Effects of selective inhibition of cyclooxygenase and lipoxygenase pathways in follicle rupture and ovulation in the rat

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Abstract

Treatment with non-steroidal anti-inflammatory drugs, either non-selective or selective cyclooxygenase-2 (COX-2) inhibitors, consistently impairs ovulation, indicating the essential role of COX-2/prostaglandins in the ovulatory process. Indomethacin, a potent inhibitor of both COX-1 and COX-2, induced several ovulatory alterations, consisting of a decrease in the number of oocytes effectively ovulated, trapping of oocytes inside the luteinized follicle, as well as abnormal follicle rupture at the basolateral sides, with release of the oocyte and follicular fluid to the interstitium. Yet, the precise role of prostaglandins in ovulation and whether some of the ovulatory defects induced by indomethacin are due to interference with additional components of the ovulatory cascade, beyond prostaglandin synthesis, are not completely understood. We have used gonadotrophin-primed immature rats to analyse whether, compared to indomethacin, selective inhibition of COX-2, with or without concomitant inhibition of COX-1, or selective inhibition of the lipoxygenase (LOX) pathway, induce similar ovulatory alterations. Immature rats (27 days of age) were injected PMSG (10 IU), and 48 h later hCG (10 IU) subcutaneously, and different anti-inflammatory drugs. Animals were killed at 21 h after hCG injection. Rats treated with the selective COX-2 inhibitor NS398 (10 mg/kg body weight, (bw)) showed alterations in follicle rupture as those treated with indomethacin (0.5 mg/rat), albeit affecting a lower number of follicles, irrespective of the concomitant inhibition of COX-1 with the selective inhibitor SC560 (10 mg/kg bw). Rats treated with the LOX inhibitor NDGA (300 mg/kg bw) did not show ovulatory alterations. These data indicate that the characteristic alterations of follicle rupture induced by indomethacin, are also induced by selective COX-2 inhibitors, strengthening the contention that prostaglandins play a crucial role in the spatial targeting of follicle rupture at the apex.

Reproduction (2006) **132** 571–577

Introduction

Ovulation is a complex, multistep process that is triggered in cycling females by the mid-cycle preovulatory LH surge. The gonadotrophin surge induces the coordinate expression of a plethora of genes, initiates a cascade of events, and finally results in follicle rupture and release of the mature oocyte from the ovary. One of the LH-induced genes in preovulatory follicles is that encoding cyclooxygenase-2 (COX-2), the inducible isoform of the prostaglandin G/H synthase, while the other main cyclooxygenase isoform, COX-1, is constitutively expressed (Sirois *et al.* 2004). A large body of evidence has accumulated, indicating the essential role of COX-2/prostaglandins in ovulation. In the first approach, classical non-steroidal anti-inflammatory

drugs (NSAIDs), inhibiting both COX-1 and COX-2 activity, as well as the more recently developed selective COX-2 inhibitors, have been repeatedly reported to inhibit ovulation in all mammalian species investigated so far (reviewed by Brännström & Janson 1991, Tsafiri *et al.* 1993, Espey & Lipner 1994). Furthermore, exogenous prostaglandin supplementation restored ovulation in NSAID-treated animals, at least under some experimental conditions (Holmes *et al.* 1983, Sogn *et al.* 1987, Gaytán *et al.* 2002a). More recently, genetic studies in mice (Lim *et al.* 1997, Matsumoto *et al.* 2000) have confirmed the obligatory role of COX-2/prostaglandins in ovulation. Mice with targeted deletion of COX-2, but not COX-1, show multiple reproductive alterations, including defective ovulation (Lim *et al.* 1997), that is restored by exogenous supplementation

with prostaglandins of the E series (Davis *et al.* 1999). Furthermore, prostaglandin E2 receptor (subtype EP2)-knockout mice also show defective ovulation (Ochsner *et al.* 2003). However, the molecular targets and the precise role of prostaglandins in the ovulatory process are not completely understood.

In most previous studies on COX-2 inhibition, ovulation rate has been evaluated by counting oocytes in the oviduct from 16 h after the preovulatory LH surge or hCG administration, and eventual histological analysis of randomly selected ovarian sections. Common features in NSAID-treated animals are decreased numbers of oocytes in the oviducts and the presence of oocytes trapped inside luteinizing follicles. On this basis, it is generally assumed that inhibition of follicle rupture is the mechanism underlying the anti-ovulatory effects of COX inhibitors (reviewed in Brännström & Janson 1991, Tsafirri *et al.* 1993, Espey & Lipner 1994). However, this assumption is closely linked to the method used to check ovulation. Although the presence of eggs in the oviduct provides unequivocal evidence on the occurrence of follicle rupture and ovulation, the absence of follicle rupture cannot be inferred from the lack of oviductal oocytes. Furthermore, the observation of randomly selected ovarian sections provided a limited view of the ovulatory process. This can be appreciated clearly by detailed histological examination of serially sectioned ovaries in rats treated during the ovulatory period with indomethacin (INDO), a potent inhibitor of both COX-1 and COX-2 (Vane *et al.* 1998). In these animals, about 1/3 of the cumulus–oocyte complexes (COCs) remained trapped inside the luteinized follicle, about 1/3 are released to the ovarian interstitium through ruptures at the basolateral follicle sides, and about 1/3 are effectively ovulated (Gaytán *et al.* 2002a,b, 2003). Furthermore, some of the luteinizing follicles with trapped COCs show rupture of the follicle wall at the basolateral sides, with release of follicular fluid into the ovarian interstitium. In addition, the release of follicular fluid and granulosa cells to the ovarian interstitium, leads to invasion of the ovarian stroma, rupture of blood and lymphatic vessels, hemorrhagia and formation of emboli of follicular fluid and granulosa cells (Gaytán *et al.* 2002a,b, 2003). These data indicate that induction of abnormal (misdirected) follicle rupture, rather than absence of follicle rupture, seems to be the main ovulatory alteration in INDO-treated rats.

Although the anti-ovulatory effects of NSAIDs are attributed to COX-2 inhibition, some studies have found a poor correlation between the inhibition of prostaglandin synthesis and the inhibition of ovulation rate (reviewed by Espey & Lipner 1994). This raises the possibility that some of the effects of INDO on follicle rupture and ovulation could be due to COX-2-independent actions. Previous studies have reported an effect of INDO on the lipoxygenase (LOX) pathway of arachidonic acid metabolism, and a decrease in the

ovulation rate after selective inhibition of LOX pathway in rats (Mikuni *et al.* 1998). In addition, although genetic deletion of COX-1 does not inhibit ovulation (Langebach *et al.* 1995), and ovulatory impairment in INDO-treated animals seems to be due to COX-2 inhibition, it is not known whether concomitant inhibition of COX-1 has a role in the induction of abnormal follicle rupture in these animals, since recent studies have reported that COX-1 is also involved in inflammation (Gilroy & Colville-Nash 2000), and ovulation has been considered an inflammatory-like reaction (Espey 1980). In this context, we aimed to analyse whether the characteristic alterations of follicle rupture and ovulation present in INDO-treated rats were specifically related to COX-2 inhibition and if they are also related to concomitant COX-1 inhibition, or to side effects of the interconnected LOX pathway.

Materials and Methods

Animals and treatments

Immature female Wistar rats were used for this study. The animals were maintained under controlled light (14 h light:10 h darkness) and temperature (22 °C) conditions, and given access to pelleted food and tap water *ad libitum*. The experiments were conducted in agreement with the National Institute of Health's Guide for the Care and Use of Laboratory Animals, and approved by the ethics committee of the University of Córdoba. Unless indicated, all products were purchased from Sigma. At 27 days of age (about 80 g body weight (bw)), the animals were injected, subcutaneously, 10 IU PMSG at 1200 h, and 48 h later 10 IU hCG, to induce follicle development and ovulation respectively. The animals were divided into six groups that were injected (at the same time as hCG injection) s.c. with: (i) vehicle (olive oil, 200 µl); (ii) indomethacin (0.5 mg/rat), a non-selective COX inhibitor, inhibiting both COX-1 and COX-2 activity; (iii) NS398 (10 mg/kg), a selective COX-2 inhibitor; (iv) SC560 (10 mg/kg), a selective COX-1 inhibitor; (v) NS398 (10 mg/kg) plus SC560 (10 mg/kg), to inhibit both COX-1 and COX-2; and (vi) nordihydroguaiaretic acid (NDGA; 300 mg/kg), a selective inhibitor of the lipoxygenase pathway. The doses were selected based on previous studies reporting inhibition of enzyme activity and/or ovulation in rats (Mikuni *et al.* 1998, Sakurai *et al.* 2003, Gaytán *et al.* 2004) and in the range of selectivity. All the animals were killed 21 h after hCG injection.

Histological procedures and evaluation of follicle rupture and ovulation

The ovaries, including the oviducts and periovarian fat pads were dissected, fixed with Bouin–Hollande's fluid for 24 h and embedded in paraffin wax after dehydration. Serial, 6 µm-thick, sections were cut, stained with haematoxylin and eosin, and viewed under the

microscope. Twenty-one hours after hCG treatment, the corpora lutea are not completely luteinized, and could be considered as luteinizing follicles, particularly when unruptured and containing the COC. Accordingly, the term luteinized follicles has been adopted, irrespective of the presence or absence of follicle rupture and release of the COC. In each ovary, all serial sections were viewed under the microscope, and the total number of luteinized follicles was counted. The number and location of COCs (either in the oviduct, inside the luteinized follicle or at the ovarian interstitium) were recorded. The number of COCs in each location was presented as a percentage, with respect to the total numbers of luteinized follicles per animal (that matched the total number of COCs per animal). No significant differences were found among groups with respect to the mean number of luteinized follicles/COCs per animal (ranging from 13.4 ± 1.3 to 15.4 ± 1.2 , mean $\pm$ S.E.M. for $n=5$). Statistical analysis was performed by ANOVA and Student–Newmann–Keuls for multiple comparisons among means, and significance was considered at the 0.05 level.

Results

Rats treated with vehicle or selective COX-1 inhibitor SC560, showed newly formed corpora lutea, COCs in the oviduct, and no alterations of the ovulatory process. In contrast, rats treated with INDO, NS398 or NS398 plus SC560, showed altered follicle rupture and ovulation that were more frequently observed in INDO-treated rats. Ovulatory defects in these animals, included trapping of the expanded COC inside the luteinized follicles (Fig. 1a,b), and abnormal follicle rupture at the basolateral sides, with release of the COC to the ovarian interstitium (Fig. 1c–f). Irrespective of the location of the COCs, the oocytes were in the metaphase II stage, and showed expanded cumulus (Fig. 1a–f) in all groups. Additional alterations such as rupture of blood vessels and blood extravasations were commonly observed around interstitial COCs (Fig. 1c–f) because of the release of the COC and follicular fluid to the ovarian interstitium, through basolateral follicle sides, in rats treated with COX-2 inhibitors. Less frequently, invasion of the ovarian bursa, with infiltration of the periovarian fat pad by granulosa cells (Fig. 1g) was observed. Otherwise, rats treated with the LOX inhibitor NDGA, did not show alterations of the ovulatory process, and COCs were located in the oviduct (Fig. 1h), although the ovarian medulla showed extensive oedema (Fig. 1i).

The quantitative data are presented in Figs 2–4. The numbers of oocytes effectively ovulated (Fig. 2), i.e. located at the oviduct, were significantly decreased in rats treated with COX-2 inhibitors: INDO, NS398 and NS398 plus SC560. This ovulatory inhibition was significantly higher in INDO-treated rats (about 50%

decrease), than NS398-treated rats (about 25% decrease), irrespective of concomitant treatment with the COX-1 inhibitor SC560. Comparison of the percentage of COCs trapped inside the luteinized follicle after COX-2 inhibition is shown in Fig. 3. No significant difference existed among animals treated with INDO, NS398 or NS398 plus SC560. In contrast, the percentage of COCs released to the ovarian interstitium through basolateral follicle ruptures (Fig. 4) was significantly higher in INDO-treated (about 27% of the COCs) than NS398-treated rats (about 7% of the COCs), irrespective of the concomitant inhibition of COX-1 in NS398-treated animals.

Discussion

Treatment with indomethacin (INDO) causes ovulatory dysfunction, characterized by the occurrence of abnormal rupture of some follicles at the basolateral follicle sides, with release of the cumulus–oocyte complex (COC), granulosa cells and follicular fluid to the ovarian interstitium, while other COCs remain trapped inside the corpus luteum, or released to the periovarian space, i.e. effectively ovulated (Gaytán *et al.* 2002a,b, 2003). This raises the possibility that some of the effects of INDO in ovulation could be due to the interference with ovulation-related mechanisms, others than COX-2/prostaglandin synthesis inhibition. For instance, although INDO effects have been attributed to COX-2 inhibition (Sirois *et al.* 2004), it is not known whether concomitant inhibition of COX-1 could also play a role in abnormal follicle rupture. Detailed morphological studies in COX-2-knockout mice have not been published, and double COX-1/COX-2-knockout mice do not survive up to reproductive age (Reese *et al.* 2001). The data from this study indicate that all the characteristic alterations of follicle rupture and ovulation observed in INDO-treated rats are also present, albeit in a lower proportion of follicles, after selective COX-2 inhibition by treatment with NS398. In addition, concomitant treatment with the selective COX-1 inhibitor SC560 did not increase the anti-ovulatory effects of NS398-treated rats, reinforcing the contention that ovulatory defects in INDO-treated rats were due to COX-2 inhibition. This is also in agreement with the lack of ovulatory defects in rats treated with the selective COX-1 inhibitor SC560, reported here, and with the normal ovarian phenotype in COX-1-knockout mice (Langebach *et al.* 1995). Quantitative differences found in this study between non-selective (INDO) and selective (NS398) COX-2 inhibitors could be due to differences in their relative potency and/or pharmacokinetics.

Inhibition of the LOX pathway with the selective inhibitor NDGA, did not affect ovulation, and the only morphological alteration in these animals was the presence of extensive oedema of the ovarian medulla. Previous studies have provided contradictory results with

Figure 1 Representative micrographs from the ovary of rats treated with indomethacin (a, c, e), NS398 (b, d, f), NS398 plus SC560 (g) or NDGA (h,i), showing trapping of the expanded cumulus–oocyte complex (COC) inside the luteinized follicle (a, b), release of the COC to the ovarian interstitium (c, d; the boxed areas are shown at higher magnification in e, f), invasion of the ovarian bursa and periovarian fat pad by granulosa cells (g), normal ruptured follicles and location of the COC in the oviduct (h), as well as extensive oedema of the ovarian medulla (i). Haematoxylin and eosin.

respect to the effects of LOX inhibitors on ovulation, ranging from inhibition (Reich *et al.* 1983, Yoshimura *et al.* 1991, Mikuni *et al.* 1998) to lack of effects (Carvalho *et al.* 1989) or to an increased rate of ovulation (Hellberg *et al.* 1990). These discrepancies could be due to the existence of interactions and overlapping functions in ovulation of both arachidonic acid metabolism pathways (Mikuni *et al.* 1998). The treatment with NDGA has been reported to reduce prostaglandin synthesis in the ovary (Mikuni *et al.* 1998), and some of the reported effects of LOX inhibitors on ovulation could be related to side effects on the COX pathway. Furthermore, LOX-derived products seem to play a less relevant role than prostaglandins in ovulation (Hellberg *et al.* 1990).

The present view of the ovulatory process decrees that proteolytic degradation of the extracellular matrix is needed to allow rupture of the follicle wall (Tsafiriri *et al.* 1993, Espey & Lipner 1994, Curry *et al.* 2001). This implies that focalised proteolysis at the follicle apex (i.e.

the follicle side facing the ovarian surface) is required to achieve effective ovulation (Reich *et al.* 1985a,b, 1991, Ny *et al.* 2002). Yet, the mechanisms responsible for the spatial targeting of follicle rupture at the apex are not known. Previous studies on several ovulation-related biochemical events (Espey & Richards 2002), including the expression of specific matrix metalloproteinases (Bakke *et al.* 2004), have not reported the existence of polarisation (i.e. differential expression at the apex versus the base) in preovulatory follicles. It is also evident, that the presence of apical tissues, such as the ovarian surface epithelium or the tunica albuginea, are not essential for follicle rupture, as it happens in INDO-treated rats at the basolateral follicle sides. In this context, still unknown mechanisms could be responsible for the focalised proteolysis and subsequent rupture from preovulatory follicles at the apical zone. The data of this, and previous studies (Osman & Dullaart 1976, Gaytán *et al.* 2002a,b, 2003), demonstrate that misdirected

Figure 2 Percentage of oocytes effectively ovulated after treatment with vehicle (VEH), INDO (a dual NSAID), NS398 (a selective COX-2 inhibitor), SC560 (a selective COX-1 inhibitor), NS398 plus SC560, or NDGA (a selective LOX inhibitor). (a) $P < 0.05$ vs vehicle; (b) $P < 0.05$ vs INDO. ANOVA and Student–Newmann–Keuls for $n = 5$.

follicle rupture occurs after pharmacological COX-2 inhibition. The only well-characterized target of prostaglandins in preovulatory follicles is the tumour necrosis factor-activated gene-6 (TSG-6), which is dependent on the expression of COX-2 in cumulus cells in response to the preovulatory LH surge (Joyce *et al.* 2001), and codifies a hyaluronan-binding protein that participates in cumulus expansion (Richards 2005). Defective cumulus expansion, due to the lack of expression of TSG-6, has been considered as a possible cause of the anti-ovulatory effects of COX-2 inhibitors, as well as a component of the anovulatory ovarian phenotype of COX-2- and EP2-knockout mice (Hizaki *et al.* 1999, Ochsner *et al.*

Figure 3 Comparison of the percentages of oocytes trapped inside the corpus luteum after treatment with INDO (a dual NSAID), NS398 (a selective COX-2 inhibitor), or NS398 plus SC560 (a selective COX-1 inhibitor). No significant difference existed among groups (ANOVA for $n = 5$).

Figure 4 Comparison of the percentages of oocytes released to the ovarian interstitium after treatment with INDO (a dual NSAID), NS398 (a selective COX-2 inhibitor), or NS398 plus SC560 (a selective COX-1 inhibitor). A $P < 0.05$ vs INDO (ANOVA and Student–Newmann–Keuls for $n = 5$).

2003, Richards 2005). Morphological cumulus expansion was evident in NSAID-treated rats (see Fig. 1), although the existence of functional defects, possibly contributing to the trapping of some COCs inside the luteinizing follicle, cannot be discarded. However, defective cumulus expansion can hardly explain the occurrence of basolateral ruptures of the follicle wall and the release of the COC to the interstitium. In this sense, the anti-ovulatory effects of COX-2 inhibitors seem to be mainly due to the disruption of the mechanisms responsible for the spatial targeting of proteolytic activity/follicle rupture at the apex. It is worthwhile noting that follicle rupture at the apex was not selectively inhibited after COX-2 inhibition, and 50–75% of oocytes were effectively ovulated. The presence of some ovulated oocytes even with the highest possible INDO dosages (Espey & Lipner 1994) or in COX-2- or EP2-knockout mice (Russell & Richards 1997, Ochsner *et al.* 2003) has been interpreted as evidence of the role of other inflammatory-related mechanisms, beyond prostanoid synthesis, in the ovulatory process (Mikuni *et al.* 1998). Without diminishing the possible relevance of prostaglandin-independent mechanisms, the lack of a complete inhibition of ovulation after pharmacological of genetic disruption of COX-2 activity can be explained by the apparent role of prostaglandins, preventing follicle rupture at the basolateral follicle sides. Under this hypothesis, COX-2 inhibition allows rupture of the follicle at any site of the follicle wall, including the apex. However, NSAIDs are pleiotropic drugs displaying many COX-independent effects (Tegeteder *et al.* 2001). The possible effects of the activation of COX-independent factors, either alone or in combination with COX-2 inhibition, on the ovulatory process are largely unexplored and whether COX-2-independent actions of NSAIDs

account for part of the anti-ovulatory effects of these drugs is yet to be determined.

It has been previously reported that gonadotrophin-primed immature rats (GPIR) show ovulatory defects identical to that found in adult INDO-treated ones, when ovulation was induced before 25 days of age (Gaytán *et al.* 2004), suggesting that the mechanisms responsible for follicle rupture at the apex are not fully established in very young rats. In this study, ovulation was induced at 29 days of age, to avoid age-dependent ovulatory defects. In good agreement, vehicle-treated rats did not show ovulatory alterations.

A previous study (Tsafiriri *et al.* 1989) has reported the intraovarian release of eggs into the follicular thecal compartment after intrabursal administration of antibodies to tissue-type plasminogen activator (tPA) and α 2-anti-plasmin (α 2AP). It is not clear, whether the inhibition of plasmin activity shares a common mechanism with COX-2 inhibition in blocking ovulation. In the rat, INDO doses that inhibit ovulation do not affect PA activity (Shimada *et al.* 1983, Reich *et al.* 1985a,b), although higher INDO doses in the rat (Espey *et al.* 1985) or intrafollicular INDO injection in bovine preovulatory follicles (Li *et al.* 2006) has been reported to inhibit plasmin activity. Antibodies to tPA or α 2AP were effective in suppressing ovulation only when injected at the same time of hCG administration (Tsafiriri *et al.* 1989), however, INDO blocks ovulation over a broad period of time, from 10 h before, to 13 h after hCG administration (Tanaka *et al.* 1992), suggesting that suppression of plasmin activity and COX-2 inhibition act at different levels in the ovulatory cascade.

In summary, abnormal follicle rupture (that seems to be the keystone of the antioovulatory effects of indomethacin) also occurred after selective COX-2 inhibition and was not influenced by concomitant selective inhibition of COX-1 or inhibition of the lipooxygenase pathway. NSAIDs are one of the most frequently used drugs, due to their analgesic, anti-inflammatory and anti-tumoural properties. Selective COX-2 inhibitors have been recently generated in order to avoid the undesirable side effects mediated by COX-1 inhibition. The data from this study indicate that, in addition to the well-known anti-ovulatory effects, the induction of abnormal follicle rupture is also induced by treatment with selective COX-2 inhibitors.

Acknowledgements

The authors are very grateful to E Tarradas and T Recio for their technical assistance. This work has been subsidized by Grant BFU 2005-01443 ER α -ER β . The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 17 April 2006

First decision 23 May 2006

Revised manuscript received 1 June 2006

Accepted 29 June 2006