Hypothyroidism prolongs corpus luteum function in the pregnant rat

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Abstract

It has been shown that hypothyroidism in the rat produces a prolongation of pregnancy associated with a delay in the fall of circulating progesterone (P_4) at term. The aim of the present work is to determine whether the delayed P_4 decline in hypothyroid mother rats is due to a retarded induction of P_4 degradation to $20\alpha\text{OH } P_4$ or to a stimulation of its synthesis, and to investigate the possible mechanisms that may underlie the altered luteal function. We determined by RIA the circulating profile of the hormones (TSH, PRL, LH, P_4 , $\text{PGF}2\alpha$, and $\text{PGE}2$) involved in luteal regulation at the end of pregnancy and, by semiquantitative RT-PCR, the expression of factors involved in P_4 synthesis (CytP450scc, StAR, $3\beta\text{HSD}$, PRLR) and metabolism ($20\alpha\text{HSD}$, $\text{PGF}2\alpha\text{R}$, iNOS and COX2). Our results show that the delay in P_4 decline and parturition is the resultant of retarded luteal regression, caused by a combination of decreases in luteolytic factors, mainly luteal $\text{PGF}2\alpha$, iNOS mRNA expression and also circulating LH, and increased synthesis or action of luteotrophic factors, such as luteal and circulating $\text{PGE}2$ and circulating PRL. All these changes may be direct causes of the decreased $20\alpha\text{HSD}$ mRNA and protein (measured by western blot analysis) expression, which in the presence of unchanged expression of the factors involved in P_4 synthesis results in elevated luteal and circulating P_4 that prolonged pregnancy and also may favor longer survival of the corpus luteum.

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Introduction

In the rat, normal function of the corpus luteum is critical for the maintenance of pregnancy during its entire length, since it is the main source of circulating P_4 (Risk & Gibori 2001, Arosh *et al.* 2004). This hormone is required for implantation, for regulating pituitary gonadotropin secretion and, most importantly, for maintaining a quiescent uterus by inhibiting the contractile activity of the myometrium, thus preventing premature parturition (Risk & Gibori 2001). The lifespan and function of the CL is regulated by complex interactions between stimulatory (luteotropic) and inhibitory (luteolytic) factors (Arosh *et al.* 2004).

There are several luteotropic factors in the rat, among them, LH or chorionic gonadotrophin (on early gestation), $\text{PGE}2$, PRL, and estradiol (Gibori & Keyes 1980, Terranova & Greenwald 1981, Risk & Gibori 2001, Arosh *et al.* 2004). Luteotropic hormones can have direct actions on the luteal cells, stimulating P_4 production by interaction with their respective receptors. They can also show indirect actions, modulating luteal synthesis of growth factors, cytokines, or other factors

that in turn can influence luteal cell function (Horvath *et al.* 1986, Christenson & Devoto 2003). In the first step of luteal steroidogenesis, the cells take up the precursor cholesterol from circulating cholesterol-rich lipoproteins. Thereafter, cholesterol is transported into the mitochondria by steroidogenic acute regulatory protein (StAR), the protein that governs the movement of cholesterol from outer to inner membranes and converted by cytochrome P450 side chain cleavage enzyme (P450scc) to pregnenolone, which in turn is transformed to P_4 by 3β hydroxysteroid dehydrogenase ($3\beta\text{HSD}$; Christenson & Devoto 2003).

At the end of pregnancy in the rat, the most evident sign of luteal regression is the abrupt decrease in P_4 secretion by the luteal cells, which is followed by their apoptotic death (Guo *et al.* 1998). The factors responsible for initiating and mediating luteal regression are very complex. $\text{PGF}2\alpha$ is accepted as a primary luteolysin in most mammals, and its main role is to induce expression of 20α hydroxysteroid dehydrogenase ($20\alpha\text{HSD}$), the enzyme that converts intraluteal P_4 to its inactive metabolite 20α -dihydro P_4 , (Arosh *et al.*

2004). It is well known that the activity of 20 α HSD rises at the end of pregnancy and that PRL represses its expression (Albarracin *et al.* 1994). The key role of 20 α HSD as a critical component of luteolysis at the end of pregnancy was evidenced most strikingly by the development of the 20 α HSD knockout mouse, in which circulating P₄ levels remain elevated and parturition is significantly delayed (Piekorz *et al.* 2005). PGF2 α , after binding to its receptor, acts through a cascade of signaling events involving hormones, cytokines, and possible disruptions of intracellular growth factor signaling (Orlicky *et al.* 1992, Olofsson *et al.* 1996). PGF2 α action is also associated with increased reactive oxygen species and free radicals, such as nitric oxide (NO), that have been shown to participate in luteal regression (Sawada & Carlson 1991, Olson *et al.* 1996, Motta *et al.* 2001).

There is evidence that LH participates in the luteolytic process at the end of pregnancy, through modulation of the activities of 3 β HSD and 20 α HSD, and of the intraluteal PGF2 α concentration (Stocco & Deis 1996, 1998).

We have recently shown that hypothyroidism in the rat produces a prolongation of gestation associated with elevated levels of circulating P₄ on day 21 of pregnancy (Hapon *et al.* 2003). Conversely, hyperthyroid pregnant rats show premature delivery caused by an early fall in serum P₄ (Rosato *et al.* 1992, 1998). Thus, alterations in the thyroid state clearly compromise the timely initiation of luteolysis. The aims of the present work are to determine whether the delayed parturition in hypothyroid mother rats is due to a postponement of the induction of P₄ catabolism or to a stimulation of its synthesis, and to investigate the possible mechanisms that may underlie the altered luteal function. With these aims, we characterized the effect of hypothyroidism on the circulating profile of the hormones (TSH, PRL, LH, P₄, PGF2 α and PGE2) involved in luteal regulation at the end of the pregnancy and on the expression of 20 α HSD as the main inducible factor mediating P₄ catabolism. We also studied the expression of factors participating in P₄ synthesis, such as P450_{scc}, StAR, 3 β HSD, and both forms of the PRL receptor (PRLR_{long} and PRLR_{short}) and of factors involved in prostaglandin action and metabolism, such as PGF2 α receptor (PGF2 α R), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2), the main enzyme regulating prostaglandin synthesis.

Materials and Methods

Animals and experimental design

Adult female Wistar rats bred in our laboratory, 3–4 months old, weighing 200–230 g at the onset of treatment and with regular 4 day cycles were used. The rats were kept in a light (lights on 0600–2000 h) and temperature (22–24 °C) controlled room. Rat chow (Cargill, Cordoba, Argentina) and tap water or

propylthiouracyl (PTU) solution were available *ad libitum*. Hypothyroidism was induced by administration of PTU at a concentration of 0.1 g/l in the drinking water. The treatment was started on the estrus day and the rats mated 8 days later, on proestrus. The presence of spermatozoa in the vaginal smears the morning after caging, with a fertile male in the night of proestrus was indicative of pregnancy and this day was counted as day 0 of pregnancy. Two or three days before delivery the rats were caged individually. The day and hour of delivery were recorded. On day 1 of lactation, the number of pups in each litter was standardized to eight.

To determine the pattern of hormonal secretion and the function of the corpus luteum during late pregnancy and postpartum, groups of 8–10 hypothyroid or control (Co) rats were killed by decapitation on days 19 of pregnancy and 2 postpartum at 1000–1200 h or on day 21 of pregnancy at 1800 h. This last time was selected because at this moment circulating P₄ has declined to values inferior to 25 ng/ml in most control rats, indicating that functional luteolysis has occurred. Trunk blood was collected and serum was separated by centrifugation and stored at –30 °C until used. The corpora lutea from each dam were rapidly removed, washed in a cold saline solution, snap-frozen in liquid nitrogen and stored at –70 °C until they were used for RNA preparation. Other groups of 8–10 hypothyroid or control (Co) rats were killed on day 21 of pregnancy at 1800 h for measurement of luteal P₄ and prostaglandin content.

Animal maintenance and handling was performed according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication N° 86-23, revised 1985 and 1991) and the UK requirements for ethics of animal experimentation (Animals Scientific Procedures, Act 1986).

Hormone and prostaglandin determinations

PRL, LH, and TSH were 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).

For luteal P₄, PGE2 and PGF2 α quantifications the pooled corpora lutea from each dam were weighed and homogenized in 1.5 volumes of absolute ethanol. Homogenates were centrifuged at 1000 *g* for 15 min and the supernatants were transferred to polypropylene tubes. The pellet was washed with the same volume of absolute ethanol, centrifuged again and the supernatant pooled with the first ethanol extract. The extracts were evaporated to dryness under nitrogen atmosphere at room temperature. The residues were stored at –20 °C until used for P₄, PGE2 and PGF2 α radioimmunoassays (Motta *et al.* 1995). For measurement of circulating prostaglandins, sera were extracted thrice with 1 ml diethyl ether, the pooled extracts evaporated to dryness

under nitrogen atmosphere at room temperature and the residues stored at -20°C until used for PGE2 and PGF2 α radioimmunoassay.

Aliquots of the tissue extracts and were reconstituted in PBS pH 7.4, containing 0.1% bovine serum albumin (BSA; Sigma Chemical Co), and 0.1% sodium azide (Merck). Prostaglandins were quantified by radioimmunoassay as described by Motta *et al.* (1999) using specific rabbit antisera from Sigma Chemical Co.

For measurement of intraluteal P₄, aliquots of the corpus luteum extracts were dried and reconstituted in 0.5 ml PBS EDTA 1 mM, gelatin 0.1%, incubated at 38°C for 2 h and aliquots of 5 μl were used for the assay. P₄ concentrations in sera and corpora lutea were measured by radioimmunoassay using commercial kits for total hormones (DSL-3400 double antibody radioimmunoassay from Diagnostic System Laboratories, Webster, TX, USA).

RNA isolation and RT-PCR analysis

Total luteal RNA was prepared using TRIZOL Reagent (Invitrogen Life Technologies), following the manufacturers instructions for RNA isolation. Ten micrograms of total RNA were reverse transcribed at 42°C using random hexamer primers and Moloney murine leukemia virus retrotranscriptase (Invitrogen/Life Technologies) in a 20 μl reaction mixture. Aliquots of the reverse transcription reaction mix cDNA corresponding to different quantities of cDNA for each reaction were amplified with primers specific for the rat and in the conditions described in Table 1. The conditions and quantities of cDNA added 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

cDNA. RNA samples were assayed for DNA contamination by performing the different PCRs without prior reverse transcription. The PCR products were analyzed on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and photographed using a Kodak DC 290 Zoom Digital 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 housekeeping gene rat ribosomal protein L 19 cDNA.

Western blot analysis of 20 α HSD

Proteins were isolated from the phenol-ethanol supernatant obtained from RNA isolation using TRIZOL Reagent (Invitrogen Life Technologies), following the manufacturer instructions for protein isolation (Chomczynski 1993). After vacuum drying the protein pellet was dissolved by pipetting in 1% SDS, containing aprotinin, pepstatin, and leupeptin (2 $\mu\text{g}/\text{ml}$ each), followed by 30-min incubation on ice and centrifugation at 10 000 *g* for 20 min at 4°C . The supernatant was aliquoted and stored at -20°C until used. Protein content in the supernatants was measured by the method of Lowry *et al.* (1951) using BSA as standard. Aliquots containing 30 μg protein were denatured by boiling for 10 min in sample buffer, electrophoresed on 12% SDS-PAGE gels and transferred to a nitrocellulose paper at 20 V for 17 h. The blots were then washed and blocked for 2 h with 5% nonfat milk in PBS-T ((pH 7.4); PBS 10 mM, Triton X-100 0.1%) and incubated with polyclonal antibody against rat 20 α HSD (1:3000 dilution, Albarracin & Gibori 1991, generously provided by Dr Geula Gibori) overnight at 4°C , and then washed and incubated with a secondary antibody

Table 1 Primer sequences and reaction conditions used in the PCR amplification of the various cDNAs.

Gene	Sense primer (5'-3')	Gene bank accessions	Size of amplification product (pb)	Quantity of cDNA added (ng)	No. of cycles	Reference
L19 S	CGCCAATGCCAACTCTCGTCA	X82202	120	100	25	Sugino <i>et al.</i> (1998)
L19 AS	TTCGCTCGGGCCAAAGGTGTTTC					
20 α -HSD S	TTCGAGCAGAACTCATGGCTA	D14424	440	100	30	Sugino <i>et al.</i> (1998)
20 α -HSD AS	CAACCAGGTAGAATGCCATCT					
3 β -HSD S	GTCTTCAGACCAGAAACCAAG	M38179	447	100	30	Sugino <i>et al.</i> (1998)
3 β -HSD AS	CCTTAAGGCACAAGTATGCAG					
P450 sccS	GGATAAACCTGTACCACTTCC	J05156.1	366	200	20	Sugino <i>et al.</i> (1998)
P450 sccAS	CTTTGATGAAGTCCTGAGCTAC					
PGF2 α R S	ACGGCGTTTATCTCCACAAC	NM013115.1	428	20	30	Engstrom (2001)
PGF2 α R AS	CCGATGCACCTCTCAATG					
COX2 S	ACGCCACCCCAACACAGTA	NM017232.2	413	200	35	Engstrom (2001)
COX2 AS	CCCAGGTCCTCGCTTCTG					
STAR S	CCAGGAGCTGTCTACATCCAG	031558	745	20	25	Dalla Valle <i>et al.</i> (2004)
STAR AS	TACTACCCTCTCGTTGTCTT					
INOS S	GCATGGACAGTATAAGGCAAGCA	S71597	219	500	35	Markewitz <i>et al.</i> (1993)
INOS AS	GCTTCTGGTCGATGTCATGAGCAA					
PRLR S	AAAGTATCTTGCCAGACTCGCTG	M74152	279	100	30	Telleria <i>et al.</i> (1997)
PRLRLong AS	AGCAGTTCTTCAGACTTGCCCTT	NM012630.1	279	100	30	
PRLRShort AS	TTGTATTGCTTGGAGAGCCAGT					

All reactions were carried out with the following cyclic parameters for L19, PGF2 α R and COX2: 95°C for 1 min, 56°C 1 min and 72°C 1 min and for the rest of the reactions: 95°C for 1 min, 65°C 1 min and 72°C 1 min. All the reactions were terminated with a 5 min extension at 72°C .

conjugated to horseradish peroxidase (1:2000 dilution goat anti-rabbit-Dako Cytomation, Glostrup, Denmark) in blocking solution for 1 h at room temperature. For β -tubulin the blots were incubated with anti β -tubulin polyclonal antibody (1:3000 dilution, Calbiochem, MA, USA) for 1 h 30 min at room temperature, washed and then incubated with a secondary antibody conjugated to horseradish peroxidase (1:1500 dilution goat anti-mouse-Dako Cytomation, Glostrup, Denmark) for 1 h at room temperature. Protein-antibody complexes were visualized using chemiluminescence (ECL, GE Biosciences, Buenos Aires, Argentina) using X-OMAT-LS (Kodak) film using and the band densities were determined by digital analysis using a Kodak DC 290 Zoom Digital Camera, and quantified by densitometry using digital image processed by the NIH Image 1.6/ppc freeware program. Relative levels of 20 α HSD protein were expressed as the ratio of signal intensity for the protein relative to that of β -tubulin.

Statistical analysis

Statistical analysis was performed using two-way analysis of variance followed by the Bonferroni *post hoc* test to compare any two individual means or using Student's *t*-test when only two groups were compared (Snedecor & Cochran 1967). When variances were not homogeneous we performed log transformation of the data. Differences between means were considered significant at the $P < 0.05$ level.

Results

Circulating levels of TSH, PRL, LH, and P_4 in pregnant and postpartum rats

Administration of PTU in the drinking water increased circulating TSH, confirming the hypothyroid state of the rats (Fig. 1). As observed previously (Hapon *et al.* 2003), in the hypothyroid rats, circulating P_4 had similar values on days 19 and 21 of pregnancy, and was significantly diminished only on the second day postpartum (Fig. 1), while in the control rats, circulating P_4 decreased significantly from days 19 to 21 of pregnancy and continued to fall on the second day of lactation. PRL levels were similar in control animals on days 19 and 21 of pregnancy and significantly increased on day 2 of lactation, while in the hypothyroid groups they increased from day 19 of pregnancy to day 2 of lactation and were significantly higher on days 19 and 21 of pregnancy when compared with the controls (Fig. 1). Circulating LH was significantly decreased in the hypothyroid rats on days 21 of pregnancy and 2 of lactation (Fig. 1). Parturition was delayed in the hypothyroid rats by approximately 15 h, confirming the previously observed (Hapon *et al.* 2003) lengthening of gestation (duration of pregnancy, controls 22.34 ± 0.12 days, hypothyroid 22.98 ± 0.25 days, $P < 0.05$).

Figure 1 Effect of PTU treatment on serum concentrations of TSH, LH, PRL, and P_4 on days 19 (G19) and 21 (G21) of pregnancy and 2 of lactation (L2). PTU was administered in the drinking water at a concentration of 0.1 g/l. See Materials and Methods section for further details. Results are expressed as the mean $\pm$ S.E.M. for groups of eight rats. * $P < 0.05$ when compared with the respective control groups using two-way ANOVA and Bonferroni *post hoc* test. a, b $P < 0.05$ when compared with G19 and G21 respectively in the same treatment group.

Effect of hypothyroidism on mRNA abundance of proteins related to P_4 synthesis and degradation

In order to determine, if the elevated circulating P_4 observed on day 21 of pregnancy in the hypothyroid rats is due to an elevated rate of P_4 synthesis, we measured the mRNA abundance of genes encoding several proteins involved in the conversion of cholesterol to P_4 , such as StAR, P450scc, and 3 β -HSD. Expression of StAR was similar in control and hypothyroid rats on late pregnancy, but increased sharply after parturition in the hypothyroid rats, resulting in values significantly higher than controls. P450scc had a pattern similar to that described for StAR, but the increase after delivery in the hypothyroid rats did not achieve significance. The mRNA abundance of 3 β -HSD decreased sharply in the control group after parturition, but this decline was not detected in the hypothyroid rats (Fig. 2).

The mRNA for 20 α HSD, as expected, increased before parturition in the control group and declined thereafter,

Figure 2 Effect of PTU treatment on luteal mRNA abundances of enzymes related to P_4 synthesis and degradation on days 19 (G19) and 21 (G21) of pregnancy and 2 of lactation (L2). (A) Measurement by RT-PCR of expression of StAR, Cytochrome P450_{scc}, 3βHSD, 20αHSD and L19. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products. (B) expression of StAR, P450_{scc}, 3βHSD and 20αHSD, relative to L19 respectively. The gel photographs were quantified using NIH Image and expressed as arbitrary units. See Materials and Methods section for further details. Results are expressed as the mean $\pm$ S.E.M. of groups of seven to eight rats. * $P < 0.05$ when compared with the respective control groups using two-way ANOVA and Bonferroni *post hoc* test. a, b $P < 0.05$ when compared with G19 and G21 respectively in the same treatment group.

while the hypothyroid groups had significantly diminished abundance on days 19 and 21 of pregnancy, and increased values on day 2 postpartum. These results indicate that the decreased expression of 20αHSD before parturition in the hypothyroid rats may be responsible for the delayed decline in circulating P_4 (Fig. 2), while the increased values on early lactation may be linked to P_4 decline at this time, in spite of the elevated expression of the factors favoring P_4 biosynthesis. Since PRL represses the expression of 20α-HSD at the end of pregnancy and a

negative correlation between luteal expression of the PRLR and 20α-HSD has been described (Albarracin *et al.* 1994), we investigated whether hypothyroidism affected the expression of the PRLR on day 21 of pregnancy. The mRNA concentration, relative to L19, of both forms of the PRLR were similar in the control and hypothyroid rats (PRLR_{long}/L19, control: 1.58 ± 0.08 , hypothyroid: 1.39 ± 0.14 ; PRLR_{short}/L19, control: 1.24 ± 0.10 , hypothyroid: 1.13 ± 0.11).

To investigate whether the decrease in 20αHSD mRNA observed in the hypothyroid rats is also reflected in the expression of the protein, we analyzed by western blot the abundance of 20αHSD protein relative to β-tubulin. 20αHSD protein was undetectable on day 19 of pregnancy and could be readily detected on day 21 in both groups, but the hypothyroid rats had significantly lower levels when compared with the controls. On day 2 postpartum, the 20αHSD protein levels decreased in control rats when compared with day 21 of pregnancy, while there was an increase in the hypothyroid rats, which reached values comparable with the controls (Fig. 3).

Figure 3 Effect of PTU treatment on luteal abundance of 20αHSD protein on days 19 (G19) and 21 (G21) of pregnancy and 2 of lactation (L2). (A) western blot of 20αHSD protein on whole luteal protein extracts separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with specific 20αHSD (37 kDa), and β-tubulin (55 kDa) antisera. B: expression of 20αHSD relative to β-tubulin. The films were quantified using NIH Image and expressed as arbitrary units. Results are expressed as the mean $\pm$ S.E.M. of groups of five rats. * $P < 0.05$ when compared with the respective control groups using two-way ANOVA and Bonferroni *post hoc* test. a, $P < 0.05$ when compared with G19 and G21 respectively in the same treatment group.

Circulating levels of prostaglandins, and intraluteal levels of P_4 and prostaglandins in pregnant and postpartum rats

Since PGF2 α is considered the main stimulatory factor for 20 α HSD expression, we measured its concentrations in circulation on late pregnancy and early postpartum. We also measured circulating PGE2, which has luteotrophic properties and may oppose PGF2 α action. Serum levels of PGF2 α were similar in all the days studied and between groups (Fig. 4). On the other hand, circulating PGE2 was always higher in the hypothyroid groups when compared with the controls. Both groups showed significant increases from days 19 to 21 of pregnancy, but while the values in control rats tended to decrease on day 2 postpartum, circulating PGE2 continued to increase in the hypothyroid rats (Fig. 4). In contrast with the lack of variation observed in serum, luteal PGF2 α concentration was significantly diminished on day 21 of pregnancy, while luteal PGE2 was also increased in the hypothyroid rats (Fig. 4). We also found significantly higher luteal P_4 content on day 21 of pregnancy (Fig. 4), confirming the delay in luteal regression of the hypothyroid rats.

Effect of hypothyroidism on mRNA expression of proteins involved in prostaglandin synthesis and action

We next investigated the mRNA abundance of factors related to the regulation of PGF2 α synthesis and actions, such as COX2, PGF2 α receptor, and iNOS, since NO has a luteolytic action through the induction of COX2. As shown on Fig. 5, there were no significant variations in the expression of COX2, while iNOS mRNA abundance was significantly diminished in the hypothyroid rats on day 21 of pregnancy. Thereafter, the expression of PGF2 α R increased sharply in the control group on day 21 and declined on day 2 postpartum. In hypothyroid rats the expression of PGF2 α R increased progressively from day 19 of pregnancy to day 2 of lactation and the values were significantly higher on days 19 of pregnancy and 2 postpartum when compared with the controls (Fig. 5).

Discussion

The present results confirm our previous findings of elevated circulating P_4 on the last day of pregnancy in hypothyroid rats (Hapon *et al.* 2003), which along with the hereby described elevated intraluteal P_4 concentration on day 21, confirm that hypothyroid rats have delayed luteal regression. The prolongation of luteal P_4 secretion may be linked directly with the reduced expression of 20 α HSD mRNA on days 19 and 21 of pregnancy, and of 20 α HSD protein on day 21. On the other hand, the unchanged expression of StAR, P450scc, and 3 β HSD suggest that the synthesis of the steroid was

Figure 4 Effect of PTU treatment on serum concentrations of PGF2 α and PGE2 on days 19 (G19) and 21 (G21) of pregnancy and 2 of lactation (L2) and luteal concentrations of P_4 , PGF2 α and PGE2 on day 21 (G21) of pregnancy. See Materials and Methods section for further details. Results are expressed as the mean $\pm$ S.E.M. for groups of eight rats. * $P < 0.05$ when compared with the respective control groups using two-way ANOVA and Bonferroni *post hoc* test or Student's *t*-test. a, b $P < 0.05$ when compared with G19 and G21 respectively in the same treatment group.

not affected by hypothyroidism. The diminished expression of 20 α HSD is most probably related to the decreased intraluteal PGF2 α concentration, although its levels in circulation and the luteal expression of its receptor were normal at this time. Although we are not aware of any previous evidence of direct or indirect effects of the thyroid hormones on PGF2 α synthesis, our present results suggest that thyroid hormones may regulate luteal PGF2 α synthesis locally.

It is well known that the initiation of luteolysis by PGF2 α in rats is associated with an increase in reactive oxygen species including superoxide radicals (Sawada & Carlson 1991). NO is synthesized in rat luteal cells and has been proposed to be a mediator of luteal regression (Olson *et al.* 1996). Moreover, during this process, PGF2 α

Figure 5 Effect of PTU treatment on luteal mRNA abundance of COX2, iNOS and PGF2 α R on days 19 (G19) and 21 (G21) of pregnancy and 2 of lactation (L2). A: Measurement by RT-PCR of expression of COX2, iNOS, PGF2 α R, and L19. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products. B: Relative expression of COX2, iNOS and PGF2 α R relative to L19 respectively. The gel photographs were quantified using NIH image and expressed as arbitrary units. See Materials and Methods section for further details. Results are expressed as the average $\pm$ S.E.M. of groups of seven to eight rats. * $P < 0.05$ when compared with the respective control groups using two-way ANOVA and Bonferroni *post hoc* test. a, b $P < 0.05$ when compared with G19 and G21 respectively in the same treatment group.

and NO participate in a positive feedback loop that leads to increased lipid peroxidation in parallel with the diminution in P_4 concentrations (Motta *et al.* 1999). It has been demonstrated that PGF2 α enhances NOS activity and also iNOS expression (Motta *et al.* 2001) that increase NO production, which in turn, increases lipid peroxidation. Additionally, it has been shown that on the regressing corpus luteum, stimulation of NO synthesis increases PGF2 α accumulation (Motta *et al.* 1999). Hypothyroidism decreases NOS activity and basal NO production and/or release in peripheral vasculature (Iwata *et al.* 2005), and hypothalamic NOS gene

expression is regulated by thyroid hormones in the rat (Ueta *et al.* 1995). Thus, the decreased luteal iNOS mRNA expression on day 21 of pregnancy could be a direct effect of the hypothyroid state and, in turn, be responsible for the decreased intraluteal PGF2 α levels. iNOS also participates in the generation of reactive oxygen species (Olson *et al.* 1996), that may play a role in triggering luteal cell death and hence, structural luteolysis, and thus its decrease may favor corpus luteum survival.

We also found diminished circulating LH on days 21 of pregnancy and 2 postpartum in the hypothyroid rats that may also have contributed to the prolongation of luteal function. It has been shown that administration of LH antiserum on day 19 of pregnancy delays luteolysis in rats (Stocco & Deis 1996) and conversely, administration of LH advances luteolysis (Stocco & Deis 1996, 1998), mediated by increases in intraluteal PGF2 α concentration and 20 α HSD activity and a decrease in 3 β HSD activity. Thus, the diminished circulating LH observed on day 21 of pregnancy in the hypothyroid rats may be another possible factor mediating the decrease in intraluteal PGF2 α and 20 α HSD expression.

P_4 , administered *in vivo* at the end of pregnancy or to cultured luteal cells, inhibits the activity and expression of 20 α HSD mRNA (Sugino *et al.* 1997, Tellería *et al.* 1999). Thus, the increased intraluteal P_4 levels, through a positive feedback loop, may also contribute to the further inhibition of 20 α HSD expression and hence to the persistence of above normal luteal P_4 concentrations.

We have also found an increase in luteotrophic factors in the late pregnant hypothyroid rats, such as elevated circulating PGE2 and PRL, two factors that contribute to the survival of the corpus luteum and to the maintenance of P_4 production. PRLR activation blocks expression of 20 α HSD, and luteal expression of the long form of the PRL receptor declines simultaneously with the increase in 20 α HSD expression at the end of pregnancy (Stocco *et al.* 2003). We did not find any effect of hypothyroidism on PRLR expression on day 21 of pregnancy, but, since thyroid hormone interferes with PRL signaling and Stat5 activation (Favre-Young *et al.* 2000), the low levels of T_3 in the hypothyroid rats (Hapon *et al.* 2003) may have facilitated signaling by the increased circulating PRL in the corpus luteum, and thus participate in the mechanism that delayed the induction of 20 α HSD.

PGE2 stimulates P_4 production in rat luteal cells (Horvath *et al.* 1986). Although there is no evidence of a direct or indirect regulation of PGE2 by thyroid hormones, there is sufficient information demonstrating that the circulating concentration of PGE2 is dependent on cholesterol-low density lipoprotein (LDL) levels (Habenicht *et al.* 1986). We have recently shown that circulating LDL-cholesterol is elevated in hypothyroid rats regardless of the reproductive state (Hapon *et al.* 2005), and this factor may be linked with the observed elevated serum PGE2. Moreover, hypercholesterolemia may directly influence P_4 production, since *in vivo* and

in vitro evidence suggests that the main source of cholesterol for steroidogenesis is derived from the circulation in most species, including the rat (Azhar *et al.* 1981, Gwynne & Strauss 1982).

In contrast with the effects observed during late pregnancy, after delivery the hypothyroid rats had increased abundance of several factors involved in corpus luteum function, survival or demise, such as PGF₂αR and particularly, of the enzymes involved in P₄ synthesis (StAR, P450_{scc}, 3βHSD) and the key degradation enzyme, 20αHSD. Taken together, these results suggest that hypothyroidism not only delays induction of 20αHSD on late pregnancy, but also may prolong corpus luteum survival after parturition. On the other hand, it is possible that the elevated circulating P₄ may be itself responsible for the longer survival of the corpora lutea, since Goyeneche *et al.* (2003) have described that elevated P₄ promotes survival of the rat corpus luteum even in the absence of the classical nuclear receptors. Recently the existence of a family of progesterin membrane receptors in the corpus luteum has been described, that may mediate this and other paracrine actions of P₄ in rat luteal tissue (Cai & Stocco 2005).

In conclusion, our results show that the delay in P₄ decline and parturition observed in hypothyroid rats is a resultant of a postponement in luteal regression, caused by a combination of decreases in luteolytic factors, mainly luteal PGF₂α and iNOS expression and circulating LH, and increased synthesis or action of luteotrophic factors, such as luteal and circulating PGE₂ and PRL. This results in decreased 20α HSD mRNA and protein levels and elevated luteal P₄ that, in turn, may favor survival of the corpus luteum.

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