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Review

Non-steroidal anti-inflammatory drugs (NSAIDs) and ovulation: lessons from morphology

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Summary. Ovulation constitutes the central event in ovarian physiology, and ovulatory disfunction is a relevant cause of female infertility. Non-steroidal anti-inflammatory drugs (NSAIDs), widely used due to their analgesic and anti-inflammatory properties, consistently inhibit ovulation in all mammalian species investigated so far, likely due to the inhibition of cyclooxygenase 2 (COX-2), the inducible isoform of COX, that is the rate-limiting enzyme in prostaglandin (PG) synthesis. COX-2 inhibition has major effects on ovulation, fertilization and implantation, and NSAID therapy is likely implicated in human infertility and could be an important, frequently overlooked, cause of ovulatory disfunction in women. Although there is compelling evidence for a role of PGs in ovulation, the molecular targets and the precise role of these compounds in the ovulatory process are not fully understood. Morphological studies from rats treated with indomethacin (INDO), a potent inhibitor of PG synthesis, provide evidence on the actions of NSAIDs in ovulation, as well as on the posible roles of PGs in the ovulatory process. Cycling rats treated with INDO during the preovulatory period show abnormal ovulation, due to disruption of the spatial targeting of follicle rupture at the apex. Noticeably, gonadotropin-primed immature rats (widely used as a model for the study of ovulation) show age-dependent ovulatory defects similar to those of cycling rats treated with INDO. These data suggest that NSAID treatment disrupts physiological mechanisms underlying spatial targeting of follicle rupture at the apex, which are not fully established in very young rats. We summarize herein the ovulatory defects after pharmacologic COX-2 inhibition, and discuss the posible mechanisms underlying the anti-ovulatory actions of NSAIDs.

Key words: Indomethacin, Ovulation, Follicle rupture, Rat

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used drugs for the treatment of inflammatory diseases, due to their effectiveness in alleviating swelling, pain of inflammation, fever and headache (Vane et al., 1998). NSAIDs inhibit the two isoforms of the prostaglandin G/H synthase or cyclooxygenase (COX), COX-1 and COX-2, which are the first rate-limiting enzymes in the byosinthesis of prostanoids from arachidonic acid (Smith et al., 1996; Vane et al., 1998). COX-1 is constitutively expressed in most cells, whereas the expression of COX-2 is regulated by hormones, growth factors, cytokines and other inflammatory mediators (Herschman, 1996; Vane et al., 1998).

It is clearly established that NSAIDs inhibit ovulation in all mammalian species investigated so far (reviewed in Brännström and Janson, 1991; Tsafriri et al., 1993; Espey and Lipner, 1994). Both the therapeutic efficacy and the anti-ovulatory properties of these drugs are attributed to their ability to suppress COX-2 activity (Cryer and Dubois, 1998; Ando et al., 1999; Reese et al., 2001; Stone et al., 2002), and hence prostaglandin (PG) synthesis. The involvement of PGs in ovulation is based on several lines of evidence. PGs are formed in preovulatory follicles in response to the preovulatory LH surge, and reach their highest concentrations around the time of ovulation (Bauminger and Lindner, 1975; Brown and Poyser, 1984; Hedin et al., 1987). Interspecies differences in the length of the ovulatory process seem to be dependent on the species-specific time course of COX-2 induction after gonadotropin treatment (Sirois and Doré, 1997). Classical NSAIDs, inhibiting both COX-1 and COX-2, as well as selective COX-2 inhibitors (Cryer and Dubois, 1998), inhibit both PG synthesis and ovulation (Brännström and Janson, 1991; Tsafriri et al., 1993; Espey and Lipner, 1994), which can be restored (at least in some experimental conditions) by exogenous PG administration (Holmes et al., 1983; Sogn et al., 1987, Gaytán et al., 2002a). Furthermore, mice lacking the genes encoding COX-2 or PGE2 receptors (subtype EP2) show defective ovulation (Lim et al.,

1997; Matsumoto et al., 2000) which is restored by treatment with exogenous PGE2 (Davis et al., 1999). However, the molecular target(s) and the precise role(s) of PGs on the ovulatory process are not fully understood. In this context, the study of the effects of NSAIDs in ovulation has a double interest. First, NSAIDs are frequently prescribed to women at child-bearing age and can lead to reversible infertility (Akil et al., 1996; Norman, 2001; Pall et al., 2001; Stone et al., 2002; Norman and Wu, 2004). The use of NSAIDs could be a possible (overlooked) cause of infertility in women (Mendonça et al., 2000) that should be considered before starting medical assisted reproduction. Second, COX-2 inhibition constitutes an excellent tool to analyse the role of PGs in ovulation, as well as to a better understanding of the ovulatory process. Knowledge of the mechanisms underlying the antiovulatory effects of NSAIDs may help the management of ovulatory disfunction.

Normal ovulatory process

Excellent reviews on the ovulatory process have been published in recent years (Brännström and Janson, 1991; Tsafriri et al., 1993; Espey and Lipner, 1994; Espey and Richards, 2002; Richards et al., 2002). Considering the plethora of factors involved in ovulation is far beyond the purposes of this review. We will consider only those factors that are (or are suspected to be) affected by NSAID treatment, emphasizing the morphological aspects of the ovulatory process. Ovulation is a complex, multi-step process that is triggered in cycling females by the mid-cycle preovulatory LH surge. The gonadotropin surge induces the coordinate expression of a series of genes whose products determine the sequential biochemical and morphological events that allow the release of mature, fertilizable, oocytes to the periovarian space. Among these genes, directly or indirectly induced by the LH surge, those encoding the progesterone receptor (PR) and COX-2 (Park and Mayo, 1991; Sirois et al., 1992; Natraj and Richards, 1993) seem to be essential for ovulation (Robker et al., 2000a,b; Richards et al., 2002). Following the LH surge, the preovulatory follicle undergoes a series of morphofunctional processes, such as resumption of the meiotic process, cumulus expansion, rupture of the follicle wall, and finally the release of the cumulus-oocyte complex (COC) to the periovarian space (Fig. 1).

Cumulus expansion (reviewed in Richards, 2005) is due to the formation of an hialuronan-rich extracellular matrix as a consequence of the induction of hialuronan synthase-2 (HAS-2), and to the binding of several proteins, such as the proteoglican versican, the serum derived inter-alpha trypsin inhibitor (IαI), and the secreted protein tumor necrosis factor-activated gene-6 (TSG-6; Carrette et al., 2001). Expression of TSG-6 is dependent on the expression of COX-2 in cumulus cells (Joyce et al., 2001) at the time of ovulation. Accordingly, TSG-6 mRNA is reduced in COX-2 and EP2 null mice, which also show defective cumulus expansion and ovulation (Ochsner et al., 2003). This, together with additional data from mice lacking other HA-binding proteins (Richards, 2005) suggest that adequate cumulus expansion is neccessary to allow the release of the COC through the rupture site at the ovarian surface.

As the COC is encased in the follicle, which in turn is located inside the ovary, the tissues separating the COC from the periovarian space (that is, the granulosa layer, the follicular basement membrane, the theca layers, the ovarian tunica albuginea, and the ovarian surface epithelium, including its basement membrane; Fig. 2) have to be degraded to allow COC release. In this sense, ovulation is a unique process in which healthy ovarian tissue has to be degraded, and could be considered as a pathophysiological process, similar to an inflammatory reaction (Espey, 1980). The extensive process of tissue remodeling involved in ovulation requires proteolytic degradation of the extracellular matrix at the follicle apex, and a series of proteolytic enzymes are activated at the time of ovulation. Several proteolytic systems such as plasminogen activator/plasmin (PA/plasmin; Tsafriri and Reich, 1991; Ny et al., 2002), matrix metalloproteinases (MMPs; Curry and Osteen, 2001; Curry et al., 2001; Goldman and Shalev, 2004) and PR-dependent proteases such as ADAMTS-1 and cathepsin L (Robker et al., 2001a,b) are activated following the LH surge, and have been proposed to be involved in ovulation. This proteolytic activity should be tightly regulated in order to allow the tissue breakdown needed for COC release, while preventing proteolytic damage to the ovarian tissues. Accordingly, several proteolytic inhibitors such as plasminogen activator inhibitors (PAIs; Ny et al., 2002) and tissue inhibitors of metalloproteinases (TIMPs; Curry et al., 2001; Ny et al., 2002) are concomitantly expressed during the ovulatory process (Goldman and Shalev, 2004). In accordance with a central role for proteolytic enzymes in ovulation, synthetic collagenase inhibitors inhibit ovulation in the perfused rat ovary (Butler et al. 1991). However, whether particular proteolytic enzymes play essential or accessory roles in degrading extracellular matrix at the apex is not known, in spite of the abundant literature data.

Effects of NSAIDs on ovulation

It has been repeteadly reported that treatment with either dual NSAIDs (inhibiting both COX-1 and COX-2) or with the more recently developed selective COX-2 inhibitors, consistently inhibits ovulation (Brännström and Janson, 1991; Tsafriri et al., 1993; Espey and Lipner, 1994; Zaragnolo et al., 1996). This inhibitory action has been reported in different mammalian species such as the mouse (Downs and Longo, 1982, 1983), rat (Parr 1974; Osman and Dullart, 1976), rabbit (Espey et al., 1981, 1986; Schmidt et al., 1986), gilt (Hall et al., 1989), cow (De Silva and Reeves, 1985), ewe (Murdoch and

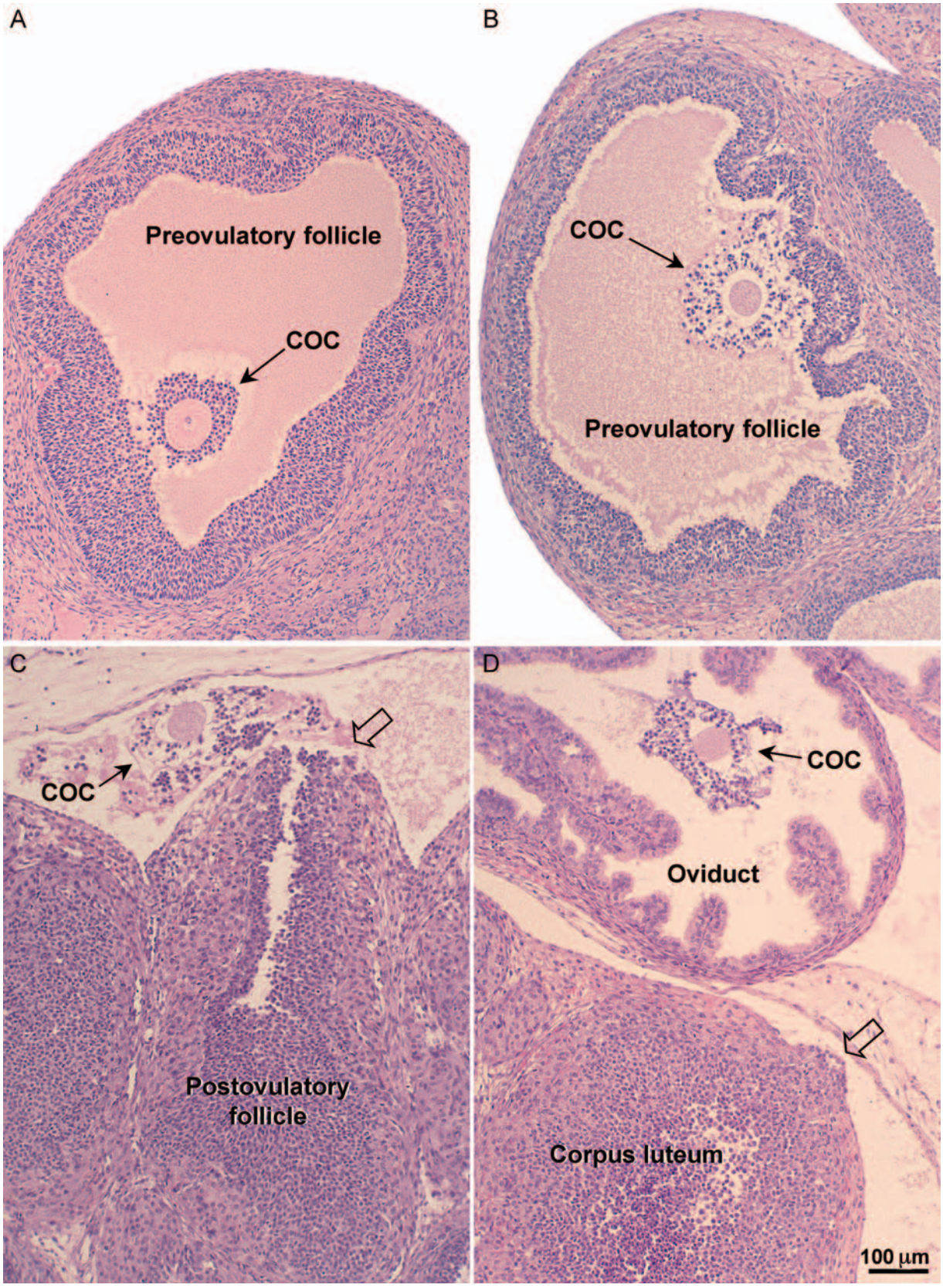

Fig. 1. Normal ovulatory process in the rat during the transition from proestrus to estrus. Preovulatory follicles show compact cumulus oocyte complex (COC) at 1200 h in proestrus **(A)**, and expanded COC at 2100 h in proestrus **(B)**, after the preovulatory LH surge. On early estrus (0300 h), rupture of the follicle at the ovarian surface (arrow) and release of the COC to the periovarian space has just happened **(C)**. At 0900 h in estrus, newly formed corpora lutea still show the rupture site at the ovarian surface (arrow), whereas the COCs are located in the oviduct.

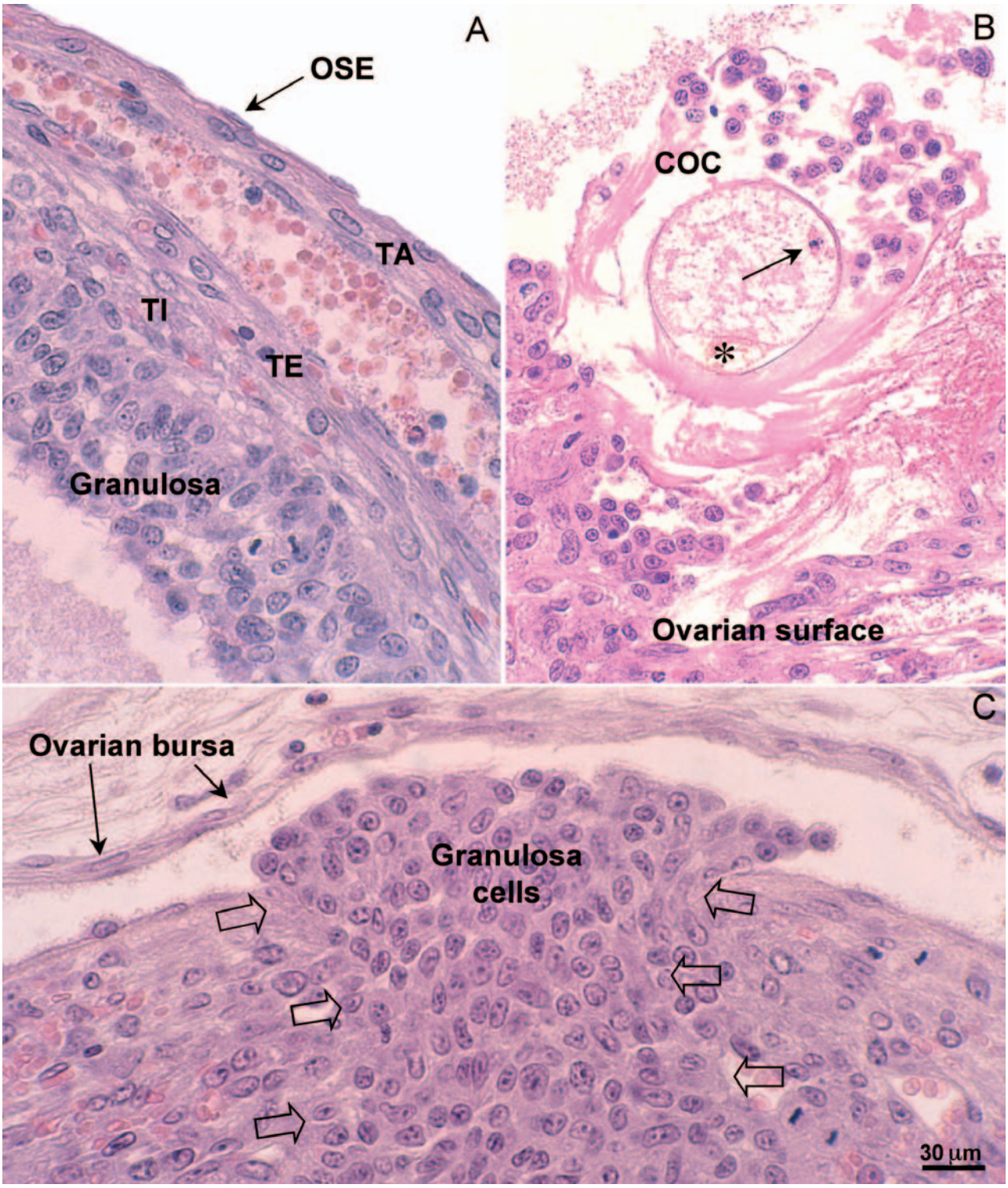

Fig. 2. Normal ovulatory process in the rat. **A.** Tissue components separating the COC from the periovarian space: the granulosa, the theca interna (TI) and theca externa (TE) layers, the tunica albuginea (TA) and the ovarian surface epithelium (OSE). **B.** Detail of the COC leaving the ovary, showing the oocyte in the metafase II stage (arrow), and the first polar body (asterisk). **C.** Detail of the rupture site at the ovarian surface. The protruding granulosa cells at the ovarian surface and the sectioned thecal and ovarian surface tissues (arrows) can be observed.

Myers, 1983; Murdoch et al., 1986), as well as monkeys (Wallach et al., 1975a,b; Duffy and Stouffer, 2002) and humans (Norman, 2001; Pall et al., 2001). The antiovulatory action of NSAIDs has been demonstrated in *in vitro* perfused ovaries (Hamada et al., 1977; Holmes et al., 1983; Schmidt et al., 1986) and, therefore, the inhibitory effects of these drugs on ovulation seem not to be mediated by general effects at central levels or ovarian blood flow. Indomethacin (INDO), a potent inhibitor of both COX isoforms (Cryer and Dubois, 1998), has been one of the most widely used NSAIDs to look for the effects of PG synthesis inhibition on ovulation (reviewed in Espey and Lipner, 1994). In most studies, ovulation was assessed by counting oocytes located in the oviduct at adequate times after the endogenous LH surge or exogenous hCG administration. This approach constitutes a reliable method to evaluate effective ovulation. However, when the number of oocytes in the oviduct is decreased, this method does not provide information about the existence or not of follicle rupture, and a decreased number of oocytes in the oviduct has been frequently interpreted as the consequence of a lack of follicle rupture. Moreover, most morphological studies have been performed on radomly selected ovarian sections, whereas more exhaustive histological studies on the effects of INDO on ovulation have been limited to the apex of the preovulatory follicles (Espey, 1967; Parr, 1974; Espey et al., 1981; Downs and Longo, 1983), reporting that changes that normally happen at the stigma, are blunted in INDO-treated animals. Studies in monkeys and women treated with COX inhibitors reported that delayed ovulation (Pall et al., 2001) or failure of the follicle to rupture (Killick and Elstein, 1987) were the main cause of NSAID-induced ovulatory disfunction. In these studies, ultrasound or visual inspection of the ovaries were used to detect ovulation, but the location of the oocyte was not determined. A more recent study (Duffy and Stouffer, 2002), assessing ovulation failure in INDO-treated rhesus monkeys by direct examination of ovarian sections, concluded that oocyte release, but not follicle rupture, was inhibited. However, the absence of an identifiable oocyte occurred in 50% of INDO-injected ovaries.

Based on the findings of reduced numbers of eggs in the oviduct, the presence of some COCs trapped inside the luteinizing follicle in randomly selected ovarian sections, and the lack of the usual morphological changes at the follicle apex (the presumed site of follicle rupture), it was concluded that the inhibitory effect of NSAIDs on ovulation was mainly due to the inhibition of follicle rupture (Brännström and Janson, 1991; Tsafriri et al., 1993; Espey and Lipner, 1994). In this sense, pharmacologic inhibition of PG synthesis has been considered as a possible cause of the luteinized unruptured follicle syndrome (LUF), either in women (Killick and Elstein, 1987; Stone et al., 2002) or experimental animals (Murdoch and Cavender, 1989). However, detailed morphological examination of serially-sectioned ovaries in INDO-treated rats provides a significantly different scenario (Gaytán et al., 2002a,b, 2003). An early study by Osman and Dullaart (1976), provides evidence on the existence of eggs that have been released from the ovulatory follicles to the ovarian interstitium and that were located under the tunica albuginea in INDO-treated rats. This indicated that ovulation and follicle rupture could be dissociated in these animals, and that the absence of follicle rupture cannot be inferred from the absence of oviductal oocytes. Nevertheless, the rule of never to ignore the unusual was not followed, and this relevant report has been almost completely ignored in the literature on ovulation. Detailed morphological studies in INDO-treated rats have been recently published (Gaytán et al., 2002a,b, 2003), demonstrating that the inhibitory action of INDO was not due to the inhibition of follicle rupture but rather to the induction of abnormal (spatially untargeted) follicle rupture. Overall, in INDO-treated rats about 35% of COCs remained trapped inside the luteinizing follicle, and about 35% were released to the ovarian interstitium through ruptures at the basolateral follicle sides (Fig. 3). Even many of the follicles in which the COC was trapped, also show rupture sites at the apex and/or basolateral follicle sides (Fig. 3B). These data indicate that an altered spatial targeting of follicle rupture is one (if not the only) mechanism underlying the anti-ovulatory action of INDO in the rat. It is worthwhile noticing that follicle rupture at the apex was not inhibited in INDO-treated rats, and about 30% of COCs were released to the periovarian space and indeed effectively ovulated. Apparently, in the presence of INDO, follicle rupture occurs at random, at any site of the follicle wall. In fact, some follicles showed several rupture sites (Gaytán et al., 2002a,b). This could explain the presence of some ovulated oocytes even with the higher possible INDO doses (Espey and Lipner, 1994), as well as in COX-2 (Russell and Richards, 1997) or EP2 (Ochsner et al., 2003) null mice.

Interestingly, INDO-treated rats also show a series of ovarian alterations, due to the release of the COC, granulosa cells, and follicular fluid to the ovarian interstitium. Follicular fluid and granulosa cells show a surprising invasive capacity. Degradation of the ovarian stroma (Fig. 3C), invasion of the blood and lymphatic vessels leading to the formation of emboli containing gel-like follicular fluid, granulosa cells and even the COC were observed (Figs. 3B, C, 4). Large emboli were frequently observed at the ovarian hilus and in the ovarian vein leaving the ovary (Fig. 5), potentially spreading through the general circulation.

Ovulatory defects in gonadotropin-primed immature rats

Gonadotropin-primed immature rats (GPIR) constitute a widely used model for the study of ovulation and, indeed, a significant part of the literature data on ovulation is derived from studies in GPIR (Mori et al.,

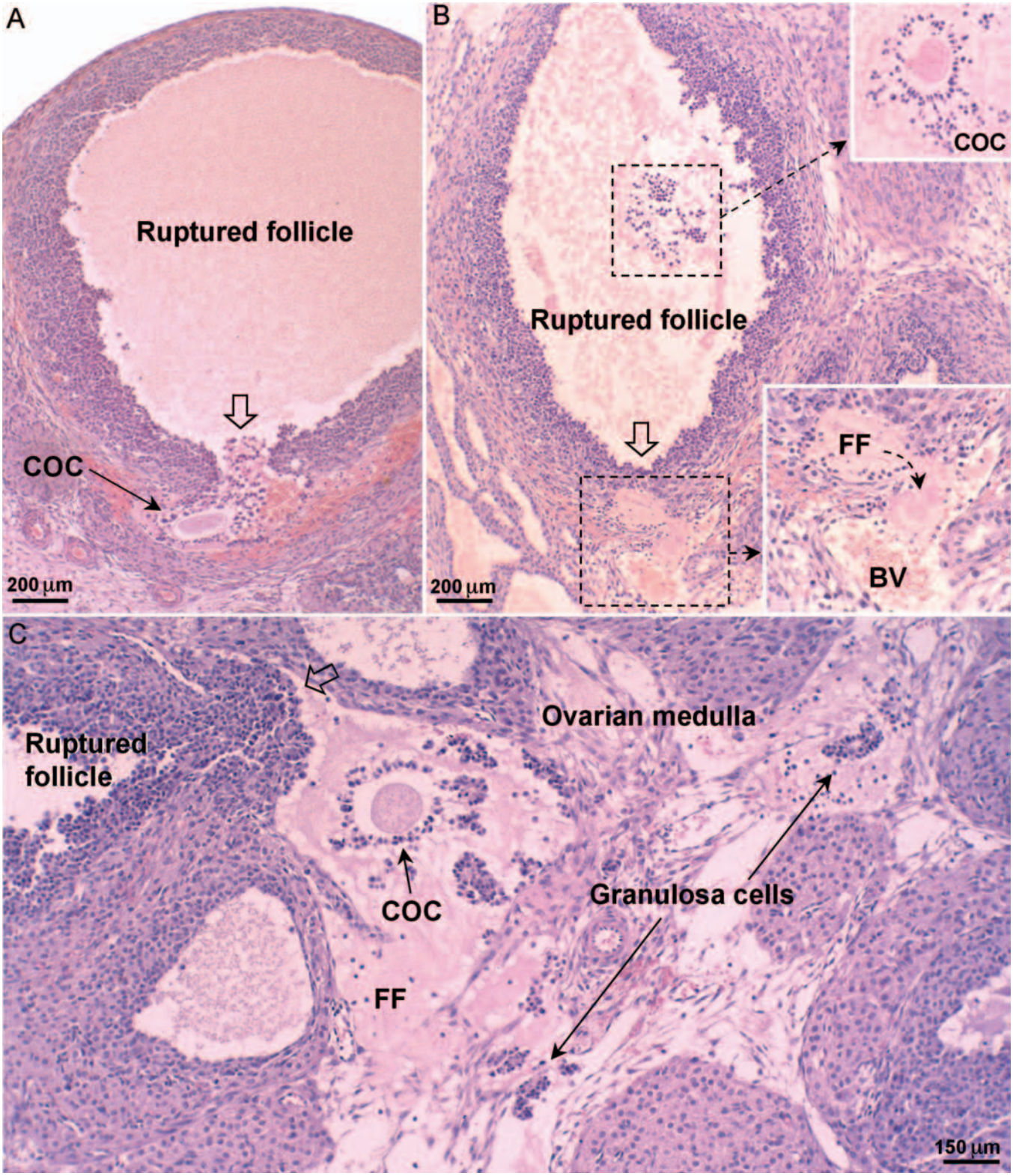

Fig. 3. Abnormal follicle rupture in INDO-treated rats. **A.** A follicle showing rupture (arrow) at the basal side and release of the COC to the ovarian interstitium. **B.** A follicle showing trapped COC (the oocyte was in an adjacent section; upper inset), and rupture (arrow) at the basolateral side with release (lower inset) of follicular fluid (FF) that is invading a blood vessel (BV). **C.** Follicle ruptured at the basal side (arrow).The COC, follicular fluid (FF), and dispersed granulosa cells can be observed in the ovarian medulla.

1977; Butler et al., 1991; Mann et al., 1993; Liu et al., 1998; Espey et al., 2000; Curry et al., 2001; Simpson et al., 2001). In this model, immature rats (from 21 to 28 days of age) are treated with a single dose of pregnant mare serum gonadotropin (PMSG) that induces the development of a large cohort of follicles that reach preovulatory size in about 48 h. Then, the administration of an ovulatory dose of human chorionic gonadotropin (hCG) induces ovulation in about 12-16 hours. This model has several advantages such as the existence of a large number (superovulation) of synchronized ovulating follicles and the absence of luteal tissue of previous cycles. However, a recent study (Gaytán et al., 2004) has reported that GPIR show age-dependent ovulatory defects identical to those found in INDO-treated rats. Immature rats primed with PMSG before 25 days of age, and therefore ovulating before 28 days of age, show disruption of the spatial targeting of follicle rupture. Similarly to what happens in INDO-treated cycling animals, follicle rupture frequently occurs at the basolateral follicle sides, and a significant proportion of COCs remain trapped inside the luteinizing follicle or are released to the ovarian interstitium (Fig. 6). As in INDO-treated animals, granulosa cells and follicular fluid released to the ovarian interstitium in GPIR are specially invasive. In addition to degradation of ovarian stroma and of blood and lymphatic vessels (Fig. 7), breakdown of the ovarian bursa (Fig. 8A,B) and invasion of the periovarian fat pad (Gaytán et al., 2004) are frequently observed. The similarity of the ovulatory defects found in INDO-treated and GPIR strongly suggests that INDO treatment disrupts a physiological mechanism controlling the spatial targeting of follicle rupture at the apex, and that this mechanism is not fully established before 28 days of age. In this context, immature animals seem not to be an adequate model for the study of the effects of COX inhibition on ovulation, for several reasons. First, the ovulation rate in 3-wk-old COX-2 or EP2–deficient mice is not as severely affected as in adult animals (Matsumoto et al., 2000), suggesting that the PG dependence of the ovulatory process is not fully established in immature animals. Second, because immature rats show multiple ovulatory defects before 28 days of age (Gaytán et al., 2004), similar to those found after COX inhibition in adult animals.

Possible mechanisms of action of NSAIDs on ovulation: open questions

In spite of the abundant literature data on the inhibitory effects of INDO or other NSAIDs on ovulation, the molecular targets of these drugs and the mechanisms underlying ovulation inhibition remain unclear. In recent studies (reviewed in Espey and

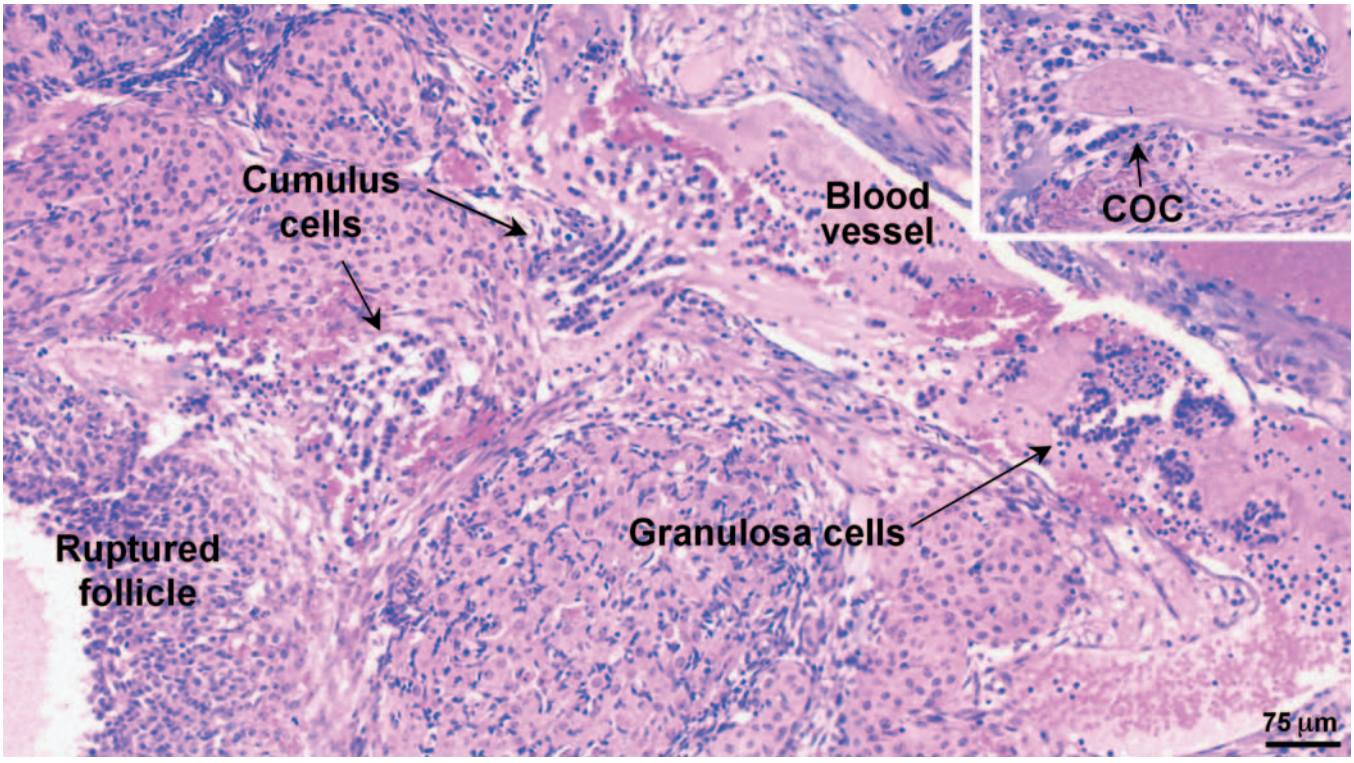

Fig. 4. Abnormaly ruptured follicle from an INDO-treated rat. Invasion of a blood vessel by follicular fluid and granulosa cells. The COC (inset) was in an adjacent section.

Richards, 2002), none of a large array of genes up-regulated by the LH surge was altered by INDO treatment. This also implies imprecise knowledge of the role of prostaglandins in ovulation. Detailed morphological studies of INDO-treated rats, as well as in GPIR, raise several questions on the mechanisms of NSAID-mediated ovulatory inhibition, and provide some clues on the specific effects of these drugs on the ovulatory process, as well as on the mechanisms of ovulation.

What are the effects of NSAID treatment on the proteolytic activity responsible for tissue breakdown during ovulation?

Although biochemical studies analysing proteolytic activity in INDO-treated rats have provided contradictory results (Reich et al., 1985, 1991; Curry et al., 1986; Murdoch and McCormick, 1991; Tanaka et al., 1992), and clear-cut evidence of decreased proteolytic activity after NSAID treatment is lacking, the current opinion is that INDO treatment inhibits the proteolytic activity needed for ovulation (reviewed in Brännström and Janson, 1991; Tsafriri et al., 1993; Espey and Lipner, 1994). The expected decrease in the proteolytic activity is based, at least in part, on the assumption that the inhibition of follicle rupture is the main ovulatory defect in INDO-treated animals. However, morphological data from INDO-treated rats showing abnormal follicle rupture, degradation of the ovarian stroma, and invasion of blood and lymphatic vessels, provide indirect, but unequivocal, evidence on the existence of effective

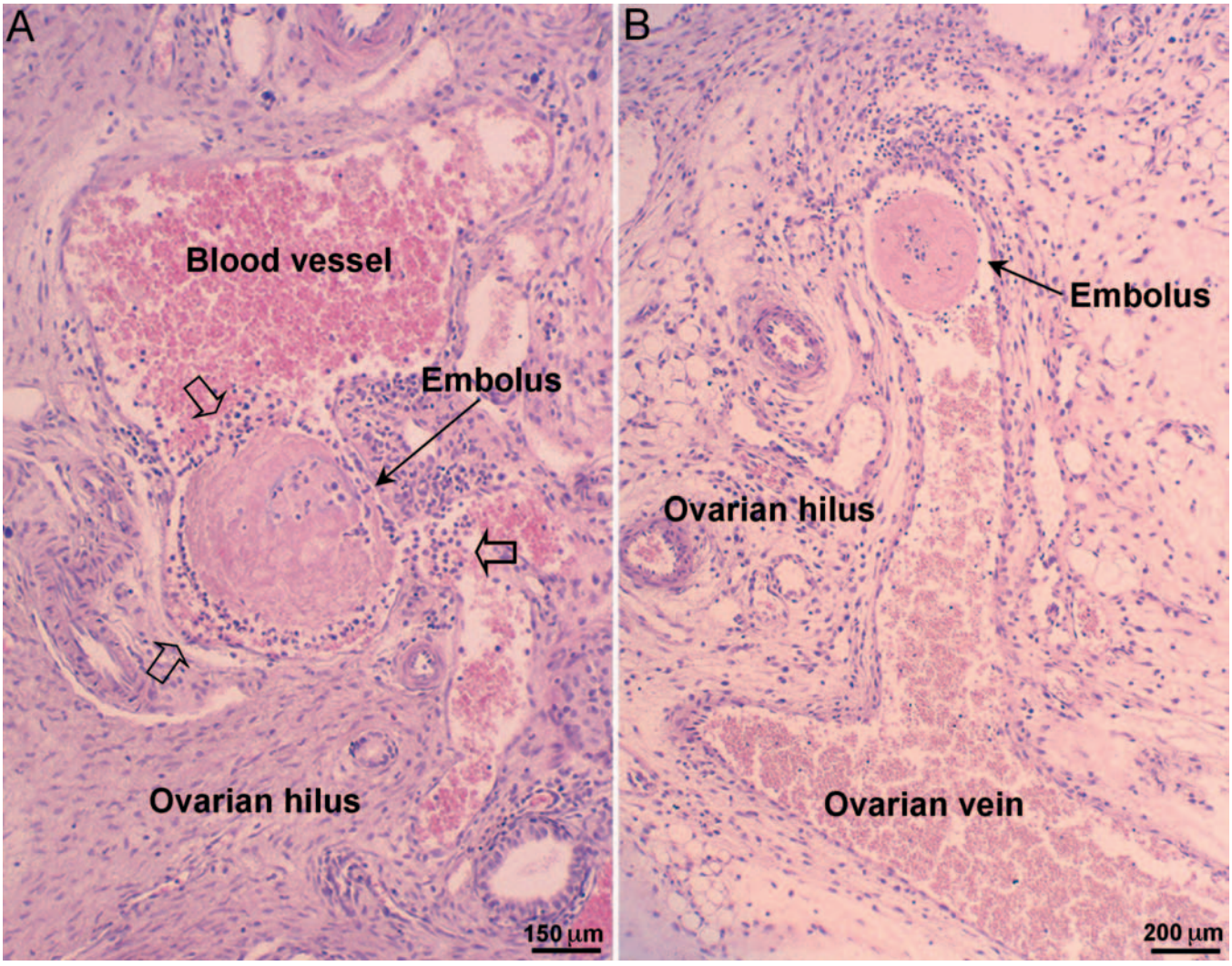

Fig. 5. INDO-treated rats. Emboli of follicular fluid and granulosa cells in ovarian blood vessels, at the ovarian medulla **(A)**, and at the ovarian vein **(B)** leaving the ovary. In **A**, abundant leukocytes (arrows) are surrounding the embolus.

proteolytic activity in INDO-treated rats. Proteolytic breakdown of basement membranes and intercellular matrix is needed for the release of granulosa cells and COC to the dense perifollicular stroma and for the invasion of blood and lymphatic vessels. Therefore, the relationship between PG synthesis inhibition and actual proteolytic activity during ovulation is unclear. Rats treated with both RU486 (a progesterone receptor antagonist) and INDO provide valuable information. The transient expression of PR in the granulosa cells of preovulatory follicles is needed for follicle rupture, although the precise role of PR activation is not fully understood. Rats treated with progesterone receptor antagonists (Van der Schoot et al., 1987; Sánchez-Criado et al., 1990), progesterone antiserum (Mori et al., 1977) or progesterone synthesis inhibitors (Snyder et al., 1984; Hibbert et al., 1996), as well as PR knockout mice (Lydon et al., 1996), showed unruptured luteinized follicles containing the oocyte, suggesting that PR activation mediates follicle rupture. Notably, the expression of some proteases such as ADAMTS-1 and cathepsin L has been found to be dependent on PR activation in granulosa cells (Robker at al., 2000a,b). Interestingly, the administration of INDO to RU486-treated rats induces follicle rupture in about 25% of the follicles, indicating that INDO treatment does not inhibit, but rather facilitates, follicle rupture even in rats lacking progesterone actions (Gaytán et al., 2003). This suggests that progesterone and PGs play opposite, complementary, roles in ovulation. Overall, INDO treatment seems to disregulate proteolytic activity, probably by inhibiting controlling mechanisms, leading to abnormal follicle rupture. This contention could also explain the apparently paradoxical inhibition of ovulation reported after treatment with PGE2, either in vivo (Espey et al., 1992) or in vitro (Schmidt et al.,

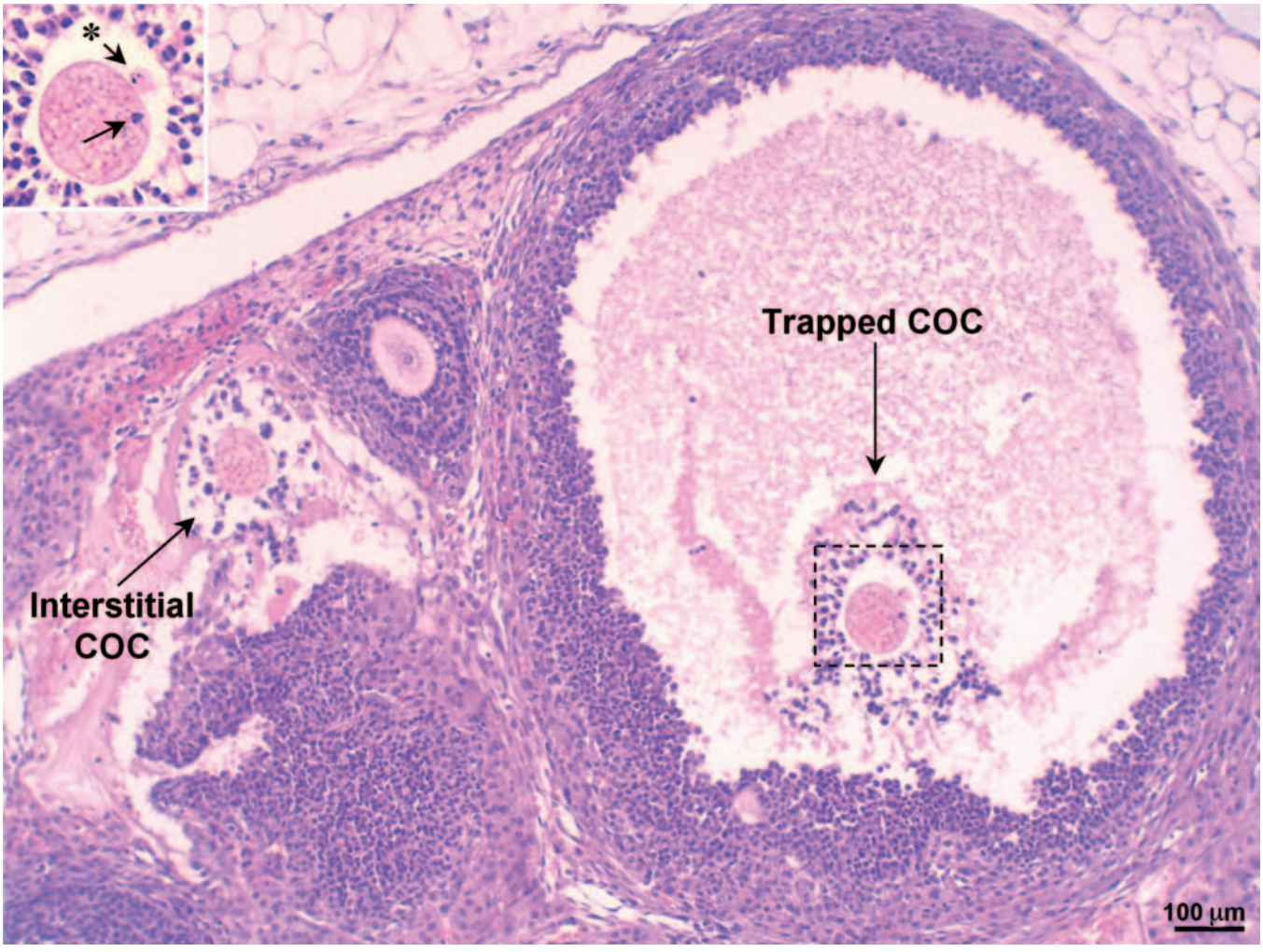

Fig. 6. Ovulatory defects in gonadotropin-primed immature rats. A COC trapped inside the follicle , with the oocyte (inset) in the metaphase II stage and formation of the first polar body (asterisk), and a COC released to the ovarian interstitium can be observed.

1986).

Another aspect that requires further consideration is what is the source of the proteolytic enzymes responsible for the rupture of the follicle wall. Most cell types are likely able to release proteolytic enzymes under adequate stimulation, and studies on mRNA expression have reported that most follicular tissue compartments, as well as the ovarian stroma express different proteolytic enzymes as well as their specific inhibitors (Bagavandoss, 1998; Chun et al., 1992; Curry and Osteen, 2001; Curry et al., 2001). The impressive invasive capacity of granulosa cells and follicular fluid, after rupture of the theca layers at the basolateral follicle sides and release to the ovarian interstitium in INDO-treated rats (Gaytán et al., 2002a, b, 2003), indicates that granulosa cells/follicular fluid have the capacity to degrade all extracellular matrix components. Furthermore, the general appearance of the rupture site (either at the apex or at the basolateral sides) showing clear cut edges of the theca layers and ovarian surface epithelium (see Fig. 2C), suggests that disruption of the theca layers and surface ovarian tissues proceeds outwards. Altogether, these data strongly suggest that granulosa cells are the main source of proteolytic enzymes responsible for the rupture of the follicle wall, and perifollicular tissues at the apex, whereas theca cells seem to be important in controlling proteolytic activity, preventing abnormal follicle rupture and proteolytic damage to the ovary.

Does defective cumulus expansion explain the effects of COX-2 inhibitors on ovulation?

Cumulus expansion seems to be critical for ovulation (reviewed in Richards et al., 2002; Richards, 2005). The formation of an expanded extracelular cumulus matrix is needed to allow detachment of the COC from mural granulosa cells, for the release of the COC through the ovulatory pore at the ovarian surface, for the transport of COCs through the oviduct, and, probably, for the protection of the oocyte from proteolytic degradation. The expression of the gene encoding one of the HA-binding proteins, the tumor necrosis factor-activated gene-6 (TSG-6) protein, is dependent on COX-2 expression in cumulus cells (Joyce et al., 2001). In this sense, defective cumulus expansion has been considered as a possible cause of ovulatory disfunction in INDO-treated or COX-2 knockout

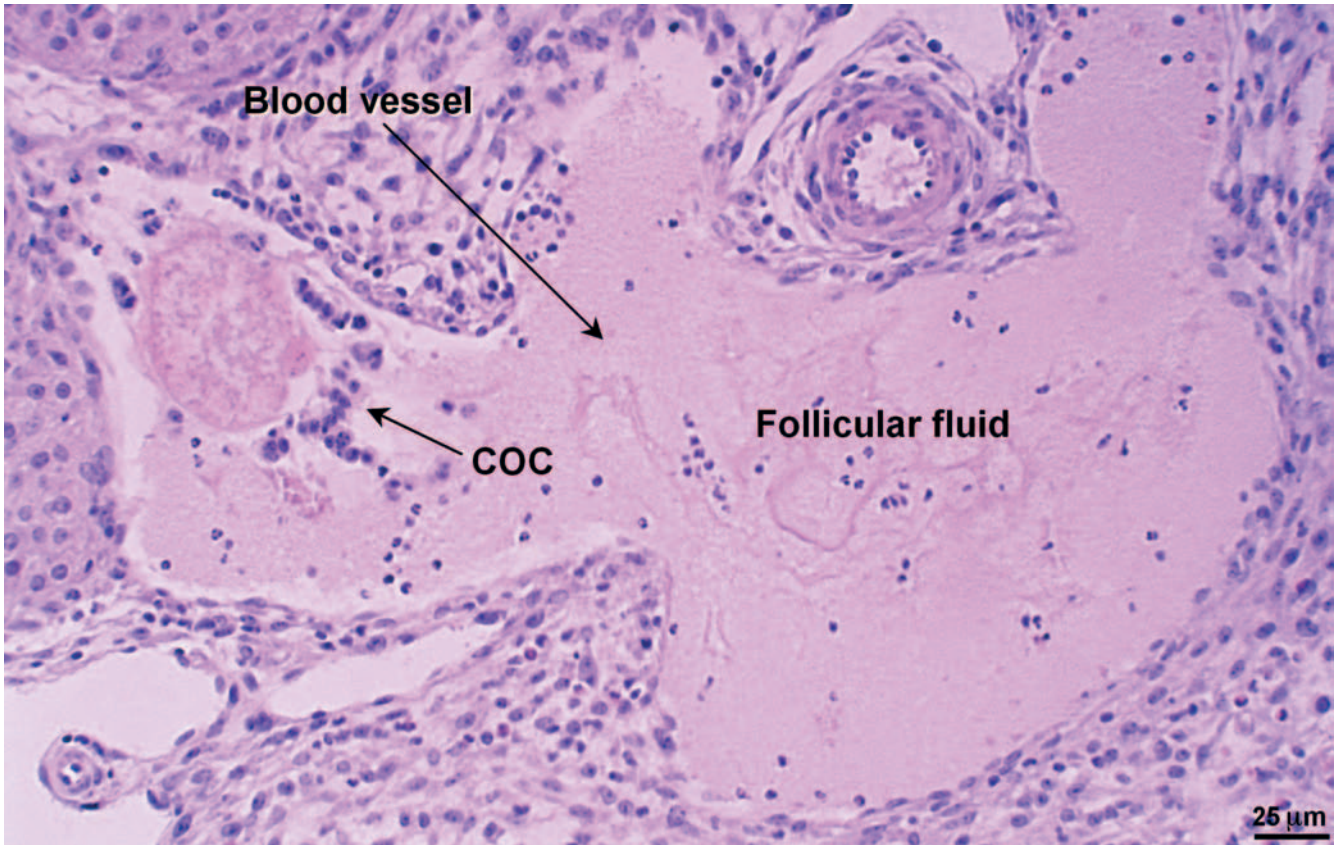

Fig. 7. Ovulatory defects in gonadotropin-primed immature rats. Embolus containing follicular fluid and the COC in a blood vessel at the ovarian medulla.

animals (Duffy and Stouffer, 2002), due to inhibition of TSG-6 expression. However, in mice lacking the prostaglandin E receptor subtype EP2 (Hizaki et al., 1999) cumulus expansion proceeds normally in preovulatory follicles, but becomes abortive in ovulated COCs, suggesting that the role of PGs in cumulus expansion is more relevant in postovulatory stages. In addition, INDO fails to inhibit FSH-induced cumulus expansion (Epigg, 1981), and the importance of defective cumulus expansion in the antiovulatory effects of INDO is not fully established. Although cumulus expansion and detachment of the COC from the mural granulosa cells are morphologically equivalent in control and INDO-treated rats (Gaytán et al., 2002a, b, 2003), the existence of morphologically undetectable functional defects in INDO-treated rats, which could contribute to the trapping of some COCs within the luteinizing follicles, cannot be discarded. However, defective cumulus expansion can hardly explain either the rupture of the follicles at the basolateral sides or the release of the COC to the ovarian interstitium and, in addition, still uncharacterized indomethacin-disrupted mechanisms are needed to explain the main ovulatory alterations found in NSAID-treated rats.

What mechanisms underlying spatial targeting of follicle rupture at the apex are disrupted by NSAID treatment?

Spatially targeted follicle rupture at the apex is necessary for ovulation to be effective. However, the mechanisms underlying the spatial targeting of follicle rupture are unknown. Studies analysing mRNA expression of several LH-induced genes (Espey and Richards, 2002) have reported that biochemical events of ovulation are not limited to the apex and that an apparent polarization (apical vs basolateral) of the preovulatory follicles is absent. Disruption of the follicular basement membrane throughout the follicle wall at ovulation is needed to allow capillary growth into the luteinizing granulosa cell layer, whereas rupture of the theca layers is limited to the apical zone. Previous hypotheses on the spatial targeting of follicle rupture have been based on the anatomical location of the follicles, protruding at the ovarian surface, as an important factor in the spatial targeting of follicle rupture. Accordingly, it was postulated that follicle rupture occurs at the apex because this is the thinnest portion of the follicle wall, whereas the basolateral sides are surrounded by dense stromal tissues that prevent the follicle from ballooning in these zones (Espey, 1967). Physical models of the mechanics of ovulation have also been based on this contention (Robbard, 1968). Nevertheless, the anatomical location of the preovulatory follicle is not modified by INDO treatment shortly before ovulation, and functional mechanisms are necessary to explain the location of follicle rupture at the follicle side facing the ovarian surface. As follicle rupture involves proteolytic degradation of the follicle wall, spatial targeting of the follicle rupture are presumably due to spatially targeted proteolytic activity. Tissue components that are present

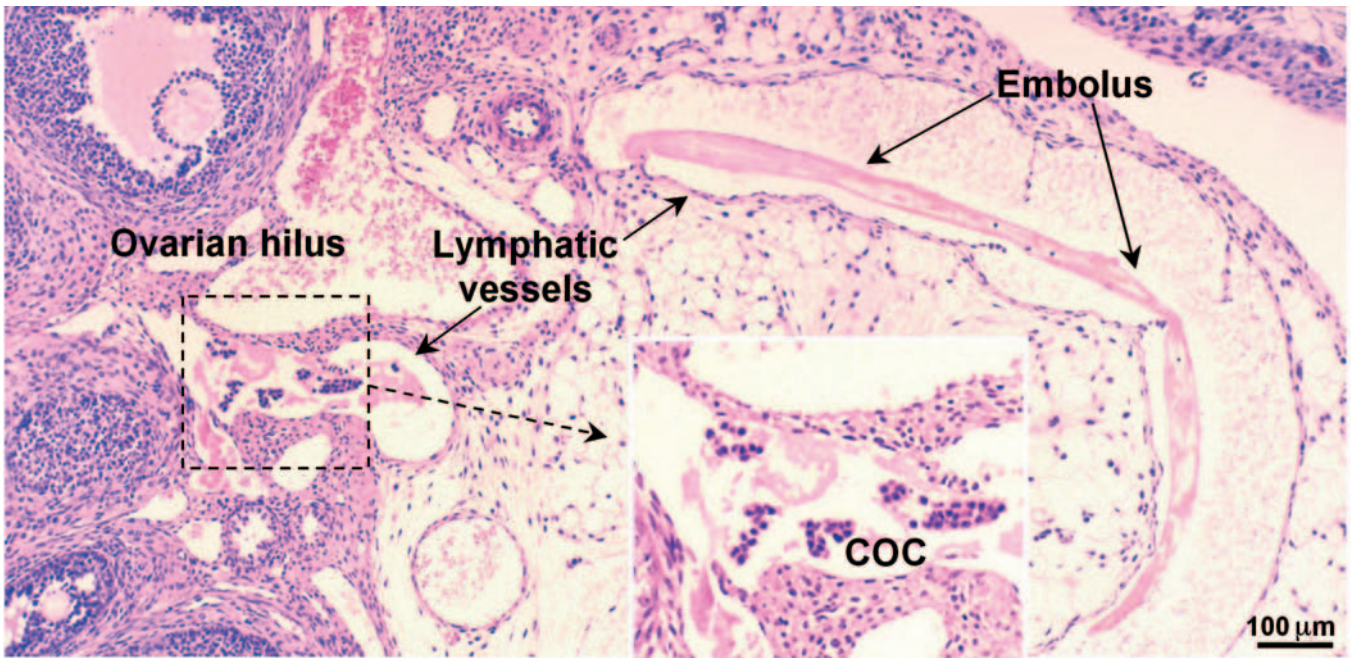

Fig. 8. Ovulatory defects in gonadotropin-primed immature rats. The COC (at higher magnification in the inset), and an embolus of follicular fluid, can be observed in the lymphatic vessels at the ovarian hilus.

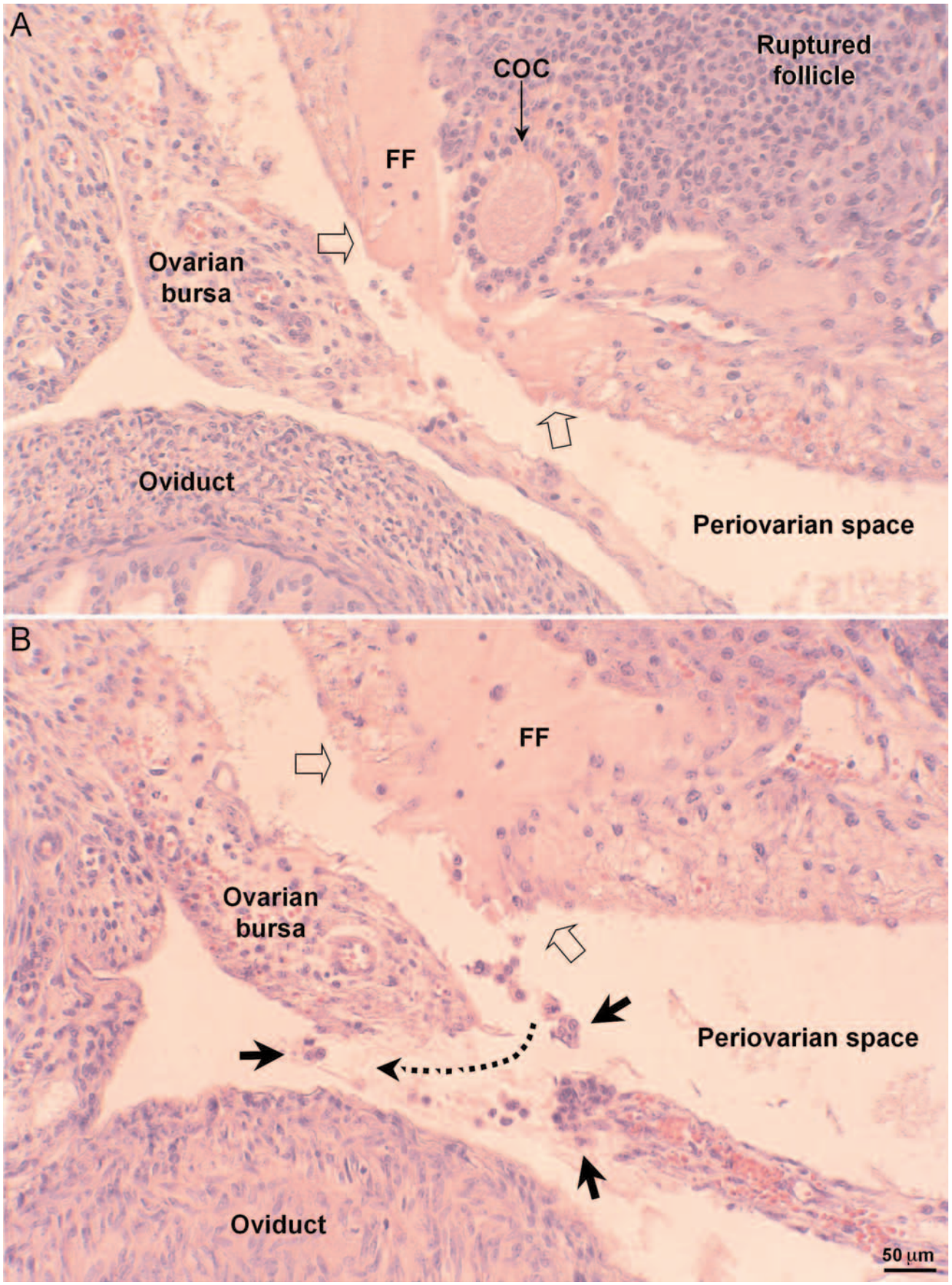

Fig. 9. Ovulatory defects in gonadotropin-primed immature rats. Breakdown of the ovarian bursa (dotted arrow in **B**) by follicular fluid (FF) and granulosa cells (arrows). The rupture site at the ovarian surface is indicated (empty arrows) The COC is trapped at the ovarian surface.

exclusively at the apical zone, that is the OSE and the tunica albuginea (TA), are obvious candidates to participate in the process of stigma formation and follicle rupture. Although early studies in the rabbit suggested that the OSE plays an active role in follicle rupture (Bjersing and Cajander, 1975), this was discounted thereafter (Rawson and Espey, 1977), as some follicles still ruptured after OSE scrapping, and the possible role of the fibroblasts of the tunica albuginea was stressed (Espey and Lipner, 1994). However, more recent studies have reported the release of proteolytic factors by OSE cells at the time of ovulation (Murdoch and McDonnel, 2002). Data from INDO treated or GPIR clearly indicate that neither the OSE nor the fibroblasts of the tunica albuginea are needed for follicle rupture, as it can occur at any site of the follicle wall (Gaytán et al., 2002a,b, 2003, 2004), irrespective of the presence or not of these tissue components. This is also supported by the observation that isolated follicles (lacking perifollicular tissues) are able to undergo rupture under adequate stimulation (Rose et al., 1999). Nevertheless, these data do not discard the possible relevance of apical tissues in the normal ovulatory process, which could contribute to the spatial targeting of follicle rupture at the apex. Based on data from INDO-treated rats we proposed a working hypothesis on the mechanism of spatial targeting of follicle rupture. The preovulatory LH surge triggers the expression of a cascade of genes in a precise temporal and spatial pattern, which leads to an acute inflammatory-like reaction resulting in oocyte release. This involves up-regulation and/or posttranslational activation of several proteolytic systems (i.e., PA/plasmin and MMPs), as well as the expression of PR-dependent proteases (i.e., ADAMTS-1). Concomitant upregulation of proteolytic inhibitors such as PAIs, TIMPs, as well as putative, INDO-sensitive, factors would maintain proteolytic homeostasis just to allow disruption of the basement membrane but preventing breakdown of the theca layers throughout the follicle wall. At the apical zone, factors derived from the OSE and/or the TA (either stimulating proteolytic activity or inhibiting proteolytic inhibitors) may cause a local imbalance of proteolytic homeostasis favouring breakdown of the theca layers and apical extrafollicular tissues. In this model, INDO treatment disrupts some still unknown proteolytic inhibitors leading to an imbalance of proteolytic homeostasis throughout the follicle wall. In these circunstances, the apex does not constitute a priviledged site for follicle rupture and disruption of the theca layers can occur at any site of the follicle wall.

Are some of the effects of NSAIDs on ovulation mediated by COX-2- independent mechanisms?

The inhibitory effects of INDO and other dual NSAIDs on ovulation have been attributed to COX-2 inhibition, a contention that is supported by the equivalent inhibitory effects of selective COX-2 inhibitors (Mikuni et al., 1998; Pall et al., 2001). However, the possible role of the concomitant COX-1 inhibition in the multiple ovulatory alterations in INDO-treated rats requires further consideration. Recent studies (Gilroy and Colville-Nash, 2000) have pointed out that COX-1 derived prostanoids also play relevant roles in inflammation. Comparison of the ovulatory defects in INDO-treated, COX-2 knockout and COX-1/COX-2 double-knockout mice, as well as selective COX-2 inhibitors, would be of interest. However, detailed morphological studies on the ovary of COX-2 deficient mice have not been published, and double-knockout mice do not survive up to reproductive age (Reese et al., 2000). Otherwise, although the existence of a still not well defined role for PGs in ovulation is clearly established, the importance of these compounds in ovulation is not free of controversy. Some studies (Espey et al., 1986; Espey and Lipner, 1994) have found a poor corrrelation between ovarian prostaglandin levels and ovulation rate in INDO-treated rabbits, and that the doses neccessary to inhibit ovulation are considerably higher than those needed to inhibit prostaglandin synthesis. It is therefore unclear whether all the reported effects of NSAIDs on ovulation are mediated by COX inhibition. NSAIDs are pleiotropic drugs displaying many COX-independent effects (Tegeder et al., 2001), which could contribute to some of the ovulatory alterations in INDO-treated rats. For instance, INDO activates the peroxisome proliferator activated receptor gamma (PPARγ; Tegeder et al., 2001), which is expressed in preovulatory follicles, is down-regulated by hCG (Komar et al., 2001), and could act as an inflammatory mediator. In addition, NSAIDs induce the expression of several genes such as the NSAID-activated gene-1 (NAG-1; Baek et al., 2002), a member of the transforming growth factor-ßsuperfamily, the transcription factor NFκB (Tegeder et al., 2001), and the nerve growth factor-inducible B (NGF-IB; Kang et al., 2000), a member of the steroid-thyroid hormone receptor family. The possible effects of the activation of these factors, either alone or in combination with prostaglandin synthesis inhibition, on the ovulatory process are largely unexplored, and whether COX-independent actions of NSAIDs account for part of the antiovulatory effects of these drugs is yet to be determined. Additional studies comparing the effects of different NSAIDs, displaying differences in their mechanisms of action, as well as the specific ovulatory defects in COX-2 knockout mice may help to address this issue.

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