THYROID HORMONE REPLACEMENT NORMALIZES RENAL RENIN AND ANGIOTENSIN RECEPTOR EXPRESSION IN THYROIDECTOMIZED FETAL SHEEP

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Running head: Thyroid hormone & RAS activity in fetal sheep

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ABSTRACT

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 utilized the technique of thyroidectomy (TX) to remove endogenous thyroid hormone from the circulation, and then replaced it with physiological amounts of exogenous thyroxin. We hypothesized that the previously observed changes in RAS activity and cardiovascular function associated with TX would be normalized. TX was performed at 120 days of gestational age (dGA), and control fetuses were sham operated. After three days recovery, TX fetuses were continuously i.v. infused with thyroxin until delivery by cesarean section close to term (around 138 dGA). Immediately prior to necropsy fetuses were infused with isoproterenol and the hemodynamic responses were noted. Thyroid hormone replacement normalized not only plasma T3 and T4 levels, but also the TX induced decreases in renal renin mRNA and renal renin content. Renal angiotensin II subtype receptor expression levels were also normalized at both the mRNA and protein level. 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 angiotensin II receptor and renin expression and secretion to normal.
**Key words:** fetus, AT1, AT2, thyroidectomy, ovine.
INTRODUCTION

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 days of gestation (dGA, where term is around 145 dGA) did not show the expected increases in renal renin mRNA, renal active renin levels, and kidney angiotensin 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 utilized the technique of TX in conjunction with physiological thyroid hormone replacement (R, 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 carts 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 similar to the procedure described by Hopkins and Thorburn (9) at approximately 120 dGA. Briefly, the fetal neck was exposed, 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, where 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 advanced to the descending aorta and inferior vena cava. Catheters were also placed in the amniotic sac to measure intra-uterine pressure, and in the maternal femoral artery and vein. Gentamicin and ampicillin were administered to the ewe at the time of surgery and for the next three days through the maternal venous catheter. After 3 days recovery, TX fetuses received a continuous i.v. infusion of thyroxine (approximately 50µg/kg/day-Sigma-Aldrich, St. Louis, MO) until delivery by cesarean section around 138dGA (these fetuses are hereafter referred to as TX+R). Control fetuses were infused with isotonic saline. Fetal plasma samples were collected following 3 days of post surgery recovery,
and just prior to necropsy for measurement of plasma triiodothyronine (T3), thyroxine (T4), active and prorenin, and arterial blood gases (O2 and CO2). Fetuses were delivered by cesarean section between 135-139 dGA (term in our flock is approximately 145 days), and kidney cortex was collected and stored at –80 °C for later renal renin content and mRNA, and angiotensin 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 fetal sheep immediately before necropsy. Fetal arterial pressures, heart rate (HR), and amniotic fluid pressure were measured with DMSI system integrators (Digi-Med, Micro-Med Enterprises, Inc. Tustin, CA) and recorded at one minute intervals on computer hard drive. All blood pressure data were corrected to corresponding amniotic pressure. Data collection was initiated after a 30 minute stabilization period. Responses to beta adrenergic stimulation were determined by measuring fetal blood pressure and heart rate changes before, during and after 10 minutes of isoproterenol (Abbott laboratories, North Chicago, IL) infusion (0.06µg/kg.min). Blood samples were obtained before isoproterenol infusion, immediately after the isoproterenol infusion was complete, and at the end of the experiment to examine plasma renin levels.

**Plasma T3, T4 and active renin**

T3 concentrations were measured by enzyme immunoassay assay using a kit from Diagnostic Systems Laboratories Inc. (Webster, TX). The minimum detectable amount of T3 was 0.4 ng/dL. Coefficients of variation were 5.7 % intra-assay 6.7 % inter-assay.
Plasma T4 concentrations were measured by RIA using a kit from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) which measures total T4. The minimum detectable amount of T4 was 0.76 ng/dL. Coefficients of variation were 5.3 % intra-assay and 7.9 % inter-assay.

Plasma active renin concentrations were measured as a function of the amount of AI generated from angiotensinogen with a kit (Perkin-Elmer Life and Analytical Sciences, Boston, MA) as described previously (6). Results are expressed as ng AI (37 °C ng/ml minus 4 °C ng/ml)/ml plasma per hour of incubation.

**Tissue active renin concentration (ARC) Measurement**

Approximately 100 mg of renal cortex was homogenized on ice for 45 seconds in 4 ml of saline, the homogenate then centrifuged at 2100 × g for 10 minutes, and the supernatant collected. An aliquot was taken for protein determination and the remainder 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 ng/per mg 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 minutes. The
activation was stopped by addition of trypsin inhibitor at room temperature for 15 minutes. The total renin concentration represented the sum of active 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 EcoR1 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 Hind III to linearize the plasmid in preparation for in vitro transcription as described previously (6).

**RNase Protection Assay (RPA) and Western blotting**

Renin, AT1 and AT2 mRNA were quantified by RPA (RPA kit III; Ambion, Austin, TX), and AT1 and AT2 protein were analyzed by Western blot as in a previous study (6).

**Densitometry**

Films were scanned and analyzed using DayOne software (PDI Imageware Systems Inc. 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 data are reported in OD units.
Statistical analysis

All data are expressed as mean plus or minus the standard error of the mean. The data for blood pressure, heart rate, and hormone levels were analyzed using two-way analysis of variance, 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 (Figure 1). Infusion of thyroxin normalized both T3 and T4 concentrations (Figure 1).

Fetal health, as assessed by arterial blood gas and pH measurements, was normal throughout the duration of the studies in both TX+R and control fetuses.

Effect of TX+R on renal and plasma renin

There were no differences in renal renin mRNA expression between TX+R and control fetuses (Figure 2). Thyroxine replacement also normalized total and active renal renin concentrations in TX+R fetuses (Figure 3).

Plasma active renin concentrations were similar in both control and TX+R fetuses at both 120-125 and 135-139 dGA (Figure 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 days of gestation.

Isoproterenol infusion increased plasma active renin concentrations equally in both groups (Figure 5).

**Effect of TX+R 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 (Figure 6,7).

**Effect of TX+R on the cardiovascular system**

TX+R fetuses had similar basal systolic blood pressure levels, but lower basal mean and diastolic blood pressure than controls (Figure 8). Fetal blood pressure and heart rate recordings are presented in figures 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 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 angiotensin 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, where 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 levels in late gestation (8) plays a critical role in regulating angiotensin 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 inotrophic effect by inducing synthesis of fast α-isoforms 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 as compared to 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 (17; 19). Reduced vascular resistance may also be secondary to increased vascularity and/or alterations in the vascular control mechanisms favoring enhanced 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 isoproterenol 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, pro- and active renin concentrations and their associated responses to isoproterenol 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.

ACKNOWLEDGEMENTS

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


Figure 1

A

Plasma T3 (ng/ml)

Control
Tx+R

0.0 0.2 0.4 0.6

120-125 dGA 135-139 dGA

B

Plasma T4 (ng/ml)

120-125 dGA 135-139 dGA

* +
Figure 2

Renal renin mRNA (pg/10ug total RNA)

Control
TX+R

0 1 2 3 4

Control TX+R
Figure 3

A

Renal Active Renin
(ng.Ang I/min.mg)

4000
3000
2000
1000
0

Control
TX+R

B

Renal Prerenin
(ng.Ang I/min.mg)

1000
800
600
400
200
0

Control
TX+R
Figure 4

A

Plasma Active Renin (ng.Ang I/ml.hour)

Control
TX+R

120-125 dGA
135-139 dGA

B

Plasma Prorenin (ng.Ang I/ml.hour)

120-125 dGA
135-139 dGA
Figure 5

Plasma Active renin (ng Ang I/ml.hour)

Control
TX+R

Minute
0'  Iso  10'  60'

*
Figure 6

A. Renal AT1 mRNA levels (pg/10ug total RNA) for Control and TX+R groups. The bar graph shows a significant increase in AT1 mRNA levels in the TX+R group compared to the Control group.

B. Renal AT2 mRNA levels (pg/10ug total RNA) for Control and TX+R groups. The bar graph indicates a moderate increase in AT2 mRNA levels in the TX+R group compared to the Control group.
Figure 7

A

AT1 protein (OD)

0.0 0.5 1.0 1.5

Control TX+R

B

78 kd AT2 protein (OD)

0.0 0.2 0.4 0.6

Control TX+R

C

44 kd AT2 protein (OD)

0.0 0.2 0.4 0.6

Control TX+R
Figure 8

![Blood Pressure Graph]

- Basal MBP
- Basal SBP
- Basal DBP

Control
TX+R

Blood Pressure (mmHg)
FIGURE LEGENDS

Figure 1. Plasma T3 (panel A) and T4 (panel B) levels during development in control and TX+R fetal sheep. Measurements were made just after surgery (120-125 dGA) and prior to sacrifice (135-139 dGA). Values are means ± SEM; n = 6 for both groups. * indicates a significant difference between the 120-125 and 135-139 dGA groups (p<0.001). + indicates a significant difference between TX+R and control fetuses (p<0.001).

Figure 2. Renal renin mRNA expression in control and TX+R fetal sheep. Values are means ± SEM; n = 6 for both groups. The insert depicts a representative gel for these results.

Figure 3. Renal active (panel A) and renal prorenin (panel B) concentrations in control and TX+R fetuses. Values are means ± SEM; n = 6 for both groups.

Figure 4. Plasma active renin (panel A) and prorenin renin (panel B) concentrations in control and TX+R fetuses. Measurements were made just after surgery (120-125 dGA) and prior to sacrifice (135-139 dGA). Values are means ± SEM; n = 6 for both groups. * indicates a significant difference between the 120-125 and 135-139 dGA groups (p<0.05). + indicates a significant difference between TX+R and control fetuses (p<0.01).

Figure 5. Plasma active renin concentrations following 10 minutes isoproterenol infusion and 50 minutes recovery in TX+R and control fetuses. Values are means ± SEM; n = 6 for both groups. * indicates a significant difference from baseline concentrations (p<0.05).

Figure 6. AT1 (panel A) and AT2 (panel B) mRNA expression levels in TX+R and control fetal kidneys at 135-139 dGA. Values are means ± SEM; n = 6 for both groups.
**Figure 7.** AT1 (panel A) and AT2 (panel B = 78 kd band, panel C = 44 kd band) protein levels in TX+R and control fetal kidneys at 135-139 dGA. Values are means ± SEM; n = 6 for both groups. The inserts on each panel depict representative blots.

**Figure 8.** Basal mean, systolic and diastolic blood pressure in control and TX+R fetal sheep. TX+R fetuses had similar systolic, but lower mean and diastolic blood pressure compared to controls (F = 4.625, p<0.05).

**Figure 9.** Heart rate before, during (10th to 20th minutes) and after isoproterenol infusion in 135-139 dGA control and TX+R fetuses. Values are means ± SEM; n = 6 for both groups. Isoproterenol significantly increased heart rate in both TX+R and control fetuses (F = 57.78, p<0.001).

**Figure 10.** Mean blood pressure before, during (10th to 20th minutes) and after isoproterenol infusion in 135-139 dGA control and TX+R fetuses. Values are means ± SEM; n = 6 for both groups. Baseline blood pressure was lower in TX+R fetuses (F = 202.5, p<0.001), and decreased significantly following isoproterenol infusion (F = 7.09, p<0.001).