THYROID HORMONE MODULATES RENIN AND ANGIOTENSIN II RECEPTOR EXPRESSION IN FETAL SHEEP

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Running head: Thyroid hormone & renin-angiotensin system activity in fetal sheep

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ABSTRACT

Fetal renin-angiotensin system (RAS) activity is developmentally regulated, increasing in late gestation towards term. At the same time fetal hemodynamic parameters change, with blood pressure increasing and heart rate decreasing. During this period, fetal plasma thyroid hormone concentrations also increase significantly. In this study we utilized the technique of thyroidectomy (TX), which removes thyroid hormone from the circulation, to investigate the importance of thyroid hormone on the developmental changes in the RAS (in plasma, kidney, heart and lung) and hemodynamic regulation in fetal sheep. TX was performed at 120 days of gestational age (dGA), and control fetuses were sham operated. Immediately prior to necropsy (around 137 dGA) fetuses were infused with isoproterenol and the hemodynamic responses noted. TX significantly decreased plasma thyroid hormone concentrations, renal renin mRNA and renal active renin levels, but did not change fetal plasma active renin levels. TX decreased both AT1 mRNA and protein levels in kidney and lung, but not in the left ventricle. TX was also associated with increased AT2 mRNA and protein at the 44 kD band in kidney, while AT2 protein was decreased at the 78 kD level in kidney and lung tissue only. TX fetuses had significantly lower basal mean arterial blood pressures (MAP) and heart rates than controls. Isoproterenol infusion decreased MAP in TX fetuses. These findings support the hypothesis that thyroid hormone is important in modulating maturation of RAS and cardiovascular function in the late gestation fetal sheep.
INTRODUCTION

The renin angiotensin system (RAS), composed of renin, angiotensinogen, angiotensin I (AI) converting enzyme (ACE), angiotensin II (AII) and the AII receptor subtypes, plays an important role in regulating fetal growth and development, particularly with respect to the cardiovascular system, and electrolyte homeostatic mechanisms (2,22,48). It is well established that components of the fetal RAS are developmentally regulated, and exhibit increased activity in late gestation (12,56,57,63). Changes in expression of the AII receptor subtypes (AT1 and AT2) levels are opposite, with AT1 mRNA being low in early gestation, raising to a plateau later, before increasing rapidly close to term, while AT2 expression is highest during mid pregnancy, and then decreases gradually thereafter (46,47). Tissue dependent differences are also apparent (18,40,66). The mechanisms underlying these ontogenic patterns of expression are not clear.

In fetal sheep, the changes in components of RAS activity appear temporally related to changes in thyroid hormone concentrations, which are low before 130-135 days of gestational age (dGA) and then markedly increased towards parturition (19). A similar pattern of change is seen in humans (44). These observations suggest that thyroid hormone may be important in regulating ontogenic changes in renin and AII receptors.

Thyroid hormone also exerts broad effects on the developing cardiovascular system, increasing cardiac contractility and output, inducing arterial relaxation, and reducing systemic vascular resistance (35). Hypothyroidism, conversely, is associated with decreased stroke volume, cardiac output, and intravascular volume (34). In adult rats, thyroidectomy (TX) significantly decreases both heart rate and blood pressure (14,20). Likewise, in fetal sheep, TX performed at 80 dGA is associated with lower blood pressure, but no change in heart rate (61). These findings suggest that thyroid hormone exerts positive regulatory effects on cardiovascular function in both adult and fetus.

The mechanisms, through which thyroid hormone influences cardiovascular system activity, are currently unclear. Previous studies in the rat have, however, confirmed that thyroid hormone alters the cardiovascular response to adrenergic agonists (14), suggesting that this interaction may be at least in part responsible.

Therefore, by utilizing the technique of TX, the present studies were designed to assess the importance of thyroid hormone in regulating the components of RAS activity, including renal renin, plasma renin and tissue AII receptor subtype expression in late gestation fetal sheep. Our
rationale for this was that the normal ontogenic change in the RAS would be attenuated in the absence of thyroid hormones in late gestation. In addition, we evaluated the impact of fetal hypothyroidism on cardiovascular function and responsiveness to beta-adrenergic stimulation in the late gestation fetal sheep.

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 TX (3 male, 3 female), and 6 controls (4 male, 2 female).

Surgical procedures and blood pressure and heart rate measurements

TX was performed similar to the procedure described by Hopkins and Thorburn (25) 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 previously 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. Fetal plasma samples were collected following 3 days of post surgery recovery, and just prior to necropsy for measurement of triiodothyronine (T3) and thyroxine (T4). Fetuses were delivered by cesarean section between 137-139 dGA (term in our flock is approximately 145 days), and tissue samples (kidney cortex, heart and lung) were collected and stored at –80 °C until assay. These specific tissues were sampled as the RAS is thought to play developmental and/or functional roles in all (11,50,53-55).

Basal fetal hemodynamic and plasma active renin and prorenin levels and their responses to beta-adrenergic stimulation were studied in both TX 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). All blood pressure data were corrected to corresponding amniotic pressure. Data collection was begun after 30 minutes stabilization period. The data were averaged and recorded every one minute automatically throughout the experiment. Responses to beta adrenergic stimulation were determined by measuring the fetal blood pressure and heart rate changes before, during and after 10 minutes of isoproterenol infusion (0.06µg/kg.min). Mean arterial blood pressure (MAP), amniotic fluid pressure, and heart rate were measured throughout the experiment and for 50 minutes after the infusion. 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 measurement**

T3 was 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 was 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 concentration was measured as a function of the amount of AI generated from angiotensinogen with a kit (Perkin-Elmer Life and Analytical Sciences. Boston, MA). In order to measure renin concentration independent of endogenous angiotensinogen, the method was slightly modified from that described for renin activity. Excess renin substrate (0.5 ml of adult nephrectomized sheep plasma) was added to each aliquot (0.1 ml) of plasma along with the enzyme inhibitors, dimercaprol, 8-hydroxyquinoline, and maleate buffer (pH 6.0, to assure a constant pH at the optimum for renin activity). One ml of this cocktail was then incubated at 37 ºC while the rest was kept at 4 ºC for one hour. The AI generated was measured by RIA with the kit. All samples from an animal were analyzed simultaneously and in duplicate, and all assays included samples from control and thyroidectomized animals. Results are expressed as ng AI (37 ºC ng/ml minus 4 ºC 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 and stored at −80 ºC until assayed. For the assay, sample was diluted with saline containing 5.2 mM BAL (2, 3 dimercapto-1-propanol), 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 ºC 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**

Total tissue RNA was extracted using standard procedures. Briefly, the tissue was homogenized in Trizol regent (Gibico BRL. Carlsbed, CA) with a high speed polytron for 30-60 seconds, chloroform was added (0.2 ml/1ml Trizol) and the mixtures centrifuged at 12 000 × g for 15 minutes at 4 ºC following 5 minutes incubation at room temperature. The aqueous phase was transferred to a fresh tube, the RNA precipitated by the addition of isopropanol (0.5 ml/1 ml Trizol), and recentrifuged at 7500 × g at 4 ºC for 5 minutes. The ethanol was removed, and the RNA pellets were allowed to air dry, before being re-dissolved in RNase-free water. RNA concentrations were determined by measuring absorbance at 260 nm. RNA sample integrity was determined by electrophoresis on a 1.0 % agarose gel containing 6.6 % formaldehyde.

**Synthesis of antisense RNA probes**

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.

In vitro transcription was performed by adding the following items in this order: 4 µl 5X transcription buffer, 2 µl 100 mM dithiothreitol, 1 µl Rnasin RNase inhibitor, 4 µl ATP, GTP, and CTP mix (25 mM each), 2.4 µl 100 µM UTP, 5µl [α-32P] UTP (3000 Ci/mm; Perkin-Elmer, Boston, MA), and 1 µl SP6 (for renin) or T7 (for AT1 and AT2) polymerase and incubating for 2 hours at room temperature. 1 µl RQ1 RNase-free DNase was added, and the reaction was incubated for an addition 15 minutes at 37 °C to remove the DNA template. Unincorporated nucleotides were removed using G-50 sephadex column chromatography (Roche Molecular Biochemicals, Indianapolis. IN). 1 µl of the purified probe was placed into a scintillation vial to determine counts per minute. Sense strand RNA used for the standard was synthesized with linearized plasmid by in vitro transcription similar to the above, however [α-32P] UTP and 100 µM UTP were replaced with 25 mM UTP.

**RNase Protection Assay (RPA)**

Renin, AT1 and AT2 mRNA were quantified by RPA (RPA kit III; Ambion, Austin, TX). Briefly, 20 µg RNA samples from kidney cortex, left ventricle and lung were mixed with 10 µl hybridization buffer and 100 000 cpm of the renin, AT1 or AT2 probe. Samples were then heated at 95 °C for 4 minutes and placed in a 48 °C water bath for overnight hybridization. RNaseA/T1 (1:150 dilution in RNase digestion buffer) was then added to the samples to digest unhybridized probe and RNA. Digestion was stopped, and the hybridized RNA precipitated by adding RNase inactivation/precipitation buffer and incubating for 30 minutes at –20 °C. Hybridized RNA was pelleted by centrifugation at 14 000 × g for 15 minutes. Samples were then run on a 5 % polyacrylamide/8 M urea denaturing gel at 250 V for 1 hour. Gels were then exposed to film (Biomax-MR, Kodak. Cealex, France) with an intensifying screen. Known quantities of sense RNA were used to construct a standard curve for each assay. Hybridization signals from the protected fragments of the unknown were quantitated by comparing with the standard curve established from the signals of the sense standards. A standard curve was made for each assay and samples from both control and TX animals were analyzed in the same assay.

**Immunoblotting**
Western blot analysis for AT1 and AT2 was performed as previously described (51). Briefly, protein concentrations of samples harvested from HPD and control animals were determined using a modified Bradford method. A standard curve was produced using known concentrations of bovine albumin, and the protein concentrations of samples were determined by comparing their optical density (OD) 595 nm value with values from the standard curve. Both AT1 and AT2 receptor-specific polyclonal antibodies (Santa Cruz Biotechnology, CA) have been used to detect the ovine AT1 and AT2 receptors respectively (51). 40 µg of protein per lane were electrophoresed on a 12 % polyacrylamide gel containing sodium dodecyl sulphate for 1.5 hours and then blotted onto a polyvinylidene fluoride membrane (Immobilon, Millipore Corp., Marlborough, MA) by semidry electrobloting. The blot was blocked overnight at 4 °C with 6 % nonfat milk in 0.05 % Tween-20 Tris-buffered saline (TTBS) and then incubated with the primary antibody using 1:2000 (AT1) or 1:6000 (AT2) dilutions in 6 % dry milk/TTBS for 2 hours at room temperature. Blots were then rinsed, washed and incubated with a 1:4000 dilution of monkey anti-rabbit horseradish peroxidase-conjugated antibody in 6 % dry milk/ TTBS for 1 hour at room temperature. Binding of the secondary antibody was detected using a chemiluminescent system consisting of horseradish peroxidase-hydrogen peroxide oxidation of luminol (ECL plus, Amersham, Arlington Heights, IL, USA). Blots were then exposed to film for 5-10 minutes before densitometric analysis.

**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.

**Data analysis**

Renal renin mRNA, renal prorenin and active renin, fetal plasma renin concentrations, fetal plasma T3 and T4 concentrations, levels of AT1 and AT2 protein in TX and control animals were compared by unpaired Student’s t test. AT1 and AT2 mRNA expression in different tissues were compared using analysis of variance followed by Newman-Keuls multiple comparison procedure. Blood pressure and heart rate measures before the isoproterenol infusion, throughout the experiment and for 50 minutes after the infusion were compared by analysis of variance.
followed by Newman-Keuls multiple comparison procedure. All values are expressed as mean ± SEM, with p<0.05 considered significant.

RESULTS

Confirmation of TX and fetal health

The completeness of TX was confirmed by visual inspection at necropsy and by the measurement of plasma T4 and T3 levels. Plasma T4 levels were significantly lower in TX than control fetuses. Plasma T3 concentrations were below the level of assay detection (<0.004 ng/ml) in all TX fetuses, while plasma T3 levels were as expected and significantly higher in controls (Figure 1).

Fetal health, as assessed by arterial blood gas and pH measurements, was normal throughout the duration of the studies in both TX and control fetuses (Table 1).

Effect of TX on renal and plasma renin

Renal renin mRNA expression was significantly decreased by TX when compared to the control values (Figure 2). TX also significantly decreased the total renal renin (3661 ± 1009 vs. 2481 ± 402 ng.Angiotensin I / min.mg, p = 0.03) and renal active renin concentrations (p < 0.05), but had no effect on renal prorenin levels (Figure 3).

Just prior to necropsy basal concentrations of plasma active and prorenin were similar, while isoproterenol infusion increased plasma active renin concentrations equally in both groups (p < 0.05, Figure 4). Prorenin levels did not change (Figure 4).

Effect of TX on AII receptor subtype expression

AT1 and AT2 mRNA were expressed in kidney cortex, lung and left ventricle in TX and control fetuses. In all tissues, and in both groups, expression of AT1 mRNA was greater than AT2 (F = 9.14, p < 0.001 AT1 mRNA vs. AT2 mRNA). TX significantly decreased AT1 mRNA expression in kidney and lung (F = 4.29, p < 0.05), but did not affect left ventricular expression (Figure 5). TX was associated with increased AT2 mRNA levels in kidney only (Figure 6, F = 5.39, p<0.05).

AT1 and AT2 receptor proteins were detected in all three tissues, with bands of expected size (51). The major AT1 band, at 67kD, tended to decrease in kidney (p=0.06), and was significantly decreased in lung (p < 0.05) following TX (Figure 7). AT2 bands were detected at
44 and 78 kD, considered to be the primary bands expressed in fetal kidney and in adult adrenal respectively (51). TX led to increased AT2 44 kD band protein expression in the kidney (p < 0.05). While at the 78 kD band, TX was associated with decreased expression in kidney and lung (p < 0.05). Neither AT1 nor AT2 protein levels were altered in the heart following TX (Figure 8).

**Effect of TX on the cardiovascular system**

Fetal blood pressure and heart rate recordings are presented in figures 9 and 10. TX fetuses had significantly lower basal heart rates than controls (F = 268.99, p < 0.001), while isoproterenol infusion significantly increased heart rate in both groups (F = 44.43, p < 0.001) with similar magnitude (Figure 9). Basal MAPs were significantly lower in TX fetuses (F = 3528.71, p < 0.001). Isoproterenol infusion decreased MAP in TX fetuses (F = 29.44, p < 0.001) (Figure 10).

**DISCUSSION**

This study examined changes in RAS activity and cardiovascular function in the late gestation sheep fetus following TX. We found that renal renin mRNA, renal renin and active renin concentrations, AII receptor subtype expression in different tissues, as well as basal blood pressure and heart rate were altered as a consequence of TX. These findings indicate that thyroid hormone plays an important role in regulating maturation of components of the RAS and cardiovascular system in late gestation.

We and others have previously noted that renal renin mRNA and renin content increase significantly in the late gestation fetal sheep (9,10,45), and that this is a consequence of increased number and content of renin containing cells (63). In fetal sheep, the concentration of thyroid hormone also increases in late gestation, suggesting that this may be driving force behind these changes (19). The findings from our study support this assertion. We observed that fetuses lacking thyroid hormone (i.e. TX fetuses) exhibited decreased renal renin mRNA expression and renin content in late gestation. Several in vitro studies shed further light on exactly how thyroid hormone may be influencing renin gene expression. For instance, in transgenic mice carrying extra copies of the Ren2 gene, thyroid hormone has been demonstrated to increase renin mRNA accumulation by directly stimulating transcription and/or stabilizing precursor renin mRNA (29,59). Additional studies using Calu-6 cells have confirmed that thyroid hormone directly
stimulates the promoter region of the human renin gene through thyroid hormone response element-dependent mechanisms (36).

Contrary to expectations, TX had no effect on either basal or isoproterenol increased plasma renin concentrations. Renal renin is considered to be the main source of the circulating plasma renin, and it has been shown that concentrations also increase in later gestation (45). Plasma renin levels also increase significantly in response to isoproterenol infusion during this period (41,64). In adult rats, TX significantly decreases plasma renin activity (6,15,40), and the renin responses to isoproterenol (5). In our studies, although renal renin mRNA and content were decreased by TX, basal plasma renin concentrations were maintained, and isoproterenol infusion increased plasma active renin concentrations similarly in both control and TX fetuses. These findings suggest that there are species differences with respect to how renin secretion is regulated, or that factors other than thyroid hormone help maintain basal and isoproterenol stimulated renal renin secretion in thyroidectomized fetal sheep. One possibility to this end is increased sympatho-adrenal activation, as has been observed in hypothyroid human subjects (17). There is some evidence in fetal sheep suggesting that sympathetic tone is altered following TX, for example, fetal sheep thyroidectomized at 80 dGA, had significantly higher plasma noradrenaline concentrations compared to controls when assessed at 125-135 dGA (61). Alternatively, the approximate 30% decline in active renal renin concentration may not have been large enough to affect secretory response to an acute stimulation.

Prior investigation has confirmed that AII receptor subtype expression in the kidney is developmentally regulated. Renal AT1 mRNA expression is lower in early and higher in late gestation, while the reverse is true for AT2 mRNA (46,47). What regulates these developmental patterns of expression is not known. We found that following TX, renal AT1 receptor expression was decreased at both the mRNA and protein level. In adult human and animal models of hypothyroidism, renal function is also altered, resulting in decreased renal blood flow, glomerular filtration rate and sodium reabsorption (7,31,32,43), as well as impaired concentrating and diluting capacity (24,60). These outcomes are likely to be mediated in part by associated changes in AT1 receptor expression. Indeed, AT1 receptor blockade also exerts similar effects with reference to sodium and water reabsorption in adult rats (42). Similarly, in the late gestation fetal sheep, AII acts via the AT1 receptor to regulate salt and water excretion, hence maintaining fetal fluid sac volumes and ensuring normal growth and development (65),
and inhibition of the system leads to anuria, and oligohydramnios (1,49). Our findings imply that thyroid hormone may be an important mediator of this phenomenon, and indeed on AT1 mediated reabsorption in general.

Some work has also been undertaken examining the relative importance of the RAS and sympathetic nervous system in hyperthyroid-induced cardiac hypertrophy. To date, the results of these studies have been inconsistent (37,38), although there may be a role for both systems in this phenomenon (26).

Until recently, little has been known about the function of the AT2 receptor. In the kidney, it has been suggested that AT2 may play an important role in early gestation nephrogenesis (21). Here we noted that following TX, expression of AT2 mRNA as well as protein at the 44kD band was elevated, while protein was decreased at the 78 kD band. It has previously been found that the 44kD is the primary band in fetal kidney, while the 78 kD is the primary band in adult adrenal (51). The present pattern of expression suggests that TX may have resulted in delayed renal maturation, and that thyroid hormone per se has the capacity to influence AT2 expression in the fetus.

Thyroid hormone is also implicated in fetal lung development and maturation (3,27,33). In fetal sheep, TX results in decreased lung size (4), and functional immaturity at term (13). This is most likely due in part to the lack of thyroid hormone stimulation of lung ACE activity, which in turn usually facilitates RAS development in preparation for postnatal life (62). TX is associated with significantly decreased lung ACE concentrations in the fetal sheep (18). AII, through the AT1 receptor, is also believed to play an important role in fetal lung development and maturation. Strong evidence for this assertion comes from human and animal studies where fetal exposure to AT1 receptor blockers has been associated with lung hypoplasia (49) and decreased lung liquid flow (58). We found that TX led to decreased expression of AT1 mRNA and protein in the fetal sheep lung, thus indicating that the previously observed impaired lung development following TX (4,13) may also be related to retarded AT1 receptors, as well as ACE expression.

TX had no effect on the expression of pulmonary AT2 mRNA. Protein expression, however, was decreased at the 78 kD band. Again this may be indicative of delayed tissue maturation.
Although TX significantly altered AT1 and AT2 receptor expression in both kidney and lung, no changes were evident in fetal sheep heart. These differential tissue specific effects have also been observed following thyroid hormone treatment in different species. Adult rats treated with triiodothyronine (T3) exhibited decreased adrenal but increased heart, liver and kidney AII receptor densities (40). Dogs similarly exposed to T3 had increased AII receptor density in the left ventricle and liver, but no change in adrenal expression (52). The mechanisms underlying these tissue differences are not clear.

In a prior investigation (40) it was found that hypothyroid adult rats had increased cardiac AT2, but unchanged AT1 receptor density. These changes are contrary to what we observed in fetal sheep. Apart from the species difference, there are several plausible explanations for the different findings. Firstly, basal AII receptor subtype expression is different between adult and fetal animals. In rats, AT2 mRNA expression is higher in fetal tissues, while AT1 mRNA is higher in adults (23). Secondly the mechanisms that regulate AII receptor expression may be different in adult and fetus. This is apparent with respect to another RAS component, renal renin, the expression of which is decreased following renal denervation in the adult, but not the fetus (28). Finally, the maturational expression of thyroid hormone receptors might be different between tissues, for example, in the human fetus, thyroid hormone receptors are expressed earlier in the brain than in liver (16).

Our studies demonstrate that in the late gestation TX fetal sheep both basal blood pressure and heart rate are decreased. The hypothyroid induced hypotension may be due to decreased blood volume, peripheral vascular resistance, myocardial Na-K ATPase activity and myocardial contractility, and/or changing adrenergic receptor number and subtype (20,39,61). In a previous investigation, TX at 80 dGA was also associated with decreased MAP at 125-135 dGA (61). Our studies also demonstrate that MAP declined following the isoproterenol infusion in TX fetal sheep. This suggests that TX inhibits or decreases the inotropic effect of isoproterenol, and may be related to decreased cardiac contractile protein ATPase, reduced calcium uptake by cardiac sarcoplasmic reticulum, altered expression of vascular adrenergic receptors and/or failed augmentation of left ventricular dP/dt (an index of left ventricular contractility) in response to the isoproterenol challenge (14).

The mechanisms underlying the decreased basal heart measures in TX fetuses are not clear, although it appears that the timing of TX is an important determinant. In a previous study...
where TX was performed at 80 dGA there was no effect on heart rate at around 125-135 dGA (61). While in two other investigations, TX performed at approximately 130 dGA (8) or at term, following caesarian section delivery (5), was associated with decreased and normal postnatal heart rate measures respectively.

In contrast to blood pressure, isoproterenol infusion increased heart rate equally in both TX and control fetuses in late gestation. This suggests that the positive chronotrophic effects of adrenergic stimulation are somehow maintained in TX fetuses. Hypothyroid induced increased sympathetic and adrenal activity (17) may help maintain the normal positive chronotrophic responsiveness to adrenergic stimulation. Another possible contributing factor is the expression levels of the AII receptors, which mediate the positive chronotrophic effects of AII (30). As previously noted, we found that neither AT1 nor AT2 receptor mRNA or protein was altered in the left ventricle as a consequence of TX in fetal sheep.

In summary, we found that in the late gestation fetal sheep renal renin mRNA, and renal total renin and active renin concentrations were decreased as a consequence of TX, while expression of the AII receptor subtypes mRNA and protein were altered in a tissue specific manner. Basal blood pressure and heart rate measures were decreased following TX, and MAP declined following isoproterenol infusion. These findings demonstrate that thyroid hormone plays an important role in mediating some of the developmental changes in the RAS and in cardiovascular function in the late gestation sheep fetus.

ACKNOWLEDGEMENTS

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REFERENCES


Table 1:

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Figure 1:

**Plasma T3**

- y-axis: Plasma T3 (ng/ml)
- x-axis: Control, TX
- Data points:
  - Control: *

**Plasma T4**

- y-axis: Plasma T4 (ng/ml)
- x-axis: Control, TX
- Data points:
  - Control: *
  - TX: *
Figure 2

Renin mRNA (pg/20ng total RNA)

Control

TX

*
Figure 3

Renal Active Renin

- Active renin (ng Angiotensin I/min/mg)

Renal Prorenin

- ProRenin (ng Angiotensin I/min/mg)

Control vs TX
Figure 4

**Plasma Active Renin**

- **Control TX**
- **Basal**
- **Isoproterenol**

**Plasma ProRenin**

- **Control**
- **TX**
Figure 5

AT1 mRNA Standard Curve

AT1 mRNA expression

Control

TX

Kidney

Lung

Left Ventricle

AT1 mRNA expression (pg/10 ug total RNA)
Figure 6

AT2 mRNA Standard Curve

AT2 mRNA expression

OD

0.0
0.1
0.2
0.3
0.4
0.5

0 2 4 6 8 10 12

Sense RNA (pg/µl)

10 pg/µl 5 pg/µl 2.5 pg/µl 1.25 pg/µl

Kidney Lung Left ventricle

* $ *

TX Control

AT2 mRNA (pg/10 µg total RNA)

0 1 2 3 4 5

Kidney Lung Left ventricle
Figure 7

**AT1 Protein Expression**

**Kidney**

![Kidney OD value graph]

**Lung**

![Lung OD value graph]

**Left ventricle**

![Left ventricle OD value graph]

**Control** vs **TX**
Figure 8

AT2 Protein Expression

- **Kidney-44kD**
  - Control vs. TX: OD values
  - TX shows a higher OD value compared to Control.

- **Kidney-78kD**
  - Control vs. TX: OD values
  - TX shows a higher OD value compared to Control.

- **Lung-44kD**
  - Control vs. TX: OD values
  - TX shows a higher OD value compared to Control.

- **Lung-78kD**
  - Control vs. TX: OD values
  - TX shows a higher OD value compared to Control.

- **Left Ventricle-44kD**
  - Control vs. TX: OD values
  - TX shows a higher OD value compared to Control.

- **Left Ventricle-78kD**
  - Control vs. TX: OD values
  - TX shows a higher OD value compared to Control.
Figure 9

![Graph showing HR (beats/min) over time for Control and TX groups.](image)

Figure 10

![Graph showing BP (mmHg) over time for Control and TX groups.](image)
LEGEND

Table 1: Arterial Blood Gases in TX and control fetal sheep during development after surgery.

Figure 1: Plasma T3 and T4 levels in control and TX fetuses just before necropsy (137-139 dGA). (*p < 0.001 control vs. TX) n=6 for both groups.

Figure 2: Renal renin mRNA expression in 137-139 dGA control and TX fetuses. (*p < 0.05 control vs TX). n=6 for both groups.

Figure 3: Renal active and prorenin levels in 137-139 dGA control and TX fetuses. (*p < 0.05 control vs. TX). n=6 for both groups

Figure 4: Plasma active and prorenin concentrations at baseline and following isoproterenol infusion in 137-139 dGA control and TX fetuses. (*p < 0.05.) n=6 for both groups.

Figure 5: Standard curve and Angiotensin II receptor subtype I (AT1) mRNA expression in kidney, lung and left ventricle from 137-139 dGA control and TX fetuses. (*p < 0.05 kidney and lung vs. left ventricle in control fetuses. $p < 0.05 control vs. TX in kidney and lung). n=6 for both groups.

Figure 6: Standard curve and Angiotensin II receptor subtype II (AT2) mRNA expression in kidney, lung and left ventricle from 137-139 dGA control and TX fetuses. (*p < 0.05 lung and left ventricle vs. kidney in control fetuses. $p < 0.05 control vs. TX in kidney). n=6 for both groups.

Figure 7: AT1 protein levels in kidney, lung and left ventricle from 137-139 dGA fetuses. (*p=0.02 control vs. TX in lung). n=6 for both groups.

Figure 8: AT2 protein levels at 44kD and 78 kD in kidney, lung and left ventricle from 137-139 dGA control and TX fetuses. (*p<0.05 control vs. TX ). n=6 for both groups.
**Figure 9:** Heart rate (HR) before, during (10th to 20th minutes) and after isoproterenol infusion in 137-139 dGA control and TX fetuses. Basal HR was lower in TX fetuses ($F=268.99$, $p<0.001$ vs. Control). Isoproterenol infusion significantly increased the heart rate in both control and TX fetuses ($F=44.43$, $p<0.001$ vs. basal HR). $n=6$ for both groups.

**Figure 10:** Mean arterial pressure (MAP) before, during (10th to 20th minutes) and after isoproterenol infusion in 137-139 dGA control and TX fetuses. MAP was lower in the TX animals ($F=3528.71$, $p<0.001$ vs. Control) and declined with isoproterenol infusion ($F=29.44$, $p<0.001$ vs. basal MAP). Isoproterenol infusion had no effect on MAP in the control fetuses. $n=6$ for both groups.