Sequence of interleukin 1 β actions on corpus luteum regression: relationship with inducible cyclooxygenase and nitric oxide synthase expression

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Corpus luteum regression has been described in terms of: (i) functional luteolysis – a reversible decline in serum progesterone concentration; and (ii) structural luteolysis – irreversible morphological changes and tissue remodelling events within the cellular membrane. In rats, PGF_{2 α} and interleukin 1 β (IL-1 β) are involved in structural luteolysis, PGF_{2 α} by increasing ovarian lipid peroxidation, and IL-1 β by reducing progesterone and increasing PGF_{2 α} concentrations. The aim of the present report was to determine whether by an early action IL-1 β is able to regulate functional luteolysis. Thus, ovarian explants from rats at the mid-stage of corpus luteum development were incubated during short periods with either 15 or 25 ng IL-1 β ml⁻¹. At 15 ng ml⁻¹, IL-1 β inhibited progesterone after

4 and 8 h of culture without affecting PGF_{2 α} production, and a longer incubation (21 h) was needed to increase PGF_{2 α} production. In contrast, IL-1 β enhanced PGF_{2 α} concentrations at 8 h only at the higher dose (25 ng ml⁻¹). The observed reduction in progesterone synthesis at the lower dose of IL-1 β before the increase in PGF_{2 α} concentrations led to the hypothesis that IL-1 β regulates functional luteolysis (progesterone diminution) before it affects structural luteolysis (PGF_{2 α} increase). The fact that the early IL-1 β action was described at 4 h but no effects on inducible nitric oxide synthase and inducible cyclooxygenase expression were found before this time led to the suggestion that these inductions were not necessary for the early IL-1 β action described.

Introduction

Luteal regression or luteolysis, a mechanism widely studied for its implication in early pregnancy dysfunction, is an example of a decrease in cell function, which is a normal and necessary event in the mammalian reproductive cycle. The demise of the corpus luteum has been described as a decline in serum progesterone concentrations (functional luteolysis) followed by morphological changes and tissue remodelling events within the luteal tissue (structural luteolysis) (Duncan, 2000).

Immune cells, a very important source of cytokines, are present as normal components of the corpus luteum in many species and have been implicated as key regulators of the lifespan of the corpus luteum (Pate and Landis Keyes, 2001). The interleukin 1 (IL-1) system has been identified in the ovary of several species, including humans (Devoto *et al.*, 2000) and rats (Wang *et al.*, 1997; Kol *et al.*, 1999), and performs a prominent role during follicular development and ovulation. Although IL-1 β is present and influences ovarian physiology, there have been controversial results concerning its action.

Prostaglandins play a very important role in corpus luteum function. PGF_{2 α} is well known for its luteolytic action (McCracken *et al.*, 1999) and can be produced by the corpora lutea of many species. Many intracellular mechanisms are involved in this action: for example, the decrease of progesterone synthesis after administration of PGF_{2 α} *in vivo*, reduction in the amount of sterol carrier protein, depletion of antioxidants from the corpus luteum and changes in the membrane fluidity of luteal cells. This last observation indicates that PGF_{2 α} is an important regulator of structural luteolysis (Niswender *et al.*, 2000).

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostanooids. There are two COX isoforms: constitutive COX (COX I) and inducible COX (COX II) and they are responsible for the synthesis of prostanooids. COX II is highly induced during cell activation by various factors, including mitogens, hormones and cytokines (Nakao, *et al.*, 2000). Prostaglandins and IL-1 β system are strongly related: IL-1 β was found to induce expression of prostaglandin receptors, to inhibit cyclooxygenase-2 mRNA degradation (Narko *et al.*, 1997) and to participate in the apoptotic mechanism.

Nitric oxide (NO) has been recognized as a paracrine molecule that evokes cell function via gas diffusion, a

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new concept in cell to cell signalling (Moncada and Higgs, 1993). NO participates in a variety of pathophysiological processes such as endothelium-derived relaxation of smooth muscle cells, inflammation and neurotransmission. In the female genital tract, NO is involved in regulating a wide variety of processes, including cervical ripening (Thomson *et al.*, 1997), oviduct motility and ovarian processes such as ovulation (Ekerhovd *et al.*, 1997). In previous studies of the mechanism of corpus luteum regression, oxytocin was shown to increase ovarian $\text{PGF}_{2\alpha}$ production during the late phase of corpus luteum development in rats (Motta *et al.*, 1996). This action was mediated by enhancing ovarian NOS activity (Motta and Gimeno, 1997). Endogenous NO increased $\text{PGF}_{2\alpha}$ synthesis during structural luteolysis (Motta *et al.*, 1999) and increased lipid peroxidation (Motta *et al.*, 2001). $\text{IL-1}\beta$ ($1\text{--}25\text{ ng ml}^{-1}$) was also found to diminish, in a dose-dependent manner, progesterone production by ovarian dispersates from rats in the mid-luteal phase of corpus luteum development cultured for 21 h but to increase $\text{PGF}_{2\alpha}$ synthesis at the highest dose of $\text{IL-1}\beta$ tested (25 ng ml^{-1}) (Estevez *et al.*, 2002). A cyclooxygenase inhibitor was used to demonstrate that only at the highest $\text{IL-1}\beta$ dose used could $\text{PGF}_{2\alpha}$ act as an intermediary metabolite of $\text{IL-1}\beta$ actions leading to the increase of ovarian lipid peroxidation and so participating in the structural luteolysis (Motta *et al.*, 2001). This previous data did not reveal whether $\text{IL-1}\beta$ could participate in functional luteolysis by means of an early action or the exact sequence of the longer lasting actions of $\text{IL-1}\beta$. Therefore, the present study was designed with a shorter incubation time (1–8 h) in addition to the incubation used previously (21 h). Ovarian explants were incubated with either 15 or $25\text{ ng IL-1}\beta\text{ ml}^{-1}$ and the functionality of the corpus luteum was evaluated by measuring progesterone and $\text{PGF}_{2\alpha}$ contents. Previous studies (Motta and Gimeno, 1997) and the inflammatory characteristics of the process (prostaglandin concentrations, macrophage infiltration) indicated the value of studying the effect of $\text{IL-1}\beta$ in the expression of the inducible isoforms of the two mainly ovarian enzymes involved (COX II and inducible nitric oxide synthase: iNOS).

Materials and Methods

Animals

The animal model used was that described by Motta *et al.* (1996). Briefly, immature (28–30 days of age) female rats of the Wistar strain were injected i.p. with 15 iu equine chorionic gonadotrophin (pregnant mare's serum gonadotropin; Sigma Chemical Co., St Louis, MO) per rat. Day 0 of pseudopregnancy was considered to be 48 h after injection. This procedure induced formation of corpus luteum that remained functional for 9 ± 1 days. Three different stages of corpus luteum development

can be established on the basis of serum progesterone concentrations: early, mid- and late luteolysis. In the present study, ovarian tissue from rats in the mid-stage of corpus luteum development (that is, those with the highest progesterone concentrations) were used. Rats were housed under conditions of controlled temperature (22°C) and illumination (14 h light:10 h dark; lights on at 05:00 h) and allowed free access to Purina rat chow and water *ad libitum*. Animals were anaesthetized with ether and killed by cervical dislocation. Ovarian tissues from rats at the mid-stage of corpus luteum development were used.

The animal protocol used was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Ovarian cultures

Culture of ovarian tissue was performed as described by Motta *et al.* (2001). Briefly, tissues were removed, placed in a Petri plaque containing sterile Dulbecco's PBS and cut into quarters. These quarters were then distributed randomly and evenly at four explants per well in a 24-well culture plate (Nunc Multidish, Frederick, MD). Ovarian explants were then cultured in serum-free medium (M-199 medium: Sigma Chemical Co.). Ovarian IL-1 concentrations had been measured previously during corpus luteum development and cytokine concentrations were found to increase during luteolysis (data not shown). Therefore, $\text{IL-1}\beta$ was added to the incubation media at 15 or 25 ng ml^{-1} . Incubations were carried out for 1, 4, 8 or 21 h at 37°C in a humidified atmosphere of 50% O_2 :5% CO_2 to determine the temporal relationship between $\text{IL-1}\beta$ treatment and both iNOS and COX II expression and, concomitantly, ovarian progesterone and $\text{PGF}_{2\alpha}$ production. Separate aliquots of culture media were collected for progesterone and $\text{PGF}_{2\alpha}$ determinations. Histological assays were made in cultured ovarian tissue to control tissue integrity.

As $25\text{ ng IL-1}\beta\text{ ml}^{-1}$ was the concentration that affected both progesterone and $\text{PGF}_{2\alpha}$ concentrations, this dose was used in immunoblot assays to study iNOS and COX II expression.

Progesterone determination

Incubation medium for hormone analysis was collected and frozen until progesterone concentration was determined by radioimmunoassay. The progesterone antiserum was provided by G. D. Niswender (Colorado State University, Fort Collins, CL); crossreactivity was $<2.0\%$ for $20\text{-}\alpha\text{-dihydro-progesterone}$ and deoxycorticosterone and $<1.0\%$ for other steroids normally in the serum. The sensitivity of the assay was 5–10 pg per tube. Results are expressed as pg per mg tissue.

Prostaglandin $F_{2\alpha}$ determination in cell culture media

PGF $_{2\alpha}$ concentrations were measured by radioimmunoassay. A rabbit antiserum (Sigma Chemical Co.) was used to evaluate the concentration of PGF $_{2\alpha}$ in culture media. Sensitivity of the assay was 5–10 pg ml $^{-1}$ and the crossreactivity was < 0.1% for PGE $_1$ and PGE $_2$. Results are expressed as pg per mg tissue.

Histological assay

Rat ovarian sections (4–6 μ m) were fixed immediately in 4% (v/v) paraformaldehyde and stained with haematoxylin and eosin (DAKO Corporation, Carpinteria, CA) for histological analysis to confirm the integrity of the tissues after the different incubation periods and treatments.

Western blot analysis for NOS and COX

Western blot analysis of ovarian homogenates was performed according to the procedure described by Jablonka-Shariff and Olson (1997) to study possible changes in protein concentrations during the different incubation periods (1, 4, 8 and 21 h). Briefly, frozen rat ovaries were placed in 370 μ l ice-cold homogenization buffer (1 mmol EDTA l $^{-1}$; 0.1% TX-100 with the addition of 1 μ g aprotinin ml $^{-1}$, 10 μ g leupeptin ml $^{-1}$, 1 mg benzamidine ml $^{-1}$ and 200 μ g soybean trypsin inhibitor ml $^{-1}$), homogenized on ice and centrifuged at 6000 g for 10 min at 4°C. Equal amounts of protein (60 μ g) from homogenized ovaries were diluted in SDS sample buffer and heated at 95°C for 4 min. Samples were loaded on to 4.0–7.5% (for NOS), 4.0–12% (for COX) gradient SDS-PAGE gels and separated by electrophoresis for 2 h at constant current (25 mA). Rainbow-coloured protein molecular mass markers (7.5–200 kDa) were always run on each gel together with the corresponding positive controls. Separated proteins were electrotransferred on to nitrocellulose membranes (Sigma Chemical Co.) using a mini electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA) at 4°C overnight at constant voltage (40 V) in a buffer containing 20% methanol, 25 mmol Tris l $^{-1}$, 192 mmol glycine l $^{-1}$, and 0.02% SDS. Membranes were washed in 0.1% (v/v) Tween 20 in a solution of 100 mmol NaCl l $^{-1}$ and 10 mmol Tris l $^{-1}$ at pH 7.5 (T-TBS) and incubated in blocking buffer (5% BSA in T-TBS) for 1 h 30 min at room temperature with gentle agitation. The presence of iNOS or COX II was confirmed by incubating the membranes with iNOS or COX II polyclonal murine antibody (Cayman Chemical, Ann Arbor, MI) at a dilution of 1:1000. After they were washed, membranes were incubated with an alkaline phosphatase-conjugated anti-rabbit IgG (1:5000) for 1 h at room temperature. Finally, the iNOS and COX II were detected in the membranes after incubating with the

Fig. 1. Effect of interleukin 1 β (IL-1 β) (15 (■) or 25 (▨) ng ml $^{-1}$) on progesterone production by rat ovarian tissue in culture. Each group represents the mean $\pm$ SEM of 10 different animals. Experiments were carried out in duplicate. * P < 0.05, ** P < 0.01 significantly different compared with controls (□).

alkaline phosphatase substrate BCIP–NBT (Sigma Chemical Co.). The relative amounts of iNOS or COX II were quantified by densitometric scanning of the membranes using Sigma Gel Software in each group (controls and treated). Positive controls for COX and iNOS included homogenates of lipopolysaccharide (LPS)-activated AW 264.7 macrophages. The proper loading was evaluated by staining the membranes with Ponceau-S.

Statistical analyses

Statistical analyses were carried out using the Instat program (GraphPAD software, San Diego, CA). Analysis of variance and Student–Newman–Keuls test were used for comparisons between values of groups (P < 0.05 was considered to be significant).

Results

Effect of IL-1 β on ovarian progesterone production

IL-1 β treatment (15 or 25 ng ml $^{-1}$) had no effect on ovarian progesterone concentrations after 1 h of incubation, but at 4, 8 and 21 h of culture, both 15 and 25 ng IL-1 β ml $^{-1}$ significantly diminished progesterone production compared with controls (Fig. 1).

Effect of IL-1 β on ovarian PGF $_{2\alpha}$ production

As for the progesterone assay, IL-1 β had no effect at 1 h of culture (Fig. 2). In contrast to progesterone, only treatment with 25 ng IL-1 β ml $^{-1}$ significantly increased PGF $_{2\alpha}$ production at 4 and 8 h (Fig. 2). Only after 21 h

Fig. 2. Effect of interleukin 1 β (IL-1 β) (15 (■) or 25 (▨) ng ml $^{-1}$) on PGF $_{2\alpha}$ production by rat ovarian tissue in culture. Experiments were carried out in duplicate. d versus f; g versus i; j versus k; j versus l; a,d versus g; a,d versus j: significantly different from controls (□); $P < 0.05$.

of incubation were both 15 and 25 ng IL-1 β ml $^{-1}$ treatments able to increase PGF $_{2\alpha}$ production (Fig. 2). It is important to note that basal PGF $_{2\alpha}$ output increased at 8 and 21 h of incubation time (Fig. 2: a,d versus g and a,d versus j) probably as a result of the increase in this prostanoid transport as has been reported by Jawerbaum *et al.* (2000).

Effect of IL-1 β on the expression of iNOS by ovarian rat tissue

Explants were cultured for 1, 4, 8 or 21 h with or without the IL-1 β (treated and control, respectively; Fig. 3) to determine the temporal relationship between IL-1 β and iNOS expression. The IL-1 β dose that modified both progesterone and PGF $_{2\alpha}$ concentrations (25 ng ml $^{-1}$) was used. Inducible NOS was detected in samples at 130 KDa.

IL-1 β treatment had no effect on iNOS expression after 1 h of incubation but promoted ovarian expression after 4 h of treatment (Fig. 3). IL-1 β treatment was able to increase iNOS expression at 8 h (Fig. 3) but had no effect at 21 h (Fig. 3).

Effect of IL-1 β on the expression of COX II by ovarian rat tissue

Ovarian explants were cultured for 1, 4, 8 or 21 h with and without 25 ng IL-1 β ml $^{-1}$ to examine the possible regulation of COX II expression. Inducible COX was detected in samples at 72 KDa. IL-1 β treatment (25 ng ml $^{-1}$) had no effect on COX II expression at 1 h of incubation, increased expression after 4 h (Fig. 4) and 8 h (Fig. 4) but did not modify COX II expression at 21 h of culture (Fig. 4).

Fig. 3. Effect of interleukin 1 β (IL-1 β) (■) on the inducible nitric oxide synthase (iNOS) by rat ovarian tissue. (a) Lane 1: positive control lipopolysaccharide (LPS)/AW 264.7 macrophages. Lanes 2, 4, 6 and 8: basal ovarian iNOS expressions. Lanes 3, 5, 7 and 9: IL-1 β -treated ovarian iNOS expressions. Experiments were carried out in duplicate ($n = 8$). (b) Semiquantitative analysis of ovarian iNOS expression after IL-1 β treatment. Density represents the area:intensity ratio. Two experiments were carried out and the data were analysed by Student–Newman–Keuls test. The proper loading was evaluated by staining the membranes with Ponceau-S. n.d.: not detected. Significant differences compared with controls (□): e versus g; $P < 0.05$; c versus d; $P < 0.001$; e versus f; $P < 0.05$; g versus h; $P > 0.05$.

Discussion

A growing body of evidence indicates the mammalian ovary may be a site of IL-1 β production (Brannstrom and Norman, 1993), reception (Scherzer *et al.*, 1996) and action (Towson and Pate, 1994). A putative regulator of ovulation and luteal function (Brannstrom and Norman, 1993), IL-1 β has been observed to modulate the functional and morphological differentiation of cultured mouse, pig and human granulosa cells (Narko *et al.*, 1997; Matsubara *et al.*, 2000). In spite of this, the exact mechanism(s) by which luteal function is regulated remains unknown.

Study of the mechanism involved in the corpus luteum regression has revealed that IL-1 β uses the NO pathway to inhibit progesterone production concomitantly with PGF $_{2\alpha}$ induction (Estevez *et al.*, 2002). Considering that luteolysis occurs as a result of multiple and complex interactions, and that previous studies were limited to one time (21 h), a temporal study was designed to investigate the exact sequence in the pathway that involves IL-1 β , PGF $_{2\alpha}$ and NO in the demise of the corpus luteum. Ovarian explants from rats in the mid-stage (functional) of corpus luteum development were cultured for different times (1, 4, 8 or 21 h) with 15 and 25 ng IL-1 β ml $^{-1}$.

The fact that both doses of IL-1 β were able to inhibit ovarian progesterone concentrations after as little as 4 h of incubation was in agreement with the findings of Townson and Pate (1994) that, at 4 h, IL-1 β inhibits progesterone concentrations of bovine luteal cells, excluding the detrimental effects of inhibitors of the cellular mechanisms of IL-1 β action. After longer incubation (8 or 21 h) IL-1 β also inhibited progesterone and this action was consistent with other mechanisms in which IL-1 β is involved but needs more time for its action, such as the ovulation process (Peterson *et al.*, 1993), fertilization outcome (Mendoza *et al.*, 2002), modulating steroidogenic acute regulatory protein (StAR) in endometriotic stromal human cells (Tsai *et al.*, 2001) and inhibiting oestrogen production in human granulosa-luteal cells from women undergoing *in vitro* fertilization (Tobai and Nishiya, 2001).

However, only the higher dose of IL-1 β increased PGF_{2 α} production when ovarian explants were incubated at 4 or 8 h. In accordance with previous findings, treatment with either 15 or 25 ng IL-1 β ml⁻¹ increased ovarian PGF_{2 α} production when cultures were carried out for 21 h (Estevez *et al.*, 2002). With respect to the increase in basal ovarian PGF_{2 α} production for up to 8 h of incubation, an enhanced release of prostanoids into the incubation media has been reported previously from the present authors' laboratory in other tissues (Franchi *et al.*, 1989; Jawerbaum *et al.*, 2000).

The fact that the actions of IL-1 β on progesterone and PGF_{2 α} occurred after shorter periods (4 and 8 h) indicates either a regulation of PGF_{2 α} receptor expression (Narko *et al.*, 1997) or a direct effect on key enzymes of steroidogenic pathway (Donesky *et al.*, 1998) by the cytokine could be abolished, as longer times have been reported for both events. It is important to point out that nuclear factor κ B (NF κ B) has been recognized as a key second messenger in rapid actions of cytokines; Zamorano *et al.* (2001) demonstrated that both IL-4 and tumour necrosis factor α were able to enhance the activation of NF κ B in a myeloid parental cell line after as little as 4 h of incubation. In addition, Kwon *et al.* (2001) described a synergistic IL-1 β activation of NF κ B pathway early at 2 h in human alveolar epithelial cells and Takahashi *et al.* (2001) reported that IL-1 β activated NF κ B and subsequently induced COX II in gastric fibroblast. Therefore, it is possible that in the system used in the present study, IL-1 β is using the NF κ B activation for a rapid action. Assays are being conducted to confirm this observation.

The fact that ovarian progesterone concentrations were decreased with the lower dose of IL-1 β and that a higher concentration or a longer IL-1 β exposure were needed to increase ovarian PGF_{2 α} production led to the suggestion that IL-1 β modulates functional luteolysis before it affects structural luteolysis.

The NO system has been involved in the actions of IL-1 β in ovarian rats (Ahsan *et al.*, 1997) but the exact

Fig. 4. Effect of interleukin 1 β (IL-1 β) on the inducible cyclooxygenase (COX II) by rat ovarian tissue. (a) Lane 1: positive control lipopolysaccharide (LPS)/AW 264.7 macrophages. Lanes 2, 4, 6 and 8: basal ovarian COX II expression. Lanes 3, 5, 7 and 9: IL-1 β -treated ovarian COX II expression. Each group represents the mean $\pm$ SEM of ten different animals. Experiments were carried out in duplicate ($n = 8$). (b) Analysis of ovarian COX II expression after IL-1 β treatment (■). Density represents the area: intensity ratio. Two experiments were carried out and the data were analysed by Student–Newman–Keuls test. The proper loading was evaluated by staining the membranes with Ponceau-S. Significance of differences compared with controls (□): a versus c: $P > 0.05$; c versus e: $P < 0.001$; e versus g: $P < 0.001$; c versus d: $P < 0.05$; e versus f: $P < 0.001$; g versus h: $P > 0.05$.

sequence of this mechanism remains unknown. In the present study, the pattern of expression of both inducible isoforms of the main enzymes involved in the pathway investigated (iNOS and COX II) were analysed. Under basal conditions, ovarian iNOS expression was present in cultures at 8 and 21 h of incubation. IL-1 β promoted the expression of the enzyme after only 4 h incubation, while maintaining the iNOS band density at 8 and 21 h.

The analysis of COX II expression showed that ovarian explants were able to express the enzyme early, after 1 h incubation. Treatment with IL-1 β (25 ng ml⁻¹) was able to enhance COX II expression when it was added to cultures carried out at 4 and 8 h incubation.

The above data indicate that, at first, IL-1 β reduces ovarian progesterone production by means of a mechanism independent of COX II or iNOS protein induction, as the expression of COX II and iNOS were not increased or induced by the cytokine before its early action. When cultures were carried out for longer than 4 h, the induction of COX II and iNOS expression by IL-1 β may amplify the mechanism increasing ovarian PGF_{2 α} concentrations and leading to the structural luteolysis. Cross-talk between inducible iNOS and COX II has been reported in rat chondrocytes (Boileau *et al.*, 2002) and

in primary hippocampal neuronal culture (Serou *et al.*, 1999).

In summary, a rapid effect of IL-1 β on rat ovarian progesterone synthesis by a mechanism(s) independent of iNOS and COX II protein induction is proposed. Later action by IL-1 β induces iNOS and COX II proteins, increasing PGF_{2 α} ovarian synthesis.

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