Molecular and Cellular Endocrinology xxx (2015) 1–11

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Hypothyroidism advances mammary involution in lactating rats through inhibition of PRL signaling and induction of LIF/STAT3 mRNAs

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ARTICLE INFO

Article history:
Received 29 June 2015
Received in revised form
22 September 2015
Accepted 23 September 2015
Available online xxx

Keywords:
PRL signaling pathway
Nuclear receptors
STAT3
LIF
Caspases
Tissue remodeling

ABSTRACT

Thyroid diseases have deleterious effects on lactation, litter growth and survival, and hinder the suckling-induced hormone release, leading in the case of hyperthyroidism, to premature mammary involution. To determine the effects of hypothyroidism (HypoT) on late lactation, we analyzed the effect of chronic 6-propyl-2-thiouracil (PTU)-induced HypoT on mammary histology and the expression of members of the JAK/STAT/SOCS signaling pathway, milk proteins, prolactin (PRLR), estrogen (ER), progesterone (PR) and thyroid hormone (TR) receptors, markers of involution (such as *stat3*, *lif*, *bcl2*, BAX and PARP) on lactation (L) day 21. HypoT mothers showed increased histological markers of involution compared with control rats, such as adipose/epithelial ratio, inactive alveoli, picnotic nuclei and numerous detached apoptotic cells within the alveolar lumina. We also found decreased PRLR, β-casein and α-lactoalbumin mRNAs, but increased SOCS1, SOCS3, STAT3 and LIF mRNAs, suggesting a decrease in PRL signaling and induction of involution markers. Furthermore, Caspase-3 and 8 and PARP labeled cells and the expression of structural proteins such as β-Actin, α-Tubulin and Lamin B were increased, indicating the activation of apoptotic pathways and tissue remodelation. HypoT also increased PRA (mRNA and protein) and *erβ* and decreased *erα* mRNAs, and increased strongly TRα1, TRβ1, PRA and ERα protein levels. These results show that lactating HypoT rats have premature mammary involution, most probably induced by the inhibition of prolactin signaling along with the activation of the LIF-STAT3 pathway.

1. Introduction

In previous studies we showed that thyroid diseases alter key events of female reproduction (Rosato et al. 1992; Jahn et al. 1995; Hapon et al. 2003, 2007a; Navas et al. 2014), in particular mammary gland function (Varas et al. 2001, 2002; Hapon et al. 2003, Hapon et al. 2007a; Campo Verde Arbocco et al. 2015). We have demonstrated previously that hypothyroidism (HypoT) has a deleterious effect on lactation (Hapon et al. 2003, 2007b; Campo Verde Arbocco et al. 2015). Thus, lactating HypoT rats exhibit increased pup mortality, reduced growth rate of the litter (Hapon et al. 2003) and diminished milk nutritional quality (Hapon et al. 2007b). We also found a partial blockade of milk ejection produced by a reduced oxytocin (OXT) response to suckling (Hapon et al. 2003), that may lead to abnormal accumulation of milk in the alveoli. Hyperthyroidism also impairs milk ejection, producing milk stasis and premature onset of mammary involution (Varas et al. 2002). Thus, thyroid hormones (THs) have significant effects on mammary function and lactation, and acting through their nuclear receptors (TRs), regulate the activity of ovarian hormone receptors and STAT5 proteins, that are key factors in mammary epithelial cell (MEC)

List of abbreviatures: prlr, Prolactin receptor mRNA; *stat5a/stat5b/stat3*, STAT5a/STAT5b/STAT3 mRNAs; STAT5a/b, STAT5a/b protein; *socs1/socs3/cis*, SOCS1/SOCS3/CIS mRNAs; *lif*, LIF mRNA; *erα/erβ*, Estrogen receptors α and β mRNAs; ERα/ERβ, Estrogen receptor α and β proteins; *pr a + b/pra/prb*, Progesterone receptors A + B, progesterone receptor isoform A and progesterone receptor isoform B mRNAs; PRA/PRB, Progesterone receptor isoform A and B proteins; *trα/trβ*, Thyroid hormone receptors α and β mRNAs; TRA/TRB, Thyroid hormone receptors α and β proteins; *bax/bcl-2*, BAX and BCL-2 mRNA; *lalba*, α-Lactoalbumin mRNA; *Csn2*, β-Casein mRNA; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

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http://dx.doi.org/10.1016/j.mce.2015.09.023
0303-7207/

differentiation (Favre-Young et al. 2000; Zhao et al. 2005; Bagamasbad and Denver, 2011).

Through lactation, MEC metabolism is directed mainly to milk synthesis. This ability comes from a massive redirection of the biosynthetic machinery to synthesis of milk components and suppression of most non-essential cellular functions, reflecting the extreme specialization of mammary tissue (Lemay et al. 2007). The transcriptional program of MEC changes gradually during gestation and more abruptly in the transition to lactogenesis. Afterwards, lactation establishment requires post-transcriptional modifications like stabilization of milk protein mRNAs (Rhoads and Grudzien-Nogalska, 2007). At weaning, the loss of suckling stimulus leads to milk stasis and mammary involution. This regression includes a dramatic change involving a rapid transcriptional switch (Bertucci et al. 2010) that proceeds in an orderly manner, in two stages (Li et al. 1997). The first step is reversible and is triggered by the accumulation of milk in the alveoli due to lack of suckling. This stage is characterized by an increase of *bax* mRNA, loss of STAT5a and STAT5b activity, decreased mRNA levels of prolactin (PRL) target genes such as α-Lactoalbumin (*lalba*) and β-Casein (*csn2*) and programmed cell death of epithelial cells (Li et al. 1997). In the second, irreversible stage, the hormonal changes induce the expression of genes related to tissue remodeling that returns the mammary gland to pre-lactational histoarchitecture (Li et al. 1997).

The regulation of the involution switch in MEC, involves multiple signaling pathways such as JAK/STAT, glucocorticoids and MAPK (Li et al. 1997; Lemay et al. 2007; Watson, 2009; Whyte et al. 2009; Bertucci et al. 2010; Zhao et al. 2012), rendering the characteristic transcriptional profile of the involuting mammary gland (Lemay et al. 2007). The control of these signaling pathways is exerted by hormones such as PRL, ovarian hormones, glucocorticoids and THs, whose interaction has been widely described (Bagamasbad and Denver, 2011).

Based on this evidence we hypothesize that HypoT may induce premature mammary involution through the ability of THs to directly or indirectly regulate the transcriptional activity of the mammary cells. In this paper we show that HypoT alters the characteristic transcriptome of the mammary gland at involution, generates premature regression and tissue restructuring through a mechanism that involves conventional and non-conventional mechanisms previously described for mammary involution.

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 h) and temperature (22–24 °C)–controlled room. Vaginal smears were performed daily and only regular 4-day cyclic rats were used. Rat chow (GEPSA Grupo Pilar SA, 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 the drinking water. The treatment was started on the day of estrus 8 days before mating. Vaginal smears were taken daily and the rats were caged with a fertile male on the night of the second proestrus after the start of the treatment. The presence of spermatozoa in the vaginal smear the following morning was indicative of pregnancy, and this day was counted as day 0 of pregnancy. Rats that did not become pregnant on this day were discarded. Between days 19 and 20 of pregnancy the rats were caged individually and the day of parturition was checked. 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. The procedures were approved by the Institutional Animal Care and Use Committee of the School of Medical Sciences, Universidad Nacional de Cuyo, Mendoza, Argentina (Protocol approval N° 17/2012). Groups of 8 PTU or control (Ctrl) rats were killed on day 21 of lactation (L21) at 09.00–12.00 h 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 were removed, snap frozen in liquid nitrogen, and stored at −70 °C until they were analyzed. The whole, frozen mammary glands were cracked with a hammer to obtain roughly powdered tissue, which was mixed to homogenize the different regions of tissue before using for RNA and protein extraction.

2.2. Hormone determinations

Estradiol (E_2) concentrations in sera were measured by radioimmunoassay (RIA) using commercial kits for total hormones (KE2D1 double antibody radio immune assay from Siemens Healthcare Diagnostic Inc USA) as described previously (Hapon et al. 2003). TSH was measured by double antibody radioimmunoassay using materials generously provided by Dr. Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA) as previously described (Hapon et al. 2003).

2.3. RNA isolation and real time RT-PCR analysis

Total RNA was isolated from 150/200 mg of mammary tissue using the guanidinium isothiocyanate-acid phenol method as modified by Puissant and Houdebine (Puissant and Houdebine, 1990). Ten micrograms of total RNA were reverse transcribed (RT) 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 (pH8.4), 75 mM KCl, 3 mM $MgCl_2$], 0.5 mM dNTPs, 5 mM DTT, 200 units M-MLV Reverse Transcriptase. 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 PRL (PRLR), thyroid hormones (TRs), estrogen (ERs) and progesterone (PRs) receptors, STAT5a, STAT5b, STAT3, LIF (leukemia inhibitory factor), suppressors of cytokine signaling 1 and 3 (SOCS1, 3) and cytokine-induced STAT inhibitor (CIS), were estimated by Real Time RT-PCR 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 (Corbett Research Pty Ltd Sydney, Australia) and 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 initiated with 5 min incubation at 95 °C, followed by 40 cycles of 95 °C for 30 s, 30 s at the annealing temperatures shown in Table 1 and 72 °C for 30 s. Melt curve analysis was used to check that a single specific amplified product was generated. Real Time quantification was monitored by

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Table 1
Sequences and conditions for the PCR reactions.

mRNAs		Primer sequence	Gen-bank 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	NM_012672.3	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			
prlr	Sense	AAAGTATCTTGTCCAGACTCGCTG	NM_001034111.1	60	(Bonafede et al. 2011)
	Antisense	AGCAGTTCTTCAGACTTGCCCTT			
stat5a	Sense	ATTCAGCGACTCGGAAATCG	NM_017064.1	60	(Conneely et al. 2001)
	Antisense	GAGAAATCCCGAGTGGTGAATG			
stat5b	Sense	CAGTTCAGTGTTGGTGGAAATGAG	NM_022380.1	60	(Conneely et al. 2001)
	Antisense	GGTAGCCGTCGCATTGTTGT			
stat3	Sense	CAATACCATTGACCTGCCGAT	NM_012747.2	60	(Wang et al. 2012)[b]
	Antisense	GAGCGACTCAAACTGCCCT			
socs1	Sense	CCGTGGGTCGCGAGAAC	NM_145879.2	63	(Simian et al. 2009)
	Antisense	AAGGAACTCAGGTAGTCACGGAGTA			
socs3	Sense	CAGCTCCAAGAGCGAGTACCA	NM_053565.1	63	(Andrews et al. 2001)
	Antisense	CGGTTACGGCACTCCAGTAGA			
cis	Sense	GCCTTCCCAGATGTGGTCA	NM_031804.1	61	(Andrews et al. 2001)
	Antisense	CCGGGTGTCAGCCGTAC			
lalba	Sense	GAATGGACCTGTGTTTTATTCCA	NM_012594.1	60	(Wellberg et al. 2010)[a]
	Antisense	TGTGCTGCCATTGTTCTT			
csn2	Sense	GGTGAAACTCATGGGACAGG	NM_017120.3	60	(Wellberg et al. 2010)[a]
	Antisense	TGACTGAATGCCGGAGTGAA			
lif	Sense	AGCTATGTGCGCCCAACATGA	NM_022196.2	60	(Wang et al. 2012)[b]
	Antisense	CGACCATCCGATACAGCTCG			
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 min 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.

[a] These sequences were obtained from Harvard PrimerBank. The original sequence was designed for *Mus musculus*. We changed the mismatched nucleotides for the corresponding correct *Rattus norvegicus* sequence to obtain 100% homology.

[b] The original sequences were designed for *Mus musculus*. We changed the mismatched nucleotides for the corresponding correct *Rattus norvegicus* sequence to obtain 100% homology.

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 RT. All measurements were performed in duplicate. The reaction conditions and quantities of cDNA added were calibrated so 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 RT. 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 of variation between treatment groups compared to β-Actin, GAPDH and HPRT1. Relative levels of mRNA were normalized to the S16 reference gene. The Real Time PCR products were analyzed on 2% agarose gels containing 0.5 μg/mL ethidium bromide and a unique band of the approximately correct molecular weight corresponded with a unique peak in the melt curve analysis.

2.4. Protein isolation and Western blot analysis

For total protein isolation, between 300/500 mg of mammary tissue were homogenized 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 12,500 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 (Simpson, 2008) and boiled 3–5 min in loading buffer. 80 μg of protein were separated by SDS-PAGE and transferred to Nitrocellulose Hybond membranes as described previously (Valdez et al. 2007). After rinsing and blocking with BSA 2% the membranes were probed with anti-TRα1 (PA1–211A, Thermo Scientific), anti-PR A + B (PR130, generated and tested in Endocrinology and Hormone Dependent Tumors Laboratory of the National University of Litoral, Santa Fe, Argentina), anti-ERα (sc-7207, Santa Cruz Biotechnology Inc Dallas TX), anti-ERβ (ab3577 Abcam, Cambridge, MA), anti- β-Actin (Sigma. St. Louis, MO, USA), anti-GAPDH (sc-47724, Santa Cruz Biotechnology Inc Dallas TX), anti-α-Tubulin (Sigma. St. Louis, MO, USA), anti-Lamin B (c-12,

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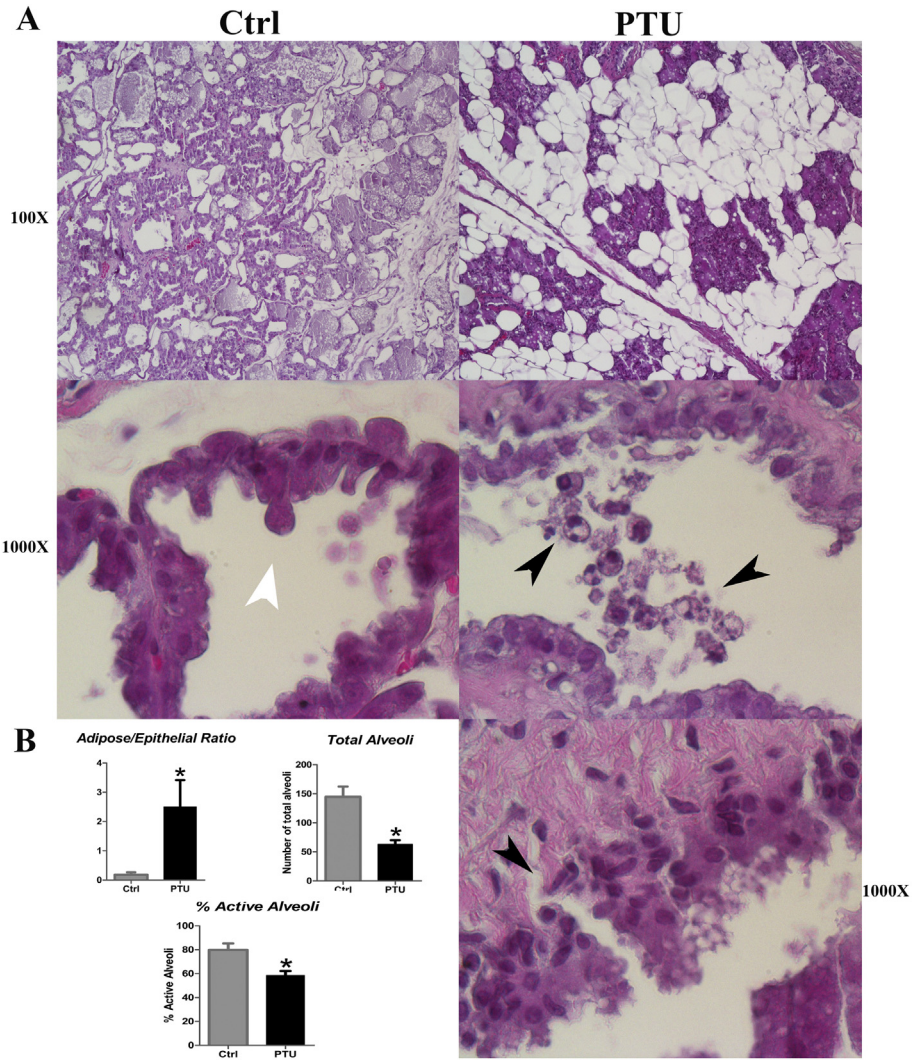

Fig. 1. Effect of PTU-induced HypoT on mammary gland histology on day 21 of lactation (L21). Mammary glands from control or PTU-treated rats were fixed and stained with hematoxylin-eosin. Panel A: representative images obtained at 100 and 1000× magnification. Panel B: quantification of adipose to epithelial tissue ratio, % of positive alveoli and total alveoli. See Materials and Methods section for further details. While control rats show abundant active alveoli with signs of MEC in active secretion (white arrow), HypoT rats show a preponderance of adipose tissue, inactive alveoli showing a loss of epithelial integrity and numerous detached apoptotic MEC within the alveolar lumen (black arrows). On Panel B: Controls (Ctrl, gray bars), HypoT (PTU, black bars), *p ⟨ 0.05 compared with the control group using Student's *t* test. For Adipose/Epithelial ratio quantitation, log transformation of the data was performed.

Santa Cruz Biotechnology Inc., Dallas TX), using Horseradish peroxidase-conjugated secondary antisera (polyclonal goat anti-rabbit, rabbit anti-goat and goat-anti mouse inmunoglobulins, 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). Since the expression of commonly used proteins for quantitation were affected by HypoT induction, we used densitometric analysis of the intensity of the Ponceau staining as loading control. For GAPDH Western blot we used as positive control 50 and 100 μL of a lysate of 1×10^6 HBL-100 cells.

2.5. Mammary gland histology

Upon sacrifice, a portion of fresh mammary tissue was fixed in buffered formol, dehydrated in ethanol and embedded in paraffin wax. Sections of 3–5 μm thickness were cut with a Carl Zeiss HYRAX M 25 Rotary microtome and stained with hematoxylin-eosin. Images were taken with a Nikon Eclipse 80i Microscope fitted with a digital still camera Nikon DS Fi1 (Nikon Instruments INC. Japan) under 100×, 400× and 1000× magnifications. Only the inguinal mammary glands were used for all the morphological analyses. Sections were evaluated histologically for changes in the extent and histoarchitecture of lobuloalveolar development and supporting adipose tissue. The morphological state of the alveoli was determined by analyzing 2 sections from three different animals per group. For quantification we analyzed 3 sections from each animal and 10 fields at 400× were counted from each section.

2.6. Immunohistochemistry

Serial sections (3–5 μm) were cut in a Carl Zeiss HYRAX M 25 Rotary Microtome and mounted onto 3-aminopropyl-triethoxysilane (Sigma–Aldrich)-coated slides for subsequent immunohistochemical (IHC) analysis. The primary antibodies used were anti-Caspase-8 (rabbit polyclonal antibody ab25901), anti-Caspase-3 (rabbit polyclonal antibody ab4051), anti-Bax (rabbit

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monoclonal antibody ab32503) and anti-PARP (rabbit monoclonal antibody ab32138) at 1:500 dilution (all from Abcam, Cambrigde, MA). An antigen retrieval protocol using heat was used to unmask the antigens (40 min in 0.01 M citrate buffer, pH 6.0). Tissue sections were incubated with the primary antibody overnight at 4 °C in humidity chambers. A commercial kit to detect mouse and rabbit antibodies was used (Dako EnVision system, horseradish peroxidase, diaminobenzidine; Dako, Carpinteria, CA, USA). Slides were lightly counterstained with hematoxylin to reveal the nuclei. Images were taken with a Nikon Eclipse E200 Microscope (Nikon Corp., Japan) fitted with a digital still camera Micrometric SE Premium (Accu-Scope, Commak, NY 11725) under 100×, 400×, and 600× magnifications. The percentage of positive cells was quantified using the Fiji's Cell Counter plugin (Schindelin et al. 2012). For quantitation we counted 1500–1900 cells per field from ten fields in each of three sections per animal.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism, and Student's *t* test or Mann–Whitney post hoc tests to compare individual means for parametric or non-parametric data respectively. When variances were not homogeneous log transformation of the data was performed. Differences between means were considered significant at the $p < 0.05$ level.

3. Results

3.1. Effect of HypoT on circulating TSH and mammary gland morphological changes at the end of lactation

The hypothyroid state of the rats was confirmed by measuring circulating TSH levels that were 0.56 ± 0.08 ng/mL in Ctrl rats and 11.86 ± 0.89 ng/mL in HypoT rats. As shown in Fig. 1, mammary histology of the HypoT group revealed high adipose/epithelial ratio compared to the Ctrl group, the presence of numerous picnotic nuclei and increased cellular debris in the lumen of the alveoli, images that were not seen in the Ctrl rats. HypoT also significantly decreased the percent of total and active alveoli (Fig. 1).

3.2. Effect of HypoT on mRNA and protein levels of JAK/STAT signaling pathway members on mammary gland at the end of lactation

Since mammary involution activates a transcriptional program that includes down-regulation of the PRL signaling pathway and up

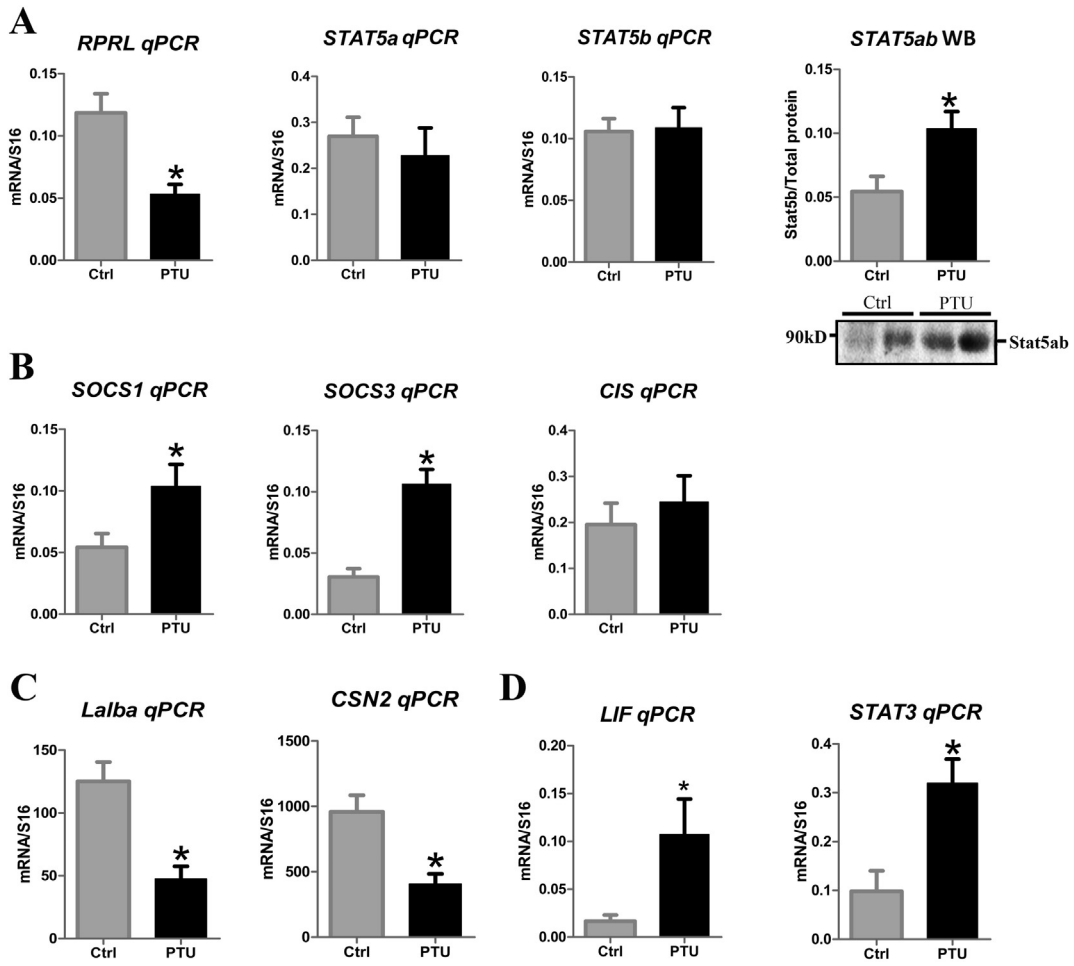

Fig. 2. Effect of PTU-induced HypoT on mammary mRNA levels of members of the PRL signaling family (*prlr. stat5a, stat5b, socs1, socs3, cis*), *lif, stat3*, α-lactalbumin (*lalba*) and β-casein (*csn2*) relative to S16, and STAT5a/b protein levels relative to total protein (Ponceau) on day 21 of lactation (L21). Controls (Ctrl, gray bars), HypoT (PTU, black bars). mRNA levels were measured by Real Time RT-PCR and protein levels by Western blot (WB) using rat specific primers and antibodies respectively. See Materials and Methods section for further details. The graphs may have different scales. Under the STAT5 protein graph is a representative WB. Bars at the left of the WB photograph mark the position and size of the molecular weight markers and at the right show the position of the band. Values are means ± SEM for groups of 8 rats for PCR and 6 rats *p ⟨ 0.05 compared with the respective control group using Student's *t* test.

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regulation of the LIF signaling pathway (Lemay et al. 2007; Kreuzaler et al. 2011; Sargeant et al. 2014), we determined the mRNA abundance of members of the PRL signaling pathway, LIF and STAT3. As shown in Fig. 2A the mRNA levels of the long form of *prlr* was significantly diminished by HypoT, while *stat5a* and *b* levels were not affected. At the same time, we found that HypoT increased the mRNA level of *lif* and *stat3*, which are clear signals of apoptosis onset (Fig. 2D). Furthermore, the mRNA of the main inhibitors of the JAK/STAT pathway, *socs1* and *socs3* increased 2 and 3 fold respectively compared with the Ctrl group, while *cis* levels were not modified significantly (Fig. 2B). This may indicate changes in the rates of transcription or degradation of these mRNAs, and suggest a down regulation of the PRL signaling pathway and up regulation of involution signaling through induction of *lif* and *stat3* mRNA transcription. Confirming this, HypoT decreased the mRNA level of *csn2* and *lalba* (target genes of the PRL signaling pathway) 2 and 3 fold with respect to the Ctrl group (Fig. 2C). We attempted to measure SOCS and CIS proteins by Western blot but were not able to detect them. On the other hand, STAT5 protein was readily detectable and, surprisingly, we found that HypoT increased the protein level compared with the Ctrl group (Fig. 2A).

3.3. Effect of HypoT on circulating E_2 and the expression of PRs, ERs and TRs on mammary gland at the end of lactation

Since cross regulation between TRs, ERs, PRs and PRLR signaling pathways has been described, we evaluated the effect of HypoT on the expression of some nuclear receptors at the end of lactation. As shown in Figs. 3–5, HypoT did not affect the mRNA levels of *prb* (Fig. 3), but increased the mRNA level of *pra* and *erβ* compared with the Ctrl group (Figs. 3 and 4 respectively). Also, HypoT decreased the mRNA level of *erα*, showing an opposite effect in both ER isoforms (Fig. 4). Circulating E_2 was increased in the HypoT rats (Fig. 4). At the same time, HypoT did not affect ERβ protein level (Fig. 4) but increased strongly PRA and ERα protein levels (Figs. 3 and 4 respectively). Fig. 5 shows that HypoT did not affect the mRNA levels of *trα1, trα2 and trβ1* compared with the Ctrl group. At the same time, HypoT increased strongly both TRs protein levels.

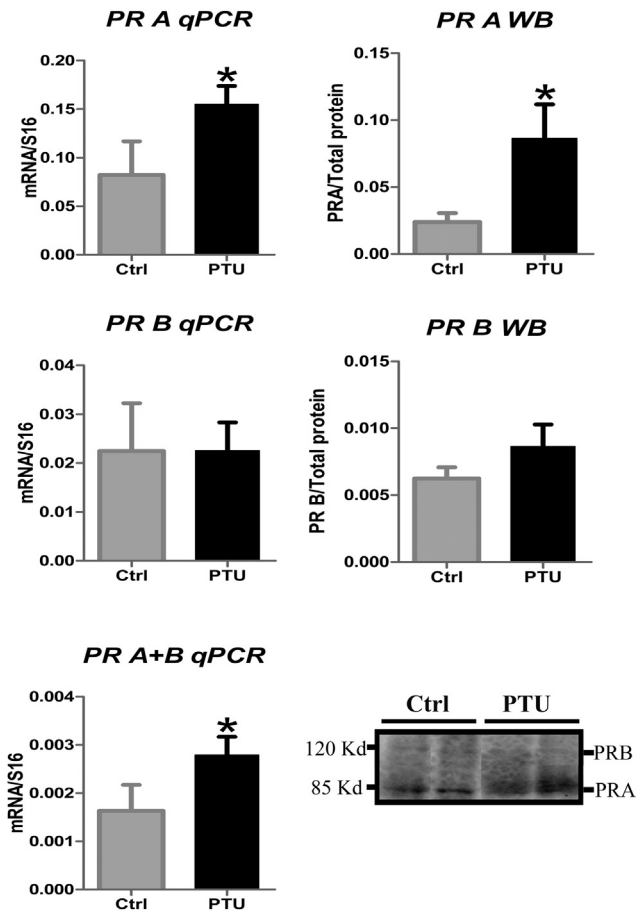

Fig. 3. Effect of PTU-induced HypoT on mammary content of PRs (PRA and PRB) at mRNA and protein levels relative to S16 and total protein (Ponceau) respectively, on day 21 of lactation (L21). Controls (Ctrl, gray bars), HypoT (PTU, black bars). mRNA levels were measured by Real Time RT-PCR and protein levels by Western blot (WB) using rat specific primers and antibodies respectively. See Materials and Methods section for further details. The graphs may have different scales. Under the protein graphs representative WBs are shown. Bars at the left of the WB photograph mark the position and size of the molecular weight markers and at the right show the position of the band. 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 using Student's *t* test. For PRA protein level quantitation, log transformation of the data was performed.

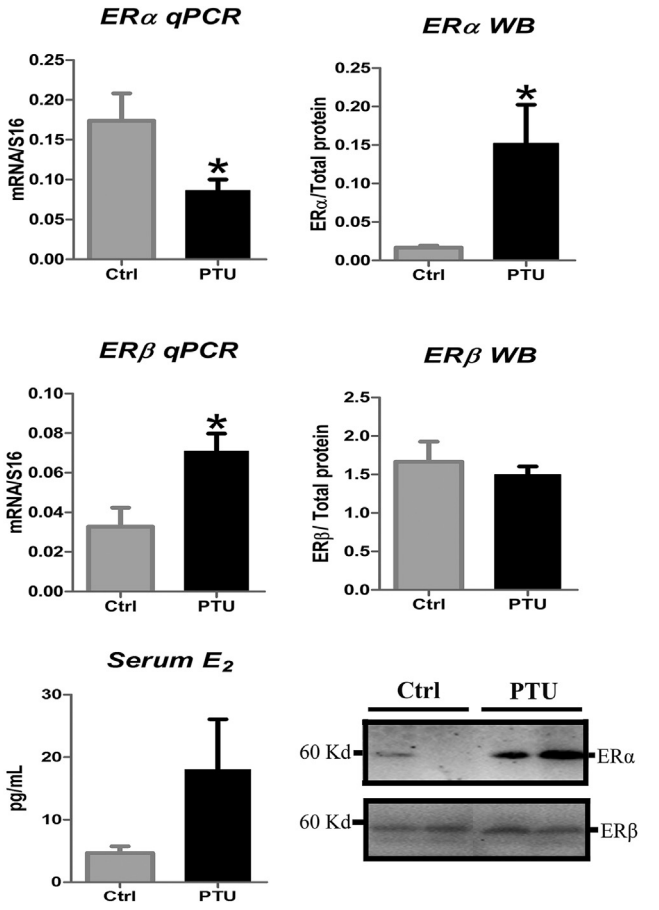

Fig. 4. Effect of PTU-induced HypoT on circulating E_2, and mammary content of ERs (ERα and ERβ) at mRNA and protein levels relative to S16 and total protein (Ponceau) respectively, on day 21 of lactation (L21). Controls (Ctrl, gray bars), HypoT (PTU, black bars). mRNA levels were measured by Real Time RT-PCR and protein levels by Western blot (WB) using rat specific primers and antibodies respectively. See Materials and Methods section for further details. The graphs may have different scales. Under the protein graphs representative WBs are shown. Bars at the left of the WB photographs mark the position and size of the molecular weight markers and at the right show the position of the band. 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, using Student's *t* test, with the exception of *erα* mRNA quantitation, in which Mann–Whitney post test was performed. For ERα protein and E_2 quantitation, log transformation of the data was performed.

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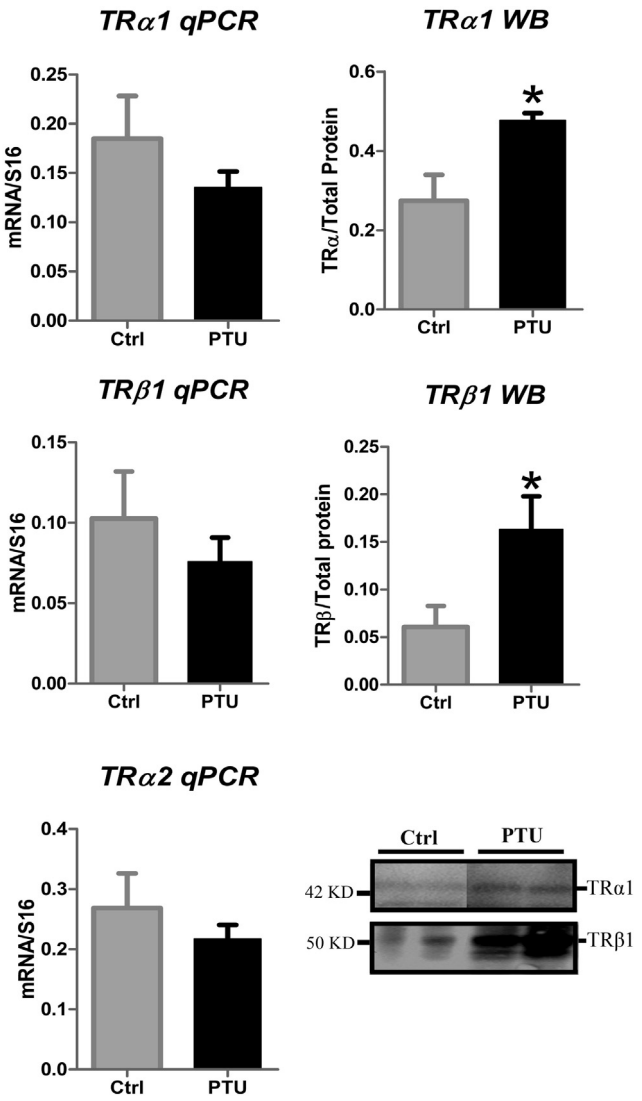

Fig. 5. Effect of PTU-induced HypoT on mammary content of TRs (TRα1, TRα2 and TRβ1) at mRNA and protein levels relative to S16 and total protein (Ponceau) respectively, on day 21 of lactation (L21). Controls (Ctrl, gray bars), HypoT (PTU, black bars). mRNA levels were measured by Real Time RT-PCR and protein levels by Western blot (WB) using rat specific primers and antibodies respectively. See Materials and Methods section for further details. The graphs may have different scales. Under the protein graphs representative WBs are shown. Bars at the left of the WB photographs mark the position and size of the molecular weight markers and at the right show the position of the band. 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 using Student's *t* test, with the exception of TRα1 protein quantitation, in which Mann–Whitney post test was performed. For *trα1, trα2* and *trβ1* mRNAs quantitation, log transformation of the data was performed.

3.4. Effect of HypoT on the expression of apoptosis related proteins and mRNAs in mammary gland at the end of lactation

Since the first stage of mammary involution is triggered by milk stasis and involves MEC programmed cell death we evaluated the effect of HypoT on MEC expression of apoptosis related proteins by IHC. As shown in Fig. 6A and B, HypoT increased the number of cell nuclei positive for Caspase-3 and Caspase-8 but not for BAX. PARP expression was strong in both groups but the number of positive cell nuclei was slightly but significantly higher in HypoT. On the other hand, HypoT did not affect *bcl-2* or *bax* mRNA levels (Fig. 6C).

3.5. Effect of HypoT on the expression of some structural proteins in mammary gland at the end of lactation

Since the second stage of mammary involution involves tissue restructuration, we evaluated by Western blot the mammary expression of structural proteins such as β-Actin, α-Tubulin, GAPDH and Lamin B. As shown in Fig. 7, HypoT strongly increased β-Actin, α-Tubulin and Lamin B protein levels, showing clear signals of tissue remodeling; however, we did not detect GAPDH neither in the Ctrl nor in the HypoT group (not shown).

4. Discussion

The present results show that in lactating rats, HypoT induces histological changes consistent with early involution of mammary tissue. The concomitant molecular changes reveal that THs may be involved in the direct or indirect regulation of the expression of genes that control mammary regression. In particular, the decrease in *prlr*, *lalba* and *csn2* expression and the increases in Caspase-3, -8 and PARP and histological signs of cessation of milk synthesis in the HypoT mothers are indicative of premature involution. We have previously found that lactating hyperthyroid rats also show premature involution (Varas et al. 2002), which along with the present results suggest that optimal levels of THs are required for normal lactation and adequate mammary function.

Lemay et al. (Lemay et al. 2007) showed that a massive transcriptional switch occurs between lactation and early involution; the mammary transcriptome is directed towards a pre-lactational state with lower STAT5 activation and higher *bax* expression. Although our results show a different profile, we found an inhibition of the PRL signaling pathway by HypoT with decreased *prlr* mRNA level and increased *socs1* and *3* mRNA levels that may be directly responsible for the decrease in *lalba* and *csn2* mRNAs, both targets of the PRL signaling pathway. *Lalba* and *csn2* mRNAs are markers of MEC differentiation and active milk synthesis, so their decrease indicates loss of the secretory phenotype, associated with premature involution (Bertucci et al. 2010).

In natural weaning (NW), expression and activation of members of the LIF signaling pathway, including STAT3 and SOCS3 is induced (Robinson et al. 2007; Kreuzaler et al. 2011), and in turn, the activation of STAT3 leads to an increase in *bax* expression (Sutherland et al. 2006, 2007). Our results show that HypoT increased the mRNA level of *stat3*, *socs3* and *lif* indicating an induction of this signaling pathway, but BAX expression was not changed. SOCS3 represses and modulates the activity of STAT5, inhibiting PRL-induced milk synthesis (Haricharan and Li, 2014), but also represses STAT3 signaling, limiting the initiation of NW mediated mammary involution. The increased levels of *socs3* mRNA found in the HypoT rats may explain the failure to find increased *bax* mRNA or protein expression in the presence of elevated *lif* and *stat3*, which in turn may induce mammary involution through alternative pathways. Considering this, the effects of HypoT in involution can be related both to inactivation of the PRL signaling pathway and induction of the LIF-STAT3 pathway. Activation of LIF is induced by milk stasis, since LIF expression is stimulated by mechanical stretching of luminal mammary cells (Quaglino et al. 2009). We previously found a partial blockade of milk ejection in HypoT lactating rats (Hapon et al. 2003) and the consequent abnormal accumulation of milk could be one cause of the increased *lif* mRNA found in the present study.

It is known that PRL binding to its receptor leads to activation of JAK2 and STAT5 phosphorylation. Phosphorylation and subsequent dimerization of STAT5 allows it to translocate to the nucleus, act as a transcription factor and be subsequently degraded by the proteolytic machinery (Chen et al. 2006). In this context, the decrease

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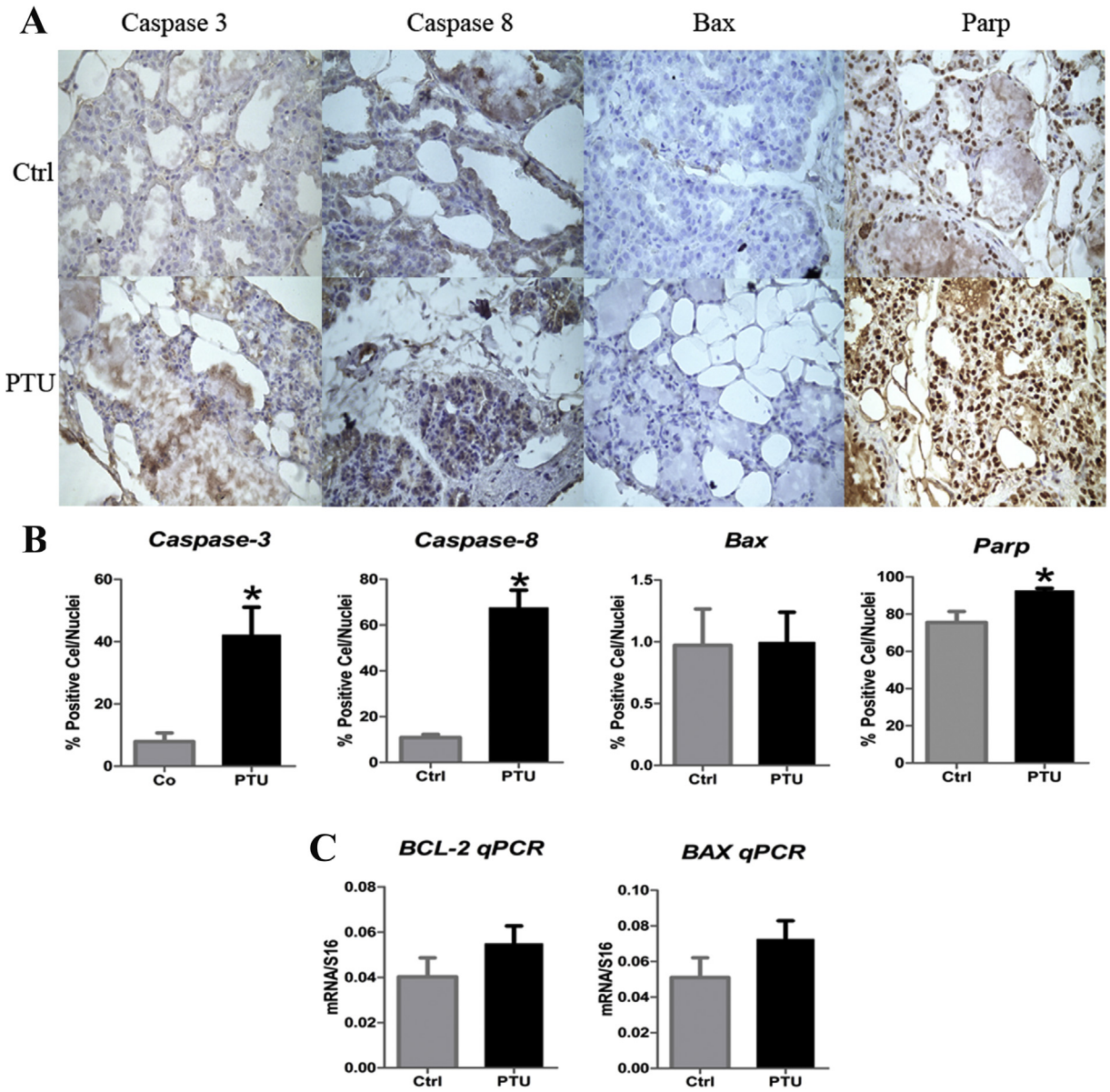

Fig. 6. Effect of PTU-induced HypoT on mammary expression of apoptosis related proteins Caspase-3, Caspase-8, BAX and PARP, measured by immunohistochemistry (IHC), and of *bax* and *bcl-2* mRNA content (relative to S16) on day 21 of lactation (L21). Controls (Ctrl, gray bars), HypoT (PTU, black bars). mRNA levels were measured by real time RT-PCR and protein expression by IHC using rat specific primers and antibodies respectively. See Materials and Methods section for further details. Panel A: Representative microphotographs of control (Ctrl) or hypoT (PTU) staining for Caspase-3, Caspase-8, BAX and PARP. Panel B: Quantification of the % of positive cells for each of the proteins. Panel C: *bax* and *bcl-2* mRNA levels. The graphs may have different scales. Values are means ± SEM for groups of 8 rats for PCR and 3 rats for IHC. *p ⟨ 0.05 compared with the respective control group using Student's *t* test.

in *prlr* and inhibition of the PRL pathway caused by HypoT could alter the turnover of STAT5. The accumulation of this protein without being degraded may result in the observed increase in STAT5 protein in the presence of unchanged levels of *stat5* mRNA. At the same time, the interaction between STAT5 and intracellular hormone receptors has been widely described. In particular, ERα and TRβ1 inhibit STAT5 activation (Favre-Young et al. 2000; Wang and Cheng, 2004), thus, the increased ERα and TRβ1 proteins and circulating E_2 could promote involution by further inhibition of STAT5 activation. Furthermore, since TRβ1 also inhibits STAT5 nuclear translocation (Favre-Young et al. 2000), this could also contribute to the increase in STAT5 protein.

Post-lactational mammary remodeling depends on lysosome-mediated cell death in a process that involves STAT3 signaling and activation of Caspases, the characteristic apoptosis indicators (Scribner et al. 2011; Hughes et al. 2012; Uejyo et al. 2015). The premature involution induced by HypoT involves activation of this pathway, since we found increased expression of Caspase-8, Caspase-3 and PARP. It has been demonstrated that the Caspase-3 gene promoter has several progesterone (P_4) response elements, and P_4 treatment increases Caspase-3 expression in uterine myocites (Jeyasuria et al. 2009). Thus, the increased PRA protein level in HypoT could stimulate Caspase-3 expression and contribute further to activation of the Caspases pathway. Furthermore, since STAT3 induces Caspase activation (Kreuzaler et al. 2011; Watson and Kreuzaler, 2011) the elevated *stat3* expression may also contribute to Caspase activation and to the observed increase in apoptotic markers.

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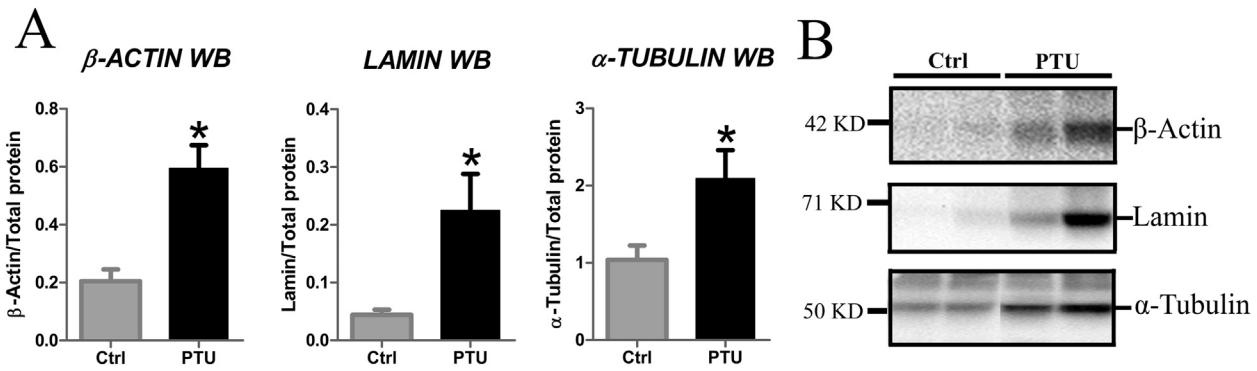

Fig. 7. Effect of PTU-induced HypoT on mammary content of the structural proteins β-Actin, α-Tubulin, and Lamin B relative to total protein (Ponceau) on day 21 of lactation (L21). Controls (Ctrl, gray bars), HypoT (PTU, black bars). Protein levels were measured by Western blot (WB) using rat specific antibodies. See Materials and Methods section for further details. The graphs (Panel A) may have different scales. Panel B shows representative WBs. Bars at the left of the WB photographs mark the position and size of the molecular weight markers and at the right show the position of the band. Values are means ± SEM for groups of 6 rats. *p ⟨ 0.05 compared with the respective control group using Student's *t* test. For Lamin B quantitation log transformation of the data was performed.

Histological analysis showed that HypoT induced mammary tissue remodeling, with increased adipose to epithelial tissue ratio and a diminution in active alveoli, changes that will ultimately lead to a pre-lactational mammary tissue architecture. At the same time, we saw that proteins commonly considered as constitutive (and thereby used as loading control for Western blot), β-Actin, α-Tubulin and Lamin B increased strongly in the HypoT group. These results are not surprising because the expression of these structural proteins is strongly affected in tissue remodeling events (Sokolowski et al. 2014). It has been proposed that Caspase activation leads to the breakdown of cytoskeletal actin and tubulin dimers (Sokolowski et al. 2014), thus, induction of Caspases in the HypoT group may lead to an increase in the dynamics of these proteins reflected in their elevated expression. Alternatively, or in conjunction with this idea, the increase in the abundance of the receptor proteins, and particularly of these constitutive proteins, may reflect a diminution in the relative abundance of milk proteins, along with the initiation of tissue remodeling directed towards an undifferentiated phenotype.

Multiple interactions among nuclear receptors altering their transcriptional capacities have been described (Wang and Cheng, 2004; Bagamasbad and Denver, 2011; Cerliani et al. 2011; Wang et al. 2014). These interactions may influence cellular function and the degree of differentiation, as well as the response to circulating hormones. The present results show that HypoT increased the protein levels of ERα and PRA, as well as circulating E_2. These phenomena can be related to the described acute inhibition of STAT5 transcriptional activity (Wang and Cheng, 2004; Hatsumi and Yamamuro, 2006) and self-repression of transcription by ERα (Hatsumi and Yamamuro, 2006; Bagamasbad and Denver, 2011). Thus, the increased serum E_2 and ERα resulting in increased ERα signaling may inhibit STAT5 activation (Wang and Cheng, 2004), thus leading to the premature involution. This may also be responsible for the decrease in ERα mRNA since it has been shown that activated ERα induces acute repression of its own transcription in mammary gland (Wang and Cheng, 2004; Bagamasbad and Denver, 2011). In control rats, ERβ mRNA increases steadily through lactation and we have found increased ERβ mRNA on day 7 of lactation in HypoT rats (Campo Verde Arbocco et al. 2015). Additionally, ERβ promoter has multiple STAT5/3 binding elements and activated STAT3 up-regulates ERβ transcription (Wang et al. 2011). Thus, the increased STAT3 found in the HypoT rats may be responsible for the increase in ERβ mRNA. STAT5 can form a complex with both PR isoforms bound to P_4, which act as comodulators of STAT5 signaling (Cerliani et al. 2011). Furthermore, P_4 bound to the PRs represses PRL-STAT5a induction of *csn2* transcription, but PRA is a stronger inhibitor than PRB (Buser et al. 2007). PR expression is finely tuned by both P_4 and E_2 (Bagamasbad and Denver, 2011). E_2 promotes PRA expression, so the above mentioned increase in ER signaling could mediate the increase in PRA in HypoT (Aupperlee and Haslam, 2007). In turn, the increased PRA augments the repression of STAT5-induced transcription constituting another mechanism by which HypoT inhibits PRL signaling in L21.

We also found that HypoT induced increases in protein levels of TRβ1 and TRα1 possibly as a mechanism compensating for the low circulating THs (Lazar, 1993). There is controversy regarding the effects of THs in mammary development and lactation. Some authors have shown that treatment with pharmacological levels of THs antagonize mammary development (Mittra, 1974, 1975; Rosato et al. 1992, Rosato et al. 1998). Favre-young et al. (Favre-Young et al. 2000) have described crosstalk between STAT5 and TRβ1 showing that upon PRL stimulation, TH inhibits STAT5a/b nuclear translocation and thereby, their transcriptional activity. However, low quantities of administered THs synergize with PRL and promote milk production in cows (Capuco et al. 1999, 2008). It could be expected that HypoT has a primary effect on PRL signaling pathways and subsequently affects involution. Then, the compensatory increase in TR proteins could be an additional input to the inhibition of PRL signaling (Favre-Young et al. 2000). Furthermore, during early and mid lactation, HypoT has a deleterious effect on mammary function (Campo Verde Arbocco et al. 2015), altering PRL signaling in a way similar to that observed in this study (unpublished work). This inhibition in the PRL signaling pathway plus the effect of HypoT on PRs, ERs and serum E_2 levels could be key factors in the onset of the de-differentiation mechanisms and trigger premature mammary involution.

Currently, various investigations aim to establish a consensus for treatment of thyroid pathologies through gestation and lactation. Hypothyroid disease affects 4–5% of women of childbearing age and 2–3% of pregnant women have overt hypothyroidism (Helfand and Force, 2004). Recent studies have shown that supplementation of hypothyroid states during gestation and lactation is inadequate in many cases (Medeiros et al. 2014; Negro and Stagnaro-Green, 2014; Taylor et al. 2014). In this work we show that HypoT induces premature mammary involution and loss of lactational performance that may be related with the previously described milk stasis (Hapon et al. 2003). The present results provide relevant information concerning the harmful effect of this pathology in lactational performance, highlighting the risks of

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inadequate TH supplementation during lactation. On the other hand, the inflammatory events produced by milk stasis is strongly related with increased mammary cancer risk (Figueroa et al. 2014; Haricharan and Li, 2014; Need et al. 2014; Silanikove, 2014; Zaragoza et al. 2015). In addition, it is known that mammary expression of PRs and ERs are key in breast cancer treatment and progression. Thus, the present findings may also offer new information about the relationship between HypoT, lactation and mammary carcinogenesis.

Acknowledgments

This work has been supported by grants PIP 2298/09 and 0863/12 from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina), and SeCTyP 06/M070, Universidad Nacional de Cuyo. Argentina. The authors are indebted to Prof. Norma B. Carreño and Elisa Pietrobon for their excellent technical assistance. GAJ, RWC and MBH are Career Scientists from CONICET, FCVA, EAA and CVS have fellowships from CONICET.

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Please cite this article in press as: Campo Verde Arboccó, F., et al., Hypothyroidism advances mammary involution in lactating rats through inhibition of PRL signaling and induction of LIF/STAT3 mRNAs, Molecular and Cellular Endocrinology (2015), http://dx.doi.org/10.1016/j.mce.2015.09.023