Endocrine Research, 31(4):357–370, (2005)

ISSN 0743-5800 print/1532-4206 online
DOI: 10.1080/07435800500454528

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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

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□ *The aim of this study was to examine, using semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR) the changes in mRNA expression of the two estrogen receptor (ER) subtypes, ERα and ERβ, prolactin receptor long and short form, and progesterone (Pg) receptor (PgR), in liver and mammary gland during gestation, early lactation, and weaning in both hyperthyroid (HT) and normal rats. Pregnancy increased long prolactin receptors ($PRL\text{-}R_L$) and ERα mRNAs in liver and $PRL\text{-}R_L$ in mammary gland. Lactation decreased $PRL\text{-}R_L$ in liver and ERβ and PgR in mammary gland. HT decreased $PRL\text{-}R_L$ at the end of pregnancy (G21), ERα (in G21 and L1) in liver and $PRL\text{-}R_L$ in L1 as well as short prolactin receptors ($PRL\text{-}R_S$) (G7, L1) and ERβ (G7, G14, L4) in mammary gland. In conclusion, our data indicated that*

1 PRL–R1 and ERα expression levels are differentially regulated in the liver, and PgR and ERβ in mammary gland during pregnancy and lactation
2 ERβ is variably expressed depending on the state of thyroid hormones, however the ERα gene expression remained constant in mammary gland.
3 PRL–R1 mRNA expression is highly induced in the mammary gland during late pregnancy and abruptly declines on the first day of lactation for the HT rats.

Keywords L-thyroxine, RT-PCR, liver, mammary gland, pregnancy, lactation

INTRODUCTION

Thyroid hormones participate in numerous processes such as growth, maturation, development, and metabolism of most tissues (1). Thyroid disorders have been implicated in a variety of reproductive problems,

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including ovulatory failures and cycling abnormalities (2,3) and two in every 1,000 pregnant women have some degree of hyperthyroidism. Most of the symptoms of hyperthyroidism (HT) are attenuated during pregnancy, but there is a marked rebound after delivery. This rebound may have a negative impact on infant development (4). In rats, pregnancy attenuates some of the metabolic effects of hyperthyroidism (5). On the other hand, daily thyroxine (T_4) given at two dose levels, (1 and 0.25 mg/kg/day), started approximately 10–15 days before mating in rats, provoked an advancement in luteolysis, lactogenesis, and parturition of roughly half a day (6,7). Delivery and maternal behavior were impaired and the mothers were unable to nurse their young, although they allowed themselves to be suckled (6). We also found that hyperthyroidism produced alterations in the mammary and liver expression of insulin–like growth factors (IGFs) and their binding proteins (8), that could be related to the impairments in lactation as well as to the changes observed in tissue metabolism and growth (5,6). Moderate hyperthyroidism, induced by a lower daily dose of T_4 (0.1 mg/kg/day) allowed the mothers to lactate, but the growth of the litters was reduced and mammary function was affected, with premature signs of involution (9).

We have shown (7) that chronic T_4 regimens increased PRL binding and decreased estrogen and progesterone binding in mammary tissue measured on day 20 of pregnancy (G20) when measured by the radioreceptor assay. We also found decreases in liver PRL and GH binding and mRNA expression of PRL and GH receptors in G20 (7). Although some of these changes may be due to the advancement in luteolysis and lactogenesis (6,7), they may also reflect alterations in the pattern of receptor expression produced during earlier stages of pregnancy.

In the mammary gland, both ERα and ERβ are expressed (10). The interaction between the two receptors in individual cells may be very important for the total effect of estrogen on the whole tissue. Saji et al. (11) have shown that ERβ protein expression at various stages of mammary gland development was not always paralleled by changes in ERβ mRNA. In lactation, although ERβ mRNA increases, ERβ protein decreases. This suggests the existence of post-transcriptional or post-translational regulatory mechanisms. PgRs are also expressed in mammary glands, where they act as negative regulators of estrogen action and block lactogenesis during pregnancy.

Both forms of PRL–R mRNA, long and short, have been identified in the mammary gland. Their expression varies during pregnancy and lactation and is negatively regulated by progesterone at the end of lactation (12,13). Mammary PRL-R expression increases at the end of pregnancy and achieves its highest values during lactation, while in liver there is a

constant increase during pregnancy, followed by an abrupt fall postpartum (12).

There are reports that examined potential estrogen-thyroid hormone interactions on the regulation of the transcription from target genes and may underlie alterations in reproductive behavior (14).

The aims of the present investigation were to examine whether the changes produced by hyperthyroidism in liver and mammary hormone binding at the end of pregnancy (7) are paralleled with changes in the expression of the different forms of the receptors, and whether these changes are also seen at early stages of pregnancy. We determined the relative (to S16) expression and developmental changes of ERα, ERβ, PRL-R_S, PRL-R_L and Pg-R genes in the rat liver and mammary gland during pregnancy and early postpartum using semi-quantitative PCR.

MATERIALS AND METHODS

Chemicals

L-Tetraiodothyronine (T_4) was a generous gift from Glaxo (Buenos Aires, Argentina). All the other chemicals were of reagent grade and were obtained from Merck Laboratory (Buenos Aires, Argentina).

Animals and Experimental Design

Adult female Wistar rats bred in our laboratory, three to four months old and weighing around 200 g at the onset of treatment, were used. The rats were kept in a 22–25°C controlled environment with a light-dark cycle of 12 hours each. Rat chow and tap water were available *ad libitum*. Hyperthyroidism was induced by daily s.c. injection of T_4 at a dose of 0.25 mg/Kg body weight dissolved in 0.9 % NaCl and alkalinized with NaOH to pH 9. The presence of spermatozoa in the vaginal smear the morning after caging with a fertile male in the night of pro-estrus was indicative of pregnancy and this day was counted as day 0 of pregnancy. The rats were mated 8–10 days after the start of the treatments.

Groups of T_4 or vehicle–treated rats were sacrificed by decapitation at 1200 hours on estrus after 18 or 35 days of treatments, on days 7, 14 and 21 of pregnancy (G7, G14, G21) or on the first day postpartum (L1). Since the litters of hyperthyroid rats rarely survive after the first day post-partum, other groups of T4- or vehicle-treated rats were isolated from the litters the day after delivery and sacrificed on the fourth day post-partum, to investigate the expression of the different receptors after premature weaning (L4). The livers and inguinal mammary glands from the dams were immediately excised, frozen on liquid nitrogen (-196°C), and stored at 80°C for RNA preparation.

RNA Isolation

Total RNA from mammary glands and liver were prepared from the frozen samples using the guanidinium isothiocyanate-acid phenol method (15) as modified by Puissant and Houdebine (16). The RNA was analyzed by denaturing agarose gel electrophoresis before use to verify integrity. The amount of RNA recovered was measured by UV spectrophotometry.

Semi-Quantitative RT-PCR Analysis

One to three micrograms of total RNA were reverse transcribed at 42°C using random hexamer primers and Moloney murine leukemia virus RT (Invitrogen Life Technologies, Buenos Aires) in a 20 µl reaction mixture. Two µl of the reverse transcription reaction mix were amplified with primers specific for rat ERα, ERβ, PRL-R_L, PRL-R_S and PgR (Table 1), as previously reported (Telleria et al., 1998, and Clarke et al. 1993). The S16 ribosomal protein gene was used as internal control. All reactions were carried out for 35 cycles and then terminated with a 5 minute extension at 72°C. The conditions were such that the amplification of the products was in the exponential phase and the assay was linear with respect to the amount of input RNA. RNA samples were assayed for DNA contamination by PCR without prior reverse transcription. The PCR products were analyzed on 2% agarose gels containing 0.5 µg/ml ethidium bromide and photographed with a Polaroid camera. Band intensities of RT-PCR products were quantified using NIH Image software. Relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to that for the ribosomal protein S16 cDNA.

Statistical Analyses

Significant differences among means were considered at a level of $P < 0.05$ and identified by one-way or two-way ANOVA and τ test. In all cases, the variances were homogeneous.

RESULTS

Regulation of PRL-R_L and PRL-R_S mRNA Expression in Liver of pregnant and Postpartum Rats

Figure 1 shows the evolution of liver prolactin receptors mRNA concentrations during pregnancy and the postpartum in control and HT rats

TABLE 1 Primer sequences used in the PCR amplification of various cDNAs

cDNA	Forward primer (5′→3′)	Reverse primer (5′→3′)	Gene Bank Accessions	Size of amplified product (pb)
S 16	TCCAAGGGTCCGCTGCAGTC	CGTTCACCTTGATGAGCCCATT	X17665	100
ERα	AATTCTGACAATCGACGCCAG	GTGCTTCAACATTCTCCCTCCTC	Y00102	344
ERβ	AAAGCCAAGAGAAACGGTGGGCAT	GCCAATCATGTGCACCAGTTCCTT	U57439	204
PRL-R_L	AAAGTATCTTGTCCAGACTCGCTG	AGCAGTTCTTCAGACTTGCCCTT	NM011169	279
PRL-R_S	AAAGTATCTTGTCCAGACTCGCTG	TTGTATTTGCTTGGAGAGCCAGT	NM011169	279
Pg-R	CCCACAGGAGTTTGTCAAGCTC	TAACTTCAGACATCATTTCCGG	L16922	326

Each combination of primers has specific cycling parameters: ERα, ERβ, PRL-R_L and PRL-R_S: 95°C, 1minute; 65°C, 1 minute; 72°C, 1 minute; Pg-R: 94°C, 1minute; 55°C, 30 seconds; 72°C, 2 minutes.

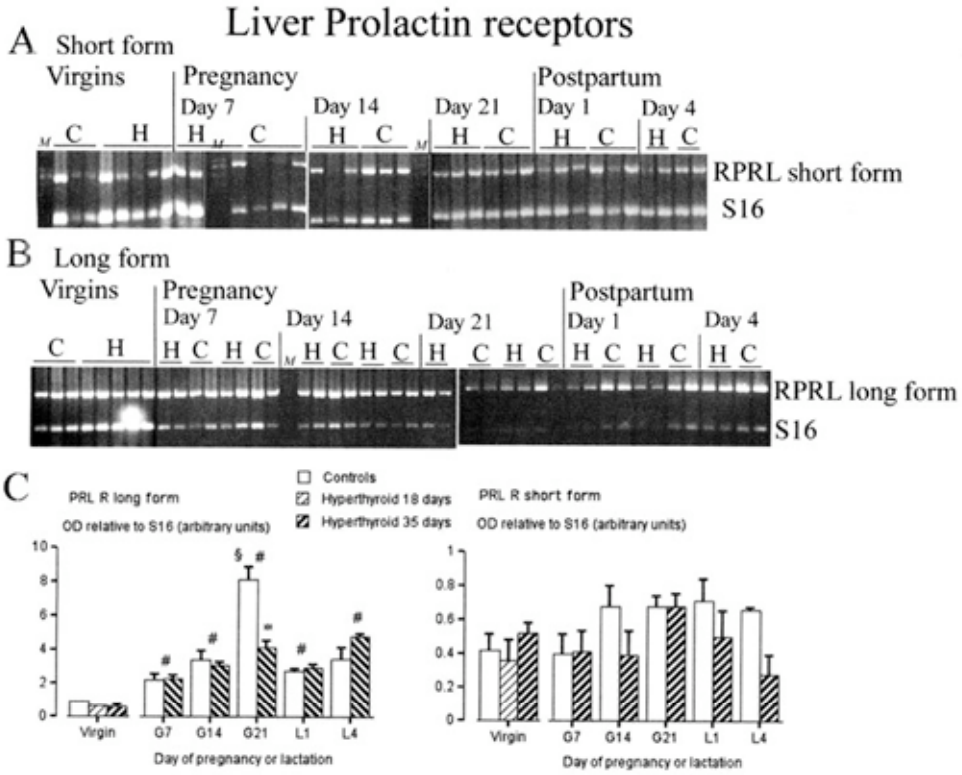

FIGURE 1 Expression of liver prolactin receptor forms in control (C) and Hyperthyroid (H) rats during days 7, 14, 21 of pregnancy and days 1 and 4 of lactation.

Panels A and B: Measurement by RT-PCR of expression of S16 and the short or long forms of the prolactin receptor, respectively. Ethidium bromide fluorescence photograph of the gel electrophoresis of the coamplification products; Lane M: molecular weight markers. Panel C: Relative expression of the receptors during pregnancy, postpartum and early weaning. The gel photographs were quantified using NIH Image and expressed as arbitrary units. Results are expressed as the average ± s.e.m of groups of 4 rats.
[a] $p < 0.05$ compared with the respective control groups using two-way ANOVA.
[b] $p < 0.05$ compared with the respective virgin group using two-way ANOVA.

and in virgin rats with 18 or 35 days of T_4 treatment. In contrast with previous results obtained with Northern blot analysis, PRL R_L was more readily detectable than PRL R_S (12). Expression of the short form of the receptor did not vary significantly with reproductive or thyroid status. In contrast, the long form of the receptor increased in control rats during pregnancy to a peak on day 21 and fell abruptly in the postpartum, confirming previous reports (12). Separation of the pups during three days (L4 group) had no effect. The T_4 treatment had no effect on virgin rats, while the increase observed during pregnancy was attenuated, with a decrease of 50% approximately on day 21. There were no differences in the postpartum.

Regulation of ERα and ERβ mRNA Expression in Liver of Pregnant and Postpartum Rats

Both forms of ER were readily detected in liver, with higher expression of the α form relative to S16 than of the β form (Figure 2). Furthermore, the expression of ERβ was highly variable and was not significantly modified by the reproductive status or the T_4 treatment. ERα mRNA concentration increased significantly on day 21 and achieved the highest values on day 1 postpartum in controls, with a subsequent fall after 3 days of pup deprivation. These increases were significantly diminished in the HT group, while there were no effects of the T_4 treatment in virgin rats (Figure 2).

Regulation of PRL-R_L and PRL-R_S mRNA Expression in Mammary Glands of Pregnant and Postpartum Rats

As previously shown, expression of the long form of the PRL receptor was higher relative to S16 than that of the short form (13). Expression of both forms was affected by the reproductive state and by T_4 treatment. Thus, in controls, the short form increased abruptly on day 1 postpartum, and fell after 3 days weaning, while the long form showed a progressive increase during pregnancy, that was significant on the first day postpartum, and was not affected by weaning. The T_4 treatment diminished markedly the increased expression on day 1 postpartum, but the weaning period paradoxically produced a recuperation of the relative mRNA concentrations to values similar to controls (Figure 3).

Regulation of ERα, ERβ and PgR mRNA Expression in Mammary Gland of Pregnant and Postpartum Rats

In mammary glands, in contrast with livers, the relative expression of ERα was very low and variable, and there were no significant variations with reproductive or thyroid status (Figure 4). Concentrations of ERβ were very high relative to S16, increased in days 14 and 21 of pregnancy with respect to day 7 and fell on day 1 postpartum. The concentrations increased again after weaning in the control rats (Figure 4). T_4 treatment diminished ERβ on days 7 and 14 of pregnancy, but on day 21 the values had recuperated to levels similar to controls. There were no differences after delivery, but the increase observed in the weaned rats was abolished in this group (Figure 4).

PgRs showed the well known decrease in early postpartum that was not modified after 3 days of weaning (Figure 5). T_4 treatment had no effect on the expression of this receptor.

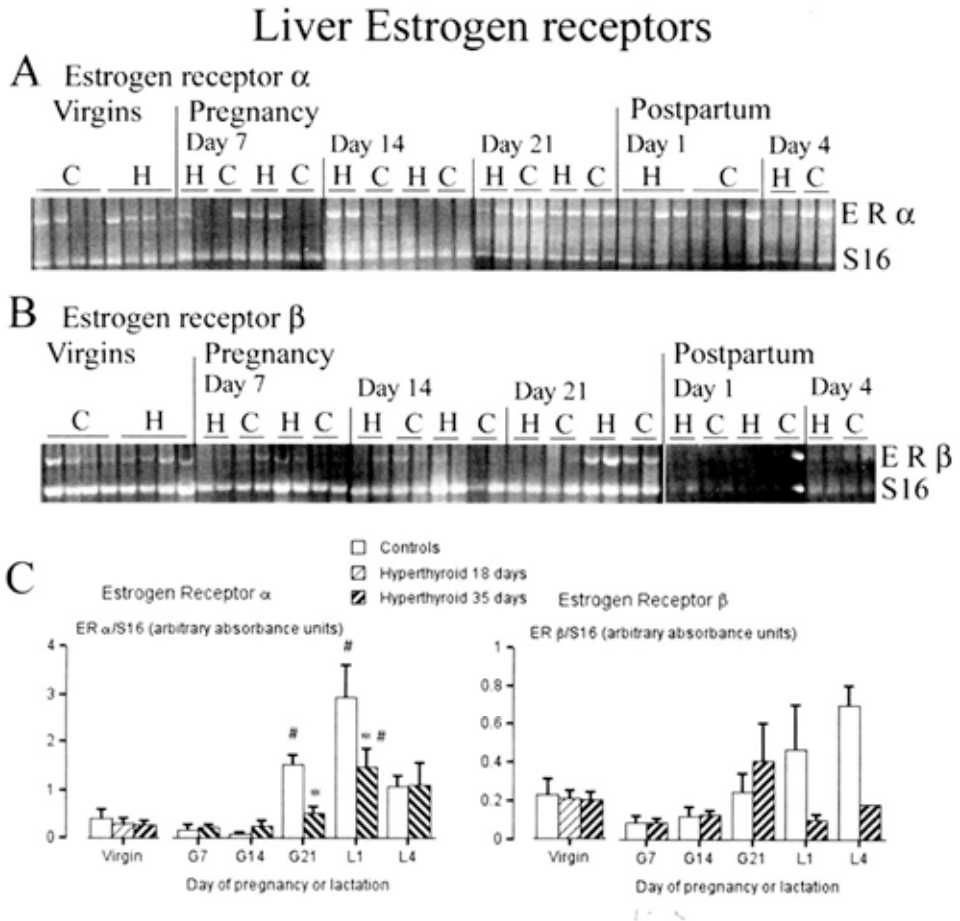

FIGURE 2 Expression of liver estrogen receptor forms in control (C) and Hyperthyroid (H) rats during days 7, 14, 21 of pregnancy and days 1 and 4 of lactation.

Panels A and B: Measurement by RT-PCR of expression of S16 and Estrogen receptor α and β respectively. Ethidium bromide fluorescence photograph of the gel electrophoresis of the coamplification products; Lane M: molecular weight markers. Panel C: Relative expression of the receptors during pregnancy, postpartum and early weaning. The gel photographs were quantified using NIH Image and expressed as arbitrary units. Results are expressed as the average ± s.e.m of groups of 4 rats.
*$p < 0.05$ compared with the respective control groups using two-way ANOVA.
#$p < 0.05$ compared with the respective virgin group using two-way ANOVA

DISCUSSION

Although the effects of hyperthyroidism are attenuated during pregnancy, it has been shown to advance parturition and to be particularly detrimental to lactation (6,7). In this study we have investigated the pattern of expression of ERα, ERβ, PRL-R_L, PRL-R_S and PgR in liver and mammary gland during pregnancy and early lactation as part of the study on the role

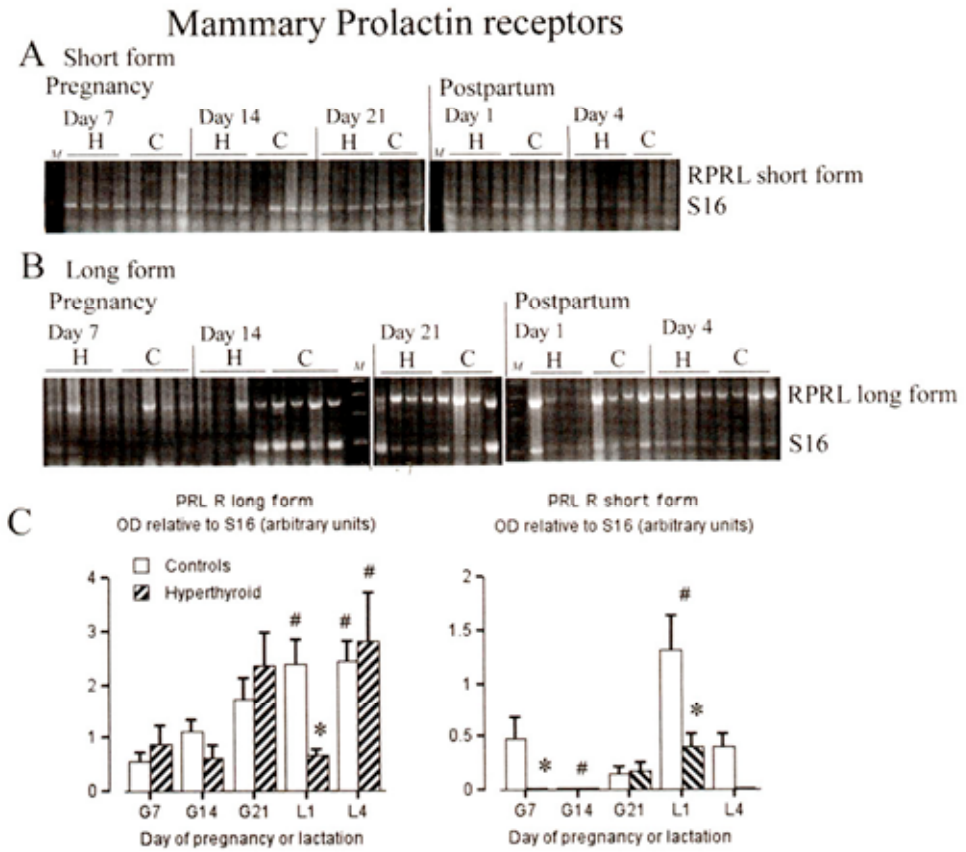

FIGURE 3 Expression of mammary prolactin receptor forms in control (C) and Hyperthyroid (H) rats during days 7, 14, 21 of pregnancy and days 1 and 4 of lactation.

Panels A and B: Measurement by RT-PCR of expression of S16 and the short or long forms of the prolactin receptor, respectively. Ethidium bromide fluorescence photograph of the gel electrophoresis of the coamplification products; Lane M: molecular weight markers. Panel C: Relative expression of the receptors during pregnancy, postpartum and early weaning. The gel photographs were quantified using NIH Image and expressed as arbitrary units. Results are expressed as the average ± s.e.m of groups of 4 rats.
*$p < 0.05$ compared with the respective control groups using two-way ANOVA.
#$p < 0.05$ compared with the respective G7 group using two-way ANOVA.

of the impact of thyroid hormone excess in mammary gland development, especially during the transition from pregnancy to lactation.

The liver is a major target organ of thyroid hormone. It has been estimated that approximately 8% of the hepatic genes are regulated by thyroid hormone *in vivo* and thus the liver is an ideal tissue to study gene regulation by thyroid hormone. Feng et al. (19) used a quantitative fluorescent cDNA microarray to identify 2,225 different hepatic genes regulated by thyroid hormone, but did not include several hormonal receptors. 17β-Estradiol (E_2), in addition to its various effects as a reproductive hormone, is a potent

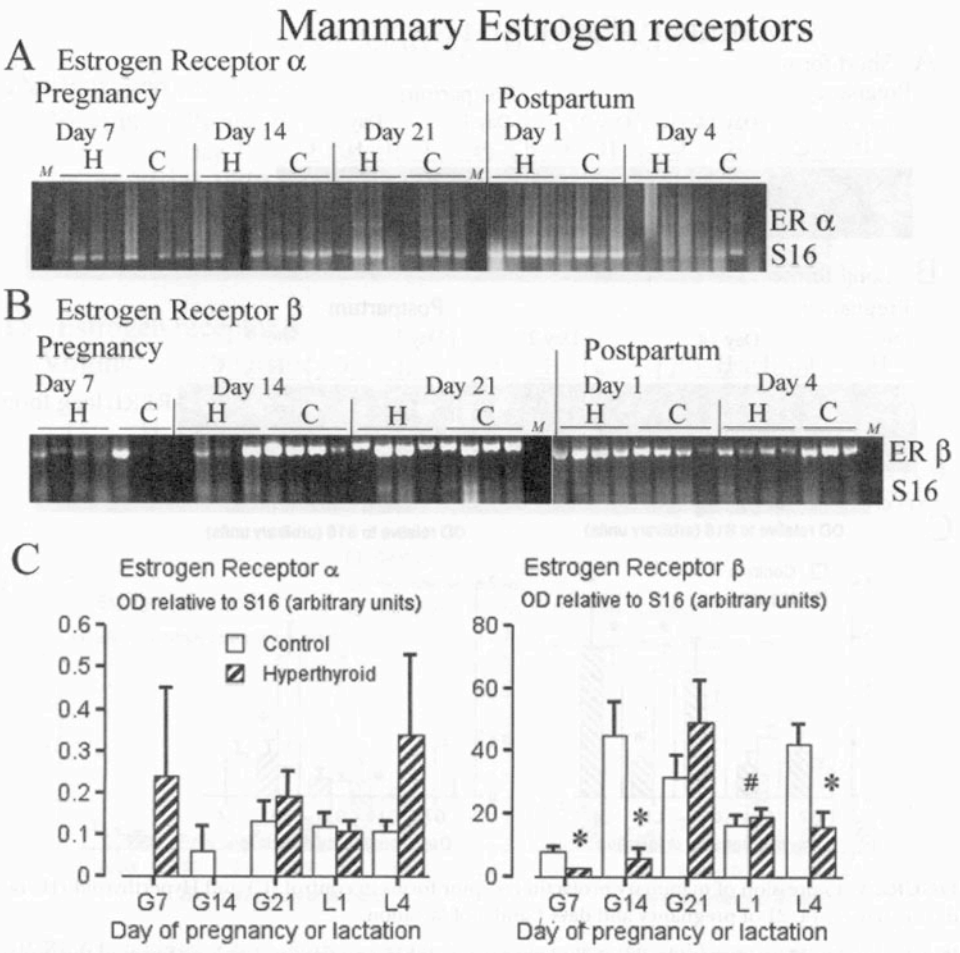

FIGURE 4 Expression of mammary Estrogen receptor forms in control (C) and Hyperthyroid (H) rats during days 7, 14, 21 of pregnancy and days 1 and 4 of lactation.

Panels A and B: Measurement by RT-PCR of expression of S16 and Estrogen receptor α and β respectively. Ethidium bromide fluorescence photograph of the gel electrophoresis of the coamplification products; Lane M: molecular weight markers. Panel C: Relative expression of the receptors during pregnancy, postpartum and early weaning. The gel photographs were quantified using NIH Image and expressed as arbitrary units. Results are expressed as the average ± s.e.m of groups of 4 rats.
*$p < 0.05$ compared with the respective control groups using two-way ANOVA.
#$p < 0.05$ compared with the respective G7 group using two-way ANOVA.

endogenous antioxidant and its actions on liver are mediated through ERα and ERβ (20). Tang et al. (21) reported that it is also possible that, in the presence of normal levels of estrogen, clinical hyperthyroidism might alter the estrogenic responses in target cells since excess thyroid hormones may mimic the action of E_2. Thyroid hormone elevation has been shown to impair maternal behavior and lactation in rats (6,9). Reproductive behavior

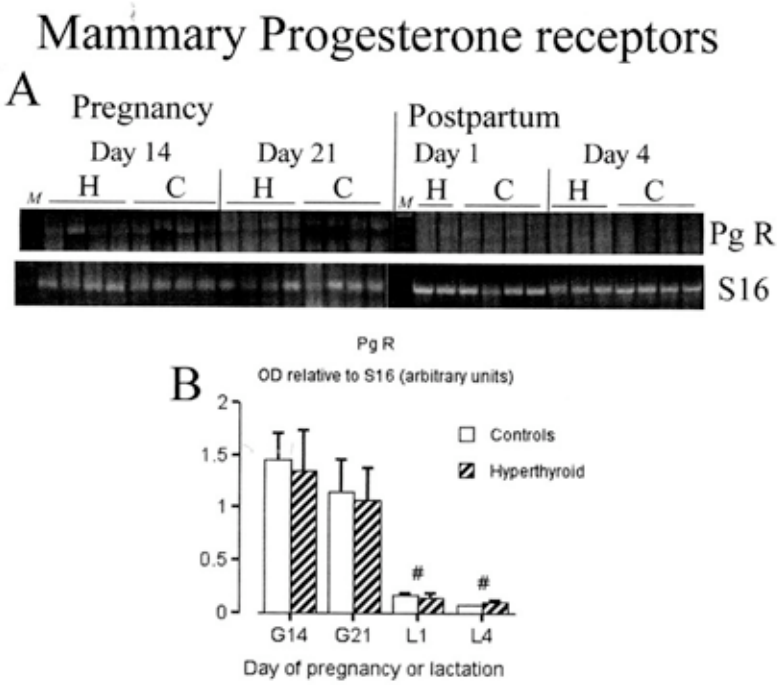

FIGURE 5 Expression of mammary progesterone receptor in control (C) and Hyperthyroid (H) rats during days 14, 21 of pregnancy and days 1 and 4 of lactation. Panel A: Measurement by RT-PCR of expression of S16 and progesterone receptor. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products; Lane M: molecular weight markers. Panel B: Relative expression of the receptor during pregnancy, postpartum and early weaning. The gel photographs were quantified using NIH Image and expressed as arbitrary units. Results are expressed as the average ± s.e.m of groups of 4 rats.

$^{\#}p < 0.05$ compared with the respective pregnant group using two-way ANOVA.

is controlled by estrogen via the ER and it is possible that reductions in ERs mediated by the thyroid hormone excess could be responsible for the observed impairments.

In concordance with results obtained by Pelletier (22) we detected that ERα is the predominant estrogen receptor in liver at all times analyzed, and seems to be the one subjected to regulation during pregnancy, since there were no significant variations in ERβ mRNA concentrations. Our results are in agreement with our previous studies on the liver (7), showing a decrease in estrogen binding on day 21 of pregnancy but no changes in the Kds and serum estradiol. Additionally, estrogen binding is very low in euthyroid pregnant rats when compared with published values from virgins (23). The present results show that the decrease in relative ERα mRNA concentrations between virgin and G14 agrees with these results, but, in contrast, in G21 and on L1 and L4 we found four to six fold

increases compared to virgin values. This increase is parallel to, and may be caused by, the marked increase in circulating E_2 observed before delivery (24) since it is well known that E_2 stimulates the expression of its receptors. We previously found no effect of T_4 treatment in liver E_2 binding on day 20 of pregnancy (7), which is in contrast with the decrease observed in ERα mRNA abundance on day 21 of pregnancy in the hyperthyroid rats. These differences may be caused by differential regulation of mRNA and protein expression, as has been shown for ERβ (10,11), plus differences in down regulation of ER protein after binding to its ligand. We have also shown that hyperthyroidism decreased circulating GH on G20 and G21 and produced a modest increase in PRL (6,7). Since these two hormones regulate ER expression in the liver, with a more important effect of GH (25,26), the observed decrease in ER mRNA abundance observed in the hyperthyroid rats on G21 and L1 may be caused by the marked diminution in serum GH.

The effects of T_4 on the relative PRL-R mRNA abundances showed variations similar to those previously observed in liver PRL binding (7), indicating that the decrease in PRL binding may be a consequence of a decrease in PRL-R_L mRNA abundance. Since hyperthyroidism advances luteolysis and delivery (6,7), and there is an abrupt fall in liver PRL-R expression after delivery (12), the decreased PRL-R_L expression on G21 in the hyperthyroid rats may be a direct consequence of the advancement in delivery.

In contrast with the previously observed attenuation of the effects of HT on liver weight, protein and lipid parameters in pregnant vs. virgin rats (5), the T_4 regimen did not modify PRL-R or ER in virgin rats, but had significant effects on the end of pregnancy, most markedly on ERα and PRL-R_L.

At the end of pregnancy, the pattern of changes in circulating hormones (most importantly, the fall in Pg and increases in PRL and E_2) trigger the initiation of milk synthesis (lactogenesis) in the mammary gland, and prepare the tissue for the subsequent lactation, enabling it to respond to the suckling stimulus. It has been extensively demonstrated that Pg is the factor that blocks lactogenesis (27). Thus the fall in circulating Pg after luteolysis enables PRL or other lactogenic factors to initiate milk synthesis, partly through induction of PRL–R synthesis (12). This increase in PRL-R (12) accompanied by a decrease in PgR (28) optimizes the capacity of the gland to respond to PRL secreted by suckling.

In contrast to liver, the predominant ER in mammary glands was ERβ, which also showed significant variations during pregnancy and after T_4 treatment. ERβ increased from early to late pregnancy and this increase was delayed by T_4 treatment but on day 21 the values were similar to controls, in contrast with the decrease previously observed in E_2 binding, which may be caused by differential timing of changes in mRNA and protein expression. The decrease observed on L1 in both groups of rats may be caused by down regulation by the

increased E_2 secretion due to the postpartum ovulation. Similarly, the long form of the PRL-R was predominant in mammary tissue, and showed significant variations during pregnancy and postpartum, with the highest values observed on L1, confirming previous results (12,13). The short form also showed the highest values on L1. Both forms were significantly diminished by T_4 treatment on L1, which along with the decreased serum PRL observed on the hyperthyroid rats on this day (8), may be one of the causes of the lactation failure.

Surprisingly, after three days weaning, relative PRL_L mRNA concentrations had remained similar to those found on L1 in the control rats, and had returned to high values in the T_4-treated rats.

On the other hand, in contrast with our previous results that showed a decrease in Pg binding in the hyperthyroid rats on G20 (7), PgR mRNA concentration was not affected by T_4, but showed the postpartum decrease that plays a role in the initiation of milk synthesis.

The inhibitory effects of HT on the expression of mammary and hepatic ER and PRL-R were most pronounced in the period immediately preceding or after delivery and may have affected the actions or metabolism of these hormones in their target tissues. Since the cell responses to hormones are believed to be related to the amount of receptors present in the cells, the decreased mammary hormone receptor expression observed at the end of pregnancy may have impaired the responsiveness of the tissue and its ability to produce milk on the subsequent lactation, thus being at least partially responsible for the lactation failure of the HT mothers.

ACKNOWLEDGEMENTS

The authors express their thanks to Dr. Carlos M. Telleria for his assistance and helpful comments in the elaboration of this work.

This work has been supported by grants PMT-PICT 06877 from the Agencia de Promoción Científica y Tecnológica, Argentina, the PLACIRH (Programa Latinoamericano de Capacitación e Investigación en Reproducción Humana) and PROIPRO 2 03–03, Universidad Nacional de San Luis. The authors are indebted to Dr. D. Gardella de Rodriguez, Glaxo, Argentina, for the gift of T_4. GAJ is Career Scientist from CONICET and SMV was financed with a fellowship from PLACIRH.

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