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Prolactin modulates luteal regression from the coeliac ganglion via the superior ovarian nerve in the late-pregnant rat

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Abstract. There is considerable evidence of the neuroendocrine control involved in luteal regression in the rat. In addition, circulating prolactin (PRL), which increases during the night before parturition, may gain access to the coeliac ganglion (CG), indirectly impacting the physiology of the ovary because of the known connection between the CG and the ovary via the superior ovarian nerve (SON). In this work we investigated in the CG–SON–ovary system and whether PRL added to the CG has an impact, indirectly via the SON, on luteal regression on Day 21 of pregnancy. The system was incubated without (control) or with PRL added to the CG. We measured the ovarian release of progesterone (P), oestradiol and prostaglandin F2 alpha (PGF2α) by radioimmunoassay, and nitrites (NO) by the Griess method. Luteal mRNA expression of *3β-hydroxysteroid dehydrogenase* (*3β-HSD*), *20α-HSD, aromatase, inducible nitric oxide synthase* (*iNOS*) and apoptosis regulatory factors was analysed by reverse transcription–polymerase chain reaction. P release, the expression of *Bcl-2* and the *Bcl-2* : *Bax* ratio was lower than control preparations, while the expression of *20α-HSD* and the release of NO and PGF2α were higher in the experimental group. In conclusion, PRL acts at the CG and, by a neural pathway, modulates luteal function at the end of pregnancy.

Additional keywords: nitric oxide, ovary, peripheral neural system, progesterone, prostaglandin F2 alpha.

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Introduction

At the end of pregnancy in the rat luteal regression occurs for parturition to take place (Albarracin *et al.* 1994; Stocco *et al.* 2000, 2007). Corpus luteum (CL) regression begins with the cessation of progesterone production because prostaglandin F2 alpha (PGF2α) induces the expression of 20α-hydroxysteroid dehydrogenase (20α-HSD), an enzyme that metabolises progesterone to an inactive metabolite (Motta *et al.* 1999, 2001). Then, the involution of luteal tissue takes place by a process of apoptosis only after parturition, although the expression of regulatory factors involved in cell survival and death such as *Bcl-2*, *Bax* and the *Fas–FasL* system have been reported in the CL during pregnancy (Sugino and Okuda 2007).

Our group has provided much evidence of the neuroendocrine control involved in luteal regression in the rat (Casais *et al.* 2001, 2012; Vallcaneras *et al.* 2009, 2011, 2013). This evidence was generated utilising an *ex vivo* coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system that was standardised in our laboratory. It should be emphasised that in the sympathetic ganglia there are special anatomical relationships between chromaffin cells, or small intense fluorescent (SIF) cells, and local fenestrated capillaries. These relationships have great physiological significance because capillaries allow extraglanglionic hormones to become available to the ganglionic cells (Matthews 1976; Williams *et al.* 1976). Thus, the sympathetic ganglia are active centres of concurrent hormonal signals

(Anesetti *et al.* 2009; Vallcaneras *et al.* 2009; Vega Orozco *et al.* 2012). Gejman and Cardinali (1983) have shown that the muscarinic cholinergic neurotransmission of the superior cervical ganglion is regulated by several hormones, including prolactin (PRL). In addition, in a previous work we have demonstrated that the coeliac ganglion (CG) is able to respond to the effect of PRL and, by a neural pathway, affect ovarian function in the postpartum lactating and non-lactating rat (Vallcaneras *et al.* 2013).

It is known that just before parturition, there is a large secretion of PRL from the pituitary needed to stimulate lactogenesis and to develop maternal behaviour required immediately after parturition (Amenomori *et al.* 1970; Grattan and Averill 1990). On the other hand, PRL, besides the known trophic effect on the CL, also has a luteolytic effect depending upon the nature of the CL and of the hormonal environment to which they are exposed. For example, there is evidence suggesting that in non-lactating postpartum rats, PRL promotes luteal regression (Takiguchi *et al.* 2004; Goyeneche and Telleria 2005). What is less clear is whether circulating PRL at the end of gestation has a role in the regression of the CL of pregnancy.

To provide further evidence about the functional interaction between the endocrine and peripheral nervous systems on the physiology of the ovary at the end of pregnancy, the purpose was to study whether PRL added in the CG, via the SON and through ovarian intermediaries, has the capacity to modulate luteal regression on Day 21 of pregnancy.

Materials and methods

Reagents

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

Animals

Virgin Holtzman strain female rats weighing 250 ± 50 g were used on Day 21 of pregnancy. To induce gestation, female rats were caged individually with fertile males beginning on the afternoon of pro-oestrus. Positive mating was verified on the following morning by identifying spermatozoa 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. Animals had free access to food (Cargill SACI; Saladillo, Buenos Aires, Argentina) and water. They were kept in a light- (lights on from 0700 to 1900 hours) and temperature-controlled room (24 ± 2°C). Animals were handled according to the procedures approve 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 (protocol number B49/10).

Extraction of CG–SON–O system of rats

Groups of six animals on Day 21 of pregnancy were used for each experimental procedure. The extraction of the CG–SON–O system was carried out at 9 a.m. The animals were anaesthetised with ketamine and xylazine (80 and 10 mg kg^{-1}, respectively, given intraperitoneally), the fetuses were removed and sacrificed in an atmosphere of ether, whereas the CG–SON–O system was extracted and the mothers sacrificed by decapitation. The entire CG–SON–O system was removed by surgery, avoiding contact between the surgical instruments and the nerve fibres in order to prevent spontaneous nerve depolarisation. The pieces of tissues 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.

Experimental procedures

The CG–SON–O system was washed with incubation medium and placed in a cuvette with two isolated 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 mL^{-1} glucose and 0.1 mg mL^{-1} albumin at 37°C in an atmosphere composed of 95% O_2 and 5% CO_2. The *ex vivo* 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 mL^{-1} ascorbic acid was added to the ganglion compartment as an antioxidant agent. At Time 0 of incubation, PRL at a concentration of 10^{-6}, 10^{-7} or 10^{-8} M was added to the ganglion compartment [$(PRL)_g$]. Control groups consisted of CG–SON–O systems that were untreated. 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 determination of P by radioimmunoassay (RIA). We selected a PRL concentration of 10^{-7} M for most experiments. Then, from the ovarian incubation liquid we measured the concentration of oestradiol, nitrites released and PGF2α released (only at 240 min). 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 and then the CL were separated, weighed and stored at −80°C. From the CL, total RNA was extracted for analysis of mRNA expression of *3β-HSD*, *20α-HSD, aromatase (oestradiol synthesis enzymes), inducible nitric oxide synthase enzyme* (*iNOS*), *Fas*, *FasL*, *Bcl-2* and *Bax* by reverse transcription–polymerase chain reaction (RT-PCR).

Progesterone and oestradiol radioimmunoassay

Steroids were measured in duplicate by RIA in the ovary incubation liquid. The P antiserum, provided by Dr R. Deis (IMBECU, Mendoza, Argentina), was produced in rabbits against P conjugated to bovine serum albumin at the 11 position. The antiserum was highly specific for P with low cross-reactivities: <2.0% for 20α-dihydro-progesterone and deoxycorticosterone and 1.0% for other steroids. The sensitivity was less than 5 ng mL^{-1} and the inter- and intra-assay coefficients of variation were less than 10%. This assay has been validated previously (Bussmann and Deis 1979). P concentration was expressed as nanogram per milligram ovary (ng mg^{-1} ovary), all against incubation time. The oestradiol levels were determined using a double-antibody RIA Diasource kit (DiagnosMed SRL,

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

Gene	Sequences (sense above, antisense below; 5′–3′)	GenBank accession no.	Amplicon length (bp)	No. of cycles
3β-HSD	GTCTTCAGACCAGAAACCAAG CCTTAAGGCACAAGTATGCAG	M38179	447	35
20α-HSD	CAACCAGGTAGAATGCCATCT TTCGAGCAGAACTCATGGCTA	D14424	440	30
Aromatase	TGCACAGGCTCGAGTATTTCC ATTTCCACAATGGGGCTGTCC	M33986	266	35
iNOS	GCATGGACCAGTATAAGGCAAGCA GCTTCTGGTCGATGTCATGAGCAA	S71597	219	40
Fas	TGTCAACCGTGTCAGCCTGGT GGGTCCGGGTGCAGTTCGTT	NM_139194	190	35
FasL	GTGCTGGTGGCTCTGGTTGGAA AGTGGGCCACACTCCTTGGCTT	NM_012908	178	35
Bcl-2	CACCCCTGGCATCTTCTCCT GTTGACGCTCCCCACACACA	L14680	349	35
Bax	TGGCGATGAACTGGACAACAAC CCCGAAGTAGGAAAGGAGGC	U32098	301	40
S16	TCCAAGGGTCCGCTGCAGTC CGTTCACCTTGATGAGCCCATT	NM_001169146	100	35

Buenos Aires, Argentina) following the manufacturer's instructions. Oestradiol was expressed as picograms per milligram of ovarian tissue (pg mg^{-1} ovary), all against incubation time. The percentages of cross-reactions were 1.8% for oestrone, 1.2% for oestriol, 0.0011% for androstenedione and 0.0002% for P. The assay sensitivity was <2.2 pg mL^{-1} oestradiol. The inter- and intra-assay coefficients of variation in all the assays were <10.0%.

Prostaglandin radioimmunoassay

Aliquots of the ovarian incubation liquid obtained at the end of the experiment (240 min) were acidified to pH 3.0 with 1 mol L^{-1} HCl and extracted three times with one volume of ethyl acetate for prostaglandin determination. Pooled ethyl acetate extracts were dried under an atmosphere of N_2 and stored at −20°C until prostaglandin RIA. Prostaglandins were quantified by RIA using rabbit antiserum from Sigma Chemical Co. The sensitivity of this assay was 10 pg per tube for PGF2α. The cross-reactivity of PGF2α was 1.0 with PGE1 and <0.1% with other prostaglandins.

Nitrite assay

Levels of nitrites, water-soluble metabolites of nitric oxide (NO), were measured spectrophotometrically in the ovary incubation liquid (Egami and Taniguchi 1974). Samples were immediately mixed with Griess reagent (sulfanilamide 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^{-1}. 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^{-1} ovary).

RNA isolation and RT-PCR analysis

Total luteal RNA was isolated using TRIZOL Reagent (Invitrogen Life Technologies, Buenos Aires, Argentina), according to the manufacturer's instructions. Purified total RNAs were then quantified and assessed for purity by measurement of the 260 : 280 ratio using an UV spectrophotometer Beckman DU-640 B (Buenos Aires, Argentina). Only samples with a 260 : 280 ratio of 1.8 to 2.0 were used. The integrity was confirmed by running 2 µg RNA on a 0.8% agarose gel. After GelRed (Biotium, Hayward, CA, USA) staining, RNA bands were visualised with a UV transilluminator and 28S and 18S rRNA band patterns were analysed. Two micrograms of total RNA were reverse transcribed at 37°C using random primers and M-MLV Reverse Transcriptase (Promega Inc., Buenos Aires, Argentina) 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, Buenos Aires, Argentina). The primer information is shown in Table 1.

The amplification of the cDNA was performed using a thermalcycler (My Cycler; BioRad, Capital Federal, Argentina); for *3β-HSD*, *20α-HSD*, *aromatase*, *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. For *Bax* and *iNOS* the parameters were 95°C for 1 min, 56°C for 1 min and 72°C for 1 min. All the reactions were terminated with a 5 min extension at 72°C. Reaction products were electrophoresed on 2% agarose gels, visualised 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://imagej.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 gene *S16*.

Statistical analysis

All data are presented as mean ± s.e.m. in each group of six rats. Differences between two groups were analysed with Student's *t*-test. For multiple comparisons made along the time of incubation, repeated-measures analysis of variance (two-way ANOVA) followed by Tukey's test was used. For multiple comparisons not involving repeated-measures, one-way analysis of variance (one-way ANOVA) followed by Tukey's test was utilised. A difference was considered to be statistically significant at $P < 0.05$.

Results

Ganglionic effect of PRL on the release of ovarian progesterone

To evaluate if PRL, through a neural pathway, has an effect on the release of P from the ovary, the CG–SON–O system obtained from rats on Day 21 of pregnancy was incubated in the presence of 10^{-6}, 10^{-7} or 10^{-8} M PRL in the ganglion compartment [$(PRL)_g$] and extractions of the ovarian incubation liquid were carried out at 30, 60, 120, 180 and 240 min for the determination of P. The addition of PRL in the ganglion compartment, at different concentrations and in most of the studied times, diminished P release when compared with the control group. The largest sensitivity demonstrated was to the dose 10^{-7} M, considering most of the time periods examined; this also approaches physiological levels of the hormone. Therefore, this concentration was selected to perform subsequent experiments (Fig. 1).

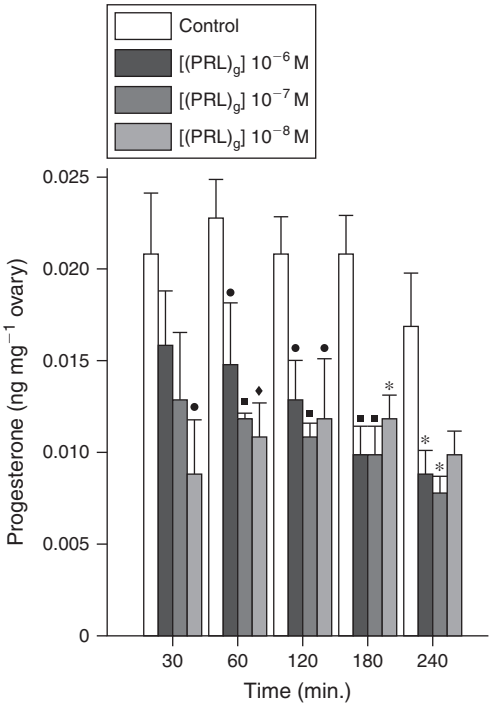

Fig. 1. Coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system on Day 21 of pregnancy: ganglionic effect of prolactin [$(PRL)_g$] at 10^{-6} M, 10^{-7} M or 10^{-8} M on the release of ovarian progesterone expressed in ng mg^{-1} ovary. The control group consisted of untreated CG–SON–O systems. Values represent the mean ± s.e.m. of six animals per experimental group. • $P < 0.05$, * $P < 0.025$, ◆ $P < 0.01$, ■ $P < 0.005$ by one-way ANOVA and Tukey's test.

Ganglionic effect of PRL on mRNA expression of luteal 3β-HSD and 20α-HSD

At the end of the incubation period of the CG–SON–O system, mRNA expression of enzymes involved in P synthesis and degradation was measured in the CL. These determinations were performed in order to investigate whether PRL from the CG has an effect on luteal P metabolism. Adding 10^{-7} M PRL to the CG compartment did not significantly change the expression of *3β-hydroxysteroid dehydrogenase* (*3β-HSD*) whereas mRNA expression of *20α-HSD* increased with respect to the control (Fig. 2).

Ganglionic effect of PRL on PGF2α ovarian release

PRL was added to the CG compartment to examine whether PRL, via the SON, affects the ovarian release of the luteolytic factor PGF2α at 240 min of incubation. The release of PGF2α increased significantly (Fig. 3).

Ganglionic effect of PRL on NO ovarian release and mRNA expression of luteal iNOS

The *ex vivo* CG–SON–O system from Day-21 pregnant rats was incubated in the presence of 10^{-7} M PRL added to the CG compartment. After 30, 60, 120, 180 and 240 min of incubation we measured the release of NO in the ovarian compartment and at 240 min of incubation we studied the luteal expression of inducible *nitric oxide synthase enzyme* (*iNOS*). These determinations were performed in order to investigate whether PRL from the CG affects NO ovarian release, given that NO is a gaseous neurotransmitter with proven involvement in the process of luteal regression (Motta *et al.* 1999, 2001). While the ovarian release of nitrite increased significantly at 30, 120, 180 and 240 min compared with the control group, the mRNA expression of luteal *iNOS* was not modified by the presence of ganglionic PRL (Fig. 4).

Ganglionic effect of PRL on ovarian oestradiol release and mRNA expression of luteal aromatase

To examine whether PRL treatment of the CG had an effect on the synthesis and release of oestradiol from ovaries containing regressing CL on Day 21 of pregnancy, CG–SON–O systems were incubated in the presence of 10^{-7} M PRL in the ganglion compartment and the release of oestradiol in the ovarian compartment was measured at 30, 60, 120, 180 and 240 min of incubation. The mRNA coding for luteal *aromatase*, which converts androgens to oestradiol, was assessed at 240 min of incubation. This was done mainly because we demonstrated in a previous work that oestradiol is a hormone with luteolytic effects at the end of gestation (Casais *et al.* 2012). Neither the

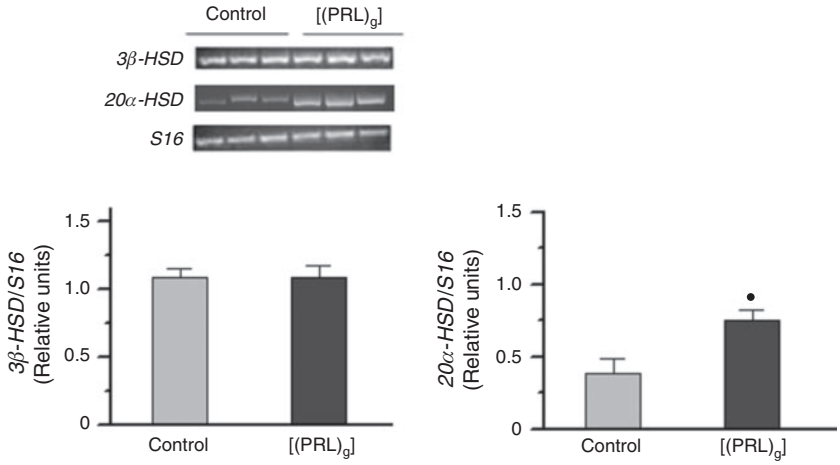

Fig. 2. Coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system on Day 21 of pregnancy: ganglionic effect of 10^{-7} M prolactin [$(PRL)_g$] on the expression (mRNA) of luteal enzymes *3β-HSD* and *20α-HSD*. The control group consisted of untreated CG–SON–O systems. The PCR products were visualised on agarose gels stained with Gel Red: *3β-HSD* (447 bp), *20α-HSD* (440 bp) and *S16* (100 bp). The average intensity value of each band was quantified using ImageJ software and expressed in arbitrary units. Values represent the mean ± s.e.m. of three animals per experimental group. • $P < 0.05$ by Student's *t*-test.

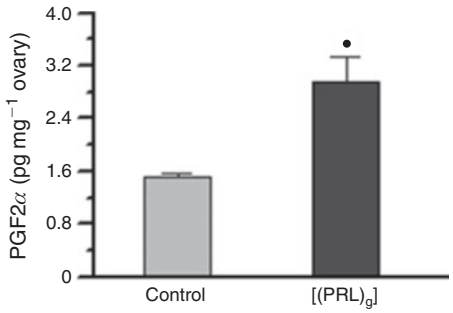

Fig. 3. Coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system on Day 21 of pregnancy: ganglionic effect of 10^{-7} M prolactin [$(PRL)_g$] on the release of ovarian prostaglandin F2α (PGF2α) expressed in pg mg^{-1} ovary. The control group consisted of untreated CG–SON–O systems. The PGF2α release values represent the mean ± s.e.m. of six animals per experimental group. • $P < 0.05$ by Student's *t*-test.

release of ovarian oestradiol nor luteal *aromatase* expression was significantly modified by ganglionic PRL (Fig. 5).

Ganglionic effect of PRL on mRNA expression of apoptosis regulatory factors: luteal Fas, FasL, Bcl-2 *and* Bax

To investigate whether ganglionic PRL modifies structural luteal regression parameters, the expression of regulatory factors of the extrinsic pathway of apoptosis (*Fas* and *FasL*) and intrinsic pathway (*Bcl-2* and *Bax*) were analysed at the end of the incubation. PRL added to the CG compartment did not change the expression of *Fas* or *FasL* with respect to the control group (Fig. 6). However, ganglionic PRL significantly decreased mRNA expression of the anti-apoptotic factor *Bcl-2*, whereas the expression of *Bax*, a pro-apoptotic factor, did not change with respect to the control. We then analysed the relative ratio of *Bcl-2* to *Bax*, because this parameter, rather than their individual absolute concentrations, is a critical marker to determine whether a cell is prone to entering apoptosis. We observed a significant decrease in the *Bcl-2* : *Bax* ratio with respect to the control group (Fig. 7).

Discussion

This study examined whether PRL added to the CG compartment in the *ex vivo* CG–SON–O system and through ovarian intermediaries has the capacity to modulate luteal regression on Day 21 of pregnancy.

PRL at various concentrations added to the ganglionic incubation medium led to reduced ovarian P release. The 10^{-7} M concentration was selected to perform the subsequent experiments since it showed the largest sensitivity at most of the studied times and also approaches physiological levels of the hormone. We associated the inhibitory effect of PRL from CG on P ovarian release with an increase in the P catabolising capacity marked by increased *20α-HSD* expression, although *3β-HSD* expression was not modified. We also found that PRL from CG increased ovarian PGF2α release. These results are in agreement with the fact that, in rodents, a decline in P release is essential for luteal regression to occur, and also agrees with Stocco *et al.* (2000), who showed that *20α-HSD* mRNA was increased by PGF2α at the end of pregnancy. Consistent with our data, in a cell culture generated from superovulated rat ovaries, PGF2α suppressed P synthesis only in cells treated with PRL, demonstrating that the luteolytic effect of PGF2α is dependent on the presence of PRL (Zetser *et al.* 2001).

In previous works, we demonstrated that different ganglionic stimuli, using the same *ex vivo* system, provoke modifications in the release of catecholamines and nitrites at the ovary level

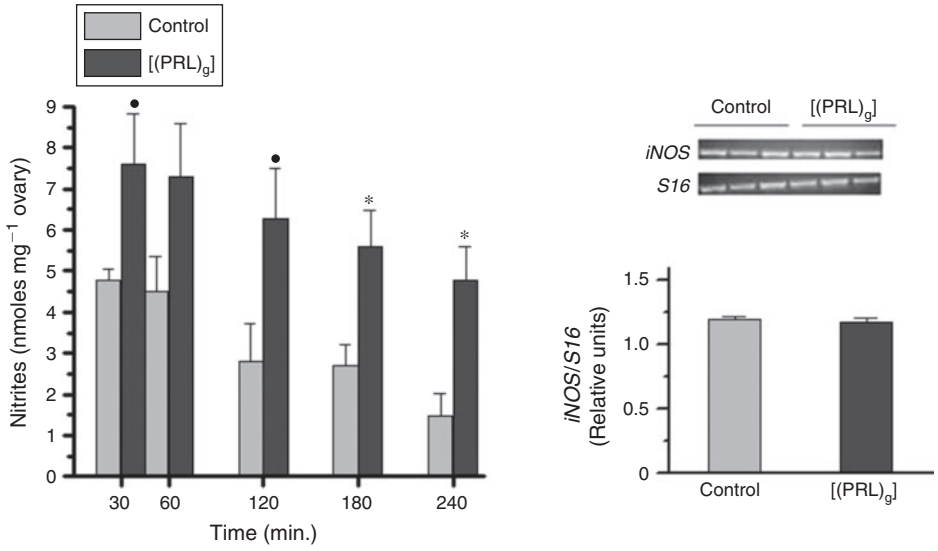

Fig. 4. Coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system on Day 21 of pregnancy: ganglionic effect of 10^{-7} M prolactin [$(PRL)_g$] on the release of ovarian nitrites (NO) expressed in nmol mg^{-1} ovary and on the expression (mRNA) of luteal *inducible NO synthase isoenzyme* (*iNOS*). The control group consisted of untreated CG–SON–O systems. The PCR products were visualised on agarose gels stained with Gel Red: *iNOS* (219 bp) and *S16* (100 bp). The average intensity value of each band was quantified using ImageJ software and expressed in arbitrary units. The NO release values represent the mean ± s.e.m. of six animals per experimental group. • $P < 0.05$, * $P < 0.025$ by one-way ANOVA and Tukey's test. The *iNOS* expression values represent the mean ± s.e.m. of three animals per experimental group. Student's *t*-test.

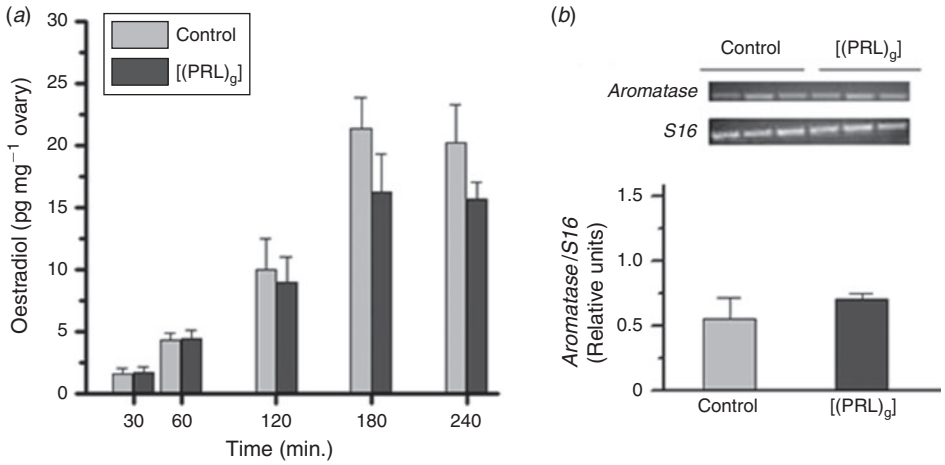

Fig. 5. Coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system on Day 21 of pregnancy: ganglionic effect of 10^{-7} M prolactin [$(PRL)_g$] on the release of ovarian oestradiol expressed in pg mg^{-1} ovary and on the expression (mRNA) of luteal *aromatase* enzyme. The control group consisted of untreated CG–SON–O systems. The PCR products were visualised on agarose gels stained with Gel Red: *aromatase* (266 bp) and *S16* (100 bp). The average intensity value of each band was quantified using ImageJ and expressed in arbitrary units. The oestradiol release values represent the mean ± s.e.m. of six animals per experimental group. One-way ANOVA and Tukey's test. The *aromatase* expression values represent the mean ± s.e.m. of three animals per experimental group. Student's *t*-test.

(Delgado *et al.* 2004; Casais *et al.* 2006; Bronzi *et al.* 2011). In the present study, ganglionic PRL increased the levels of the gaseous NO neurotransmitter in the ovarian compartment without causing modifications in the luteal mRNA expression of its inducible isoenzyme, at least in the time of study. In addition, the fact that *iNOS* mRNA was not modified by ganglionic PRL suggests that other ovarian NOS isoenzymes, such as endothelial (eNOS) or neuronal (nNOS) enzymes, may be involved

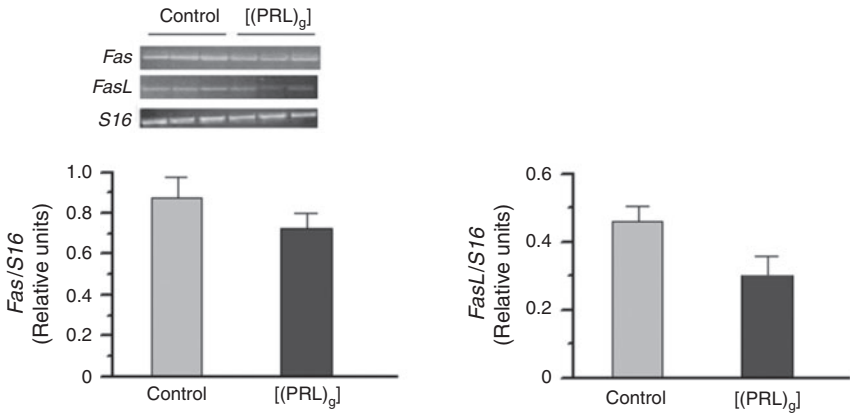

Fig. 6. Coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system on Day 21 of pregnancy: ganglionic effect of 10^{-7} M prolactin [$(PRL)_g$] on the expression (mRNA) of apoptosis regulatory factors: luteal *Fas* and *FasL*. The control group consisted of untreated CG–SON–O systems. The PCR products were visualised on agarose gels stained with Gel Red: *Fas* (190 bp), *FasL* (178 bp) and *S16* (100 bp). The average intensity value of each band was quantified using ImageJ software and expressed in arbitrary units. Values represent the mean ± s.e.m. of three animals per experimental group. Student's *t*-test.

(Yang *et al.* 2003) or that the increase of NO in the ovary compartment has a neural origin through the SON (Casais *et al.* 2007).

The negative effects of NO on ovarian steroidogenesis have been reported in granulosa–luteal cell culture, where the addition of a NO donor caused a decrease in the release of P (Ishimaru *et al.* 2001). Furthermore, the ovarian NO increase can be linked to the observed increase in the release of PGF2α. Our results are in agreement with those published by Motta *et al.* (1999, 2001), who showed in a pseudopregnant rat model that administration of a NO donor to rats in the luteal regression phase increased the synthesis of PGF2α and decreased serum P concentration. Furthermore, when they injected a luteolytic dose of PGF2α, they found increased activity of the NO synthesis enzyme and decreased serum levels of P, thus suggesting the existence of a feedback mechanism between PGF2α and NO.

In the present study we also analysed the ovarian release of oestradiol, a steroid hormone of great interest because it has a possible luteolytic effect at this stage of pregnancy (Casais *et al.* 2012). In agreement with this concept, monkey CL were sensitive to oestradiol locally produced in association with luteal regression (Duffy *et al.* 2000), whereas Vaskivuo and Tapanainen (2003) demonstrated that oestradiol promotes apoptosis in human luteal cells. Furthermore, in another study, oestradiol given to rats on Day 4 postpartum accelerated luteal regression through a mechanism that possibly involves the secretion of pituitary PRL (Goyeneche and Telleria 2005). However, in the present work, the presence of PRL in the CG compartment did not modify ovarian oestradiol levels or the expression of aromatase, indicating that oestradiol may not be a mediator of the luteolytic effect of PRL via a neural pathway, at least at the incubation times studied.

The structural regression process in CL of pregnancy, which takes place mainly via programmed cell death by apoptosis, is slow and occurs over a period of several days after the decline in the luteal progesterone-producing capacity, although genes associated with apoptosis are upregulated before morphological changes are evident (Guo *et al.* 1998). We investigated whether the action of PRL in the CG affects the mRNA expression of critical regulatory factors of cell survival and death. It should be noted that the effect of PRL is indirect and possibly NO or PGF2α are responsible for the observed effect. Yadav *et al.* (2005) showed in a pseudopregnant rat model that the extrinsic pathway of apoptosis has a significant role in luteal regression induced by PGF2α. However, the presence of PRL in the ganglionic compartment did not modify the expression levels of luteal *Fas* and *FasL*, suggesting that the extrinsic apoptotic pathway is not affected under these experimental conditions.

On the other hand, PRL in the CG diminished the luteal expression of *Bcl-2* (anti-apoptotic factor) without modifying *Bax* expression (pro-apoptotic factor). Moreover, when we assessed the *Bcl-2* : *Bax* ratio, which is a critical determinant that marks the entrance of the cell into apoptosis (Antonsson 2001), we observed a diminution after ganglionic stimulation with PRL, which tilts the balance towards a pro-apoptotic environment. In this respect, Vega *et al.* (2000) have demonstrated, through an *in vitro* study in the human CL during the medium and late luteal phase, that NO induces a decrease of Bcl-2, together with an increase in the number of cells with DNA fragmentation.

Several studies have detected the presence of two PRL receptors isoforms, short and long, in numerous parts of the central nervous system and peripheral organs (Freeman *et al.* 2000). At the moment, according to the literature available, the presence of receptors for PRL at the level of the peripheral sympathetic nervous system has not been confirmed. However, there is evidence suggesting that PRL might act on sympathetic ganglions to affect different aspects of their metabolism. For example, a study of Cardinali *et al.* (1981) showed that the treatment of spayed rats with PRL augments tyrosine

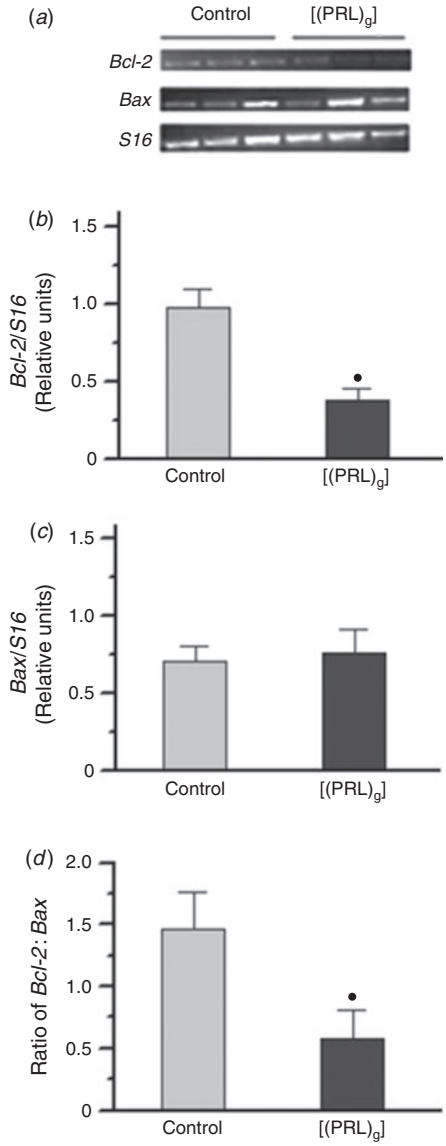

Fig. 7. Coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system on Day 21 of pregnancy: ganglionic effect of 10^{-7} M prolactin [$(PRL)_g$] on the expression (mRNA) of apoptosis regulatory factors: luteal *Bcl-2* and *Bax*. The control group consisted of untreated CG–SON–O systems. The PCR products were visualised on agarose gels stained with Gel Red: *Bcl-2* (454 bp), *Bax* (473 bp) and *S16* (100 bp). The average intensity value of each band was quantified using ImageJ and expressed in arbitrary units. Values represent the mean ± s.e.m. of three animals per experimental group. • $P < 0.05$ by Student's *t*-test.

hydroxylase activity at ganglionic perikarya and nerve terminals of the superior cervical ganglion. Furthermore, in a previous work we demonstrated in non-lactating animals on Day 4 postpartum that PRL added to the CG via the SON promotes the regression of CL of pregnancy that still remains in the postpartum rat ovary, and this was associated with a decrease in the ratio of *Bcl-2*:*Bax* and an increased release of ovarian PGF2α and NO (Vallcaneras *et al.* 2013).

This work provides evidence of the functional interaction between the endocrine and peripheral neural systems that contribute to the knowledge of vertebrate reproductive biology. The findings presented indicate that PRL may gain access to the CG impacting indirectly on ovarian physiology and inducing the release of PGF2α and NO, which favours luteal regression, at the end of rat pregnancy.

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