Regulation of Mammary Gland Sensitivity to Thyroid Hormones During the Transition from Pregnancy to Lactation

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Thyroid hormones are galactopoietic and help to establish the mammary gland’s metabolic priority during lactation. Expression patterns for genes that can alter tissue sensitivity to thyroid hormones and thyroid hormone activity were evaluated in the mammary gland and liver of cows at 53, 35, 20, and 7 days before expected parturition, and 14 and 90 days into the subsequent lactation. Transcript abundance for the three isoforms of iodothyronine deiodinase, type I (DIO1), type II (DIO2) and type III (DIO3), thyroid hormone receptors alpha1 (TRα1), alpha2 (TRα2) and beta1 (TRβ1), and retinoic acid receptors alpha (RXRα) and gamma (RXRγ), which act as coregulators of thyroid hormone receptor action, were evaluated by quantitative RT-PCR. The DIO3 is a 5-deiodinase that produces inactive iodothyronine metabolites, whereas DIO1 and DIO2 generate the active thyroid hormone, triiodothyronine, from the relatively inactive precursor, thyroxine. Low copy numbers of DIO3 transcripts were present in mammary gland and liver. DIO2 was the predominant isoform expressed in mammary gland and DIO1 was the predominant isoform expressed in liver. Quantity of DIO1 mRNA in liver tissues did not differ with physiological state, but tended to be lowest during lactation. Quantity of DIO2 mRNA in mammary gland increased during lactation (P < 0.05), with copy numbers at 90 days of lactation 6-fold greater than at 35 and 20 days prepartum. When ratios of DIO2/DIO3 mRNA were evaluated, the increase was more pronounced (>100-fold). Quantity of TRβ1 mRNA in mammary gland increased with onset of lactation, whereas TRα1 and TRα2 transcripts did not vary with physiological state. Conversely, quantity of RXRγ mRNA decreased during late gestation to low levels during early lactation. Data suggest that increased expression of mammary TRβ1 and DIO2, and decreased RXRγ, provide a mechanism to increase thyroid hormone activity within the mammary gland during lactation.


Key words: iodothyronine deiodinase; thyroid hormone receptor; thyroid hormone; lactogenesis; retinoid receptor

Introduction

Thyroid hormones are important regulators of mammalian development, cellular differentiation and metabolism (1). In vivo studies as early as 1934 demonstrated that thyroid hormone administration can increase milk production in dairy cows and in vitro studies have shown that 3,3′,5-triiodothyronine (T3) potentiates the activity of other lactogenic and galactopoietic hormones (2, 3). Tissue sensitivity to thyroid hormones can be altered by iodothyronine deiodination and changes in expression of nuclear receptors for thyroid hormones.

The primary hormone secreted by the thyroid, thyroxine (T4), has little intrinsic biological activity due to low affinity for nuclear thyroid hormone receptors (4). Mono-deiodination of the outer ring (5′-deiodination) of T4 yields a thyroid hormone with much greater biological activity, T3; whereas deiodination of the inner ring (5-deiodination) converts T4 and T3 to the biologically inert compounds, 3,3′,5′-triiodothyronine (reverse T3, rT3) and 3,3′-diodothyronine (3,3′-T2), respectively. Therefore, biological activity of thyroid hormones may be enhanced by 5′-deiodination or diminished by 5-deiodination. Three deiodinases regulate local and systemic activity of thyroid hormones. The 5′-deiodination is catalyzed by type-I (DIO1) and type-II (DIO2) deiodinases present in many tissues. DIO1 is expressed predominantly in thyroid, liver and kidney, and DIO2 in pituitary, brain, brown adipose tissue and placenta (5, 6). Depending upon species, DIO1 or DIO2 may be expressed in mammary gland, with DIO2 being the isoform expressed in bovine mammary tissue (7–9). The inner ring deiodinase (5-deiodinase), DIO3, is present in placenta, uterus and fetus and appears to protect...
developing embryonic and neonatal tissues from excessive
T3 levels (5), where its presence is critical for the maturation and
function of the thyroid axis (10). High levels of DIO3
mRNA expression have also been reported for bovine
mammary gland (11).

During the transition from pregnancy to lactation,
adjustments in metabolism of thyroid hormones appear
important in establishing metabolic priority for lactation. It
was demonstrated in rats (7, 9, 12) and cows (13) that 5'-
deiodinase activity in liver decreased during the transition
from pregnancy to lactation, while 5'-deiodinase activity in
mammary tissue increased. Moreover, the magnitude of
these changes in rats was proportional to lactation intensity,
which was manipulated by adjusting litter size (7, 9). Thus,
changes in the extent of T4 to T3 conversion by liver and
mammary gland appear to be related to establishment of
lactation. Additionally, thyroid ablation and hormone
replacement in mice showed that thyroid hormones are
necessary for a galactopoietic response to prolactin and
replacement in mice showed that thyroid hormones are
necessary for a galactopoietic response to prolactin and
mammary gland (11).

Thyroid hormone receptors (TRs) are members of the
superfamily of nuclear receptors (15, 16). In the absence of
ligand, these receptors bind to thyroid response elements
(TREs) and repress expression of target genes by virtue of
interaction with co-repressors (17). There are two TR genes,
THRA and THRβ, each of which produces two mature
transcripts. TRα1 and TRα2 (c-erbAα-2) transcripts and
proteins are derived as alternative splice variants of THRA.
Although TRα2 binds to TREs, it is unable to bind ligand
and therefore represses transcription. TRβ1 and TRβ2 are
transcript/protein variants of THRβ generated by use of
alternative promoters. The TRs bind to TREs as homo-
dimers, or as heterodimers with 9-cis retinoic acid receptors
(RXRs). TR/RXR heterodimers likely play an important
role in mediating response to thyroid hormones; for
although TRs bind to TREs as either homo or heterodimers
in absence of bound ligand, the ligand-bound TRs bind to
TREs primarily as heterodimers with RXR (16).

Neither expression of DIO3 nor thyroid hormone
receptors has been evaluated in liver and mammary gland
during the transition from pregnancy to lactation. To more
fully evaluate changes in expression of genes in liver and
mammary gland that are likely associated with altering
tissue sensitivity to thyroid hormones during lactogenesis
and establishment of lactation, we evaluated expression of
the iodothyronine-deiodinases, nuclear thyroid hormone
receptors and retinoic acid receptors in liver and mammary
gland of cows during the period of transition from
pregnancy to lactation.

Material and Methods

Experimental Design. Tissue samples were ob-
tained from the mammary gland and liver of 24 euthanized
multiparous Holstein cows. Tissues were obtained from 4
nonpregnant, lactating cows on day 14 and 90 of lactation
and from 3 pregnant cows at 7, 25, 40, and 53 days after
cessation of milking. Because milking was terminated 60
days before expected parturition, these sampling times
were equated to approximately 53, 35, 20, and 7 days before
expected parturition. Tissues were snap frozen in liquid
nitrogen for subsequent RNA isolation and real-time
quantitative RT-PCR. Mammary tissues were also fixed in
10% neutral buffered formalin and processed for paraffin
embedding, sectioning and immunohistochemical analyses
as described subsequently. Use of animals for these
investigations was approved by the Beltsville Agricultural
Research Center’s Animal Care and Use Committee.

RNA Preparation and Real-Time Quantitative
RT-PCR. Total RNA was isolated using RNeasy isolation
kits with on-column DNase digestion (Qiagen Inc.,
Valencia, CA). The RNA quality was evaluated using the
Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip
kits (Agilent Technologies, Palo Alto, CA) and concen-
tration was determined using a NanoDrop ND-1000
spectrophotometer (NanoDrop Technologies, Rockland,
DE). Reverse transcription was performed using the iScript
cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA)
with 1 μg of RNA per 40-μl reaction volume. A parallel
reaction was performed in the absence of reverse tran-
scriptase enzyme to serve as a negative control. Incubation
conditions were those suggested by the manufacturer: 25°C
for 5 min, 42°C for 30 min, 85°C for 5 min.

Transcript abundance was determined by absolute real-
time quantitative RT-PCR using the Bio-Rad iCycler with a
MyiQ Real-time PCR Detection System, and using iQ
SYBR Green Supermix (Bio-Rad Laboratories). Table 1
summarizes primer sequences and annealing temperatures
used for quantitative real-time PCR and the amplicon size of
each gene target. Identity of amplification products was
confirmed by direct sequencing of gel-purified PCR-
amplification products (QIAGen, Valencia, CA) using a CEQ8000 automated
DNA sequencer and DTCS Quickstart Chemistry (Beckman
Coulter, Fullerton, CA). Amplicon concentrations were
determined using the Agilent 2100 Bioanalyzer and DNA
500 kits (Agilent Technologies). Cycling conditions used
for real-time PCR were 95°C for 3 min followed by 45
cycles of 94°C for 15 s, annealing temperature for 30 s, and
72°C for 30 s with fluorescence measurement during the
extension step. Melting curve analysis was also performed
after PCR amplification using the MyiQ Real-time PCR
Detection System. External calibration curves were gener-
ated for each gene using known quantities of purified
double-stranded cDNA containing the amplification region
of interest and included with each assay. Standards ranged
from 1 × 10^2 to 1 × 10^6 molecules. Standards and samples
were analyzed in duplicate for each assay. In addition to
sample controls (reverse transcription reaction without
reverse transcriptase), a negative control reaction (blank)
containing water as template was included for each standard curve. Quantities of transcripts were expressed as the number of molecules per unit of total RNA used in the reverse transcription reaction.

**Immunohistochemistry.** Tissues were fixed in 10% neutral buffered formalin overnight at 4°C and transferred to 70% ethanol. Samples were dehydrated and embedded in paraffin according to standard techniques and sectioned at 5 μm onto Superfrost™ plus slides (Erie Scientific Co., Portsmouth, NH). Slides were dewaxed in xylene and hydrated in a graded series of ethanol to phosphate buffered saline (PBS, pH 7.4). Tissue sections were quenched with 3% H2O2 in PBS for 10 min and then washed in PBS (3 times). Tissue sections were incubated in microwave antigen retrieval buffer (citrate buffer 5 mM, pH 6.0) for 5 min, left undisturbed for 5 min, and then were microwaved for an additional 5 min. Slides remained in the buffer for a 30-min cooling period. Then they were washed in PBS (3 x 2 min) and blocked with 5% non-immune goat serum in PBS (30 min) prior to overnight incubation at 4°C with the following rabbit polyclonal antibodies (Affinity BioReagents, Golden, CO) used at 1:200 dilution in 5% normal goat serum: anti-TR (PA1–214), anti-TRβ1 (PA1–213), anti-TRα1 (PA1–211), and anti-TRα2 (PA1–212). Negative controls were similarly incubated using a non-immune rabbit IgG at appropriate concentration. After incubation, tissue sections were washed in PBS (3 x 2 min) and processed for immunohistochemical detections using Zymed’s Picture Plus™ polymer detection kit (Zymed Laboratories, San Francisco, CA). Sections were incubated for 30 min at room temperature with the secondary antibody horseradish peroxidase polymer conjugate. After washing in PBS (3 x 2 min), sections were incubated with diaminobenzidine, washed, counter-stained with hematoxylin and mounted with Permaslip (Alban Scientific Inc., St. Louis, MO).

**Statistical Analyses.** Data were analyzed using a one-way analysis of variance. Bonferroni’s multiple comparison test was used for pair-wise comparisons (Prism, version 4; GraphPad Software, Inc., San Diego, CA).

### Table 1. Summary of Gene Targets Evaluated by Real-Time Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon size (bp)</th>
<th>Sense primer (5’→3’)</th>
<th>Antisense primer (5’→3’)</th>
<th>Annealing temp. (°C)</th>
<th>PCR eff. (%)</th>
<th>Corr. coeff. (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRα1</td>
<td>133</td>
<td>AGGCTGTGCTGCTAATGTC</td>
<td>CAGAAATCGCGGAATTTGTTG</td>
<td>54.7</td>
<td>110.3</td>
<td>0.996</td>
</tr>
<tr>
<td>TRα2</td>
<td>105</td>
<td>ATTACTCAAGATGAGCTCT</td>
<td>GGGAGATGGTTAATAGGG</td>
<td>54.7</td>
<td>105.8</td>
<td>0.999</td>
</tr>
<tr>
<td>TRβ1</td>
<td>115</td>
<td>CGCCCACTCCGCCATTCC</td>
<td>AAGTTCTGCTTTGCTACATCTTC</td>
<td>54.7</td>
<td>106.2</td>
<td>0.996</td>
</tr>
<tr>
<td>RXRα</td>
<td>130</td>
<td>CCTAGCCCTTTCTTCTCAG</td>
<td>CTGCTCTGGTCTCAGACTAG</td>
<td>61.0</td>
<td>99.3</td>
<td>0.999</td>
</tr>
<tr>
<td>RXRβ</td>
<td>144</td>
<td>AAGACTGCTCCTCAGCAAG</td>
<td>CACACTGCTGCTACCTTTC</td>
<td>61.0</td>
<td>98.9</td>
<td>0.998</td>
</tr>
<tr>
<td>DIO1</td>
<td>106</td>
<td>TGCGGTAAGACAAATGACGA</td>
<td>GCCAGATTTACCCTTGTAGGA</td>
<td>52.0</td>
<td>95.6</td>
<td>1.000</td>
</tr>
<tr>
<td>DIO2</td>
<td>134</td>
<td>CCACCTTCTGGACTTGTGCA</td>
<td>GGAAGTCAGCCAGGAATGGAG</td>
<td>54.0</td>
<td>96.3</td>
<td>1.000</td>
</tr>
<tr>
<td>DIO3</td>
<td>120</td>
<td>TCACCTCTGAGGCTCTTG</td>
<td>CCCAGTAATGCTTACGGATG</td>
<td>61.0</td>
<td>97.3</td>
<td>0.999</td>
</tr>
</tbody>
</table>

**Results and Discussion.**

The quantity of transcripts for DIO1, DIO2, and DIO3 during the period of transition from pregnancy to lactation are depicted in Figure 1. Dairy cows are typically pregnant during most of lactation and milking is terminated 60 days prior to expected parturition. Therefore physiology of the mammary gland during the prepartum period (as depicted) is influenced by the cessation of milking, which promotes involution, and the mammosogenic and lactogenic effects of late pregnancy, promoting a process that we have referred to as regenerative involution (18, 19).

As previously reported for protein level, DIO1 is the predominant 5′-deiodinase transcript isoform expressed in bovine liver and DIO2 is the predominant isoform in bovine mammary gland (8, 20). DIO1 transcript abundance in liver changed during the prepartum period (Fig. 1A; P = 0.027), with low levels throughout most of the period, but with a brief rise in abundance the final week of pregnancy. In mammary gland, abundance of DIO2 transcripts increased from low levels during pregnancy through early lactation (day 14) to peak lactation (day 90; Fig. 1B; P < 0.0001). These later changes are consistent with changes in mammary DIO2 enzyme in cow (13). Levels of DIO1 mRNA in mammary tissue and DIO2 mRNA in liver were near the limit of detection and did not change during this transition period (P > 0.10). DIO3 mRNA was present in both liver and mammary gland, with higher concentrations evident in mammary tissues (Fig. 1C). In mammary gland, DIO3 transcript abundance decreased as gestation advanced, with a nadir during early lactation (P = 0.019). To visualize relative changes in thyroid hormone activation and inactivation pathways, the ratios of DIO1/DIO3 and DIO2/DIO3 were calculated for liver and mammary tissues, respectively (Fig. 1D). When viewed in this manner, changes in DIO1/DIO3 mRNA levels in liver (P = 0.038) suggest increased thyroid hormone activation during late gestation, followed by a decline during established lactation. Because liver 5′-deiodination of T4 is the primary source of T3 in circulation, this conclusion is consistent with the higher increased systemic concentrations of thyroid hormones during late gestation and the decline during lactation (12, 13, 21). Opposite to changes in liver, alterations in DIO2/DIO3 mRNA levels (P = 0.0005) suggest an increase...
in local conversion of T4 to T3 within the mammary gland during lactation. This local generation would help to maintain a euthyroid condition in mammary gland in face of the functional hypothyroid state that is characteristic of lactation (12, 22).

The observed changes in expression of deiodinase transcripts are consistent with previously reported transcriptional regulation of these genes. The DIO1 gene contains SP1 promoters and two thyroid response elements (TRE) in the 5’ flanking region, imparting T3 and retinoic acid responsiveness to the gene (23). DIO1 is particularly sensitive to thyroid status, with T3 enhancing transcription. Thus, decreased activity of the thyroid hypothalamic-pituitary axis during lactation promotes a systemic decrease in DIO1 expression with advancing gestation/lactogenesis and the onset of lactation (12). DIO2 responds to thyroid status in opposite direction to the effect on DIO1. Although a negative TRE has not been identified in the DIO2 promoter region, transcription of DIO2 is decreased by T3 (5, 6). Thyroid ablation and hormone replacement demonstrated that growth hormone and prolactin specifically increased DIO2 expression in the mammary glands of mice (14), regulation that is consistent with increased expression of DIO2 during lactation. Recent studies indicate that bile acids may provide a linkage between food consumption and metabolic control via DIO2. Postprandial increases in serum bile acids have been shown to bind to TGR5, a membrane G-protein, eliciting an increase in intracellular cAMP and induction of DIO2 transcription in muscle and fat (24, 25). Whether bile acids increase mammary DIO2 expression and thereby adjust mammary metabolism to the increase in feed intake that accompanies lactation in cattle remains to be determined, but are consistent with the changes observed. Several factors are known to affect DIO3 expression, but the DIO3 binding sites and mechanisms of regulation are largely undefined. Consistent with the decline in DIO3 transcript abundance observed during the relatively hypothyroid state of lactation, thyroid hormones increase abundance of DIO3 transcripts (26), whereas glucocorticoids and growth hormone reduce expression of DIO3 in several in vitro systems (27, 28). Fibroblast and epidermal growth factors (27, 29) and transforming growth factor beta (30) induce DIO3 expression in a number of cell lines. Potential regulation by these factors would be consistent with the greater expression of DIO3 during late pregnancy when extensive epithelial growth, differentiation and angiogenesis occur within the mammary gland. Thus, changes in expression of the three deiodinases during the transition from pregnancy to lactation are consistent with known regulation in various species and cell types.

The quantity of transcripts for the thyroid hormone receptors, TRα1, TRα2 and TRβ1, and for retinoid receptors, RXRα and RXRγ, in bovine mammary gland are summarized in Figure 2. The TR present in greatest abundance was TRα1; however, its expression was unaltered during the transition period (Fig. 2A; P > 0.10). TRβ1 mRNA was present in

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**Figure 1.** Expression of transcripts for iodothyronine deiodinases type I (D1), type II (D2) and type III (D3) in mammary gland (MG) and liver, during the periparturient transition from pregnancy to lactation. Values are expressed as number of molecules per unit of total RNA in the reverse transcription reaction. D1 and D2 (Panels A and B) are 5’-deiodinases that enhance thyroid hormone activity by converting T4 to T3. D3 (Panel C) is a 5-deiodinase that diminishes thyroid hormone activity by metabolizing T4 and T3 to the inactive iodothyronines, rT3 and T2. Ratios of activating to inactivating deiodinases in mammary tissue (D2/D3) and liver (D1/D3) are depicted in panel D. Number of cows = 3 to 5 per time point. Within tissue, means without common superscripts differ (P < 0.05).
reverse transcription reaction. Number of cows expressed as number of molecules per unit of total RNA in the periparturient transition from pregnancy to lactation. Values are point. Means without common superscripts differ (P = 0.0006). RXRs form heterodimers with TRs, which then serve as TRE transcription factors. A limited number of naturally occurring TRE have been identified that do not require the RXR proteins for function (31, 35). Our results suggest that RXRα may play a role in local regulation of thyroid hormone-responsive genes in the bovine mammary gland, particularly during late pregnancy. But its decline during late pregnancy and lactation, appears at odds with increased mammary sensitivity to thyroid hormones. Heterodimers of TR/RXR often mediate transcription of thyroid hormone-responsive genes, but may also block transcription of other genes via RXR (16). There is extensive cross-talk between nuclear receptors and the nature of these and other interactions that impact the transition from pregnancy to lactation remain to be fully elucidated.

Expression and localization of TR proteins were evaluated by immunohistochemistry (data not shown). Use of an antibody that recognizes both TRα1 and TRβ1, showed that these TR proteins are expressed in mammary tissues from pregnant and lactating cows, and that the TRs are localized in nuclei of both mammary epithelium and stroma. Tissues stained using antibodies specific for single isoforms of TR showed the same patterns of staining (TRα1, TRα2 and TRβ1). The extensive expression indicates that a variety of cell types are receptive to thyroid hormone regulation.

In conclusion, we have shown that the transition from pregnancy to lactation in dairy cows is accompanied by changes in abundance of transcripts for activating and deactivating iodothyronine deiodinases, consistent with decreased T4 to T3 conversion in liver and enhanced T4 to T3 conversion in mammary gland. Transcripts for TRα1, TRα2 and TRβ1 were expressed in mammary gland, as were transcripts for the related receptor, RXRα. Increased abundance of TRβ1 transcripts and decreased abundance of RXRα transcripts during the transition from pregnancy to lactation may alter signaling and increase the sensitivity of lactating mammary tissue to thyroid hormones. Such changes are consistent with establishing metabolic priority for the lactating mammary gland and enhancing direct and indirect galactopoietic effects of thyroid hormones.


