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Further Evidence that Prostaglandin F2-alpha is the Obligatory Eicosanoid in Porcine Ovulation

by

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Prostaglandin F2-alpha in Porcine Ovulation

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Abstract

Further Evidence that Prostaglandin F2-alpha is the Obligatory Eicosanoid in Porcine Ovulation

Metabolites of arachidonic acid, known as eicosanoids, result from two enzymatic pathways: cyclooxygenase (COX) and lipoxygenase (LOX). Indomethacin (INDO), at a dose level of approximately 10 mg/kg, has been commonly used in studies on the role of prostaglandins (PG) in the porcine ovulatory process since it was believed to be specific in its inhibition of the COX pathway. Other evidence suggests that INDO also has inhibitory effects on the formation of LOX metabolites including 12- and 15-hydroxyeicosatetraenoic acid (HETE). There is also evidence that LOX metabolites may have a role in the ovulatory process of the rat and the pig. In the previous studies on PGs in porcine ovulation, the effect of INDO on LOX metabolites was not examined. It has been shown that progesterone has a role in the formation of eicosanoids in the ovary in the rat and the ewe. The results of this study indicate that an intramuscular injection of INDO, at dose levels as high as 5 mg/kg, reduces ovulation rat and preovulatory follicular fluid concentrations of PGF2α without affecting concentrations of 12- and 15-HETE in the gonadotropin-primed pig. Indomethacin, at dose levels as low as 1 mg/kg, reduced ovulation rate and intrafollicular PGF2α concentrations but 0.1 mg/kg had no effect on ovulation rate or PGF2α. In cultured granulosa and theca interna cells, 10 ng or greater of INDO per $3X10^5$ cells reduces PGF2α accumulation in the media. Accumulation of 12- and 15-HETE in the media was reduced by 100 ng or greater of INDO per $3X10^5$ cells. To examine the role of progesterone in the formation of eicosanoids, danazol, an inhibitor of steroidogenesis, was added to cultures of porcine follicular cells. This treatment had no effect on progesterone production by either cell type and, similarly, had no effect on eicosanoid production. In summary, PGF2α is required for ovulation in the pig since inhibition of its synthesis completely blocks ovulation. Similar dose levels of INDO fail to alter LOX metabolites 12- and 15-HETE *in vivo*; *in vitro* data indicate that INDO does have an inhibitory effect on the production of 12- and 15-HETE but at dose levels greater than those required to reduce PGF2α production.

(Supported by NSERC of Canada)

Résumé

Évidence Supplémentaire que la Prostaglandine F2-alpha est l'Eicosanoide Nécessaire pour l'Ovulation chez la Truie

Les eicosanoides, produits du métabolisme de l'acide arachidonique, dérivent de deux voies enzymatiques: la cyclooxygénase (COX) et lipoxygénase (LOX). Comme l'indométhacine (INDO) est reconnue comme inhibiteur specifique de la COX, elle a fréquemment été ultilisée à des doses d'environ 10 mg/kg lors des études sur le rôle des prostaglandines (PG) pendant l'ovulation chez la truie. D'autres évidences suggèrent aussi que l'INDO inhibe la production des métabolites de la voie LOX incluants les acides 12- et 15-hydroxyeicosatetraenoique (HETE). Il apparait évident que les produits de la voie LOX sont impliqués dans le processus ovulatoire chez la rate et chez la truie. Lors des expériences précédentes sur le rôle des PGs pendant l'ovulation chez la truie, l'effet de l'INDO sur les produits de la voie LOX n'a pas été étudié. La progestérone a un rôle lors de la formation des eicosanoides dans l'ovaire de la rate et de la brebis. Chez la truie, les résultats de cette étude indiquent qu'une injection intramusculaire de l'INDO, jusqu'à une dose de 5 mg/kg, ont réduit les concentrations de PGF2α dans le liquide folliculaire ainsi que le taux d'ovulation, sans changer les concentrations de 12- et 15-HETE. Le taux d'ovulation et la concentration de PGF2α dans le liquide folliculaire ont été réduits par des doses aussi faibles que 1 mg/kg mais 0.1 mg/kg de l'INDO n'a eu aucun effet. L'indométhacine à une concentration d'au moins 10 ng/ml ($3X10^5$ cellules) a réduit la production de PGF2α par les cellules granulosa et theca interna. L'accumulation de 12- et 15-HETE dans le milieu a été réduite par au moins 100 ng/ml de l'INDO. Pour examiner l'effect de la progestérone sur la production des eicosanoides, un inhibiteur de la stéroidogénèse, le danazol, a été ajouté dans le milieu de culture de cellules folliculaires de truie. Ce traitement n'a eu aucun effet sur la production de la progestérone par ces cellules, de plus il n'a eu aucun effet sur la production des eicosanoides. En conclusion, la PGF2α est nécessaire à l'ovulation chez la truie car, si la PGF2α est inhibée, l'ovulation est ausssi complètement inhibée. L'INDO, aux doses rapportées ci-haut, n'a eu aucun effet sur la production de 12- et 15-HETE *in vivo*; les résultats de l'expérience *in vitro* indiquent que l'INDO inhibe la voie LOX mais à des doses supérieures à celles requises pour inhiber la PGF2α.

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Figures

Tables

Abbreviations

Cyclooxgenase	COX
Dulbecco's modified Eagle's medium	DMEM
Follicle stimulating hormone	FSH
Follicular fluid	FF
Granulosa cells	GC
Indomethacin	INDO
Human chorionic gonadotropin	hCG
3β-hydroxy steroid dehydrogenase	3β-HSD
Lipoxygenase	LOX
Luteinized unruptured follicle	LUF
Luteinizing hormone	LH
Nordihydroguaiaretic acid	NDGA
Pregnant mare serum gonadotropin	PMSG
Prostaglandin	PG
Theca interna cells	TIC

Introduction

Ovulation may be considered the central occurrence in the reproductive cycle such that ovulation marks both the end of follicular development and the beginning of the luteal phase. Metabolites of arachidonic acid, known as eicosanoids, have a role in the mammalian ovulatory process. Eicosanoids result from two enzymatic pathways: cyclooxygenase (COX) and lipoxygenase (LOX). The prostaglandins (PG), products of the COX pathway, especially PGE2 and PGF2α, and their role in ovulation have been studied in great detail, but other eicosanoids, especially those of the lipoxygenase pathway, have had less focus on their role as potential mediators in the ovulatory process.

In the pig, it has been demonstrated that inhibition of PG synthesis with a single intramuscular injection of indomethacin (INDO) inhibits ovulation (Ainsworth *et al.*, 1979; Downey and Ainsworth, 1980). It has also been demonstrated that indomethacin-inhibited ovulation in the pig can be partially restored by administration of PGF2α (Downey and Ainsworth, 1980). Moreover, premature ovulation in the pig can be induced with PGF2α analogues (Srikandakumar and Downey, 1989). The role of lipoxygenase products, such as the hydroxyeicosatetraenoic acids (HETE), has not been studied extensively in the pig, although it has been demonstrated that follicular fluid concentrations of 15-HETE increase prior to ovulation and that nordihydroguaiaretic acid (NDGA), an inhibitor of 12- and 5-HETE (Tanaka *et al.*, 1991), can reduce ovulation rate when injected into the ovarian stalk (Mootoo, 1995). Evidence does exist in the rat, which is indicative of a role for these LOX metabolites in ovulation (Espey *et al.*, 1991; Tanaka *et al.*, 1989, 1991).

Indomethacin was believed to be a specific inhibitor of the COX enzyme but evidence shows that it also inhibits both 12- and 15-LOX enzymes (Seigel *et al.*, 1979, 1980; Tanaka *et al.*, 1991). Studies in the past regarding the role of prostaglandins in the porcine ovulatory process have used intramuscular injections of INDO at a dose level of approximately 10 mg/kg at 24 h after hCG treatment (Ainsworth *et al.*, 1979; Downey and Ainsworth, 1980; Hansen *et al.*, 1991). The effect of INDO on follicular fluid levels of LOX metabolites in these studies was not examined. The effect of dose level of INDO in the pig has not been studied but, in the rat, dose studies demonstrated that INDO inhibits the preovulatory increase in PGs at lower doses than those required to reduce ovulation rate (Tanaka *et al.*, 1991).

An interrelationship between progesterone and the eicosanoids may exist as it does for luteolysis and parturition. Evidence for this exists in the rat (Espey *et al.*, 1991; Tanaka *et al.*, 1991) and in the ewe (Murdoch *et al.*, 1986). This relationship, to my knowledge, has not been examined in the porcine follicle.

The prepubertal gonadotropin-primed gilt provides a good model for the study of reproduction in polytocous species, such that the time of ovulation can be reasonably predicted and is similar morphologically and biochemically to the naturally cycling sow (Ainsworth *et al.*, 1982). This is advantageous in that the information obtained can be directly applied in the development of reproductive strategies in this species. A better understanding of the ovulatory process in the pig can aid us in improving the reproduction efficiency via better heat detection, increased ovulation rates and better control over the estrous cycle. A better understanding of the ovulatory process in general can also aid in the development of birth control and fertility products.

The objectives of the following study were: 1) to determine the minimum effective dose of indomethacin that will inhibit ovulation in the pig.

2) to measure preovulatory follicular fluid concentrations of PGF2α, 12-HETE and 15-HETE associated with indomethacin treatment and to assess the effect on ovulation.

3) to determine the differential production of PGF2α, 12-HETE and 15-HETE by cultured porcine granulosa (GC) and theca interna cells (TIC) treated with different levels of INDO in the media.

4)to determine if progesterone influences eicosanoid production by cultured porcine granulosa and theca interna cells.

Literature Review

Eicosanoids

Eicosanoids are the 20 carbon non-saturated fatty acid products derived from arachidonic acid. These arachidonic acid products include prostaglandins, prostacyclin (PGI2), thromboxanes, leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids (HETE). The formation of eicosanoids is initiated by the release of arachidonic acid from cell membrane phospholipids by the action of phospholipase (PL) A_1, A_2, C or D, but PLA_2 seems to be the most important (reviewed by Murdoch *et al.*, 1993). The release of arachidonic acid is the rate-limiting step in the formation of the eicosanoids. Once the arachidonic acid is free, it may be oxygenated by the cyclooxygenase or the lipoxygenase pathway (Figure 1).

The cyclooxygenase pathway leads to the formation of PGE2, PGF2α, prostacyclin and the thromboxanes. The formation of these products is accomplished by the enzyme cyclooxygenase (COX), also known as prostaglandin G/H synthase (PGS) or prostaglandin endoperoxide synthase, which forms the unstable intermediate PGH2 which undergoes further enzyme action by tissue specific enzymes that compete for this unstable intermediate. The COX enzyme exists in two forms: COX-1 which is involved in constitutive production of PGH2 and COX-2 which is involved in regulated production of PGH2. Prostaglandins can also be inter-converted by additional enzymes; for example, prostaglandin E2-9-ketoreductase can convert PGE2 to PGF2α.

The lipoxygenase (LOX) pathway leads to the formation of the unstable intermediates 5-, 12- and 15-hydroperoxyeicosatetraenoic acids (HPETE) via 5-, 12-, and 15-

lipoxygenase enzymes, respectively. The 5-, 12-, and 15-HPETEs give rise to the more stable 5-, 12- and 15-HETE. The metabolite 15-HETE can be further acted upon by 5-lipoxygenase to form the lipoxins and 5-HPETE can form the various leukotrienes. It is not known if there is further metabolism of 12-HETE. Eicosanoids are synthesized upon demand and do not accumulate within cells.

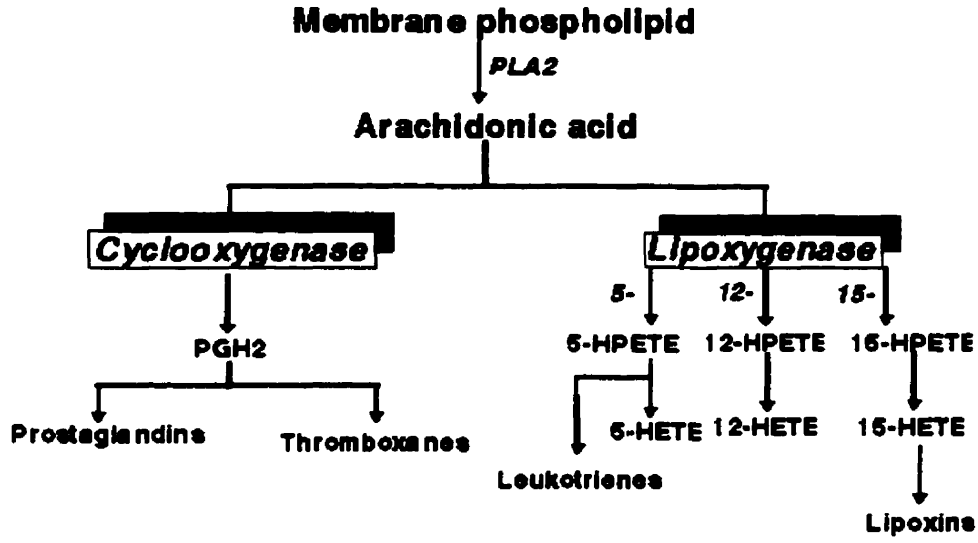

Figure 1 Metabolism of arachidonic acid.
PL=Phospholipase, HPETE=Hydroperoxyeicosatetraenoic acid, HETE=hydroxyeicosatetraenoic acid.

Cyclooxygenase Products and Ovulation

The prostanoids are commonly associated with inflammation. The ovulatory process is comparable to the inflammatory process in several ways (reviewed by Espey, 1980 and 1994), one such common feature being the activation of the arachidonic acid cascade. Prostanoids have been associated with ovulation for more than two decades. One of the first reports of PG involvement in ovulation was in 1971, when Labhsetwar found that PGF2α induced ovulation and corpus luteum formation in hamsters. Follicular fluid (FF) concentrations of PG increase shortly after the LH surge and reach a maximum near the time of ovulation in several species (rat: Espey *et al.*, 1989a, 1991; Higuchi *et al.*, 1995; sheep: Murdoch *et al.*, 1986, 1993; pig: Tsang *et al.*, 1979a; human: Patwardhan and Lanthier, 1981). Prostaglandins are produced in the follicle and act upon the follicle itself.

In the pig, both cellular compartments of the follicle produce PGs. Ovarian levels of PGE2, PGF2α and 8-keto-PGF1α (stable metabolite of PGI2) increase with the development of the follicle (Evans *et al.*, 1983). Compared to granulosa cells, theca cells are the predominant source of PGs (Evans *et al.*, 1983, and Ainsworth *et al.*, 1984). *In vitro*, LH and FSH have no significant effect on PG production by porcine follicular cells (Evans *et al.*, 1983; Ainsworth *et al.*, 1984; Tsang *et al.*, 1988) although addition of arachidonic acid to the culture media significantly enhances PG production by both cell types (Ainsworth *et al.*, 1984; Tsang *et al.*, 1988). In the pig, prior to ovulation, intrafollicular levels of PGF2α and PGE2 increase (Tsang *et al.*, 1979a). Cultured porcine follicular cells produce more PGE2 than PGF2α (Tsang *et al.*, 1988). In the porcine follicle, theca cells are the primary source of PG prior to LH exposure *in vivo*; after LH exposure, there is an increase

in PG production by the follicle characterized by a general increase in PG production by the granulosa cells and an increase in PGF2α production by both cell types (Tsang *et al.*, 1988).

Cyclooxygenase-2, a key rate-limiting enzyme in PG synthesis, increases prior to ovulation (Sirois, 1994 [bovine], 1995 [equine]). In the bovine follicle, COX-2 is located in the granulosa cells but not the theca interna and, as the follicle matures, COX-2 but not COX-1 levels progressively increase (Sirois, 1994). The transcription factor C/EBP$_\beta$, which is involved in the expression of COX-2, increases in preovulatory rat follicles induced by hCG (Pall *et al.*, 1995). LH stimulates the production of prostaglandins in follicles and LH and GnRH act synergistically to induce COX-2 in cultured rat granulosa cells (Morris and Richards, 1995). In cultured rat granulosa cells, it has been shown that inhibition of the PKA pathway blocks the induction of COX-2 when the cells are stimulated with LH but not GnRH; blocking the PKC pathway also inhibits COX-2 induction but only under the stimulation of GnRH and not LH (Morris and Richards , 1995). This implies that the induction of COX-2 is through both the PKA and the PKC pathways. It is known that LH binding to its receptor causes an increase in cellular cAMP, which acts to stimulate various enzymes involved in steroidogenesis in the preovulatory follicle. It has been shown, *in vitro*, that in preovulatory rat follicles, cAMP alone can stimulate PGE2 accumulation and that other compounds such as ATP, ADP, 3'-AMP, 5'-AMP and cGMP do not (Clark *et al.*, 1978), which supports the concept that the effect of LH on PG synthesis is mediated through cAMP. It has been suggested that ovarian PG production is regulated by LH in two different but complementary modes of action, these being increased arachidonic acid availability and increased levels of COX-2 (Tsang *et al.*, 1988). Contradictory to most of the evidence

supporting the requirement for PGF2α in mammalian ovulation, the mRNA for the PGF2α receptor in bovine granulosa cells did not increase until after ovulation occurred (Tsai and Wiltbank, 1995) although no literature was found that reported the levels of receptor or receptor mRNA in other species.

It has been demonstrated repeatedly that the non-steroidal anti-inflammatory drug (NSAID), indomethacin (INDO), which inhibits PG synthesis, also blocks ovulation in several mammalian species (pigs: Ainsworth *et al.*, 1979; Downey and Ainsworth, 1980; sheep: Murdoch *et al.*, 1986, 1993; rabbits: YoungLai, 1978; rats: Tanaka *et al.*, 1991). Acetylsalicylic acid (aspirin) and naproxen sodium (naproxin), NSAIDs with less potency than INDO, both, at high doses, reduce ovulatory efficiency of rabbits *in vivo* and in ovarian perfusion systems; a decrease in ovarian PGF2α occurs in both models (Zanagnolo *et al.*, 1996). Cloprostenol, a PGF2α analogue, induces premature ovulation and luteinization in the gonadotropin-primed gilt (Srikandakumar and Downey, 1989). Administration of PGF2α can partially overcome INDO induced blockade of ovulation in the pig, but PGE2 does not (Downey and Ainsworth 1980), which suggests that PGF2α is required for ovulation in the pig. Indomethacin-blocked ovulation can be restored with exogenous PGF2α in other species as well(sheep: Murdoch *et al.*, 1986, 1993; rat: Espey *et al.*, 1992;) but results obtained with PGE2 replacement have been less consistent. In the perfused rabbit ovary, PGE2 was unable to restore indomethacin-blocked ovulation (Espey *et al.*, 1992) whereas, in the perfused rat ovary, it was shown that PGE2 or PGF2α could restore ovulation (Sogn *et al.*, 1987). It has also been observed, in the rat, that exogenous PGE2 alone (no NSAID treatment) may actually reduce ovulation rate (Espey *et al.*, 1992). In the cases where ovulation was restored with PGE2, the PGE2 may provide an increase in substrate for

prostaglandin E_2-9-ketoreductase, the enzyme that converts PGE2 to PGF2α, thus leading to an increase in PGF2α. In the pig, ovarian activity of PGE2-9-ketoreductase is less than that of the human (Watson *et al.*, 1979), which may be why PGE2 does not restore ovulation in the INDO treated pig. Another theory why PGE2 may give conflicting results on whether or not it restores INDO blocked ovulation is the ratio between PGE2 and PGF2α, which is a factor that may influence follicular rupture (Murdoch *et al.*, 1993).

Different PGs can have opposing functions. For example, the prostanoids are commonly associated with the inflammatory process but PGE1 may have anti-inflammatory actions, such that PGE1 was shown to have an inhibitory effect on collagenase mRNA levels while PGE2 had no effect in non-ovarian cells (Salvatori *et al.*, 1992). It has also been found that PGF2α is likely to have a role in the activation of collagenase leading to the degradation of the follicle wall aiding in follicular rupture (Murdoch *et al.*, 1986). These opposite actions of prostaglandins suggest that one PG may be responsible for the degradative events leading to ovulation while another PG is acting to repair the tissue after follicular rupture. Another example of opposing effects of the prostanoids is the fact that PGF2α can induce ovulation (Labhsetwar, 1971; Srikandakumar and Downey, 1989) while PGE2 may inhibit or suppress ovulation (Espey *et al.*, 1992; reviewed by Espey, 1980; Murdoch *et al.*, 1993). Some of the actions suggested for PGF2α in ovulation include vasoconstriction, effects on the contractile elements of the follicle and stimulation of collagenolytic enzymes (review by Espey, 1980; Murdoch *et al.*, 1993; Espey and Lipner, 1994). Prostaglandin E_2 is a vasodilator and may be a factor leading to ovarian hypermia (Espey, 1980; Murdoch *et al.*, 1993). As reviewed by Murdoch *et al.* (1993), PGE2 may have a stimulatory effect on cAMP, which could be working as positive feedback on the synthesis of the cyclooxygenase enzyme; the

mechanism for increased cAMP by PGE2 is likely different from the mechanism of LH to increase cAMP, since cells that have become refractory to LH remain sensitive to PGs. Although PGE2 may not influence the collagenase enzyme directly, since it does not stimulate mRNA for collagenase (Salvatori *et al.*, 1992), PGE2 may have a stimulatory effect on plasminogen activator which may participate in the activation of procollagenase (reviewed by Espey, 1980). Lipoxygenase products may also play a role in the expression of collagenases, since rats treated with either INDO or nordihydroguaiaretic acid (NDGA, a lipoxygenase inhibitor) show a decrease in interstitial collagenase (Reich *et al.*, 1991).

Although PGs are required for ovulation, they are not required for luteinization, in that the unruptured follicles form luteinized unruptured follicles (LUF). The occurrence of LUFs has been seen in pigs (Ainsworth *et al.*, 1979) and rats (Plas-Roser *et al.*, 1984). Luteinized unruptured follicles in the rat secrete less progesterone than the post ovulatory corpus luteum (Plas-Roser *et al.*, 1984). In the INDO treated pig, plasma progesterone profiles do not differ from untreated control animals during the luteal phase of the cycle, indicating that luteal function is not impaired by INDO (Ainsworth *et al.*, 1979).

Several studies also indicate that PGI2 is produced by the ovaries (reviewed by Espey and Lipner, 1994) but, to my knowledge, evidence of a mandatory role for this proinflammatory, arachidonic acid metabolite in ovulation has not been demonstrated.

Lipoxygenase Products and Ovulation

Both PGF2α and PGE2 are significantly inhibited at doses of INDO that have no effect on ovulation in the rat (Tanaka *et al.*, 1989, 1991), which demonstrates that ovulation can occur even when intrafollicular concentrations of PGs are significantly reduced. Intrafollicular levels of 15-HETE and 12-HETE increase prior to ovulation in the rat (Tanaka *et al.*, 1989; Espey *et al.*, 1991; Higuchi *et al.*, 1995,). It has been demonstrated in the rat that blocking the synthesis of LOX metabolites also blocks ovulation (Espey *et al.*, 1991; Tanaka *et al.*, 1991). INDO was traditionally considered to be a cyclooxygenase blocker, but is now known to also have an inhibitory effect on the lipoxygenase pathway (Seigel *et al.*, 1979, 1980; Tanaka *et al.*, 1991; Mootoo, 1995). In the rat, the lowest dose of indomethacin that significantly inhibits ovulation is also the lowest dose that significantly inhibits 15-HETE biosynthesis (Tanaka *et al.*, 1989, 1991). Tanaka *et al.* (1991), found that 15-HETE is the eicosanoid most correlated to ovulation in the rat, followed by 12-HETE.

In the gonadotropin-primed pig, follicular fluid concentrations of 15-HETE increase significantly from the time of hCG treatment (LH surge) to the time of ovulation (Mootoo, 1995). In pigs that were treated with the specific lipoxygenase blocker nordihydroguaiaretic acid (NDGA), ovulation was inhibited but no significant reduction in the intrafollicular levels of 15-HETE nor PGF2α occurred (Mootoo,1995). Nordihydroguaiaretic acid also had no effect on ovarian levels of 15-HETE, although it did cause a moderate reduction in 5-HETE and greatly reduced 12-HETE in the rat *in vivo* (Tanaka *et al.*, 1991). This suggests that lipoxygenase products, other than 15-HETE are of importance in porcine ovulation. In

the pig, when ovulation was blocked by injecting INDO into the ovarian stalk 24 h prior to ovulation, 15-HETE levels in the follicle were reduced (Mootoo, 1995), so a role for this metabolite cannot be ruled out. In cultured porcine follicular cells, 100 ng/ml of INDO reduced the production of 15-HETE in TIC but not GC, whereas 1000 ng/ml of NDGA, reduced 15-HETE by both cell types (Mootoo, 1995).

In the rat, 5-HETE levels in ovarian homogenates decrease from the time of LH initiation of ovulation to the time of follicular rupture (Espey *et al.*, 1991). Other metabolites of the 5-LOX pathway, known as the leukotrienes (LT), have also been measured in ovarian homogenates of rats. Preovulatory ovarian profiles for LTB4 and LTC4/D4/E4 have been examined in the rat; LTB4 increases sharply shortly after hCG treatment, then decreases until levels plateau shortly after ovulation, while LTC4/D4/E4 peak twice after hCG inducement of ovulation (Espey *et al.*, 1989b; Higuchi *et al.*, 1995). Indomethacin treatment in the gonadotropin-primed rat induces an increase in ovarian leukotriene levels (Espey *et al.*, 1989b) which may indicate the metabolism of arachidonic acid is being shunted to this pathway.

Lipoxins were not examined in any of the literature reviewed. The different profiles of the various eicosanoids over time may indicate a different role for each of eicosanoid in the ovulatory process.

The biological effects of lipoxins and leukotrienes were reviewed by Samuelson *et al.* (1987), which include bronchial contraction and roles in the inflammatory response such

as leukocyte degranulation, PMN extravation and plasma exudation for lipoxins and lung tissue contraction and possibly intracellular messenger for leukotrienes.

Steroidogenesis in the Porcine Follicle

Steroidogenesis in the sow and the two cell theory was reviewed by Ainsworth *et al.* (1990). Receptors for FSH are found on GC and LH receptors are found on both GC and TIC prior to ovulation. Both FSH and LH stimulate adenylate cyclase through their respective receptors to produce cAMP. The increased intracellular levels of cAMP stimulate several of the enzymes in the steroidogenic pathway (Figure 2). Progesterone is made from pregnenelone by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). The major site of progesterone production is GC although TIC are capable of production also. Progesterone may then be converted to androgens by the enzyme 17α-hydroxylase. Granulosa cells can not produce androgens so the progesterone produced by these cells is used by TIC to produce androgens. The androgens can then be converted by the enzyme aromatase to estrogens. In the sow, estrogen is produced by both cell types which is one of the key differences between follicular steroidogenesis in the rat and the pig. Estrogen production by GC depends on androgens provided by TIC. When the preovulatory LH surge occurs, the LH receptors become less sensitive and decrease in number. Progesterone and androgens are still produced but are no longer under the influence of LH. After the LH surge, both TIC and GC lose their ability to produce estrogen due to a decrease in aromatase activity.

In support of the two cell theory, only TIC produce androgens in culture, and estrogens are produced by both GC and TIC while GC cells are dependent on androgens for estrogen production (Evans *et al.*, 1981); and estrogen production appears to be more dependent on androgen availability than aromatase activity (Foxcroft and Hunter, 1985). In culture, both follicular cell types produce progesterone but GC are the principal producer.

Histological studies, in the pig, have shown that the 17α-hydroxylase enzyme is located in the TIC, except for a lining of cells surrounding the basement membrane, and ovulation is associated with a decline in this enzyme (Conley *et al.*, 1994, 1995). Aromatase was located in both cell types but, surprisingly, 3β-HSD was only found in TIC; the authors suggested that progesterone production by cultured GC may reflect *in vitro* luteinization and that there is no preovulatory progesterone synthesis by GC, which contradicts the two cell theory (Conley *et al.*, 1994, 1995).

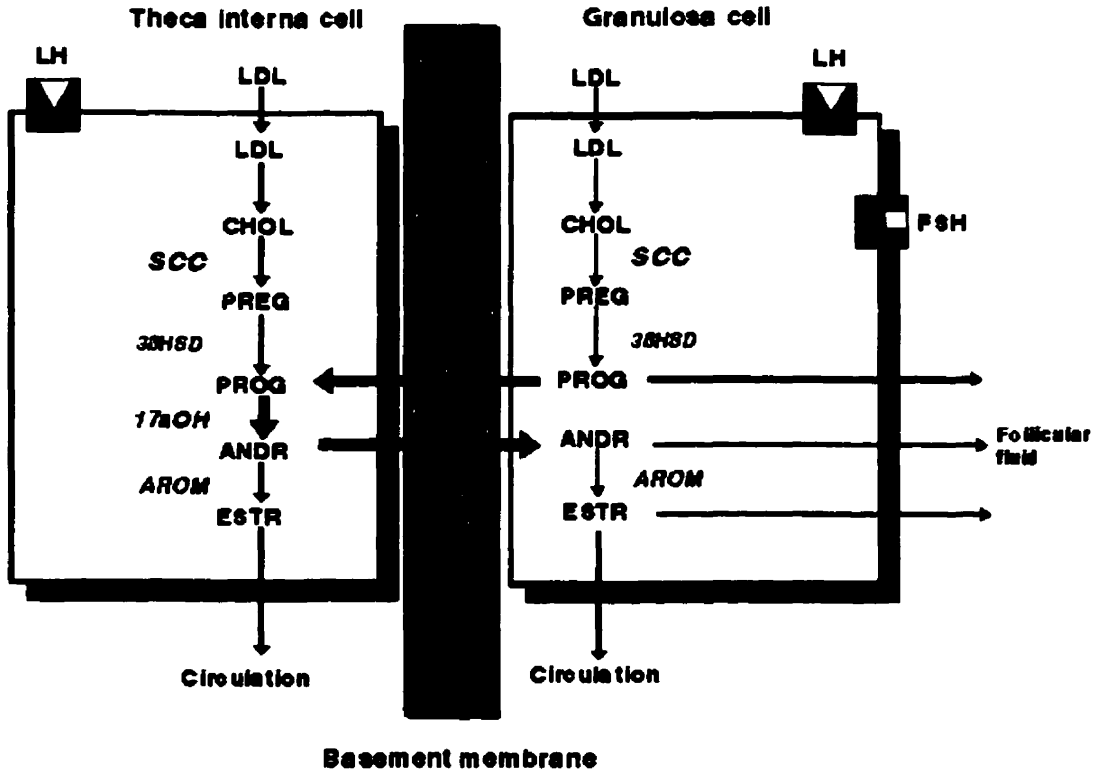

Figure 2 The two cell model of steroidogenesis by porcine follicular cells.
LDL= low density lipoprotein; CHOL= cholesterol; PREG= pregnenelone; PROG= progesterone; ANDR= androgen; ESTR= estrogen; SCC= side chain cleavage enzyme; 3βHSD= 3β-hydroxysteroid dehydrogenase; 17αOH= 17α-hydroxylase; AROM= aromatase. Adapted from Ainsworth *et al.*, 1990.

Progesterone and Ovulation

After the LH surge and prior to ovulation, ovarian accumulation of progesterone increases in the rat (Espey *et al.*, 1989a, 1990, 1991). Several studies have shown, and it is generally well accepted that progesterone plays an important role in the ovulatory process of several species (rabbit: Bränström and Janson, 1989; rat: Espey *et al.*, 1990; Tanaka *et al.*, 1991; Snyder *et al.*, 1984; sheep: Murdoch *et al.*, 1986). Several inhibitors of progesterone synthesis exist including: epostane, trilostane, aminoglutethimide, isoxazol, danazol and progesterone receptor blockers RU486 and onapristone. Epostane is an inhibitor of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) which is the enzyme that converts pregnenelone into progesterone. A dose dependent relationship exists between ovulation rate and epostane dose level in rats (Espey *et al.*, 1990; Tanaka *et al.*, 1991); progesterone replacement in epostane-treated rats restores ovulation (Espey *et al.*, 1990). Intraovarian injections of isoxazol, another 3β-HSD inhibitor, in sheep, blocked ovulation and a systemic dose of progesterone restored ovulation (Murdoch *et al.*, 1986).

It should be noted that the 3β-HSD blocker, epostane, has not only been used to inhibit ovulation but has also been used to inhibit progesterone in attempts to improve ovulation rate in gilts (Fu *et al.*, 1990) and ewes (Hoefler *et al.*, 1986; Webb, 1987; Webb *et al.*, 1992). To increase the number of follicles and ovulation rate using epostane, the treatments must be given over an extended period of time and during a different stage in the estrous cycle (luteal phase), whereas to inhibit ovulation the epostane must be administered just prior to the ovulatory increase in progesterone.

Interactions between Progesterone and the Eicosanoids

In the perfused rabbit ovary, it was found that ovulation could be blocked using Compound A (a 3β-HSD inhibitor) and that progesterone, but not testosterone, could restore the LH induced ovulations (Brännström and Janson, 1989). In contrast, progesterone alone could not induce ovulation indicating that, although progesterone plays a role in mediating the ovulatory process, progesterone is not capable of causing ovulation on its own. It has been shown that the prostanoids, PGE2, PGF2α, and 6-keto-PGF1α, increase with progesterone production in the preovulatory rat follicle, suggesting that there may be some interdependency between these substances (Espey *et al.*, 1989a). An experiment done in rats, by Espey *et al.* (1989a), compared the effect of three anti-ovulatory drugs: indomethacin, aminoglutethimide and cycloheximide, the latter two being inhibitors of steroidogenesis. The ovarian levels of PGF2α, PGE2, 6-keto-PGF1α, progesterone and estradiol were measured, and it was found that indomethacin reduced ovarian levels of the three prostanoids without affecting the steroids, aminoglutethimide reduced both the prostanoids and the steroids, while cycloheximide had little effect on any of the metabolites measured. These results show that when progesterone is inhibited by steroid inhibiting drugs, PGs are also inhibited which implies that progesterone may be involved in regulating the metabolism of the prostanoids but the prostanoids do not have an effect on progesterone production by the ovary. In contrast, in the perfused rabbit ovary, aminoglutethimide significantly reduced progesterone and estradiol production without affecting ovulation rate (Yoshimura *et al.*, 1986). A possible explanation for this result may be that, although progesterone was significantly reduced there still was sufficient progesterone for PG synthesis but PGs were not measured in this study. Alternatively, since the significant reduction in progesterone

occurred eight hours after hCG induction of ovulation this may have not been a time when progesterone was critical for PG formation.

In the ewe, intraovarian injections of isoxazol block ovulation and reduce ovarian levels of progesterone and PGF2α, but not PGE2, and replacement of PGF2α or progesterone is capable of restoring ovulation (Murdoch *et al.*, 1986). This indicates that PGF2α is important for follicular rupture but the levels of PGF2α are dependent on the presence of progesterone in the ovine follicle. In rats, it was found that epostane had a rapid and transient effect on ovarian progesterone synthesis and that ovarian levels of PGF2α and PGE2 decreased by approximately 30%. However, this decrease was not considered, by the authors, to be sufficient to block ovulation and they suggested that, since progesterone returned to preovulatory levels, it may be involved in other metabolic events leading to follicular rupture in the rat (Espey *et al.* 1990; Tanaka *et al.*, 1991). Tanaka *et al.* (1991), found that the lowest dose of epostane to inhibit ovulation in rats significantly reduced ovarian levels of progesterone and 15-HETE; higher doses of epostane significantly reduced PGE2, PGF2α, and 12-HETE, whereas INDO was capable of significantly reducing eicosanoid levels without affecting any of the steroids, even at doses much greater than the standard. Similar results were seen in the rat by Espey *et al.* (1991), and it was suggested that formation of 15-HETE may be dependent on progesterone synthesis. In the gonadotropin-primed rat, if INDO is administered 10 hours post hCG, there are no significant changes in plasma levels of the steroids but, if INDO is given at the same time as the hCG, it results in the reduction of plasma levels of progesterone and testosterone but not estrogen (Mori *et al.*, 1980). This evidence suggests that prostaglandins may mediate ovulation through two mechanisms at different stages during the preovulatory period where, at the early stages, their role may involve stimulation of steroidogenesis.

If progesterone is given concurrently with hCG and INDO, it partially restores ovulation (Kohda *et al.*, 1983). In the perfused rat ovary, INDO inhibits ovulation and reduces progesterone release and, although PGE2 and PGF2α both could restore ovulation, only PGE2 could restore progesterone levels indicating that, in this system, PGE2 is required for steroidogenesis (Sogn *et al.*, 1987). One way that progesterone may mediate PGs is through the enzyme PGE2-9-ketoreductase. In the ewe, the ovarian activity of PGE2-9-ketoreductase increases six fold before the time of ovulation; INDO has no effect on the conversion of PGE2 to PGF2α, but the progesterone inhibitor, isoxazol, suppresses the conversion, and the enzyme activity can be restored by progesterone replacement (Murdoch and Farris, 1988).

Contradictory to the above evidence, some research suggests that prostaglandins may play a role in follicular steroidogenesis. For example, in isolated rabbit follicles, PGE2 enhanced LH induced progesterone and testosterone production but blocking PG synthesis failed to inhibit steroidogenesis, implying that PGs may play a minor role in the formation of the sex steroids (YoungLai, 1978). In the perfused rabbit ovary, there was no difference in progesterone levels between LH-treated and LH + indomethacin-treated ovaries, while PGE2 treatment alone caused a slight increase in progesterone in the perfusate; LH + PGE2 treatment showed less of an increase (Holmes *et al.*, 1983). In cultured bovine granulosa cells, PGF2α stimulates progesterone production and acts synergistically with low doses of insulin and IGF-I; in contrast, PGF2α inhibits progesterone accumulation in luteal cells (McArdle, 1990). PGF2α may inhibit progesterone through the steroid acute regulatory protein (StAR). StAR is thought to be involved with the transport of cholesterol to the inner mitochondrial membrane where the side chain cleavage enzyme can convert it to pregnenelone. In the rat ovary , a luteolytic dose of PGF2α causes a reduction in the

transcript of StAR and a corresponding reduction in progesterone (Sandhoff and McLean, 1996). In the pig, there is no correlation between PGs and steroidogenesis, indicating that in this species PGs do not play a role in steroidogenesis (Evans *et al.*, 1983). The differences seen in the ability of PGs to influence steroidogenesis may be due to species differences or due to differences in the systems used to study the effects.

Indomethacin

Evidence exists which indicates that the inhibitory effects of indomethacin are not specific for the COX pathway as previously believed and that it has inhibitory effects on the LOX pathway as well. In rat neutrophil cells, INDO inhibits 15-HETE and 11-HETE formation (Seigel *et al.*, 1980) and INDO inhibits 12-HETE production by human platelets (Seigel *et al.*, 1979). In the rat ovary, *in vivo*, INDO reduces 15-HETE and 12-HETE (Tanaka *et al.*, 1991) and in the porcine ovary, when injected into the ovarian stalk, it reduces 15-HETE (Mootoo, 1995). Modes of action, other than inhibition of arachidonic acid metabolism, which may contribute to the anti-inflammatory effects of INDO include interference with Ca^{++} mobilization and other early signalling events in the cell (Abramson *et al.*, 1985).

Danazol

Danazol (17α-pregn-4-en-20-yno-(2,3-d)isoxazol-17-ol) inhibits steroidogenesis in several tissues including rat leydig cells (Barbieri *et al.*, 1977), human endometrium (Kokko *et al.*, 1982), human corpus luteum (Barbieri *et al.*, 1981), human placenta (Rabe *et al.*, 1983) and porcine granulosa and luteal cells (Tsang *et al.*, 1979b). Danazol was originally thought to inhibit steroidogenesis through interference with the pituitary gonadotropins, but it has now been demonstrated in women treated with daily doses of 600, 400 or 200 mg of danazol that no significant changes in serum levels of FSH and LH occur while serum progesterone levels are reduced (Rannevik and Thorell, 1984). The inhibition of steroidogenesis is due to the competitive inhibition of 17,20-lyase, 17α-hydroxylase, 17β-HSD, and 3β-HSD but not aromatase (Barbieri *et al.*, 1977 ,1981; Rabe *et al.*, 1983). In cultured porcine granulosa and luteal cells, danazol inhibits progesterone and estradiol production but estradiol can be restored if testosterone is added to the media (Tsang *et al.*, 1979b).

Materials and methods

Experiment 1

Objective: To determine the minimum effective dose of indomethacin that will inhibit ovulation in the pig.

Experimental design: Nineteen prepubertal PMSG/hCG-treated gilts, four per treatment except the 600 mg per pig dose level where three animals were treated, were given INDO (0, 6, 60, 300, or 600 mg) via i.m. injection 24 h post hCG. Ovulation rate was determined at 46-48 h post hCG, by examining the ovaries through a mid-ventral incision while the animal was under isoflurane anaesthesia. Ovulation rate was determined by calculating the number of recently ovulated follicles over the sum of recently ovulated and non-ovulated follicles greater than 6 mm in diameter.

Experiment 2

Objective: To measure preovulatory follicular fluid concentrations of PGF2α, 12-HETE and 15-HETE associated with indomethacin treatment and to assess the effect on ovulation.

Experimental design: Twenty prepubertal PMSG/hCG-treated gilts, five per treatment, were given INDO (0, 6, 60, or 300 mg) via i.m. injection 24 h post hCG. Follicular fluid was collected at 38 h post hCG. The samples were assayed for PGF2α, 15-HETE and 12-HETE using commercial RIA kits. Each eicosanoid was assayed using a single kit.

Experiment 3

Objective: To determine differential production of PGF2α, 12-HETE and 15-HETE by cultured porcine granulosa and theca interna cells treated with different dose levels of indomethacin in the media.

Experimental design: Theca interna and granulosa cells were collected from PMSG/hCG-treated gilts 38 h post hCG. The cells were pooled from two gilts for each of four replicates. In 24 well culture plates, 3 X 10^5 cells were placed in each well with 1 ml DMEM and treated, in duplicate, with INDO (0, 10, 100, or 500 ng/ml) dissolved in ethyl alcohol (untreated control was alcohol alone). The cells were cultured for 42 h based on preliminary studies of eicosanoid production over time (see Appendix A).

Experiment 4

Objective: To determine if progesterone influences eicosanoid production by cultured porcine granulosa and theca interna cells.

Experimental design: Theca interna and granulosa cells were collected from PMSG/hCG-treated gilts 38 h post hCG. The cells from two animals were pooled for each of four replicates. In 1 ml of DMEM, 3 X 10^5 cells were treated in duplicate with the steroidogenic inhibitor, danazol (0, 10 or 100 ng/ml), dissolved in ethanol (untreated control received ethanol alone). The cells were cultured for 42 h and the media was collected as described.

Animals

Prepubertal Landrace X Yorkshire gilts, weighing 60 to 70 kg and approximately 4 mos of age, were fed a commercial ration containing 15% crude protein and water *ad libitum* and were housed, during the experiments, at the Large Animal Research Unit on the Macdonald Campus. To induce follicular growth and ovulation, i.m. injections of 750 IU pregnant mare serum gonadotropin (PMSG; Equinex®, Ayerst Laboratories, Montreal, QC) and 72 hours later 500 IU of human chorionic gonadotropin (hCG; APL®, Ayerst Labs) were given , in an effort to synchronize ovulation within 42 ± 2 hours after hCG administration (Ainsworth *et al.*, 1982). Animals were randomly assigned to treatment groups.

Indomethacin treatments in vivo

In experiment 1 and 2, indomethacin (Merck Frost Canada Inc., Kirkland, QC) suspended in propylene glycol was given by intramuscular injection to PMSG/hCG-primed gilts 24 hours after hCG.

Animal surgeries

All surgeries were performed while the animals were under isoflurane anaesthesia. In experiment 1, 46 hours after hCG administration, ovaries were examined by mid ventral laparotomy and ovulation rate was determined. In experiment 2, 38 hours post hCG, via mid ventral laparotomy, follicular fluid (FF) samples were collected by aspiration of the follicles with a 26G needle and syringe. For experiment 3 and experiment 4, the animals were ovariectomized at 38 h post hCG.

Follicular fluid samples

The follicular fluid was pooled by gilt, centrifuged at 1000 X g for 15 min, and frozen in liquid nitrogen. The samples were stored at -70°C until assayed.

Cell culture

Animals were ovariectomized at 38 h post hCG and the preovulatory follicles were dissected free from the ovaries in Hanks buffered saline solution (HBSS; Gibco BRL, Burlington, ON) without magnesium nor calcium, containing 50 mg/L gentamycin (Gibco, BRL). The granulosa cells were scraped from the follicles using a fine wire loop, and the theca interna cells peeled from the follicle using fine forceps and then minced using small scissors prior to enzymatic dispersion. The cells were centrifuged for 3 min at 150 X g at 4°C to remove the HBSS and then were resuspended in 10 ml/g of tissue of freshly prepared enzyme solution to disperse the cells . The enzyme solution contained 0.1% hyaluronidase (Sigma Chemical, St. Louis, MO), 0.5% type II collagenase (Sigma Chemical) and 1% chicken serum (Gibco BRL) in Moscona's buffer (Moscona, 1961). The cells were placed in a 37°C shaking water bath and after 15 min, approximately 1 mg of type I DNase (Sigma

Chemical) was added to the solution. The cells were agitated throughout the dispersion by repeated drawing and expelling with a Pasteur pipet. The dispersion was terminated when the cells were dispersed (approximately 35 - 45 min) by adding 2 ml of heat inactivated FBS (Gibco BRL) and diluting the suspension 1:1 with HBSS. The cells were filtered through three layers of sterile surgical gauze to remove clumps of cells and then washed 3 times with Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 50 mg/L gentamycin. The cells were then resuspended in DMEM at a concentration of 3 X 10^5 live cells/ml and 1 ml was placed in each well of 24 well cell culture plates (Linbro®; Flow Laboratories Inc., McLean, VA).

Indomethacin and danazol treatments in vitro

Indomethacin and danazol (Sanofi Research, Toulouse Cedex, France) were dissolved in 50% ethanol at the appropriate concentrations such that when 0.01 ml was added to 1 ml of media, 10, 100, or 500 ng/ml of INDO or 10 or 100 ng/ml of danazol was achieved without having the ethanol concentration greater than 0.5% of the total. Each treatment was done in duplicate and four replicates were performed. Each replicate was from the pooled cells of two animals.

Measurement of eicosanoids and progesterone

Eicosanoid and progesterone concentrations were determined using commercial radioimmunoassay (RIA) kits; 15-HETE and 12-HETE, Perspective Biosystems, Cambridge, MA; PGF2α, Amersham, Oakville, ON; progesterone,Coat-a-count®,Diagnostic Products Corporation, Los Angeles, CA. The sensitivity of the assays (at 95% B/Bo displacement of tracer) for 12-HETE, 15-HETE, PGF2α and progesterone were 0.0275, 0.025, 0.03 and 0.02

ng/ml, respectively. The cross reactivity of the progesterone assay with danazol, as stated in the package insert, is 0.006% when 2500 ng/ml of danazol is added to the sample. The intra-assay coefficients of variation were 2.7, 1.5, 3.3 and 3.6% and the inter-assay coefficients of variation for culture media samples were 4.5, 8.0, 9.1 and 6.9% for 12-HETE, 15-HETE, PGF2α and progesterone respectively.

Statistical analysis

The design used for the *in vivo* experiments was a complete randomized design and for the *in vitro* experiments a randomized block design was used. The ovulation data were analyzed using the GENMOD procedure and the rest of the data were analyzed using the GLM procedure of the Statistical Analysis Systems Institute (SAS). Pearson's Chi square was used to determine if treatment had an effect on ovulation rate compared to the control and Student's t test was used to determine differences between the mean eicosanoid concentrations for each treatment. Treatment means were considered significantly different at $p < 0.05$.

Results

Effect of indomethacin on ovulation

The effect of INDO dose level on ovulation rate is presented in Table 1 and Figure 3. Treatments of 300 and 600 mg per pig completely inhibited ovulation and 60 mg per pig significantly reduced ovulation rate compared to the untreated control. The 6 mg per pig treatment had no apparent effect on ovulation.

Table 1 Effect of indomethacin on ovulation rate of individual animals.

treatment	ovulation rate				total (%)
control	6/6	8/12	12/12	14/14	90.9
6 mg	4/5	5/9	19/20	6/6	85.0
60 mg	0/11	2/4	0/28	7/7	18.0*
300 mg	0/9	0/24	0/8	0/12	0*
600 mg	0/19	0/11	0/20		0*

Indomethacin was given 24 h after hCG treatment and ovulation rate was determined 46 h after hCG treatment. Ovulation rate is the number of ovulated follicles over the sum of recently ovulated follicles and non-ovulated follicles greater than 6 mm in diameter.
* significantly different from the control ($p < 0.05$)

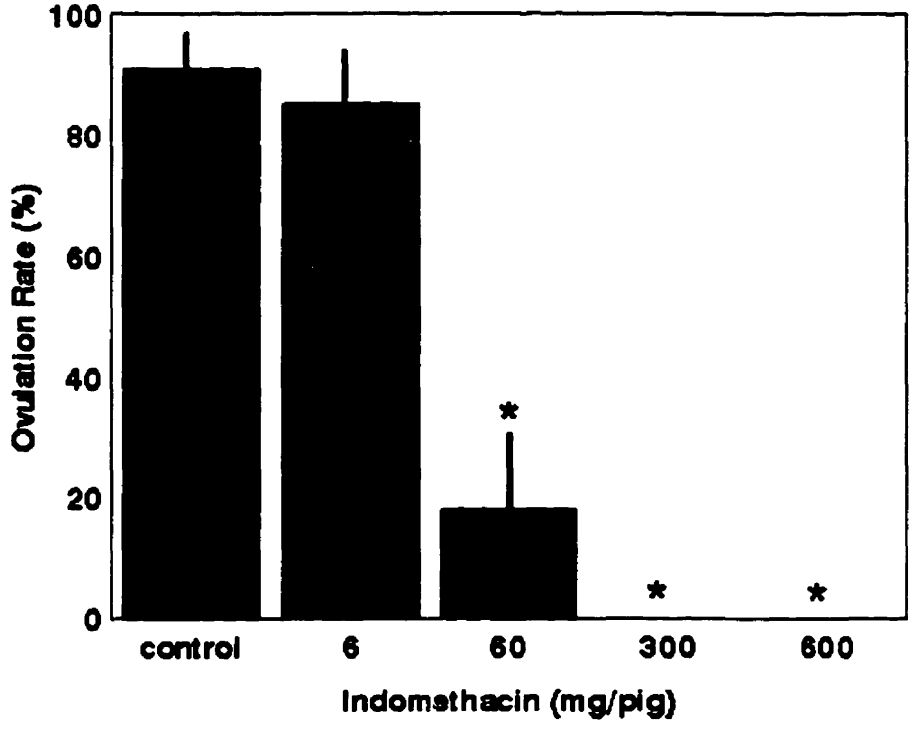

Figure 3 The effect of indomethacin on ovulation rate. Error bars represent the confidence interval.
* significantly different from the control ($p < 0.05$).

Effect of indomethacin on follicular fluid concentrations of eicosanoids

The effect of indomethacin dose on FF concentration of PGF2α, 12-HETE and 15-HETE is presented in Figure 4. Under these experimental conditions, INDO had no effect on the concentrations of LOX products 15-HETE and 12-HETE; in contrast PGF2α was significantly reduced with the 60 and 300 mg per pig treatments.

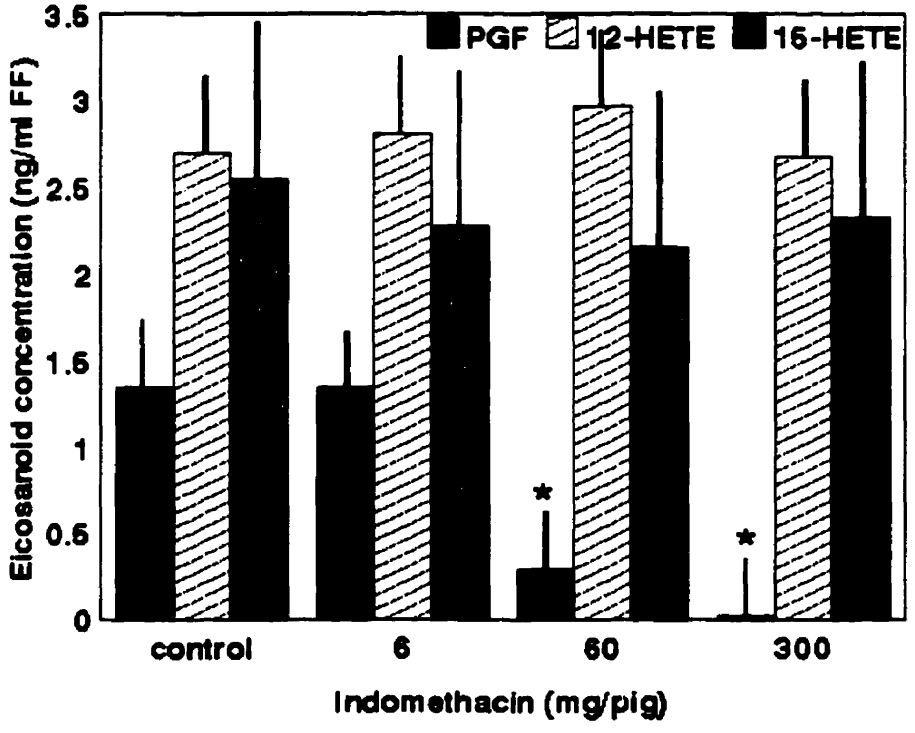

Figure 4 The effect of indomethacin on follicular fluid concentrations of PGF2α, 12-HETE and 15-HETE. Bars represent LSMeans of five animals (± s.e.).
* significantly different from respective control ($p < 0.05$).

Effect of indomethacin on eicosanoid production by follicular cells *in vitro*

The effect of INDO dose on eicosanoid production by GC and TIC is presented in Figures 5 and 6, respectively. In both cell types, INDO significantly reduced PGF2α at all doses compared to the untreated control. 15-HETE was significantly reduced with 100 and 500 ng/ml treatments in both cell types compared to the untreated controls. 12-HETE was not significantly changed by any treatment except in GC at 100 ng/ml.

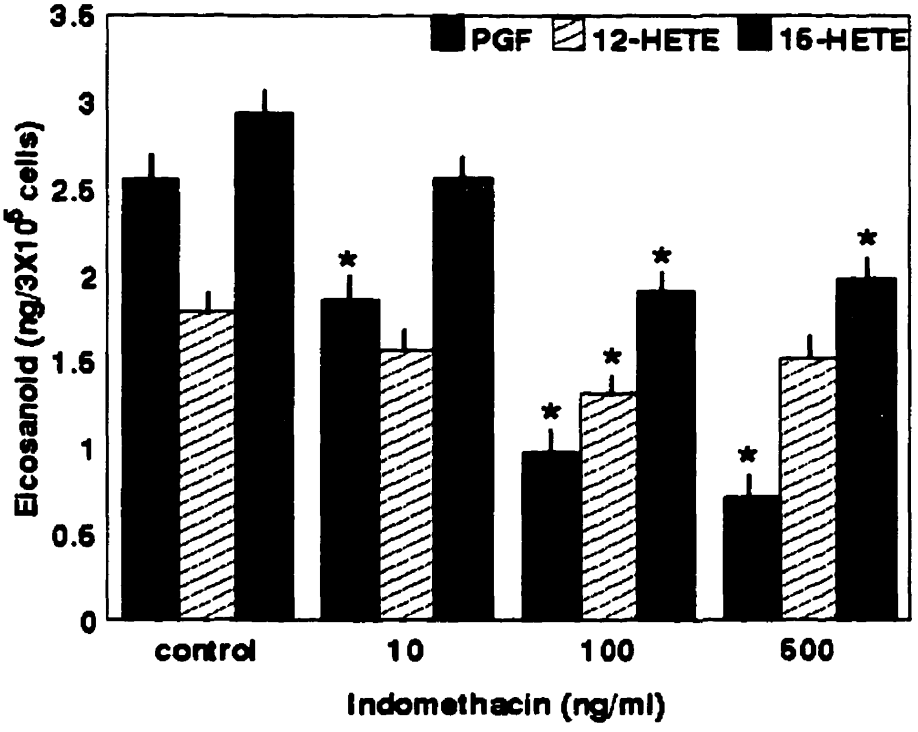

Figure 5 The effect of indomethacin on PGF2α, 12-HETE and 15-HETE production by granulosa cells *in vitro*, after 42 h of incubation.
Bars represent LSMeans (±s.e.) of four replicates in duplicate.
* significantly different from respective control ($p < 0.05$)

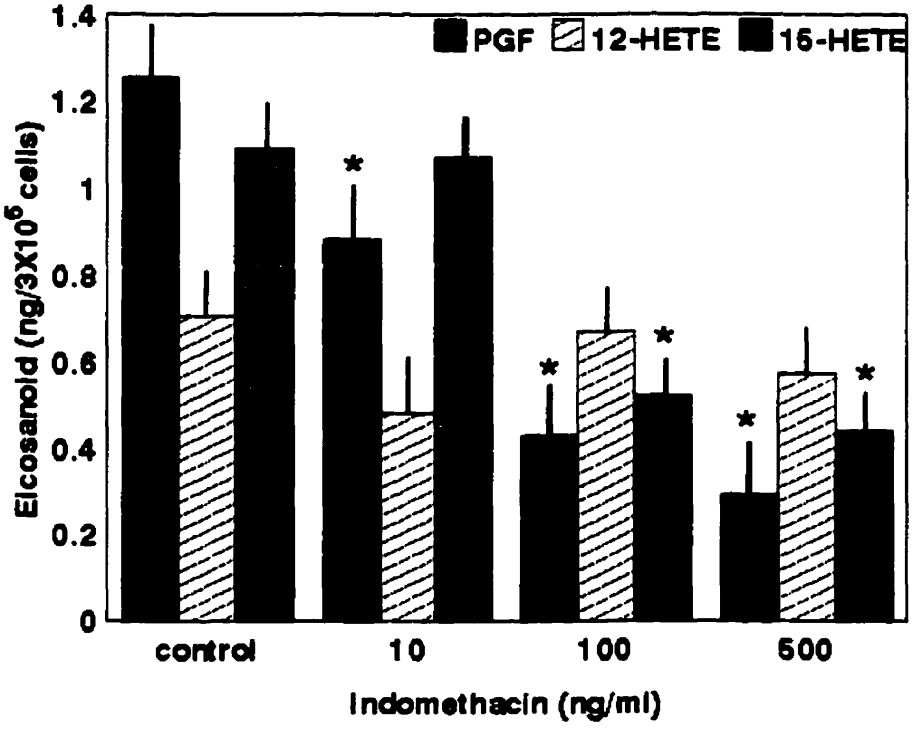

Figure 6 The effect of indomethacin on PGF2α, 12-HETE and 15-HETE production by theca interna cells *in vitro*, after 42 h of incubation.
Bars represent LSMeans (±s.e.) of four replicates in duplicate.
* significantly different from respective control ($p < 0.05$)

Effect of danazol on progesterone and eicosanoid production by follicular cells *in vitro*

The effect of danazol on progesterone production by GC and TIC is presented in Figures 7 and 8, respectively. The effect of danazol on eicosanoid production by GC and TIC is presented in Figures 9 and 10, respectively. Danazol, under the conditions of this experiment, had no effect on progesterone production by either cell type. Similarly, no changes in eicosanoid production were observed.

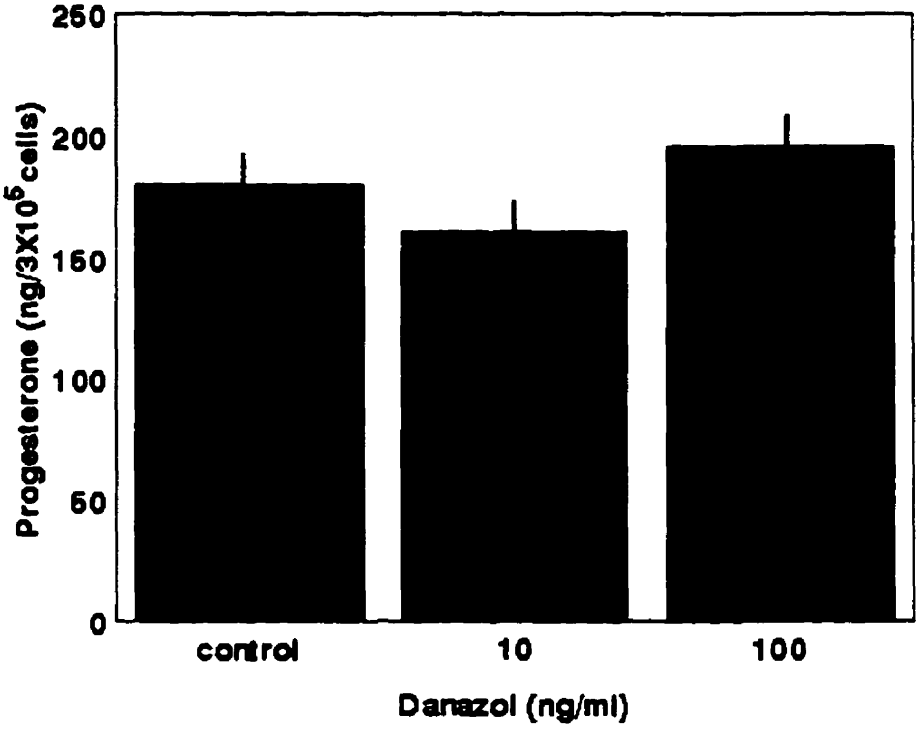

Figure 7 The effect of danazol on progesterone production by granulosa cells *in vitro*, after 42 h of incubation. Bars represent LSMeans (± s.e.) of four replicates in duplicate.

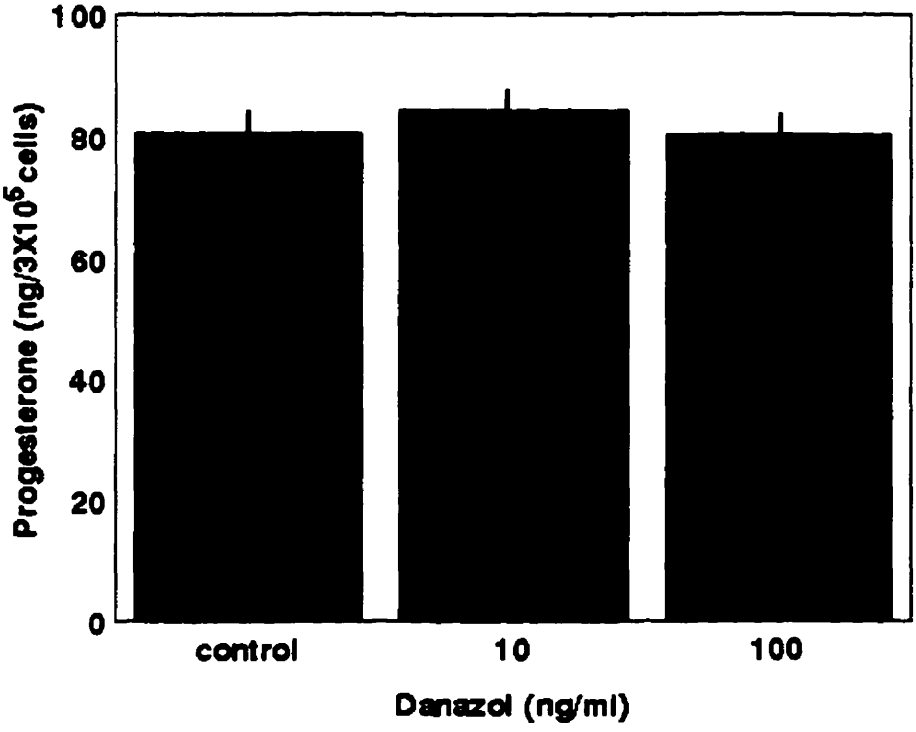

Figure 8 The effect of danazol on progesterone production by theca interna cells *in vitro*, after 42 h of incubation. Bars represent LSMeans (± s.e.) of four replicates in duplicate.

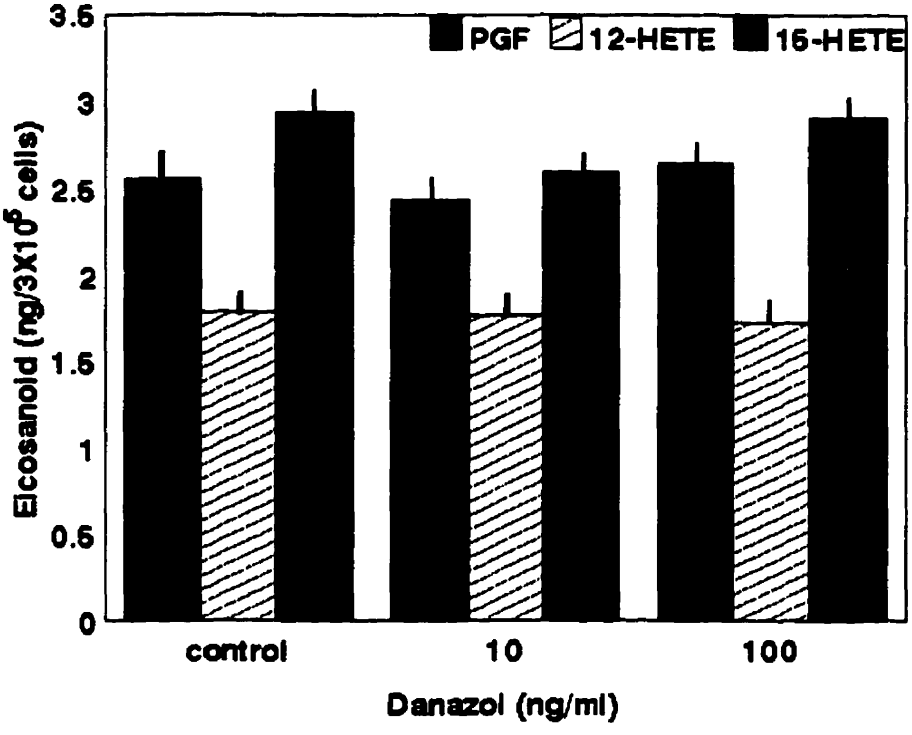

Figure 9 The effect of danazol on PGF2α, 12-HETE and 15-HETE production by granulosa cells *in vitro*, after 42 h of incubation. Bars represent LSMeans ($\pm$ s.e.) of four replicates in duplicate.

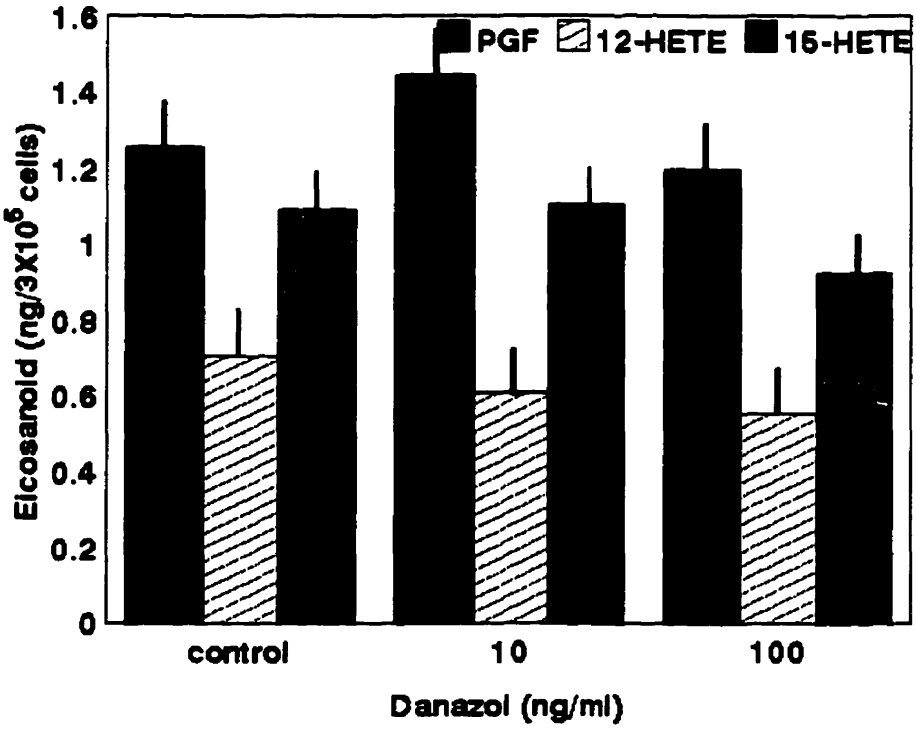

Figure 10 The effect of danazol on PGF2α, 12-HETE and 15-HETE production by theca interna cells *in vitro*, after 42 h of incubation. Bars represent LSMeans (± s.e.) of four replicates in duplicate.

Discussion

As indomethacin dose level increased, ovulation rate decreased. This is similar to what occurs in the gonadotropin-primed rat model (Tanaka *et al.*, 1991) and the rabbit (Espey *et al.*, 1986). In this study, 60 mg of INDO per pig significantly reduced ovulation and 300 mg per pig completely inhibited ovulation; these doses correspond to approximately 1 mg/kg and 5 mg/kg, respectively. Doses as low as 2.5 mg/kg significantly reduced ovulation in the rat (Tanaka *et al.*, 1991) and in the rabbit (Espey *et al.*, 1986). In the rabbit, 1.25 mg/kg reduced ovulation rate from 73 ± 8% to 47 ± 12% but was not found to be statistically significant, while 10 mg/kg completely inhibited ovulation and 5 mg/kg significantly reduced ovulation (Espey *et al.*, 1986).

Indomethacin treatment, *in vivo*, caused significant reduction in the FF concentration of PGF2α with the 60 mg and 300 mg per pig doses. However, no significant suppression of 12-HETE and 15-HETE was observed as compared to the untreated control. This indicates that, under the conditions of this experiment, INDO has no significant effect on the formation of LOX products *in vivo*. In contrast, it has been demonstrated that injecting 10 mg of INDO into the ovarian stalk of a gonadotropin-primed gilt reduces FF concentration of 15-HETE by approximately 50% (Mootoo, 1995). In the rat, INDO significantly reduced PGF2α, 15-HETE, and 12 HETE at 0.75, 2.5, and 7.5 mg/kg, respectively, compared to the untreated control (Tanaka *et al.*, 1991).

In this present experiment, PGF2α was significantly suppressed at the same doses of INDO which significantly suppressed ovulation, unlike the rat where PGF2α was significantly reduced at lower doses than the dose required to inhibit ovulation; hence,

ovulation in the rat can occur without the preovulatory rise in PGF2α (Tanaka *et al.*, 1991). The results of this study indicate that this is not true for the pig. The lowest level of INDO to reduce FF concentrations of PGF2α also reduced ovulation; hence, providing further evidence that PGF2α is necessary for ovulation in the pig. Although indomethacin also reduces levels of PGE2 in the porcine follicle, exogenous PGE2α does not restore ovulation whereas PGF2α does (Downey and Ainsworth, 1980) and, in the rat, exogenous PGE2 reduces ovulation rate (Espey *et al.*, 1990). Thus, PGF2α is likely to be the prostanoid of greater significance in the induction of ovulation.

In the pig, higher doses of INDO may affect 12-HETE and/or 15-HETE *in vivo* but they were not examined in this study. In the rat, 15-HETE increases by 12.4 fold prior to ovulation (Tanaka *et al.*, 1991) whereas in the pig it only increases by 2.3 fold (Mootoo, 1995); hence, the lack of effect of indomethacin on the LOX metabolites of arachidonic acid may be due to a lesser production in the pig. Furthermore, the eicosanoid concentrations were determined from ovarian homogenates in the rat whereas, in this study, the concentrations were determined in the follicular fluid such that intracellular and extrafollicular eicosanoids were not measured.

Mootoo (1995) speculated that in the pig, LOX products, other than 15-HETE, may be involved in porcine ovulation since NDGA, a inhibitor of LOX, injected into the ovarian stalk, inhibited ovulation but did not inhibit 15-HETE. NDGA is a strong inhibitor of 12-HETE and a moderate inhibitor of 5-HETE but does not inhibit 15-HETE in the rat ovary (Tanaka *et al.*, 1991); hence, either 12- or 5-HETE may be the LOX metabolite of importance in the porcine ovulatory process. A role for 15-HETE cannot be ruled out since it was not inhibited with NDGA and the effect of specifically inhibiting 15-HETE on

ovulation was not examined. A role for LOX products in porcine ovulation was not demonstrated under these experimental conditions, although this does not rule out the possibility that they may play a role since the effect of inhibiting these metabolites on ovulation was not observed in this study.

Future studies concerning a role for lipoxygenase metabolites in the porcine ovulatory process could include inhibiting these metabolites specifically and observing the effect on ovulation. Inhibition of ovulation with NDGA in the pig was observed by Mootoo (1995) but only 15-HETE was measured in the follicular fluid; a similar experiment could be performed but measuring the follicular concentrations of 5-HETE, 12-HETE and leukotrienes. Experiments using inhibitors of the LOX pathway other than NDGA could also be useful in determining if LOX metabolites are essential to the ovulatory process. An example of another LOX inhibitor is BW 755C which inhibits 5-HETE, 12-HETE and 15-HETE (Tanaka *et al.*, 1991). Profiles of the changes in concentrations of 5-HETE, 12-HETE and leukotrienes over time in the preovulatory porcine follicle, to my knowledge, have not been determined.

Previous studies in the pig regarding the role of prostaglandins in ovulation commonly used 10 mg/kg of INDO to block ovulation (Ainsworth *et al.*, 1979; Downey and Ainsworth, 1980; Hansen *et al.*, 1991); studies using less than 10 mg/kg of INDO have not been conducted. The results of this research indicate lower dose levels of INDO are appropriate in future studies of this kind.

Synthesis of PGF2α by both TIC and GC was inhibited when 10 mg of INDO or greater was added to the culture media per well, compared to the untreated control. Although INDO did not affect 12-HETE and 15-HETE *in vivo*, these LOX products were reduced *in*

vitro. 15-HETE was inhibited with 100 ng/ml or greater of INDO in both TIC and GC. This differs from what was found by Mootoo (1995), where 100 ng/ml did not inhibit 15-HETE production by porcine GC. The possible reason for this difference may be the length of time the cells were left in culture (24 versus 42 h). The longer culture time may have allowed the untreated controls in this experiment to synthesize more 15-HETE. INDO significantly suppressed 12-HETE production by GC at a dose of 100 ng/ml, but the 10 ng/ml and 500 ng/ml treatments had no effect. Indomethacin did not significantly suppress 12-HETE production in TIC, but a 43% decrease was seen with 10 ng/ml compared to the untreated control. In both cell types, it appears that 12-HETE decreases and then increases as the dose of INDO increases; a larger number of samples may have demonstrated more definite changes in 12-HETE.

Under these experimental conditions, INDO is not COX specific such that not only was PGF2α reduced but the LOX metabolites, 12-HETE and 15-HETE, were also suppressed. This is in agreement with the literature (Seigel *et al.*, 1979, 1980; Tanaka *et al.*, 1991). Possible reasons for this effect on LOX *in vitro* but not *in vivo* are: 1) at the level of the ovary, INDO concentration is not great enough to have an effect on LOX since, as was demonstrated *in vitro*, only higher concentrations of INDO suppress LOX or 2) FF concentrations do not reflect intra-cellular and extra-follicular 12-HETE and 15-HETE.

In the rat, inhibition of ovulation can be achieved by blocking progesterone synthesis with epostane, a 3β-HSD competitive inhibitor (Tanaka *et al.*, 1991, and Espey *et al.*, 1991). Attempts to inhibit ovulation in the pig with oral doses of danazol, an inhibitor of steroidogenesis, were unsuccessful (see appendix B). This may be due to a failure to find the correct dose and/or timing or to the fact that danazol may be too weak an inhibitor of

steroidogenesis to influence the preovulatory increase in progesterone. Intraovarian treatments may overcome the effect of low concentrations of danazol at the level of the ovary but they were not attempted in this study. Since danazol did not reduce progesterone *in vitro*, it is unlikely to do so *in vivo*.

In the rat, *in vivo*, inhibition of ovarian progesterone was associated with suppression of PGF2α, 15-HETE and 12-HETE (Espey *et al.*, 1991; Tanaka *et al.*, 1991). Under the conditions of this experiment, porcine GC and TIC, *in vitro*, treated with danazol showed no differences in progesterone production compared to untreated control cells and, similarly, no changes in eicosanoids were observed. Tsang *et al.* (1979b), demonstrated that danazol could inhibit progesterone synthesis in cultured porcine GC and luteal cells but this was not found in the present study.

Danazol was used in this research because other inhibitors of progesterone synthesis such as epostane and isoxazol were unavailable or, in the case of aminoglutethimide, had undesirable side effects if used *in vivo*. Based on the literature, the ideal way to inhibit actions of progesterone would have been to use a progesterone receptor blocker such as RU486 or onapristone. By blocking the progesterone receptor the effects of progesterone are inhibited but the progesterone itself is available as a substrate for androgen and estrogen synthesis; although it is unlikely that androgens and estrogens are involved during the time just prior to ovulation (Espey and Lipner, 1994), they should not be ignored. The reason a progesterone receptor blocker was not used in this research was these substances were not permitted for use in research or otherwise in Canada at the time the study was conducted.

To determine if progesterone has a role in the formation of eicosanoids during the porcine ovulatory process further research is required. Such research may include the use

of progesterone inhibitors other than danazol or the addition of progesterone to the culture media of porcine follicular cells to evaluate the effect of progesterone on eicosanoid production. The effect of intraovarian treatments of progesterone synthesis inhibitors on ovulation rate and eicosanoid concentrations in the follicular fluid could also demonstrate if progesterone has a role in the formation of eicosanoids.

Summary and Conclusions

In vivo, INDO can completely block ovulation in the prepubertal gonadotropin-primed gilt model at 300 mg and 600 mg per pig and significantly inhibits ovulation at 60 mg per pig. Under the conditions of this experiment, INDO inhibited FF concentrations of PGF2α, but did not cause significant suppression of 12-HETE and 15-HETE. This indicates that PGF2α is necessary for ovulation in the pig but does not rule out a role for 12-HETE and 15-HETE. Furthermore these data indicate that the 10 mg/kg dose level of indomethacin used in prior research on the role of PG in the porcine ovulatory process was greater than required and future studies can use lower dose levels which do not affect LOX metabolites. Roles for LOX metabolites in ovulation require further study.

In vitro, INDO was not specific for COX, such that LOX products, 12- and 15-HETE were also suppressed but at doses greater than required for inhibition of PGF2α.

Danazol, an inhibitor of steroidogenesis, under these experimental conditions had no significant effect on progesterone nor eicosanoid synthesis by porcine follicular cells *in vitro*; hence, no conclusions can be made on the requirements for progesterone in eicosanoid synthesis based on this study.

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Appendix A

Eicosanoid production by GC and TIC *in vitro* over time.

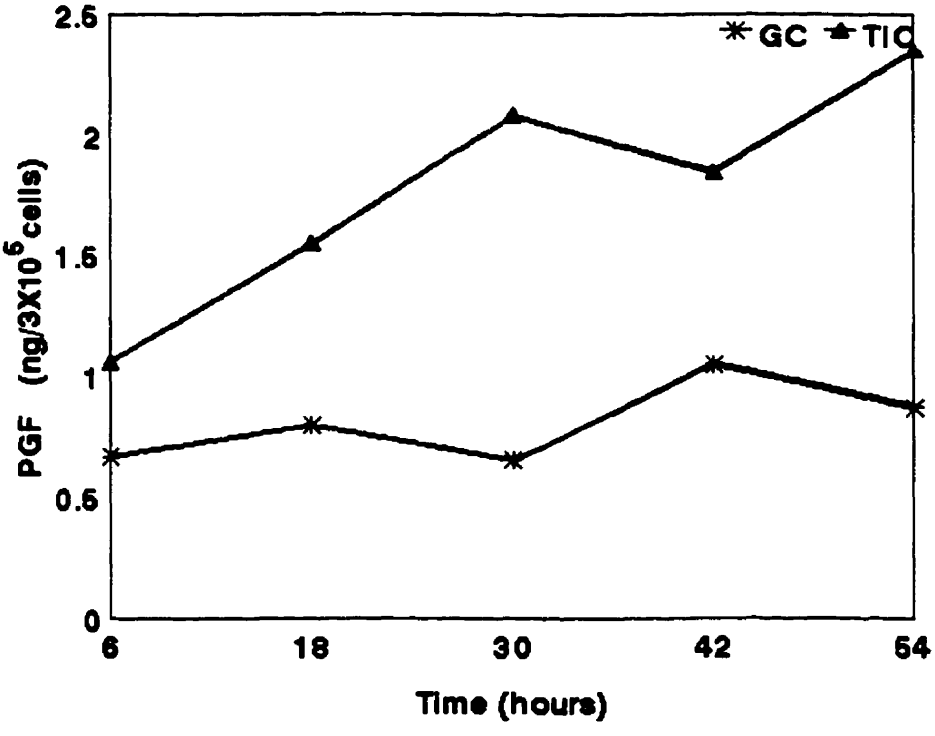

Figure 11 PGF2α production over time by GC and TIC. Points represent mean concentration of PGF2α in the culture media from cells pooled from two pigs in triplicate.

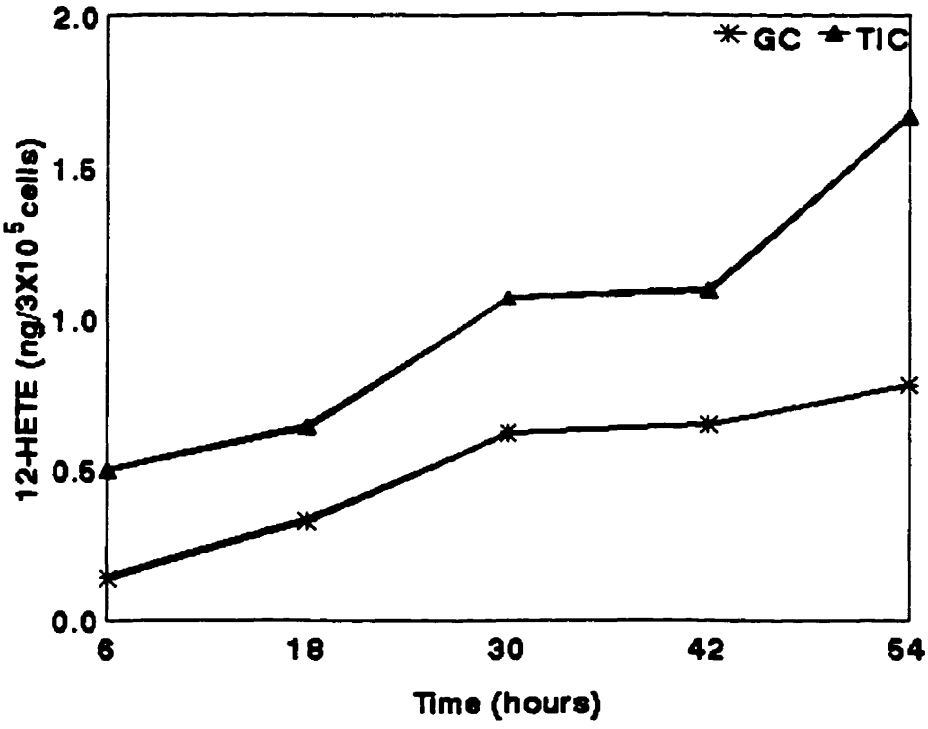

Figure 12 12-HETE production by GC and TIC over time. Points represent mean concentration in the culture media from cells pooled from two pigs in triplicate.

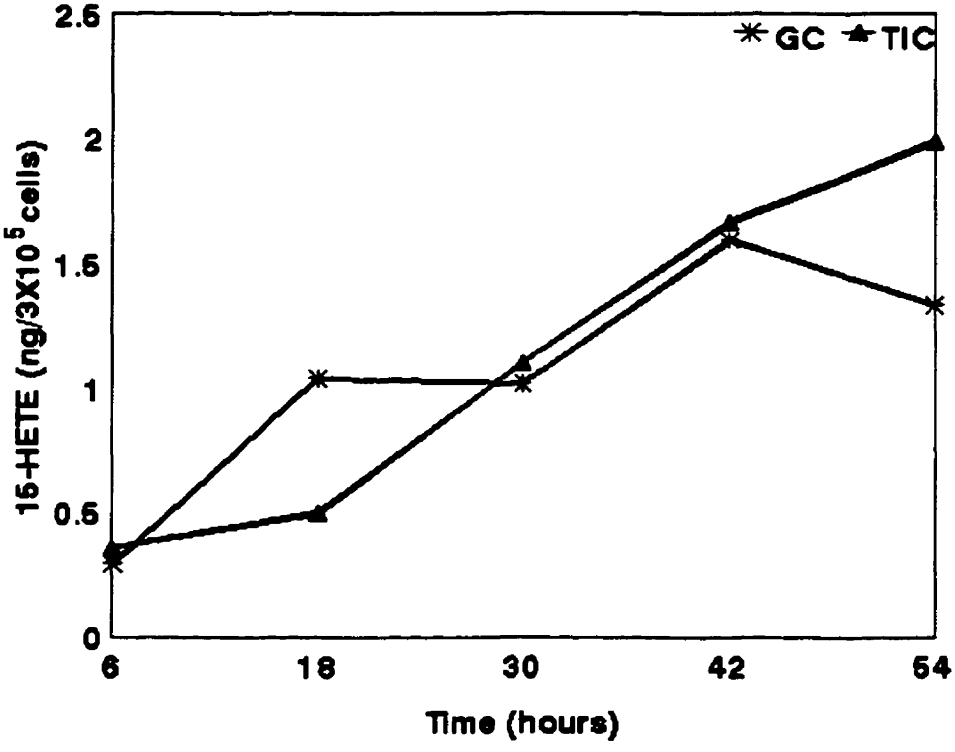

Figure 13 15-HETE production over time by GC and TIC. Points represent mean concentration in the cultur media from pooled cells of two pigs in triplicate.

Based on these data cells should be incubated for 36-42 h for experiment 3. The cells were incubated for 42 h for convenience

Appendix B

The effect of oral dose of danazol on ovulation rate at different times and doses.

Table 2 Effect of oral dose of danazol on ovulation rate.

dose (mg/pig)	time (h post hCG)	follicles (>6 mm), n	corpora lutea, n
200 X 3	12, 18, 24	3	15
400 X 4	12, 16.5, 21, 24.5	3	18
800 X 4	12, 16.5, 21, 24.5	4	8
1600 X 1	16	4	11

Observed numbers of follicles and corpora lutea are from one animal per treatment. Corresponding untreated control animals ovulated normally.

Appendix C

General pig data.

Table 3 Age, body weight and ovarian stimulation of PMSG/hCG treated prepubertal gilts.

	n	mean	sd	minimum	maximum
body weight (kg)	65	64.0	2.8	57	73
age (d)	64	126	9.9	106	154
sum of follicles and CL on both ovaries	63	13.0	6.25	1	28
left ovary	60	6.9	3.5	0	17
right ovary	60	6.3	3.6	0	15

n = number of animals observed; sd= standard deviation; minimum and maximum respectively correspond to the least and greatest observation made on an individual animal.

Not included with the above data were five pigs with no stimulation of the ovaries and one pig which only had one uterine horn and one ovary. Of the pigs examined, three had honeycomb type (follicles embedded in the ovary opposed to appearing grape-like) ovaries, one had one honeycomb type ovary and one grape type, and the rest all had grape type ovaries.

Table 4 Follicular fluid volumes collected.

	n	mean	sd
volume per pig (ml)	21	2.1	0.8
calculated volume per follicle (ml)	21	0.13	0.05

Table 5 Cell collection data.

		mean	sd
Granulosa cells	% viability	86	3.0
	No. of cells $X10^5$/ follicle	10.5	2.5
Theca cells	% viability	84.5	3.0
	No. of cells $X10^5$/follicle	8.0	2.6

The percent viability was determined using trypan blue staining. The number of cells per follicle was calculated by dividing the total number of cells collected by the number of follicles from which they were collected.