Prolactin inhibits oocyte release after gonadotropin stimulation in the rat: Putative mechanism involving ovarian production of beta-endorphin and prostaglandin

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Objective: To evaluate whether prolactin (PRL) is able to inhibit ovulation induced with exogenous gonadotropins in the rat and whether this effect could be mediated by the ovarian production of beta-endorphin, prostaglandin, and nitric oxide (NO).
Design: Controlled in vivo and in vitro experiments.
Setting: Academic research laboratories.
Animal(s): Immature female rats undergoing ovulation induction with equine gonadotropins and hCG.
Intervention(s): Prolactin (100 or 200 μg), PRL + the opioid antagonist naloxone (200 μg each), or placebo were injected SC 4 hours after hCG administration for ovulation induction. In the in vitro experiments, isolated preovulatory ovaries were incubated with or without PRL in a final concentration of 100 or 200 ng/mL.
Main Outcome Measures(s): Number of oocytes ovulated in vivo, ovarian beta-endorphin, PGE_2 and NO_2^-/NO_3^- release, and NO synthase activity in vitro.
Result(s): Prolactin reduced significantly the number of oocytes ovulated at the doses of 100 and 200 μg, and this effect was partially reversed by naloxone administration together with 200 μg PRL. PRL also induced a twofold increase in the ovarian release of beta-endorphin and a threefold decrease in the ovarian production of PGE_2. Ovarian NO synthase activity and the concentrations of NO_2^-/NO_3^- in the incubation medium were not modified by PRL.
Conclusion(s): Prolactin is able to reduce the number of oocytes released and modulate ovarian beta-endorphin and PGE_2 release, which may account for its peripheral anovulatory effects. This local effect of PRL could interfere in the process of ovulation induction by exogenous gonadotropins. (Fertil Steril® 2005;83(Suppl 1): 1119–24. ©2005 by American Society for Reproductive Medicine.)

Key Words: Prolactin, endogenous opioid, beta-endorphin, prostaglandin, nitric oxide, ovulation induction

Hyperprolactinemia is a frequent cause of chronic anovulation and infertility (1). High circulating levels of prolactin (PRL) may inhibit ovarian function and ovulation by central and peripheral mechanisms. Central pathways include the stimulation of endogenous opioid peptide release in the hypothalamus (2, 3) and the consequent blockade of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) pulsatility (4–6). The peripheral mechanisms of PRL-induced anovulation are still not clear.

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It has been observed that PRL inhibits E_2 production (7) and the luteinization of granulosa cells in vitro (8) and arrests follicular growth in vivo (9, 10). In the isolated rabbit ovary, PRL inhibits the plasminogen activator activity and thereby limits plasmin generation in preovulatory follicles (11, 12). In the human ovary, PRL inhibits steroidogenesis (13, 14) and suppresses follicular maturation and luteinization (15). The presence of PRL receptors in the ovary (16) provides additional evidence for the direct ovarian effect of the suppression of ovulation by PRL.

Despite this evidence, it remains uncertain whether hyperprolactinemia affects the response to ovulation induction in a clinical context. While earlier reports sustain that hyperprolactinemic women can respond to ovulation induction despite constantly high PRL levels (17, 18), a poor response to hMG has been observed in women with this condition (19).

Local production of peptides derived from proopiomelanocortin (POMC), which includes beta-endorphin, has been extensively demonstrated in the ovary (20, 21). However, the

possible regulation of ovarian POMC-derived peptides by PRL remains unknown. The more recent finding that beta-endorphin inhibits prostaglandin (PG) release and blocks ovulation in the rat (22) suggests that the antiovulatory effect of PRL might be mediated via stimulation of ovarian production of endogenous opioids. These, in turn, could regulate PG and nitric oxide (NO) production (23) and thereby inhibit ovulation.

The present study was designed to test this hypothesis by evaluating whether the IP injection of PRL reduces the number of oocytes ovulated by immature rats stimulated with exogenous gonadotropins and whether the effect of PRL is reversed by an opioid antagonist, naloxone. In addition, we investigated the effect of PRL on the ovarian production of beta-endorphin, prostaglandin E_2 (PGE_2), and stable NO metabolites (NO_2^-/NO_3^-), as well as on the activity of NO synthase in isolated preovulatory ovaries incubated in vitro.

MATERIALS AND METHODS

In Vivo Experiments

Fifty prepubertal (28–30 days of age) female Wistar rats weighing 60–70 g were obtained from Centro de Bioterismo da Universidade Federal de Minas Gerais (CEBIO-UFMG) (Belo Horizonte, Brazil) and were cared for according to the international guidelines for animal care, after approval by the local animal care committee. They were housed in a light- and temperature-controlled environment (lights on from 5:00 A.M. to 7.00 P.M., 23°C ± 2°C) and were fed with commercial chow and tap water ad libitum.

Ovulation was induced with 20 IU equine gonadotropins (pregnant mare serum gonadotropin [PMSG], supplied by the National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK, Bethesda, MD]) in 0.2 mL saline given SC. Forty-eight hours later (1:00 P.M.), the animals were injected IP with 20 IU hCG (Serono, Geneva, Switzerland) dissolved in 0.2 mL saline to trigger ovulation. Four hours after the hCG injection (5:00 P.M.), the rats were divided into four groups, which received the following treatments IP: 0.2 mL saline (control group, n = 19), 100 μg PRL (rat PRL, NIDDK-rPRL-B-9, n = 10), 200 μg PRL (n = 11), or 200 μg PRL plus 200 μg naloxone (n = 10). These doses of PRL were chosen on the basis of previous pharmacokinetic studies (24) and were expected to achieve peak plasma concentrations of approximately 50 and 100 ng/mL, respectively. In addition, further experiments showed that immature rats injected with saline vehicle (n = 4), 100 μg PRL (n = 4), and 200 μg PRL (n = 4) after the same protocol of ovulation induction had mean plasma PRL concentrations of 11.8, 38.7, and 91.4 ng/mL, respectively, 2 hours after PRL injection.

Twenty-two hours after the hCG injection (11:00 A.M.), the rats were killed by decapitation and the oviducts were dissected, compressed between two glass slides, and examined under a light microscope (×100 magnification) to assess the number of oocytes ovulated. Fallopian tube examination was performed by the same investigator, who was not aware of the rat's treatment group.

The ovaries were then removed, fixed in 4% buffered formalin, embedded in paraffin, and cut into 4 μm sections that were stained with hematoxylin-eosin. Serial sections of each ovary were evaluated under light microscopy with ×200 magnification to identify preovulatory and atretic follicles.

In Vitro Experiments

Immature female Wistar rats were stimulated as described above. Four hours after the hCG injection the rats were killed by cervical dislocation and the ovaries were removed and dissected out of adjacent connective tissues in a Petri dish containing incubation medium (Sigma 199; Sigma Chemical Co., St. Louis, MO). Each isolated ovary was cut into quarters and incubated for 4 hours in one of the wells of a 24-well culture plate containing 500 μL of incubation medium (control wells) or 450 μL of incubation medium plus 50 μL of the substance to be tested (PRL in a final concentration of 100 or 200 ng/mL). These PRL concentrations were chosen on the basis of previous experiments with hypothalamic explants (3) and were planed to approach serum PRL levels found in the clinical condition of hyperprolactinemia (25). The plates were incubated in an incubator with a humidified chamber and gassed with carbogen (5% CO_2 in 95% O_2) at 37°C. After the incubation period, the ovaries were removed and weighed and aliquots of culture media were collected and stored at −20°C until beta-endorphin, PGE_2, and NO metabolites (NO_2^-/NO_3^-) determinations.

A second experimental design was used to specially evaluate the NO synthase activity in ovarian homogenates after tissue incubation with PRL in vitro. Superovulation was induced as described before. Four hours after hCG administration, the rats were sacrificed by cervical dislocation and the ovaries were removed, trimmed of visible fat, and weighed. Isolated ovaries were incubated in tubes containing 1 mL of KREBS Ringer bicarbonate solution. Fifteen minutes later, the ovaries were transferred to tubes containing fresh media and the test substance (PRL in a final concentration of 100 or 200 ng/mL). Both preincubation and incubation were carried out in a Dubnoff metabolic shaker, gassed with carbogen (5% CO_2 in 95% O_2) at 37°C. After 30 minutes of incubation, ovaries were homogenized using a tissue homogenizer with Teflon pestle (Eberbach, Ann Arbor, MI) in 500 μL of Hepes buffer, pH 7.4, containing 0.45 mM $CaCl_2$ and 1 mM dithiotreitol.

Radioimmunoassays

Beta-endorphin concentrations were measured in the stored samples of incubation media by radioimmunoassay as in previous studies (23) using a highly specific antiserum kindly provided by Dr. George Chrousos (National Institute

of Child Health and Human Development, Bethesda, MD). The sensitivity of the assay was 20 pg per tube, and the curve was liner up to 1,000 pg of beta-endorphin. The antibody cross-reacts on an equimolar basis with rat beta-endorphin and its immediate precursor, β-lipotropin. The intra- and interassay coefficients of variation were 8.5% and 12.1%, respectively.

Prostaglandins were quantified by radioimmunoassay as described previously (22) using rabbit antiserum from Sigma Chemical (St. Louis). The sensitivity of this assay was 12.5 pg/tube, and the cross-reactivity was 100% with PGE_2 and lower than 0.1% with other prostaglandins. The intra- and interassay coefficients of variation were 8.2 and 12%, respectively.

Nitric Oxide Synthase Activity Assay

Nitric oxide synthase activity was measured using the conversion of L-[^{14}C]-arginine into L-[^{14}C]-citrulline assay according to Bredt and Snyder (26) and modified as in previous studies (27). The enzyme activity was expressed in picomoles of NO per gram of wet weight per minute. Intra- and interassay variations were lower than 10%.

Nitrite/Nitrate Assay

Nitric oxide metabolite (NO_2^-/NO_3^-) levels were measured using the Griess reagent as described elsewhere (28). This assay measures nitrite derived from the reduction of both nitrate and endogenous nitrite, therefore we report our results as NO_2^-/NO_3^-. Overall NO_2^-/NO_3^- recovery by this method is 88% (29) and the intra-assay coefficient of variation is under 3%.

Statistical Analysis

Data were tested for normality and for homogeneity of variances and are reported as means ± SEM. Differences between groups were assessed by one-way analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons. $P<.05$ was considered statistically significant.

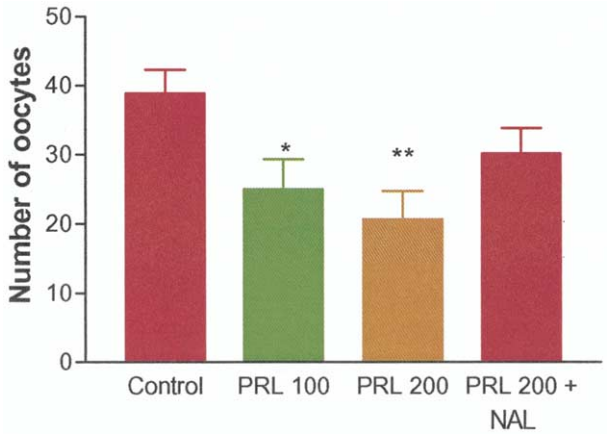
RESULTS

Effect of PRL on Ovulation

The administration of PRL 4 hours after the induction of ovulation with hCG inhibited significantly the number of oocytes within the oviducts, either at the dose of 100 μg ($P<.05$) or at the dose of 200 μg ($P<.01$) (Fig. 1). The numbers of oocytes shown in Figure 1 represent the oocytes ovulated by the two ovaries of the same animal, and the inhibition was 35.63% (number of oocytes, 25.0 ± 4.03) when 100 μg of PRL was given IP and 46.85% (20.64 ± 4.10) when 200 μg of PRL was injected, compared with the control group (38.84 ± 3.43). As shown in Figure 1, naloxone partially reversed the effect of PRL because the number of oocytes was not significantly reduced in the animals

FIGURE 1

Effects of prolactin (PRL) (100 or 200 μg) and PRL + naloxone (200 μg each) on the number of oocytes recovered in the oviducts of superovulated rats. *$P<.05$; **$P<.01$ vs. control (Student-Newman-Keuls test). Other comparisons between treatments were not statistically significant.



Polisseni. Effects of prolactin on the rat ovary. Fertil Steril 2005.

treated with both PRL 200 μg and naloxone (30.20 ± 3.64, $P>.05$ vs. control group).

Histological examination of the ovaries revealed the presence of follicles in all developmental stages, corpora lutea, and rare atretic follicles. The preovulatory follicles that failed to release their oocytes were morphologically preserved with a well-developed granulosa layer and a central, fluid-filled antral cavity. We did not observe any signs of atresia, premature luteinization, or oocyte maturation arrest in such follicles, and their appearance was similar in the various treatment groups (Fig. 2).

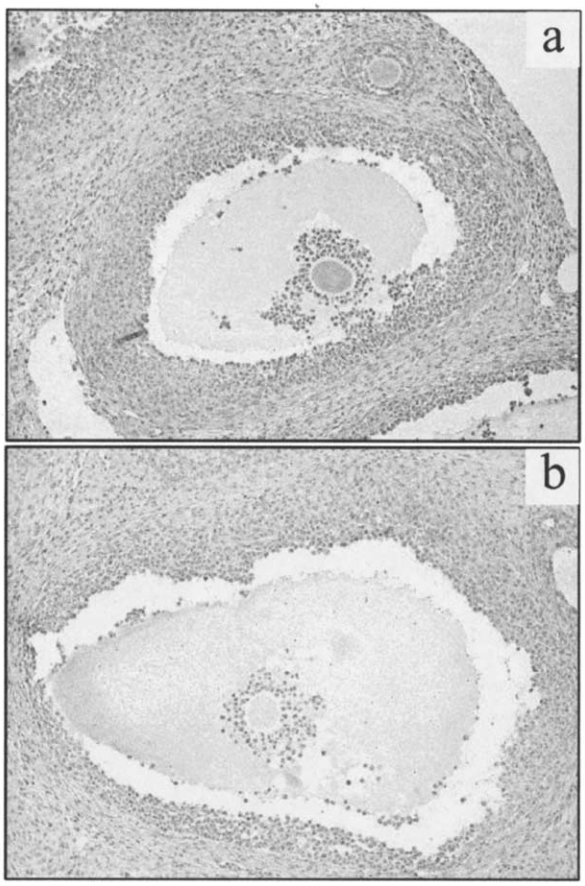
Effects of PRL on the Ovarian Production of Beta-Endorphin, PGE_2, and NO

Figure 3 shows that when the ovaries were incubated in the presence of 100 ng/mL of PRL no significant increase in the ovarian synthesis of beta-endorphin (3.18 ± 0.53 pg/mg wet weight [w.w.]) was noticed compared with the control group (3.67 ± 0.09 pg/mg w.w., $P>.05$). When 200 ng/mL of PRL was used in the incubation medium, the beta-endorphin production increased significantly ($P<.001$) and reached levels more than 100% higher when compared with the control group (8.17 ± 0.66 pg/mg w.w.).

Moreover, Figure 3 shows that the amount of PGE_2 produced was significantly lower in the PRL 200 ng/mL group than in the control group ($P<.05$). After 4 hours of incubation in this concentration of PRL, the ovarian PGE_2 release had decreased by one-third compared with the control group

FIGURE 2

Representative photomicrographs of preovulatory follicles from rats treated with **(a)** saline vehicle or **(b)** 200 μg prolactin (PRL) 4 hours after hCG injection for ovulation induction. Magnification was $\times$200.



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(319 ± 71 and 912 ± 124 pg/mg w.w., respectively). At the concentration of 100 ng/mL, PRL was not able to significantly decrease the production of PGE_2 (644 ± 30 pg/mg w.w.).

Nitric oxide synthase activity in the ovarian tissue was not significantly altered by PRL either at the concentration of 100 ng/mL (8.92 ± 0.65 pmol NO/g w.w./minute) or 200 ng/mL (8.71 ± 0.44 pmol NO/g w.w./minute) when compared with the control group (8.57 ± 0.49 pmol NO/g w.w./minute). The concentrations of NO_2^-/NO_3^- in the ovary incubation medium were not modified by the presence of PRL in both concentrations either (Fig. 3).

DISCUSSION

The results of the present study indicate that PRL is able to inhibit ovulation through mechanisms independent of the control of the hypothalamus-pituitary axis. The injection of PRL shortly after ovulation induction with PMSG/hCG reduced the number of oocytes ovulated, and this effect was more remarkable with a higher dose of PRL. Because we have used immature rats and ovulation was induced with exogenous gonadotropins, it seems likely that PRL can act locally on the ovaries, preventing them from properly responding to hCG. Naloxone, an opioid antagonist, partially reversed the effect of PRL. This might suggest that at least part of the inhibitory effect of PRL is mediated by endogenous opioids. Alternative mechanisms may include the down-regulation of the plasmin-generating system (11) and other intrinsic ovarian pathways.

In physiological concentrations, PRL is required for normal ovarian function in rodents (11) and possibly also in humans (30). During the estrous cycle in the rat, there is a brief preovulatory PRL surge with an approximately twofold increase of its plasma levels (31). A transient PRL surge has also been observed in women undergoing controlled ovarian hyperstimulation (32).

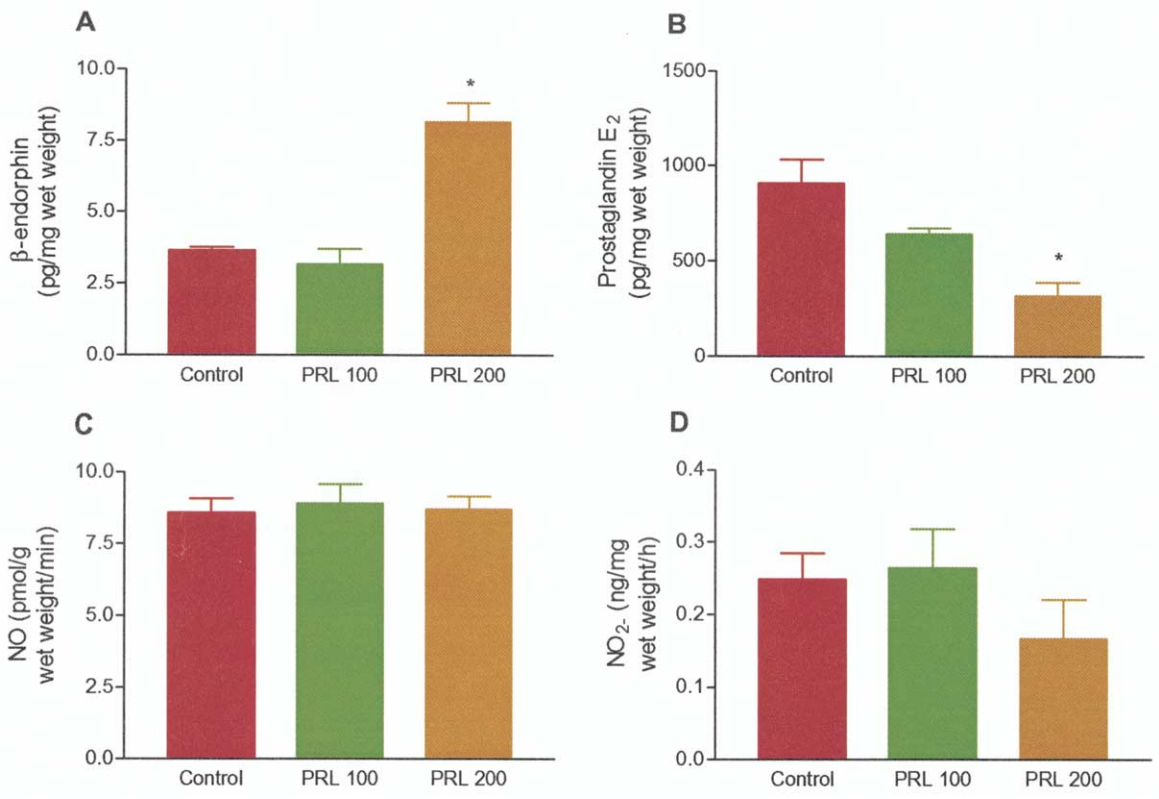
In excessive amounts, however, PRL turns out to be deleterious to follicle development and ovulation. In this regard, PRL inhibits gonadotropin-stimulated ovarian weight gain and decreases the number and the mean diameter of antral follicles in adult rats (10). It is reasonable to conclude that this effect of high PRL concentrations, combined with the antiovulatory effects demonstrated in the present study, could interfere in the process of ovulation induction by exogenous gonadotropins.

This fact could be relevant to the management of hyperprolactinemic patients undergoing ovulation induction for infertility treatment. In accordance with this view, it has been reported that women with chronic hyperprolactinemia are refractory to ovarian hyperstimulation with human menopausal gonadotropins (19). Nevertheless, case series from the 1970s showed that noncompensated hyperprolactinemic women underwent ovulation induction with clomiphene citrate and that hCG may achieve good pregnancy rates (17, 18). Since then, medical therapy for hyperprolactinemia has evolved and it became current practice to treat anovulation in hyperprolactinemic women with dopamine agonists (25). However, future clinical trials may clarify whether treating hyperprolactinemia improves the results of infertility treatments that include ovarian stimulation.

On isolated preovulatory ovaries, PRL increased the synthesis of beta-endorphin and reduced the synthesis of PGE_2. Previously, it had been shown that beta-endorphin has a clear inhibitory action on PG production by isolated ovaries and that this effect impairs the ovulation process (22). In the present study, it was demonstrated, for the first time, that PRL could activate this mechanism of ovulation inhibition. Faletti et al. (23) have recently explored the interaction between these molecular systems in vivo, and there seems to be a mutual regulation by which beta-endorphin treatment

FIGURE 3

Effects of prolactin (PRL) (100 or 200 ng/mL) on the isolated rat ovary in vitro. **(A)**, Beta-endorphin release; **(B)**, prostaglandin E_2 release; **(C)**, NO synthase activity (expressed as picomoles of NO per gram w.w. per minute); and **(D)**, NO_2^-/NO_3^- release (expressed by the NO_2^- concentration after NO_3^- reduction). *$P<.05$ vs. control (Student-Newman-Keuls test).



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inhibits PG production while PG exerts a tonic inhibition on the local production of beta-endorphin.

The role of NO in ovulation seems to be complex and to involve multiple functions, such as the control of oocyte maturation (33), ovarian blood flow (34), and PG production (27). The activity of NO synthase increases after hCG treatment, and the NO produced stimulates the synthesis of PGs, which are believed to be the critical signal for achieving follicle rupture (27, 35). Our results suggest that the inhibitory effect of PRL on ovarian PGE_2 release may not involve the NO pathway, as no change was induced either on NO synthase activity or NO metabolite accumulation in PRL-treated ovaries. However, this mechanism cannot be excluded and deserves further evaluation at different times from hCG treatment.

We did not observe any morphological differences between the remaining preovulatory follicles of animals that received or did not receive PRL treatment. Our hypothesis is that PRL might have caused a delay in oocyte release, rather than induced follicular atresia. This hypothesis is supported by our in vitro findings that PRL inhibited PG production, which is a final step in the cascade leading to follicle rupture and oocyte release. Moreover, PRL has been shown to inhibit the punctual epithelial degenerative changes at the site of follicular rupture (11).

In summary, we have shown that PRL reduces the number of oocytes released after treatment with PMSG/hCG and that this effect is partly mediated by the action of endogenous opioids. In addition, PRL stimulates ovarian beta-endorphin and inhibits ovarian PGE_2 release. These local effects of PRL on the ovaries could interfere in the process of ovulation induction by exogenous gonadotropins.

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