Inhibition of *in vitro* maturation of equine oocytes by interleukin 1 β via specific IL-1 receptors

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Interleukin 1 β (IL-1 β) inhibits the LH-induced resumption of meiosis of equine oocytes *in vitro*. The present study was performed to clarify this inhibitory effect of IL-1 β by testing increasing concentrations of IL-1 β , and by measuring the effect of addition of IL-1 receptor antagonist (IL-1RA) to the culture medium. The effect of IL-1 β on epidermal growth factor (EGF)-induced resumption of meiosis was also studied. Cumulus–oocyte complexes (COCs) were collected from subordinate follicles on ovaries obtained from an abattoir. In five distinct experiments, COCs were cultured for 30 h and nuclear maturation of oocytes was evaluated by DNA staining. In Expt 1, seven different media were tested: medium 1 (TCM199 + BSA); medium 2 (medium 1 + 50 ng IL-1 β ml⁻¹); medium 3 (medium 1 + eLH); and media 4, 5, 6 and 7 (medium 3 containing 0.1, 1.0, 10.0 and 50.0 ng IL-1 β ml⁻¹, respectively). In Expt 2, four different media were tested: medium 1 (TCM199 + BSA + eLH); medium 2 (medium 1 + 50 ng IL-1 β ml⁻¹); and media 3 and 4 (medium 2 + IL-1RA at 50 and 100 ng ml⁻¹, respectively). In Expt 3, three different media were tested: medium 1 (TCM199 + BSA + eLH); medium 2 (medium 1 + 50 ng IL-1RA ml⁻¹); and medium 3 (medium 2 + 50 ng IL-1 β ml⁻¹). In Expt 4, four different media were tested: medium 1 (TCM199 + BSA + eLH); and media 2, 3 and 4 (medium 1 + IL-1RA at 50, 100 and 150 ng ml⁻¹, respectively). In Expt 5, three different media were tested: medium 1 (TCM199 + BSA + EGF); medium 2 (medium 1 + 50 ng IL-1 β ml⁻¹); and

medium 3 (medium 2 + 50 ng IL-1RA ml⁻¹). In Expt 1, LH alone induced an increase in the rate of *in vitro* maturation (IVM) of equine oocytes ($P < 0.05$), whereas IL-1 β alone did not have any effect compared with medium 1. IL-1 β (50 ng ml⁻¹) significantly inhibited the eLH-induced IVM of oocytes ($P < 0.05$) compared with medium 3. A decrease in rate of maturation was observed from a concentration of 10 ng IL-1 β ml⁻¹ onwards. In Expt 2, the presence of IL-1RA in the culture medium inhibited the effect of IL-1 β and restored the rate of oocyte maturation ($P < 0.05$) observed in the presence of LH alone. In Expts 3 and 4 it was demonstrated that IL-1RA alone had no positive effect on the eLH-induced rate of maturation. In Expt 5, IL-1 β inhibited the EGF-induced resumption of meiosis ($P < 0.05$). The addition of IL-1RA inhibited this effect and restored the rate of oocyte maturation ($P < 0.05$) observed with EGF alone. In conclusion, the present data confirm the inhibitory effect of IL-1 β on IVM of equine oocytes induced by eLH and demonstrate its inhibitory effect on EGF-induced oocyte maturation. The rate of maturation decreased in a dose-dependent way and the lowest rate of maturation was observed at 50 ng IL-1 β ml⁻¹ ($P < 0.05$). The use of IL-1RA inhibited these effects, demonstrating that the action of IL-1 β is receptor-mediated. Moreover, the results clearly show that, in equine species, IL-1 β is involved in the physiology of COCs by regulating resumption of meiosis.

Introduction

Ovulation is assimilated to a cyclic inflammatory-like process (Espey, 1980) in which interleukin 1 (IL-1), an established mediator of inflammation, may play an intermediate role (Hurwitz *et al.*, 1991). IL-1 is organized as a gene system including two bioactive ligands, (IL-1 α and IL-1 β ; Dinarello, 1994), two types of receptor (IL-1R1 and IL-1R2; Colotta *et al.*, 1991; Sims and Dower, 1994) and a natural receptor antagonist (IL-1RA) that regulates

the biological activity of IL-1 ligand (Arend, 1991). Several lines of evidence support the intermediate role of IL-1 at the ovarian follicle level. First, the supply of IL-1 β to an *ex vivo*-perfused ovary promotes ovulation in rat and rabbit models and synergizes with LH (Brännström *et al.*, 1993a; Takehara *et al.*, 1994). In parallel, the supply of IL-1RA attenuates LH-supported ovulation *ex vivo* (Peterson *et al.*, 1993) and *in vivo* (Simon *et al.*, 1994a). Second, the expression of some components of the intraovarian IL-1 system (for example, IL-1 α , IL-1 β , IL-1RA, IL-1R1 and IL-1R2) was detected in ovarian follicles of rats (Hurwitz *et al.*, 1991; Kol *et al.*, 1999a,b,c), mice (Simon *et al.*, 1994b), humans (Hurwitz *et al.*, 1992;

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Chen *et al.*, 2000) and mares (Martoriati *et al.*, 2002; Martoriati and Gerard, 2003). Finally, IL-1 β regulates several ovulation-associated events *in vitro*, such as the activation of nitric oxide synthase (Ben-Shlomo *et al.*, 1994a,b) and the synthesis of proteases (Hurwitz *et al.*, 1993), plasminogen activator (Bonello *et al.*, 1995; Karakji and Tsang, 1995), hyaluronic acid (Kokia *et al.*, 1993) and prostaglandins (Kokia *et al.*, 1992; Brännström *et al.*, 1993b) in the preovulatory follicle. Moreover, IL-1 ligands (IL-1 α and IL-1 β) stimulate gonadotrophin-supported steroidogenesis in rat, hamster and human granulosa cells (Gottschall *et al.*, 1987; Nakamura *et al.*, 1990; Best and Hill, 1995).

We recently tested the potential role of IL-1 β on *in vitro* maturation (IVM) of equine oocytes to determine the involvement of IL-1 β in ovarian cell function (Martoriati *et al.*, 2002). IL-1 β was found to display an inhibitory effect on the nuclear maturation of oocytes induced by equine LH (Martoriati *et al.*, 2002). The aim of the present study was to clarify the action of IL-1 β on nuclear maturation of equine oocytes *in vitro*. Thus, the inhibitory effect of increasing doses of IL-1 β on LH-induced resumption of meiosis *in vitro* was studied; the effect of IL-1 β on the EGF-induced rate of maturation of equine oocytes was also measured. Furthermore, the *in vitro* effect of IL-1RA alone or in the presence of IL-1 β was investigated

Materials and Methods

Recovery and culture of cumulus–oocyte complexes (COCs)

Equine COCs were collected from ovaries at an abattoir (Goudet *et al.*, 2000). Subordinate follicles were punctured and aspirated with an 18.5 gauge needle under 30 mm Hg of vacuum pressure. The follicular fluids recovered during collection were maintained at 32°C. After puncture, the follicular fluids were examined under a stereomicroscope for the presence of COCs. The morphology of the cumulus of recovered COCs was examined and they were classified as compact, expanded or denuded. Compact COCs were washed four times in PBS with gentamycin (50 μ g ml⁻¹; Sigma, Saint Quentin Fallavier) and once in maturation medium (see below). The COCs were cultured individually in a humidified atmosphere (95% air and 5% CO₂) at 38.5°C for 30 h in 20 μ l maturation medium covered with mineral oil (Sigma).

Examination of nuclear stage of oocytes

After culture, the COCs were rinsed twice in PBS at 37°C and their cumulus cells were stripped off with a small glass pipette. Totally denuded oocytes were rinsed once in PBS and DNA was stained with 1 μ g bis-benzimide solution ml⁻¹ (Hoechst 33342; Sigma).

The oocytes were observed in a droplet on a slide under both a light and a fluorescence microscope to determine the nuclear stage. Oocytes with a polar body, an intact membrane (light microscopy) and two distinct spots of chromosomes stained by Hoechst (fluorescence microscopy) were considered to be in metaphase II (Goudet *et al.*, 1997).

Experiment 1: effect of dose of IL-1 β on rate of nuclear IVM

Seven different maturation media were used. Medium 1 contained tissue culture medium 199 (TCM-199; Sigma) supplemented with 0.5% (w/v) BSA (Sigma). Media 2 and 3 contained medium 1 supplemented with either recombinant human interleukin-1 β (rhIL-1 β ; Eurobio, Les Ulis; 50 ng ml⁻¹; medium 2) or equine LH (eLH; NIH, Bethesda, MD; 5 μ g ml⁻¹; medium 3). Media 4, 5, 6 and 7 were composed of medium 3 supplemented with 0.1, 1.0, 10.0 and 50.0 ng rhIL-1 β ml⁻¹, respectively.

Experiment 2: effect of IL-1RA on nuclear IVM of oocytes exposed to IL-1 β

Four maturation media were used. Medium 1 contained TCM-199 supplemented with 0.5% (w/v) BSA and 5 μ g eLH ml⁻¹. Medium 2 was composed of medium 1 supplemented with 50 ng rhIL-1 β ml⁻¹. Media 3 and 4 contained medium 2 supplemented with 50 and 100 ng recombinant human IL-1RA ml⁻¹ (rhIL-1RA; R and D systems, Abington), respectively.

Experiment 3: effect of IL-1RA on rate of nuclear IVM

Three maturation media were used. Medium 1 was TCM-199 supplemented with 0.5% (w/v) BSA and 5 μ g eLH ml⁻¹. Medium 2 was composed of medium 1 supplemented with rhIL-1RA (50 ng ml⁻¹). Medium 3 was medium 2 supplemented with 50 ng rhIL-1 β ml⁻¹.

Experiment 4: effect of dose of IL-1RA on rate of nuclear IVM

Four maturation media were used. Medium 1 was TCM-199 supplemented with 0.5% (w/v) BSA and 5 μ g eLH ml⁻¹. Media 2, 3 and 4 were composed of medium 1 supplemented with 50, 100 and 150 ng rhIL-1RA ml⁻¹, respectively.

Experiment 5: effect of IL-1 β on nuclear IVM of oocytes induced by EGF

Three different maturation media were used. Medium 1 contained TCM-199 supplemented with 0.5% (w/v) BSA and 50 ng mouse epidermal growth factor ml⁻¹ (EGF; Sigma). Media 2 and 3 contained medium 1 supplemented with either 50 ng rhIL-1 β ml⁻¹ (medium 2)

Fig. 1. Effect of interleukin 1β (IL-1β) on nuclear *in vitro* maturation of equine oocytes induced by eLH. Medium 1 contained TCM-199 supplemented with 0.5% (w/v) BSA. Media 2 and 3 contained medium 1 supplemented with either recombinant human IL-1β (rhIL-1β; 50 ng ml⁻¹; medium 2) or equine LH (eLH; 5 μg ml⁻¹; medium 3). Media 4, 5, 6 and 7 were composed of medium 3 supplemented with 0.1, 1.0, 10.0 and 50.0 ng rhIL-1β ml⁻¹, respectively. The histogram represents the percentage of oocytes in metaphase II. The number of oocytes analysed in each medium is indicated in brackets at the bottom of each bar. ^{a-c}Different letters indicate significant differences according to the Pearson's chi-squared test ($P < 0.05$).

or 50 ng rhIL-1β ml⁻¹ and 50 ng rhIL-1RA ml⁻¹ (medium 3).

Statistical analysis

In each experiment, the Pearson's chi-squared test was performed to compare rates of oocyte maturation using StatXact 5 software (CYTEL, Cambridge, MA; www.cytel.com).

Results

Experiment 1: effect of dose of IL-1β on rate of nuclear IVM

A total of 295 COCs from 102 ovaries was used in this experiment. Increasing doses of IL-1β were tested on equine oocytes. The rate of oocyte maturation was not significantly different between medium 1, which contained BSA only (control medium), and medium 2, which was supplemented with 50 ng IL-1β ml⁻¹ (Fig. 1). The rate of oocyte maturation was significantly higher in medium 3, which contained eLH, than in media 1 and 2 ($P < 0.05$). Addition of IL-1β to the culture medium containing eLH resulted in a dose-dependent decrease in rate of oocyte maturation from a concentration of 10 ng ml⁻¹ onwards. A dose of 50 ng IL-1β ml⁻¹ totally

Fig. 2. Effect of interleukin 1 receptor antagonist (IL-1RA) on nuclear *in vitro* maturation of equine oocytes in the presence of eLH and IL-1β. Medium 1 contained TCM-199 supplemented with 0.5% (w/v) BSA and 5 μg eLH ml⁻¹. Medium 2 was composed of medium 1 supplemented with 50 ng recombinant human IL-1β ml⁻¹. Media 3 and 4 contained medium 2 supplemented with 50 and 100 ng recombinant human IL-1RA ml⁻¹ (rhIL-1RA), respectively. The histogram represents the percentage of oocytes in metaphase II. The number of oocytes analysed in each medium is indicated in brackets at the bottom of each bar. ^{ab}Different letters indicate significant differences according to the Pearson's chi-squared test ($P < 0.05$).

inhibited the positive effect of LH on oocyte maturation. Indeed, similar rates of maturation were observed in medium 1 and medium 7.

Experiment 2: effect of IL-1RA on nuclear IVM of oocytes exposed to IL-1β

A total of 186 COCs from 58 ovaries was used in this experiment. As observed in Expt 1, the rate of oocyte maturation was significantly higher in medium 1, which contained eLH, than in medium 2, which contained eLH and 50 ng IL-1β ml⁻¹ ($P < 0.05$; Fig. 2). Addition of IL-1RA at 50 ng ml⁻¹ (medium 3) or 100 ng ml⁻¹ (medium 4) significantly increased the rate of oocyte maturation compared with medium 2 ($P < 0.05$), and restored the rate of maturation to that observed in medium 1.

Experiment 3: effect of IL-1RA on rate of nuclear IVM

A total of 118 COCs from 46 ovaries was used in this experiment. Despite a slight increase in medium 2 (eLH + IL-1RA), the rates of maturation were similar in the three media tested (Fig. 3). This finding demonstrates that IL-1RA, in the absence of IL-1β, has no effect on LH-induced oocyte maturation.

Fig. 3. Effect of interleukin 1 receptor antagonist (IL-1RA) on nuclear *in vitro* maturation of equine oocytes induced by eLH (5 µg ml⁻¹). Medium 1 was TCM-199 supplemented with 0.5% (w/v) BSA and 5 µg eLH ml⁻¹. Medium 2 was composed of medium 1 supplemented with recombinant human IL-1RA (rhIL-1RA, 50 ng ml⁻¹). Medium 3 was medium 2 supplemented with 50 ng rhIL-1β ml⁻¹. The histogram represents the percentage of oocytes in metaphase II. The number of oocytes analysed in each medium is indicated in brackets at the bottom of each bar. No significant difference between groups was observed (Pearson's chi-squared test).

Experiment 4: effect of dose of IL-1RA on rate of nuclear IVM

A total of 181 COCs from 40 ovaries was used in this experiment. The rate of oocyte maturation was not different between medium 1, which contained eLH alone (control medium), and media 2 and 3, which were supplemented with IL-1RA at 50 and 100 ng ml⁻¹, respectively (Fig. 4). The rate of oocyte maturation was significantly decreased in medium 4, which contained the highest concentration of IL-1RA (150 ng ml⁻¹; $P < 0.05$). This finding confirms that IL-1RA has no positive effect on LH-induced oocyte maturation.

Experiment 5: effect of IL-1β on nuclear IVM of oocytes induced by EGF

A total of 95 COCs from 28 ovaries was used in this experiment. Addition of IL-1β (50 ng ml⁻¹) to COCs in the presence of EGF resulted in a significant decrease in the rate of oocyte maturation ($P < 0.01$; Fig. 5). IL-1RA at a concentration of 50 ng ml⁻¹ inhibited this negative

Fig. 4. Effect of dose of interleukin 1 receptor antagonist (IL-1RA) on nuclear *in vitro* maturation of equine oocytes induced by eLH (5 µg ml⁻¹). Medium 1 was TCM-199 supplemented with 0.5% (w/v) BSA and 5 µg eLH ml⁻¹. Media 2, 3 and 4 were composed of medium 1 supplemented with 50, 100 and 150 ng recombinant human IL-1RA ml⁻¹, respectively. The histogram represents the percentage of oocytes in metaphase II. The number of oocytes analysed in each medium is indicated in brackets at the bottom of each bar. ^{ab}Different letters indicate significant differences according to the Pearson's chi-squared test ($P < 0.05$).

effect and restored the rate of maturation to that observed in medium 1, which contained EGF only.

Discussion

Martoriati *et al.* (2002) demonstrated that IL-1β inhibits the eLH-induced resumption of meiosis of equine oocytes *in vitro*. The aim of the present study was to clarify this inhibitory effect.

Expt 1 was designed to determine the lowest dose of IL-1β that is required to inhibit eLH-induced oocyte maturation. The results demonstrate that completion of meiosis is influenced by IL-1β in a dose-dependent way. The inhibitory effect was observed from 10 ng ml⁻¹

onwards and was significant at 50 ng ml^{-1} . It is possible that IL-1 β may act directly on LH or on its receptor. This hypothesis was tested by investigating the effect of IL-1 β in the presence of EGF, another factor involved in IVM of oocytes (Goudet *et al.*, 2000). In Expt 5 it was observed that IL-1 β inhibited the resumption of meiosis induced by EGF as observed with LH. This finding indicates that IL-1 β may be involved in the regulation of meiosis downstream of the LH and EGF receptors rather than on the LH signal transduction, as was suggested in our previous work (Martoriati *et al.*, 2002). Although the mechanism by which IL-1 β regulates oocyte maturation is unclear, it can be hypothesized that IL-1 β could act at a common pathway between LH and EGF, and inhibit resumption of meiosis. Indeed, IL-1 β could exert an effect on meiosis by regulating the synthesis or the activity of some key proteins such as p34cdc2, cyclin B or MAP kinase.

IL-1 β concentration in follicular fluid has been measured at between 53 pg ml^{-1} and 255 pg ml^{-1} in humans (Machelon *et al.*, 1995), and the production of IL-1 β by granulosa cells in culture has been estimated at 350 pg ml^{-1} (24 h^{-1}). No data are available on the intrafollicular concentration of IL-1 β in horses, but the results of the present study show that IL-1 β influences IVM of equine oocytes from 10 ng ml^{-1} onwards. This dose is higher than the intrafollicular physiological concentration measured so far in humans (Machelon *et al.*, 1995) but corresponds to the dose used to study the effect of IL-1 β on human steroidogenesis *in vitro* (Best and Hill, 1995) and on biosynthesis of rat ovarian prostaglandins (Ando *et al.*, 1998). Moreover, it is possible that the effective dose used *in vitro* in the present study could be explained by some species-specific features. Indeed, it has already been shown in the equine species that *in vivo* maturation conditions are somewhat different from those in other mammals: there is no LH surge, but a slow increase lasting several days, with a maximum value observed at 1 day after ovulation (Whitmore *et al.*, 1973; Irvine and Alexander, 1994).

The aim of the second part of the present study was to determine how IL-1 β acts on oocyte maturation. IL-1RA is a competitive inhibitor of the actions of both IL-1 α and IL-1 β . It regulates IL-1 ligand biological activity by binding to IL-1 receptors without inducing any cellular effect (Hannum *et al.*, 1990; Arend, 1991; Dripps *et al.*, 1991). In several studies, IL-1RA has been used to demonstrate that the effect of IL-1 β is mediated via IL-1-specific receptors. For example, the presence of IL-1RA in culture media limits IL-1 β fixation on its receptor and thus limits the effect of IL-1 β on prostaglandin production by human (Hurwitz *et al.*, 1995) and rat (Ando *et al.*, 1998) granulosa cells. In Expt 2, IL-1RA was used in the culture media and it was observed that IL-1RA reversed the inhibitory effect of IL-1 β on the oocyte maturation induced by LH. However, in Expt 5 it was shown that IL-1RA neutralized the IL-1 β inhibitory effect and restored the EGF positive action on oocyte maturation. Moreover,

Fig. 5. Effect of interleukin 1 β (IL-1 β) and interleukin 1 receptor antagonist (IL-1RA) on nuclear *in vitro* maturation of equine oocytes induced by epidermal growth factor (EGF). Medium 1 contained TCM-199 supplemented with 0.5% (w/v) BSA and 50 ng ml^{-1} mouse EGF. Media 2 and 3 contained medium 1 supplemented with either 50 ng ml^{-1} recombinant human IL-1 β (rhIL-1 β) (medium 2) or $50 \text{ ng rhIL-1}\beta \text{ ml}^{-1}$ and $50 \text{ ng rhIL-1RA ml}^{-1}$ (medium 3). The histogram represents the percentage of oocytes in metaphase II. The number of oocytes analysed in each medium is indicated in brackets at the bottom of each bar. ^{a-c}Different letters indicate significant differences according to the Pearson's chi-square test ($P < 0.05$).

equine oocytes expressed IL-1R2 and cumulus cells expressed the two types of IL-1 receptor (IL-1R1 and IL-1R2; Martoriati *et al.*, 2002). Taken together, these results demonstrate that the inhibitory effect of IL-1 β on oocyte maturation is receptor-mediated and is a biological effect of IL-1 β rather than a cytotoxic effect of IL-1 β in the culture conditions used.

As equine COCs contain IL-1 β mRNA (Martoriati *et al.*, 2002), it can be hypothesized that COCs are capable of producing some biologically active IL-1 β . This local IL-1 β could then interact with eLH or EGF and thus limit the rate of oocyte maturation in the culture conditions used. This hypothesis was tested in Expts 3 and 4 of the present study. In both experiments, IL-1RA was added to the culture medium containing LH to neutralize the potential inhibitory action of some locally produced IL-1 β . In Expt 3, a slight, but not significant, positive effect of IL-1RA (50 ng ml^{-1}) on the rate of oocyte maturation induced by eLH was observed. In Expt 4, the supplementation of culture medium with IL-1RA at 50 and 100 ng ml^{-1} confirmed the absence of a

positive effect of IL-1RA on the rate of oocyte maturation induced by eLH. This observation could be explained by the absence of active IL-1 β production by COCs in the culture conditions used. It is possible that the IL1 β mRNA that is present in equine COCs (Martoriati *et al.*, 2002) may not be translated (or is translated at very low level), or is translated in a non-biologically active protein, or is not secreted. Thus, the effect of IL-1RA cannot be visualized. The absence of a positive effect of IL-1RA on rate of oocyte maturation induced by eLH can also be explained by the fact that the rate of maturation cannot be increased in our culture conditions, as non-matured oocytes are incompetent to resume IVM. Finally, considering that equine COCs contained some IL-1RA mRNA (Martoriati *et al.*, 2002), the absence of a significant effect of IL-1RA might also be explained by the neutralization of endogenous IL-1 β by some locally produced IL-1RA. The significant decrease in rate of maturation was observed in medium supplemented with IL-1RA at 150 ng ml⁻¹, indicating that IL-1RA probably has a cytotoxic effect at high concentration.

In conclusion, the results of the present study confirm that IL-1 β has an inhibitory effect on the IVM of equine oocytes induced by eLH. This effect is dose-dependent and is significant at a dose of 50 ng IL-1 β ml⁻¹. The use of IL-1RA allowed us to demonstrate indirectly that IL-1 β acts by binding to specific IL-1 receptors. Finally, the inhibitory effect of IL-1 β was not exclusive to eLH, as it was also observed with EGF. This result indicates that the action of IL-1 β is probably on the regulation of meiosis. Although the mechanism by which IL-1 β regulates oocyte maturation is unclear, the IL-1 family may play an essential role in the physiology of COCs in equine species.

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