

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 (*TR α 1*), alpha2 (*TR α 2*) and beta1 (*TR β 1*), and retinoic acid receptors alpha (*RXR α*) and gamma (*RXR γ*), 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 *TR β 1* mRNA in mammary gland increased with onset of lactation, whereas *TR α 1* and *TR α 2* transcripts did not vary with

physiological state. Conversely, quantity of *RXR α* mRNA decreased during late gestation to low levels during early lactation. Data suggest that increased expression of mammary *TR β 1* and *DIO2*, and decreased *RXR α* , provide a mechanism to increase thyroid hormone activity within the mammary gland during lactation. Exp Biol Med 233:1309–1314, 2008

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'-diiodothyronine (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

This work was funded by the United States Department of Agriculture, Agricultural Research Service, CRIS 1265-31000-086-00.

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Received March 12, 2008.
Accepted May 14, 2008.

DOI: 10.3181/0803-RM-85
1535-3702/08/23310-1309\$15.00
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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 somatotropin, and demonstrated that these galactopoeitic hormones increased 5'-deiodinase activity (*DIO2*) specifically in mammary gland (14).

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 *THRB*, 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 *THRB* generated by use of alternative promoters. The TRs bind to TREs as homodimers, 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 obtained 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 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 concentration 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 transcriptase 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 (QIAquick Gel Extraction Kit, Qiagen Inc., 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 generated 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)

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

Gene	Amplicon size (bp)	Sense primer (5' → 3')	Antisense primer (5' → 3')	Annealing temp. (°C)	PCR eff. (%)	Corr. coeff. (r)
<i>TRα1</i>	133	AGGCTGTGCTGCTAATGTC	CAGAAGTGC GGAATGTTGTG	54.7	110.3	0.996
<i>TRα2</i>	105	ATATCCAAGATGAGCCTCTG	GGGAGAGTGGTTTAAATAGGG	54.7	105.8	0.999
<i>TRβ1</i>	115	CCGCAACTCAGCCCATTC	AAGTTCTGTCTTGTCCATCCTC	54.7	106.2	0.996
<i>RXRα</i>	130	CCTACGCCTTCTTCTTCC	CTGCTCTGGGTCTCAATG	61.0	99.3	0.999
<i>RXRγ</i>	144	AAGACTGCCTCATTGACAAG	CACACTCTGCCTCACTTTC	61.0	98.9	0.998
<i>DIO1</i>	106	TGGGGTAGACACAATGACGAA	GGCCAGATTTACCCTTG TAGGA	52.0	95.6	1.000
<i>DIO2</i>	134	CCACCTTCTGGACTTTGCCA	GGAAGTCAGCCACGGATGAG	54.0	96.3	1.000
<i>DIO3</i>	120	TCACTCCCTGAGGCTCTG	CCCAGTAAATGCTTACGGATG	61.0	97.3	0.999

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 μ m 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 $\times$ 2 min). Microwave antigen retrieval was then used. Slides were heated in a microwave at high power (650 W) in 400 ml of 10 mM citrate buffer (pH 6.0) for 5 min, remained undisturbed for 5 min, and then were microwaved for an additional 5 min. Slides remained in the buffer for a 30-min cooling period. They were then washed in PBS (3 $\times$ 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 in phosphate buffered saline: anti-TR (PA1–214), anti-TR β 1 (PA1–213), anti-TR α 1 (PA1–211), and anti-TR α 2 (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 $\times$ 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 $\times$ 2 min), sections were incubated with diaminobenzidine, washed, counter stained with hematoxylin and then 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).

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 mammogenic 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

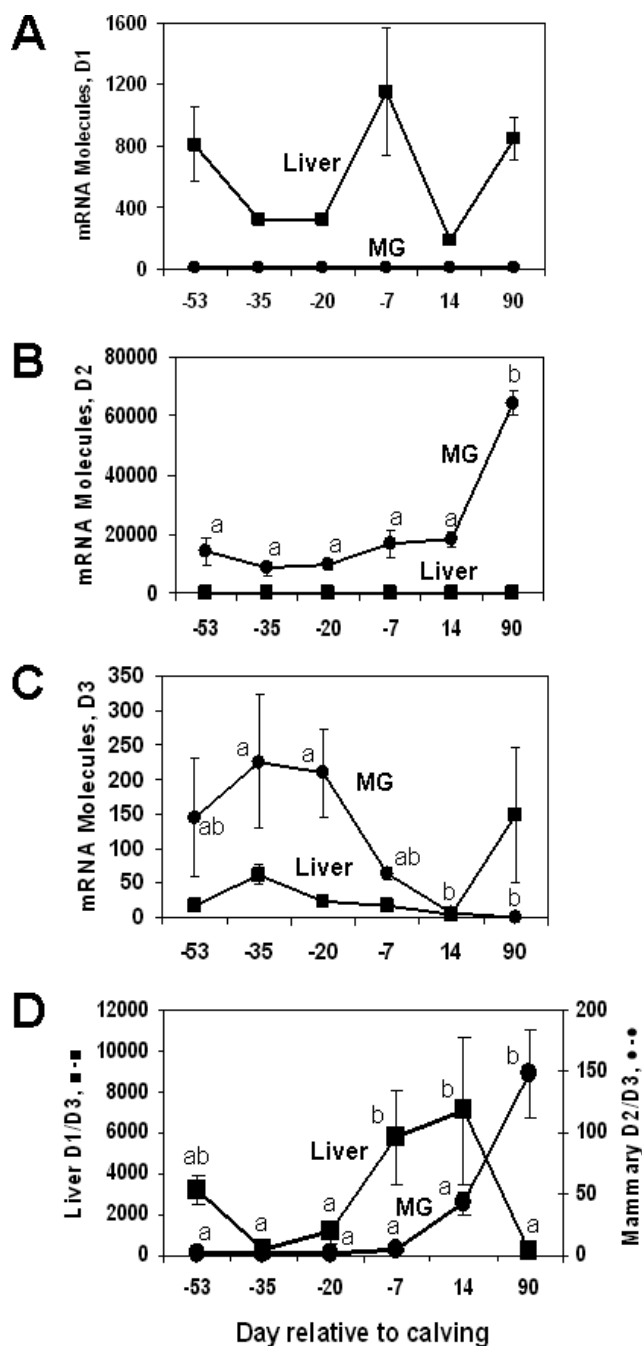


Figure 1. Expression of transcripts for iodothyronine deiodinases type I (D1), type II (D2) and type III (D3) in mammary gland (MG) and liver, during the periparturient transition from pregnancy to lactation. Values are expressed as number of molecules per unit of total RNA in the reverse transcription 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 ($P < 0.05$).

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

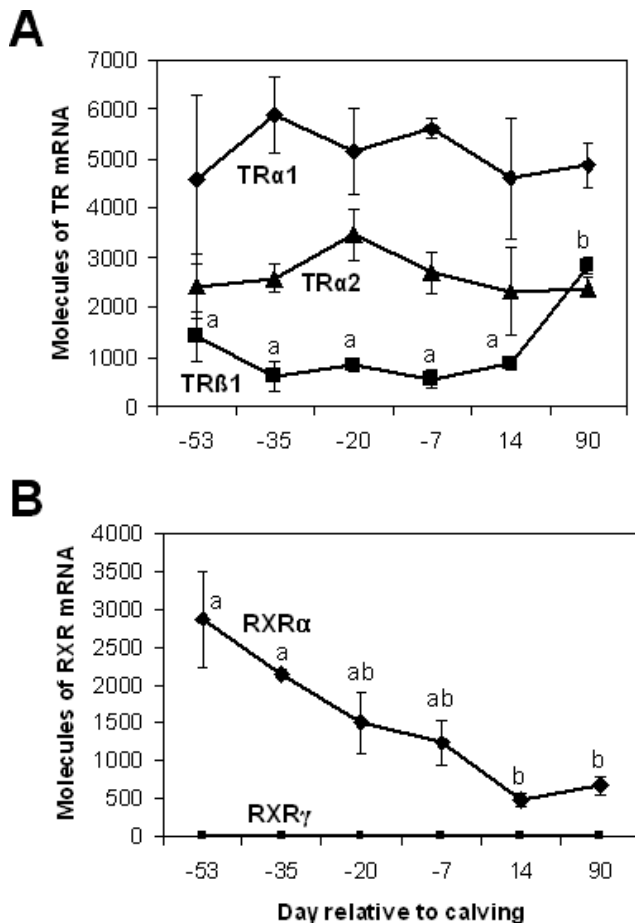


Figure 2. Expression of transcripts for thyroid hormone receptor isoforms, *TRα1*, *TRα2*, and *TRβ1* (Panel A), and retinoid receptor isoforms, *RXRα* and *RXRγ* (Panel B), in mammary gland during the periparturient transition from pregnancy to lactation. Values are expressed as number of molecules per unit of total RNA in the reverse transcription reaction. Number of cows = 3 to 5 per time point. Means without common superscripts differ ($P < 0.05$).

lower abundance, but the quantity increased from pregnancy to established lactation ($P = 0.0001$). Expression of these TRs is consistent with general expression patterns for these receptors in other tissues and species. Both *TRα1* and *TRβ1* mRNAs and proteins are expressed in almost all tissues, whereas *TRβ2* is expressed almost exclusively in anterior pituitary and hypothalamus (31–33). Thus, expression of *TRβ2* was not evaluated in the current study. Interestingly, *TRα2* mRNA was present in significant quantity in bovine mammary gland. Because it does not bind thyroid hormones but does bind to TREs, it can serve as a potential repressor of thyroid hormone-responsive genes. However, because the quantity of *TRα2* mRNA was not impacted by the transition from pregnancy to lactation ($P > 0.10$) it does not appear to serve as a regulator of thyroid hormone-responsive genes in mammary gland during this period. These results are similar to the expression patterns of TRs in rat mammary gland. Using semi-quantitative RT-PCR, *TRβ1* was shown to be expressed in rat mammary gland mainly during lactation,

whereas *TRα1* appeared to be expressed primarily during pubescent, pregnant and weaning periods (34). Data suggest that *TRβ1* mediates metabolic adjustments within the lactating mammary gland that are promoted by thyroid hormones.

Expression of *RXRα* and *RXRγ* in mammary tissue is depicted in Figure 2B. *RXRγ* mRNA was present at low and invariant concentrations ($P > 0.10$). Alternatively, *RXRα* mRNA was expressed at high concentrations 53 days prior to expected parturition (7 days after cessation of milking) and declined throughout the prepartum period and into the succeeding lactation ($P = 0.0006$). 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.

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