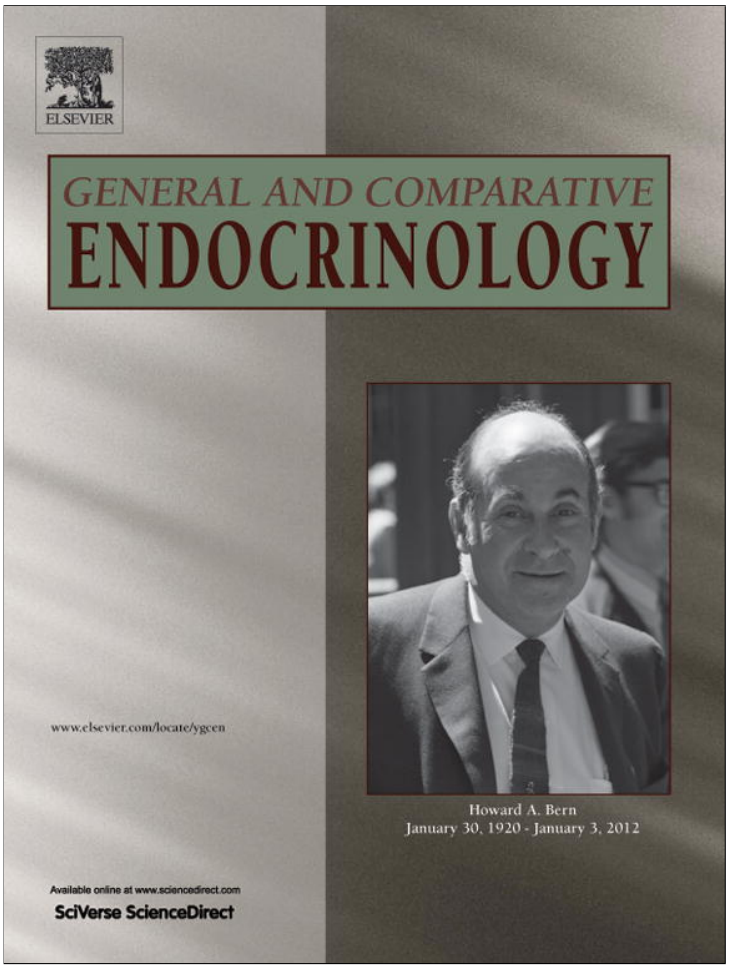

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General and Comparative Endocrinology 184 (2013) 1–8

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

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

Effect of prolactin acting on the coeliac ganglion via the superior ovarian nerve on ovarian function in the postpartum lactating and non-lactating rat

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ARTICLE INFO

Article history:
Received 25 September 2012
Revised 22 December 2012
Accepted 24 December 2012
Available online 10 January 2013

Keywords:
Prolactin
Luteal regression
Coeliac ganglion
Post-partum rat

ABSTRACT

Whether prolactin (PRL) has a luteotrophic or luteolytic effect in the rat ovary depends on the nature of the corpora lutea present in the ovaries and the hormonal environment to which they are exposed. The aim was to investigate the effect of PRL acting on the coeliac ganglion (CG) on the function of the corpora lutea on day 4 postpartum under either lactating or non-lactating conditions, using the CG–superior ovarian nerve–ovary system. The ovarian release of progesterone (P), estradiol, PGF2α, and nitrites was assessed in the ovarian compartment at different incubation times. Luteal mRNA expression of 3β-HSD, 20α-HSD, aromatase, PGF2α receptor, iNOS, Bcl-2, Bax, Fas and FasL was analysed in the corpus luteum of pregnancy at the end of the experiments. Comparative analysis of control groups showed that the ovarian release of P, nitrites, and PGF2α, the expression of PGF2α receptor, and the Bcl-2/Bax ratio were lower in non-lactating rats, with increased release of estradiol, and higher expression of aromatase, Fas and FasL, demonstrating the higher luteal functionality in ovaries of lactating animals. PRL added to the CG compartment increased the ovarian release of P, estradiol, nitrites and PGF2α, and decreased the Bcl-2/Bax ratio in non-lactating rats; yet, with the exception of a reduction in the release of nitrites, such parameters were not modified in lactating animals. Together, these data suggest that the CG is able to respond to the effect of PRL and, via a neural pathway, fine-tune the physiology of the ovary under different hormonal conditions.

1. Introduction

In rats ovulation occurs immediately following parturition; therefore, in the postpartum rat ovary, the corpora lutea of pregnancy that are undergoing regression coexist with the newly-formed corpora lutea after postpartum ovulation. In non-lactating rats, this new cohort of corpora lutea belong to a new estrual cycle and have very short life before regressing [13,43]. Postpartum luteal regression and apoptosis in non-lactating animals might be triggered by an increase in the circulating estradiol at the end of pregnancy [44], likely in response to pituitary PRL [21]. In this regard, Casais et al. [10] demonstrated that estradiol has a direct effect on the ovary favoring regression of the luteal tissue and can certainly mediate PRL-induced luteal regression.

If lactation is established after parturition, however, high and sustained concentrations of PRL are induced as a consequence of the suckling reflex; in this case PRL has a luteotrophic effect on the newly formed corpora lutea after postpartum ovulation – the corpora lutea of lactation – leading to the production of high levels of P, which prevents apoptosis in both the corpora lutea of pregnancy and the corpora lutea of lactation [20,43]. Overall, the previous findings suggest that the dual response (luteotrophic/luteolitic) of the luteal tissue to PRL is dependent on the nature of the corpora lutea and of the hormonal environment to which they are exposed.

Other agents involved in the process of luteal regression are prostaglandin F2α (PGF2α) and the gaseous neurotransmitter nitric oxide (NO). The existence of a feedback mechanism between PGF2α and NO has been shown in a model of rat pseudopregnancy. Administration of NO donors during luteal regression increases the synthesis of ovarian PGF2α and decreases serum P concentrations

Abbreviations: CG, coeliac ganglion; CLP, corpus luteum of pregnancy; iNOS, inducible nitric oxide syntase; NO, nitric oxide; P, progesterone; PGF2α, prostaglandin F2α; PGF2αR, prostaglandin F2α receptor; PRL, prolactin; RIA, radioimmunoassay; SON, superior ovarian nerve.

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http://dx.doi.org/10.1016/j.ygcen.2012.12.012

[34]; furthermore, administration of a luteolytic dose of PGF2α increases the activity of NO synthase (NOS) in the ovary, while it reduces P production [35].

In mammals, the ovary is controlled by endocrine and neural factors. The latter come from two routes: the ovarian plexus and the superior ovarian nerve (SON). While the former is important for the innervation of the blood vessels of the ovary, the latter is critically relevant to regulate the overall physiology of the ovary, including ovulation and steroidogenesis. The SON is mainly constituted by adrenergic fibers, most of which originate in the coeliac ganglion (CG) [28,25,5–7]. The CG is included in the sympathetic pre-vertebral chain and it is constituted by principal neurons and interneurons [18,1,30,37]. Sympathetic ganglia possess a diversity of neurotransmitters, among them catecholamines [24], neuropeptides [14] and NO [33], receptors for hormones, neurotransmitters [48,49], and a profuse capillary plexus that constitutes a microcirculation among the different ganglionic structures [12]. This suggests that bioactive substances such as hormones can reach through this plexus and modulate the ganglionic activity. In addition, in rats, the muscarinic cholinergic neurotransmission of the superior cervical ganglion is affected by several hormones, including PRL [19].

The aim of this study was to investigate the effect of PRL acting on the CG in the *ex vivo* CG–SON–O system previously standardized in our laboratory [9] on the physiology of the corpora lutea found in the ovary on day 4 postpartum under two different endocrine environments: non-lactating and lactating conditions.

2. Materials and methods

2.1. Reagents

The following drugs: ovine PRL (L6520), dextrose (D9434), ascorbic acid (A5960), bovine serum albumin fraction V (BSA) (A2153), sulphanilamide (S9251) and *N*-1-naphthyl-ethylenediamine (N9125) were purchased from the Sigma Chemical Co. 1,2,6,7-[^{3}H] Progesterone (107.0 Ci/mmol) was provided by New England Nuclear Products (Boston, MA, USA). Other reagents and chemicals were of analytical grade.

2.2. Animals

To induce pregnancy, virgin Holtzman female rats weighing 250 ± 50 g were caged individually with fertile males beginning on the afternoon of proestrus. Positive mating was verified on the following morning by identifying sperm or copulation plugs in the vagina. This day was designated as day 0 of pregnancy. In our laboratory, rats usually give birth on day 22. Pregnant rats were divided into two groups at parturition: non-lactating rats encompassing mothers whose pups were removed immediately after delivery, and lactating rats, including mothers that were normally kept with their newborn pups (the number of pups was adjusted to 8). Animals had free access to food (Cargill SACI, Saladillo, Buenos Aires, Argentina) and water. They were kept in a light (lights on from 07:00 to 19:00 h) and temperature controlled room (24 ± 2 °C). Animals were handled according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory Animals. The experimental protocol was approved by the University of San Luis Animal Care and Use Committee (number protocol: B49/10).

2.3. Extraction of CG–SON–O system of rats

The extraction of CG–SON–O system was carried out at 9 am on day 4 postpartum. Thus, in non-lactating animals, the system is only influenced by the preovulatory PRL surge occurring at 3 h postpartum, but not by that of afternoon of proestrus (day 4 postpartum).

Groups of six animals were used for each experimental procedure. The surgical procedure to remove the CG–SON–O system and the incubation conditions were carried out according to Casais et al. [9]. Animals were anaesthetized with ketamine and xylazine (80 and 10 mg kg^{-1}, respectively, I.P.). The system was extracted and the mothers were sacrificed by decapitation. The complete system was removed by surgery, avoiding contact between the surgical instruments and the nerve fibres in order to prevent spontaneous depolarisation of the nerves. The piece of tissue removed consisted of the left ovary, the fibres that constituted the SON, inserted in the suspensory ligament, and the CG accompanied by some small ganglia surrounding it. The total surgical procedure was completed in 1–2 min.

2.4. Experimental procedures

The system was washed with incubation medium and placed in a cuvette with two compartments, one for the CG and the other for the ovary, both joined by the SON. The incubation medium used was Krebs–Ringer bicarbonate buffer, pH 7.4 with the addition of 0.1 mg glucose/ml and 0.1 mg albumin/ml at 37 °C in an atmosphere composed by 95% of O_2 and 5% of CO_2.

The system was preincubated for 30 min, and the end of this period was considered incubation time 0. After this time, buffer was changed in both compartments and 0.1 mg of ascorbic acid/ml was added to the ganglion compartment as antioxidant agent. At time zero of incubation, 10^{-7} M PRL was added to the ganglionic compartment [$(PRL)_g$]. Control groups consisted of untreated CG–SON–O systems. Periodic extractions (250 μl) of the ovary incubation liquid were carried out at 30, 60, 120, 180 and 240 min and kept at −20 °C until dosage of P, estradiol and PGF2α (only at 240 min) by radioimmunoassay (RIA) and determination by Griess technique of nitrite, the soluble metabolite of NO. The corresponding corrections were made in all cases, taking into consideration the volume extracted in each tested period. After incubation (240 min), whole ovaries were weighed, the CLP were separated and stored at −80 °C. From the CLP of both groups on day 4 postpartum, total RNA was extracted for analysis of mRNA expression of 3β-hydroxysteroid-dehydrogenase (3β-HSD), 20α-hydroxysteroid-dehydrogenase (20α-HSD), aromatase (P synthesis and degradation and estradiol synthesis enzymes, respectively), PGF2α receptor (PGF2αR), inducible nitric oxide synthase enzyme (iNOS), Bcl-2, Bax, Fas and FasL by RT-PCR.

2.5. Progesterone and estradiol radioimmunoassay

Steroids were measured in duplicate by RIA in the ovary incubation liquid. The progesterone antiserum, provided by Dr. R. Deis (IMBECU, Mendoza, Argentina), was produced in rabbits against progesterone conjugated to bovine serum albumin at the 11 position. The antiserum was highly specific for progesterone with low cross-reactivities, <2.0% for 20α-dihidro-progesterone and deoxycorticosterone and 1.0% for other steroids. The sensitivity was less than 5 ng/ml and the inter and intraassay coefficients of variation were less than 10%. This assay has been validated previously [4]. Progesterone concentration was expressed as nanogram per milligram ovary (ng/mg ovary), all against incubation time. The estradiol levels were determined using a double antibody RIA Diasource kit (DiagnosMed SRL) following the manufacturer's instructions. Estradiol was expressed as picograms per milligram of ovarian tissue (pg/mg ovary), all against incubation time. The percentages of cross-reactions were 1.8% for estrone, 1.2% estriol, 0.0011% androstenedione and 0.0002% for progesterone. The assay

sensitivity was <2.2 pg estradiol/ml. The inter- and intra-assay coefficients of variation in all the assays were <10.0%.

2.6. Prostaglandin radioimmunoassay

Aliquots of ovarian incubation liquid obtained at the end of incubation (240 min) were acidified to pH 3.0 with 1 mol/l HCl and extracted for PGF2α determination three times with 1 vol. of ethyl acetate. Pooled ethyl acetate extracts were dried under an atmosphere of N_2 and stored at −20 °C until prostaglandin radioimmunoassay. PGF2α was quantified by radioimmunoassay using rabbit antiserum from Sigma Chemical Co. (St. Louis, MO, USA). Sensitivities of these assays were of 10 pg/tube for PGF2α. The cross-reactivity of PGF2α was 1.0% with PGE_1 and <0.1% with other prostaglandins.

2.7. Nitrite assay

Levels of nitrites, water-soluble metabolites of nitric oxide, were measured spectrophotometrically in the ovary incubation liquid [16]. Samples were immediately mixed with Griess reagent (sulphanilamide with N-1-naphthyl-ethylendiamine/HCl). After a 10 min incubation period at room temperature, the optical readings at 540 nm were measured and nmol of nitrite were determined using a standard curve. The assay sensitivity was <2.5 nmol/ml. The intra-assay coefficients of variation for all the assays were <10.0%. The results were expressed as nmol of nitrite per milligram of ovarian tissue (nmol/mg ovary).

2.8. RNA isolation and RT-PCR analysis

Total luteal RNA was isolated using TRIZOL Reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Purified total RNAs were then quantified and assessed for purity by measurement of the 260/280 and 260/230 ratio using an UV spectrophotometer Beckman DU-640 B (CA, USA). Only samples with 260/280 ratio of 1.8–2.0 and 260/230 ratio of 2.0–2.2 were used. La integrity was confirmed by running 2 μg RNA on a 0.8% agarose gel. After GelRed™ (Biotium) staining RNA bands were visualized with a UV transilluminator and 28S and 18S rRNA band patterns were analyzed. Two micrograms of total RNA were reverse transcribed at 37 °C using random primers and M-MLV Reverse Transcriptase (Promega Inc.) in a 26 μl reaction mixture. For amplification of the reverse transcription (RT) products, the reaction mixture consisted of 1× Green Go Taq reaction buffer, 0.2 mM deoxynucleoside triphosphates, 0.5 μM specific oligonucleotide primers and 1.25 U Go Taq DNA polymerase (Promega Inc.) in a final volume of 50 μl. The PCR primers were designed using Primer Express 3.0 software (Applied Biosystems, USA) on the basis of the respective published rat DNA sequences. The primers information is shown in Table 1.

The amplification of the cDNA was performed using a thermalcycler (My Cycler, BioRad); for 3β-HSD, 20α-HSD, aromatase, PGF2αR, Fas, FasL, Bcl-2 and S16 the reactions were carried out at 95 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min by 35 cycles. For Bax and iNOS the parameters were 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min by 40 cycles. All the reactions were terminated with a 5 min extension at 72 °C. Reaction products were electrophoresed on 2% agarose gels, visualized with GelRed, and examined by ultraviolet transillumination. Band intensities of RT-PCR products were quantified using ImageJ (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). Relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to that for the housekeeping genes S16.

2.9. Statistical analysis

All data are presented as means ± S.E.M in each group of six rats. Differences between two groups were analyzed with Student's *t*-test. For multiple comparisons made along the time of incubation, repeated measures analysis of variance followed by Tukey's test was used. Instead, for multiple comparisons not involving repeated measures, one-way analysis of variance followed by Tukey's test was utilized. A difference was considered to be statistically significant at $p < 0.05$.

3. Results

3.1. CG–SON–O systems of day 4 postpartum in non-lactating and lactating rats: ovarian P release and mRNA expression of 3β-HSD and 20α-HSD

We analyzed the release of P in the ovarian compartment and the mRNA expression of enzymes involved in P synthesis and degradation in the control groups of non-lactating and lactating rats. The ovarian release of P in non-lactating animals was lower than in the lactating group at 30′, 180′ and 240′ (a, $p < 0.005$), 60′ and 120′ (b, $p < 0.001$). On the other hand, the expressions of 3β-HSD and 20α-HSD enzymes were similar in both studied groups (Fig. 1A and B). In non-lactating rats, addition of PRL in the CG [$(PRL)_g$] significantly increased the release of ovarian P at all incubation times tested (•, $p < 0.05$) (Fig. 1A). Such stimulatory effect on P release was not observed in lactating animals (Fig. 1B). In both groups studied [$(PRL)_g$] did not change the mRNA expression of 3β-HSD and 20α-HSD when compared to the control group (Fig. 1A and B).

3.2. CG–SON–O systems of day 4 postpartum in non-lactating and lactating rats: ovarian estradiol release and mRNA expression of aromatase in the CLP

The comparative analysis of estradiol release in the ovarian compartment and semi-quantitative mRNA expression of aromatase between ovaries of both lactating and non-lactating animals showed that in non-lactating rats the levels of estradiol were higher than those in lactating rats at 30′, 60′ and 240′ (c, $p < 0.05$); 120′ and 180′ (d, $p < 0.025$). In turn, the expression of mRNA coding for aromatase is higher in non-lactating ovaries when compared with that of ovaries from lactating animals (e, $p < 0.01$) Fig. 2A and B). Moreover, [$(PRL)_g$] in non-lactating rats significantly increased the ovarian estradiol release at 30′, 120′ and 180′ (•, $p < 0.05$), 60′ and 240′ (∗, $p < 0.025$) in relation to the control group; while in lactating rats [$(PRL)_g$] did not modify estradiol release as compared to controls. In both lactating and non-lactating animals, [$(PRL)_g$] did not change aromatase mRNA expression in the ovaries (Fig. 2B).

3.3. CG–SON–O systems of day 4 postpartum in non-lactating and lactating rats: ovarian PGF2α and NO release, mRNA expression of iNOS and PGF2αR

The release of PGF2α at 240 min in the ovarian compartment and semi-quantitative mRNA expression of its receptor in the control groups were compared in non-lactating and lactating rats. Both parameters were lower in non-lactating rats (c, $p < 0.05$ and b, $p < 0.001$, respectively) (Fig. 3A and B). In non-lactating rats, addition of PRL on the CG [$(PRL)_g$] significantly increased ovarian PGF2α release with respect to non-lactating control ovaries (•, $p < 0.05$) without modifying the mRNA expression of PGF2αR. In lactating rats, neither ovarian PGF2α release nor mRNA expression of PGF2αR were modified by [$(PRL)_g$] (Fig. 3A and B). The comparative

Table 1
Primer used for semi-quantitative RT-PCR amplification.

Gen	Sequences (sense above, antisense below; 5–3′)	GenBank accession #	Amplicon length (pb)	Sequence reference
3β-HSD	GTCTTCAGACCAGAAACCAAGCCTTAAGGCACAAGTATGCAG	M38179	447	[42]
20α-HSD	CAACCAGGTAGAATG CCATCT TTCGAGCAGAACTCATGGCTA	D14424	440	[42]
Aromatase	TGCACAGGCTCGAGTATTTCC ATTTCCACAATGGGGCTGTCC	M33986	266	[22]
iNOS	GCATGGACCAGTATAAGGCAAGCA GCTTCTGGTCGATGTCATGAGCAA	S71597	219	[29]
PGF2αR	ACGGCGTTTATCTCCACAAC CCGATGCACCTCTCAATG	NM_013115.1	428	[17]
Fas	TGTCAACCGTGTCAGCCTGGT GGGTCCGGGTGCAGTTCGTT	NM_139194	190	[23]
FasL	GTGCTGGTGGCTCTGGTTGGAA AGTGGGCCACACTCCTTGGCTT	NM_012908	178	[39]
Bcl-2	CACCCCTGGCATCTTCTCCTGTTGACGCTCCCCACACACA	L14680	349	[38]
Bax	TGGCGATGAACTGGACAACAACCCCGAAGTAGGAAAGGAGGC	U32098	301	[46]
S16	TCCAAGGGTCCGCTGCAGTC CGTTCACCTTGATGAGCCCATT	NM_001169146	100	[11]

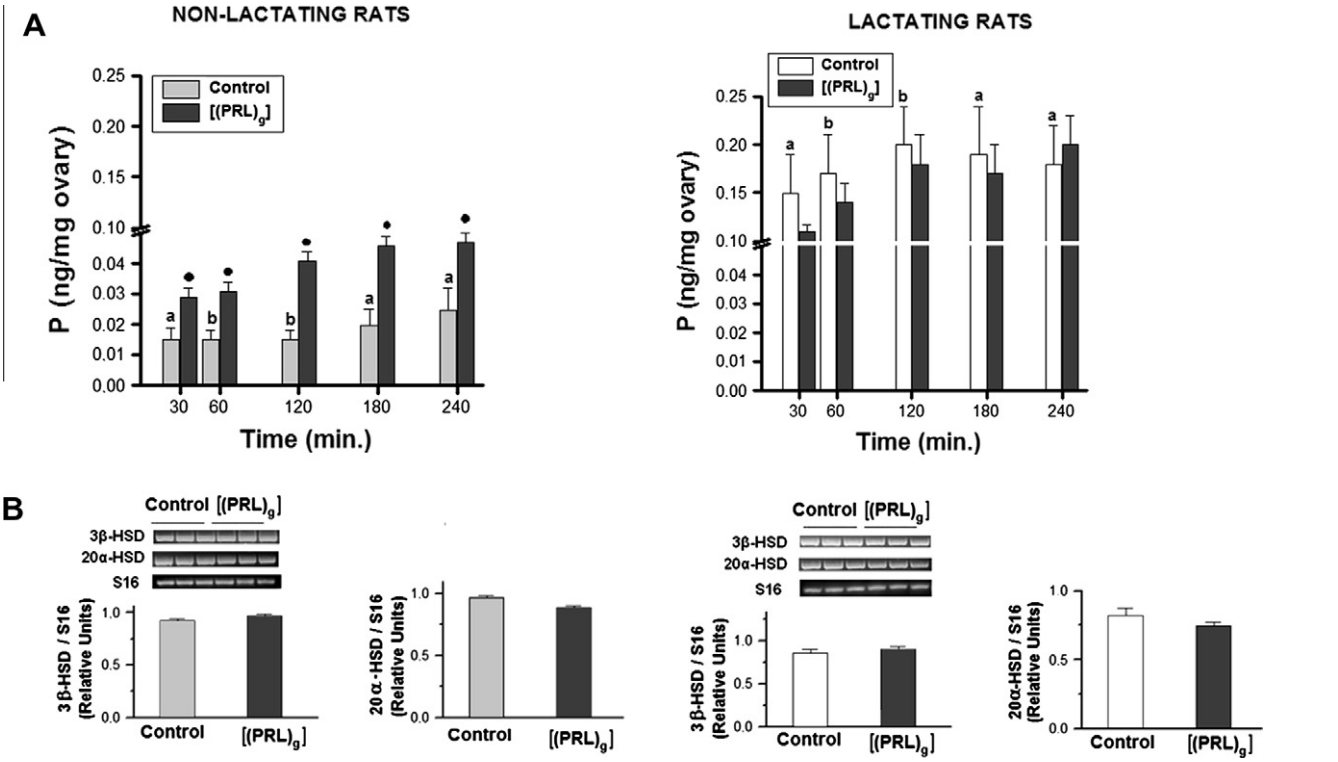

Fig. 1. Coeliac ganglion–superior ovarian nerve–ovary system on day 4 postpartum obtained from non-lactating and lactating rats. The comparison of basal levels of P between non-lactating and lactating rats is represented by letters. The same font denotes statistical significance between both studied groups (a) $p < 0.005$ and (b) $p < 0.001$. Ganglionic effect of PRL [$(PRL)_g$] on the ovarian release of P, expressed in ng/mg ovary (superior panel A). Ganglionic effect of PRL [$(PRL)_g$] on the mRNA expression of 3β-HSD and 20α-HSD luteal enzymes (lower panel B). The respective systems were incubated without [control] and with the addition of 10^{-7} M PRL in the ganglionic compartment [$(PRL)_g$]. Results are expressed as mean ± S.E.M of six animals per group for *P* determination. (●) $p < 0.05$. ANOVA I–Tukey test. PCR products: 3β-HSD (447 bp), 20α-HSD (440 bp) and S16 (100 bp) as endogenous control, were visualized on agarose gels stained with Gel Red. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± S.E.M of three animals for the expression analysis.

analysis of nitrites released in the ovarian compartment and semi-quantitative mRNA expression of luteal iNOS enzyme between both control groups showed that in non-lactating rats the levels of nitrites were lower than in lactating rats at 30′ and 60′ (b, $p < 0.001$), 120′ (a, $p < 0.005$), 180′ and 240′ (c, $p < 0.05$). In relation to mRNA expression of iNOS, there was no difference between both groups (Fig. 3C and D). In the CG–SON–O system of non-lactating rats, [$(PRL)_g$] significantly increased the ovarian release of nitrites at all incubation times studied when compared to the control group (•, $p < 0.05$), without modifying the expression of luteal iNOS. In contrast, in lactating rats, [$(PRL)_g$] significantly decreased the release of nitrites at 30′ (∗, $p < 0.025$) and 60′ (•, $p < 0.05$) with respect to the control group (Fig. 3C and D).

3.4. CG–SON–O systems of day 4 postpartum in non-lactating and lactating rats: mRNA expression of regulatory factors of apoptosis

The comparative analysis of the semi-quantitative expression of Bcl-2 and Bax between the lactating and non-lactating groups not treated with ganglionic PRL showed that in non-lactating rats the expression of both factors was higher than in the lactating group (b, $p < 0.001$); however, the ratio of Bcl-2:Bax in the latter group was higher (b, $p < 0.001$). In the non-lactating group [$(PRL)_g$] significantly decreased the mRNA expression of Bcl-2 (■, $p < 0.001$), whereas it did not change the expression of Bax in relation to control. As a consequence, the ratio of Bcl-2:Bax decreased with respect to control (♦, $p < 0.01$). In lactating rats, nonetheless, [$(PRL)_g$] did

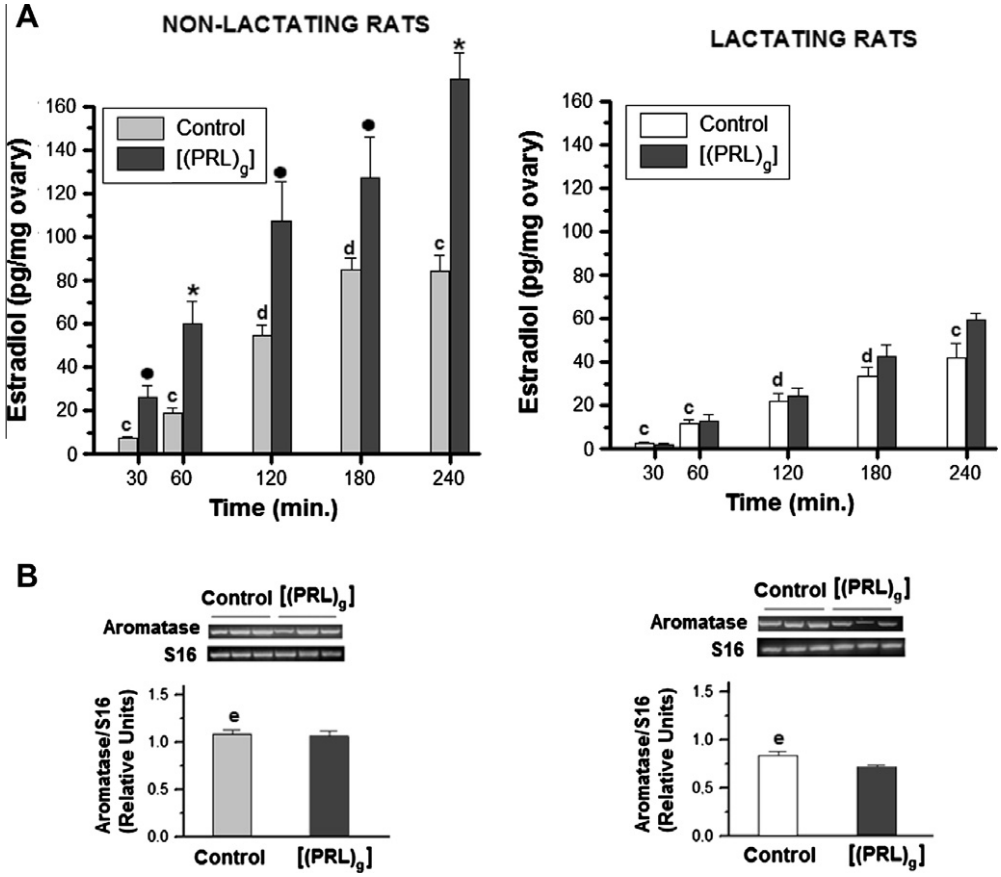

Fig. 2. Coeliac ganglion–superior ovarian nerve–ovary system on day 4 postpartum obtained from non-lactating and lactating rats. Comparison of basal levels of estradiol and mRNA expression of aromatase between non-lactating and lactating rats is represented by letters. The same font denote differences of statistical significance (c) $p < 0.05$, (d) $p < 0.025$ and (e) $p < 0.01$. Ganglionic effect of PRL [$(PRL)_g$] on the release of ovarian estradiol, expressed as pg/mg ovary (superior panel A). Ganglionic effect of PRL [$(PRL)_g$] on the mRNA expression of aromatase luteal enzyme (lower panel B). The respective systems were incubated without [control] and with the addition of 10^{-7} M PRL in the ganglionic compartment. Results are expressed as mean ± S.E.M of six animals per group for estradiol determination (●) $p < 0.05$ (∗) $p < 0.025$. ANOVA I–Tukey test. PCR products: aromatase (266 bp) and S16 (100 bp) as endogenous control, were visualized on agarose gels stained with Gel Red. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± S.E.M of three animals for the expression analysis.

not change either Bcl-2 expression or Bax expression. The comparative analysis of the semi-quantitative mRNA expression of Fas and FasL between the control non-lactating and lactating groups showed an increase in the expression of Fas (b, $p < 0.001$) and FasL (a, $p < 0.005$) in non-lactating rats with respect to lactating rats. Such parameters were not modified by [$(PRL)_g$] in either of the two groups. (Fig. 4).

4. Discussion

This study examined the effect of PRL acting on the CG, through the SON, on the functionality of the ovaries on day 4 postpartum in two different endocrine environments: lactating vs. non-lactating animals. Basal levels of ovarian P release were markedly lower in the non-lactating group when compared to lactating animals, in agreement with a previous study showing that serum levels of P are higher in lactating rats on day 4 postpartum when compared to non-lactating animals [44]. In addition, these authors compared the rate of production of P from both corpora lutea of pregnancy and newly formed corpora lutea after postpartum ovulation when incubated *in vitro* and observed that in the non-lactating group, the corpora lutea of pregnancy are the ones releasing more P while in the lactating group, most P is released by the newly formed corpora lutea. Because our studies were conducted with the entire ovary as part of the CG–SON–O system, we cannot conclude which type of corpora lutea contributes the most to the increased ovarian P release observed in the lactating group.

It has been shown that the suckling stimulus increases not only PRL secretion from the pituitary, but also the secretion of P from the corpora lutea of lactation [47,44,20]. PRL given in the CG of non-lactating rats caused increased ovarian P release via the SON, which, however, does not even reach the basal levels observed in lactating rats. The ovarian gland in lactating rats is developed in an environment characterized by high levels of PRL and may be unable to respond to further input of PRL from the CG mediated via the SON.

Under the experimental conditions described, increased P release is not consequence of changes in the mRNA expression of enzymes involved in P synthesis and degradation. In this regard, the presence of a pool of P ready to be released without the need for new synthesis has been previously reported in the corpora lutea of various mammalian species including rats, thus perhaps explaining why the release of P may be disengaged from the synthetic capacity of the gland [2,3,32,45].

Gejman and Cardinali [19] showed that muscarinic cholinergic neurotransmission in sympathetic superior cervical ganglia is affected by several hormones, including PRL. In our experimental scheme, PRL added to the CG likely caused the release of neurotransmitters, which may act promoting the release of P reserves into the ovarian incubation compartment, without affecting the expression of enzymes involved in the metabolism of P at the analyzed times.

The basal release of ovarian estradiol and the mRNA expression of aromatase were higher in the non-lactating group when compared to the lactating group. On the other hand, PRL added

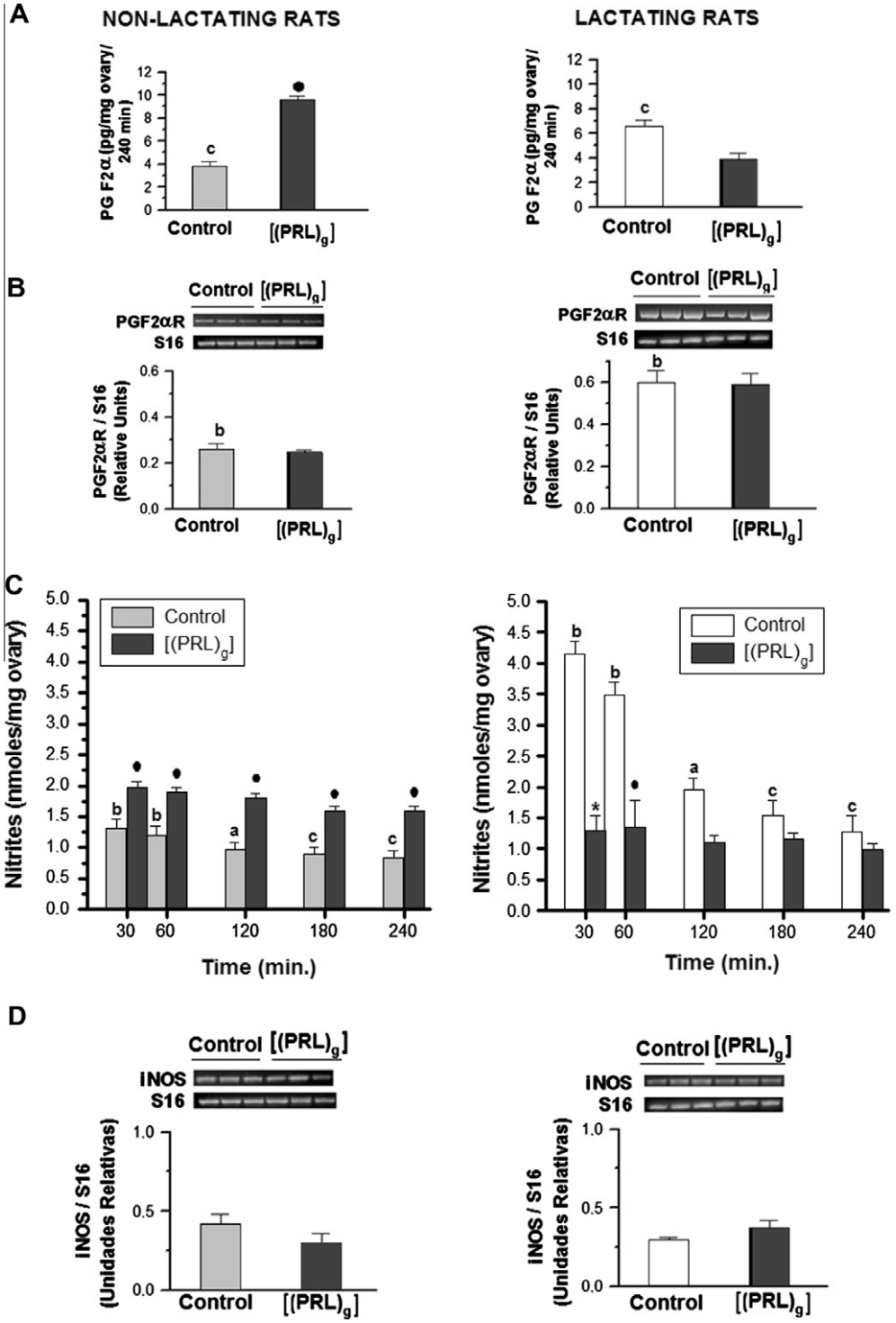

Fig. 3. Coeliac ganglion–superior ovarian nerve–ovary system on day 4 postpartum obtained from non-lactating and lactating rats. The comparison of basal levels of ovarian PGF2α release, PGF2αR luteal mRNA expression and ovarian nitrites release between non-lactating and lactating rats is represented by letters. The same font denotes differences of statistical significance (c) $p < 0.05$, (a) $p < 0.005$ and (b) $p < 0.001$. Ganglionic effect of PRL [$(PRL)_g$] on: (A) ovarian PGF2α release (B) PGF2αR luteal mRNA expression, (C) ovarian nitrites release and (D) iNOS luteal mRNA expression. The respective systems were incubated without [control] and with the addition of 10^{-7} M PRL in the ganglionic compartment. Results are expressed as mean ± S.E.M of six animals per group for PGF2α and nitrites determinations. (●) $p < 0.05$ (∗) $p < 0.025$ ANOVA I–Tukey test. PCR products: PGF2αR (428 bp), iNOS (219 bp) and S16 (100 bp) as endogenous control, were visualized on agarose gels stained with Gel Red. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± S.E.M of three animals for the expression analysis.

to the CG only increased ovarian estradiol release in non-lactating rats, without modifying the expression of its synthesis enzyme. It has been previously proposed that PRL has different effects on the corpora lutea depending upon the prevailing steroid concentrations within the environment of the target tissue [21]. These authors observed in non-lactating postpartum rats receiving subcutaneous estradiol that PRL promoted luteal apoptosis in the presence of high P. By contrast during lactation PRL prevents apoptosis in the corpora lutea probably because of the high concentrations of P together with low levels of estradiol.

In non-lactating rats, both the basal release of PGF2α and the expression of its luteal receptor were lower than in lactating rats.

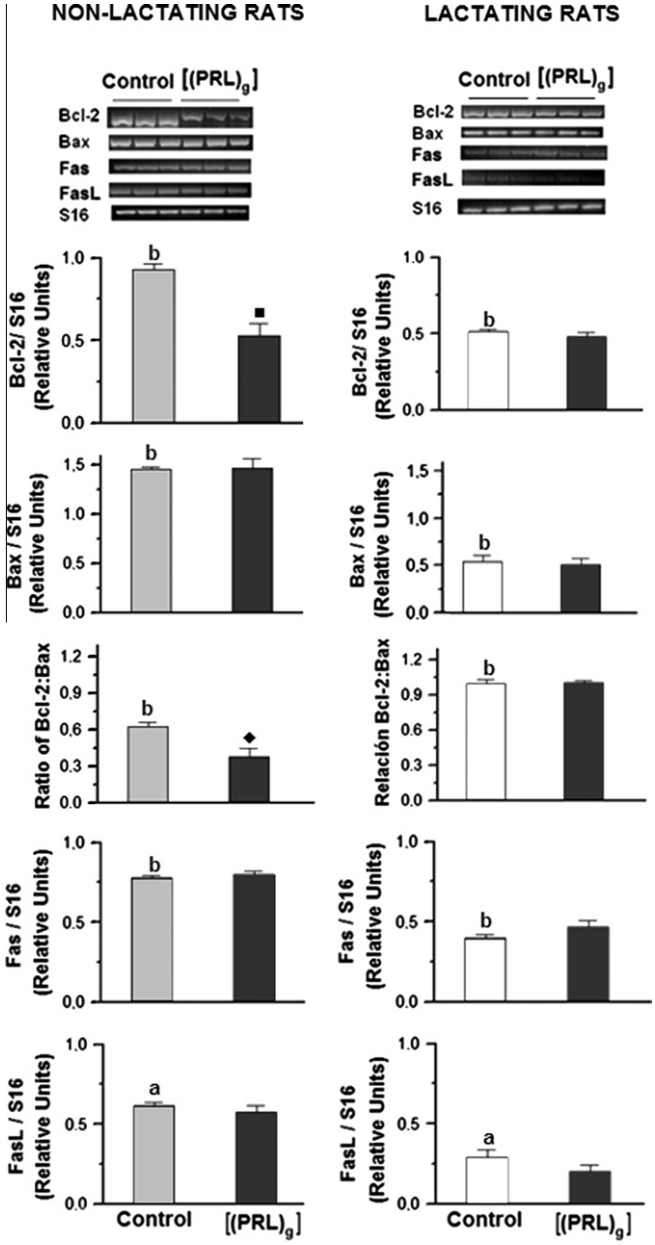

Fig. 4. Coeliac ganglion–superior ovarian nerve–ovary system on day 4 postpartum obtained from non-lactating and lactating rats. The comparison of basal mRNA expression of Bcl-2, Bax, Fas and Fas L between non-lactating and lactating rats is represented by letters. The same font denote differences of statistical significance (a) $p < 0.005$ and (b) $p < 0.001$. Ganglionic effect of PRL [$(PRL)_g$] on luteal expression of regulators of apoptosis. The respective systems were incubated without [control] and with the addition of 10^{-7} M PRL in the ganglionic compartment. PCR products: Bcl-2 (454 bp), Bax (473 bp), Fas (190 bp), FasL (178 bp) and S16 (100 bp) as endogenous control, were visualized on agarose gels stained with Gel Red. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± S.E.M of three animals per group. (♦) $p < 0.01$, (■) $p < 0.001$ (Student's *t*-test).

In addition, PRL given in the CG of non-lactating animals increased ovarian PGF2α release without modifying the expression of its receptor. Our results are in agreement with those published by Kurusu et al. [27] who observed in non-lactating rats that the exogenous administration of PRL increases the levels of PGF2α. It is known that PGF2α is a luteolytic agent in a number of mammalian species [31,36]. Supporting this concept parturition does not occur in PGF2αR knock-out mice due to the absence of luteolysis associated with PGF2αR activation [40]. In the postpartum ovary, however, there is little information about the role played by PGF2α and its receptor on luteal function.

NO is a neurotransmitter of known involvement in ovarian physiology and has been implicated in PGF2α action [34,8]. In the present study, basal NO levels were lower in the non-lactating group than in the lactating group, whereas PRL added to the CG of non-lactating animals increased ovarian release of NO without modifying iNOS mRNA expression. This would indicate that the increase of NO in the ovary compartment has a neural origin, through the SON, depending upon the endocrine environment [8]. On the other hand, the fact that iNOS mRNA was not modified by ganglionic PRL suggests that other ovarian NOS isoenzymes, such as endothelial (eNOS) or neuronal (nNOS), may be involved in the increase observed in NO [50].

The mechanism driving structural regression of the corpora lutea populating the ovaries has been intensively studied with a main focus on apoptosis [15,41]. Results from the comparative analysis between basals (i.e., preparations not receiving PRL on the CG) on both studied groups (i.e., lactating and non-lactating animals) indicates that in non-lactating rats the expression of pro-apoptotic factors is higher than in the lactating group. In non-lactating animals the ratio of Bcl-2 to Bax expression, which is indicative of the entry of the corpora lutea into the apoptosis pathway, was lower than in the lactating group. In turn, the expression of both Fas and FasL was higher in the non-lactating group. In addition, it has been shown that P is able to suppress the expression of Fas in a wide variety of cells; such mechanism would be responsible for inhibiting apoptosis of the corpora lutea induced by PRL [26]. Thus, elevated levels of basal P in the lactating group would have an antiapoptotic effect associated with the decrease observed in the expression Fas/FasL system.

In addition, the evidence indicates that a high estrogenic environment observed in non-lactating rats may favor a pro-apoptotic effect of PRL through a neural pathway. Thus, in non-lactating animals, the addition of PRL to the CG decreased the expression of Bcl-2 and also the ratio of Bcl-2 to Bax expression. This effect can be linked to the observed increase in the release of estradiol, despite the presence of increased levels of ovarian P. Even though the steroidogenic activity of the newly formed corpora lutea in non-lactating rats is low [44,43], their possible contribution to this increase of P is not discarded. It should be emphasized that the effect of PRL on the ovary observed is indirect and that the neurotransmitters released from the SON, including NO, might be responsible for the observed changes in ovarian physiology.

The overall results of this work suggest that endogenous levels of PRL released as a consequence of the establishment of lactation protect the corpora lutea of previous pregnancy through the increase in the ratio of Bcl-2 to Bax expression, in the presence of high levels of P and low levels of estradiol, despite the capacity of the ovaries to produce PGF2α and NO. On the other hand, in non-lactating animals, PRL added to the CG impacts the ovaries via the SON and favors the regression of luteal tissue. This regression is associated with the decrease in the ratio of Bcl-2:Bax and the increased release of ovarian PGF2α, NO and estradiol, although the ovary retains the capacity to produce P. It is highly possible that in lactating rats, the direct effect of PRL on the ovaries prevails over that of PRL-CG-mediated neural stimulation. PRL plays a critical role in the reproductive neuroendocrine function. We provided data supporting the feasibility of PRL to impact the CG and, by a neural pathway, affect ovarian function. This peripheral, neural indirect effect of PRL may be operational in times of transition when the corpora lutea needs to be either abruptly interrupted or rescued from apoptosis.

Acknowledgments

This manuscript is dedicated to the memory of Dr. Luis I Aguado (1946–2003). We thank the Instituto de Medicina y Biología Experimental de Cuyo (IMBECU)-CONICET-Mendoza that provided prolactin and progesterone antiserum and Gerardo Randazzo and Luis Villegas for technical support. This work is part of the doctoral thesis of Sandra Vallcaneras.

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