Reproductive Experience Alters Prolactin Receptor Expression in Mammary and Hepatic Tissues in Female Rats[1]

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ABSTRACT

Recent studies have reported that reproductive experience in female rats alters prolactin (PRL) receptor gene expression in the brain as well as neural sensitivity to PRL. Given PRL's actions in nonneural tissues, that is, mammary tissue and liver, it was asked whether reproductive experience may also alter prolactin receptor (Prlr) gene expression in these tissues. Groups of age-matched female rats were generated with varying reproductive histories. Separate groups of primiparous (first lactation) and multiparous (second lactation) had mammary tissue and liver samples collected on Day 3 or 10 of lactation. A fifth group raised one litter to weaning and then resumed estrous cyclicity. This group and a final group of age-matched, virgin controls were killed on diestrus. Tissue was processed by quantitative PCR for expression rates of the long and short forms of Prlr mRNA as well as casein beta mRNA (mammary tissue only). Western blots were performed to quantify receptor protein content. Multiple lactations as well as lactation itself resulted in alterations in Prlr expression. Prlr gene expression in mammary tissue was increased in primiparous mothers compared with that in multiparous dams, whereas in the liver, Prlr expression was reduced during an initial lactation. In contrast, PRLR protein levels declined during lactation in mammary, but not hepatic, tissues. Overall, the results demonstrate that the prolactin receptor system is altered in nonneural tissues as a result of the female's reproductive history. The findings are discussed in the context of milk and bile production and PRL's possible role in breast cancer.

lactation, liver, mammary glands, pregnancy, prolactin/prolactin receptor, reproductive experience

INTRODUCTION

The pituitary hormone prolactin (PRL) plays a key role in the physiological adaptations of pregnancy and lactation that prepare the female for energetic and behavioral demands postpartum [1–3]. Key roles that PRL plays include modulating stress responsivity during pregnancy and lactation [4], inducing the onset of maternal care [5], and stimulating lactogenesis [6]. Earlier research has shown that reproductive experience results in reductions in circulating levels of PRL in both women [7] and female rats [8, 9]. The functional significance of these reductions in circulating hormone is not known. However, it may involve an actual increase in responsiveness to PRL at the receptor level. In agreement with this concept are the findings that a long-term increase in the expression of the long form of the prolactin receptor (PRLR$_L$) as well as its responsiveness to exogenous PRL treatment occur in the brain of experienced female rats after normal estrous cyclicity resumes [10]. Likewise, more recent research has found that basal as well as PRL-stimulated phosphorylated STAT5 activities in the brain are enhanced in reproductively experienced female rats [11].

In addition to PRL's actions within the brain, PRL acts on a number of key peripheral target tissues that include the mammary gland [1, 12] and liver [13, 14]. Primary functions of PRL in these tissues involve the stimulation of lactogenesis in mammary tissue [6] and the facilitation of bile transport in the liver [15]. Given that PRL acts at these nonneural sites, it was asked whether reproductive experience might produce shifts in PRLR expression in mammary and hepatic tissues as it does in selected neural sites. Earlier studies have demonstrated that prior parity can alter the responsiveness of mammary tissue to hormonal challenges [16] as well as result in changes in estrogen receptor expression [17]. Therefore, in the present study the effects of reproductive experience on the expression of both the long (Prlr$_L$) and short (Prlr$_S$) forms of the receptor [18] were measured by real-time RT-PCR in mammary tissue and liver in female rats that had varying amounts of reproductive experience. Moreover, receptor protein content was measured by Western blot analysis. Specifically, PRLR expression and content were compared between age-matched rats during an initial versus a second lactation and in nonlactating rats that had experienced one prior pregnancy and lactation or were inexperienced virgins. Finally, in order to compare possible changes in mammary function, expression of the milk protein, casein beta [19], was measured in these same treatment groups to see whether reproductive experience modified expression of this PRL-regulated protein.

MATERIALS AND METHODS

Animals

Fifty nulliparous Sprague-Dawley (Crl:CD[SD]BR) females (176–200 g) were purchased from Charles River Breeding Laboratories and triple-housed (45 × 25 × 20 cm polypropylene cages) in a light-controlled (lights on 0500–1900) and temperature-controlled (21°C–24°C) room with food and water available ad libitum throughout the current study. All the rats were maintained in accordance with the guidelines of the Division of Teaching and Research Resources at Tufts University, Cummings School of Veterinary Medicine (AAALAC accredited) following the procedures for animal care prepared by the National Research Council Committee of the Care and Use of Laboratory Animals Resources. The animal protocol (G911-07) was approved by Tufts University Institutional Animal Care and Use Committee. Twenty-five nulliparous females were mated with breeding males from our colony 3 wk after arrival at approximately 10 wk of age. On the day following parturition, each litter was culled to five males and five females. Females were allowed to raise their litters until weaning at 21 days postpartum. Approximately 11 wk after primiparous, females weaned their first litter (28 wk of age); 17 of these primiparous females together with 17 nulliparous females were mated with colony males to generate lactating multiparous and primiparous dams. The liver

and mammary tissues were harvested from these rats at one of two points: D3 (Day 3) or D10 of lactation. The remaining nulliparous and primiparous females served as nonlactating controls. Vaginal smears were taken daily from both groups of nonlactating controls to characterize the individual estrous cycles. Tissue samples were collected from these subjects on the day of diestrus of the estrous cycle. Thus, the six treatment groups consisted of age-matched, nulliparous diestrous (N-Di), primiparous diestrous (P-Di), lactating primiparous D3 (P-D3) and D10 (P-D10), and lactating multiparous D3 (M-D3) and D10 (M-D10) subjects.

Tissue Collection

All the tissues were collected between 0900 and 1100 h on the designated collection day. After the animals were killed, the left lateral lobe of the liver together with the inguinal and abdominal mammary glands from one side were removed and kept frozen on dry ice under RNase-free conditions.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from 100 mg of both mammary and liver tissues using TRI Reagent (Ambion) according to the manufacturer's instructions. Total RNA was measured using a NanoDrop ND-1000 spectrophotometer. M-MLV Reverse Transcriptase (Invitrogen) was used to make first strand cDNA from 200 ng of total RNA from each sample.

Real-Time PCR

TaqMan Gene Expression assays were purchased from Applied Biosystems for the long (Rn01525470_ml, RefSeq NM_001034111) and short (Rn01525458_ml, RefSeq NM_012630.1) forms of the rat *Prlr* as well as the casein beta gene (Rn00567460_ml, RefSeq NM_017120.2). Actin beta (*Actb*) was used as the housekeeping gene (Rn00667869_m1, RefSeq NM_031144.2; Applied Biosystems), and each sample was run in duplicate. For liver samples, TaqMan gene expression assays for both the long and short forms of *Prlr* as well as the *Actb* housekeeping gene were run simultaneously, and for the mammary tissue, the casein beta assay was included. Two animals from the nulliparous diestrous group were randomly selected to serve as controls and were included in all the reaction plates. RT-PCR was performed on an Applied Biosystems 7500 Real-Time PCR System. To calculate relative expression, ΔCt was calculated by subtracting the Ct (cycle threshold) of the housekeeping gene from each target. This calculation [20] was also used to determine the difference in Ct expression between the housekeeping and target genes from the mean Ct level from the two controls included in each plate (mean Ct target minus mean Ct housekeeping). The $\Delta\Delta Ct$ was calculated by subtracting ΔCt control from ΔCt target. Finally, relative expression was calculated using the formula $2^{-\Delta\Delta Ct}$.

Western Blot Analyses

Individual samples of 100 mg of mammary or hepatic tissues were homogenized in 1 ml of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) prior to centrifugation; 10% of the homogenate was used to measure total protein content with a Coomassie Plus Protein Assay kit (Pierce Biotechnology). Dilutions of liver (5 μg/μl) and mammary (1 μg/μl) homogenates were taken, and equal volumes of sample, lysis buffer (catalog no. 89900; Thermo Scientific), and loading buffer (catalog no. 161-0737; Bio-Rad) were boiled for 5 min. These samples (15 μl/well) were loaded on 12% Tris-HCl gels together with molecular weight markers (37.8, 85.1, and 127.6 kDa). Gels were run at 90 V for approximately 3.5–4 h in running buffer (catalog no. 161-0771; Bio-Rad) and transferred to a polyvinylidene fluoride membrane at 33 V for 2 h in transfer buffer (catalog no. LC3675; Invitrogen).

The membrane was placed in 5% skim milk and 3% bovine serum albumin (BSA) for 1 hr prior to adding the primary antibody, mouse anti-PRLR U-5 (diluted 1:1000; all antibodies were purchased from Abcam), in 1% skim milk and 0.6% BSA overnight. This primary antibody binds to the extracellular domain of the PRLR and therefore recognizes both the long and short isoforms of the receptor. The second antibody, goat anti-mouse horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (diluted 1:10 000 in 1% skim milk and 0.6% BSA), was added for 1 h followed by ECL Western blot detection reagent (40A:1B) for 5 min; the membrane was exposed to film and the Restore Stripping Buffer (Pierce/Thermo Scientific) for 15 min. The membrane was subsequently placed in 5% skim milk for 1 h, and a primary antibody, goat anti-actin (diluted 1:200 in 1% skim milk) was added for 1 h followed by the addition of a secondary antibody, rabbit anti-goat HRP-conjugated IgG (diluted 1:10 000 in 1% skim milk), for 1 hr. The membrane was then exposed to 40A:1B for 5 min prior to being exposed to film.

The film was then scanned, and gel intensities were determined using a Kodak 1D 3.6 detector. The intensities of the bands for both isoforms of PRLR
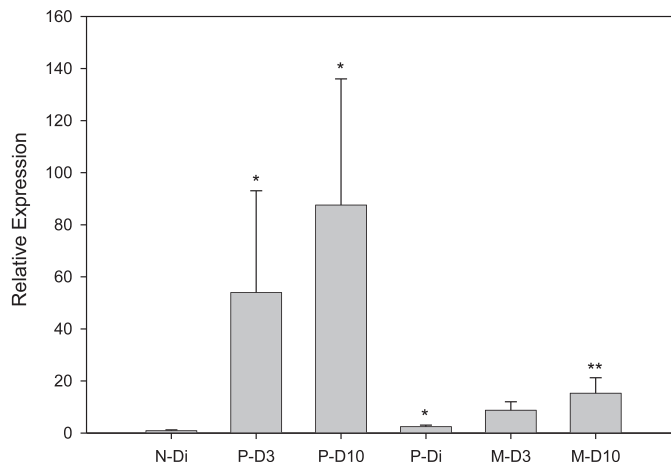
Prlr Long mRNA Expression in Mammary Tissue

FIG. 1. Mean (± SEM) mRNA expression for *PrlrL* in rat mammary tissue as a function of reproductive state and experience. N-Di, nulliparous, diestrous group; P-D3, primiparous, Day 3 lactating group; P-D10, primiparous, Day 10 lactating group; P-Di, primiparous, nonlactating, diestrous group; M-D3, multiparous, Day 3 lactating group; M-D10, multiparous, Day 10 lactating group. *Denotes significant difference versus the N-Di group ($P < 0.05$). **Denotes significant difference versus the P-Di group ($P = 0.01$); n = 5–8/group.

and actin were identified according to the molecular weight markers, and the values reported as relative expression of PRLR to actin.

Data Analysis

Data were initially analyzed by a two-way ANOVA using two sets of groups for analysis. Comparisons were first made within reproductive experience groupings (e.g., N-Di, P-D3, and P-D10) and then across lactational states independent of the diestrous groups (P-D3, P-D10, M-D3, and M-D10). Because the gene expression data from the mammary tissue were not normally distributed, the data were transformed to a log scale. Transformed data from the mammary tissue and normally distributed, nontransformed data from liver samples were analyzed for overall group effects and subsequently by post hoc *t*-tests or Fisher least significant difference tests. The data comparing protein density ratios were analyzed using nonparametric tests, including the Kruskal-Wallis and Mann-Whitney *U* tests, because the equal variance test failed for these comparisons. An α value of ≤ 0.05 was set as the criterion for statistical significance for all the tests.

RESULTS

Effects of Reproductive Experience on Prolactin Receptor and Casein Beta Expression and Content in Mammary Tissue

Prlr expression changed both as a function of lactation and reproductive experience ($P < 0.05$). As shown in Figure 1, in mammary tissue the relative expression of $PRLR_L$ was significantly increased on P-D3 ($P = 0.002$) and P-D10 ($P < 0.001$) of lactation in all the females when compared to diestrous controls. In experienced females, $Prlr_L$ expression was significantly higher in the M-D10 than in P-Di controls ($P = 0.01$). In addition, the level of expression of the $Prlr_L$ in mammary tissue was significantly higher in primiparous rats sampled on D3 and D10 combined when compared with the level of expression present at the comparable times in multiparous dams ($P = 0.05$). In cycling diestrous females, basal levels of expression for the $Prlr_L$ females with prior reproductive experience, that is, a pregnancy and lactation, although low, were statistically higher than that detected in their age-matched, virgin controls ($P = 0.015$).

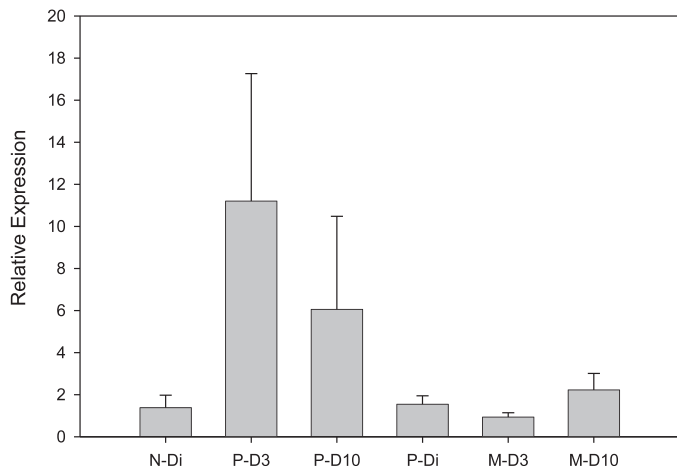## *Prlr* Short mRNA Expression in Mammary Tissue

FIG. 2. Mean (± SEM) mRNA expression for *Prlr$_S$* in rat mammary tissue as a function of reproductive state and experience. N-Di, nulliparous, diestrous group; P-D3, primiparous, Day 3 lactating group; P-D10, primiparous, Day 10 lactating group; P-Di, primiparous, nonlactating, diestrous group; M-D3, multiparous, Day 3 lactating group; M-D10, multiparous, Day 10 lactating group; n = 5–8/group.

No overall differences were detected in the expression of the *Prlr$_S$* in mammary tissue (Fig. 2). As with the *Prlr$_L$*, on both D3 and D10, primiparous lactating rats tended to have increased levels of *Prlr$_S$* expression when compared with virgin, diestrous controls. These differences did not reach statistical significance. It is noted that as with the expression of *Prlr$_L$*, the relative expression of the *Prlr$_S$* declined between the first and second lactation in these age-matched groups ($P = 0.029$). Comparisons between levels of expression of the *Prlr$_L$* and *Prlr$_S$* isoforms revealed that *Prlr$_L$* was expressed significantly more than *Prlr$_S$* in the P-D10 ($P = 0.015$), M-D3 ($P = 0.008$), and M-D10 ($P = 0.003$) groups.

Western blot analysis of PRLR$_L$ and PRLR$_S$ is shown in Figure 3. Analysis of PRLR protein expression relative to ACTB indicates that the densities of PRLR$_L$ are generally higher than those for PRLR$_S$ in all but the N-Di group ($P < 0.05$). Moreover, the ratio of PRLR$_L$:PRLR$_S$ was higher during lactation (average ratio = 1.6) than in the diestrous groups (ratios < 1.0). An analysis of the PRLR$_L$:PRLR$_S$ ratio among groups demonstrated an overall group effect ($P = 0.029$). A subsequent comparison between nonlactating diestrous and lactating subjects found that the PRLR protein ratios were significantly higher in the lactating animals ($P = 0.001$). The ratios of receptor protein densities were comparable in both diestrous groups as they were among the lactating groups. The Western blot depicted in Figure 4 demonstrates reduced densities for the PRLR$_S$ isoforms on D3 of lactation in the primiparous animals and on both lactation days in the multiparous groups when compared with the diestrous groups. ACTB band densities were similar across all the treatment groups (Fig. 4).

Casein beta mRNA expression levels in mammary tissues are shown in Figure 5. There was an overall effect of reproductive state on casein beta expression ($P < 0.001$). Relative casein beta expression levels were significantly higher during lactation than in either nonlactating diestrous group ($P = 0.003$). On both D3 and D10 of lactation, casein beta expression was markedly elevated in the primiparous dams versus virgin controls ($P < 0.01$). Comparable elevations in casein beta expression were present in rats undergoing their
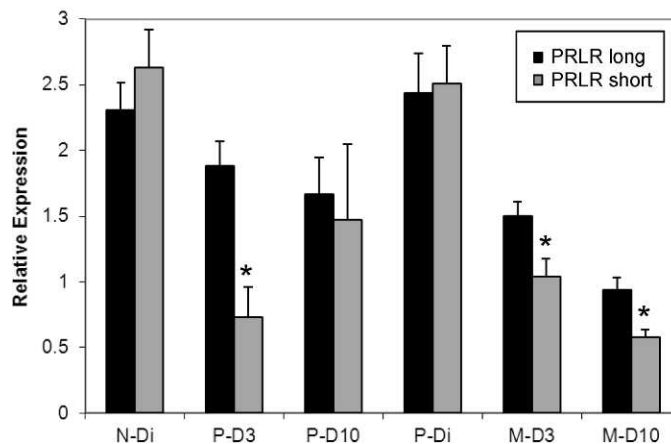
PRLR Protein in Mammary Tissue

FIG. 3. Relative protein expression of the PRLR$_L$ and PRLR$_S$ in mammary tissue. Values are expressed as means (± SEM). *Denotes significant difference versus PRLR$_L$ ($P < 0.05$); n = 4/treatment group.

second lactation (i.e., the multiparous rats) versus diestrous primiparous controls ($P = 0.002$). Comparisons of casein beta expression between age-matched primiparous and multiparous mothers revealed similar levels of expression as a function of both day of lactation and amount of reproductive experience. Finally, expression of mRNA for casein beta was statistically higher in the age-matched, primiparous diestrous rats when compared with those in the nulliparous diestrous rats ($P = 0.016$), reflecting an effect of prior reproductive experience on casein beta expression in mammary tissue.

Effects of Reproductive Experience on Prolactin Receptor Expression and Content in Liver

Prlr expression in liver changed as a function of reproductive experience. As shown in Figure 6, expression for *Prlr$_L$* mRNA was lower during a first lactation when compared to levels of expression during a second lactation ($P = 0.024$). No differences in *Prlr$_L$* gene expression were found as a function of day of lactation with levels comparable on D3 and D10 in both reproductive experience groups. Likewise, no day of lactation by reproductive experience interactions were found. As shown in Figure 7, the initial lactation was also associated with significantly lower levels of *Prlr$_S$* expression ($P = 0.039$), which was reduced on both D3 ($P = 0.027$) and D10 ($P = 0.035$) when compared with diestrous virgin rats. *Prlr$_S$* mRNA expression was similar in the multiparous lactating groups and their primiparous diestrous controls. Thus, the primary shift in *Prlr* expression in the liver involved a significant reduction in *Prlr$_S$* and decreased *Prlr$_L$* mRNA expression during an initial lactation. Comparisons of expression levels of long versus short isoforms of the *Prlr* in liver indicate that lactation is associated with increased ratios of *Prlr$_L$*:*Prlr$_S$*. When comparing *Prlr* expressions (Figs. 6 and 7), this ratio increased from about 1:1 in the diestrous groups to approximately 2:1 during lactation.

An analysis of Western blot densities in liver for the long and short forms of the PRLR for treatments is shown in Figure 8. The PRLR$_L$ concentrations were consistently higher than those of the PRLR$_S$ form. Examination of the ratios of hepatic PRLR$_L$:PRLR$_S$ revealed that the two diestrous groups had similar ratios of about 2.6, whereas these ratios were higher in the lactating groups, reflecting shifts in PRLR densities in
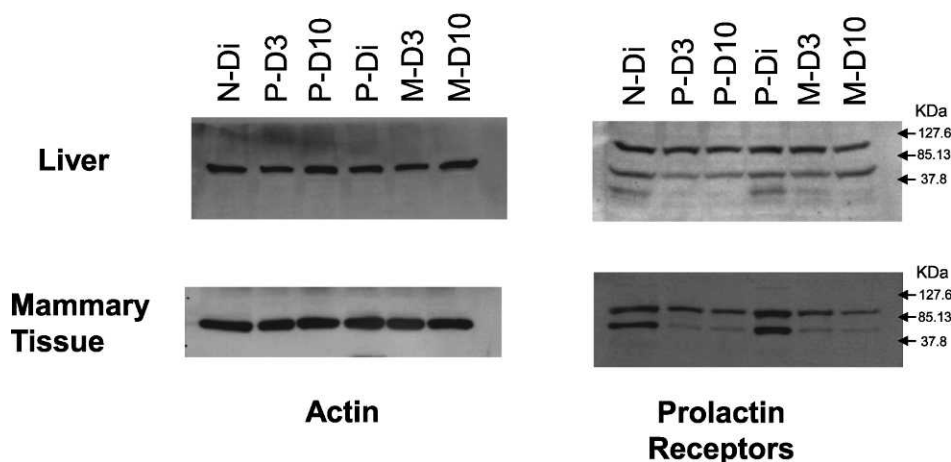
Western Blots of Treatment Groups

FIG. 4. Western blots of mammary and liver tissue pools (pools of 4–6 animals/treatment group) for PRLRs and actin. Two distinct bands are present in mammary samples processed for the PRLRs. The larger band that is slightly greater than 85.13 kDa is consistent with $PRLR_L$, while the bands between 85.13 and 37.8 kDa are consistent with the molecular weight of the $PRLR_S$ isoform. A third faint band of less than 37.8 kDa is also present in the diestrous groups of the hepatic pools.

lactating rats. An analysis comparing group receptor ratios revealed a nonsignificant trend toward a group effect ($P = 0.108$). A subsequent comparison between the diestrous and lactating groups found higher ratios of $PRLR_L:PRLR_S$ in the lactating groups ($P = 0.011$). The average ratio of $PRLR_L:PRLR_S$ for nonlactating diestrous subjects was 2.6, whereas for lactating subjects, the ratio was 4.0. Examination of the Western blots (Fig. 4) indicates that these shifts appear to be associated with reductions in the densities of $PRLR_S$ as a function of lactation. No differences were found in hepatic PRLR densities as a function of reproductive experience, that is, primiparous versus multiparous lactating states. Densities of the ACTB bands were similar for all the treatment groups.

DISCUSSION

The results of the present study demonstrate that both the states of lactation and prior reproductive experience alter *Prlr* gene expression in mammary and hepatic tissues. Receptor expression patterns in both mammary and hepatic tissues shifted as a function of reproductive state.

Comparisons of the level of *Prlr* expression in mammary tissue indicate that during an initial lactation the long form of the receptor is increased and expressed at a higher level than during a second lactation in age-matched rats. Likewise, gene expression of the short form of the receptor is higher in primiparous, lactating rats compared with those of the multiparous mothers. Nevertheless, the ratio of $Prlr_L:Prlr_S$ expression remained fairly constant on D3 and D10 of lactation in both the primiparous and multiparous groups. Comparison of the ratio of long to short form of the *Prlr* mRNA among the groups revealed that this ratio was higher in the lactating groups. Whereas the ratios of $Prlr_L:Prlr_S$ were approximately 1:1 in both tissues in the virgin diestrus and nonlactating, primiparous, diestrous groups, this ratio increased during lactation to a range from 3:1 to 5:1 in mammary tissue and 1.8:1 to 2.7:1 in hepatic tissue. These changes in the ratios of *Prlr* gene expression may reflect an increase in PRL activity; it has been proposed that $PRLR_S$ can act as a dominant negative and thereby reduce PRL signal transduction activity at the receptor level [21, 22]. Thus, a decline in $PRLR_S$ availability would be expected to potentiate the actions of the $PRLR_L$ and its associated biological actions.

It was unexpected to find that receptor protein product in mammary tissue in general did not parallel that of *Prlr* gene expression. For example, in mammary tissue the band densities for both forms of the PRLR in the lactating groups were generally lower than in the diestrous groups. In contrast, *Prlr* gene expression was elevated during lactation compared with diestrous animals. Further studies are warranted to identify the mechanisms involved in this differential *Prlr* gene expression and protein content.

Casein beta expression, which is, as expected, under PRL regulation [19, 23], was increased during lactation. During lactation, casein beta constitutes a major portion of milk protein. Casein beta is assembled into micelles within milk, which help provide some of the milk's physical characteristics, including stabilizing the milk, reducing the viscosity of the milk, and being able to carry large amounts of calcium phosphate in suspension. Although the levels of casein beta gene expression did not differ between the primiparous and multiparous groups on D3 or D10, there was a slight, yet
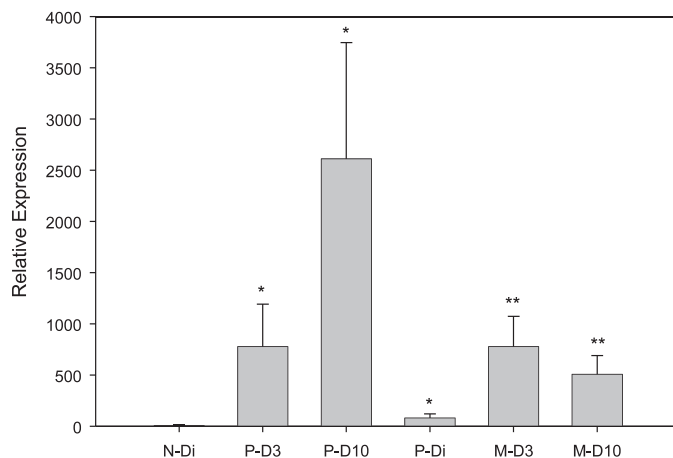
Casein Beta mRNA Expression in Mammary Tissue

FIG. 5. Mean (± SEM) casein beta mRNA expression in mammary tissue samples as a function of reproductive state and experience. *Denotes significant difference versus N-Di group ($P < 0.05$). **Denotes significant difference versus P-Di group ($P < 0.05$); n = 5–8/group.
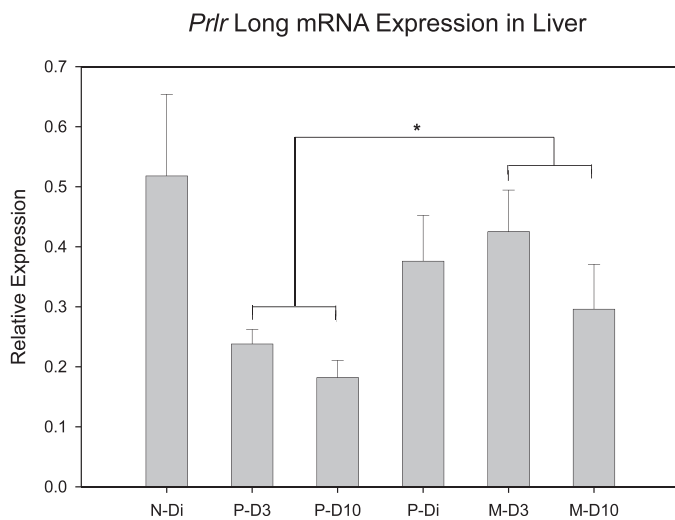
Prlr Long mRNA Expression in Liver

FIG. 6. Mean (± SEM) mRNA expression for the $Prlr_L$ in hepatic tissue as a function of reproductive state and experience. *Denotes significant difference between P-D3 plus P-D10 versus M-D3 plus M-D10 ($P < 0.05$); n = 5–8/group.

significant, higher level of casein beta gene expression in the diestrous rats that had previously given birth and raised a litter. Thus, prior reproductive experience appears to affect basal levels of casein beta gene expression. This reproductive experience effect is similar to that reported in neural tissue for $Prlr_L$, that is, nonlactating, cycling rats that have previously raised a litter were found to have higher levels of $Prlr$ expression in both the medial preoptic area and the arcuate region compared to levels present in age-matched virgin rats [10]. Further work is needed to assess the effects of reproductive experience on casein beta protein content because measurement of mRNA expression provides only one perspective of the effects of reproductive experience on casein beta's biological activity.

In the liver, gene expression of both forms of the $Prlr$ was lower during an initial lactation when compared with levels of expression in both the diestrous groups and the multiparous
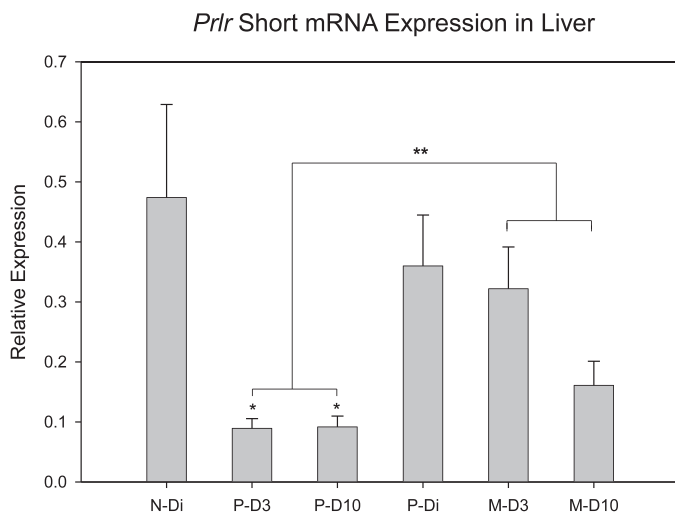
Prlr Short mRNA Expression in Liver

FIG. 7. Mean (± SEM) mRNA expression for the $Prlr_S$ in hepatic tissue as a function of reproductive state and experience. *Denotes significant difference versus N-Di ($P < 0.05$). **Denotes significant difference between P-D3 plus P-D10 versus M-D3 plus M-D10 ($P < 0.05$); n = 5–8/group.
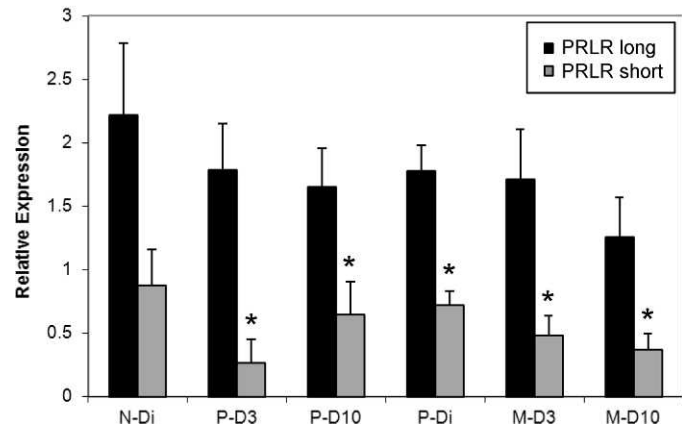
PRLR Protein in Liver

FIG. 8. Relative protein expression of the $PRLR_L$ and $PRLR_S$ in hepatic tissue. Values are expressed as means (± SEM). *Denotes significant difference versus $PRLR_L$ ($P < 0.05$); n = 4/treatment group.

subjects. Messenger RNA expression levels for both forms of $Prlr$ were comparable between the diestrous groups and the multiparous animals on both D3 and D10 of lactation. The most pronounced change in hepatic $Prlr$ gene expression was present in $Prlr_S$ mRNA expression in the primiparous groups on D3 and D10 of lactation when it declined. Those factors responsible for the decline in hepatic mRNA expression for $Prlr_S$ or the consequences of these declines are unknown. However, such changes may affect bile production/transport and lipid metabolism differentially during an initial lactation.

What is the overall impact of reproductive experience on PRLR systems? When the results of the present study are taken together with prior studies that focused on the neural PRLR system, some overall patterns are found. Notably, the $Prlr$ expression levels change as a function of the number of pregnancies and lactations in multiple PRL target tissues. In both brain and liver, there is an increase in $Prlr_L$ gene expression with increasing amounts of reproductive experience. In mammary tissue, in contrast, expression of both $Prlr_S$ and $Prlr_L$ decline between the initial and second lactation. The physiological significance of these changes is not clearly understood. However, given the higher level of PRL-stimulated milk production associated with a second lactation [1, 24], it is possible that these changes in mammary $Prlr$ expression and receptor protein may participate in the elevations in lactogenesis in multiparous animals. It is possible that the changes in receptor isoform protein ratios found in this study may underlie a shift in sensitivity of the mammary glands with the $PRLR_S$ acting as a dominant negative. In mice, both pregnancy and prior parity render the mammary glands more responsive to hormonal induction of lactose synthetase activity [16]. Related studies in rats have reported that parity results in increased estrogen receptor expression in the mammary gland with increased expression of both ESR1 (previously ER alpha) and ESR2 (previously ER beta) detected in multiparous dams [17]. In humans, milk intake in neonates of multiparous mothers is greater than that in neonates of primiparous mothers [24]. Moreover, body weight gains of the neonates of multiparous mothers are higher, presumably reflecting some combination of greater PRLR occupation and activity during the first few days of life and/or enhanced milk letdown and altered nursing patterns. Multiple lactations also enhance lactogenesis in mice, that is, in mice heterozygous for the $Prlr$ gene, lactational performance is poor following an initial birth but is capable of

sustaining the growth of young after a second birth [25, 26]. Thus, it is apparent that the ability of mammary tissue to respond to the endocrine and sensory signals during a second lactation may be enhanced compared with that present in primiparous mothers.

The shift in PRLR expression in hepatic tissue between the first and second lactation may functionally affect bile transport. Prolactin increases maternal bile secretion in postpartum as compared with pregnant or nonpregnant controls [15]. Moreover, increased expression of the bile salt transporters *Slc10a1* (formerly *Ntcp*) and *Abcb11* (formerly *Bsep*) and proteins have been reported to occur in postpartum lactating rats, an increase potentiated by PRL treatment [27]. Prolactin also increases ATP-dependent taurocholate transport in canalicular plasma membrane from rat liver that is associated with increased bile transport [28]. It has been suggested that the increased bile secretion and the activity of these transporters may function to meet the maternal nutritional needs to increase absorption of fats and fat-soluble vitamins [27]. An increase in hepatic function in response to elevated lipid levels may help modulate excessive fat content in the maternal circulation.

That multiple pregnancies resulted in a higher level of $Prlr_L$ mRNA expression in hepatic tissue parallels the effects of a prior parity on the expression of $Prlr_L$ in brain [10]. A similar increase in $Prlr_S$ expression was found in hepatic tissues. The physiological consequences of these shifts are unknown given that these changes in gene expression were not associated with comparable changes in PRLR protein in liver. This was surprising but may reflect differences in posttranslational processing of the receptors or alterations in degradation rates. Future studies that examine these shifts are warranted in order to gain a better understanding of the dynamics between receptor gene expression, receptor protein content, and the biological significance of these changes in the PRLR systems.

It is noted that measurements of *Prlr* gene expression in the present study did not examine the possible intracellular compartmentalization of PRLRs within either mammary or hepatic tissues. Posner et al.'s [29] study of hepatic PRLR localization in rats identified receptors both on the cell surface membrane and internalized in the Golgi. Moreover, during lactation a higher percentage of PRLRs were localized in the Golgi. These authors proposed that the location of the PRLR sites may affect the actions of ligand bound to the receptor in the liver. Similar studies have identified both an acid-sensitive and acid-insensitive subset of PRLRs in mammary tissue [30]. Specifically, during lactation the level of acid-sensitive receptors increase more slowly than that of acid-insensitive receptors located primarily on intracellular membranes. Likewise, it is noted that PRLRs are expressed in both mammary epithelial cells and stroma with increases in the long form of the receptor present in the epithelial compartment during lactation [31, 32]. It is possible that the differences in expression of receptors in the mammary gland in the present study result from changes in the relative number of epithelial cells associated with the various reproductive states examined. Thus, whereas our determinations of PRLR subtype content in these tissues revealed alterations in the isoforms of the PRLR during lactation, future studies are needed to examine the precise compartmentalization of these receptors.

The regulation of PRLR expression likely reflects a time-dependent, ligand-receptor interaction. It is well established that PRL stimulates PRLR concentrations in mammary [33], hepatic [34, 35] and neural [36] tissues. The stimulatory effects of PRL on receptor numbers in these tissues likely are accompanied by temporally dependent up-regulation [10, 11, 33–35] and down-regulation [37] in PRLR protein and gene expression. Thus, assessments of ligand concentrations, receptor numbers, occupation, and turnover as well as receptor gene expression are important determinants in clarifying the biological impact of shifts and levels in *Prlr* gene expression.

Finally, that reproductive experience reduces circulating PRL levels [7–9] and alters *Prlr* expression in mammary tissue is of interest in light of the possible role of PRL in breast cancer [38, 39] and the protective effects of giving birth when a woman is in her twenties upon the subsequent incidence of breast cancer [40, 41]. Whether reproductive experience in women induces similar shifts in the sensitivity of PRLRs and PRL actions in mammary tissue that participate in protecting the female from the development of breast cancer is not known. It would be of interest to determine if a shift in PRL activity occurs in women that may play a role in the development of breast cancer.

In summary, the present study demonstrates that multiple reproductive experiences modify *Prlr* gene expression and receptor protein densities at two key sites of PRL action, the mammary gland and liver. The precise physiological consequences of these changes likely include improved milk production in multiparous mothers as well as alterations in fat metabolism. These findings together with earlier ones that demonstrate shifts in receptor expression and activation of PRL transduction pathways in neural tissues as a consequence of reproductive experience [10, 11] emphasize the importance of weighing the impact of single and multiple reproductive experiences on a range of endocrine functions related to the health status of the female across her lifespan.

ACKNOWLEDGMENT

REFERENCES

1. Forsyth IA. Variation among species in the endocrine control of mammary growth and function: the roles of prolactin, growth hormone, and placental lactogen. J Dairy Sci 1986; 69:886–903.
2. Woodside B, Augustine RA, Ladyman SR, Naef L, Grattan DR. Role of prolactin in the metabolic adaptations to pregnancy and lactation. In: Bridges RS (ed.), Neurobiology of the Parental Brain. San Diego: Academic Press; 2008: 249–268.
3. Grattan DR. The actions of prolactin in the brain during pregnancy and lactation. Prog Brain Res 2001; 133:153–171.
4. Torner L, Toschi N, Nava G, Clapp C, Neumann ID. Increased hypothalamic expression of prolactin in lactation: involvement in behavioural and neuroendocrine stress responses. Eur J Neurosci 2002; 15:1381–1389.
5. Bridges RS, DiBiase R, Loundes DD, Doherty PC. Prolactin stimulation of maternal behavior in female rats. Science 1985; 27:782–784.
6. Tucker HA. Endocrinology of lactation. Semin Perinatol 1979; 3:199–233.
7. Musey VC, Collins DC, Musey PI, Martino-Saltzman D, Preedy JRK. Long-term effect of a first pregnancy on the secretion of prolactin. New Engl J Med 1987; 316:229–234.
8. Bridges RS, Byrnes EM. Reproductive experience reduces circulating estradiol-17β and prolactin levels during proestrus and alters estrogen sensitivity in female rats. Endocrinology 2006; 147:2575–2582.
9. Bridges RS, Henriquez BM, Sturgis JD, Mann PE. Reproductive experience reduces haloperidol-induced prolactin secretion in female rats. Neuroendocrinology 1997; 66:321–326.
10. Anderson GM, Grattan DR, van den Ancker W, Bridges RS. Reproductive experience increases prolactin responsiveness in the medial preoptic area and arcuate nucleus of female rats. Endocrinology 2006; 147:4688–4694.
11. Sjoeholm A, Bridges RS, Grattan DR, Anderson GM. Region, neuron and signaling pathway-specific increases in prolactin responsiveness in reproductively experienced female rats. Endocrinology 2011; 152:1978–1988.
12. Oakes SR, Rogers RL, Naylor MJ, Ormandy CJ. Prolactin regulation of mammary gland development. J Mammary Gland Biol Neoplasia 2008; 13:13–28.

13. Bogorad RI, Ostroukhova TY, Orlova AN, Rubtsov PM. Long isoforms of prolactin receptor predominates in rat intrahepatic bile ducts and further increases under obstructive cholestasis. J Endocrinol 2006; 188:345–354.

14. Varas SM, Jahn GA. The expression of estrogen, prolactin, and progesterone receptors in mammary gland and liver of female rats during pregnancy and early postpartum: regulation by thyroid hormones. Endocr Res 2005; 31:357–370.

15. Liu Y, Hyde J, Vore M. Prolactin regulates maternal bile secretory function postpartum. J Pharmacol Expr Ther 1992; 261:560–566.

16. Bolander FF. Persistent alterations in hormonal sensitivities of mammary glands from parous mice. Endocrinology 1983; 112:1796–1800.

17. Kass L, Durando M, Ramos JG, Varayoud J, Powell CE, Luque EH, Munoz-de-Toro M. Association of increased estrogen receptor β2 expression with parity-induced alterations in the rat mammary gland. Steroid Biochem Mol Biol 2004; 91:29–39.

18. Okamura H, Zachwieja J, Raguet S, Kelly PA. Characterization and applications of monoclonal antibodies to the prolactin receptor. Endocrinology 1989; 124:2499–2508.

19. Lee CS, Oka T. Progesterone regulation of a pregnancy-specific transcription repressor of β-casein gene promoter in mouse mammary gland. Endocrinology 1992; 131:2257–2262.

20. Byrnes EM, Bridges RS. Reproductive experience and expression of dopamine D2 receptor mRNA: a possible mechanism for reduced prolactin secretion in primiparous rats. J Neuroendocrinol 2007; 19:773–778.

21. Berlanga JJ, Garcia-Rutz JP, Perrot-Applanat M, Kelly PA, Edery M. The short form of the prolactin (PRL) receptor silences induction of the β-casein gene promoter. Mol Endocrinol 1997; 11:1449–1457.

22. Perrot-Applanat M, Gualilio O, Pezet A, Vincent V, Edery M, Kelly PA. Dominant negative and cooperative effects of mutant forms of prolactin receptor. Mol Endocrinol 1997; 11:1020–1032.

23. Guyette WA, Matusik RJ, Rosen JM. Prolactin-mediated transcriptional and post-transcriptional control of casein gene expression. Cell 1979; 17:1013–1023.

24. Zuppa AA, Tornesell A, Papacci P, Tortorolo G, Segni G, Lafuenti G, Moneta E, Diodato A, Sorcini M, Carta S. Relationship between maternal parity, basal prolactin levels and neonatal breast milk intake. Biol Neonate 1988; 53:144–147.

25. Ormandy C, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N, Kelly PA. Null mutation of the prolactin gene produces multiple reproductive defects in the mouse. Genes Dev 1997; 11:167–178.

26. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocrinol Rev 1998; 19:225–268.

27. Cao J, Huang L, Lui Y, Hoffman T, Stieger B, Meier PJ, Vore M. Differential regulation of hepatic bile salt and organic anion transporters in pregnant and postpartum rats and the role of prolactin. Hepatology 2001; 33:140–147.

28. Liu Y, Suchy FL, Silverman JA, Vore M. Prolactin increases ATP-dependent taurocholate transport in canalicular plasma membrane from rat liver. Am J Physiol 1997; 272:G46–G53.

29. Posner BI, Josefsberg Z, Bergerson JJM. Intracellular polypeptide hormone receptors. J Biol Chem 1979; 254:12494–12499.

30. Sakai S, Mizoguchi Y, Kim JY. Characterization of plasma and intracellular membrane prolactin receptor in lactating mouse mammary cells. Endocr J 1994; 41:249–256.

31. Camarillo IG, Thordarson G, Moffat JG, Van Horn KM, Binart N, Kelly PA, Talamantes F. Prolactin receptor expression in the epithelia and stroma of the rat mammary gland. J Endocrinol 2001; 171:85–95.

32. Hovey RC, Trott JF, Ginsburg E, Goldhar A, Sasaki MM, Fountain SJ, Sundararajan K, Vonderhaar BK. Transcriptional and spatiotemporal regulation of prolactin receptor mRNA and cooperativity with progesterone receptor function during ductal branch growth in the mammary gland. Dev Dyn 2001; 222:192–205.

33. Djiane J, Durand P. Prolactin-progesterone antagonism in self regulation of prolactin receptors in the mammary gland. Nature 1977; 266:641–643.

34. Posner BI, Kelly PA, Friesen HG. Induction of lactogenic receptor in rat liver: influence of estrogen and the pituitary. Proc Natl Acad Sci U S A 1974; 71:2407–2410.

35. Barash T, Cromlish W, Posner BI. Prolactin (PRL) receptor localization in cultured rat hepatocytes: dual regulation by PRL and growth hormone. Endocrinology 1988; 122:1151–1158.

36. Muccoli G, DiCarlo R. Modulation of prolactin receptors in the rat hypothalamus in response to changes in serum concentrations of endogenous prolactin or to ovine prolactin administration. Brain Res 1994; 663:244–250.

37. Djiane J, Clauser H, Kelly PA. Rapid down-regulation of prolactin receptors. Biochem Biophys Res Com 1979; 90:1371–1378.

38. Ewertz M, Duffy SW. Risk of breast cancer in relation to reproductive factors in Denmark. Br J Cancer 1988; 58:99–104.

39. Stuebe AM, Willett WC, Xue F, Michels KB. Lactation and incidence of premenopausal breast cancer. Arch Intern Med 2009; 169:1364–1371.

40. Kampert JB, Whittemore AS, Paffenbarger RS. Combined effect of childbearing, menstrual events, and body size on age-specific breast cancer risk. Am J Epidemiol 1988; 128:962–979.

41. Leon DA. A prospective study of the independent effect of parity and age at first birth on breast cancer incidence in England and Wales. Int J Cancer 1989; 43:986–991.