Effect of hypothyroidism on the expression of nuclear receptors and their co-regulators in mammary gland during lactation in the rat

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ABSTRACT

Thyroid hormones (TH) regulate mammary function. Hypothyroidism (HypoT) has deleterious effects on lactation, litter growth and survival. We analyzed the effect of chronic 6-propyl-2-thiouracil (PTU)-induced HypoT in the expression of nuclear receptors, co-regulators and oxytocin receptor (OTR) on lactation (L) days 2, 7 and 14. TH receptors (TRs) were increased on L7 at mRNA and protein levels, except TRα protein, that fell on L14. HypoT decreased TRα2 mRNA on L7 and TRα1 protein on L2, while TRβ1 protein increased on L14. HypoT increased estrogen receptor β (ERβ) mRNA on L7 but decreased its protein levels on L14. Progesterone receptor A (PRA) mRNA decreased from L2 to L14 while PRB increased, and at protein levels PRA levels showed a nadir on L7, while PRB peaked. HypoT decreased PRA mRNA and protein and increased PRB mRNA at L14. Nuclear receptor co-activator (NCOA) 1 and RXRα mRNA showed an opposite pattern to the TRs, while NCOA2 increased at L14; HypoT blocked the variations in NCOA1 and NCOA2. HypoT increased NCOR1 on L2 and decreased OTR at L2 and circulating estradiol and NCOR2 at L14.

In controls the most notable changes occurred on L7, suggesting it is a key inflection point in mammary metabolism. The low levels of TRα1, NCOA1 and OTR, and increased NCOR1 produced by HypoT on L2 may hinder the mammary ability to achieve normal milk synthesis and ejection, leading to defective lactation. Later on, altered ER and PR expression may impair further mammary function.

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1. Introduction

The lactating mammary cell can be considered as a major example of cell differentiation (Lemay et al., 2007), driven by hormonal signals that control its proliferation and differentiation and lead to the acquisition of the ability to produce, store and secrete milk. Nuclear receptors and their ligands such as ovarian and adrenal steroids, thyroid hormones (TH), etc. comprise, along with lactogenic hormones and their receptors, the main factors controlling mammary development and function. Understanding the mechanisms by which nuclear receptors control their expression, activation and interactions is critical for the knowledge of hormone action (Bagamasbad and Denver, 2011).

TH have significant effects on mammary function and lactation. The maintenance of a precise TH balance at systemic and mammary levels is essential for the adequate initiation and progress of lactation. Both hypothyroidism (HypoT) and hyperthyroidism

(HyperT) impair milk ejection, diminish milk quality and alter mammary and hepatic lipid metabolism, resulting in increased pup mortality and impaired growth (Hapon et al., 2003, 2005, 2007; Rosato et al., 1992a, 1992b; Varas et al., 2002). They also affect the mammary gland indirectly, through effects on circulating hormones, such as corticosterone, prolactin (PRL) and progesterone (P$_4$) (Hapon et al., 2003; Rosato et al., 1992a) and produce precocious involution of the mammary gland (Varas et al., 2002). HypoT virgin rats show mammary development equivalent to midgestation with increased casein and lactose content (Hapon et al., 2005). It has been demonstrated that triiodothyronine (T$_3$) antagonizes PRL signaling through inhibition of signal transducer and activator of transcription 5 a (STAT5a) mediated transcriptional activity (one key component of PRL signaling pathway) (Favre-Young et al., 2000). Highlighting the necessity of exposure of the mammary tissue to precise TH concentrations, it has been shown that PRL regulates mammary deiodinase 1 (Dio1) and TH receptors (TR) α1 and β1 expression during the transition from pregnancy to lactation as well as in established lactation (Aceves et al., 1999; Anguiano et al., 2004).

The regulation and interactions among estrogen receptors (ER) and P$_4$ receptors (PR) (Mehta et al., 2005) have been amply described in the mammary gland. Thus, ER regulation is exerted mainly

through auto repression; in lactating mammary gland estrogen treatments decrease gene expression of ERα and ERβ (Hatsumi and Yamamuro, 2006). PR expression is induced mainly by estrogens through binding to the estrogen response element (ERE) in the PR gene (Bagamasbad and Denver, 2011), and is inhibited at transcriptional and posttranscriptional levels by self-repression. However, less is known of the regulation of TRs at mammary level and the interaction between TRs and steroid hormone receptors. In mammals, regulation of TR expression is mainly isoform dependent, and possibly also tissue dependent (Bagamasbad and Denver, 2011). THs enhance ER induced gene expression in rat liver (Freyschuss et al., 1994) and ERα mRNA in GH3 cells (Fujimoto et al., 2004). TRs and ERs also can interact at the level of their response elements in target genes or compete for the binding to co-activators (Vasudevan et al., 2002a).

The mammary gland also expresses numerous nuclear receptor co-regulators, which can interact with ERs, PRs and TRs. Thus, slight changes in the expression rates of these proteins may have significant effects on mammary differentiation, function or pathophysiology. For example, estrogens regulate the mRNA levels for several co-regulators (Frasor et al., 2003; Lai et al., 2003; Lauritsen et al., 2002; Shi et al., 2001; Thenot et al., 1999). Estrogens also downregulate nuclear co-repressor (NCOR) protein expression in mammary cancer cells and thus alters the actions of other nuclear receptors in the same cell (Frasor et al., 2005). Nuclear co-activator 1 (NCOA1), NCOR1 and NCOR2 are present in mammary tissue and their abundance is higher in glands from post lactating compared with virgin rats (Macejova et al., 2005). Retinoid X receptor (RXR) heterodimerizes with the ligand-bound TRs modulating their transcriptional activity (Yen et al., 2001) and its levels vary with the physiological state of the animal, suggesting a role in the maintenance of mammary function (Capuco et al., 2008; Macejova et al., 2005).

Based on this evidence, we hypothesize that the lactation deficit produced by HypoT may be mediated by changes in the expression of the nuclear receptors and their regulating proteins, whose interactions finely tune the metabolism and function of the mammary cells. In order to explore this hypothesis, we analyze in this work the effect of propylthiouracyl (PTU)-induced HypoT in the expression of nuclear receptors and transcription co-regulators during lactation in key stages of mammary cell differentiation such as the beginning of the differentiation or early lactation (L2), mature lactation (L7) and maintenance lactation (L14).

2. Materials and methods

2.1. Animals

Adult female Wistar rats bred in our laboratory, 3–4 months old and weighing 190–210 g at the onset of treatment, were used. The rats were kept in a light (lights on 06:00–20:00 hours) and temperature (22–24 °C) controlled room. Rat chow (Cargill, Cordoba, Argentina) and tap water or 6-propyl-2-thiouracil (PTU) solution were available ad libitum. HypoT was induced by administration of PTU at a concentration of 0.1 g/L in drinking water. The treatment was started 8 days before the onset of pregnancy. Vaginal smears were taken daily and the rats were caged with a fertile male on the night of proestrus. The presence of spermatozoa in the vaginal smear the morning after was indicative of pregnancy, and this day was counted as day 0 of pregnancy. On day 1 of lactation, the number of pups in each litter was standardized to eight, and mothers and litters were weighed weekly. Animal maintenance and handling was performed according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication N8 86-23, revised 1985 and 1991), the UK requirements for ethics of animal experimentation (Animals

Scientific Procedures Act 1986), and the FRAME guidelines of 1999. Groups of 8 PTU or control (Ctrl) rats were killed on days 2, 7 and 14 of lactation at 09:00–12:00 hours by decapitation. After decapitation, trunk blood was collected, and serum was separated by centrifugation and stored at –20 °C until used. The inguinal mammary glands from the dams were removed, snap frozen in liquid nitrogen, and stored at –70 °C until they were analyzed.

2.2. Determination of hormone concentration

Estradiol (E_2) concentrations in sera were measured by radioimmunoassay using commercial kits for total hormones (Ke2d1 double antibody radio immune assay from Siemens Healthcare Diagnostic Inc USA).

2.3. RNA isolation and RT-real time PCR analysis

Total RNA from mammary glands was prepared using the guanidinium isothiocyanate-acid phenol method as modified by Puissant and Houdebine (1990). Ten micrograms of total RNA was reverse transcribed at 37 °C using random hexamer primers and Moloney murine leukemia virus retrotranscriptase (Invitrogen-Life Technologies, Buenos Aires, Argentina) in a 20 μL reaction mixture. The RNA was first denatured at 70 °C for 5 min. in the presence of 2.5 μg of random hexamer primers (Invitrogen). For the subsequent RT reaction the following mixture was added: RT buffer [50 mM Tris–HCl (pH 8.4), 75 mM KCl, 3 mM MgCl$_2$], 0.5 mM dNTPs, 5 mM DTT, 200 units M-MLV Reverse Transcriptase (Invitrogen). The reaction was incubated at 37 °C for 50 min., next, the reaction was inactivated by heating at 70 °C during 15 min. The cDNA was stored at –20 °C.

The mRNA levels of TRs, ERs, PRs, NCOA1/2, NCOR1/2, RXRα and oxytocin receptor (OTR) were estimated by RT real time PCR with a Corbett Rotor Gene 6000 Real-Time Thermocycler (Corbett Research Pty Ltd (Sydney, Australia) using rat-specific primers and reaction conditions described in Table 1. The PCR reactions were performed using a Corbett Rotor Gene 6000 Real-Time Thermocycler using Eva-Green™ (Biotium, Hayward, CA) in a final volume of 20 μL. The reaction mixture consisted of 2 μL of 10×PCR Buffer, 1 μL of 50 mM MgCl$_2$, 0.4 μL of 10 mM dNTP Mix (Invitrogen), 1 μL of 20× Eva Green, 0.25 μL of 5 U/μL Taq DNA Polymerase (Invitrogen) 0.1 μL of each 2.5 mM primer (forward and reverse primers) and 10 μL of diluted cDNA. The PCR reactions were performed at the conditions described on Table 1. Melt curve analysis was used to check that a single specific amplified product was generated. Real time quantification was monitored by measuring the increase in fluorescence caused by the binding of EvaGreen dye to double-strand DNA at the end of each amplification cycle. Relative expression was determined using the Comparative Quantitation method of normalized samples in relation to the expression of a calibrator sample, according to the manufacturer's protocol (Pfaffl, 2001). Each PCR run included a no-template control and a sample without reverse transcriptase. All measurements were performed in duplicate. The reaction conditions and quantities of cDNA added were calibrated such that the assay response was linear with respect to the amount of input cDNA for each pair of primers. RNA samples were assayed for DNA contamination by performing the different PCR reactions without prior reverse transcription. To select the reference gene, we estimated the expression stability of four candidate reference genes, β-actin, S16, GAPDH and HPRT1 using the freely available online software BestKeeper version 1 [http://gene-quantification.com/bestkeeper.html]. This approach allowed us to select S16 as the reference gene since it showed the lowest coefficient variation along lactation and between treatment groups compared to β-actin, GAPDH and HPRT1. Relative levels of mRNA were normalized to S16 reference gene. The

Table 1
Sequences and conditions for the PCR reactions.

Primer		Sequence	GenBank accession no	Annealing T°	Reference
TRα1	Sense	TGCCCTTACTCACCCCTACA	NM_001017960.1	60	Navas et al. (2014)
	Antisense	AAGCCAAGCCAAGCTGTCCT			
TRα2	Sense	TGAGCAGCAGTTTGGTGAAG	NM_031134.2	60	Navas et al. (2014)
	Antisense	GAATGGAGAATTCCGCTTCG			
TRβ1	Sense	AGCCAGCCACAGCACAGTGA	J_03819.1	60	Navas et al. (2014)
	Antisense	CGCCAGAAGACTGAAGCTTGC			
ERα	Sense	TGCCTCTGGCTACCATTATGG	NM_012689.1	62	Bonafede et al. (2011)
	Antisense	TATGTCCTTGAATGCTTCTCTTAAAGAA			
ERβ	Sense	TGAGCAAAGCCAAGAGAAACG	NM_012754.1	62	Bonafede et al. (2011)
	Antisense	CCAGTTGCTCTGGACTCAAGGT			
PR A + B	Sense	GGTCTAAGTCTCTGCCAGGTTTCC	NM_022847.1	60	Bonafede et al. (2011)
	Antisense	CAACTCCTTCATCCTCTGCTCATTC			
PR B	Sense	GCATCGTCTGTAGTCTCGCCAATAC	NM_022847.1	60	Bonafede et al. (2011)
	Antisense	GCTCTGGGATTTCTGCTTCTTCG			
OTR	Sense	TGTTCGCCTCCACCTACC	NM_012871.2	58	Feng et al. (2009)
	Antisense	TTCACCGCCTGCCTCAGA			
NCoA1	Sense	TGCTCCCGAGGAGGTTAAA	NM_01108012.1	55	(*)
	Antisense	ATCAAACTGGTCAAGGTCAGC			
NCoA2	Sense	CTGTGAAGGAGGAGGTGAGC	NM_031822.1	60	(*)
	Antisense	TCCAAAATCTCTTCCAAGTTGTC			
NCoR1	Sense	AGTCGCTACAGCCCAGAGTC	NM_01271103.1	60	Malik et al. (2010)
	Antisense	CTCCTCTCTGGGGATTTTCC			
NCoR2	Sense	CCGAACACCACTCACCAA	NM_001108334.1	60	(*)
	Antisense	ACCTGCCTGTAGCCTCATA			
RXRα	Sense	GAGGACATGCCTGTAGAGAAGATT	NM_012805.2	62	Nasrollahzadeh et al. (2008)
	Antisense	ACAGATGTTGGTAACAGGGTCATT			
S16	Sense	TCCAAGGGTCCGCTGCAGTC	NM_001169146.1	60	Navas et al. (2014)
	Antisense	CGTTCACCTTGATGAGCCCATT			

The real-time PCR reactions were carried out for 40 cycles with an initial step of 5 minutes at 95 °C followed by a three step scheme: 30 s at 95 °C, 30 s at the annealing temperature shown above for each primer pair, and a final step at 72 °C for 30 s, except for OTR, in which the final step was carried out for 60 s.
(*) These primers were designed with the software Beacon Design 7.92, of Premier Biosoft International, Palo Alto, CA.

real time PCR products were analyzed on 2% agarose gels containing 0.5 mg/mL ethidium bromide and a unique band of the approximately correct molecular weight corresponded with a unique peak in melt curve analysis.

2.4. Protein isolation and western blot analysis

Total proteins were extracted from mammary gland by homogenization in 2 volumes of homogenization buffer (50 mM Tris, pH 7.5, 250 mM sucrose, 10 mM benzamidine, 10 mM NaF, 5 mM sodium pyrophosphate, 20 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM p-nitrophenylphosphate, and aprotinin, leupeptin, and pepstatin at 2 mg/L) in an ice bath. The homogenate was centrifuged at 10,900 *g* for 30 min and the supernatant was separated and frozen in several aliquots at –80 °C until used. Proteins were quantified using the BCA method and boiled 3–5 min in loading buffer. Eighty micrograms of protein was separated by SDS–PAGE and electrotransferred to Nitrocellulose Hybond membranes as described previously (Valdez et al., 2007). After rinsing and blocking with BSA 2% the membranes were probed with anti-TRs, PR, ERs (anti-TRα/β rabbit polyclonal antibody sc-772, anti-TRβ mouse polyclonal antibody sc-738, anti-ERα rabbit polyclonal antibody sc-7207, Santa Cruz Biotechnology Inc Dallas TX, anti-ERβ rabbit polyclonal antibody AB3577 Abcam, Cambridge, MA; and anti-PR A + B rabbit polyclonal antibody PR130 was generated and tested in Endocrinology and Hormone Dependent Tumors Laboratory of the National University of Litoral, Santa Fe, Argentina), using horseradish peroxidase-conjugated secondary antisera (1/2000 polyclonal goat anti-rabbit, rabbit anti-goat and goat anti mouse inmunoglobulin (Dako Cytomation-CA) and chemiluminiscence (Amersham ECL™, GE Healthcare, Buenos Aires, Argentina) to detect specific bands that were quantified by densitometry using FIJI Image processing package (Schindelin et al., 2012). The membranes were reprobed with anti-β-actin (1/10,000 mouse monoclonal antibody, Sigma. St. Louis, MO, USA) and horseradish peroxidase-conjugated secondary antisera (1/2000 polyclonal goat anti-mouse immunoglobulins Dako Cytomation-CA) as loading and transfer control.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism™ (GraphPad Software Inc., La Jolla, CA) and two way ANOVA followed by the Bonferroni post hoc test to compare any two individual means. Differences between means were considered significant at the $p < 0.05$ level.

3. Results

3.1. Effect of HypoT on the expression of mammary TR isoforms through early and midlactation

The mRNA levels of the three isoforms analyzed (TRα1, TRα2 and TRβ1) were much higher in L7 compared to values on L2 and L14 (Fig. 1A). HypoT did not alter this pattern for TRβ1 and TRα1, but blocked the increase in TRα2 mRNA levels on L7, that remained similar to the other 2 days of lactation (Fig. 1A).

In the control group TRα2 and TRβ1 protein levels showed a similar pattern to the mRNA level, with an increase in L7 compared to L2 and L14 (Fig. 1B,C). HypoT did not alter the TRα2 protein level pattern but TRβ1 protein levels remained elevated on L14, with values that were similar to those seen on L7, although with more variability (Fig. 1B,C). In contrast with the pattern observed in the other isoforms, in control rats TRα1 level was similar in L2 and L7 but declined in L14, while in the HypoT group this pattern was inverted, with values significantly lower than controls on L2, that increased slightly on L7 to reach maximum levels at L14 (Fig. 1B,C).
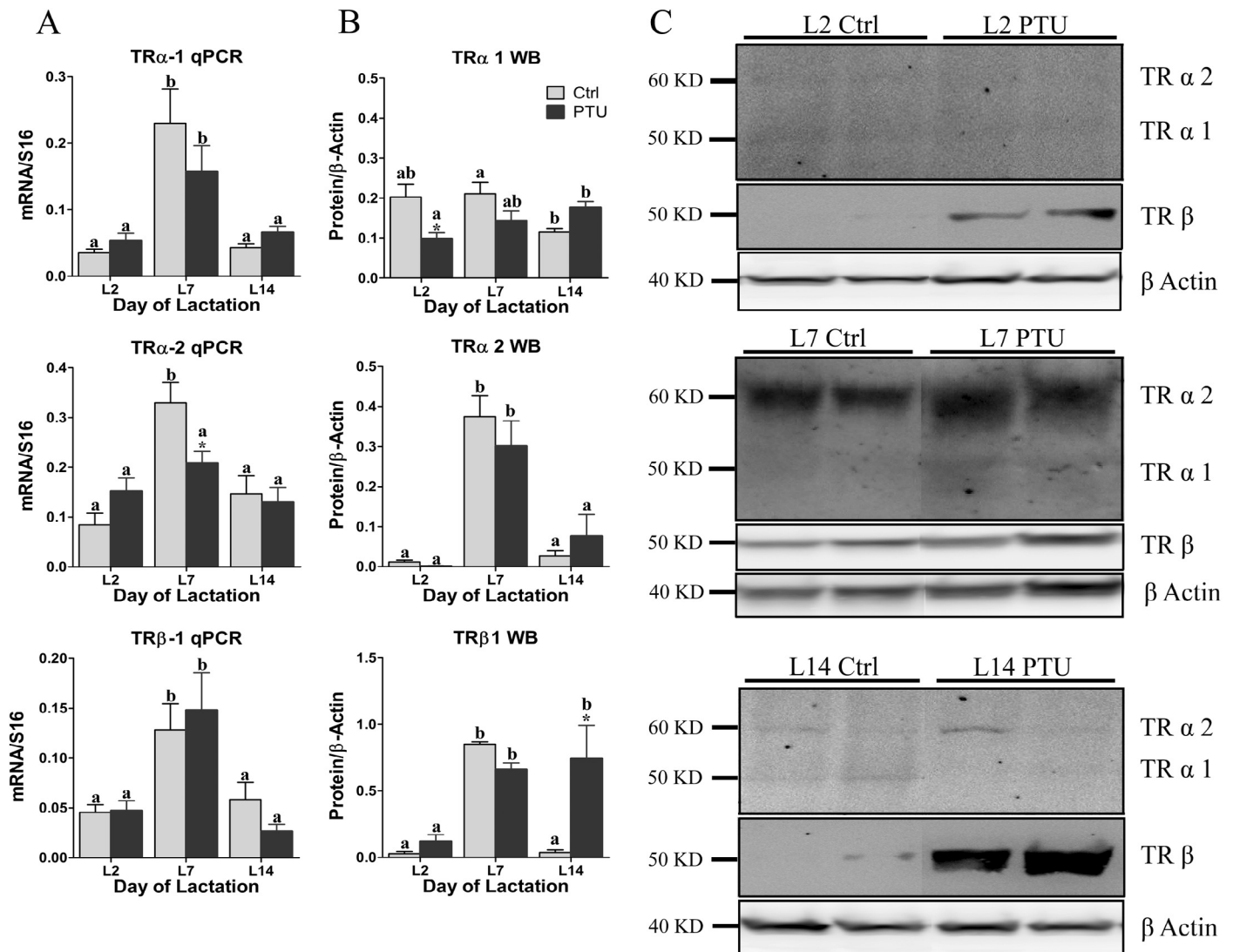
Fig. 1. Effect of PTU-induced HypoT on mammary mRNA (A) and protein (B,C) levels of thyroid hormone receptors (TRα1, TRα2 and TRβ1) relative to S16 and β-actin respectively on days 2–14 of lactation. Controls (gray bars), HypoT (PTU, black bars). mRNA levels were measured by real time RT-PCR and protein levels by Western blot using rat specific primers and antibodies respectively. The graphs may have different scales. Panel C shows representative Western blots for each day of lactation. Bars at the left of the images mark the position and size of the molecular weight markers. Arrows at the right show the position of the bands for each isoform. Values are means ± SEM for groups of 8 rats for PCR and 6 rats for WB. *p < 0.05 compared with the respective control group. Different superscript letters represent significant differences at p < 0.05 between different days of lactation within the same treatment groups.

3.2. Effect of HypoT on the expression of mammary ER isoforms through early and midlactation

During lactation in control rats ERα and ERβ mRNA levels increased gradually making L14 levels significantly higher than those found in L2 (Fig. 2A). HypoT did not alter ERα mRNA levels, but advanced the increase in ERβ mRNA levels to L7, when they were significantly higher compared with controls on the same day and with L2 values (Fig. 2A). ERβ protein levels followed a pattern similar to that of the mRNA levels in the control rats, with low values on L2 and L7 that increased significantly on L14. HypoT blocked this increase, keeping the levels constant on the 3 days of lactation (Fig. 2B,C). In contrast with the mRNA levels, ERα protein levels remained constant throughout lactation and HypoT had no significant effect, showing that HypoT does not alter protein level of this receptor in any stage of functional mammary differentiation (Fig. 2B,C).

3.3. Effect of HypoT on the expression of mammary PR isoforms through early and midlactation

Since the expression of PRs on mammary tissue is regulated by ER activation, and we found that HypoT decreased ERβ level, we also evaluated the expression level of PRA and B at both transcriptional and translational levels. Total PR (PR A + B) mRNA levels did not differ significantly among the 3 days studied, and there were no significant effects of HypoT (Fig. 3A). Receptor isoform B (PRB) mRNA, in contrast, increased with progression of lactation, with L14 expression levels in controls two-fold higher than in L2. Furthermore, HypoT increased even further the expression level of PRB mRNA, with values significantly higher than controls on L7 and L14 (Fig. 3A). Using the method proposed by Hayashi et al. (2012), we calculated the levels of PRA mRNA, finding an opposite pattern to PRB levels. Thus, PRA levels decreased gradually through lactation in the control group, with values at L14 significantly lower than on
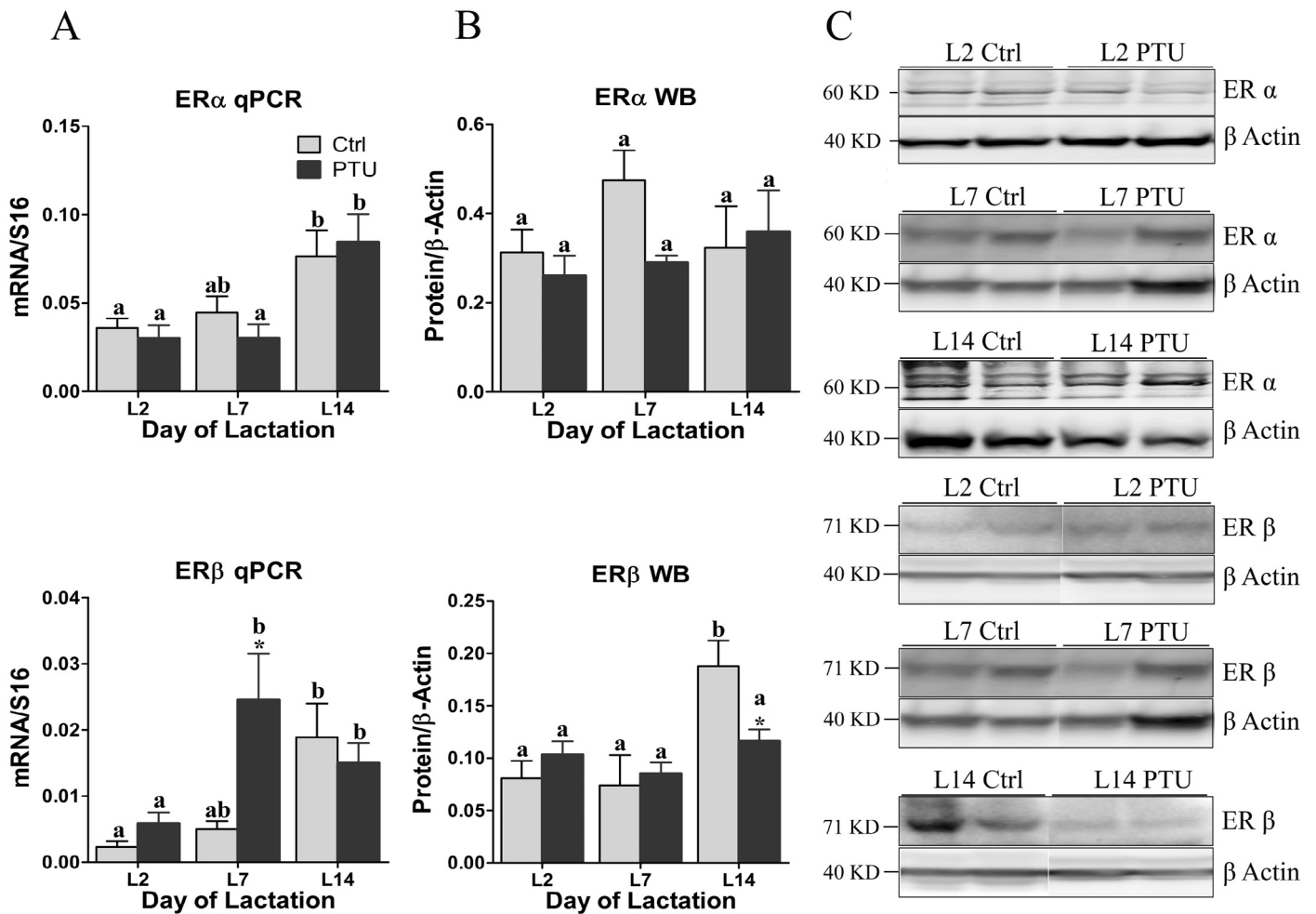
Fig. 2. Effect of PTU-induced HypoT on mammary mRNA (A) and protein (B,C) levels of ERs (ERα and ERβ) relative to S16 and β-actin respectively on days 2–14 of lactation. Controls (gray bars), HypoT (PTU, black bars). mRNA levels were measured by real time RT-PCR and protein levels by Western blot using rat specific primers and antibodies respectively. The graphs have different scales. Panel C shows representative Western blots for each day of lactation. Bars at the left of the images mark the position and size of the molecular weight markers. Arrows at the right show the position of the bands for each isoform. Values are means ± SEM for groups of 8 rats for PCR and 6 rats for WB. *$p < 0.05$ compared with the respective control group. Different superscript letters represent significant differences at $p < 0.05$ between different days of lactation within the same treatment groups.

L2 and HypoT magnified the decrease with values at L14 significantly lower that controls (Fig. 3A).

The pattern of PR expression at protein levels was markedly different from that observed at mRNA level; in controls, PRB protein level increased from L2 to L7 followed by a decrease in L14, while PRA showed the opposite variation, with high levels on L2 and L14 and significantly lower levels at L7. HypoT had no effect on PRB protein values, but the increase observed between L7 and L14 was partially blocked (Fig. 3B,C).

3.4. Effect of HypoT on the expression of OTR and serum levels of estradiol throughout lactation

We have previously shown that HypoT decreased oxytocin release and milk ejection in response to the suckling stimulus (Hapon et al., 2003), and the present results show that it also disturbs the expression profile of receptors that regulate OTR expression, such as ERs. There is also evidence that THs via TRα1 decrease ERα induction of OTR levels in neural cell lines (Vasudevan et al., 2001). For these reasons we also evaluated mammary OTR expression at the transcriptional level and circulating estradiol (E_2). Our results show that throughout lactation the mammary gland has a constant and high level of OTR mRNA in the control group. Although OTR mRNA

tended to be lower in the 3 days of lactation in the HypoT group, the values were significantly different from controls only on L2 (Fig. 4A). Circulating E_2 increased on L14 compared with the previous days in the control rats, and HypoT blocked this increase (Fig. 4B).

3.5. Effect of HypoT on the mammary expression of co-regulators of intracellular receptors through early and midlactation

One of the most important mechanisms of cross-regulation between intracellular receptors include squelching of co-regulated receptor expression through competition for binding to the co-regulators. To estimate the impact of HypoT on this regulation point, we assessed the mRNA level of the major receptor co-activators and co-repressors throughout lactation, as well as RXRα mRNA, that also regulates TR function through heterodimerization.

In control rats, NCOA1 mRNA levels were significantly lower on L7 compared with L2, but on L14 values increased to levels similar to L2. HypoT decreased NCOA1 levels on L2 and L14, resulting in constant mRNA levels throughout lactation (Fig. 5). In controls, NCOA2 mRNA levels increased significantly in L14, and as well as for NCOA1, HypoT blocked the increase in L14 (Fig. 5).
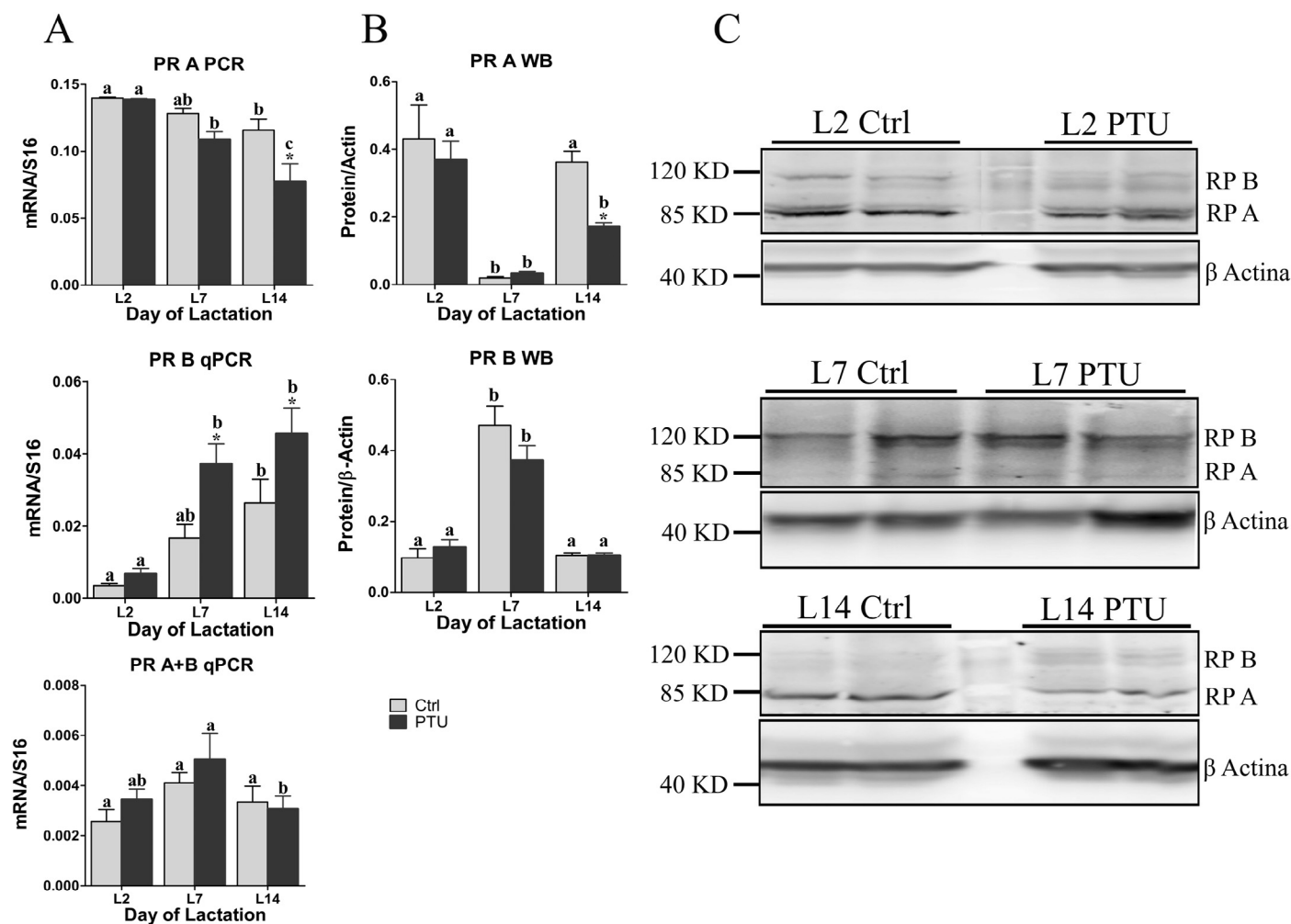
Fig. 3. Effect of PTU-induced HypoT on mammary mRNA (A) and protein (B,C) levels of PRs (PRA and PRB) relative to S16 and β-actin respectively on days 2–14 of lactation. Controls (gray bars), HypoT (PTU, black bars). mRNA levels were measured by real time RT-PCR and protein levels by Western blot using rat specific primers and antibodies respectively. The graphs may have different scales. Panel C shows representative Western blots for each day of lactation. Bars at the left of the images mark the position and size of the molecular weight markers. Arrows at the right show the position of the bands for each isoform. Values are means ± SEM for groups of 8 rats for PCR and 6 rats for WB. *$p < 0.05$ compared with the respective control group. Different superscript letters represent significant differences at $p < 0.05$ between different days of lactation within the same treatment groups.

In control rats, NCOR1 mRNA levels tended to decrease on L7, without achieving a significant difference. The HypoT group had significantly higher values on L2 that returned to values not different from controls on L7 and L14 (Fig. 5). NCOR2 mRNA levels were similar on the 3 days of lactation studied in control rats and the only effect of HypoT was a significant decrease on L14 (Fig. 5). RXRα mRNA levels showed a pattern similar to NCOA1, and opposite to the pattern observed in the TR isoforms mRNA (Fig. 1A) with values on L7 significantly lower than on L2 and L14, and in this case, HypoT had no significant effect on this pattern (Fig. 5).

These results reflect that HypoT dampens the fluctuation of co-activators mRNA during lactation but accentuates the variations of co-repressors mRNA.

4. Discussion

Normal thyroid balance is necessary for successful lactation, since HyperT and HypoT have deleterious effects in mammary development and milk production and ejection (Hapon et al., 2003; Varas et al., 2002). In addition to the changes in circulating THs, during early post-partum and lactation PRL finely tunes mammary Dio1 expression and activity, providing the optimal T₃ concentrations

(Aceves et al., 1999; Anguiano et al., 2004). Another potential regulatory point is differential expression of the TR isoforms expressed in mammary tissue. In mammary gland TRα has been linked to cell differentiation and apoptosis while TRβ is linked to metabolic functions (Brent, 2000; Yen, 2001); thus, changes in the differential expression of the isoforms may be important for mammary function at the different stages of lactation.

Between L2 and L7, the different expression patterns of TRα1 protein level compared with TRα2 and TRβ1 may be related to a specific function of TRα1, such as a promoter of mammary cell differentiation. We have previously described elevated levels of T₃ on L2, followed by much lower levels during the rest of lactation (Hapon et al., 2003). In this hormonal condition, TH binding to TRα1 could repress TRα2 and TRβ1 transcription in response to negative autoregulation similar to that observed in GH1 cells (Samuels et al., 1977). The low values, relative to L7, of TRα2 and TRβ1 mRNA and protein observed at L2 support the hypothesis of a protective mechanism raised by Anguiano et al. (2004). These authors proposed that in the peripartum the mammary gland is protected against deleterious actions of TH such as inducing premature involution (Varas et al., 2002) or blocking PRL-induced STAT5 activation (Favre-Young et al., 2000). Thus, the low relative protein levels of the TR isoforms
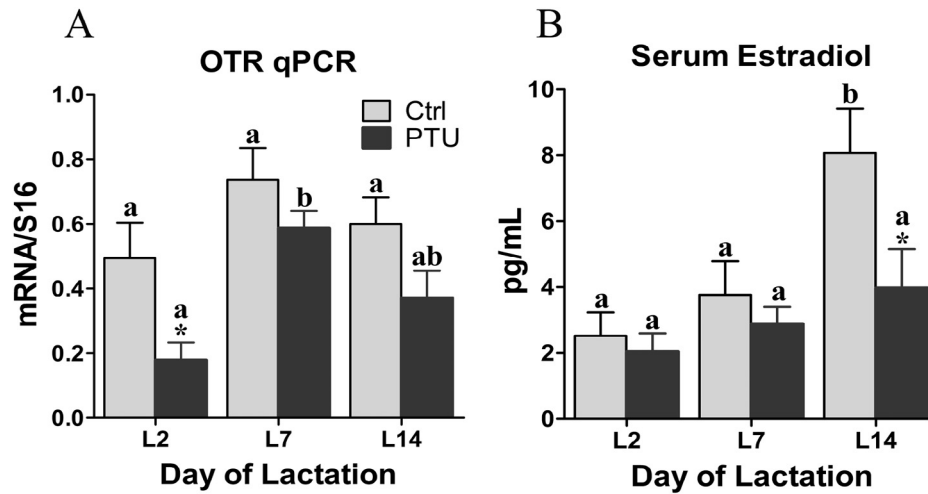
Fig. 4. Effect of PTU-induced HypoT on mammary mRNA levels of OTR relative to S16 and circulating estradiol on days 2–14 of lactation. Controls (gray bars), HypoT (PTU, black bars). mRNA levels were measured by real time RT-PCR using rat specific primers and estradiol was measured by RIA. Values are means ± SEM for groups of 8 rats. *p < 0.05 compared with the respective control group. Different superscript letters represent significant differences at p < 0.05 between different days of lactation within the same treatment groups.
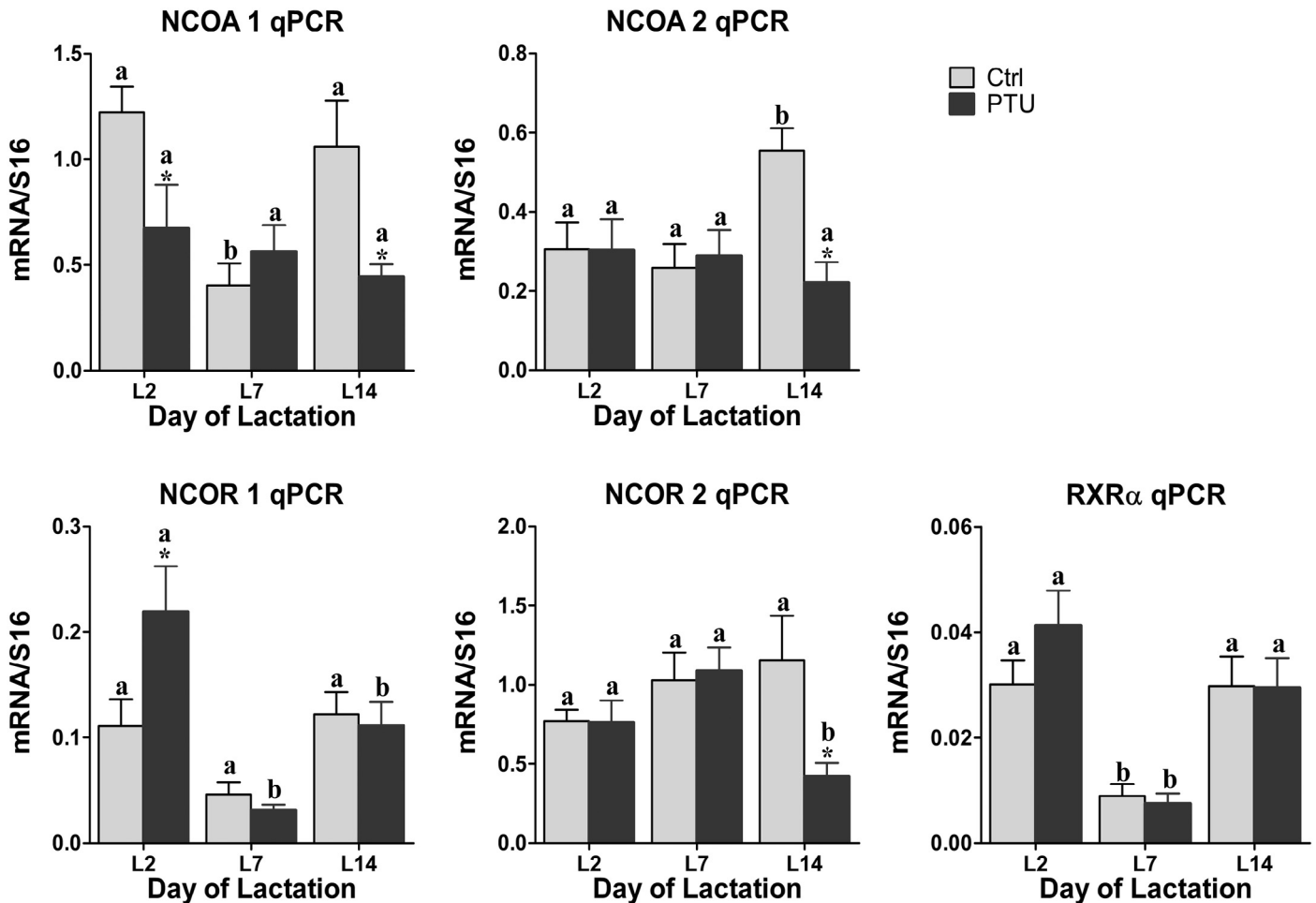

Fig. 5. Effect of PTU-induced HypoT on mammary mRNA level of co-regulators of intracellular receptors and RXR receptor relative to S16 on days 2–14 of lactation. Controls (gray bars), HypoT (PTU, black bars). mRNA levels were measured by real time RT-PCR. The graphs have different scales. Values are means ± SEM for groups of 8 rats. *p < 0.05 compared with the respective control group. Different superscript letters represent significant differences at p < 0.05 between different days of lactation within the same treatment groups.

we found in L2, along with the low Dio1 expression (Anguiano et al., 2004), protect the mammary tissue against any harmful effect of the elevated circulating T_3. On the other hand, the changes in TRα1, TRα2 and TRβ1 mRNA expression between L2 and L7 may be related to lower circulating TH levels in L7 (Hapon et al., 2003), that may lift the self-repression and allow for increased transcription of all isoforms of the receptor.

Between L7 and L9 mature lactation is achieved, when the mammary gland produces copious amounts of milk in response to the demands of the growing litter; this is a key stage between early and maintenance lactation (Anderson et al., 2007; Lemay et al., 2007). L7 represents a stage of high metabolic demand of the mammary cell, which coincides with increased protein levels of TRα2 and TRβ1 that may sensitize the mammary tissue in order to meet the high metabolic demand of this stage. Furthermore, from day 5 of lactation onwards, mammary Dio1 expression increases (Anguiano et al., 2004) providing additional T_3 from serum T_4 in a moment when circulating T_3 levels are lower. These results show that the protective mechanism against TH overexposure seen earlier on lactation may be no longer needed during this stage, when mammary tissue most possibly requires of TH action to achieve maximum milk production (Anderson et al., 2007).

In L14 or maintenance lactation, maximum alveolar development has been reached (Anguiano et al., 2004) and the lactome, the set of genes responsible for the synthesis of milk, is constitutively active (Lemay et al., 2007). At this stage the mammary cell function and lactome operation becomes largely independent of circulating PRL, that is much lower compared with earlier stages of lactation (Hapon et al., 2003). These events may reflect less metabolic demand in a period of low serum TH levels. Indeed the absence of self-induction may explain the decrease of mRNA and protein level of the three TR isoforms on L14 in control rats.

HypoT had differential effects on the expression of the mRNA and protein of the different isoforms. In L2, the decreased TRα1 protein levels may explain the reduced lobuloalveolar development shown in our earlier studies at the moment of lactogenesis (Hapon et al., 2005), since TRα1 has been linked to mammary cell differentiation (Brent, 2000; Yen, 2001). In L14 there are elevated levels of TRβ1 protein compared with the control group, even with low levels of TRβ1 mRNA. These differences between protein and mRNA levels indicate that HypoT may prevent the decrease of the protein by a transcription independent mechanism. The proteolytic machinery is reduced on early lactation (L1–L9) and up regulated afterwards, determining one of the most important molecular events in lactation (Lemay et al., 2007). Also, it has been shown that THs promote TR ubiquitination and proteosomal degradation in order to regulate the transcriptional activity of the receptors (Dace et al., 2000). Thus, the sustained TRβ1 expression in the HypoT group at L14 may be related to an inhibition of proteolysis of this receptor in order to promote cell sensitization to the low TH levels; however, this hypothesis should be further investigated.

On the other hand, HypoT had no effect on TRα2 protein levels, and a very modest effect on the mRNA levels of the three isoforms, only blocking the increase in TRα2 mRNA on L7 that was observed in the controls. TRα2 cannot bind THs, but can bind to thyroid response elements (TREs), and thus recruits co-repressors of transcription, acting as a repressor of the other isoforms (Izumo and Mahdavi, 1988). Thus, the differential effect of HypoT could be a compensatory mechanism for the low circulating TH levels, in which the expression is directed toward those isoforms capable of TH binding.

Cross-regulation within members of the different nuclear receptor families has been described. The TR capacity to control transcription of ERα and β, as well as self-regulation by E_2, is crucial in the control of reproductive function, and is tissue and reproductive stage dependent (Bagamasbad and Denver, 2011). Cross-regulation between

TRs and ERs is isoform and promoter dependent and implicates the participation of co-regulatory proteins (Vasudevan et al., 2001). Our results show that in control rats, circulating E_2, ERβ protein levels and mRNA levels of both ERs follow the same pattern, with maximum values in L14, suggesting self induction of ER mRNAs by E_2. HypoT did not alter mRNA or protein level of ERα but, as we have previously demonstrated in the ovary (Hapon et al., 2010), increased ERβ mRNA level in L7. It has been shown that liganded TRα1 and TRβ1 inhibit ERα induction of estrogen target genes, such as OTR in neural cell lines, through competition for the co-activator NCOA1 (Vasudevan et al., 2001). Thus, the low circulating T_3 levels in the HypoT rats may be responsible for the abrupt increase in ERβ mRNA on L7 as a consequence of reduced inhibition by TRs of ERα induction of ERβ transcription, according to the mechanism suggested by Vasudevan and collaborators (Vasudevan et al., 2002b; Zhao et al., 2005).

On the other hand, HypoT decreased circulating E_2 and ERβ protein levels in L14 without modifying mRNA levels. This can allow us to infer that HypoT may alter not only ER transcription but also degradation and translation as proposed by Alarid et al. (2003). Thus, the effects of HypoT on ER expression may be a combination of its direct actions on ER expression and effects on circulating E_2 that in turn influence ER expression.

Several mechanisms have been involved in the regulation of PR transcription, mainly regulation by estrogens and P_4 itself. Estrogens induce PRA expression, which is repressed by P_4, while P_4 induces PRB expression that is enhanced by estrogen (Aupperlee and Haslam, 2007). Also, PRA inhibits transcription of both PRA and PRB and the latter promotes self-induction of transcription (Aupperlee and Haslam, 2007; Jacobsen and Horwitz, 2012; Kariagina et al., 2008). Thus, since PRA inhibits PRB expression, the high PRB protein levels observed on L7 in both groups may be a consequence of the low PRA protein levels present at the same time. This same mechanism may also explain the increased PRB mRNA levels observed on L14 in the HypoT rats. Furthermore, the fall in PRA protein and mRNA contents induced by HypoT on L14 may be a consequence of the diminished levels of circulating E_2 and of ERβ protein on the same day. The discrepancy between the patterns of mammary PRA mRNA and protein contents through lactation may be caused by regulatory mechanisms acting at posttranscriptional levels that may modulate translation, processing or degradation of the protein (Jacobsen and Horwitz, 2012). ERα mediates mammary proliferation and development, while ERβ is associated with terminal differentiation of the mammary tissue (Forster et al., 2002). An abnormal PRA/PRB ratio has also been associated with abnormal mammary differentiation (Shyamala et al., 1998, 2000). Thus, the decreased protein expression of PRA and ERβ in the HypoT mothers may be involved in deregulation of mammary function, and contribute to abnormal milk composition (Hapon et al., 2003) and the signs of precocious involution we found at later stages of lactation (unpublished observations).

PRA and B are localized to the epithelial cells of rat and mouse mammary glands in virgin and pregnant animals and in involuted glands, but have not been detected in lactating animals (Aupperlee et al., 2005; Kariagina et al., 2007). In contrast, we were able to detect both isoforms in lactating rats using Western blots. The inverse pattern of expression of PRA and B proteins shown here may indicate a role in the differentiation of the lactating mammary gland around the first week postpartum, when mature lactation is achieved.

It has already been suggested that TR inhibition of ER activity involves silencing or squelching of co-activators (Vasudevan et al., 2002b). Our results show that HypoT decreased mRNA level of NCOA1 and NCOA2, abolishing their normal variations during lactation, while it had relatively opposite effects on NCOR1 (increasing its expression on L2) and NCOR2 (decreasing its expression on L14). On L2, the differential effects of HypoT on NCOA1 and NCOR1 may result in a decrease in the activation of transcription (Watson et al.,

2012), that in turn could be responsible for the decrease in the level of ER induction of OTR mRNA. OTR activation is a key component in milk ejection, and may explain the impaired response to suckling observed previously on HypoT mothers (Hapon et al., 2003).

The present work shows a pattern of changes that can be correlated with the already described effects of hypoT on lactational performance. However, to confirm the cause–effect relationships further work is needed. To our knowledge, this is the first work that studies the effects of HypoT on the expression of nuclear receptor families along with some of their co-regulators during lactation, showing a complex pattern of changes. In control rats, these variations were most marked on L7 compared with the other stages of lactation studied, suggesting this stage as a key inflection point when the mammary glands are reaching their maximum capacity to produce milk. On the other hand, in the early lactating HypoT rats, the combination of low levels of TRα1, NCOA1 and OTR, along with increased NCOR1 may hinder the capacity of the mammary tissue to achieve its full capacity for synthesis and ejection of milk in response to lactogenic hormones. Early lactation is a crucial time point that will influence the future course of lactation. Later on, the altered ER and PR expression may also contribute to impair milk synthesis and induce premature involution.

Acknowledgments

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