($F = 11.94, P < 0.01$) with the levels being higher in the older group. For prorenin there were age ($F = 35.2, P < 0.001$), treatment ($F = 14.88, P < 0.01$) and interaction ($F = 14.49, P < 0.01$) effects. Prorenin concentrations were lower in the older group, while replacement animals had higher prorenin values at 120–125 dGA. Isoproterenol infusion increased plasma active renin concentrations equally in both groups (Fig. 5).

**Effect of TX/H11001R on renal ANG II subtype receptors expression.** Renal AT1 and AT2 mRNA and protein expression levels were not different in TX+R and control fetuses (Figs. 6 and 7).
Effect of TX\(^{-}/\text{H}11001\)R on the cardiovascular system. TX\(^{-}/\text{H}11001\)R fetuses had similar basal systolic blood pressure levels but lower basal mean and diastolic blood pressure than controls (Fig. 8). Fetal blood pressure and heart rate recordings are presented in Figs. 9 and 10. Basal heart rate and responsiveness to isoproterenol infusion were not different between TX+R and control fetuses. Infusion of isoproterenol decreased mean arterial pressure in TX+R but not in control fetuses.

**DISCUSSION**

In this study, we examined the effect of thyroidectomy followed by thyroid hormone replacement on RAS and cardiovascular activity in late-gestation fetal sheep. We found that replacement normalized the previously observed (6) changes in renal renin mRNA and content, renal ANG II receptor subtype mRNA, and protein expression levels, basal heat rate, and systolic blood pressure associated with TX. These findings establish that thyroid hormone is an important mediator of both RAS and cardiovascular system development in the late-gestation fetal sheep.

Renal renin mRNA and renin content increase significantly in the late-gestation fetal sheep (1, 2, 20) as a consequence of increased number and content of renin-containing cells (27). In a prior study of fetal sheep thyroidectomized at 120 dGA, we noted that the expected increases in renal renin mRNA expression and renal renin content were attenuated in late gestation (6), implicating thyroid hormone in a regulatory role. In accordance with this observation, we found in the present study that TX+R normalized renal renin content and mRNA expression. Also indicative of the importance of thyroid in regulating renin expression is the observation that hypothyroid human individuals treated with thyroxine exhibit increased plasma renin activity (18).

Precisely how thyroid hormone influences renin gene expression is unclear; however, studies in transgenic mice carrying extra copies of the Ren2 gene indicate that thyroid hormone...
can directly stimulate transcription and/or stabilize precursor renin mRNA (12, 25). It has also been noted in human Calu-6 cells that thyroid hormone interacts with the promoter region on the renin gene via specific thyroid hormone response elements (15).

The AT1 and AT2 receptors are also developmentally regulated and play important roles in mediating fetal kidney development and function (10, 21, 23). We previously noted that both renal AT1 and AT2 receptor mRNA and protein levels were altered in TX fetuses (6). Findings from the current investigation, in which there were no differences in AT1 and AT2 receptor mRNA and protein levels between TX+R and control fetuses, suggest that the increase in thyroid hormone concentrations in late gestation (8) plays a critical role in regulating ANG II receptor subtype expression, and hence renal development and function.

We found in late-gestation TX fetal sheep that thyroxine replacement normalized the previously observed decreases in basal heart rate and systolic blood pressure, but interestingly, not mean and diastolic blood pressure (6). It has been established that T3 exerts a direct positive inotropic effect by inducing synthesis of fast α-isofoms of heavy myosin chains and by increasing calcium-adenosine triphosphatase and cAMP levels, together with the number and sensitivity of β-adrenergic receptors (7, 11, 13, 16, 24). Hence, the normal T3 concentrations in the TX+R fetuses in the present study may explain, at least in part, the normalization of systolic blood pressure compared with TX fetuses, where T3 levels are significantly decreased (6). With regard to the decreased diastolic blood pressure observed, another response of the cardiovascular system to thyroid hormone administration is decreased peripheral vascular resistance (14). This is thought to be a consequence of T3-mediated relaxation of vascular smooth muscle cells, leading to general arteriolar vasodilatation (26), such as increased muscular capillary vessel number (4, 5) and heightened local release of vasodilators in peripheral tissue (3, 22). Therefore, thyroid hormone-induced decreases in peripheral vascular resistance may underlie the lower diastolic blood pressures observed in TX+R fetuses. The mechanisms underlying the effect of thyroid hormone on heart rate are at the present time unknown and warrant further investigation.

The previously noted decline in MAP following isoprotrenanol infusion in late-gestation TX fetal sheep (6) was not prevented by thyroid hormone replacement in the present study. The precise mechanisms mediating this effect are unclear and worthy of further investigation.

In summary, we have found that thyroid hormone replacement normalizes plasma T3, T4, preprorenin, and active renin concentrations and their associated responses to isoprotrenanol infusion in thyroidectomized fetuses. Basal heart rate and expression levels of AT1 and AT2 receptor mRNA and protein in these same fetuses also returned to normal. These findings demonstrate that thyroid hormone plays an important regulatory role in RAS and cardiovascular system development/function in the late-gestation sheep fetus.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Child Health and Human Development Grant HD-17644.

REFERENCES


