Thyroid Hormone Coordinates Respiratory Control Maturation and Adenine Nucleotide Translocator Expression in Heart In Vivo

Michael A. Portman, MD; Yun Xiao, MD; Kun Qian, MD; Russell L. Tucker, DVM; Steven M. Parish, DVM; Xue-Han Ning, MD

Background—The signal transduction mechanism linking mitochondrial ATP synthesis with cytosolic ATP utilization in heart changes during postnatal development in vivo. This maturational process occurs in parallel with accumulation of mitochondrial adenine nucleotide translocator (ANT), which provides a possible site for respiratory control. We postulated that thyroid hormone regulates these maturational processes.

Methods and Results—We used 31P MR spectroscopy to determine the relationship between myocardial high-energy phosphates, phosphocreatine, and ADP and oxygen consumption (MV02) during epinephrine stimulation in 32- to 40-day-old lambs thyroidectomized after birth (THY) and age-matched controls. Steady-state protein and mRNA levels for ANT isofoms and β-F1-ATPase were assessed from left ventricular tissues by Western and Northern blotting. With greater doses of epinephrine, THY attained lower peak MV02 than controls (P<0.05). Controls maintained high-energy phosphate levels, unlike THY, which demonstrated significantly decreased phosphocreatine/ATP and increased cytosolic ADP despite lower peak MV02. No significant differences in β-F1-ATPase protein or mRNA occurred between groups. However, ANT isoform mRNA levels were 2-fold greater and protein levels 4-fold greater in control hearts.

Conclusions—These data imply that the maturational shift away from ADP-mediated respiratory control is regulated by thyroid hormone in vivo. Specific thyroid-modulated increases in ANT mRNA and protein imply that this regulation occurs in part at a pretranslational level. (Circulation. 2000;102:1323-1329.)

Key Words: mitochondria ■ magnetic resonance imaging ■ metabolism

Efficient coupling between cytosolic ATP utilization and mitochondrial ATP production occurs in myocardium in vivo.1-3 The modes of signal transduction that operate between these processes are developmentally regulated.4,5 Maturational changes in transduction efficiency and mode have been demonstrated in sheep heart in vivo with 31P NMR spectroscopy1,4,5 and in isolated rat heart mitochondria with control flux experiments.6 Although cytosolic ADP concentrations are maintained during elevated oxidative phosphorylation rates in mature myocardium in vivo, the newborn myocardium demonstrates increases in ADP during similar changes in oxygen consumption. Kinetic studies demonstrate that regulation in the immature heart conforms to simple Michaelis-Menten models of respiratory control through ADP via the adenine nucleotide translocator (ANT).5 Results from these kinetic studies in vivo corroborate findings in isolated rat heart mitochondria6 that demonstrate that control flux through ANT decreases with age. Changes in the respiratory regulation pattern and control through ANT parallel mitochondrial accumulations of this mitochondrial protein during cardiac maturation.5 Furthermore, protein expression for ANT coordinates with mRNA expression of ANTβ, the predominant heart skeletal isoform.7 This coordination implies that ANT regulation occurs at least in part at a pretranslational level. Regulators of these developmental processes in vivo have not been clearly defined. Thyroid hormones promote mitochondrial membrane expansion through transcriptional activation of specific nuclear-encoded genes, including ANT isoforms.8-10 Furthermore, a postnatal thyroid hormone surge has been well documented in the lamb.11 Thus, thyroid hormone is a likely candidate for regulation of respiratory control maturation in the postnatal period. This study tests the hypothesis that neonatal thyroid hormone deficiency delays ANT accumulation and mitochondrial respiratory control maturation.

Methods

Thyroidectomy

Total thyroidectomy was performed in sheep 1 to 24 hours old through a subcricoid incision under anesthesia.11 Triiodothyronine...
(T₃) and thyroxine (T₄) levels were performed before thyroidectomy and after 24 hours, 7 days, and 28 days to confirm the effectiveness of the procedure.

**Surgical Preparation**

At 32 to 40 days, thyroidectomized lambs (n=9) and controls of comparable age (n=8) were sedated (intramuscular ketamine and xylazine), intubated, and ventilated with room air and oxygen, followed by intravenous α-chloralose (40 mg/kg). A femoral arterial cannula was placed for monitoring systemic blood pressure and sampling blood. After median sternotomy, pacing electrodes were sutured to the right atrial appendage. Coronary sinus flow was measured via an extracorporeal shunt fashioned between the coronary sinus and the superior vena cava as previously described. Hemizygous vein ligation directed coronary venous flow into the shunt located within the proximal coronary sinus. A cannulating ultrasonic transit time probe provided continuous flow data. A 2-cm-diameter round NMR surface coil was sutured to the pericardium overlying the left ventricle. The lamb, wrapped in a heating blanket to maintain body core temperature at ~38°C, was placed inside a Lucite cradle and transferred into a spectrometer 26-cm clear bore.

**NMR Measurements**

The NMR surface coil was tuned to 81 MHz and matched to 50 Ω. NMR data were collected with a spectrometer operating at 4.7 T and using resident software. Shimming on the ¹H free induction decay at 200 MHz and acquisition of ³¹P spectra were done with cardiac gating. The interpulse delay was ~2 seconds, and the pulse width was optimized for the phosphocreatine (PCr) signal. Spectra were acquired with a simple 1-pulse sequence, 5000-Hz sweep width, and 2048 data points. Thirty-two spectra were collected in data acquisition blocks. Spectra were analyzed with a least-squares fitting program and integration. Intracellular pH was determined from the chemical shift difference of inorganic phosphate (Pi)-PCr. The NMR surface coil was tuned to 81 MHz and matched to 50 Ω. NMR data were collected with a spectrometer operating at 4.7 T and using resident software. Shimming on the ¹H free induction decay at 200 MHz and acquisition of ³¹P spectra were done with cardiac gating. The interpulse delay was ~2 seconds, and the pulse width was optimized for the phosphocreatine (PCr) signal. Spectra were acquired with a simple 1-pulse sequence, 5000-Hz sweep width, and 2048 data points. Thirty-two spectra were collected in data acquisition blocks. Spectra were analyzed with a least-squares fitting program and integration. Intracellular pH was determined from the chemical shift difference of inorganic phosphate (Pi)-PCr.

**Protocol**

After completion of an 8-minute baseline acquisition period in control sheep, intravenous epinephrine infusion was initiated at 1 μg·kg⁻¹·min⁻¹ and slowly increased over 8 minutes to 8 μg·kg⁻¹·min⁻¹. The epinephrine dose was titrated to maintain a steady coronary sinus flow rate for 8 minutes. After this stage, epinephrine was slowly decreased until near-baseline coronary flow and blood pressure were acquired, and 8 minutes' worth of data was then collected as recovery. Preliminary experiments indicated that larger doses of epinephrine were necessary to stimulate oxygen consumption in thyroidectomized lambs. The protocol in thyroidectomized lambs was identical to the control, except that doses to 30 μg·kg⁻¹·min⁻¹ epinephrine were infused. Arterial and coronary venous blood was sampled during the steady-state coronary flow periods at baseline, under epinephrine infusion, and at recovery.

Oxygen content was determined by use of data obtained from an hemoximeter. MVO₂ was calculated from the coronary arteriovenous difference times coronary sinus flow rate. At completion of the experiment, the heart was rapidly excised, and left ventricular tissue was removed for Western blotting or snap-frozen in liquid nitrogen and stored at ~80°C for Northern blotting and ATP and creatine analysis.

**Metabolite Analysis**

Tissue ATP and creatine concentrations were determined by perchloric acid extraction and chemical analyses with high-performance liquid chromatography as previously described. These values, along with PCr/ATP obtained from recovery MR spectra, were used to calculate free cytosolic ADP from the creatine kinase equilibrium reaction equation.

**Northern Blot Analysis**

RNA isolation was performed by previously described methods. RNA (15 μg) was denatured and electrophoresed into a 1% form-
recovery. Baseline and peak heart rates are significantly lower in thyroidectomized sheep. No significant difference was found during recovery. The dynamic heart rate range in these experiments is greater in the control sheep, possibly reflecting increased $\beta_2$-adrenergic receptor sensitivity. Although baseline mean arterial pressure is higher in the control group, differences subside at peak and recovery. Coronary blood flow increases substantially in both groups during epinephrine infusion but is greater in control sheep at baseline and peak epinephrine stimulation.

$\text{MV}_2$ is slightly higher in control sheep at baseline and increases to substantially higher levels at peak than attained in the thyroidectomized sheep. These data indicate that greater $\text{MV}_2$ elevations in the control group relate to the greater peak heart rates. The marked differences in peak oxygen consumption between groups cannot be explained entirely by coronary flow discrepancies. Diminished arterio-venous extraction in thyroidectomized sheep also contributes to differences in oxygen consumption. Hemoglobin and arterial saturation values were similar between the 2 groups, indicating that differences in oxygen content and carrying capacity were not responsible for this oxygen extraction limitation.

### Response of Hemodynamic Indices to Epinephrine

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Peak</th>
<th>Recovery</th>
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</thead>
<tbody>
<tr>
<td>Arterial mean pressure, mm Hg</td>
<td>64±3</td>
<td>103±7†</td>
<td>58±4</td>
</tr>
<tr>
<td>Control (n=8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THY (n=9)</td>
<td>48±2*</td>
<td>90±6†</td>
<td>44±2</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>145±7</td>
<td>231±13†</td>
<td>178±14</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THY</td>
<td>124±6*</td>
<td>179±5†</td>
<td>156±9</td>
</tr>
<tr>
<td>Coronary flow, mL • min$^{-1}$ • g$^{-1}$</td>
<td>0.50±0.04</td>
<td>2.01±0.15</td>
<td>0.56±0.07</td>
</tr>
<tr>
<td>Control</td>
<td>0.32±0.03*</td>
<td>1.46±0.21†</td>
<td>0.59±0.16</td>
</tr>
<tr>
<td>THY</td>
<td></td>
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</tr>
<tr>
<td>$\text{MV}_2$, $\mu$mol • min$^{-1}$ • g$^{-1}$</td>
<td>1.85±0.14</td>
<td>8.52±0.93</td>
<td>2.05±0.37</td>
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<tr>
<td>Control</td>
<td>1.08±0.21*</td>
<td>4.96±0.68†</td>
<td>1.74±0.59</td>
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<td>THY</td>
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</tbody>
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*P<0.05 vs Control.
†P<0.05 vs Baseline.

### High-Energy Phosphates

The increase in PCr during elevation in $\text{MV}_2$ is apparent in spectra obtained from an individual experiment in a thyroidectomized lamb (Figure 2). No difference in saturation-corrected PCr/ATP between the groups occurs at baseline or recovery (Figure 3). However, PCr/ATP decreases significantly at peak in the thyroidectomized sheep only. ATP does not change significantly during epinephrine infusions in either group. Thus, changes in PCr/ATP that occur in thyroidectomized sheep are due to decreases in PCr. Recovery values for PCr/ATP are similar to baseline for both groups. There were no significant differences in tissue ATP or creatine levels between groups. No significant differences in free cytosolic ADP calculated through the creatine kinase reaction occur at baseline (Figure 3). However, ADP rises significantly during increased $\text{MV}_2$ in the thyroidectomized group but exhibits no change in the control group. ADP at peak $\text{MV}_2$ is also greater in thyroidectomized sheep.

### Intracellular pH

Although there is a trend toward lower myocardial intracellular pH (pHi) in thyroidectomized sheep (Figure 4), comparisons reveal no statistical differences between the groups. Furthermore, no significant changes in pHi occur through the experiments in either group.

### Steady-State mRNA Levels

Semi quantitative analyses of steady-state mRNA levels for ANT isoforms and $\beta$-F$_{1}$-ATPase were performed for control (n=5) and thyroidectomized (n=5) sheep hearts. The repre-
sentative Northern blot (Figure 5) demonstrates the 28S ribosomal RNA band, as well as bands for the 3 ANT isoforms and β-F1-ATPase obtained from successive probergs. Relative intensities normalized to 28S intensity are shown in Figure 6. Steady-state β-F1-ATPase mRNA levels for control and thyroidectomized sheep hearts are similar. In contrast, control heart ANT1, ANT2, and ANT3 mRNA levels are each >2-fold greater than levels in hearts from thyroidectomized sheep.

Protein Levels
ANT and β-F1-ATPase protein levels were assessed semi-quantitatively by Western blotting. A representative Western blot is shown in Figure 7, and relative normalized densitometric intensities are shown in Figure 8. Like mRNA levels, protein levels for β-F1-ATPase in heart are not perturbed by thyroidectomy. In contrast, and coordinately with steady-state mRNA levels, cardiac ANT protein content in control sheep hearts is >4-fold greater than in thyroidectomized sheep.

ANT Exchange Efficiency
Respiratory control indices were obtained by standard techniques to confirm mitochondrial viability. Respiratory control index values were >6.0 in all heart mitochondrial suspensions. There was no significant difference in percent exchange between the 2 groups: control, 77±3% and thyroidectomized, 67±5%.

Discussion
Developmental changes in cardiac mitochondrial ANT accumulation and respiratory regulation have not previously been linked to thyroid hormone. Studies performed with isolated liver mitochondria and using control flux analysis provide limited data, which contribute to formulation of a hypothesis invoking thyroid modulation of respiratory control maturation. Mitochondria isolated from hypothyroid liver demonstrate deficits in adenine nucleotide transport and alterations in respiratory control through ANT. These deficits cannot be rapidly reversed by T3 administration. This implies that translocator deficits occur through long-term mechanisms that limit ANT protein synthesis and not through immediate T3 action at the mitochondrial membrane.

The present experiments were thus designed to examine the role of thyroid in altering respiratory control patterns in heart in vivo and promoting ANT accumulation during a previously documented period of developmental transition. The newborn lamb provides such a model for investigation, considering that Breall et al previously documented the normal ovine perinatal thyroid hormone surge and the diminished contractile response to isoproterenol in thyroidectomized lambs. Furthermore, the change in the relationship between high-energy phosphates and oxidative phosphorylation rate that occurs during maturation in this model has been well established, as has the maturational accumulation of ANT.

Data from this study support the hypothesis that respiratory control maturation in this neonatal model is thyroid hormone–dependent. The coordinate increases in myocardial free cytosolic ADP and oxidative phosphorylation rate indicate that an immature respiratory control pattern persists in thyroidectomized sheep. This deviation from the normal mature relationship occurs at submaximal oxidative phosphorylation levels, because catecholamine stimulation in an open-chest anesthetized animal does not achieve the maximal oxygen consumption rates seen in exercising animals of the same species. Thus, mitochondrial or extramitochondrial factors, such as myosin ATPase isoform switching or reductions, which might limit maximal oxygen consumption rates in the thyroidectomized sheep, should not confound interpretation of these relationships.

Timing of thyroidectomy might have diminished the magnitude of the high-energy phosphate changes observed in these experiments. Thyroid hormone (T3 and T4) levels normally surge immediately after birth in lambs. Thus, the present model using thyroidectomy within the first 24 hours may not prevent the initial postnatal rise in these levels. Conceivably, differences in cardiac mitochondrial respiration that were apparent between control and thyroidectomized lambs would be greater if thyroidectomy had been performed before birth. Nevertheless, we were still able to establish that thyroidectomy caused substantial changes in myocardial respiratory patterns and ANT accumulation.

Maturation of respiratory control has previously been linked to mitochondrial accumulation of ANT. Thus, we
pursued ANT accumulation as one plausible explanation for the respiratory pattern changes observed after thyroidectomy. The present Western and Northern blot analyses indicate that reductions in ANT protein and mRNA expression parallel thyroidectomy-induced changes in mitochondrial respiratory control mode. Summation of the metabolic and kinetic data together with protein and gene expression data from both previous and current experiments further supports the contention that alterations in respiratory control occur through ANT accumulation. The lack of change in \( \beta\)-F1-ATPase protein demonstrates that the thyroid-dependent increase in ANT is not part of a ubiquitous mitochondrial membrane protein response. Thyroid does promote other specific mitochondrial membrane proteins, such as cytochrome \( c \). Deficiencies of those components, however, would be more likely to limit the maximal inherent oxidative capacity than to alter the respiratory control pattern at submaximal oxygen consumption rates. Although \( \beta\)-F1-ATPase content is not altered by thyroidectomy, Scholz and Balaban demonstrated that the activity of this protein, as measured in subendocardial biopsy samples, does increase during phenylephrine-induced elevations in \( \text{MV}_2 \) in dogs in vivo. Conclusions relating these changes to F1-ATPase regulation of respiratory control remain speculative. Nevertheless, because thyroid hormone deficiency might alter calcium-induced activation of this or other mitochondrial proteins, such a process cannot be excluded as a contributor to the observed changes in respiratory regulatory pattern.

In addition to ANT protein content, conformation or function of individual ANT sites might be thyroid-dependent. Such dependency could offer an adjunctive or alternative explanation for persistence of the newborn-type respiratory control pattern in thyroidectomized sheep. Differences in relative isoform distribution might cause ANT functional discrepancies between our experimental groups. Dummler et al demonstrated \( T_3 \)-induced upregulation of the tissue ubiquitous isoform, ANT2, in normal rat hearts. The importance of this isoform in heart is unclear, because ANT1 greatly predominates in most species. The data in sheep heart indicate that ANT2 and ANT3 mRNA levels are also present and thyroid-dependent. However, reductions in transcript levels for these isoforms match ANT1 changes after thyroid-
Assessments of ANT exchange in isolated mitochondria do not necessarily reflect overall ANT function under conditions in vivo. Exchange values reflect the percentage of $[^{14}C]$ADP loaded into the mitochondria that was transported back to the extramitochondrial milieu during a specific time period. Because the available number of ANT sites can influence $[^{14}C]$ADP loading, percent exchange is not synonymous with traditional definitions of enzyme activity. However, the high percentage exchange values indicate that almost the entire $[^{14}C]$ADP was transported back across the mitochondrial membrane within a short time interval after loading. Individual exchanger sites are then highly and similarly efficient in both sheep groups. These data imply that although thyroid deficiency decreases the number of exchange sites, functional efficiency at an individual translocator site is not altered by assessments made from isolated mitochondria. Thyroid deficiency might cause functional limitations at ANT sites in vivo that are not detectable by studies in vitro and contribute to the observed changes in high-energy phosphate.

The influence of thyroid hormone on the mitochondria is not restricted to ANT. Conceivably, thyroid hormone action on enzymes, such as pyruvate dehydrogenase, or at peripheral tissues could alter reducing equivalent supply and cause the observed differences in relationships between ADP and oxidative phosphorylation rate.22 Studies performed with restricted forms of substrate supply in perfused heart in vitro suggest that cytosolic ADP levels and phosphorylation potential as well as NADH redox potential depend on the substrate available to the heart.22 Such substrate limitations do not normally occur under in vivo conditions. However, myocardial PCr/ATP in vivo can be altered under severe conditions of ketosis or pharmacological inhibition of fatty acid uptake also did not differ between the 2 groups during baseline or peak epinephrine conditions. These similarities in uptake support the contention that no substantial differences in substrate or reducing equivalent supply occur between the 2 groups during epinephrine infusion. Undetected changes in reducing equivalent supply or utilization in thyroidectomized sheep heart might occur and produce subtle changes in PCr/ATP and ADP during epinephrine infusion. However, these should not cause the magnitude of high-energy phosphate response observed in this group.

Thyroid regulation of gene expression for the nuclear-encoded mitochondrial proteins ANT and $\beta$-F$_1$-ATPase in heart has not previously been described in detail. Transcript levels for these genes are coordinately expressed during development in both sheep and rabbit myocardium.5,12 Chung et al9 reported that T$_4$ coordinately regulates expression of these genes in myoblast cell lines through a shared muscle-specific promoter element. However, the present findings indicate that these shared sites are not regulated by thyroid in heart. Instead, thyroid appears to specifically regulate ANT transcript levels, which closely coordinate with the protein levels. This coordination provides a potential pathway for nuclear-mediated regulation of myocardial respiratory control maturation.

Conclusions
These studies demonstrate that thyroid regulates postnatal maturation of myocardial respiratory control. Thyroidectomy-induced delays in maturation of respiratory control and/or efficiency of ADP phosphorylation occur in conjunction with decreased accumulation of mitochondrial ANT. The coordination between these processes conforms to kinetic modeling of respiratory control through ANT and strongly suggests that these phenomena are causally linked. However, considering the complexities involved in thyroid regulation, this hypothesis cannot be proved without a doubt in an in vivo model. As discussed previously, thyroid regulates other mitochondrial proteins and processes involved in respiration, including membrane composition.23 These remain considerations for future research.

These results noted in vivo present possible clinical implications for the newborn who undergoes artificial disruption of thyroid homeostasis during such procedures as mechanocirculatory support.26,27 Conceivably, delays in thyroid-induced maturation cause decreases in mitochondrial efficiency and regulation, as well as limitations in ATP synthesis and utilization capacities.

Acknowledgment
This work was supported by NIH grant 1R01-HL-60666.

References


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Circulation. 2000;102:1323-1329
doi: 10.1161/01.CIR.102.11.1323

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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