STUDIES ON PROSTAGLANDIN AND THROMBOXANE

PRODUCTION BY REPRODUCTIVE TISSUES

by

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I declare that this thesis was totally composed by myself and that all the experimental work described herein was performed by myself with the following exceptions:

The LH assay in Secion 2.2f was performed by Ms A. Horn and Ms L. Nairn. Animals used in Section 2.4 were ovariectomized and treated with steroid hormones by Dr. R.G. Gosden.

Carol G. Brown

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Some of the results presented in this thesis have been
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ABBREVIATIONS

AH-POA	-	Anterior hypothalamus and pre-optic area
LH	-	Luteinizing hormone
LHRH	-	Luteinizing hormone-releasing hormone
ME	-	Median eminence
NA	-	Noradrenaline
OVX	-	Ovariectomized
PG	-	Prostaglandin
TX	-	Thromboxane
min	-	minutes
h	-	hours
PMSG	-	Pregnant Mare serum gonadotrophin
GC-MS	-	Gas chromatography-Mass Spectrometry
NRS	-	Normal Rabbit serum
DARS	-	Donkey anti-rabbit serum
Ci	-	Curie ≡ 3.7×10^{10} Becquerels
Froben	-	2-(2-fluoro-4-biphenylyl) proprionic acid
BSTFA	-	bis (trimethylsilyl) trifluoracetamide

ABSTRACT OF THESIS

Measurement of PG and TX synthesis in reproductive tissues of the rat were carried out using radioimmunoassay. The study was in 3 main parts.

1. Measurement of PG and TX synthesis by ovarian tissue in relation to ovulation. Between pro-oestrus and oestrus, PGE-2, PGF-2α, 6-keto-PGF-1α production increased but there was a preferential increase in PGE-2. This was not due to altered arachidonic acid availability, decreased PGE-2 metabolism or increased conversion of PGF-2α to PGE-2. It is proposed that increased ovarian cyclooxygenase activity could increase the ratio of PGE-2 to the other PGs and TX at the time of ovulation.

2. Measurement of PG and TX synthesis by the hypothalamus in relation to LH release. PG and TX production by ME homogenates showed a diurnal variation, being high at 06:00h and low at 18:00h. There was an extra peak in PGE-2 production at 18:00h on pro-oestrus which is the time of LH release in the rat. Treatment of long-term OVX oestrogen-primed rats with progesterone, stimulated PGE-2 production by the ME. While 2-hydroxy oestradiol had no effect, NA stimulated PG and TX production by the ME. Since there are similarities in ME PGE-2 production and plasma progesterone concentrations it is proposed that progesterone stimulates PGE-2 synthesis in this tissue - possibly by causing NA release from noradrenergic nerve endings.

PG and TX production by AH-POA homogenates peaked at 22:00h on each day. However, there was no clear correlation between PG and TX production by the AH-POA and LH release.

PG synthesis by the brain has been proposed to be essential for LH release in the rat. This hypothesis was tested by examining the effect of a PG synthesis inhibitor, Froben, on the pro-oestrous LH surge. Froben completely inhibited the surge in 33% of treated animals and delayed the surge in the remaining animals. These results are in support of a physiological role for PGs in LH release.

3. Measurement of PG and TX synthesis by the uterus during the oestrous cycle and in relation to the effects of ageing on the process of implantation. Uterine PG and TX production increased on pro-oestrus and peaked at 02:00h on oestrus. Endometrial PGF-2α and TXB-2 showed the greatest percentage increase. Treatment of long-term OVX oestrogen-primed rats with progesterone stimulated uterine PGF-2α and TXB-2 production suggesting that ovarian steroids may be the physiological stimulus for uterine PG and TX synthesis. It is speculated that the peak in uterine PG and TX production at 02 :00h on oestrus may be associated with changes in uterine motility at that time.

The failure of the uterus to support pregnancy may be a factor which contributes to the reduced fertility of aged animals. Since PGs have been implicated in the process of blastocyst implantation the hypothesis that aged uteri show a deficiency in PG synthesis was tested. Uterine PGE-2 synthesis was reduced in the aged animal and there was a diminished response to oestrogen and progesterone treatment with ageing. Reduced PGE-2 synthesis may have significant implications for fertility in ageing uteri.

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GENERAL INTRODUCTION

Prostaglandins (PGs) have several characteristics which allow them to be distinguished from the classical hormones. Firstly, it appears that most, if not all, cell types have the capacity to synthesize PGs. In the female reproductive tract, PGs have been identified in the ovary, uterus, decidual tissue, menstrual fluid, placenta and amniotic fluid (see Poyser, 1981).

A second distinguishing feature of PGs is that they are not stored in tissues (with the only exception of PGs stored in primate seminal vesicles) and their synthesis must therefore immediately precede their release into tissue fluids or into the circulation. Many stimuli, including simple mechanical damage, cause PG release (Pace-Asciak and Wolfe, 1968; Flower and Blackwell, 1976).

A third distinguishing feature of PGs is their short half-life. PGE and PGF are effectively metabolized in the circulation by one passage of the blood through the lungs (Ferreira and Vane, 1967). This short half-life suggests that they may act as local mediators or modulators of cell function but are not hormones by the classical definition since they cannot circulate in biologically active concentrations. Thromboxane A-2 (TXA-2) is rapidly hydrolyzed into the almost inactive TXB-2 with a half-life of about 30 sec in aqueous solution. PGI-2 escapes lung metabolism and has recently been proposed as a circulating hormone (Moncada, Korbut, Bunting and Vane, 1978).

Chemically, the PGs are 20-carbon fatty acids consisting of 2 side chains of 7 and 8 carbon atoms respectively, linked by a cyclopentane ring. PGs of the 2-series are derived from arachidonic acid which is obtained from the diet or synthesized from another dietary fatty acid linoleic acid. The first stage in the biosynthesis of PGs of the 2-series requires the release of free arachidonic acid from cholesterol esters, phospholipids or triglycerides by the action of the appropriate esterases and lipases. Arachidonic acid is then converted by the action of a membrane-bound enzyme complex (PG synthetase) initially by the cyclooxygenase component into PGH-2, an unstable endoperoxide intermediate. PGH-2 is then converted by other enzymes and factors in the PG synthetase complex into the different PGs and TX. The latter compound is distinguished chemically by the presence of an oxygen atom in the ring system. Lipoxygenation of arachidonic acid gives rise to another group of compounds, the leukotrienes.

Since PGs were first identified in seminal fluid (von Euler, 1932) much attention has been directed towards their connection with reproductive function. The particular reproductive processes which have been the subject for study in this thesis are ovulation and the release of gonadotrophins. In addition, uterine PG and TX production either during the oestrous cycle or by uterine tissue which has been sensitized for the decidual cell response has been investigated.

The unifying aim of these studies is to investigate the physiological significance of PG and TX in these processes and also to investigate the regulation of PG and TX synthesis by reproductive tissues in the rat.

Prostaglandins and Ovulation

The involvement of PGs in ovulation was first proposed by Armstrong (1970), as an extension of the hypothesis of Pharris and Wyngarden (1969). The latter authors suggested that PGF-2α could precipitate luteolysis by virtue of its potent venoconstrictive activity (Du Charme and Weeks, 1968). Luteolysis was proposed to arise as the result of luteal ischaemia. Armstrong (1970) proposed that luteinizing hormone (LH), by increasing cholesterol esterase activity (Behrman and Armstrong, 1969) may cause increased release of arachidonic acid from cholesterol arachidonate and hence may increase PGF-2α production. He suggested that the increased PGF-2α levels, acting in concert with increased capillary permeability in response to LH (Zachariae, 1958) may cause intrafollicular fluid accumulation and hence follicular rupture.

Further support for the hypothesis that PGs are involved in ovulation arose from the observation that inhibitors of PG synthesis can block ovulation. Indomethacin (Armstrong and Grinwich, 1972) or aspirin (Orczyk and Behrman, 1972) administered to rats at the time of the LH surge, blocks ovulation. Since exogenous LH did not overcome this block it was concluded that indomethacin was not acting by preventing LH release from the brain but rather, inhibited ovulation by a direct action on the ovary (Armstrong and Grinwich, 1972). Tsafriri, Lindner, Zor and Lamprecht (1972) confirmed that the anti-ovulatory action of indomethacin was by a direct action on the ovary, preventing follicular rupture while permitting ovum maturation.

In rabbits, indomethacin prevented ovulation when injected either 30 min before administering exogenous LH (Grinwich, Kennedy and Armstrong, 1972) or 8h after mating (O'Grady, Caldwell, Auletta and Speroff, 1972). Indomethacin neither interfered with follicular luteinization in the rabbit (Grinwich et al., 1972), nor prevented the elevation of progesterone levels to the usual post-ovulatory range (O'Grady et al., 1972), suggesting that indomethacin affects only the physical process of ovulation, while follicular development and secretory potential remain intact. Likewise, indomethacin inhibited human chorionic gonadotrophin (hCG) - induced ovulation in the Rhesus monkey, without interfering with follicular maturation or inhibiting steroid production (Wallach, de la Cruz, Hunt, Wright and Stevens, 1975). These initial findings were confirmed for several other species including mice (Lau, Saksena and Chang, 1972) marmoset monkeys (Maia, Barbosa and Continho, 1978) and pigs (Ainsworth, Tsang, Downey, Barker, Marcus and Armstrong, 1979). The only study in women, using aspirin to block PG synthesis failed to demonstrate ovulation blockade (Chaudhuri and Elder, 1976). However, the dosage of aspirin may have been insufficient to block PG synthesis in the ovarian follicles, a parameter which was not assessed in the study. The toxicity of indomethacin in all of these experiments is an unknown factor, and the action of the drug as an inhibitor of PG synthesis, may not be its only effect. However, since these early observations, it has been reported that superovulation, induced by pregnant mare serum gonadotrophin (PMSG) or hCG in immature rats is inhibited by anticyclooxygenase antiserum in spite of continued ovarian progesterone production (Satoh, Fukoka, Wu, Mitsuhashi, Kinoshita and Sakamoto, 1981).

These studies indicate that ovarian PG synthesis is necessary for ovulation to occur.

Effects of PG administration on ovulation

In rats in which ovulation had been blocked by indomethacin, PGE-2 administration overcame this block in the majority of animals treated (Tsafriri et al., 1972). In nembutal-treated rats (where the LH surge is prevented), PGE-2 treatment during the afternoon of pro-oestrus, induced final maturation of the ovum as well as ovulation (Tsafriri et al., 1972). However it had previously been shown that indomethacin will block ovulation while ovum maturation proceeds normally. These observations suggest that, although PGE-2 can mimic LH in inducing ovum maturation and ovulation, the former process does not require endogenous PG production (Tsafriri et al., 1972). In monkeys (Wallach, Bronson, Hamada, Wright and Stevens, 1975) and rabbits (Diaz-Infante, Wright and Wallach, 1974), exogenously administered PGF-2α can reverse the inhibitory effect of indomethacin on ovulation induced by gonadotrophin.

A species difference is apparent when considering which PG is most effective in causing ovulation. Treatment of rabbits with PGE-2 actually delayed hCG-induced ovulation (Richman, Wright and Wallach, 1972) whereas PGE-2 was most effective in causing ovulation in the rat (Tsafriri et al., 1972). More recently, it has been shown that PGF-2α alone induced ovulation in the absence of gonadotrophin in the isolated perfused rabbit ovary (Wallach, Wright and Hamada, 1978; Kobayashi, Santutti, Wright and Wallach, 1981).

Ovarian PG Synthesis

During the process of ovulation, the endogenous levels of PGs in the ovary undergo marked changes. Follicles isolated from rabbits at various times between the ovulatory LH surge and ovulation showed a striking increase in PG levels as ovulation approached (Le Maire, Yang, Behrman and Marsh, 1973). PGF and PGE levels were similar although PGF levels had increased 50-fold whereas PGE levels had only increased 11-fold.

In a study, extended to include follicles obtained from rabbits up to 48h after hCG injection, Yang, Marsh and Le Maire (1974) found that PGF levels fell rapidly after ovulation whereas PGE levels continued to rise for several hours after ovulation before declining. This suggests differential roles for these two PGs in ovulation in the rabbit. Moreover, the increase in both PGs was confined to the follicles which went on to ovulate (Yang et al., 1974).

Indomethacin administration just prior to hCG or LH treatment, or after mating abolished the increase in PG levels in rabbit follicles as well as preventing ovulation (Yang et al., 1973). Armstrong, Grinwich, Moon and Zamecnik (1974) demonstrated that the anti-ovulatory action of indomethacin was local by micro-injection of indomethacin into rabbit follicles. As well as blocking ovulation, the injection prevented the LH-induced increase in follicular PGF. Additionally they demonstrated that while treatment with PGF antiserum blocked ovulation, PGE antiserum was only partially effective (Armstrong et al., 1974). This further suggests that PGF may be more important than PGE in mediating LH-induced ovulation at the follicular level in the rabbit.

Levels of PGE and PGF in rat ovaries increased to reach a peak at 05.00h on oestrus, i.e. shortly after the time of ovulation (Bauminger and Lindner, 1975). PGF levels increased 6-fold whereas PGE levels increased 30-fold. There was a concomitant increase in PG synthetase levels. By 10.00h on oestrus, levels of PGE, PGF and PG synthetase had declined to basal values. Administration of nembutal prevented the pre-ovulatory rise of both PGE and PGF. LH administration restored the rise in PGE and PGF levels (Bauminger and Lindner, 1975), suggesting that LH stimulates PG synthesis by the ovary.

Intrafollicular PGE and PGF levels also increased towards the time of ovulation in the rat (Le Maire, Leidner and Marsh, 1975). Early on pro-oestrus, PG levels were low and PGF levels exceeded PGE levels. Towards the time of ovulation, the levels of both PGE and PGF increased and at the time of ovulation, PGE levels exceeded PGF levels. Within 4h both PGE and PGF levels had returned to basal, and the ratio of PGE to PGF had reverted back in favour of PGF (Le Maire et al., 1975).

Pre-ovulatory increases in ovarian PG levels have also been demonstrated for other species. In the pig, PGF and PGE levels in follicular fluid increased a few hours prior to ovulation (Tsang, Ainsworth, Downey and Armstrong, 1979). Ovulation and the increase in PG levels were prevented by indomethacin treatment (Ainsworth et al., 1979) and, as in the rabbit, PGF-2α but not PGE-2 overcame the inhibition of ovulation by indomethacin (Ainsworth et al., 1979; Downey and Ainsworth, 1980). In guinea-pigs, ovarian synthesis of PGE-2 and PGF-2α increased markedly 3 days before ovulation and reached a peak on the day of ovulation. PGF levels exceeded PGE levels throughout the pre-ovulatory period (Sharma, Wilson and Pugh, 1976).

In the ewe, follicular PGE and PGF levels were increased 8h after the initiation of the LH surge, again PGE-2 showing a greater percentage increase in the pre-ovulatory period (Murdoch, Dailey and Inskeep, 1981). Increased levels of PGF-2α have been observed in venous blood draining the human ovary containing the developing follicle when compared to the inactive ovary (Askel, Shromberg and Hammond, 1977). The concentration of PGF-2α was higher in corpora lutea immediately after ovulation than at any time during the luteal phase (Swanston, Mcnatty and Baird, 1977). Similarly the concentrations of PGF-2α in fluid taken from pre-ovulatory follicles was higher than in fluid taken from three less mature follicles (Edward, 1973). In a large-scale study, PGF-2α concentrations measured in human follicular fluid rose 2 to 3 days before ovulation, and reached a peak at the time of ovulation, before falling after mid-cycle (Darling, Jogee and Elder, 1982). These observations are consistent with the proposal that PGs play a role in the ovulatory process at the follicular level.

The stimulus for ovarian PG synthesis

The stimulus for ovarian PG synthesis appears to be gonadotrophin since in all these species, the increase in PG concentrations follows either the ovulatory surge of LH or the exogenous administration of LH or hCG. Indeed, injection of antiserum to LH suppressed PG synthesis by ovarian homogenates, whereas simultaneous injection of LH or addition of LH to the homogenates restored PG synthesis (Chasalow and Pharriss, 1972).

Bauminger and Lindner (1975) also reported that pre-treatment of rats with antiserum to the β-subunit of LH prevented both the increase in PG levels and the increase in PG synthetase levels which normally occur between late pro-oestrus and early oestrus . PG synthetase levels in granulosa cells are also increased 4 to 8h after the ovulatory LH surge. This increase in PG synthetase levels can be stimulated by treating PMSG-primed rats with hCG (Clark, Chainy, Marsh and Le Maire, 1979).

In addition to LH, follicle stimulating hormone (FSH) stimulated follicular PGF and PGE synthesis in the rat (Bauminger et al., 1975). This effect was not due to contamination with LH, as a stimulatory effect of FSH was still present after treatment of the gonadotrophin preparation with antiserum to the sub-unit of LH.

In follicles isolated from rabbits at oestrus PGE synthesis could be stimulated by LH or hCG whereas FSH, prolactin or bovine serum albumin were without effect (Marsh, Yang and Le Maire, 1974). The synthesis of PGF in bovine follicular tissue *in vitro* was stimulated by LH (Shemesh and Hansel, 1975), and human follicular tissue synthesizes PGF *in vitro* after exposure to gonadotrophin (Plunkett, Moon, Zamecnik and Armstrong, 1975). More recently, Veldhuis, Klase and Demers (1982) reported that LH elicited a dose-dependent increase in 6-keto-PGF-1α accumulation in granulosa cells isolated from mature swine Graafian follicles, while FSH, prolactin, adrenaline, oestradiol-17β and PGE-2 were devoid of this activity. LH also stimulated 6-keto-PGF-1α synthesis by rat granulosa cells incubated *in vitro* (Koos and Clark, 1982). All these studies indicate that LH released from the anterior pituitary gland is the physiological stimulus for increased PG production by the ovary.

Luteinizing hormone-releasing hormone (LHRH) may mimic the actions of LH on the ovary. Analogues of LHRH have been reported to stimulate follicular luteinization (Hseuh, Wang and Erickson, 1980) oocyte maturation (Hseuh et al, 1980; Hillensjo and Le Maire, 1980) and progesterone accumulation (Knecht, Katz and Catt, 1981; Jones and Hseuh, 1981). Furthermore, an LHRH-like peptide has been identified in ovarian follicular fluid (Ying, Ling, Bohlen and Guilleman, 1981). Therefore LHRH may be the intra-ovarian stimulant of PG synthesis. In a study of the effects of LHRH and LHRH analogues on ovarian granulosa cell PG synthesis, Clark, Thibier and Marsh (1980a), observed that these compounds, when added *in vitro* to cell incubates from PMSG-treated rats, stimulated PGE and PGF accumulation. A stimulatory effect of an LHRH analogue $[\text{D-Ala}^{6}, \text{des-Gly-NH}^{2-10}]$ LHRH-ethylamide on ovarian PGE synthesis was also observed when administered to hypophysectomised PMSG-treated immature rats (Ekholm, Clark, Magnusson, Isaksson and Le Maire, 1982). Thus although the physiological role of locally produced LHRH-like peptides is unclear, they may control ovulation, in addition to LH, by stimulating ovarian PG synthesis. Indeed ovulation induced by $[\text{D-Ala}^{6}, \text{des-Glyn-NH}^{2-10}]$ LHRH ethylamide in PMSG-treated rats was blocked by indomethacin administration (Ekholm et al., 1982).

Involvement of cAMP in ovarian PG synthesis

.Kuehl, Humes, Tarnoff, Cirillo and Ham (1970) suggested initially that PGs function as a necessary intermediate in the effect of LH upon ovarian adenosine 3' 5' monophosphate (cAMP) production. They observed a dose-related increase in cAMP production by mouse ovaries in response to PGE-1.

The PG-induced increase in cAMP was blocked by a PG antagonist, 7-oxa-13-prostynoic acid, and in addition the antagonist blocked LH-induced cAMP formation. However, when the time courses of the accumulation of ovarian cAMP and PGE in response to LH were compared, no changes in ovarian PGE content could be detected after injection of LH at a time when cAMP accumulation was markedly enhanced (Rigler, Peake and Ratner, 1976). Moreover, indomethacin did not inhibit the LH-induced rise in cAMP (Raj, 1974). These results suggest that it is unlikely that PGs are mediators of LH action on follicular cAMP production as suggested by Kuehl et al, (1970). It is more likely that the converse is true, in that cAMP mediates the stimulation of ovarian PG accumulation by LH. cAMP added to incubates of ovarian follicles from rabbits (Marsh, Yang and Le Maire, 1974) and rats (Clark, Marsh and Le Maire, 1978a) mimics the effect of LH in stimulating the synthesis of both PGE and PGF. This effect was specific for cAMP, as other nucleotides such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), 3' adenosine monophosphate (3' AMP), 5' AMP, cyclic guanosine monophosphate (cGMP) and O '-monobutyryl cAMP did not stimulate PGE accumulation (Clark et al., 1978a). However, a cAMP analogue N^6 -monobutyryl cAMP produced a similar increase in PGE accumulation to that seen with LH (Clark et al., 1978a). Although Clark et al., (1978a) did not observe a stimulation of PGE synthesis with cGMP, it has been reported that 8-bromo- cGMP increases PGE production by isolated rat Graafian follicles during a 6h incubation (Zor, Strulovici, Nimrod and Lindner, 1977). Information concerning the influence of LH on cGMP levels is conflicting however.

Whereas hCG has been reported to stimulate ovarian cGMP levels (Rao and Carman, 1973), other workers report that LH decreased cGMP levels in rat ovaries in vitro (Ratner, 1976). Therefore, it is difficult to interpret the effects of 8-bromo-cGMP on ovarian PGE accumulation.

Ovarian PGE synthesis is also stimulated by two other agents which elevate follicular cAMP levels, namely, 1-methyl-3 isobutyl xanthine (Zor et al., 1977) and cholera toxin (Clark et al., 1978a). With the exception of the anomalous result of Zor et al., (1977), these results suggest that the stimulation of follicular PG synthesis is specific for cAMP-like nucleotides.

The increase in PGE accumulation by cAMP in rat follicles occurred after 3h which is similar to the time course of PG stimulation by LH (Clark et al., 1978a). In the mouse, cAMP stimulated ovarian PG production (Kuehl, Cirillo, Ham and Humes, 1972), and likewise in pig follicles, increased cAMP levels preceded the pre-ovulatory increase in follicular PG production (Tsang et al., 1979).

As well as the stimulation of ovarian PGE synthesis by cAMP, it has already been stated that PGE in turn stimulates cAMP formation (Kuehl et al., 1970) indicating that a positive feedback mechanism may operate. Quenching of this feedback mechanism could be brought about by the refractoriness of ovarian tissue to PGE which has been observed after continued exposure to this PG (Zor, Lamprecht, Misulovin, Koch and Lindner, 1976).

All these results suggest that LH-induced PG production within the follicle is mediated by cAMP. However, increased ovarian cAMP levels do not appear to be a pre-requisite for increased PG synthesis.

Concanavalin A induced PGE production by isolated Graafian follicles after a 2h incubation with no detectable rise in cAMP levels until 6h of incubation (Strulovici, Weinstein and Zor, 1981). Moreover, colchicine blocked the effect of Concanavalin A on cAMP formation with no inhibition of follicular PGE synthesis (Strulovici et al., 1981). Additional support for a cAMP-independent mechanism for ovarian PGE synthesis came from the observation that [D-Ala^{6}, des-Gly-NH$^{2-10}$] LHRH-ethylamide stimulated PGE accumulation in rat granulosa cells without an associated effect on cAMP accumulation in these cells (Clark et al., 1980a).

PG synthesis and ovarian steroid secretion

It has been suggested (Le Maire and Marsh, 1975) that the effect of LH and cAMP on PG synthesis might be mediated by increasing steroidogenesis. However, Bauminger, Lieberman and Lindner (1975), have shown that stimulation of PG accumulation in rat follicles by LH and FSH was unimpaired during suppression of progesterone and oestrogen synthesis by aminoglutethimide. Consequently, increased steroid production is not necessary for increased PG production in the ovary.

The mechanism of LH-stimulated PG synthesis in the ovary

When considering the time course of follicular cAMP and PGE accumulation in response to LH, it is apparent that the two responses differ markedly.

Whereas cAMP accumulation was augmented within minutes (Marsh, Mills and Le Maire, 1972) there was a delay of 2 to 3h between the exposure to LH (<u>in vivo</u> or <u>in vitro</u>) and the earliest detectable increases in follicular PGs (Bauminger and Lindner, 1975). This lag period suggests that macromolecular synthesis may participate in the mechanism of the LH-stimulated PG accummulation. To test this hypothesis, Clark, Marsh and Le Maire (1976) incubated follicles from PMSG-treated rats with LH in the presence of various concentrations of the protein synthesis inhibitor puromycin. The LH-stimulated PGE accumulation was lowered in the presence of 10μM puromycin and was completely inhibited at a concentration of 100μM. Puromycin aminonucleoside, which does not inhibit protein synthesis (Nathans and Neidle, 1963) had no effect on LH-stimulated PGE accumulation (Clark et al., 1976). Further studies showed that both the LH- and cAMP-induced increased in follicular PGE production were blocked by inhibitors of protein and nucleic acid synthesis (Zor et al., 1977). Indeed it has been known for several years that protein and ribonucleic acid (RNA) synthesis inhibitors will suppress ovulation (Pool and Lipner, 1964; 1966). This effect of protein synthesis inhibitors could be explained by their inhibitory effects on ovarian PG synthesis. Increases in follicular RNA and protein synthesis precede ovulation (Mills, 1975). Although the proteins affected have not been identified, one of them may be PG synthetase. Bauminger and Lindner (1975) have observed an increase in ovarian PG synthetase activity in the rat ovary immediately before ovula tion. They found a 50 to 80% increase in activity of the enzyme and this was associated with the pre-ovulatory increases in ovarian PG concentrations.

In granulosa cells from immature PMSG-treated rats, LH was found to stimulate PG synthetase activity approximately 5-fold (Clark, Marsh and Le Maire, 1978b). A similar marked stimulation of granulosa cell PG synthetase activity was observed 5h and 10h after in vivo exposure of immature PMSG-treated rats to hCG (Clark, Chainy, Marsh and Le Maire, 1979). These authors also found that in mature rats, granulosa cell PG synthetase activity increased late on pro-oestrus by approximately 10-fold, concomitantly with a 5-fold increase in follicular fluid PG levels. It is apparent that an increase in PG synthetase concentrations does take place following the LH surge, but prior to ovulation.

Mechanism of PG-induced follicular rupture

PG synthesis in ovulatory follicles may be important in the connective tissue decomposition and subsequent thinning of the follicle wall. A comparison of the ultrastructure of ovulatory follicles with follicles from indomethacin-treated rabbits showed that, by 9h after coitus (near the time of ovulation), the tissue at the apex of normal follicles begins to thin out, whereas follicles from indomethacin-treated animals showed no signs of thinning as late as 12h post-coitus (Espey, Coons, Marsh and Le Maire, 1981). In fact the normal transformation of follicular fibroblasts from a quiescent to a proliferative state was inhibited in indomethacin-treated animals and it has been suggested that the normal elevation in PGs might be necessary to stimulate the synthesis or release of proteolytic enzymes from the fibroblasts (Espey et al., 1981).

In support of this suggestion, PGE-1 and PGE-2, but not PGF-2α, have been shown to stimulate the production of the enzyme, plasminogen activator, from rat granulosa cells (Strickland and Beers, 1976). LH also stimulates granulosa cells to produce this enzyme *in vitro*, although FSH is more active in this respect. Plasminogen activator converts the inactive protein plasminogen to plasmin which is capable of weakening the follicular wall (Strickland and Beers, 1976).

In addition to the enzymatic weakening of the follicular wall, a role for smooth muscle contractions in follicular rupture has been proposed. The wall of the Graafian follicle has contractile properties (Guttmacher and Guttmacher, 1921) and the theca externa layer of rat Graafian follicles contains the contractile proteins actin and myosin (Walles, Groschel-Stewart, Owman, Sjoberg and Unsicker, 1978). In rabbits, ovarian contractility is reduced by indomethacin and is restored following PGF-2α administration (Diaz-Infante, Wright and Wallach, 1974). It was proposed that, by stimulating ovarian contractility, follicular rupture would be promoted. However, in another study in rabbits, no association between ovarian contractility, ovulation and the number of ovulation points could be found (Demers and Gable, 1976).

Figure 1 shows a model for the biochemical mechanism of ovulation.

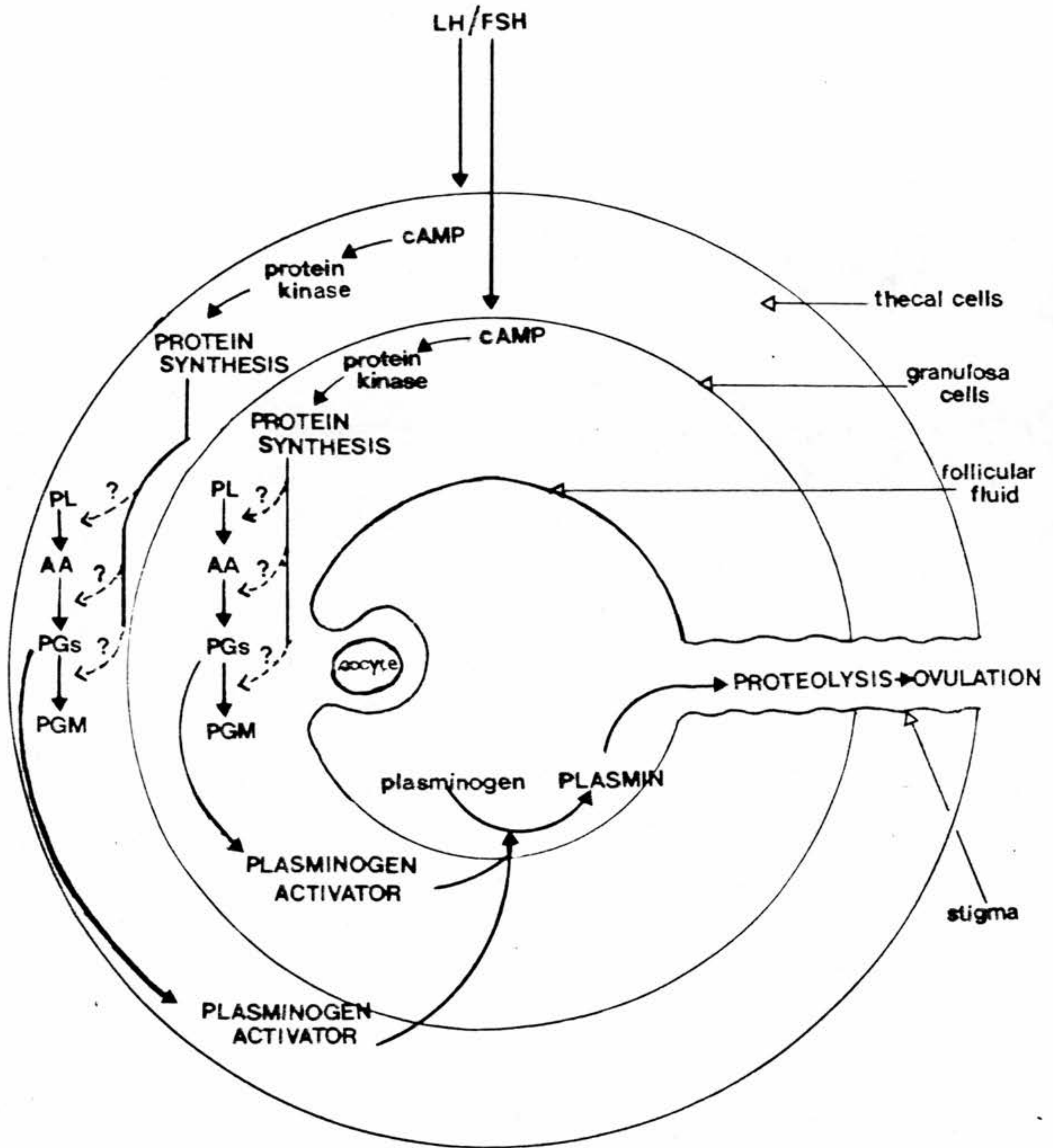

Figure 1. A model for the biochemical mechanism of ovulation in the rat.
PL = phospholipids
AA = arachidonic acid
PGM = PG metabolites

Summary and aims

In summary, PGs (particularly PGE-2) mimic many of the biological actions of LH on the ovary - ovum maturation, luteinization, cAMP accumulation and steroidogenesis - but PGs are not essential for these processes. However, PGs appear to be indispensable for the process of follicular rupture. PGE-2 seems to be the PG involved in ovulation in the rat, while PGF-2α is involved in ovulation in other species.

Many features of the control of ovarian PG biosynthesis remain unexplained. For example, the cellular mechanism which brings about an apparently directional synthesis of PGs in the rat requires investigation. In addition, the study of ovarian PG synthesis has largely been confined to the E-series PGs, and therefore the study of other cyclo-oxygenase products of arachidonic acid metabolism in the ovary is required.

The majority of evidence suggests that LH stimulates an increase in ovarian PG synthetase. However another possibility is that the site of action of LH may be at the level of catabolism of the PGs. In this case LH may inhibit PG breakdown thereby increasing the accumulation of these substances.

The experiments described in Section 2.1 were aimed at the study of ovarian PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 synthesis during the oestrous cycle and at the elucidation of the control of their synthesis by the rat ovary.

PGs and Gonadotrophin Secretion

The secretion of anterior pituitary hormones is under specific control by hypothalamic releasing factors, and by the feedback action of gonadal, adrenal and thyroid hormones. The releasing factors are themselves released from nerve endings in the median eminence (ME) (Pelletier, Labrie, Puviani, Arimura and Schally, 1974) and are transported to their site of action by the hypophysial portal vascular system.

In the rat (Sarkar, Chiappa, Fink and Sherwood, 1976) and monkey (Neill, Dailey, Tson, Patton and Tindrill, 1976) it has been demonstrated that the pre-ovulatory LH surge coincides with a surge of luteinizing hormone-releasing hormone (LHRH) in hypophysial portal blood. The LHRH surge depends upon the pre-ovulatory surge of circulating oestradiol-17β (Sarkar and Fink, 1979).

Much evidence is accumulating which suggests an involvement of PGs in the control of gonadotrophin release from the brain and it has been postulated that PGs are an essential link between the stimulatory effect of oestradiol and the LH surge (Craig, 1975).

PG synthesis by the brain

Hypothalamic tissue contains a relatively higher concentration of PGs than other regions of the brain (Holmes and Horton, 1968). These authors also found that, in general, the concentration of PGF was greater than PGE.

In agreement, Wolfe, Pappius and Marion (1976) found that incubates of rat, cat and human cerebral cortex slices synthesized more PGF-2α than PGE-2. Moreover, noradrenaline (NA), added in vitro, stimulated the formation of PGF-2α in slices and homogenates. In addition to PGE-2 and PGF-2α, PGH-2 was converted to PGD-2 and 6-keto-PGF-1α by rat brain homogenates (Pace-Asciak and Nashat, 1976). Subsequent studies have shown PGD-2 to be the major PG formed in rat brain (Saeed Abdel-Halim, Hamberg, Sjoquist and Anggard, 1977; Saeed Abdel-Halim and Anggard, 1979). Higher concentrations of PGD-2 were formed in the pineal body, hypothalamus and pituitary gland than elsewhere in rat brain (Narumiya, Ogorochi and Hayaishi, 1982).

Using a bioassay technique, Cseh, Szabo, Lang and Palkovits (1978), demonstrated that PG concentrations were higher in the hypothalamus than in other areas of the rat brain. PGF-2α levels exceeded PGE-2 levels in most tissues with the exception of the median eminence (ME) where PGE-2 predominated. Moreover, in the median eminence, PGE-2 concentrations were approximately 10-fold higher than elsewhere in the brain. Using radioimmunoassay (RIA), Ojeda, Naor and McCann (1978) were able to confirm the results of Cseh et al, (1978). They showed that the PGE content of the ME was much higher than in the remainder of the medial basal hypothalamus (MBH) in both male and female rats. However, no difference in PGE content of the ME, MBH or anterior pituitary gland was observed between male and female animals.

When considering the 'newer' arachidonic acid cyclooxygenase metabolites, it has been reported that TXB-2 formation by incubates of guinea pig and rat cerebral cortex was 5-6 fold greater than that of PGF-2α or PGE-2 (Wolfe, Rostworoski and Marion, 1976).

However, divergent results exist as other workers report that rat brain homogenates synthesize less TXB-2 than PGF-2α (Saeed Abdel-Halim et al., 1977). The reason for differences in these results is at present unclear, as both groups used a similar GC-MS technique. The formation of 6-keto-PGF-1α in homogenates of mouse, rat and rabbit brain was low in relation to that of PGD-2, PGE-2 and PGF-2α, but 6-keto-PGF-1α was the major PG present in cerebral blood vessels (Saeed Abdel-Halim, Lindner, Cseh and Anggard, 1980). Likewise, in the rat, 6-keto-PGF-1α formation by homogenates of isolated brain microvessels was much higher than in whole cortical homogenates, suggesting that 6-keto-PGF- 1α synthesis by the brain is of vascular, rather than of neuronal origin (Birkle, Wright, Ellis and Ellis, 1981). Information is lacking on the regional distribution of 6-keto-PGF-1α and TXB-2 in the brain.

Since PG synthesis by brain tissue has been demonstrated, the proposal that PGs produced by the brain may be involved in gonadotrophin secretion has gained support. In particular, the higher concentration of PGE in the ME compared to other areas of the brain suggests that PGE synthesis by this tissue (which is also rich in releasing factors (Krulich, Quijada, Wheaton, Illner and McCann, 1977) may play a role in the physiological control of hypothalamo-pituitary function.

Effects of indomethacin on ovulation

Indomethacin can block ovulation by a direct action on the ovary (see earlier). In addition, indomethacin may also block ovulation by inhibiting LH release (Orczyk and Behrman, 1972). Ovulation in rats was blocked by indomethacin given as a single injection 6h before the expected LH surge and this block was overridden by LH administered 3h after the indomethacin (Orczyk and Behrman, 1972). This dose of indomethacin reduced the hypothalamic and pituitary PGF levels. This experiment suggests that indomethacin blocked ovulation by preventing the release of LH. However, the site of the blocking action of indomethacin could have been either at the hypothalamic or pituitary level. Support for a hypothalamic site of action of PG synthesis inhibitors arose from experiments demonstrating that an aspirin-induced blockade of ovulation was consistently reversed by LHRH given twice to rats either 3h and 4h or 2h and 3h after aspirin administration. However, LHRH did not reverse indomethacin-induced ovulation block (Behrman, Orczyk and Greep, 1972). This is inconsistent with the effects of LH on indomethacin-blocked ovulation as reported by Orczyk and Behrman (1972) and suggests that aspirin may exert its blocking effect on ovulation primarily at the hypothalamic level, whereas indomethacin appears to block ovulation at both the ovarian and hypothalamic levels. The fact that indomethacin was equally effective in blocking ovulation when given to rats before or after the pre-ovulatory LH surge (Armstrong and Grinwich, 1972) argues primarily for an ovarian site of action for indomethacin.

However, the latter authors noted a considerably higher incidence of follicular luteinization and loss of uterine lumenal fluid when indomethacin administration was delayed until 16:00h, i.e. after the LH surge. The lack of follicular luteinization or of evidence for progesterone secretion (as indicated by loss of uterine lumen fluid) in rats which received indomethacin before the critical period for LH release, suggests that LH secretion was inhibited by indomethacin. Obviously, the timing of indomethacin administration in relation to the LH surge is crucial as to the overall effects produced.

Effects of indomethacin on LH release

Although it is clear that inhibitors of PG synthesis are capable of interfering with ovulation probably by inhibiting LH release, in studies so far, indomethacin has failed to suppress the pre-ovulatory LH surge (Tsafriri, Koch and Lindner, 1973; Sato, Taya, Jyujo and Igarashi, 1974). However, on the basis of these two "indomethacin experiments" alone, it is not possible to rule out an involvement of PGs in LH release as the lack of effect of indomethacin treatment on the LH surge in these two studies might be attributable to its relatively poor uptake into the CNS (Hucker, Zacchei, Cox, Brodie and Cantwell, 1966). Although no reports are available demonstrating suppression of the pro-oestrous LH surge, indomethacin will suppress plasma LH concentrations in ovariectomized rats (Ojeda, Harms and McCann, 1975; Sato, Jyujo, Hirono and Iesaka, 1975).

The intravenous injection of indomethacin 3 to 4 days after ovariectomy failed to alter the pulsatile pattern of LH release in rats, but a subcutaneous injection of indomethacin 20h before blood sampling reduced the pulsatile rhythm of LH. A higher dose given 30h prior to blood sampling completely abolished the LH pulsatile rhythm (Ojeda et al., 1975). In further support of a hypothalamic site of action of indomethacin, Harms, Ojeda and McCann (1973), Spies and Norman, (1973), Harms, Ojeda and McCann (1974) and Ojeda et al., (1975) reported that indomethacin did not affect the LHRH-induced LH surge in ovariectomized rats. In male rats, indomethacin reduced LH concentrations in intact animals (Saksena, Lau, Bartke and Chang, 1975) and suppressed the rise in plasma LH levels following orchidectomy (Ojeda et al., 1975).

Concerning species other than the rat, indomethacin given subcutaneously every 4h for 48h abolished the oestradiol-induced LH surge in anoestrous sheep (Roberts, Carlson and McCracken, 1976). A hypothalamic action of indomethacin in preventing the LH surge was concluded, as LHRH-induced LH release was unimpaired during indomethacin treatment (Roberts, Glew and McCracken, 1975). In monkeys, indomethacin inhibited oestradiol-induced LH release (Carlson, Wong and Perrin, 1977a). The administration of aspirin to bulls abolished the episodic release of LH and this block was reversed by giving PGF-2α (Haynes, Kiser, Hafs and Marks, 1977).

In contrast to the effects on plasma LH levels, indomethacin given to ovariectomized rats actually raised FSH levels but had no effect on the post-castration rise in plasma FSH in male rats.

Indomethacin abolished the LH surge and partially inhibited the rise in plasma FSH following the injection of oestradiol and progesterone into ovariectomised rats (Ojeda et al., 1975). This result suggests that this treatment regime of oestrogen and progesterone stimulates PG synthesis in the hypothalamus and that this stimulation of PG synthesis is necessary for LH and FSH release. It must be noted, however, that in these studies, relatively high doses of indomethacin were employed (12.5 to 50 $mgkg^{-1}$ body weight). Hypothalamic PG synthesis has been shown to be inhibited by as little as 1.5 $mgkg^{-1}$ indomethacin (Orczyk and Behrman, 1972).

Effects of indomethacin on pituitary responsiveness to LHRH

Pituitary responsiveness to LHRH increased following indomethacin treatment in the ewe (Roberts et al., 1976), in the rat, (Sandberg, Fawcett, Illner and McCann, 1975) and in women with amenorrhoea or oligomenorrhoea (Craig, Ginsburg, Gore and Isaacs, 1975). The mechanism whereby indomethacin exerts a facilitatory effect is not known. One possible explanation is that indomethacin also suppresses the formation of a PG which reduces pituitary responsiveness to LHRH. In this regard, PGD-2 has been shown to inhibit pulsatile LH release in orchidectomized male rats (Kinoshita, Nakai, Katakami, Imura, Shimizu and Hayaishi, 1982). Another possibility is that a lipoxygenase product is involved in sensitizing the pituitary gland to LHRH. Thirdly, it is widely known that indomethacin inhibits phosphodiesterase activity and therefore would reduce cyclic nucleotide degradation (Ciosek, Ortel, Thamassi and Newcombe, 1974; Zor, Koch and Naor, 1976).

Cyclic nucleotides have been implicated in the mechanism by which LHRH stimulates gonadotrophin release (Labrie, Pelletier, Borgeat, Drouin, Ferland and Belanger, 1975).

In contrast, a longer term schedule of indomethacin treatment depressed pituitary responsiveness to exogenous LHRH in ovariectomised rats (Sato et al., 1975). Clearly the effect of indomethacin depends upon the hormonal status of the animals as pituitary responsiveness is depressed in ovariectomized rats but is enhanced in intact rats.

Effects of indomethacin injection into the brain

The intraventricular administration of 10 μg indomethacin or its implantation into the MBH of ovariectomized rats decreased the plasma LH levels by approximately 60% and 50% respectively (Ojeda et al., 1975). Eicosatetraynoic acid, another PG synthesis inhibitor, was effective in depressing LH levels following intraventricular injection, but the effect was less sustained than that of indomethacin. Microinjection of aspirin into the anterior hypothalamic area (AHA) of rats, interfered with progesterone-induced ovulation in 87% of animals (Labhsetwar and Zolovick, 1973). Simultaneous injection of PGF-2α restored the incidence of ovulation, suggesting that aspirin blocks ovulation by preventing PG synthesis in the hypothalamus. The possibility that aspirin had diffused to the pituitary gland and acted therein, was excluded by demonstrating that an intrapituitary injection of aspirin was ineffective in blocking ovulation.

In a separate study Linton and Whitehead (1980) reported that indomethacin injected into two different brain areas, namely the AHA and the pre-optic area (POA), blocked ovulation. The PGI-2 synthetase inhibitor, 15-hydroperoxy-arachidonic acid, and the TXA-2 synthetase inhibitor, imidazole, were both ineffective in this respect, suggesting that PGI-2 and TXA-2 are not involved in LH release.

All these studies suggest that, providing the PG synthesis inhibitors are acting solely by inhibiting PG synthesis, that hypothalamic PGs are required for LH release. However, it still has to be demonstrated that the inhibition of hypothalamic PG synthesis can block the pro-oestrous LH surge.

Effects of PGs on gonadotrophin release

Numerous reports have indicated that PGs injected systemically or intraventricularly, can elicit gonadotrophin release. PGE given subcutaneously or intraventricularly, on the afternoon of pro-oestrus to phenobarbitone-blocked rats overcame the block and caused an LH surge (Tsafriri et al., 1973; Spies and Norman, 1973). In the latter study, PGE-1 was more potent than PGE-2 or PGF-2α. Similarly, Harms, Ojeda and McCann (1974) demonstrated that intravenous PGE-2 produced a slight increase in plasma LH levels in ovariectomized rats. However, when injected into the 3rd ventricle, PGE-2 induced a much greater increase in plasma LH levels, as well as a smaller increase in plasma FSH levels.

When ovulation in rats was blocked by intraventricular phentolamine, a further intraventricular injection of PGE-2 overcame the effect of the α-receptor antagonist (Linton, Perkins and Whitehead, 1977). PGE-1 was less effective and PGF-2α completely ineffective in this respect (Linton et al, 1977). Intracarotid injections of PGE-1 into ovariectomized oestrogen-pretreated rats were followed by a rapid increase in plasma LH levels which were maintained for up to 30h with no change in FSH levels (Batta, Zanisi and Martini, 1973). In male rats, intracarotid PGE-1 (Batta et al., 1973), subcutaneous PGE-1 or PGE-2 (Castracane and Saksena, 1974), or intravenous PGE-2 (Chobsieng, Naor, Koch, Zor and Lindner, 1975) increased circulating LH levels. The infusion of PGE-2 into non-receptive or pseudopregnant rabbits produced a 2-to 4-fold increase in plasma LH levels (Carlson, Wong and Perrin, 1977b). In cycling or ovariectomized oestrogen-treated monkeys, PGE-1 or PGE-2 stimulated LH release (Batta, Niswender and Brackett, 1978).

The effects of systemically administered PGF-2α on LH release seem more variable. Thus, PGF-2α induced an increase in LH levels in pentobarbitone-blocked pro-oestrous rats (Spies and Norman, 1973), ovariectomized oestrogen-treated rats (Batta et al., 1973), and pseudopregnant oestrogen-treated rabbits (Carlson et al., 1977b) and hamsters (Saksena, Lau and Chang, 1974). In ovariectomized, oestrogen-treated monkeys (Batta et al., 1978) or in male monkeys (Kimball et al., 1979), injection of PGF-2α increased plasma LH levels. In human females, the intravenous infusion of PGF-2α (up to 250 μg min^{-1}) during the luteal phase resulted in a transient elevation in plasma LH concentrations (Hillier, Dutton and Corker, 1973).

However, other workers have reported that PGF-2α infusion (50 μgmin^{-1}) in similar women, produced a transient fall in plasma LH levels (Elias, Newton and Collins, 1975). These conflicting results in women may relate to the differing infusion rates of PGF-2α.

PGF-2α injected into the 3rd ventricles of ovariectomized rats failed to affect LH levels (Harms et al., 1973). Likewise, a subcutaneous injection of PGF-2α into ovariectomized hamsters (Labhsetwar, 1973) or ovariectomized sheep (Chamley and Christie, 1973) failed to elicit LH release. Systemic injection of PGF-2α into male rats (Batta et al., 1974; Warberg, Eskay and Porter, 1976) and humans (Craig, 1975) did not affect LH levels. In heifers, the systemic administration of PGF-2α increased plasma LH levels, but not until 1.5 to 6h after administration (Louis, Stellflug, Tucker and Hafs, 1974). Results of this type suggest that part of the effect of PGF-2α on LH release is subsequent to alterations in gonadal steroid production as the effect is absent in either males or ovariectomized females. Alternatively, the failure of PGF-2α to enhance LH release in ovariectomized animals may be due to a poor pituitary responsiveness to LHRH. Experiments in cycling Rhesus monkeys (Carlson et al., 1977b) and rats (Carlson and Kehl, 1979) have demonstrated that PGE-2 and PGF-2α were most effective in stimulating LH release during the late follicular phase and at pro-oestrus respectively. However, these results are perhaps not surprising since the PGs are thought to act via the release of LHRH from the hypothalamus, and pituitary responsiveness to the releasing hormone itself is highest on these days (Aiyer, Chiappa and Fink, 1974).

Although in the rat, PGE-2 appears to be more effective than PGF-2α in stimulating LH release, intracarotid infusion of PGF-2α in the cycling ewe caused a rapid increase in plasma LH (Roberts, Carlson and McCracken, 1976). Since this effect was absent in ovariectomized animals (Chamley and Christie, 1973) PGF-2α probably acted at the hypothalamic level and not by altering gonadal steroid production.

There is general agreement that PGF-1α does not affect LH (Harms et al., 1973; Ojeda et al., 1974; Warberg, Eskay and Porter, 1976) or FSH (Harms et al., 1973; Ojeda et al., 1974) release from the pituitary gland. The work of Warberg et al., (1975) has included ten different PGs which were ranked according to their LH-stimulating potency as PGE-2 =, PGF-2α = PGF-2β > PGA-2 = PGB-2 = PGE-1 = PGF-1β > PGA-1 = PGB-1 = PGF-1α = vehicle. From these findings it was concluded that the cis-double bond at the 5, 6 position and the 11-hydroxyl group are essential for this supra-pituitary stimulation of LH secretion. Ojeda et al., (1976) confirmed that PGA-2 and PGB-2 given intraventricularly into ovariectomized oestrogen-treated rats were less potent than PGE-2. Also the injection of two endoperoxide analogues (U-44069 and U-46619) into the 3rd ventricle was followed by a transient increase in plasma LH levels with no effect on FSH levels.

Site of action of PGs in eliciting LH release

Substantial evidence exists to show that PGs do not stimulate LH release by a direct action on the pituitary gland. In male rats (Chobsieng et al., 1975) and in pro-oestrous rats (Drouin, Ferland and Labrie, 1976) prior administration of antiserum to LHRH prevented the increase in circulating LH levels following intravenous injection of PGE-2. The direct infusion of PGE-2 into hypophysial portal vessels of male rats failed to cause LH release, whereas the intraventricular infusion of PGE-2 stimulated a 2-to 3-fold increase in the concentration of LHRH in hypophysial portal plasma (Eskay et al., 1975). An increase in circulating LHRH was found after injection of PGE-2 into the 3rd ventricle of conscious ovariectomized rats (Ojeda, Wheaton and McCann, 1975).

In vitro studies with pituitary glands also demonstrated that PGs do not act directly on the gland to cause LH release. Neither PGE-1 nor PGE-2 altered the time-course of basal, or LHRH-induced LH release from cultured pituitary glands (Drouin et al., 1976). Similarly addition of LHRH to cultured hemipituitary glands from male rats stimulated cAMP accumulation and LH release, but did not affect either PGE-2 levels or PG synthetase activity in the glands (Naor, Koch, Bauminger and Zor, 1975). Moreover, the stimulatory action of LHRH on these parameters was not impaired during PG synthetase inhibition with aspirin or indomethacin.

In contrast, however, Sato, Hirono, Jyujo, Iseka, Taya and Igarashi (1975) favour a pituitary site of action of PG based on their observation that PGE-1, PGE-2 and PGF-2α could elevate plasma LH levels upon intraventricular injection into rats with M.E. lesions, or upon intrapituitary injection into intact rats. However, failure to observe altered LH secretion following intrapituitary injection of PGs has also been described (Harms et al., 1974, Spies and Norman 1973, and Hedge, 1977). These discrepancies may be due to different doses of PGs employed. Sato et al., (1975) used 50 μg and 100 μg whereas most other studies used about 5 μg. It seems therefore, that although under certain circumstances PGs can act directly on the pituitary gland to evoke gonadotrophin release, their primary and most important site of action is within the hypothalamus.

<u>Hypothalamic sites of action of PGs in eliciting gonadotrophin release</u>

By implanting crystalline PGs into the hypothalamus of ovariectomized rats, Ojeda, Jameson and McCann (1977a) have defined more clearly the anatomical sites at which PGs evoke LH release. When PGE-2 was placed in the arcuate nucleus - median eminence region (ARH-ME), a 4-to 5-fold increase in plasma LH levels ensued within 60 min. PGE-2 placed more dorsally, close to the ventromedial nucleus, caused a similar increase in plasma LH levels but only after 2h. When implanted in the caudal portion of the ARH-ME or dorsally in the ventromedial hypothalamus (VMH) and dorso-medial nuclei, PGE-2 barely increased plasma LH levels, whereas its implantation into the anterior portion of the ARH-ME clearly elevated plasma LH levels.

The POA or anterior ventral portion of the anterior hypothalamic area are other important sites at which PGE-2 act as here the PGE-2 induced a large increase in plasma LH levels within 20 min of injection. Areas where PGE-2 implants were relatively ineffective include the para ventricular nuclei and the dorsal portion of the AHA. PGF-2α appeared less potent than PGE-2 as a smaller increase in LH levels followed the implantation of PGF-2α into the ARH-ME or POA. The finding that PGs are effective in releasing LH when implanted in the ARH-ME is significant as this area is rich in LHRH-containing nerve endings (Ibata, Watanabe, Kimura, Sano, Sin, Hashimura and Imagawa, 1978). Studies of this type are useful in that they overcome the problem of pulmonary metabolism of PGs administered intravenously. It is difficult, however, to assess the results of the implant experiments quantitatively as the amount of PG diffusing into the tissues is unknown. Using a similar technique, the hypothalamic areas where PGs stimulate FSH release have been outlined (Ojeda, Jameson and McCann, 1977b). The results indicate that PGE-2 and PGF-2α act mainly on the POA and AHA regions and that PGE-2 is more effective than PGF-2α in this respect.

Information on the sites of action of PGs within the hypothalamus in other species is lacking.

Interaction of PGs and neurotransmitters

There have been few attempts to determine the possible relationships between PGs and some of the hypothalamic neurotransmitters thought to regulate gonadotrophin secretion. It has been reported, however, that the inhibition of progesterone-induced ovulation by aspirin injected into the hypothalamus can be reversed by either PGF-2α or dopamine (DA) (Labhsetwar and Zolovick, 1973). Furthermore NA, DA or adrenaline stimulated PGE formation by rat brain synaptosomes (Hillier, Roberts and Woolard, 1976). In addition PGE-2 in turn has been shown to facilitate NA release from rat brain synaptosomes (Roberts and Hillier, 1976) indicating that PGE-2 may exert a positive feedback on NA release on central nerve terminals. Although these findings are consistent with an interaction between PGs and the dopaminergic and noradrenergic systems, pharmacological evidence has been provided to suggest that PGE-2 acts directly on LHRH secreting neurons. Thus, α or β-adrenergic receptor antagonists, dopaminergic, serotonergic or muscarinic receptor antagonists failed to inhibit the LH release induced by intraventricular injection of PGE-2 in ovariectomized rats (Harms et al., 1976). Moreover, PGE-2 will overcome the ovulatory block induced by phentolamine (Linton et al., 1976) and nembutal (Tsafriri et al., 1973; Spies and Norman, 1973). However, PGE-2 failed to stimulate rat hypothalamic synaptosomes to release LHRH (Linton, Bennet and Whitehead, 1979). It is possible, therefore, that PGs stimulate LHRH release by acting on the cell bodies of the LHRH neurones.

Indeed, iontophoretically applied PGE-2, increased the firing rate of hypothalamic neurones (Poulain and Carette, 1974). It would be interesting to study the effects of PGs applied iontophoretically to specific hypothalamic areas involved in LHRH release.

PG synthesis by the brain in relation to the LH surge

The demonstration that endogenous PG levels change concomitantly with alterations in LH and LHRH secretion have further substantiated the idea that PGs play a physiological role in the control of gonadotrophin release. In the ewe, Roberts, Carlson and McCracken (1976) have shown a temporal association between the release of PGF-2α and of LH from the brain. Thus, circulating PGF-2α levels varied in a pulsatile manner and LH release was also pulsatile. The arterio-venous difference in PGF-2α concentrations across the brain showed that significant amounts of PGF-2α were released by the brain. Furthermore, an infusion of oestradiol resulted in an initial suppression of the PGF-2α pulses, followed by a distinct rise in their amplitude at a time coincident or slightly prior to the steroid- induced LH surge. While these experiments do not prove that PGF-2α mediates the LH surge, they are consistent with this suggestion.

In the rat, the ME of both male and female animals showed a higher PGE content than either the MBH or the anterior pituitary gland (Ojeda et al., 1978). No sex differences were observed.

The elevated content of PGE in the ME is associated with a 5 to 6-fold greater capacity of the tissue to release PGE *in vitro* compared to the MBH of male rats (Ojeda, Negro-Vilar, McCann, 1979). The *in vitro* addition of indomethacin, which completely suppressed basal PGE release by both the MBH and ME, also depressed basal LHRH release from these tissues, suggesting that PG synthesis is required for basal LHRH release to occur. NA or DA added *in vitro*, elicited both LHRH and PGE release with NA being the more potent catecholamine. Moreover, the *in vitro* suppression of PG synthesis by indomethacin, completely abolished the release of LHRH from the ME induced by DA or NA. In contrast, indomethacin did not prevent release of LHRH induced by PGE-2 (Ojeda, Negro-Vilar and McCann, 1979). These results suggest that the stimulatory effect of DA and NA on LHRH release by the ME and MBH is mediated by PGs. When comparing catecholamine induced PGE release, NA, DA and adrenaline are more effective in causing release of PGE from the ME than from the MBH (Ojeda et al., 1979). It should be noted that since the ME contains the terminals of LHRH neurones, that high concentrations of LHRH coincide with areas which have a high capacity to release PGE in response to NA. NA-induced PGE-2 and LHRH release *in vitro* from the ME of male rats requires activation of an α-adrenergic receptor as the effect of NA was blocked by phentolamine. Neither propanolol nor pimozide had any effect on NA-induced PGE-2 or LHRH release, indicating that stimulation of β-adrenergic or dopaminergic receptors are not involved (Ojeda, Negro-Vilar and McCann, 1982).

Since the effect of PGE-2 on LH release cannot be prevented by monoaminergic receptor blockers (Harms et al., 1976) but the effect of catecholamines on LHRH release is inhibited by preventing PG synthesis, it appears that PGE-2 mediates the effect of catecholamines on hypothalamic LHRH release. These observations may explain the inhibitory effect of α-antagonists on LH release and ovulation (see Barraclough and Wise, 1982).

There is a diurnal variation in the response of rat ME fragments to NA or PGE-2 on met-oestrus, pro-oestrus and oestrus. (De Paolo, Ojeda, Negro-Vilar and McCann, 1982). The release of PGE-2 in response to NA was higher at 13.00h than at 16.00h on each day. However, on di-oestrus the NA-induced PGE-2 release showed no diurnal variation. Since, with the exception of di-oestrus, fluctuations in NA-induced PGE-2 release from the ME were independent of the day of the cycle, it is unlikely that these changes were secondary to cyclical changes in steroid hormones. As it has been suggested that the release of LHRH caused by NA is mediated by PGE-2 (Harms et al., 1976) it would be expected that the responsiveness of the LHRH terminals to NA should parallel the NA induced PGE-2 response during the cycle. This did not appear to be the case however, as on pro-oestrus, NA-induced LHRH secretion in the ME increased between 13.00h and 16.00h, which is the converse trend for NA-induced PGE-2 secretion. Cyclical changes in NA-induced LHRH release more closely resembled the changes in the response of the LHRH terminals to PGE-2. Therefore, it seems likely that the response of the ME to NA with respect to LHRH release is dependent upon the response to PGE-2 and not on the amount of PGE-2 released by NA (De Paolo et al; 1982).

Progesterone given at 09.30h on pro-oestrus advanced both the onset of the LH surge and the in vitro response of the LHRH terminals to PGE-2 (De Paolo et al., 1982). As well as a diurnal pattern of responsiveness of the ME to PGE-2, a similar diurnal rhythm in plasma progesterone levels occurs during the rat oestrous cycle with highest levels at 03.00h and lowest at 10.00h on each day (Mann and Barraclough, 1973). Thus progesterone may alter the responsiveness of the LHRH terminals to PGE-2, although this suggestion remains speculative at present. A diagrammatic representation of the involvement of PGs in hypothalamic LHRH release is shown in Figure 2.

Summary and Aims

In conclusion, the concept that PGs (particularly PGE-2) are involved in the physiology of LHRH secretion has gained support in recent years. There are, however, several unresolved issues. The possibility of cyclical changes in hypothalamic PG production has not been investigated. A major argument against the hypothesis that PGs are essential for LH release arises from the failure to demonstrate that inhibition of hypothalamic PG synthesis results in suppression of the pre-ovulatory LH surge. It is possible that the use of a more specific inhibitor of PG synthesis or perhaps a PG antagonist could suppress the LH surge and this area requires investigation. Lastly, if PGs are an essential link between the positive feedback effect of oestradiol and the LH surge, it may be possible to demonstrate that the steroid will affect hypothalamic PG synthesis. The experiments described in section 2.2. were aimed at testing these possibilities.

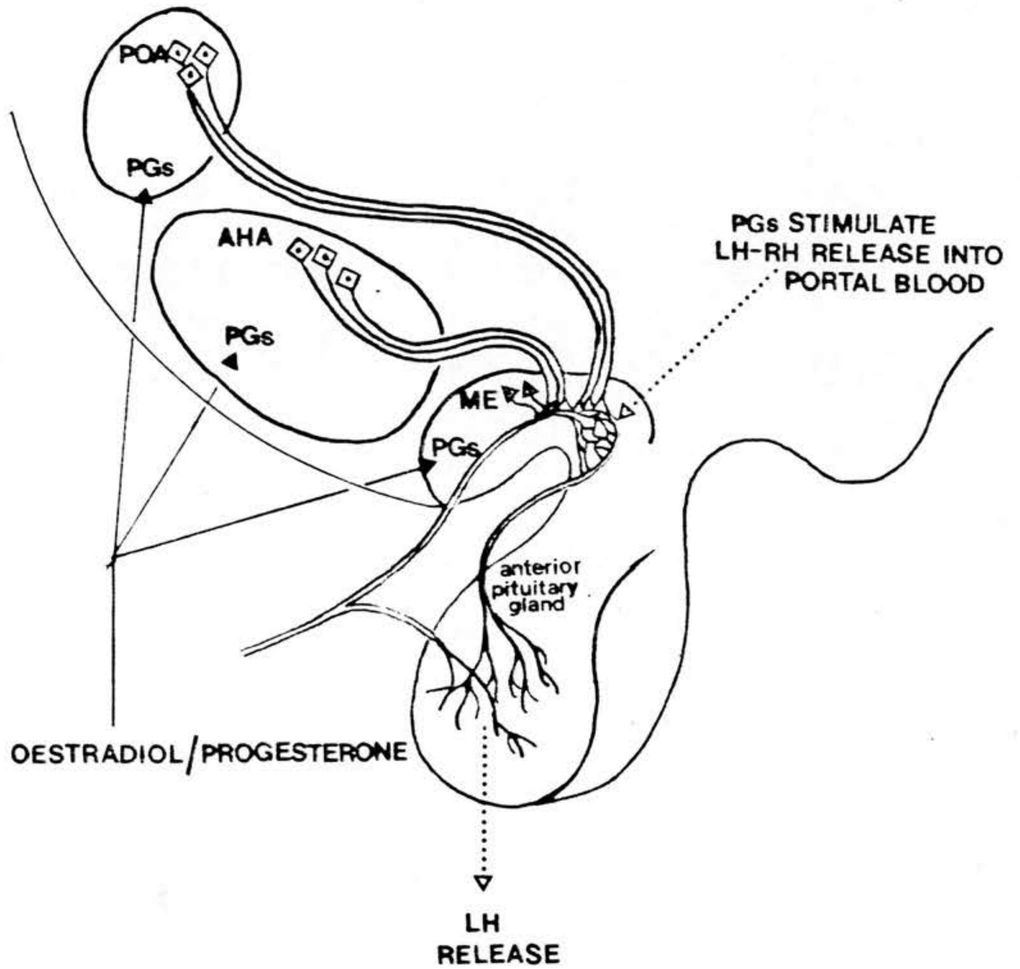

Figure 2. Possible sites of action of PGs in the hypothalamo-pituitary axis in the rat.

Uterine PG Synthesis

A. Non-pregnant uterus

Functional significance of uterine PG synthesis

The presence of PGE and PGF in uterine tissue of many species has been widely demonstrated (see Horton and Poyser, 1976). The majority of studies on uterine PG synthesis concern the increased production of PGF-2α towards the end of the cycle in relation to luteolysis. There is also a significant increase in endometrial PGF-2α production during the luteal phase of the menstrual cycle in women (Downie, Poyser and Wunderlich, 1974; Le Vitt, Tobon and Josimovich, 1975; Singh, Baccarini and Zuspan, 1975, Maathius and Kelly, 1978). However, luteal regression in women does not require uterine PGF-2α synthesis as ovarian activity continues after hysterectomy (Whitelaw, 1958) or in cases of congenital absence of a uterus (Fraser, Baird, Hobson, Michie and Hunter, 1973). As in the human, uterine PG synthesis is not required for luteal regression during the rat oestrous cycle although uterine PGF-2α synthesis may be required for luteolysis in the pseudopregnant rat (Chatterjee, 1973).

In addition to PGE and PGF, Poyser and Scott (1980) have identified PGI-2, PGD-2 and TXA-2 in the uterus during the oestrous cycle of the rat. In fact PGI-2 (measured as 6-keto-PGF-1α) was the major PG produced by the uterus of the rat (Fenwick et al., 1977; Poyser and Scott, 1980), sheep (Jones, Poyser and Wilson, 1977) and human (Abel and Kelly, 1979). It is possible that these PGs play a role in uterine physiology during the oestrous cycle.

<u>Uterine PG synthesis during the oestrous cycle</u>

Saksena, Shaikh and Shaikh (1973) found that, in rats, uterine tissue concentrations of PGF correlated with PGF concentrations in uterine venous plasma in that they were highest on met-oestrus and also showed a smaller peak on pro-oestrus. In general however, other workers (see below) have reported highest uterine tissue PG levels on pro-oestrus or oestrus in the rat. The anomalous result of Saksena et al., (1973) may be attributed to the frequency of sampling (once on met-oestrus and di-oestrus, twice on oestrus and four times on pro-oestrus). In sheep, more frequent sampling (every 2h) from the utero-ovarian vein revealed the presence of pulses of PGF-2α lasting up to 2h and, which increased in amplitude towards the end of the oestrous cycle (Barcikowski, Carlson, Wilson and McCracken, 1974). Clearly, the variation in the pattern of uterine PG production depends greatly on the resolution of the experimental sampling.

In the rat, there is general agreement (except for the report by Saksena et al., 1973) that uterine levels of PGF and PGE are highest on pro-oestrus or between pro-oestrus and early oestrus (Ham, Cirillo, Zanetti and Kuehl, 1975; Ishikawa and Fuchs, 1978; Poyser and Scott, 1980; Van Orden Goodale, Baker and Farley, 1980; Thaler-Dao, Saintot, Ramonatxo, Chavis and Crastes de Paulet, 1982). PGI-2 and PGD-2 synthesis by rat uterine homogenates, measured between 10:00h and 12:00h on each day, paralled that of PGE-2 and PGF-2α in being highest on oestrus and lowest on pro-oestrus with no change in the ratios of PGs formed (Poyser and Scott, 1980).

However, Thaler-Dao et al., (1982) report an additional peak in uterine PGE-2 synthesis at 15:00h on di-oestrus. Also in contrast to the results of Poyser and Scott (1980), Ham et al., (1975) found that PGF synthesis increased at the expense of PGE synthesis and, during di-oestrus, PGE synthesis exceeded that of PGF by 3-fold. It is difficult to compare these conflicting reports as differing methodologies were employed. Poyser and Scott (1980) incubated uterine homogenates with no added co-factors, whereas Ham et al., (1975) used a microsomal preparation and added arachidonic acid and glutathione. Likewise Thaler-Dao et al., (1982) added arachidonic acid, glutathione and adrenaline to uterine homogenates. Consequently the addition of a co-factor (i.e. glutathione) which promotes PGE synthesis may upset the natural pattern of PG synthesis by the uterus.

B. Pregnant uterus

In the pregnant rat, the endometrium is the major site for PGF-2α synthesis while the myometrium synthesizes mainly PGI-2 (Williams, Sneddon and Harney, 1974; Williams, Dembinska-Kiec, Zmuda and Gryglewski, 1978). The significance of myometrial PGI-2 production is at present unknown. Myometrial blood vessels are unlikely to be the main source of PGI-2 as PGI-2 synthesis by uterine arteries and veins did not change significantly during pregnancy in the rat, whereas myometrial PGI-2 synthesis increased markedly towards the end of gestation (Williams et al., 1978). Although PGI-2 itself was not oxytocic, oxytocin-induced contractions of the pregnant rat uterus were potentiated by PGI-2 (Williams et al., 1979) and thus PGI-2 may play a role in stimulating myometrial activity at parturition.

Both the endometrium and myometrium produce PGE-2 while the endometrium is a major site for PGD-2 synthesis in the pregnant rat (Downing and Williams, 1977).

Uterine PG synthesis and implantation

In a study of PG production by the rat uterus during pseudopregnancy, Fenwick et al., (1977) found highest production of both PGF and PGE on day 5 after mating. This day is when blastocyst implantation takes place and hence the suggestion is that PGs may be involved in this process.

Indomethacin inhibited implantation in rats (Gavin, Dominguez Fernandez-Tejerina, Montanes de las Heras and Vijil Maeso, 1974) mice (Saksena, Lau and Chang, 1976), hamsters (Evans and Kennedy, 1978) and rabbits (Hoffmann, Di Pietro and McKenna, 1978).

One of the earliest signs of blastocyst implantation is the appearance of localized sites of increased endometrial vascular permeability (Psychoyos, 1973). These sites can be visualized experimentally by intravenous injection of Evans blue dye. Indomethacin administration effectively inhibited the uterine dye site response in ovariectomized rats treated with exogenous steroids, adequate for the initiation of implantation (Kennedy, 1977) and delayed the appearance of uterine dye sites in pregnant rats (Phillips and Poyser, 1981b). When PG synthesis at implantation sites in rats was examined, PGF, PGE and PGI-2 levels were found to be higher than in surrounding endometrial tissue with PGI-2 being present in greatest concentration (Kennedy, 1977; Kennedy and Zamecnik, 1978).

Similarly, PGE levels were high at implantation sites in the hamster (Evans and Kennedy, 1978). Since PGE-2 but not PGI-2 or PGF-2α increased vascular permeability in the rat uterus (Kennedy, 1979a) this suggests that PGE-2 mediates this process during implantation.

Increased endometrial vascular permeability is thought to be pre-requisite for the decidual cell reaction (Psychoyos, 1973). Decidualization of endometrial stromal cells can be obtained during a limited period of pregnancy, pseudopregnancy or experimentally by artificial stimuli such as uterine scratching or injection of oil or phosphate-buffered saline containing gelatin (PBSG). Kennedy (1979b) showed that increased endometrial vascular permeability which normally follows the unilateral injection of PBSG, was inhibited by indomethacin and in addition, uterine concentrations of PGE and PGF were elevated following PBSG-injection. Moreover, the intraluminal injection of PGE-2 resulted in increased endometrial vascular permeability in rats in which endometrial PG production had been inhibited. Artificial deciduogenic stimuli (trauma or the injection of sesame oil) increased the uterine content of PGE and PGF in mice (Jonsson, Rankin, Ledford and Baggett, 1978; Rankin, Ledford, Jonsson and Baggett, 1979). In addition, artificially-induced decidualization was inhibited by indomethacin treatment in rats (Castracane, Saksena and Shaikh, 1974; Sananes, Baulieu and Le Goascogne, 1976; Tobert, 1976) and in mice (Rankin et al., 1979). All these results suggest that PG synthesis by the endometrium in these species is essential for increased vascular permeability and the subsequent decidualization of the endometrium during the implantation process.

There is evidence that PGs affect the decidual cell reaction per se, and not solely by inducing increased vascular permeability. When indomethacin administration was delayed until 8h after the artificial stimulus (by which time increased vascular permeability had already occurred), decidualization was still inhibited (Tobert, 1976). PGF-2α elicited a decidual response when instilled into the uterus of prepubertal rats (Sananes et al., 1976), and in pseudopregnant rabbits, the insertion of implants containing PGE-2, PGF-2α or arachidonic acid into the uterus elicited a decidual response. Similar implants releasing histamine or oestradiol had a slight effect in some animals, while dibutyryl cAMP was inactive (Hoffman et al., 1977).

PG synthetase activity in deciduomata, induced by traumatization of the pseudopregnant rat uterus, was 7-to 10-fold higher than in untraumatized tissue (Anteby, Bauminger and Lindner, 1975). PGE-2 concentrations in the deciduomata exceeded those in control tissue by 10-to 20-fold on the third or fourth day after decidual induction and by about 5-fold on the ninth and tenth day. However, PGF concentrations did not differ between decidualized and non-decidualized tissue (Anteby et al., 1975).

PGF-2α production by the pregnant rat uterus was lower on day 5 compared with the pseudopregnant rat uterus (Fenwick et al, 1977) which indicates that the presence of a blastocyst has an effect on uterine PG synthesis. However, PGE-2 and 6-keto-PGF-1α production by homogenates of pregnant rat uterus were highest on day 5 as in pseudopregnancy (Phillips and Poyser, 1981b).

This day 5 peak in uterine PG synthesis in the pregnant rat coincides with the time at which the uterus is most sensitive to deciduogenic stimuli (De Feo, 1963). However, when ovariectomized rats were treated with oestradiol and progesterone so that they were at the equivalent of days 4, 5 or 6 of pseudopregnancy, uterine concentrations of PGE and PGF did not differ between the three days in response to intrauterine PBSG. In contrast, endometrial vascular permeability was highest in animals on the equivalent of Day 5 of pseudopregnancy (Kennedy, 1979b). It appears therefore that the timing of uterine sensitivity to deciduogenic stimuli is not related to the ability to respond to such stimuli with increased PG production. Alternatively, changes in the uterine PG receptor population may be related to the changes in sensitivity to deciduogenic stimuli. In support of this suggestion, Kennedy (1979b) showed that when PGE-2 was administered into the uterine lumen of indomethacin-treated rats on the equivalent of days 4, 5 or 6 of pseudopregnancy, the greatest change in endometrial vascular permeability (as indicated by the uterine radioactivity levels after intravenous ^{125}I-labelled bovine serum albumin) was in animals on day 5. Using a receptor binding assay, Kennedy (1982) has recently demonstrated greater PGE receptor numbers in rats on the equivalent of day 5 of pseudopregnancy, compared with other days.

The suggestion that PGI-2 may be the mediator of decidualization has come from Rankin et al., (1979) who found that tranylcypromine, an inhibitor of PGI-2 synthesis (Gryglewski et al., 1976) inhibited decidualization in mice.

However, the selectivity of the inhibitory action of tranylcypromine has been questioned (Rajtar and de Gaetario, 1979) and also the inhibition has not been overidden with PGI-2. Nevertheless, uterine levels of 6-keto-PGF-1α were elevated following the application of an artificial deciduogenic stimulus to the sensitized rat uterus (Kennedy, 1979). In addition, 6-keto-PGF-1α was the major PG produced by uterine homogenates from rats in early pregnancy or pseudopregnancy (Phillips and Poyser, 1981b). 6-Keto-PGF-1α , PGF-2α, and PGE-2 production peaked on day 5 of pseudopregnancy, whereas only 6-keto-PGF-1α and PGE-2 peaked on day 5 of pregnancy (Phillips and Poyser, 1981b). However, PGI-2 failed to increase endometrial vascular permeability following the application of an artificial stimulus to the rat uterus sensitized for the decidual cell reaction (Kennedy, 1979a). Failure to observe an effect of PGI-2 may be due to the instability of this PG at neutral pH. Injection of PGF-2α into the uterine lumen of rats had no effect on endometrial vascular permeability although the intralumenal infusion of the same PG from an osmotic minipump resulted in increased vascular permeability and subsequent uterine decidualization (Kennedy and Lukash, 1982). Clearly the mode of administration of PGs is important and the infusion of PGI-2 in a similar manner requires investigation.

In indomethacin-treated rabbits, PGE-2 or PGF-2α only partially overcame the reduction in the number of implanted blastocysts (Hoffmann, 1978).

Similarly, in indomethacin-treated mice, the administration of PGE-2 or PGF-2α alone or in combination resulted in implantation only in 60% of the mice (Lau, Saksena and Chang, 1973). If, however, the mice were treated with PGF-2α and histamine, then a 100% success rate for implantation was achieved (Saksena, Lau and Chang, 1976). A role for histamine was further supported by the observation that the histamine H_1 and H_2 receptor antagonists, mepyramine and burimamide reduced the number of implantation sites in rats (Brandon and Willis, 1977). It is possible that an interaction between histamine and PGs is involved in implantation. An analogy between the process of implantation and the inflammatory response is apparent. Acute inflammation is also characterised by both vasodilation and increased vascular permeability and the involvement of PGs and histamine is well documented (see Williams, 1977).

Another observation regarding implantation, is that ageing of the uterus is associated with decreased frequency of blastocyst implantation in many species. In ageing hamsters, a diminished local oedema in the endometrial stroma at implantation sites has been reported (Sorger and Soderwall, 1981). Since PGs are implicated in the production of an 'inflammatory response' at implantation sites, this has stimulated the study of the uterine PG production in uterus sensitized for the decidual cell reaction in the aged animal. (These studies are described in more detail in Section 2.4).

c) The control of uterine PG synthesis

Oestrogen appears to be a potent stimulant for uterine PG synthesis. The secretion of PGF into the utero-ovarian vein increased significantly following oestrogen treatment of guinea pigs (Blatchley, Donovan, Poyser, Horton, Thompson and Los, 1971). Similarly, oestrogen treatment of hamsters (Saksena and Harper, 1972), ovariectomized mice (Saksena and Lau, 1973), monkeys (Demers, Yoshinaga and Greep, 1974) and rats (Ryan, Clark, Van Orden, Farley, Edvinsson, Sjoberg Van Orden, and Brody, 1974) increased uterine levels of PGF. Oestradiol- 17β infused into the arterial supply of the autotransplanted sheep uterus increased PGF-2α levels in utero-ovarian venous plasma 100-fold within 90 min (Barcikowski et al., 1974). Ham et al., (1975) found that oestradiol treatment of ovariectomized rats stimulated uterine PGF-2α synthesis but reduced PGE-2 synthesis. Co-administration of progesterone with oestradiol completely inhibited the stimulatory effect of oestrogen on PGF synthesis in the rat uterus (Ham et al., 1975). In contrast, a stimulatory role for progesterone in the production of PGE and PGF by the rat uterus was demonstrated by Castracane and Jordan (1975). After two days of progesterone priming, the PGF and PGE levels in uterine venous plasma of ovariectomized rats were higher than in unprimed animals. Furthermore, progesterone priming prolonged the stimulatory effect of oestradiol on PG production. In the study of Ham et al., (1975) no progesterone priming was employed and this may account for differences in results between the groups.

It has been shown that progesterone is required for oestradiol-stimulated PGF production by the sheep uterus (Barcikowski et al., 1974). They showed that, although peaks of oestradiol in utero-ovarian plasma occurred throughout the cycle in ewes, it was not until days 13 and 14 (i.e. after progesterone priming) that small peaks of PGF-2α were associated with the oestradiol peaks. Similarly, prior treatment with progesterone greatly enhanced the effect of oestrogen on uterine PGF release in ovariectomized sheep (Scaramuzzi et al., 1977) and guinea pigs (Blatchley and Poyser, 1974). However, in ovariectomized guinea-pigs, prior treatment with progesterone failed to affect the stimulatory effect of oestradiol treatment on PGF-2α synthesis by uterine homogenates (Naylor and Poyser, 1975).

The disparity in results reported by various groups probably arises from the variations in the dose regimes of steroids used. It would seem important to attempt to replicate the plasma levels and sequential order of hormones in the circulation during the oestrous cycle. However, many of these studies have involved treating animals with progesterone followed by oestrogen. While this regime mimics the <u>in vivo</u> situation in many animals including the guinea pig and sheep, it does not do so for the rat.

The temporal pattern of changes in plasma steroid hormones during the rat oestrous cycle have been characterized by many workers (Goldman, Kamberi, Sitteri and Porter, 1969; Barraclough, Collu, Massa and Martini, 1971; Feder, Brown-Grant and Corker, 1971; Naftolin, Brown-Grant and Corker, 1972; Butcher, Collins and Fugo, 1974; Shaikh and Shaikh, 1975; Nequin, Alvarez and Schwartz, 1979).

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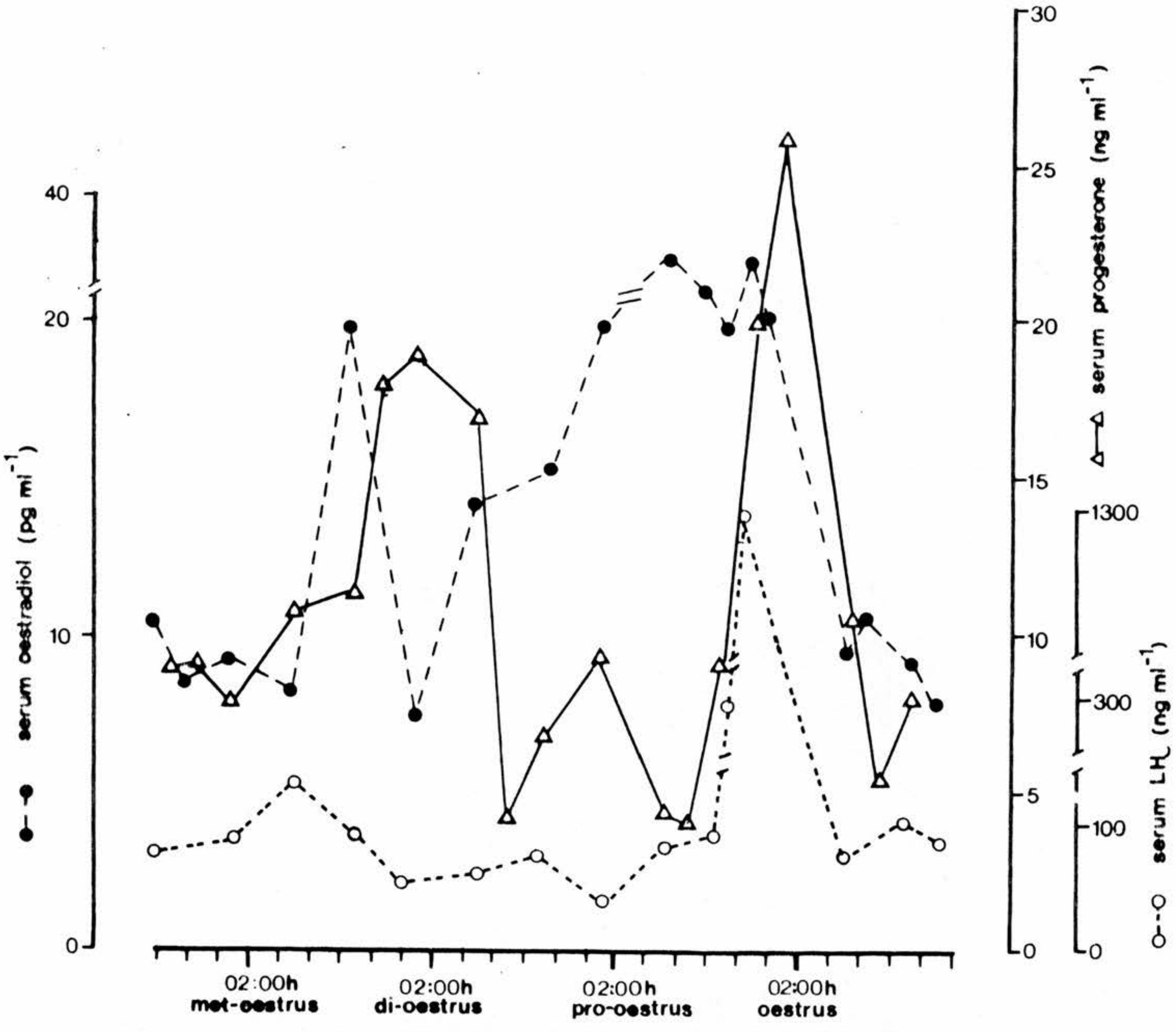
Figure 3 shows the plasma concentrations of LH, progesterone and oestradiol-17β throughout the 4-day oestrous cycle of the rat. Oestradiol rises on the evening of met-oestrus and reaches a peak on the morning of pro-oestrus, before falling to a nadir on the morning of the next met-oestrus. Nequin et al., (1979) found that progesterone was not released on pro-oestrus prior to the LH surge, but rather, began to rise between 12:00h and 18:00h on pro-oestrus to reach a peak at 22:00h on that night. However, other workers (Feder et al., 1971; Karla and Karla, 1974) report that during pro-oestrus plasma progesterone levels increase slowly from 09:30h to 13:30h then more rapidly after LH release to peak at 21:00h. It has been suggested that this rise in circulating progesterone on the morning of pro-oestrus facilitates LH release (Feder et al., 1971). Therefore in the rat, the effect of progesterone given after oestrogen on uterine PG synthesis requires re-evaluation.

Following ovariectomy, PGI-2 production by chopped strips of rat uteri increased (Gimeno, Batta, Lazzari and Gimeno, 1980). Subsequent injection of oestradiol, progesterone or both into ovariectomized rats diminished uterine PGI-2 production (Gimeno et al., 1980). In this study, however, 50ug of oestradiol was administered 24h before the animal was killed. Since oestradiol is normally released from the ovary over a period of 48h it would have been more instructive to have assessed the effects of this dose of oestrogen later than 24h after administration. Likewise, 4mg of progesterone was given 48h and 24h before sacrifice, whereas in the cycling rat, the ovulatory surge in plasma progesterone is relatively transient, lasting approximately 12h.



serum oestradiol (pg ml-1)
40
20
10
0
serum progesterone (ng ml-1)
30
25
20
15
10
5
0
serum LH (ng ml-1)
1300
300
100
0
02:00h
met-oestrus
02:00h
di-oestrus
02:00h
pro-oestrus
02:00h
oestrus

Thaler-Dao et al., (1982a) found that in the cycling rat, a rise in uterine PGE-2 and PGF-2α production occurred 5h after the highest value for plasma oestradiol and was superimposed on the peak of plasma progesterone. This suggests that oestradiol output by the ovary could stimulate uterine PGF-2α and PGE-2 synthesis but that in addition progesterone could facilitate this process.

In contrast to the cyclical fluctuations in uterine PGF-2α and PGE-2 production, uterine PGI-2 production did not change during the oestrous cycle (Thaler-Dao et al., 1982a). This observation suggests that the synthesis of each of PGE-2, PGF-2α and PGI-2 by the uterus is modulated independently (possibly by activating PGH-2 reductase, PGH-2 isomerase or PGI-2 synthetase) and not via a common intermediate such as the phospholipase or cycloxygenase enzymes.

Mechanism of action of oestrogen and progesterone in stimulating uterine PG synthesis

There are several ways in which ovarian steroids could modulate uterine PG production. Since PGs are not stored in tissues, it is likely that the steroid hormones affect PG synthesis either by a) induction of lipases, thereby providing arachidonic for PG synthesis. In this regard it is interesting to note that in the ewe, progesterone administration caused the appearance of lipid droplets in the endometrium which decreased following oestrogen treatment (Brinsfield and Hawk, 1973) b) direct induction of PG synthetase.

There is evidence that oestradiol treatment increases the level of PG synthetase in the uterus of the rat (Ham et al., 1975) and guinea-pig (Naylor and Poyser, 1975; Wlodawer, Kindall and Hamberg, 1976; Thaler-Dao, 1982).

c) affecting co-factor availability thereby providing optimal conditions for enzyme and substrate interaction.

Protein synthesis is required for oestradiol-induced PG synthesis in the guinea pig uterus, as intra-uterine administration of actinomycin D abolished the increase in uterine PG synthesis which normally occurs at the end of the oestrous cycle (Poyser, 1979). Inhibition of ovarian oestradiol output following treatment with the oestrogen antagonist Tamoxifen was probably responsible for the attenuation of the increase in uterine PGF, PGD and PGE production normally seen on day 5 of pseudopregnancy in the rat (Fenwick et al., 1977). Tamoxifen also antagonizes the effects of oestrogen at the tissue receptor level (Harper and Walpole, 1967). These findings suggest that oestrogen release and receptor activation is required for the stimulation of uterine PG synthesis.

PG synthesis by the endometrium and myometrium

Most of the preceeding studies have concerned PG synthesis by the whole uterus. However, the uterus contains two types of tissue (the endometrium and myometrium) which differ greatly in structure. There is general agreement that the main site of PGI-2 synthesis is the myometrium, whereas the endometrium synthesizes mainly PGF-2α and PGD-2 (Williams, Sneddon and Harney, 1974; Williams, Dembinska-Kiec and Zmuda, 1978; Abel and Kelly, 1979; Alwachi, Bland and Poyser, 1980; Poyser, 1983). The study by Abel and Kelly (1979) demonstrated that in vitro, human endometrial tissue synthesized less PGI-2 when incubated separate from the myometrium.

The differential PG production by endometrial and myometrial tissue may reveal differing roles for PGs in these tissues, although there may be an interaction between the two tissue types.

Summary and aims

Uterine PG synthesis has been widely investigated. However, many of the studies which have been described have involved relatively infrequent measurements of only PGE and PGF synthesis. In the experiments described in section 2.3 uterine synthesis of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 has been studied at 4-hourly intervals throughout the rat oestrous cycle. In addition PG and TX production by the separated endometrium and myometrium has been measured. As to the control of uterine PG synthesis, it has been pointed out that existing studies showing an effect of ovarian steroids on uterine PG synthesis have used steroid treatments which do not attempt to mimic the physiological situation. Consequently, the effects of oestrogen and progesterone on uterine PG synthesis has been re-examined.

The experiments described in section 2.4 were performed to investigate the hypothesis that diminished uterine PG synthesis in the aged animal could be responsible for the failure of the uterus to support blastocyst implantation which has previously been described in aged animals.

The control of PG biosynthesis in reproductive tissues

In the preceeding pages, the evidence for and against the involvement of PGs in several reproductive processes has been reviewed. The information largely deals with the effects of indomethacin on these processes and the pharmacological effects of PGs. In addition, evidence has been presented to show that PG synthesis by the uterus and ovary (and possibly the hypothalamus) changes throughout the oestrous cycle. The stimulus for PG synthesis in the ovary, appears to be LH and in the uterus, oestrogen and progesterone. However, relatively little is known of precisely how these hormones exert their actions on the PG synthetic pathway.

It has already been mentioned that PGs are not stored in tissues and therefore by controlling PG synthesis, a hormone could affect PG release. In addition, during PG synthesis the cyclooxygenase enzyme is destroyed (Lands, Le Tellier and Vanderhoek, 1973) and thus fresh enzyme must be synthesized for further PG release. Indeed there is evidence that protein synthesis is an integral part of LH-induced (Clark et al., 1976) and estrogen-induced (Poyser, 1979) PG synthesis by the ovary and uterus, respectively. In addition, PG synthetase levels have been shown to increase during periods of increased PG production by the ovary and uterus (Bauminger and Linder, 1975; Anteby et al., 1975, Wlodawer et al., 1976). However, when the synthesis of several PGs in the ovary (Bauminger and Lindner, 1975) and in the uterus (Poyser and Scott, 1980; Thaler- Dao et al, 1982) is considered, it is apparent that the ratios of products changes during the oestrous cycle.

This suggests that there must be specific mechanisms which direct PG synthesis towards the production of one PG in preference to another. It is possible that this is achieved by controlling the relative quantities of the specific enzymes and/or co-factors which convert the endoperoxides into PGs.

Fig 4 shows the pathway for PG and TX synthesis and breakdown and outlines the various stages at which control of PG synthesis could be exerted. In addition to controlling PG synthetase levels, changes in PG levels in tissues could be brought about by altering PG metabolism. There is evidence that PGE-2 metabolism in rat lung tissue is increased in pregnancy and is decreased by oestradiol treatment (Blackwell and Flower, 1975). Also PGE-2 and PGF-2α levels may be altered by the variations in activity of the enzyme PGE-2-9-ketoreductase which has been identified in ovarian tissue (Watson, Shepherd and Dodson, 1979; Shepherd and Dodson, 1980) as well as in rat brain incubates (Wolfe, Pappius and Marion, 1976).

It is widely held that the release of arachidonic acid is the rate-limiting step for PG synthesis in tissues (see Vogt, 1978). Indeed, the levels of free arachidonic acid are too low for continued PG synthesis in the guinea pig uterus (Leaver and Poyser, 1980). Consequently arachidonic acid release from some bound source commonly assumed to be phospholipids, is necessary to allow PG synthesis to occur. However it is difficult to imagine that alterations in phospholipase activity per se, can account for the changes in PG ratios within tissues.

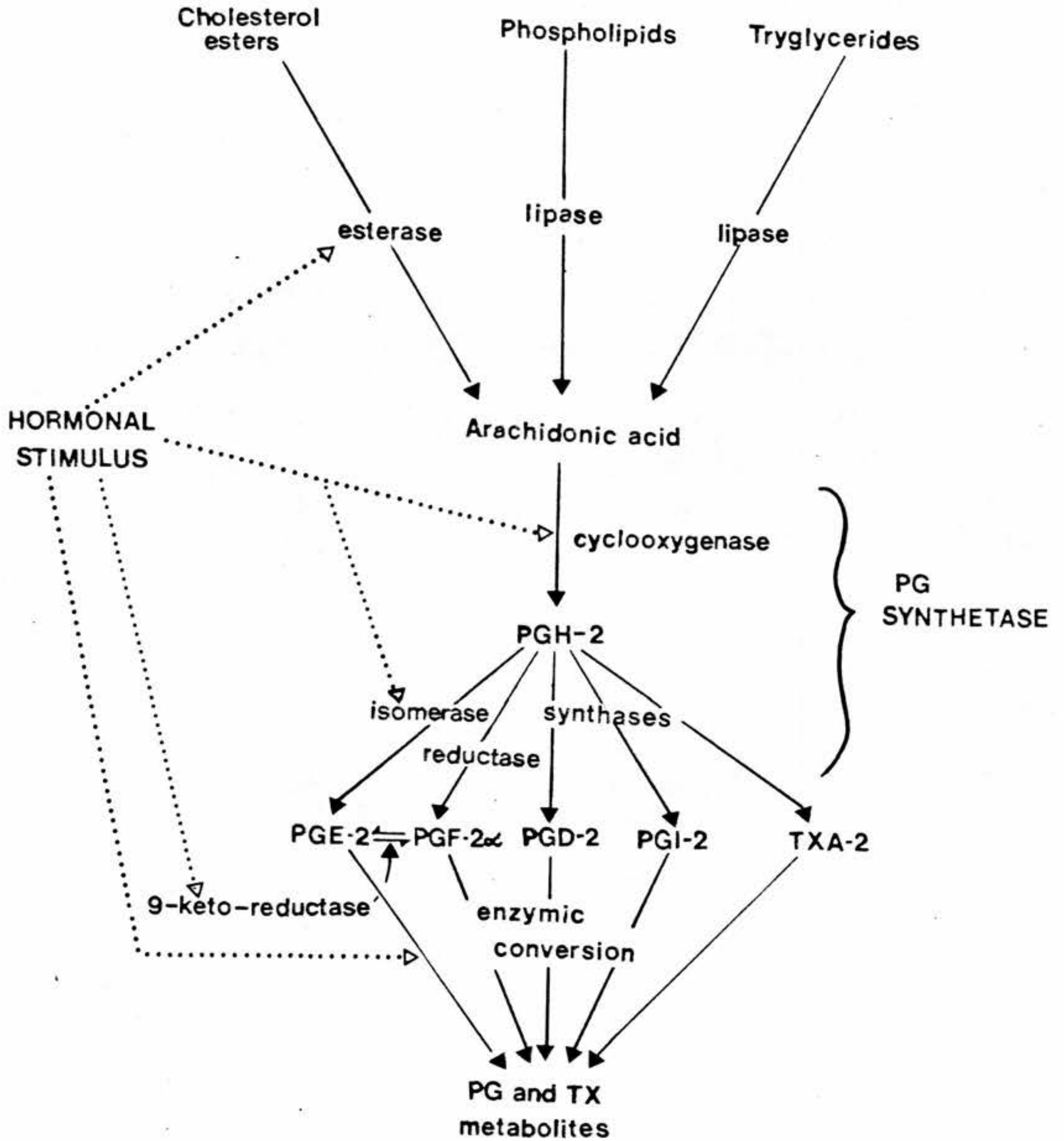


Figure 4. The biochemical pathways for PG and TX synthesis and metabolism showing possible stages at which hormonal control could be exerted.

In the following sections, several aspects of PG and TX synthesis by the ovary, uterus and hypothalamus have been studied. These include:

a) the measurement of PG and TX levels in tissues. This gives information on the endogenous concentrations of PGs and reflects which PGs the tissue synthesizes <u>in vivo</u>

b) the measurement of PG synthetic capacity following homogenization of the tissue. This procedure liberates arachidonic acid which is then present in excess amounts in the homogenate. The relative amounts of PGs and TX which have been synthesized at the end of the incubation period, gives information about the activity of the enzymes which convert the arachidonic acid into PGs and TX

c) metabolism of PGE-2 and PGF-2α and

d) the measurement of the interconversion of PGE-2 and PGF-2α. This gives a measure of the activity of the PGE-2-9-ketoreductase enzyme.

e) release of PGs from the superfused uterus. This gives an indication of which PGs are released from uterine tissue after synthesis within the cells of the uterus.

It is anticipated that these studies will provide further insight into the significance of, and into the control of, PG biosynthesis in these reproductive tissues.

SECTION 1. MATERIALS AND GENERAL METHODS

This thesis presents work which has largely involved the measurement of PGs and thromboxane in the uterus, ovaries, median eminence and hypothalamus of female rats.

PGs and thromboxane have been extracted from homogenized tissues or superfusates into organic solvents and have been measured by RIA. Where possible, the PGs and thromboxane were identified by combined gas chromatography and mass spectrometry (GC-MS). The experimental results have been divided into four sections. This section describes the general procedures for extracting, measuring and identifying PGs and thromboxane which are common to each section.

SECTION 1.1 MATERIALS

Solvents

Chloroform (analar)	B.D.H. Chemicals Ltd.
1,2-Dichloroethane (HPLC grade)	Rathbone Chemicals
Ethanol	J. Boroughs Ltd.
2-Ethoxyethanol (analar)	B.D.H. Chemicals Ltd.
*Ethyl acetate (reagent grade)	B.D.H. Chemicals Ltd.
Glacial acetic acid (analar)	B.D.H. Chemicals Ltd.
Hexane (analar)	B.D.H. Chemicals Ltd.
Methanol (analar)	B.D.H. Chemicals Ltd.
*Petroleum spirit, b.p 40-60°C (reagent grade)	B.D.H. Chemicals Ltd.
Toluene	Koch Light Laboratories Ltd.

(*) These solvents were re-distilled prior to use.

Radioactive compounds

(5,6,8,11,12,14,15 (n)-^{3}H) Prostaglandin E-2 (Sp. act. 160 Ci $mmol^{-1}$)
Amersham International.

(5,6,8,11,12,14,15 (n)-^{3}H) Prostaglandin F-2α (sp. act. 160 Ci $mmol^{-1}$)
Amersham International.

(5,8,9,11,12,14,15 (n)-^{3}H) 6-keto-Prostaglandin F-1α (sp. act. 150 Ci $mmol^{-1}$)
Amersham International.

(5,6,8,9,11,12,14,15 (n)- ^{3}H)	Thromboxane B-2 (sp. act. 100 Ci mmol^{-1}).
	New England Nuclear Inc.

All radioactive compounds were stored at 5 μCi ml^{-1} in methanol at -20°C.

Other Chemicals

Most authentic PGs, thromboxanes and their metabolites were the gift of Dr. J. Pike, Upjohn Company, Kalamazoo, U.S.A. PGH-2 was prepared by chemical synthesis by Dr. R.L. Jones and Dr. N.H. Wilson in this department.

Althesin	Glaxo, U.K.
Arachidonic acid	Sigma Chemical Company, U.K.
Flurbiprofen	Boots Company, U.K.
Heparin	Evans Medical Ltd., U.K.
2-hydroxyoestradiol	Sigma, Chemical Company, U.K.
Noradrenaline	Sigma Chemical Company, U.K.
Oestradiol benzoate	Sigma Chemical Company, U.K.
Progesterone	Sigma Chemical Company, U.K.
Sagatal	May & Baker Ltd., U.K.

Krebs' solution was made up daily as required in 1l quantities:-

2g Glucose

2.1g Sodium dihydrogen phosphate

6.9g Sodium Chloride

1.4 ml 10% Magnesium sulphate (1.47g)

1.6 ml 10% Potassium dihydrogen orthophosphate (0·81g)

2.5 ml 1 M Calcium chloride (1·86g)

3.5 ml 10% Potassium chloride (1·77g)

All reagents were supplied by B.D.H. Chemicals Ltd., U.K.

Chemicals and Solutions for Radioimmunoassay (RIA)

Gelatin	B.D.H. Chemicals Ltd., U.K.
Sodium Azide	Hopkin and Williams Ltd., U.K.
Polyethylene glycol	Sigma Chemical Company, U.K.
PPO (2, 5-diphenyloxazole)	Fisons, U.K.
Tris (hydroxymethyl) methylamine	B.D.H. Chemicals Ltd., U.K.

Diluents for Radioimmunoassay

Diluent 1	0.05M Tris buffer pH 8.0
	0.1 gl^{-1} Sodium azide
	1.0 gl^{-1} Gelatin
Diluent 2	0.05M Phosphate buffer pH 7.5
	0.1 gl^{-1} Sodium azide
	1.0 gl^{-1} Gelatin
Diluent 3	0.05M Tris buffer pH 6.8
	0.1 gl^{-1} sodium azide
	1.0 gl^{-1} Gelatin
Scintillation fluid I	10.5 g PPO
	1.5l Toluene

Scintillation fluid II 10.5 g PPO
1.5 l Toluene
0.9 l 2-ethoxyethanol

Donkey anti-rabbit serum (DARS) Wellcome Reagents Ltd., and Scottish Antibody Production Unit, U.K.

Normal rabbit serum (NRS) was obtained from male New Zealand white rabbits by the method of Dighe, Emslie, Henderson and Simon, 1975.

SECTION 1.2 EXTRACTION OF PGS AND TX FROM BIOLOGICAL FLUID

Section 1.2a Solvent extraction of PGs and TX

PGs were extracted from homogenates using the method described by Poyser (1972). The pH of the homogenate was first lowered to 4 to 4.5 by the dropwise addition of IN HCL. The homogenates were then partitioned three times with two volumes of redistilled ethyl acetate. The ethyl acetate fractions were combined and evaporated to dryness under reduced pressure at 45°C on a rotary evaporator. Residues were redissolved in known volumes of ethyl acetate and stored at -20°C until assayed.

Section 1.2b Recovery of tritiated PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 from tissue homogenates

Method

Four albino Wistar rats were killed by decapitation. Each uterus was removed and divided into 4 segments each weighing approximately 120 mg. Each segment was homogenized in 2 ml Krebs' solution using a Fisons glass homogenizer, and the homogenate was added to a 25 ml flask. The homogenizer was washed with two 1 ml volumes of Krebs' solution and the washings added to the flask. 0.2 μCi of ^{3}H PGF-2α was added to one flask and also to 4 scintillation vials. These scintillation vials acted as counting standards. The other three flasks contained 0.2 μCi ^{3}H PGE-2, 0.2 μCi ^{3}H 6-keto-PGF-1α and 0.2 μCi ^{3}H TXB-2 respectively. Counting standards were also set up as for the flask with ^{3}H PGF-2α. The homogenates were incubated for 90 min at 37°C in a Grant shaking water bath gassed with 5% CO_2 in O_2. Homogenates were then extracted as described in section 1.2a.

After taking each sample to dryness, the residue was redissolved in 2 ml ethyl acetate. The ethyl acetate was transferred to a scintillation vial and evaporated to dryness under nitrogen at 45°C. 10 ml scintillation fluid I was added to each vial and the radioactivity monitored using the sample channels ratio method. The percentage recovery was calculated from the formula:

$$\% \text{ recovery} = \frac{\text{disintegrations per min (dpm) per vial}}{\text{average dpm of counting standards}} \times 100$$

Results

The percentage recoveries (mean + s.e.m., n=4) at pH 4

were 96.3 ± 0.53% for PGF-2α, 89.7 ± 1.44% for PGE-2, 81.5 ± 2.01% for 6-keto-PGF-1α, and 95.1 ± 1.3% for TXB-2.

It is presumed that similar recovery values would be obtained after extraction of PGs from ovarian and brain tissue.

Conclusion

Extracting PGs by the method described in section 1.2a gives high recovery for PGF-2α, PGE-2, 6-keto-PGF-1α and TXB-2. PG concentrations measured in tissue homogenates have not been corrected for procedural losses.

SECTION 1.3 MEASUREMENT OF PGS BY RADIOIMMUNOASSAY

Section 1.3a PGE-2 Radioimunoassay

Introduction

PGE-2 antiserum was raised byDr. K.K. Dighe in rabbits immunized with prostaglandin-bovine serum albumin conjugates using the procedure described by Dighe, Jones and Poyser (1978). The double antibody method was used for the separation of free- from antibody-bound prostaglandin (Levine, Gutierrez Cernosek and Van Vunakis, 1971).

PGE-2 Standard Curve

Method

Standard solutions were made up in diluent 2 from a stock solution of 1 μg ml^{-1} PGE-2 in methanol.

Standard PG solutions were dispensed in triplicate into plastic tubes as detailed in Table 1.

Table 1. Concentrations of PGE-2 standards used in setting up a PGE-2 standard curve together with the volume of standard dispensed and the amount of PGE-2 in each tube

Concentration PGE-2 standards $(ng\ ml^{-1})$	Volume tube (ml)	ng PGE tube^{-1} (ng)
5.12	0.5	2.56
2.56	0.5	1.28
1.28	0.5	0.64
0.64	0.5	0.32
0.32	0.5	0.16
0.16	0.5	0.08
0.08	0.5	0.04
0.04	0.5	0.02
0.02	0.5	0.01

Tubes were also set up in quadruplicate for counting standards, zero standards and non-specific binding standards. Zero standards contained 0.5 ml diluent 2 and non-specific binding standards contained 0.5 ml of 10 ng ml^{-1} standard PGE-2. One tube was also set up for background counting.

Before use, the methanol containing radioactive PGE-2 (tracer) was evaporated to dryness at 45°C under a stream of air. The residue was redissolved in diluent 2 to give a concentration of approximately 0.26 μCi ml^{-1} (0.6 ng ml^{-1}). Antiserum was diluted in diluent 2. The dilution of antiserum chosen was that which bound 60% of tracer PGE-2 in the absence of non-radioactive standard PG. All tubes were treated as in Fig. 5.

The counting standards indicate the average amount of radioactivity (i.e. the average number of counts) added to each tube. The zero standards indicate the binding of the tracer PG to the antibody in the absence of non-radioactive PG. Non-specific binding is assessed by calculating the degree of binding of tracer in the presence of excess non-radioactive prostaglandin which is present in sufficient amounts to completely prevent binding of ^{3}H-PGE-2 to the specific sites on the antibody. If the non-specific binding exceeded 10% in any assay, the assay was repeated.

<u>Preparation of samples for radioimmunoassay</u>

Tissue homogenates were extracted into ethyl acetate as described previously (section 1.2a). Two different volumes of 50 to 500 μl were dispensed in duplicate, (to check for parallelism between volumes) and the ethyl acetate was blown off under a stream of air at 45°C. A volume of 0.5 ml of diluent 2 was added to each tube and the tubes treated according to Fig. 5. In addition to the assay tubes, four tubes were set up containing 0.5 ml ethyl acetate.

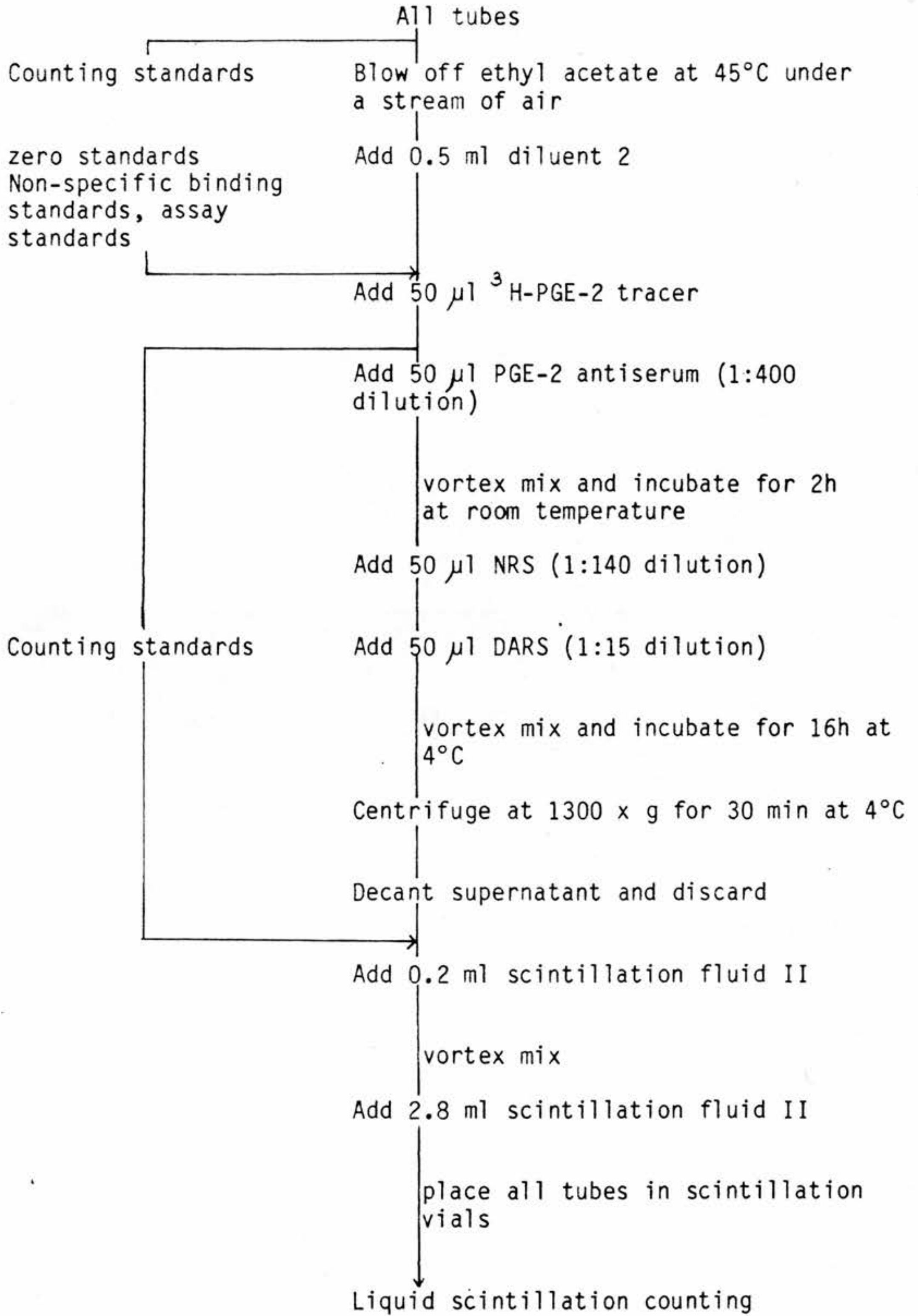


Fig. 5. Procedure for setting up a PGE-2 standard curve and assay.

These tubes monitored any possible interference of a residue in the ethyl acetate with the binding of tracer to antibody. If any binding did occur, the assay was repeated. Two standard PG solutions were also set up in duplicate at the end of the assay tubes. These tubes were used to calculate the interassay coefficient of variation. The tubes were treated as in Fig. 5.

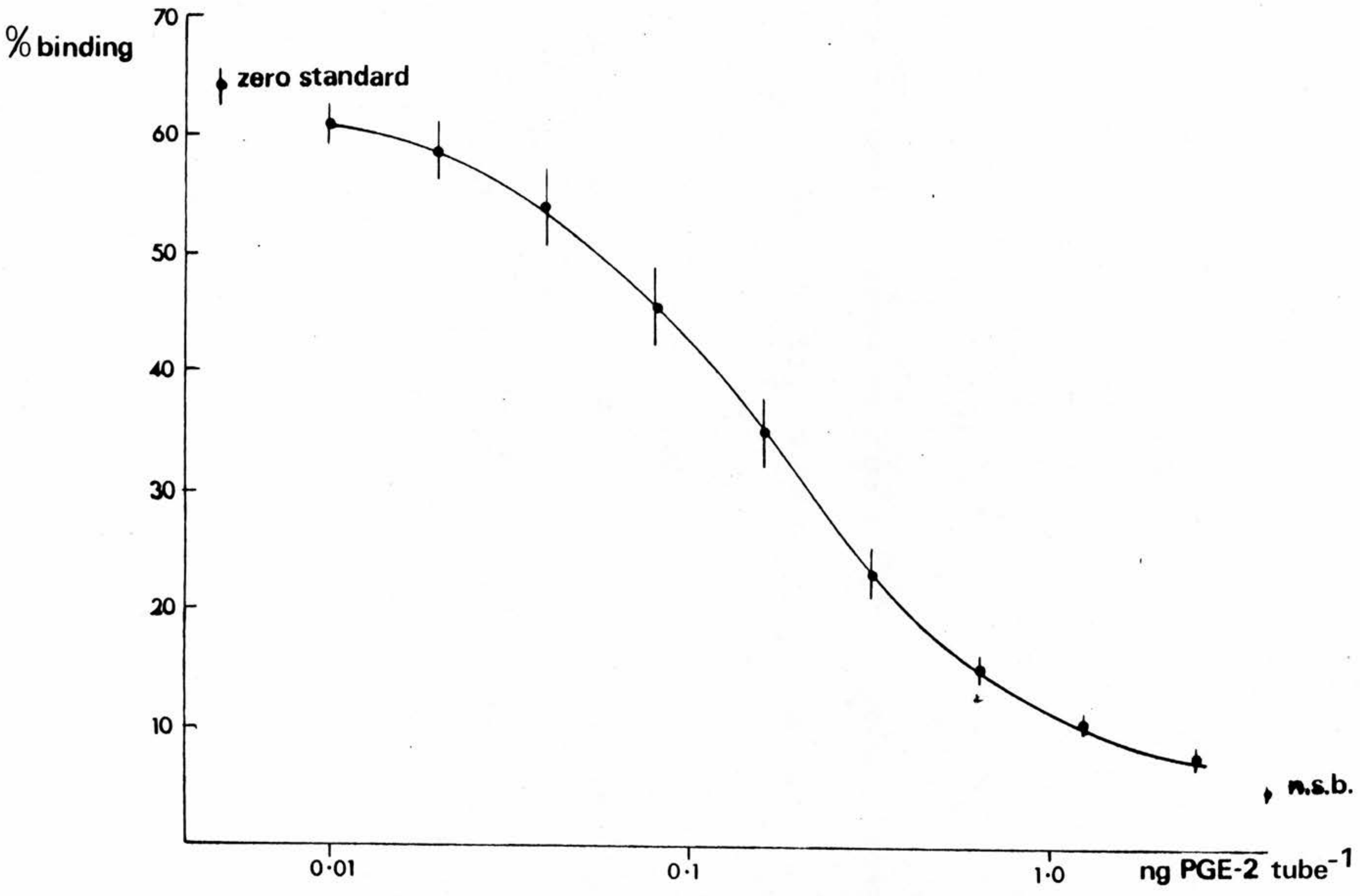
The vials were monitored for radioactivity in a Nuclear Chicago Liquid Scintillation counter, counting each vial for 4 min. Counts from the liquid scintillation counter were recorded on paper tape which was fed into a PDP 8 computer, programmed to calculate the percentage binding. The percentage of tracer bound was calculated using the formula:

$$\% \text{ bound} = \frac{\text{sample counts - background counts}}{\text{average counting standard counts - background counts}} \times 100$$

The computer applied a logistic curve fitting formula to the observed values of percentage tracer bound in the standard curve and calculated the best fitting curve. Two sets of values, observed and calculated, were compared, and, if the variation between them was less than 5%, the curve was judged to be acceptable.

Results

The standard curves of 6 consecutive PGE-2 assays are shown in Fig. 6 (mean $\pm$ s.e.m.). The detection limit of the assay, defined as the amount of standard PG required to give a 10% fall in binding from the zero standard, was 40 pg. The gradient of the linear portion of the standard curve for PGE-2 was 11.5% measured over a doubling of the concentration of PGE-2 per tube. If the gradient is less than 8%, small differences in PGE-2 concentrations in samples, produce changes in binding of tracer that are too small to be measured accurately.


% binding
70
60
50
40
30
20
10
zero standard
n.s.b.
0·01
0·1
1·0
ng PGE-2 tube-1

If the gradient of the standard curve was less than 8%, the assay was repeated. The intra-assay coefficient of variation was calculated from the formula:-

$$\text{intra assay coefficent of variation} = \frac{\text{standard deviation of sample quadruplicate}}{\text{mean value of sample quadruplicate}} \times 100$$

The intra-assay coefficient of variation for the PGE-2 radioimmunoassay was 9.2%, and the working range of the assay was 40-500 pg. The inter-assay coefficient of variation was calculated from the results obtained by incorporating a known amount of PGE-2 into each assay, using the formula.

$$\text{Inter-assay coefficient of variation} = \frac{\text{standard deviation of PGE-2 standard}}{\text{mean of PGE-2 standard}} \times 100$$

The inter-assay coefficient of variation for the PGE-2 assay was 12.6%.

The accuracy of this assay was assessed by adding known amounts 0, 50, 200, 500 and 1000 ng of PGE-2 to 20 ml Krebs' solution, extracting the PG as described in Section 1.2a and measuring the amount of PGE-2 recovered by radioimunoassay. The mean values obtained were respectively (mean $\pm$ s.e.m., n=3) 0, 45 $\pm$ 7 ng, 208 $\pm$ 19 ng, 490 $\pm$ 37 ng and 987 $\pm$ 35 ng. This shows that the PGE-2 assay can measure PGE-2 extracted from Krebs' solution with sufficient accuracy.

Cross reactivity of the PGE-2 antiserum

Method

A standard curve for PGE-2 was set up. In parallel, standard curves for different PGs and PG metabolites were prepared. The percentage cross reactivity of the antiserum was determined by finding the concentration of PG or PG metabolite which produced a 10% fall in binding obtained with the zero standard. The following calculation was then applied:

$$\% \text{ cross reactivity} = \frac{\text{concentration of PGE-2 giving a 10\% (or 30\%) fall in zero-binding}}{\text{concentration of PG or PG metabolite giving a 10\% fall in zero-binding}} \times 100$$

Results

The cross reactivities of antiserum obtained from rabbit 5, 7 bleed with various prostanoids are given in Table 2.

Conclusion

The PGE-2 antibody cross reacts significantly with PGE-1, PGA-2 and PGB-2. Samples obtained by extraction of tissue homogenates were therefore subjected to further analysis by gas chromatography-mass spectometry (GC-MS) to determine if these samples contained significant quantities of PGE-1, PGA-2 and PGB-2 which might interfere with the measurement of PGE-2 in them. The GC-MS analysis is described in Section 1.4.

Table 2. Cross reactivities of prostanoids with PGE-2 antiserum from rabbit 5, 7th bleed, measured at 10% fall in zero binding of tracer.

Prostanoid	% cross reactivity
PGE-2	100
PGE-1	94
PGA-2	13.6
PGB-2	72.7
PGF-2α	1.5
PGD-2	0.4
15-keto-PGE-2	0.2
13,14-dihydro,15-keto-PGE-2	0.1
15-keto-PGF-2α	1.1
13,14-dihydro,15-keto-PGF-2α	0
6-keto-PGF-1α	1.1
TXB-2	0.2

Section 1.3b PGF-2α Radioimmunoassay

Introduction

Samples were assayed for PGF-2α using an antiserum raised in rabbits immunized with a PGF-2α-bovine serum albumin conjugate (Dighe, Emslie, Henderson, Rutherford and Simon, 1975).

Method

Reagents: Diluent 1

^{3}H-PGF-2α 0.26 μCi ml^{-1} (0.6 $ngml^{-1}$) in diluent 1

PGF-2α antiserum (rabbit 6, 4th bleed) 1:1500 dilution in diluent 1.

NRS 1:140 dilution in diluent 1

DARS 1:15 dilution in diluent 1.

PGF-2α Standard Curve and Assay

A standard curve for PGF-2α was set up as for PGE-2. Standard solutions of PGF-2α were made up in diluent 1 in concentrations from 0.2 ng ml^{-1} to 5.12 $ngml^{-1}$. Standards were dispensed in triplicate into tubes in 0.5 ml volumes. Counting standards, non-specific binding standards and zero standards were set up in quadruplicate. One tube was also set up for background counting. All tubes were treated as in Fig. 7.

Results

Fig. 8 shows the standard curve for the PGF-2α radioimmunoassay drawn from the results of 6 consecutive assays (mean $\pm$ s.e.m.). The detection limit was 40 pg PGF-2α. Intra- and inter-assay coefficients of variation were 8.6% and 11.1% respectively. The gradient of the standard curve measured over a doubling of the concentration of PGF-2α in the tube was 13% and the working range of the assay was 40 to 500 pg. The accuracy of the assay was demonstrated by adding known amounts of PGF-2α, 0, 50, 200, 500 and 1000 ng PGF2α to 20 ml Krebs' solution, extracting the PGF-2α using the method described in Section 1.2a and measuring the amount of PGF-2α by radioimmunoassay. The values obtained were 0, 52 $\pm$ 2 ng, 187 $\pm$ 15 ng, 478 $\pm$ 31 ng and 978 $\pm$ 40 ng (mean $\pm$ s.e.m.) respectively.

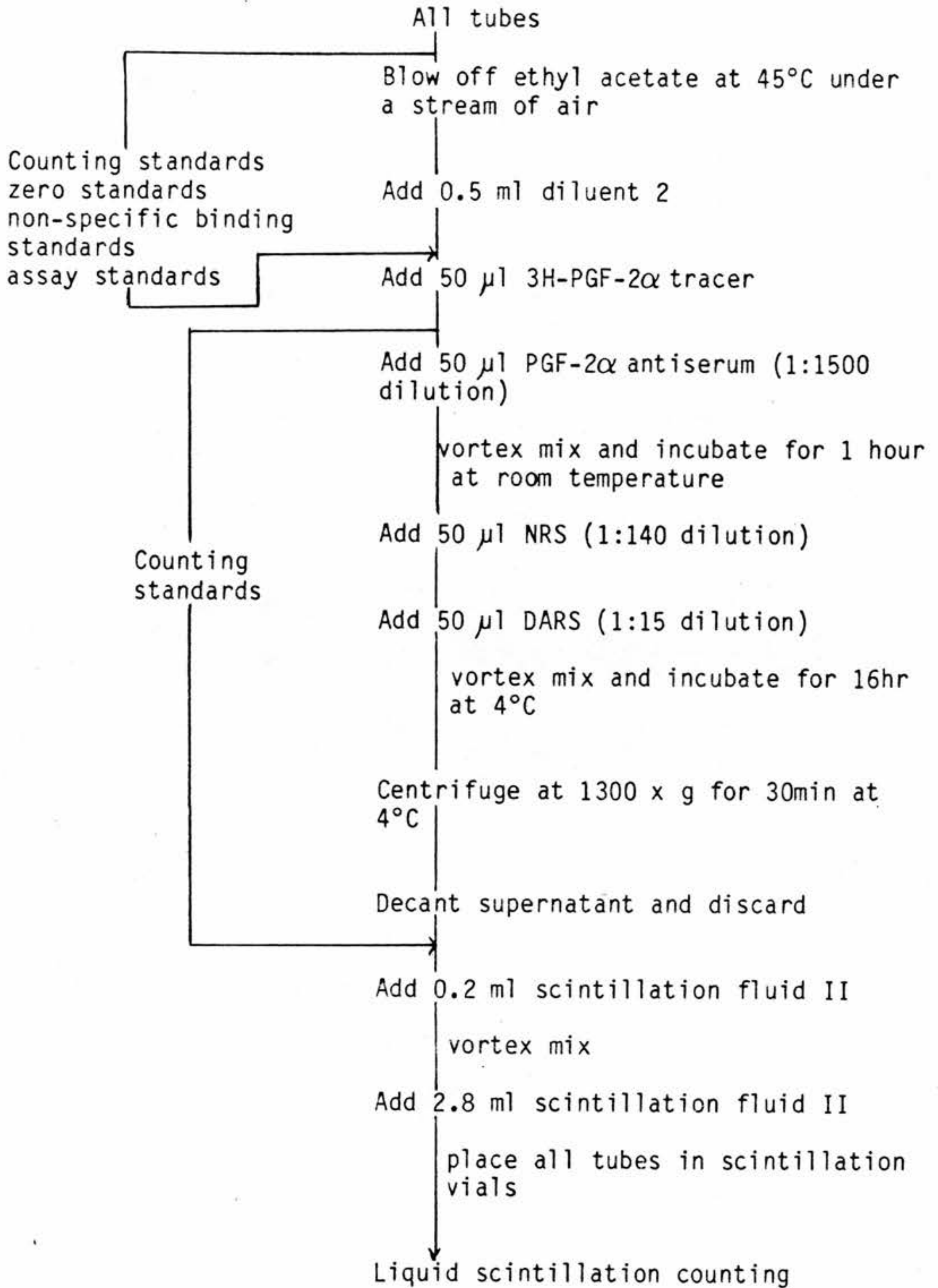
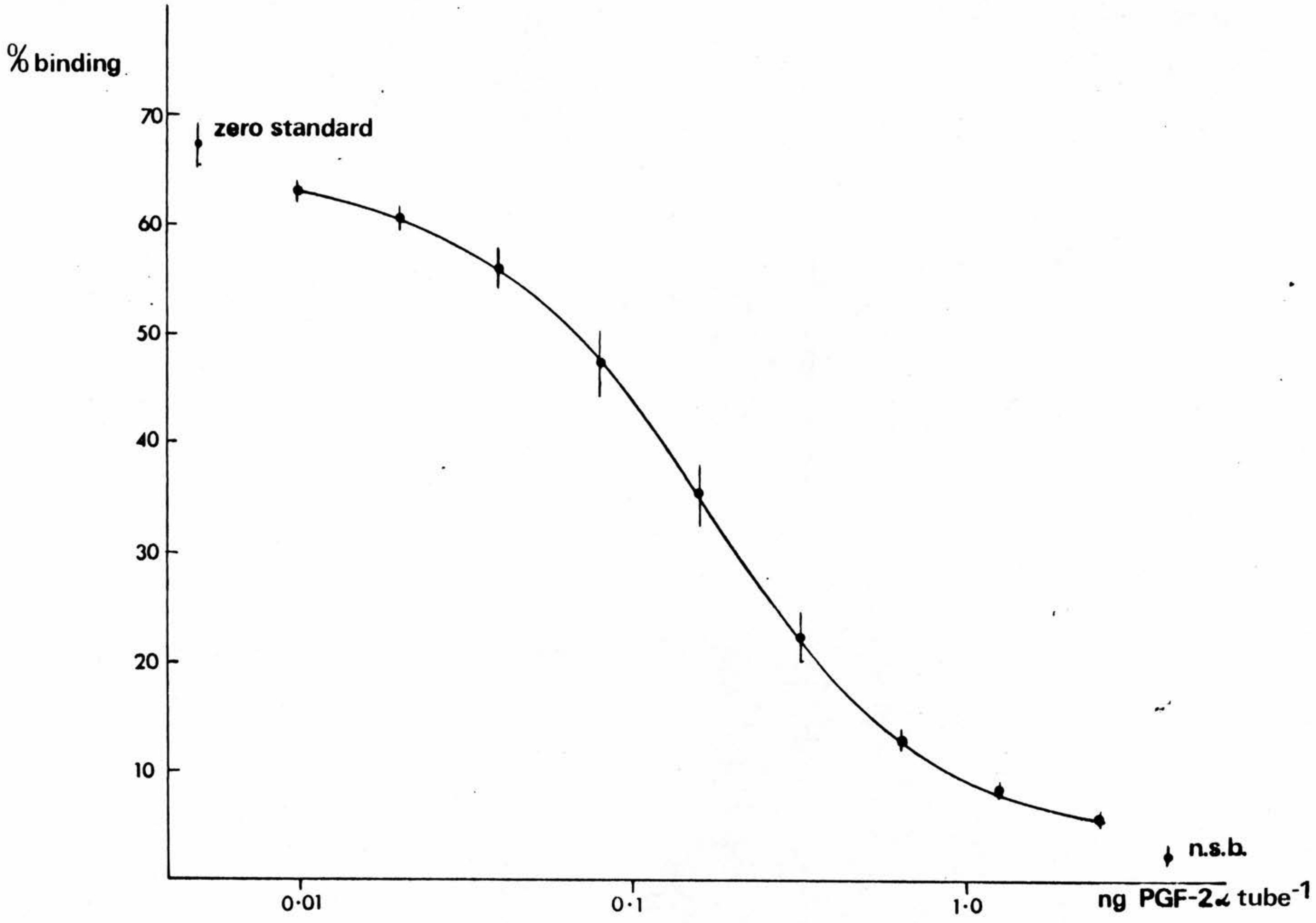


Fig. 7. Procedure for setting-up a PGF-2α Standard Curve and Assay.



% binding
70
60
50
40
30
20
10
zero standard
0·01
0·1
1·0
n.s.b.
ng PGF-2α tube⁻¹

The cross reactivities of antiserum from rabbit 6, 4th bleed with other prostanoids as determined by Miss I. Ramsay are given in Table 3.

Table 3. Cross reactivities of prostanoids with PGF-2α antiserum from rabbit 6, 4th bleed, measured at 30% fall in zero binding of tracer.

Prostanoid	% cross reactivity at 30% binding of tracer
PGF-2α	100
PGF-1α	100
PGE-2	0.56
PGE-1	0.60
PGA-2	0.07
PGB-2	0.01
PGD-2	0.30
15-keto-PGF-2α	0.10
13,14-dihydro,15 keto-PGF-2α	0.02
6-keto-PGF-1α	1.1
TXB-2	0.1

Conclusion

The PGF-2α antibody cross reacts completely with PGF-1α. Samples obtained by extraction of tissue homogenates were therefore subjected to further analysis by GC-MS to determine if these samples contained significant quantities of PGF-1α as described in section 1.4.

Section 1.3.c 6-keto-PGF-1α Radioimmunoassay

6-keto-PGF-1α was measured using an antibody raised in rabbits immunized with a 6-keto-PGF-1α-thyroglobulin conjugate (Dighe, Jones and Poyser, 1978).

Method

Reagents: Diluent 3

^{3}H-6-keto-PGF-1α 0.24 μCi ml^{-1} (0.6 $ngml^{-1}$) in diluent 3.

6-keto-PGF-1α antiserum (rabbit 1, 6th bleed) 1:1500 dilution in diluent 3

NRS 1:140 dilution in diluent 3

DARS 1:15 dilution in diluent 3.

6-keto-PGF-1α Standard Curve and Assay

A series of standard solutions of 6-keto-PGF-1α ranging from 0.2 $ngml^{-1}$ to 5.12 $ngml^{-1}$ were made up in diluent 3. The procedure for this assay was as described for the PGF-2α assay.

Results

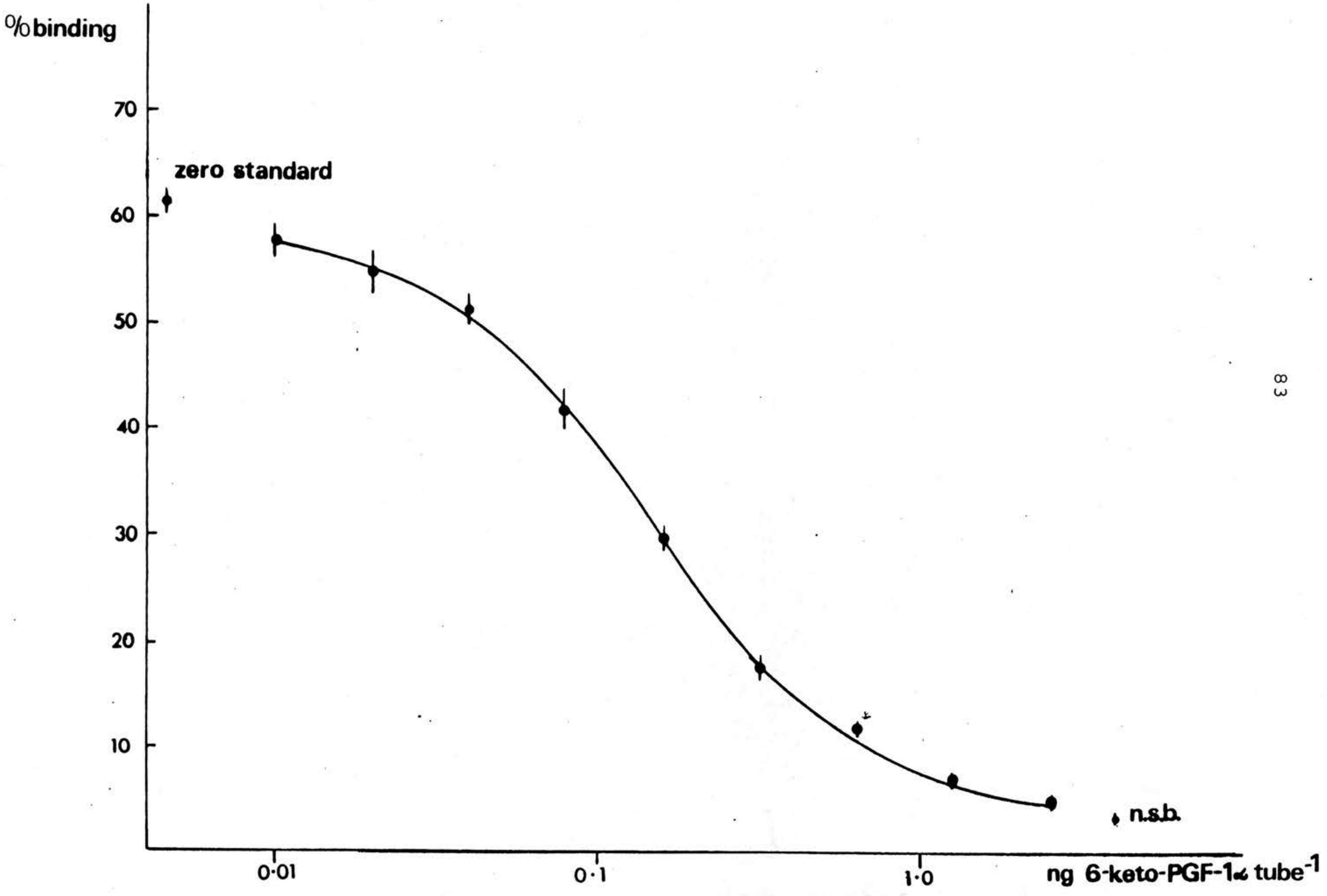
Fig. 9 shows the standard curve for the 6-keto-PGF-1α radioimmunoassay drawn from the results of 6 consecutive assays (mean $\pm$ s.e.m.). The detection limit was 40 pg 6-keto-PGF-1α . Intra- and inter-assay coefficients of variation were 8.2% and 9.7% respectively. The gradient of the standard curve measured over a doubling of the concentration of 6-keto-PGF-1α in the tube was 12% and the working range of the assay was 40 to 400 pg. The accuracy of the assay was assessed by incorporating two tubes, each containing 0.32 ng of 6-keto-PGF-1α standard into the assay. The mean value obtained was 0.310 $\pm$ 0.019 ng (mean $\pm$ s.e.m.).

Deterioration of ^{3}H-6-keto-PGF-1α was indicated by lower percentage binding of tracer to antibody. When this occurred, the tracer was purified by column chromatography as described below.

Purification of ^{3}H-6-keto-PGF-1α by Column Chromatography

Method

^{3}H-6-keto-PGF-1α was purified by straight-phase, liquid-gel partition, column chromatography on Lipidex 1000. The column was first equilibrated overnight with the solvent mixture hexane, 1,2 dichloroethane, ethanol and acetic acid in the ratio 100:100:15:0.1 (Brash and Jones, 1974). Effluent from the column was collected in a L.K.B. ultronic fraction collector. Ten drops from the column were collected and transferred into a scintillation vial to determine background radiation.



%binding
70
60
50
40
30
20
10
zero standard
n.s.b.
0·01
0·1
1·0
ng 6-keto-PGF-1α tube⁻¹

The tritiated 6-keto-PGF-1α was taken to dryness at 40°C under reduced pressure on a rotary evaporator. The tracer residue was redissolved in 0.5 ml methanol and applied dropwise to the top of the column. The flask containing the tracer was then washed three times with 0.5 ml methanol and each washing, applied to the top of the column. 500 ml of the solvent system was added to the reservoir above the column and allowed to flow through the column at a rate of 10ml h^{-1}. Fractions were collected each containing 180 drops.

Samples (10μl) of each fraction were transferred to individual scintillation vials each containing 10 ml scintillation fluid I and counted for 1 min on a scintillation counter. Fig. 10 shows a histogram of the amount of radioactivity in 17 fractions collected from the column. The fractions containing the greatest number of c.p.m. (fractions 7, 8 and 9) were assumed to contain pure tracer and these fractions were pooled and transferred to a pear shaped flask. The fraction tubes were washed three times with 0.5 ml methanol and the washings added to the flask. The pure tracer was taken to dryness at 45°C under reduced pressure and the residue was dissolved in 5 ml methanol. Two 10μl aliquot volumes were transferred to vials containing 10 mls scintillation fluid I and counted in a scintillation counter for d.p.m. The volume of remaining tracer was adjusted with methanol to give a final dilution of 5μCiml^{-1}.

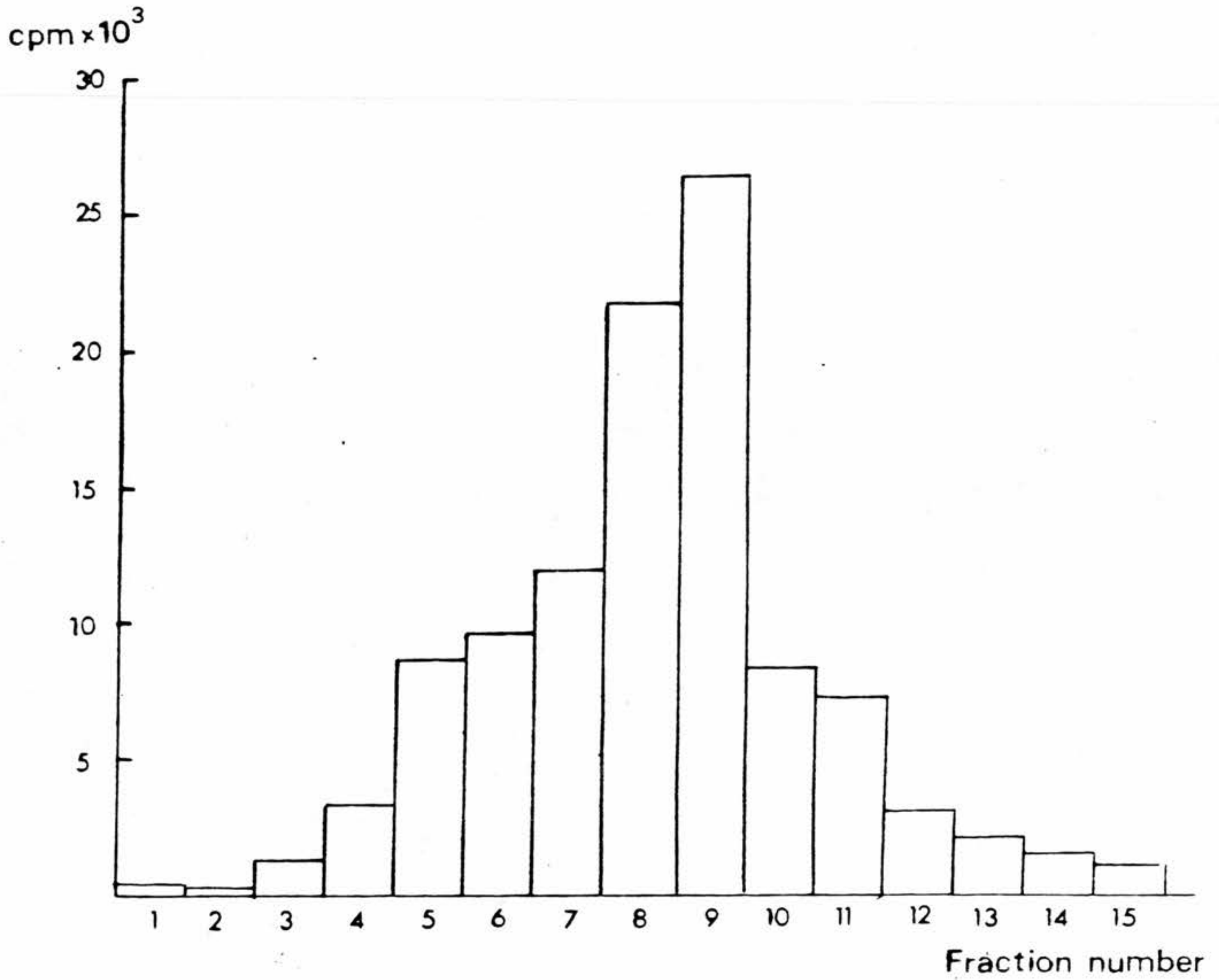


Figure 10. Histogram of the amount of radioactivity in serial fractions collected during purification of ^{3}H-6-keto-PGF-1α by column chromatography on Lipidex 1000.

Results

Following purification of ^{3}H--6-keto-PGF-1α by column chromatography percentage binding of ^{3}H-6-keto-PGF-1α in zero standards was between 60 and 70%.

Table 4 shows the cross reactivities of the 6-keto-PGF-1α antiserum from rabbit 1, 6th bleed with various other prostanoids as determined by Miss I. Ramsay. Cross reactivity was measured at 30% binding of tracer.

Conclusion

Antiserum from rabbit 1, 6th bleed raised against 6-keto-PGF-1α shows minimal cross reactivity with other prostanoids and is considered suitable for the measurement of 6-keto-PGF-1α by radioimmunoassay.

Table 4. Cross reactivities of prostanoids with 6-keto-PGF-1α antiserum from rabbit 1, 6th bleed, measured at 30% fall in binding of tracer.

Prostanoid	% cross reactivity at 30% binding of tracer
6-keto-PGF-1α	100
PGF-1α	0.43
PGE-2	4.2
PGE-1	1.1
PGA-2	0.065
PGB-2	0.03
PGF-2α	0.01
PGD-2	0.01
15-keto-PGF-2α	0.04
13,14-dihydro,15-keto-PGF-2α	0.065
15-keto-PGE-2	0.08
13,14-dihydro,15-keto-PGE-2	0.09
TXB-2	0.01

Section 1.3.d. TXB-2 Radioimmunoassay

Introduction

TXB-2 was measured by an antibody raised in rabbits immunized against a TXB-2-bovine serum albumin conjugate by N.L. Poyser.

Method

Reagents: Diluent 2.

^{3}H-TXB-2 0.16 μCiml^{-1} (0.6 ngml^{-1}) in diluent 2.

TXB-2 antiserum (rabbit 11, 1st bleed) 1:400 dilution in diluent 2.

NRS 1:140 in diluent 2

DARS 1:8 in diluent 2.

Standard solutions of TXB-2 ranging from 0.2 ngml^{-1} to 5.12 ngml^{-1} were made up and the radioimmunoassay set up as for PGE-2.

Results

The results of 6 standard curves for TXB-2 are given in Fig. 11 (mean ± s.e.m.). The limit of detection was 30 pg. Intra- and inter-assay coefficients of variation were 8.0% and 6.1% respectively. The gradient of the standard curve was 12% as measured over a doubling of the concentration of TXB-2 in the tube. The working range of the assay was 30 to 400 pg. The accuracy of the assay was demonstrated by adding known amounts of TXB-2 standard (0, 50, 100, 200, 500 ng) to 20 ml Krebs' solution, extracting the TXB-2 by the method described in Section 1.2a and measuring the amount of TXB-2 by radioimmunoassay. The values obtained were (0, 49 ± 2.1, 103 ± 3.2, 189 ± 7.9, 507 ± 10.3 ng).

Table 5 shows the cross reactivities of TXB-2 antiserum with various prostanoids as measured by Miss I. Ramsay.

Conclusion

Antiserum from rabbit 11, 1st bleed raised against TXB-2 shows negligible cross reactivity with other prostanoids and the accuracy of the assay has been demonstrated. The assay is considered to be suitable for the measurement of TXB-2.

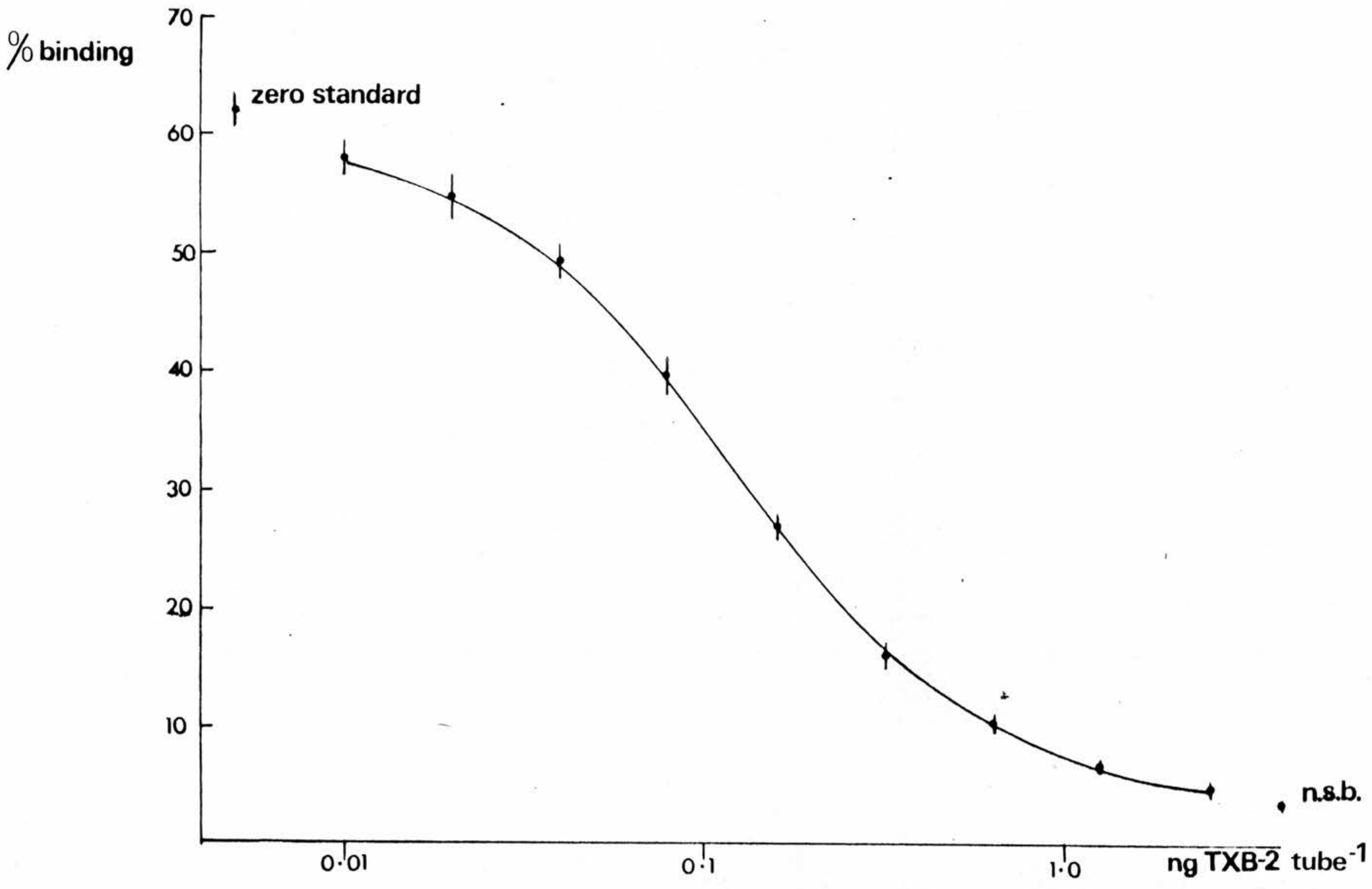

% binding
70
60
50
40
30
20
10
zero standard
0·01
0·1
1·0
ng TXB-2 tube-1
n.s.b.

Table 5. Cross reactivities of prostanoids with TXB-2 antiserum from rabbit 11, 1st bleed, measured at 30% fall in binding of tracer.

Prostanoid	% cross reactivity at 30% binding of tracer
TXB-2	100
PGE-2	0.011
PGE-1	0.037
PGA-2	0.020
PGB-2	0.008
PGF-2α	0.091
PGF-1α	0.048
PGD-2	0.034
15-keto-PGE-2	0.007
13,14-dihydro,15-keto-PGE-2	0.008
15-keto-PGF-2α	0.043
13,14-dihydro,15-keto-PGF-2α	0.023
6-keto-PGF-1α	0.030
13,14-dihydro,15-keto-TXB-2	0.080

SECTION 1.4 COLUMN CHROMATOGRAPHY AND GAS CHROMATOGRAPHY - MASS SPECTROMETRY

Introduction

Although RIA provides a method for the determination of picogram quantities of PGs in biological samples, the identification of these compounds is not definitive. Extraction of the PGs from the biological sample decreases the likelihood of compounds present in the sample cross-reacting with the antibody and cross reactivity studies provide further indication of the specificity of the RIA. However, even if cross reactivity is low, the ability of an antiserum to measure a specific PG will depend on the relative amounts of that PG and cross reacting substances present in the sample.

The cross reactivities of the antisera have been outlined in the previous section and it was noted that the PGF-2α antiserum cross-reacts significantly with PGF-1α and that the PGE-2 antiserum cross-reacts significantly with PGA-2, PGB-2 and PGE-1.

Uterine and ovarian samples which had been assayed for PGs and TX by RIA were therefore subjected to further analysis by GC-MS firstly to positively identify PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 in the samples and secondly to determine if these samples contained significant quantities of PGA-2, PGB-2, PGE-1 and PGF-1α. Unfortunately there was insufficient samples from median eminence or anterior hypothalamus for analysis by GC-MS. Prior to GC-MS analysis, lipids were separated from the tissue extracts by column chromatography.

Section 1.4.a Purification of tissue extracts by column chromatography

Four columns containing 4g silicic acid (Unisil 100-200 mesh) were prepared and each washed with 100 ml diethyl ether.

The extracts of one tissue type (uterus or ovaries) were combined to provide two pooled samples. Each sample was evaporated to dryness under reduced pressure at 45°C, redissolved in 30 ml of 67% ethanol, washed twice with 60 ml petroleum ether (b.p. 40°-60°) and evaporated to dryness again. Each sample was then dissolved in 0.5 ml diethyl ether and applied, dropwise, to the top of each column. The columns were eluted in succession by 100 ml diethyl ether, 100 ml 80% diethyl ether and 20% methanol, and 100 ml methanol. The second solvent mixture elutes PGs and TX and this fraction from each column was evaporated to dryness, and redissolved in 1 ml ethyl acetate.

Section 1.4b Gas chromatography - Mass spectrometry

GC-MS analysis was carried out on a VG-micromass 7070F double focussing gas chromatogram-mass spectrometer. The column (3m x 4 mm) was packed with 3% OV1 on supelcoport 100-120 mesh (Supelco Inc., Bellefonte, U.S.A.). The column temperature was 275°C and the helium carrier gas flow rate was 30 ml min^{-1}. Conditions for operation of the mass spectrometer were:-

Ion source temperature	250°C
Electron energy	70 eV
Accelerating voltage	4 kV
Signal amplification	2×10^{7} amps.

Full mass spectra were recorded on photographic paper by a light beam oscillograph (S.E. Labs. (EMI) Ltd., Feltham, England). A mixture of straight chain saturated fatty acid methyl esters with 16, 18, 20, 22, 24 and 26 carbon atoms was used to calibrate the G.C. column. By plotting the retention times of each fatty acid against the number of carbons in the fatty acid on a logarithmic scale, the observed retention times of the standard PGs can be converted to carbon values. The size and shape of the G.C. peak corresponding to each fatty acid standard also indicates the efficiency of the G.C. column.

Derivitization of PGs for GC-MS

Introduction

Derivitization of PGs is essential to provide a molecule

which has suitable volatility and high thermal stability which will run on the GC column. Carboxylic acid groups on the PGs are protected by forming methyl ester derivatives, hydroxyl groups are protected by forming trimethylsilyl ethers and ketone groups are protected by conversion to butyl oxime derivatives. Butyl oximation produces two geometrical isomers with most ketonic PGs.

Method

Two 0.5 ml aliquot volumes of each sample were evaporated to dryness in 1 ml eppendorf tubes at 20°C under a stream of nitrogen. Drying was completed by vacuum dessication for 15 min.

Methyl ester formation

0.5 ml of diazomethane solution (9 parts diethyl-ether: 1 part methanol) were added to the two tubes from each sample and the contents of the tubes allowed to react for 10 min. The solvent was evaporated at 60°C under a stream of nitrogen and the process was repeated. Following evaporation of the solvent again, the samples were vacuum dessicated for 10 min. The methyl esters of standards - PGA-2, PGB-2, PGD-2, PGE-1, PGE-2, PGF-1α, PGF-2α , 6-keto-PGF-1α and TXB-2 were also prepared by this method.

n-Butyloxime formation

The butyloxime derivatives were prepared by adding 3 drops of 5 ng ml^{-1} O-butyl hydroxylamine hydrochloride in dry pyridine to one tube from each sample and to tubes containing PGD-2, PGE-1, PGE-2, 6-keto-PGF-1α and TXB-2. The tubes were stoppered and the contents allowed to react at room temperature overnight. The following morning the tubes were placed in an oven at 60°C for 15 min to complete the reaction. The pyridine was then evaporated off under a stream of nitrogen at 60°C and drying completed by vacuum dessication for 15 min.

Trimethylsilyl ether formation

30μl bis (trimethylsilyl) trifluoracetamide (BSTFA) was added to all standards and 20μl BSTFA to all samples. After closing the tubes and vortex mixing the tubes were heated for 15 min at 60°C.

Each standard PG was injected, in turn, into the instrument. After noting their retention times on the GC, a full mass spectrum was taken. Each sample was then injected into the instrument and a mass spectrum taken at the appropriate retention time. The mass spectra from the samples and standards were compared. The limit of detection of the mass spectrum is approximately 50 ng. Table 6 shows the characteristic ions used to determine the presence of PGs and TXB-2 in biological samples.

Table 6. Characteristic ions of methyl trimethylsilyl ether (Me, TMS) and methyl trimethylsilyl butoxime (Me, BuO, TMS) derivatives of PG and TX standards.

Compound	Derivative	Characteristic ions (m/e value)
PGA-2	Me,TMS	420,349,190
PGB-2	Me,TMS	420,349,173
PGF-1α	Me,TMS	586,513,497,425,406
PGF-2α	Me,TMS	584,513,494,423,404
TXB-2	Me,BuO,TMS	301,211
6-keto-PGF-1α 1st isomer	Me,BuO,TMS	671,656,600,598,510,508 491
PGD-2 2nd isomer	Me,BuO,TMS	581,510,508,420,418,295
PGE-1 1st isomer	Me,BuO,TMS	583,568,510,512,297
PGE-2 2nd isomer	Me,BuO,TMS	581,566,510,508,295,225

Section 2.1 PGS AND OVULATION

Introduction

Ovarian PGE and PGF levels rise from late afternoon on pro-oestrus, reach a peak at midnight and return to basal levels within 5h. This increase in PG levels follows the surge of LH from the pituitary gland and is a consequence of that surge (Bauminger and Lindner, 1975).

The biochemical mechanism whereby LH stimulates PG synthesis in the ovary is not fully understood. PG synthetase activity in whole ovaries (Bauminger and Lindner, 1975) and in cultured granulosa cells (Clark, Marsh and LeMaire, 1978b) increases concurrently with the increase in PG levels at the time of ovulation. Subsequent studies have indicated that LH stimulates PG synthesis in the ovary by increasing PG synthetase activity rather than by increasing the availability of arachidonic acid for PG synthesis (Bauminger, Liberman and Lindner, 1975; Zor, Strulovici, Nimrod and Lindner, 1977; Clark, Marsh and LeMaire, 1978). Characterization of the ovarian PG synthetase enzyme complex indicates that the apparent Km for arachidonic acid is not altered by hCG treatment, but that hCG increases the specific activity of the enzyme (measured as the amount of PGE produced min^{-1} mg $protein^{-1}$). These findings suggest that hCG and presumably LH increase ovarian PG levels either by increasing the quantity of the enzyme complex or its catalytic efficiency (Clark, Chainy, Marsh and LeMaire, 1980).

Experiments described in this section were performed to elucidate further, the mechanism of the increased ovarian PG synthesis at the time of ovulation.

Section 2.1a Measurement of the ability of ovarian tissue to produce PGs and TX during the oestrous cycle

Introduction

The PG synthetase enzymes form several metabolites of arachidonic acid. In addition to PGE-2 and PGF-2α, rat ovaries synthesize 6-keto-PGF-1α (reflecting PGI-2 synthesis), TXB-2 (reflecting TXA-2 synthesis) and PGD-2 (Poyser and Scott, 1980). This study showed that PGI-2 is the major PG present in, and synthesized by, rat ovarian homogenates. Ovarian 6-keto-PGF-1α and TXB-2 synthesis did not show any daily variations, however PG and TX measurements were only determined at 10:00h on each day of the oestrous cycle. As PG synthesis has previously been shown to rise and fall within a 12h period between pro-oestrus and oestrus (LeMaire, Leidner and Marsh, 1975), such changes in PG synthesis would have been overlooked in the experiment of Poyser and Scott (1980).

The following experiment has involved measurement of the synthesis of 6-keto-PGF-1α and TXB-2 as well as PGE-2 and PGF-2α by the ovary, at more frequent intervals (4-hourly) throughout the oestrous cycle.

Method

A total of 144 mature female Albino Wistar rats weighing between 200g and 250g were used (These animals also provided tissue for use in experiments described in Section 2.2a and Section 2.3a).

The animals were housed under controlled lighting conditions (light period 06:00h to 20:00h) and were offered both a standard diet and water without restriction. After an initial period of 2 days, vaginal smears were taken daily and examined microscopically. Oestrus was determined as the day of maximum cornification of a smear of vaginal epithelial cells, preceeding the day of leucocyte infiltration. Only females showing at least 2 consecutive 4-day oestrous cycles were included in the study.

Animals were killed by a blow to the thorax and decapitation at 02:00h, 06:00h, 10:00h, 14:00h, 18:00h and 22:00h on a particular day of the cycle, a total of six animals being killed for each time of the day. The ovaries from each rat were dissected from the ovarian side of the ovarian-oviductal junction and placed into Krebs' solution. After drying the ovaries, their weight was recorded. The ovaries from each rat were homogenized in 5 ml Krebs' solution and the resulting homogenate added to a 50ml conical flask. The homogenizer was washed twice with two 5ml volumes of Krebs' solution and the washings added to the flask. The homogenate was incubated (but without ^3H-label) for 90 min as described in Section 1.2b. Following incubation, the pH was adjusted to 4 with HCl and the lipids were extracted with ethyl acetate as described in Section 1.2.a. Samples were stored in 10 ml ethyl acetate at -20°C until they were assayed for PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 content by RIA as previously described in sections 1.3a, b, c and d. Results were compared initially by an F-ratio test and subsequently by Duncan's Multiple Range test for equal variances. Significance was tested at the 5% level.

Identification of extracted PGs by GC-MS

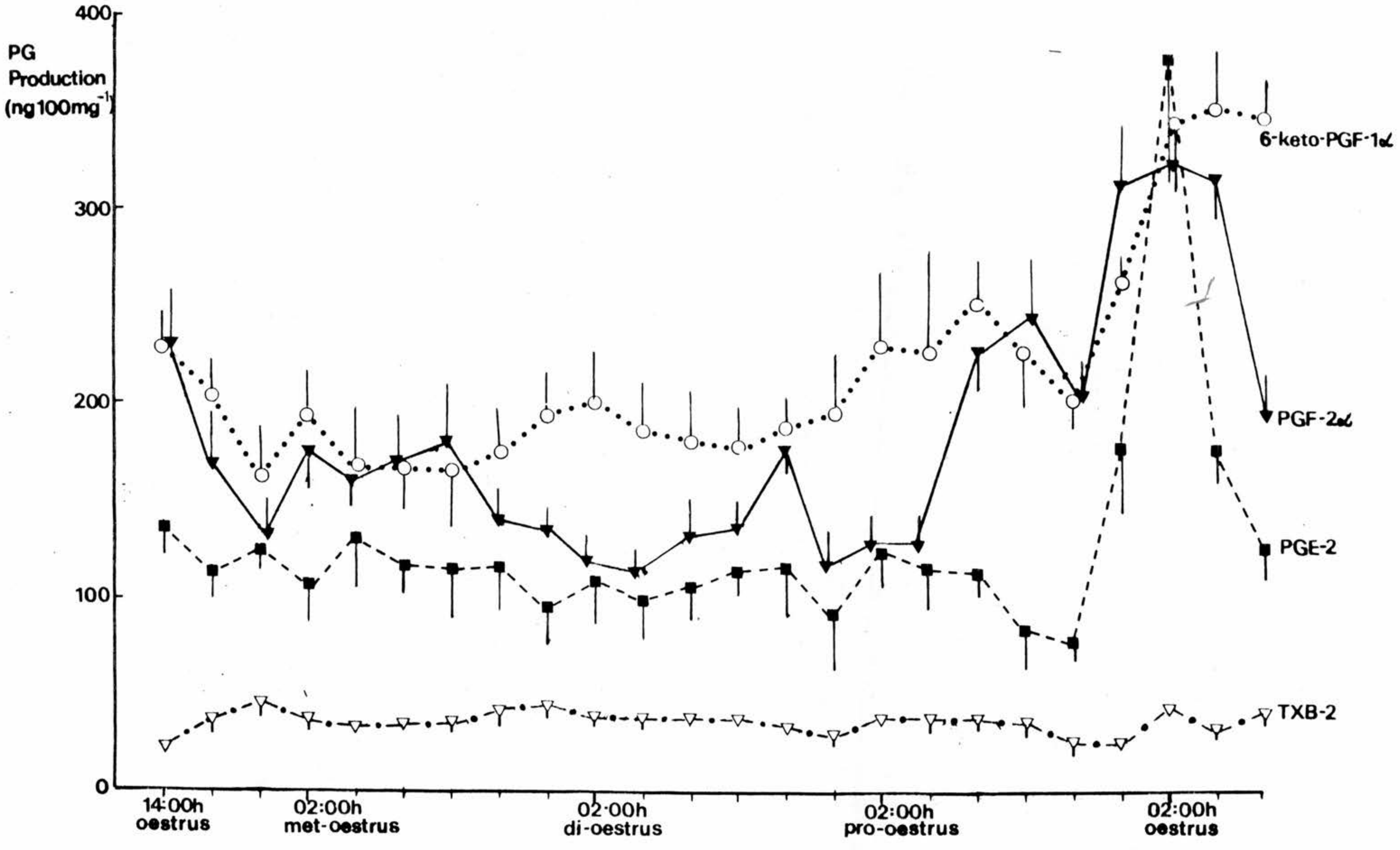
Samples which had been assayed for PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 by RIA were pooled, purified by silicic acid column chromatography and the appropriate derivatives prepared as described in section 1.4. Following derivitization, samples were injected into the GC in 10μl BSTFA. Full mass spectra were taken at the appropriate retention times on the GC corresponding to the carbon values of PGA-2, PGB-2, PGD-2, PGE-1, PGE-2, PGF-1α, PGF-2α, 6-keto-PGF-1α and TXB-2.

Results

Analysis by GC-MS showed that PGF-2α, PGE-2, 6-keto-PGF-1α, PGD-2 and TXB-2 were present in extracts from rat ovaries, by noting the presence of their characteristic ions in the mass spectra. PGA-2, PGB-2, PGE-1 and PGF-1α were not detected in these extracts.

Figure 12 shows the amounts of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 formed from endogenous precursors in ovarian homogenates at 4-hourly intervals throughout the oestrous cycle. A statistical analysis of these results is given in Table 7. The major PG produced by ovarian homogenates throughout most of the cycle was PGI-2 (measured as 6-keto-PGF-1α) with lesser quantities in decending order of PGF-2α, PGE-2 and TXA-2 (measured as TXB-2). At oestrus, PGE-2 was the major PG formed.

PGE-2 production was low throughout most of the cycle and was lowest at 18:00h on pro-oestrus.



PG Production (ng100mg^{-1})
400
300
200
100
0
6-keto-PGF-1α
PGF-2α
PGE-2
TXB-2
14:00h oestrus
02:00h met-oestrus
02:00h di-oestrus
02:00h pro-oestrus
02:00h oestrus

Table 7. Statistical analysis of PG and TX production by rat ovarian homogenates as shown in Fig. 12. Significance was tested by Duncan's multiple range test for equal variances. Values with the same superscript within each column are not significantly different ($p > 0.05$). Values are expressed as mean $\pm$ s.e.m., n=6 for each time.

Time	PG production ng 100 mg^{-1}	PGE-2	PGF-2α	6-keto-PGF-1α	TXB-2
14:00h	oestrus	$138+15^{ab}$	$230+22^{cde}$	$230+17^{a}$	$25+4^{a}$
18:00h	oestrus	$114+14^{ab}$	$169+25^{abcd}$	$205+17^{a}$	$38+7^{a}$
22:00h	oestrus	$125+23^{ab}$	$128+16^{ab}$	$162+23^{a}$	$45+8^{a}$
02:00h	met-oestrus	$108+25^{ab}$	$171+22^{abcd}$	$194+22^{a}$	$38+5^{a}$
06:00h	met-oestrus	$130+25^{ab}$	$160+12^{abcd}$	$165+32^{a}$	$33+4^{a}$
10:00h	met-oestrus	$116+12^{ab}$	$168+20^{abcd}$	$164+20^{a}$	$34+5^{a}$
14:00h	met-oestrus	$113+18^{ab}$	$170+17^{abcd}$	$163+25^{a}$	$35+6^{a}$
18:00h	met-oestrus	$112+18^{ab}$	$138+17^{ab}$	$175+20^{a}$	$39+8^{a}$
22:00h	met-oestrus	$91+20^{ab}$	$135+10^{ab}$	$192+25^{a}$	$44+7^{a}$
02:00h	di-oestrus	$107+20^{ab}$	$121+11^{ab}$	$201+24^{a}$	$38+5^{a}$
06:00h	di-oestrus	$98+20^{ab}$	$114+9^{a}$	$184+25^{a}$	$38+5^{a}$
10:00h	di-oestrus	$104+15^{ab}$	$127+17^{ab}$	$181+25^{a}$	$37+4^{a}$
14:00h	di-oestrus	$115+12^{ab}$	$148+12^{abc}$	$178+20^{a}$	$38+5^{a}$
18:00h	di-oestrus	$119+20^{ab}$	$178+10^{abcd}$	$185+15^{a}$	$31+4^{a}$
22:00h	di-oestrus	$90+32^{ab}$	$120+15^{ab}$	$193+30^{a}$	$28+5^{a}$
02:00h	pro-oestrus	$125+15^{ab}$	$126+12^{ab}$	$233+36^{a}$	$38+4^{a}$
06:00h	pro-oestrus	$118+17^{ab}$	$126+20^{ab}$	$227+48^{a}$	$39+7^{a}$
10:00h	pro-oestrus	$115+10^{ab}$	$225+18^{cd}$	$250+22^{ab}$	$38+7^{a}$
14:00h	pro-oestrus	$84+17^{ab}$	$240+22^{def}$	$226+30^{a}$	$36+7^{a}$
18:00h	pro-oestrus	$75+9^{a}$	$200+20^{bc}$	$201+12^{a}$	$25+3^{a}$
22:00h	pro-oestrus	$175+35^{b}$	$313+30^{ef}$	$262+12^{ab}$	$24+4^{a}$
02:00h	oestrus	$380+65^{c}$	$320+10^{f}$	$344+25^{b}$	$42+2^{a}$
06:00h	oestrus	$172+20^{b}$	$315+20^{f}$	$350+32^{b}$	$35+5^{a}$
10:00h	oestrus	$126+13^{ab}$	$190+17^{abcd}$	$345+20^{b}$	$40+5^{a}$

Production of PGE-2 increased significantly ($p<0.05$) between 18:00h and 22:00h on pro-oestrus. By 02:00h on oestrus, PGE-2 production had increased 5-fold when compared to production at 18:00h on pro-oestrus and was significantly ($p<0.05$) higher than at any other time during the cycle. Within 4 to 8h, PGE-2 production had fallen to a value which did not differ significantly from that throughout most of the cycle.

PGF-2α production was lowest at 06:00h on di-oestrus. It had increased significantly by 10:00h on pro-oestrus and like PGE-2 production had reached a maximum at 02:00h on oestrus, although the rise and fall in PGF-2α production was more gradual than for PGE-2 production. By 10:00h on oestrus PGF-2α production had fallen to a value which was not significantly different from PGF-2α production at most other times in the cycle. Between 18:00h on pro-oestrus and 02:00h on oestrus PGF-2α production had increased 1.6-fold.

6-keto-PGF-1α production was lowest at 22:00h on oestrus and fluctuated, without significant differences until 22:00h on pro-oestrus. A significant increase in 6-keto-PGF-1α production occurred between 18:00h on pro-oestrus and 02:00h on oestrus. This increase being 1.7 fold. Production of 6-keto-PGF-1α remained elevated for 8h and had decreased by 14:00h on oestrus to a value which was not significantly different from those throughout most of the oestrous cycle. There were no significant differences in ovarian TXB-2 production throughout the oestrous cycle.

Conclusion

Analysis of ovarian extracts by GC-MS confirmed the presence of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 as had been previously estimated by RIA. PGA-2, PGB-2, PGE-1 or PGF-1α were not detected in these extracts. Thus, although the antisera used in the RIA to measure PGE-1 shows cross reactivity with PGA-2, PGB-2 and PGE-1, and that used to measure PGF-2α shows cross reactivity with PGF-1α, it is probable that the assays measured PGE-2 and PGF-2α respectively since no PGA-2, PGB-2, PGE-1 or PGF-1α were detected in the extracts.

The major PG produced by ovarian homogenates throughout most of the cycle was PGI-2 (measured as 6-keto-PGF-1α) with lesser quantities of PGF-2α, PGE-2 and TXA-2 (measured as TXB-2). Ovulation in these rats is known to occur around 02:00h on oestrus. Towards the time of ovulation, production of PGE-2, PGF-2α and 6-keto-PGF-1α increased and reached a maximum at 02:00h on oestrus (or 06:00h, in the case of 6-keto-PGF-1α). PGE-2 production showed the greatest percentage increase between 18:00h on pro-oestrus and 02:00h on oestrus. Between these two times, mean TXB-2 production also showed an increase, however, due to the large variance associated with the data, this difference was not significant.

Section 2.1b Measurement of ovarian PG and TX levels at 10:00h on pro-oestrus and 02:00h on oestrus and PG and TX production by ovarian homogenates incubated in the presence of arachidonic acid

Introduction

Hitherto, the PG and TX content of extracts of ovarian homogenates after a 90 min incubation period has been termed 'production'. In order to equate 'production' with 'synthesis' it is necessary to determine a) that before homogenization and incubation, the levels of PG and TX are low and b) that metabolism of PG and TX in the homogenate is low. Homogenization of tissues releases arachidonic acid from cellular phospholipids (Pace-Asciak and Wolfe, 1968; Flower and Blackwell, 1976) and incubation of tissue homogenates will further increase arachidonic acid release (Mitchell, Poyser and Wilson, 1977). As the level of free arachidonic acid is rate limiting in the biosynthesis of PGs (Kunze, 1970; Flower and Blackwell, 1976), it is important to determine if changes in PG production by homogenates are due to changes in arachidonic acid availabiliy.

The following experiments were performed to determine a) that PG and TX levels in rat ovaries are low and that b) arachidonic acid availability in ovarian homogenates at a time of low PG production was not rate-limiting in the biosynthesis of PGs and TX.

PG metabolism in ovarian homogenates is described in Section 2.2.c

Measurement of ovarian prostaglandin and thromboxane levels at 10:00h on pro-oestrus and 02:00h on oestrus

Method

Rats were killed (as described previously) at 10:00h (n=3) and at 02:00h (n=3). The ovaries of each rat were removed, weighed and homogenized in 5 ml ethanol. After transferring the homogenate to a centrifuge tube the homogenizer was washed twice with 5 ml ethanol and the washings added to the tube. Each homogenate was centrifuged at 1000 x g for 15 min and the supernatant liquid withdrawn. The precipitate was washed with 5 ml ethanol, centrifuged at 1000 x g for 15 min and the resulting supernatant combined with the original extract. Each extract was evaporated to dryness, under reduced pressure at 45°C, then dissolved in 10ml distilled water, and the PGs and TX extracted as described in section 1.2.a. Samples were stored and assayed by RIA as described in sections 1.3.a, b, c and d. Results were expressed as mean ± s.e.m. and are given in Table 8. Appropriate comparisons were made using Student's 't' test.

Results

Table 8. Concentrations of PGs and TX in ethanolic homogenates of ovaries from rats at 10:00h on pro-oestrus and at 02:00h on oestrus (mean $\pm$ s.e.m.)

Time \ PG concentration ng $100mg^{-1}$	PGE-2	PGF-2α	6-keto-PGF-1α	TXB-2
10:00h pro-oestrus (n=3)	8.22$\pm$ 0.42	2.40$\pm$ 0.20	2.87$\pm$ 0.04	1.23$\pm$ 0.03
02:00h oestrus (n=3)	*21.40$\pm$ 5.70	*3.70$\pm$ 0.80	*5.81$\pm$ 1.06	1.61$\pm$ 0.31

* significantly different ($p<0.05$) from 10:00h on pro-oestrus.

Concentrations of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 in ovaries were low when compared to the amounts of PGs and TX formed during incubation of the homogenates. At 02:00h on oestrus, ovarian PGE-2, PGF-2α and 6-keto-PGF-1α concentrations were significantly increased by 160%, 54% and 102% respectively, ($p<0.05$) when compared to 10:00h on pro-oestrus. Ovarian TXB-2 concentrations did not change significantly between these two times.

Conclusion

Ovarian levels of PGs and TX were low in comparison to PG and TX production after incubation of homogenates as shown previously. It can be concluded therefore, that the increased PG and TX production following homogenization and incubation reflects fresh synthesis of PGs and TX by the ovarian tissue. Because PGE-2, PGF-2α and 6-keto-PGF-1α levels were significantly higher at the time of ovulation than at 10:00h on pro-oestrus, this suggests that these PGs may be important in the mechanism of ovulation in the rat.

Measurement of PG production by ovarian homogenates incubated in the presence of arachidonic acid

Method

4 rats were killed at 10:00h on pro-oestrus, the ovaries were dissected out, homogenized and incubated as described in section 2.2.a except that arachidonic acid was added to the homogenate immediately prior to incubation. The final concentration of arachidonic acid was 2 μgml^{-1}. Homogenates were extracted and assayed for PG and TX by RIA as previously described (Sections 1.2a and 1.3.a, b, c and d). Results (shown in Fig. 13) are expressed as mean $\pm$ s.e.m. and were compared using Student's 't' test.

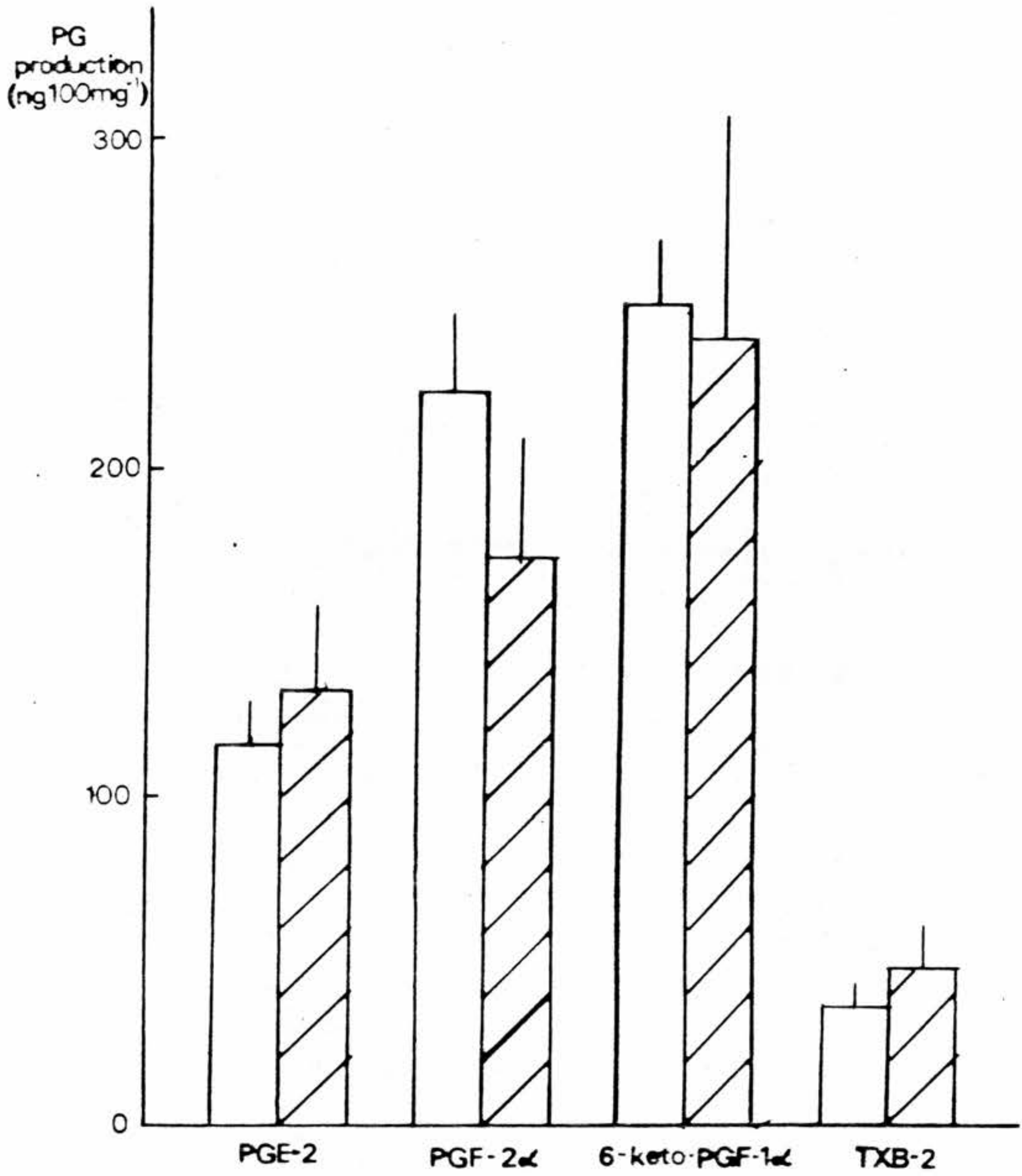


Figure 13

PG and TX production by ovarian homogenates incubated in the absence ☐ (n=6) or presence ▨ (n=4) of $2\mu g ml^{-1}$ arachidonic acid. Rats were killed at 10:00h on pro-oestrus.

Results

PG and TX production by homogenates of ovaries from rats killed at 10:00h on pro-oestrus did not differ significantly whether incubated in the absence or presence of arachidonic acid.

Conclusion

Incubation of ovarian homogenates from rats killed at 10:00h on pro-oestrus with arachidonic acid did not increase PG or TX production when compared to PG and TX production by homogenates incubated without the addition of arachidonic acid. Thus it can be concluded that the relatively low PG production at 10:00h on pro-oestrus was not due to lack of endogenous free arachidonic acid.

Section 2.1.c. Measurement of the ability of ovarian tissue to metabolize PGE-2 and PGF-2α

Introduction

The previous sub-sections have described experiments which measured PG and TX production by ovarian homogenates when incubated for 90 min, and which showed that the PGs and TX produced reflected fresh synthesis during the incubation. However, ovarian tissue has the ability to metabolize PGs (Anggard, Larsson and Samuelsson, 1971; Maule Walker, Patek, Leaf and Watson, 1977; Aizawa, Inazu and Kogo, 1980).

Therefore the amount of PG synthesized after 90 min may be affected by the extent of PG metabolism in the homogenate.

PGs are metabolized by oxidation of the secondary alcohol group at carbon atom 15 to a ketone, by 15-hydroxy prostaglandin dehydrogenase (15 PGDH) followed by reduction of the Δ-13 double bond by prostaglandin Δ-13 reductase. The former conversion requires nicotinamide adenine dinucleotide (NAD+) as a cofactor (Anggard, 1971; Samuelsson, 1972). The 15-keto- and 13,14-dihydro-15-keto PG metabolites show greatly reduced biological activity (Anggard, 1966; Crutchley and Piper, 1975). Interconversion of primary PGs has also been demonstrated in several tissues resulting in compounds of different or even enhanced biological potency. Watson, Shepherd and Dodson (1979) have demonstrated the existence of prostaglandin E-2-9-ketoreductase, an NADPH dependant enzyme, which converts PGE-2 to PGF-2α in rat ovarian tissue, and that this conversion is reversible.

The following experiments were performed to study the metabolism of PGE-2 and PGF-2α by ovarian homogenates with and without the addition of cofactor (NAD+) at times when PG production by ovarian homogenates was low (10:00h on pro-oestrus) and high (02:00h on oestrus). The possible interconversion of PGE-2 and PGF-2α in homogenates in the absence and presence of the appropriate cofactor reduced nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide phosphate (NADP) was also studied at 02:00h on oestrus.

Methods

The ovaries were removed from rats killed at 10:00h on pro-oestrus and 02:00h on oestrus (n=4 for each time). The two ovaries from each rat were separated. Each ovary was homogenized in 5 ml Krebs' solution. NAD+ was added to one ovarian homogenate to give a final concentration of 2mM. PGF-2α (10μg) was also added to this homogenate. Each homogenate was then incubated in the presence of 0.5μCi ^{3}H-PGF-2α (sp. act. 160 μCimmol^{-1}) for 90 min. The PGs were extracted as described in section 1.2a. This experiment was repeated except that 10μg PGE-2 was added to one homogenate instead of PGF-2α, and that each homogenate was incubated in the presence of 0.5μCi ^{3}H-PGE-2 (sp. act 160 μCimmol^{-1}) instead of ^{3}H-PGF-2α. The extracts were evaporated to dryness by a stream of air and the residues were redissolved each in 0.15 ml methanol, and subjected to thin-layer chromatography. ^{3}H-PGE-2, ^{3}H-PGF-2$\alpha$and non-radioactive marker standards (10μg each of PGE-2, PGF-2α, 15-keto-PGE-2, 15-keto-PGF-2α, 13,14-dihydro-15-keto-PGE-2 and 13,14-dihydro-15-keto-PGF-2α) were applied to silica gel precoated TLC plates (Merck, UK). All plates were developed in the solvent system chloroform:methanol: glacial acetic acid: water, 90: 9: 1: 0.65, to give a solvent front 15 cm from the origin. Standard spots on the control plates were visualized by exposure of the plates to iodine vapour for 30 min and the Rf values of these standard were recorded. The silica gel was scraped off the radioactive plates in 0.5 cm intervals into scintillation vials. After adding 0.5 ml methanol, 10 ml of scintillation fluid I was dispensed into each vial.

Radioactivity was monitored in a Phillips scintillation counter using sample channels ratio counting. The Rf values of radioactive peaks on the plate were compared to Rf values of standard PGs and metabolites. Metabolism was calculated by expressing the radioactivity associated with each PG or metabolite as a percentage of the total radioactivity on the plate. Radioactivity associated with metabolites was corrected for the loss of tritium from the 15-hydroxyl group during its conversion to a ketone group.

The possible inter-conversion of PGE-2 and PGF-2α was studied using essentially the same method as above. Three rats were killed at 02:00h on oestrus. The ovaries were dissected out and processed as described previously except that to one ovarian homogenate, NADP (2mM) and 10μg PGF-2α were added. Each ovarian homogenate was incubated in the presence of 0.5 μCi ^{3}H-PGF:2α. This experiment was repeated replacing PGE-2 for PGF-2α, ^{3}H-PGE-2 for ^{3}H-PGF-2α and using NADPH (2mM) as a cofactor. Extracts were subjected to thin layer chromatography as above. The radioactivity was localized using a Panax radio thin layer chromatographic plate scanner.

Results

The Rf values for the non-radioactive marker standards are shown in Table 9. Thin layer chromography of ^{3}H-PGE-2 and ^{3}H-PGF-2α had previously shown that at least 98% of the radioactivity was associated with PGE-2 and PGF-2α respectively.

The percentage metabolism of PGE-2 and PGF-2α by ovarian homogenates at 10:00h on pro-oestrus and 02:00h on oestrus incubated in the absence and presence of NAD+ is shown in Table 10 (mean $\pm$ s.e.m. n=4).

Table 9. Rf values of non-radioactive marker standards

Compound	Rf value
PGF-2α	0.14
15-keto-PGF-2α	0.25
PGE-2	0.26
13,14-dihydro-15-keto-PGF-2α	0.35
15-keto-PGE-2	0.42
13,14-dihydro-15-keto-PGE-2	0.43

The % metabolism results have not been corrected for tissue weight. However ovarian weights did not change significantly between the two times (52 $\pm$ 7.2 mg at 10:00h pro-oestrus, and 56 $\pm$ 9.4 mg at 02:00h on oestrus).

Table 10. Percentage metabolism of PGE-2 and PGF-2α at 10:00h on pro-oestrus and 02:00h on oestrus in ovarian homogenates incubated in the absence and presence of NAD+ (mean $\pm$ s.e.m., n=4)

Time	PGE-2 Metabolites		PGF-2α metabolites	
	ovary without NAD+	ovary with NAD+	ovary without NAD+	ovary with NAD+
10:00h pro-oestrus	10.1$\pm$1.02	*51.5$\pm$13.1	11.3$\pm$1.5	*19.5$\pm$3.5
02:00h oestrus	11.6$\pm$2.2	*30.4$\pm$1.6	9.3$\pm$1.6	*15.0$\pm$2.0

* significantly different from the percentage metabolism without NAD+ added ($p < 0.05$)

Incubation of ovarian homogenates with ^{3}H-PGE-2 or ^{3}H-PGF-2α resulted in the formation of metabolites with Rf values corresponding to the 15-keto- and 13,14-dihydro-15-keto-metabolites of these PGs. The percentage metabolism of PGE-2 and PGF-2α is expressed as the sum of these two metabolites in each case. In the absence of NAD+, the percentage metabolism of PGE-2 and PGF-2α was approximately 10%. This percentage did not differ significantly between 10:00h on pro-oestrus and 02:00h on oestrus.

The addition of NAD+ significantly ($p<0.05$) increased both PGE-2 and PGF-2α metabolism at both times. PGE-2 and PGF-2α metabolism in the presence of NAD+ was greater at 10:00h on pro-oestrus than 02:00h on oestrus. However, due to the small numbers in the group, this difference was not significant.

The ability of the ovaries to convert ^{3}H-PGF-2α to ^{3}H-PGE-2 at 02:00h on oestrus was undetectable whether NADP was present in the incubation medium or not. Figure 14 shows the thin-layer radiochromatogram obtained by use of the Panax scanner showing the conversion of ^{3}H-PGE-2 to ^{3}H-PGF-2α in the absence and presence of NADPH in the homogenate. Similar radiochromatograms were obtained for all 3 experiments. Conversion of ^{3}H-PGE-2 to ^{3}H-PGF-2α at 02:00h on oestrus was less than 2% in the absence of NADPH. Addition of NADPH increased the conversion to 6.1 $\pm$ 1.7% for the 3 experiments. The main metabolites of PGE-2 as identified by T.L.C. were 15-keto-PGE-2 and 13,14-dihydro-15-keto-PGE-2 and the percentage conversion of PGE-2 to these metabolites did not differ significantly in the absence (19.8 $\pm$ 3.3%) or presence (15.2 $\pm$ 1.1%) of NADPH.

Conclusion

Metabolism of PGE-2 and PGF-2α in ovarian homogenates in the absence of NAD+ is low. As it has been previously demonstrated in this section that ovarian PG levels are also low, and that the addition of arachidonic acid does not increase PG production by homogenates, it can be concluded that the amounts of PGs produced by these homogenates reflects the PG synthesizing capacity of the ovarian tissue.

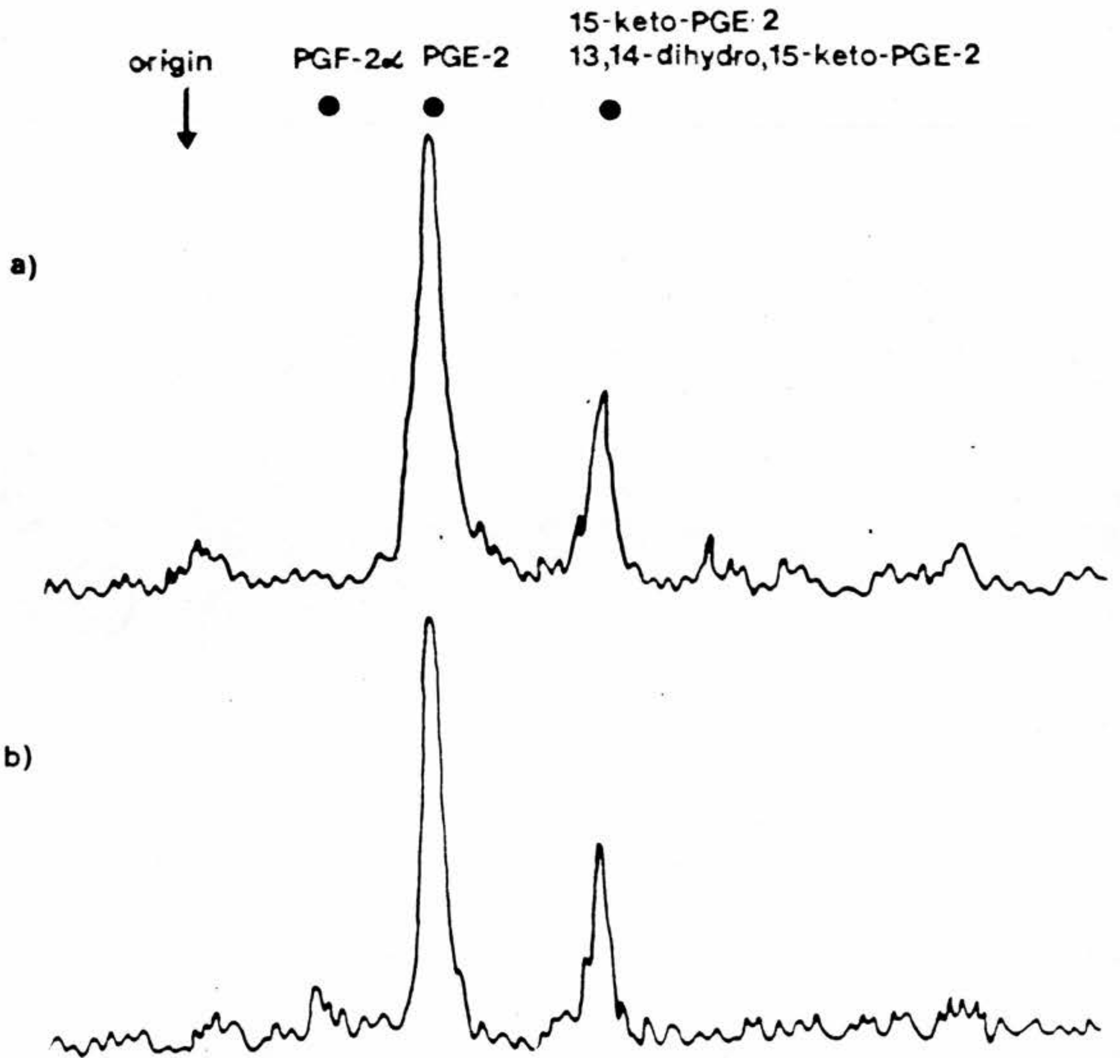


Figure 14

Profiles of thin-layer radiochromatograms from a typical experiment showing conversion of ^{3}H-PGE-2 to PGE-2 metabolites and PGF-2α by ovarian homogenates incubated a) in the absence of NADPH and b) in the presence of NADPH. Similar radiochromatograms were obtained in all 3 experiments.

There was no significant difference in the ability of ovarian homogenates to metabolize ^{3}H-PGE-2 or ^{3}H-PGF-2α at 10:00h on pro-oestrus compared to 02:00h on oestrus. Therefore, the increase in PGE-2 and PGF-2α production by ovarian homogenates seen at the time of ovulation cannot be due to decreased metabolism. PGI-2 and TXA-2 metabolism has not been studied here but PGI-2 is also metabolized by the NAD-dependent enzyme, 15-hydroxyl PG dehydrogenase, to form 15-keto-PGI-2 (McGuire and Sun, 1978) which is subsequently reduced to form 6,15-diketo-13,14-dihydro-PGF-1α (Sun and Taylor, 1978). Likewise 15-keto-13,14-dihydro-TXB-2 has been identified as the major product released after immunological challenge of sensitized guinea pig lungs (Mallen, Osborne, Cockerill, Boot and Dawson, 1978). It is likely that the enzymes which metabolize PGI-2 and TXB-2 are the same as those which metabolize PGE-2 and PGF-2α (although the affinity for the substrates may differ) and that PGI-2 and TXA-2 metabolism in ovarian tissue is equally as low as that of PGE-2 and PGF-2α.

The addition of NAD+ increased both PGE-2 and PGF-2α metabolism and this increase tended to be greater at 10:00h on pro-oestrus than at 02:00h on oestrus. This may suggest that the supply of NAD+ may control PG metabolism in the ovaries, however more experiments of this nature would be required in order to confirm this suggestion.

Although the existence of an ovarian enzyme PGE-2-9-keto reductase which can convert PGE-2 to PGF-2α has been indicated (Watson, Shepherd and Dodson, 1979), experiments in this section have shown that the activity of this enzyme in ovarian homogenates in the absence or presence of NADPH is low at 02:00h on oestrus. There was no detectable conversion of ^{3}H-PGF-2α to ^{3}H-PGE-2 at this time. Therefore, the high PGE-2 production at the time of ovulation cannot be due to conversion of PGF-2α to PGE-2 by the reversible action of the enzyme PGE-2-9-ketoreductase.

Section 2.1.d Measurement of the conversion of PGH-2 to PGE-2 prior to and at the time of ovulation

Introduction

The ratio of PGE-2: PGF-2α: 6-keto-PGF-1α: TXB-2 production by ovarian homogenates was approximately 3:6:6:1 at 10:00 on pro-oestrus. This ratio had changed to 8:7:7:1 by 02:00h on oestrus. Thus, at the time of ovulation the synthesis of PGs and TX was directed towards PGE-2. Previous experiments in this section have demonstrated that the increased PG production at the time of ovulation was not due to alterations in arachidonic acid availability, PG metabolism or interconversion of PGF-2α and PGE-2. This suggests that there is an increase in the conversion of the available arachidonic acid into PGs, i.e. increased PG synthetase activity. In addition, these experiments suggest that a mechanism exists in the ovary which specifically converts arachidonic acid via PGH-2 into PGE-2 at the time of ovulation.

Following the action of cyclooxygenase, which converts arachidonic acid into PGH-2, the endoperoxide is converted to PGE-2 by PGE isomerase. The isomerase enzyme has been isolated from bovine seminal vesicle microsomes (Ogino, Miyamoto, Yamamoto and Hayashi, 1977). PGH-2 also decomposes in aqueous solution into a mixture of PGD-2 and PGE-2 with a half-life of 10 min at 37°C (Nugteren and Christ-Hazelhof, 1979). The increased PGE-2 synthesis at the time of ovulation could be due to either increased cyclooxygenase activity, or increased isomerase activity, or both. Increased isomerase activity would be accompanied by increased conversion of PGH-2 to PGE-2. In the following experiment the conversion of PGH-2 to PGE-2 at 10:00h on pro-oestrus and 02:00h on oestrus has been compared, thus giving an index of PGE isomerase activity. Homogenization of the ovarian tissue was carried out in the presence of indomethacin in order to inhibit endogenous PG synthesis.

Method

Rats were killed by a blow to the thorax and decapitation at 10:00h on pro-oestrus (n=6) or 02:00h on oestrus (n=7). The ovaries were dissected out and placed into Krebs' solution containing 50 μgml^{-1} indomethacin. Indomethacin was made up as a concentrated stock solution of 2.5 $mgml^{-1}$ in ethanol and added to Krebs' solution in 100μl or 200μl volumes of ethanol as required. The ovaries from each rat were homogenized in 9ml Krebs' solution containing 50μgml^{-1} indomethacin. The homogenate was then divided into 3 volumes and each 3 ml of homogenate was transferred to separate conical flasks.

A sample of 100μl of each homogenate was taken for estimation of protein content using the method of Lowry, Rosebrough, Farr and Randall, (1951). One flask was suspended in a boiling water bath for 10 min. During this period, the remaining two flasks were kept on ice. After this 10 min period, the flask containing the boiled homogenate was cooled on ice. Each of the three flasks were transferred to a shaking water bath and incubated therein for an initial period of 3 min which allowed the temperature of the homogenates to reach 37°C. Flasks were gassed with a mixture of 95% O_2 and 5% CO_2.

PGH-2 was stored in hexane: ethyl acetate at -20°C. Before use, the solvent was evaporated in a pear-shaped flask, under reduced pressure, on ice. The flask was then connected to a vacuum pump and drying was completed under reduced pressure on ice. The residue was redissolved in 400μl of ice cold Krebs' solution. 200μl of PGH-2 in Krebs' solution was added to the flask containing the boiled homogenate and to one other flask. Each flask contained approximately 1μg PGH-2. The former flask measured non-enzymatic breakdown of PGH-2 and the latter measured enzymatic transformation. The third flask was used to check that indomethacin had effectively inhibited cyclooxygenase activity in the homogenates. Initial experiments of this kind involved incubating the homogenates for 5 min at 37°C. After this incubation period 0.5 ml of Stannous chloride in ethanol (50 μgml^{-1}) was added to flasks containing PGH-2. Stannous chloride acts as a reducing agent in the conversion of any PGH-2 remaining after the incubation into PGF-2α (Hamberg and Samuelsson, 1973).

All flasks were extracted for PGs as described in section 1.2.a. Samples were stored in ethyl acetate at -20°C until assayed for PGE-2 by RIA as described in section 1.3.a.

Results

Initial experiments showed that using the method described above, PGE-2 synthesis from PGH-2 did not differ between unboiled and boiled homogenates. Possible reasons for this result include:

a) that the incubation time was too long and that non-enzymatic transformation of PGH-2, as well as enzymatic transformation had occurred, and
b) that the incubation temperature was too high, which would also accelerate non-enzymatic breakdown of PGH-2 and
c) that indomethacin could be inhibiting PGE isomerase as well as cyclooxygenase and as a consequence of this, the enzymatic transformation of PGH-2 was inhibited.

The experiment was therefore repeated using slightly different conditions. Indomethacin was replaced by another PG synthesis inhibitor Froben (2-(2-fluoro-4-biphenyl)propionic acid, Boots, Co., Nottingham) also used at a concentration of 50 μgml^{-1}. Homogenates were incubated at 25°C for 1 min. Using these conditions PGE-2 synthesis from PGH-2 was greater in the unboiled than in the boiled homogenate. These results are shown in Table II.

Column b shows PGE-2 synthesis expressed as ng PGE-2 mg protein in homogenates incubated with the addition of PGH-2 and was used to measure PGH-2 to PGE-2 isomerase activity.

Table 11. PGE-2 synthesis by ovarian homogenates using PGH-2 as a precursor.

Flask a. no PGH-2 added

b. unboiled homogenate, PGH-2 added

c. boiled homogenate, PGH-2 added.

Results are expressed as ng PGE-2 per mg protein.

(mean ± s.e.m. n=6).

Flask	a	b	c	b-(a+c) enzymic synthesis
rat	ng PGE-2 mg protein^{-1}			
10:00h pro-oestrus 1	5.5	55.4	17.5	32.4
2	3.7	43.8	31.9	8.2
3	7.4	71.8	44.3	20.1
4	1.0	39.4	11.3	27.1
5	8.3	51.6	21.5	21.8
6	7.5	23.5	12.6	2.4
mean ± s.e.m.				18.6 + 4.2
02:00h oestrus 1	10.5	36.2	16.9	8.8
2	1.0	33.0	23.3	8.7
3	4.6	46.7	37.0	5.1
4	5.5	25.4	5.8	14.1
5	2.3	36.1	14.7	19.1
6	1.0	41.0	29.1	10.9
mean ± s.e.m.				11.1 + 1.8

Column a shows PGE-2 synthesis in homogenates without the addition of PGH-2 which gave an index of the effectiveness of Froben in inhibiting PG synthesis in the homogenate. The results show that Froben did not completely inhibit PGE-2 synthesis. Therefore the contribution of PGE-2 synthesis from endogenous PGH-2 had to be subtracted from the values in column b. PGE-2 synthesis by boiled homogenates is shown in column c. The values given in this column represent PGE-2 produced by non-enzymic decomposition of PGH-2 and these values were subtracted from the figures given in column b. The last column shows the calculated enzymic synthesis of PGE-2 from PGH-2. There was no significant difference in the calculated enzymic PGE-2 synthesis from PGH-2 between 10:00h on pro-oestrus and 02:00h on oestrus.

Conclusion

As there was no significant difference in the ability of ovarian homogenates to synthesize PGE-2 from PGH-2 at 10:00h on pro-oestrus and at 02:00h on oestrus, this suggests that the increased PGE-2 production by ovarian homogenates at the time of ovulation cannot be due to increased PGH-2 to PGE-2 isomerase activity. The limitations of this experiment have to be realized however. A fixed volume of solvent containing PGH-2 was used in each experiment, and although an initial concentration was estimated by reduction to PGF-2α followed by quantification by mass spectrometry, the exact concentration before each experiment was unknown.

Therefore, although PGH-2 is stable for several weeks under the storage conditions used, slight variations in the quantity of PGH-2 used in each experiment may have occurred. It was assumed that Froben did not inhibit the conversion of PGH-2 to PGE-2. Confirmation of this would require isolation of the isomerase enzyme and ideally this experiment should be carried out. Crude homogenates contain thiol compounds and haemoproteins which act as reducing agents in the conversion of PGH-2 to PGF-2α and boiling of the homogenate may even increase PGF-2α formation (Nugteren and Christ-Hazelhof, 1980). Thus the transformation of PGH-2 to PGE-2 in the boiled homogenate may be under-estimated due to increased PGF-2α formation.

In conclusion, this experiment shows that increased PGH-2 to PGE-2 isomerase activity does not occur at the time of ovulation in the rat. This suggests that the increase in PGE-2 synthesis in the ovary is the result of increased cyclooxygenase activity with a greater proportion of the increased PGH-2 formed being converted into PGE-2.

Discussion

Levels of PGE-2, PGF-2α in the ovary were increased at the time of ovulation and this agrees with previous reports (Bauminger and Lindner, 1975; LeMaire, Leidner and Marsh, 1975). PGI-2 levels (measured as 6-keto-PGF-1α) were also increased at the time of ovulation, a finding which has not been previously reported for the rat.

In the swine, it has been demonstrated that LH will stimulate PGI-2 production by cultured granulosa cells and that this stimulation is susceptible to inhibition by inhibitors of protein synthesis (Veldhuis, Klase and Demers, 1982). The dependence of PGI-2 production on protein synthesis suggests that the stimulation of ovarian PGI-2 production by LH is similar to that of PGE-2 production. It is not known whether in these experiments PGI-2 was of vascular origin or not, but it is possible that increased PGI-2 synthesis is responsible for the vasodilation which occurs in the walls of the pre-ovulatory follicles prior to ovulation (Jones, 1979).

Homogenates of rat ovaries were also found to produce PGD-2. Production of PGD-2 by the ovary shows no daily variation when measured at 10:00h (Poyser and Scott, 1980). It would be interesting to determine whether changes occur in ovarian PGD2 production in the periovulatory period.

Ovarian PG synthesizing capacity was shown to increase at the time of ovulation. The increase in synthesizing capacity for PGE-2, PGF-2α and PGI-2 (measured as 6-keto-PGF-1α) was not due to increased arachidonic acid availability. This agrees with the results of other workers. Bauminger, Lieberman and Lindner, (1975) showed that addition of arachidonic acid to cultured Graffian follicles failed to enhance PG accumulation or to act synergistically with LH in this respect. LH increases the PGE concentration in cultures of ovarian fragments by 300% over a 6h period with no change in the free arachidonic acid concentration in the tissue (Zor, Strulovici, Nimrod and Lindner, 1977). Exogenous arachidonic acid increased PGE accumulation in cultured granulosa cells only after a 5h exposure to LH.

LH was without effect on ^{3}H-arachidonic acid release from pre-labelled granulosa cell lipids or on ^{3}H-arachidonic acid uptake into these lipids from the incubation medium (Clark, Marsh and LeMaire, 1978b).

These experiments also showed that decreased metabolism of PGE-2 and PGF-2α is not responsible for the increased synthesis at 02:00h on the day of oestrus as metabolism in the absence of NAD+ was low and it did not differ between 10:00h on pro-oestrus or 02:00 on oestrus. This work confirms the result of Poyser and Scott (1980) who also found that metabolism of PGF-2α in homogenates in the absence of NAD+ was low and that no significant daily variations occurred.

An enzyme PGE-2-9-ketoreductase has been reported to be present in rat tissue (Watson, Shepherd and Dodson, 1979). It is thought that this enzyme is reversible and will catalyse the conversion of PGF-2α to PGE-2 in the presence of NADP. More recently it has been demonstrated that hCG administered to PMSG-primed rats 1 day before killing, increased the activity of this enzyme over threefold. The hypothesis that gonadotrophin can control ovarian PGE-2 and PGF-2α levels by altering the activity of 9-ketoreductase would seem an attractive one. However, the results presented here do not demonstrate any appreciable interconversion of these two PGs. Differences in experimental design may account for these discrepancies. Watson et al., used a relatively highly purified enzyme preparation and added NADPH, glucose-6-phosphate dehydrogenase and dithiothreitol.

It is unlikely that PGE-2-9-ketoreductase could exert fine control over the balance between PGE and PGF in the ovary in view of the fact that it is not a PG-specific enzyme.

Chang and Tai (1981) have classed the enzyme as a general aldo-keto reductase which will also catalyse the reduction or oxidation of steroids, aldo sugars, quinones and carbonyl compounds.

Following injection of rats with LH on the morning of pro-oestrus, Bauminger and Lindner (1975) observed a lag period of 3h before the in vivo changes in ovarian PGs occurred. A result of this type suggested that there might be increased synthesis of an enzyme in the PG synthetic pathway. Resolution of PG synthetase into cyclooxygenase and isomerase components has been achieved by Miyamoto, Yamamoto and Hayaishio (1974). The results presented in this section showed that no increase in PGH-2 to PGE-2 isomerase activity occurred at the time of ovulation. Collectively, these experiments suggest that the control of PG biosynthesis in the ovary is exerted at the level of the cyclooxygenase enzyme.

Section 2.2 PROSTAGLANDINS AND GONADOTROPHIN RELEASE

Introduction

In the previous section, it has been pointed out that indomethacin will block ovulation in the rat by preventing follicular rupture in the ovary, an action which is not surmountable by the administration of LH (Armstrong and Grinwich, 1972; Tsafriri et al., 1972). However, the block of ovulation induced by a single lower dose of indomethacin given 3h before the expected LH surge, could be reversed by the administration of LH 3h after indomethacin (Orczyk and Behrman, 1972). Moreover, Behrman, Orczyk and Greep (1972) showed that the aspirin blockade of ovulation was reversed by LHRH administration. These results suggest that indomethacin and aspirin, in addition to blocking ovulation by an action at the ovary, can inhibit the release of LH as the result of the inhibition of LHRH release from the brain.

The stimulatory effect of progesterone in oestrogen-primed rats on LH release is blocked by indomethacin administration (Ojeda, Harms and McCann, 1975) and it has been suggested that when plasma oestrogen reaches a critical level, oestrogen-induced release of PGs occurs within the hypothalamus, thus promoting an LH surge (Craig, 1975). However, while there is evidence that PGs may act in the hypothalamus to stimulate LHRH release (Chobsieng et al., 1975; Ojeda, Wheaton and McCann, 1975, Eskay et al., 1975; Linton et al., 1979, Ojeda, Negro-Vilar and McCann, 1979; 1982), the evidence so far accumulated in support of a physiological role for PGs in this process is essentially indirect.

It has yet to be demonstrated that inhibition of PG synthesis in the hypothalamus will block the LH surge in cycling rats. In addition, the measurement of PG synthesis by the hypothalamus would help us to further understand the role of PGs in LHRH release. However, although it has been demonstrated that the PGE content of the median eminence (ME) is much higher than that of the medial based hypothalamus (MBH) in male and female rats (Ojeda, Naor and McCann, 1978), no information on PG synthesis by the hypothalamus during the rat oestrous cycle is available.

Experiments described in this section were designed to study PG and TX synthesis by the hypothalamus throughout the oestrous cycle and in response to treatment with oestrogen and progesterone. The effects of 2-OH oestradiol, an oestrogen metabolite formed by the hypothalamus (Fishman and Norton, 1974) and NA, a neurotransmitter associated with LH release (see Barraclough and Wise, 1982) on hypothalamic PG and TX synthesis were also investigated. To further elucidate whether PGs play a physiological role in LHRH and LH release, the effect of pretreating rats with a PG synthesis inhibitor 2-(-fluoro-4-biphenylyl) proprionic acid, on the pro-oestrus surge of LH was studied.

Section 2.2.a Measurement of the ability of the median eminence and anterior hypothalamus/pre-optic area to produce PGs and TX during the oestrous cycle

Introduction

The site of action of PGs in stimulating gonadotrophin release appears to be at the hypothalamus and PGE-2 is more potent than PGF-2α in this respect (Harms, Ojeda and McCann, 1974; Batta, Zanisi and Martini, 1974). Ojeda, Jameson and McCann (1975) showed that PGE-2 was most effective in causing a rise in plasma LH levels when implanted in the AHA in the POA and, to a lesser extent, in the ARH-ME. PGF-2α induced LH release when implanted in the POA or ARH-ME.

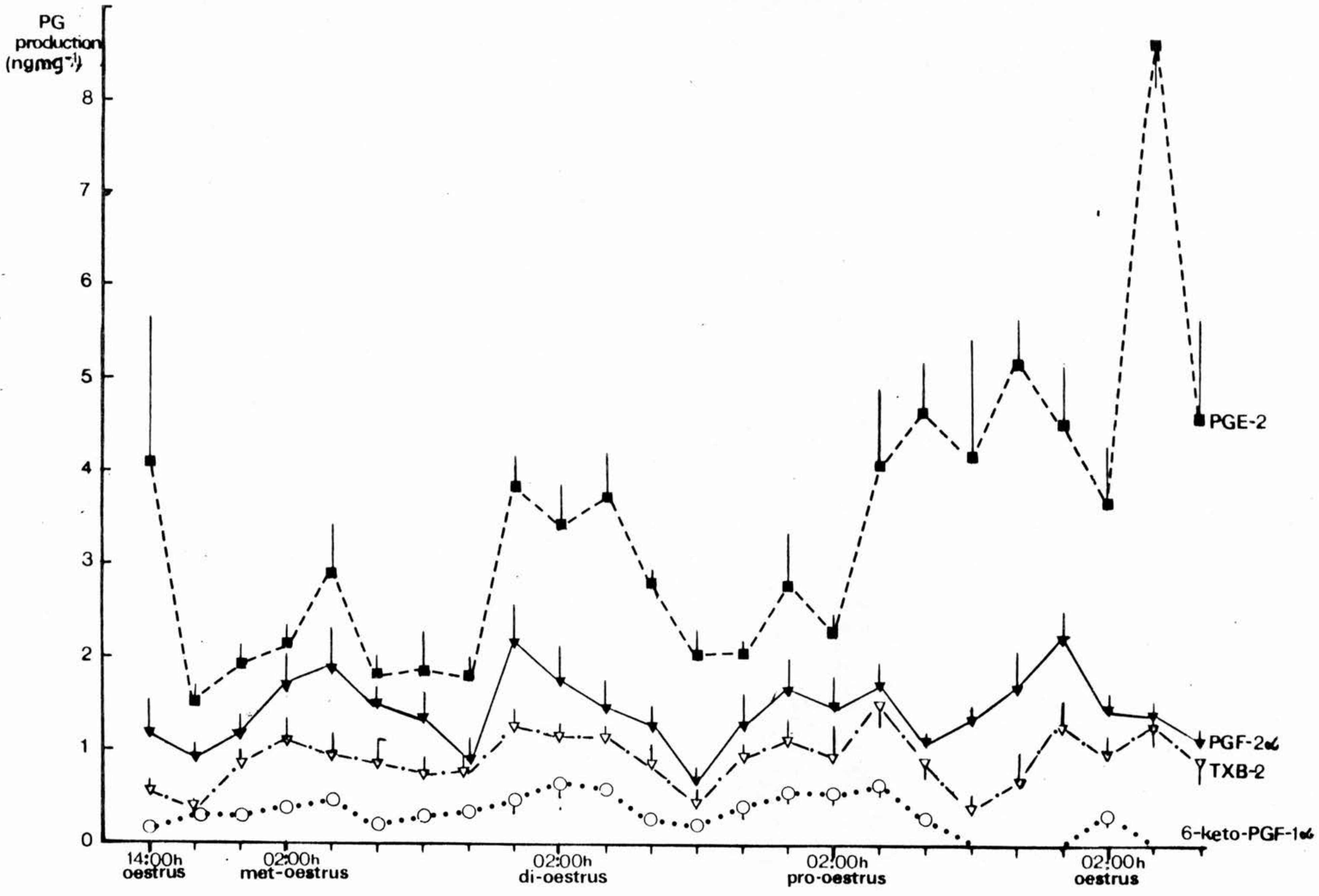
More recently, it has been demonstrated that intraventricular injection of the endoperoxide analog 11,9-epoxymethano-PGH-2, which is a TXA-2 mimic (Ojeda, Jameson and McCann, 1976) and intravenous injection of PGI-2 (Kimball, Porteus, Kirkton, Frielink, Creasy and Dayan, 1979) into oestrogen-treated ovariectomized rats stimulated LH release. Therefore the synthesis of PGI-2 and TXA-2, as well as PGE-2 and PGF-2α in the AH-POA and ME may be important in the control of LH-RH release from the hypothalamus.

In the following experiments the production of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 by homogenates of the ME and AH-POA has been measured at 4-hourly intervals throughout the oestrous cycle.

Method

Measurement of PG and TX production by homogenates of ME and AH-POA

A total of 144 mature female Albino Wistar rats weighing between 200g and 250g were used (these animals also provided tissue for use in experiments described in Section 2.1a and Section 2.3a). The animals were housed, fed, smeared and killed as described in Section 2.1a. The brain was removed from each rat and was placed on ice. Dissection of the ME and AH-POA was carried out under a stereoscopic microscope. The ME is a well delineated structure; it appears as an ovoid shape on the ventral surface of the brain, and forms the floor of the IIIrd ventricle. Using a pair of fine forceps, the remnant of the pituitary stalk was lifted. Two longitudinal cuts oriented forward and slightly convergent were performed using a pair of iris scissors. A cut was made at the point where the optic tracts enter the brain and the ME was lifted from the brain with fine forceps. The wet weight of this structure was approximately 0.3 mg. The dissection of the AH-POA was as follows. The optic chiasm and optic tracts were removed from the basal surface of the brain. A cut was made 2 mm anterior to the optic chiasm. Two lateral cuts were performed along the borders of the hypothalamic sulci. The final transverse cut was made along the anterior edge of the mamillary bodies. Each of these cuts were 2 mm in depth. The weight of this tissue was between 17 and 20 mg.



PG production (ng mg⁻¹)
0
1
2
3
4
5
6
7
8
14:00h oestrus
02:00h met-oestrus
02:00h di-oestrus
02:00h pro-oestrus
02:00h oestrus
PGE-2
PGF-2α
TXB-2
6-keto-PGF-1α

Table 12. Statistical analysis of PG and TX production by the ME at 4 hourly intervals throughout the oestrous cycle as in Fig. 15. Values with the same superscript within each column are not significantly different ($p > 0.05$). Values are expressed as mean ± s.e.m. n = 6 each time.

PG Production ng $100mg^{-1}$	PGE-2	PGF-2α	6-keto-PGF-1α	TXB-2
14:00h oestrus	4.11+1.60^{ghi}	1.28+0.37^{bcd}	0.14+0.06^{a}	0.58+0.12^{ab}
18:00h oestrus	1.51+0.20^{a}	0.94+0.11^{ab}	0.36+0.09^{bcd}	0.37+0.05^{a}
22:00h oestrus	1.93+0.15^{a}	1.17+0.18^{bc}	0.27+0.07^{abc}	0.86+0.25^{bcde}
02:00h met-oestrus	2.15+0.20^{abcd}	1.73+0.35^{def}	0.39+0.04^{bcde}	1.11+0.26^{de}
06:00h met-oestrus	2.94+0.40^{def}	1.92+0.41^{efg}	0.47+0.06^{cdefg}	0.98+0.22^{cde}
10:00h met-oestrus	1.84+0.19^{a}	1.50+0.20^{cd}	0.16+0.05^{ab}	0.89+0.22^{bcde}
14:00h met-oestrus	1.89+0.37^{a}	1.36+0.27^{bcd}	0.27+0.07^{abc}	0.77+0.15^{bcd}
18:00h met-oestrus	1.78+0.17^{a}	0.93+0.24^{ab}	0.31+0.10^{abc}	0.79+0.15^{bcd}
22:00h met-oestrus	3.85+0.27^{ghi}	2.18+0.42^{fg}	0.48+0.19^{c}	1.29+0.19^{e}
02:00hdi-oestrus	3.42+0.42^{efg}	1.74+0.39^{defg}	0.68+0.16^{g}	1.19+0.12^{e}
06:00h di-oestrus	3.71+0.55^{fgh}	1.48+0.26^{cde}	0.58+0.05^{efg}	1.18+0.11^{e}
10:00h di-oestrus	2.84+0.11^{cdef}	1.30+0.20^{bcd}	0.26+0.07^{ab}	0.89+0.22^{bcde}
14:00h di-oestrus	2.02+0.25^{ab}	0.66+0.10^{a}	0.19+0.06^{ab}	0.44+0.11^{a}
18:00h di-oestrus	2.05+0.12^{abc}	1.28+0.36^{bcd}	0.40+0.12^{bcdef}	0.97+0.10^{cde}
22:00h di-oestrus	2.81+0.6^{bcde}	1.64+0.32^{de}	0.55+0.12^{defg}	1.15+0.21^{e}
02:00h pro-oestrus	2.29+0.15^{abcde}	1.48+0.32^{cde}	0.55+0.11^{defg}	0.96+0.32^{cde}
06:00h pro-oestrus	4.08+0.79^{ghi}	1.71+0.24^{def}	0.63+0.17^{f}	1.50+0.25^{e}
10:00h pro-oestrus	4.62+0.59^{hi}	1.09+0.08^{abc}	0.27+0.04^{abc}	0.90+0.20^{bcde}
14:00h pro-oestrus	4.16+1.25^{ghi}	1.35+0.13^{bcd}	0.44+0.08^{bcdef}	0.41+0.11^{a}
18:00h pro-oestrus	5.17+0.43^{i}	1.66+0.36^{de}	undetectable	0.70+0.29^{b}
22:00h pro-oestrus	4.51+0.59^{ghi}	2.23+0.28^{g}	undetectable	1.27+0.26^{e}
02:00h oestrus	3.7+0.59^{fgh}	1.48+0.18^{cde}	0.31+0.10^{abc}	1.0+0.17^{cde}
06:00h oestrus	8.64+0.55^{j}	1.41+0.14^{bcd}	undetectable	1.30+0.25^{e}
10:00h oestrus	4.51+1.06^{ghi}	1.13+0.14^{bc}	undetectable	0.90+0.21^{bcde}

After recording the tissue weights, each was homogenized in Krebs' solution (2 ml for the ME and 5 ml for the AH-POA) and each transferred to conical flasks. The homogenates were incubated for 90 min as described in Section 1.2.b. Following incubation, the pH was adjusted to 4 and the PGs and TX extracted as described in section 1.2.a. Samples were stored in 2 ml ethyl acetate at -20°C until they were assayed for PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 content by RIA as previously described in sections 1.3a, b, c and d. Results were compared initially by an F-ratio test and subsequently by Duncan's Multiple Range test for equal variances. Significance was tested at the 5% level.

Results

Fig. 15 shows PG and TX production by homogenates of ME at 4-hourly intervals throughout the oestrous cycle, and a statistical analysis of these results is given in Table 12. The major PG produced by the ME was PGE-2 with lesser quantities (in descending order) of PGF-2α, TXA-2 (measured as TXB-2) and PGI-2 (measured as 6-keto-PGF-1α). Both PG and TX production by the ME showed a diurnal pattern, production being highest in the early hours of the morning (02:00h and 06:00h) and lowest in the afternoon (14:00h and 18:00h). PGE-2 production showed peaks at 06:00h on each day of the oestrous cycle. Each value at 06:00h was significantly greater ($p<0.05$) than that at 14:00h on met-oestrus, di-oestrus and pro-oestrus. At 06:00h on oestrus, PGE-2 production by the ME was significantly different from that at any other time in the oestrous cycle.

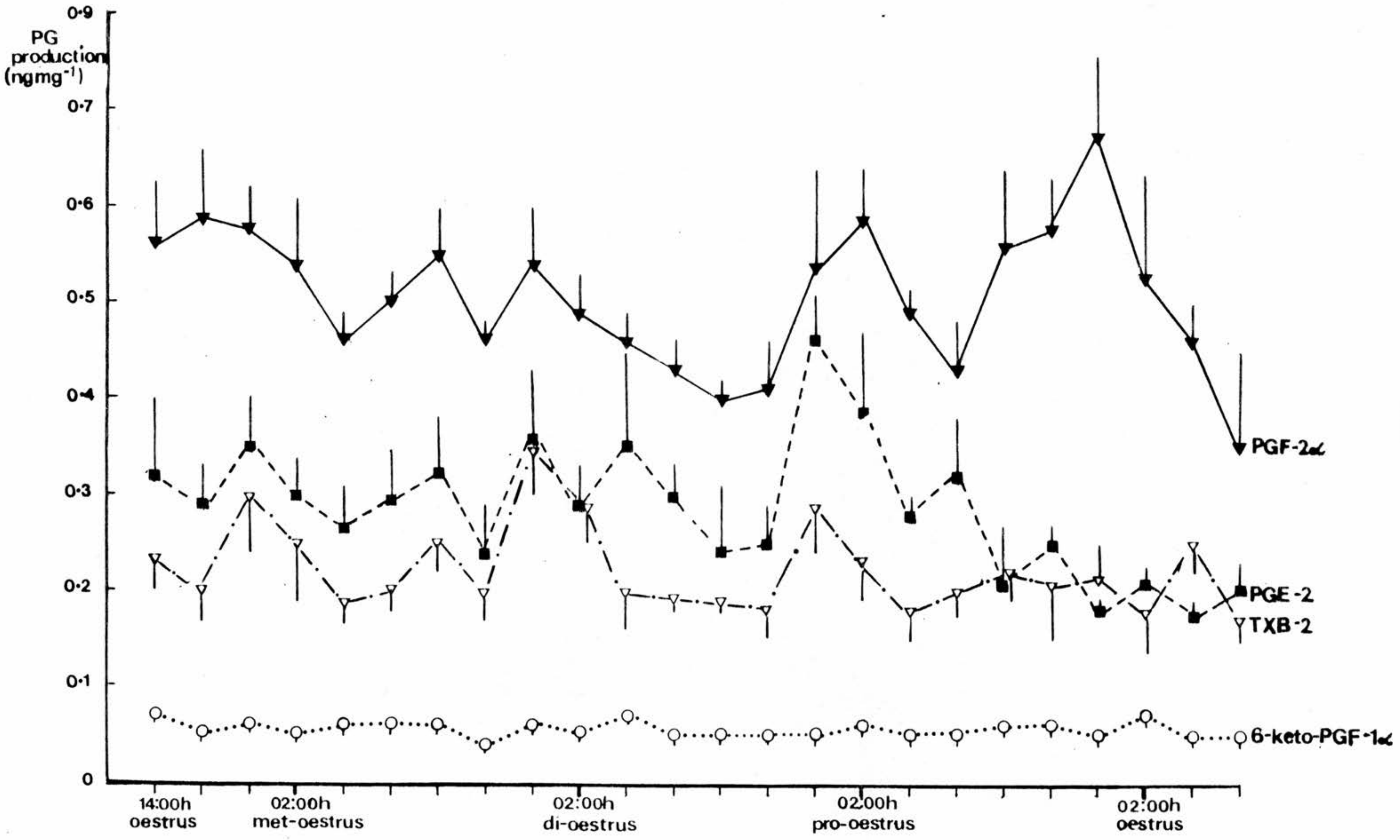

PG production (ngmg-1)
0·9
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
14:00h oestrus
02:00h met-oestrus
02:00h di-oestrus
02:00h pro-oestrus
02:00h oestrus
PGF-2α
PGE-2
TXB-2
6-keto-PGF-1α

Table 13. Statistical analysis of PG and TX production by the AH-POA at 4-hourly intervals throughout the oestrous cycle as in Fig. 16. Values with the same superscript within each column are not significantly different ($p > 0.05$). Values are expressed as mean ±s.e.m.

time \ ng.100 mg^{-1}	PGE-2	PGF-2α	6-keto-PGF-1α	TXB-2
14:00h oestrus	$0.32+0.08^{de}$	$0.56+0.07^{bc}$	$0.07+0.01^{a}$	$0.23+0.03^{ab}$
18:00h oestrus	$0.29+0.04^{bcde}$	$0.59+0.07^{c}$	$0.05+0.01^{a}$	$0.20+0.03^{ab}$
22:00h oestrus	$0.35+0.05^{ef}$	$0.58+0.04^{c}$	$0.06+0.01^{a}$	$0.30+0.06^{bc}$
02:00h met-oestrus	$0.30+0.03^{cde}$	$0.54+0.07^{bc}$	$0.05+0.01^{a}$	$0.25+0.06^{abc}$
06:00h met-oestrus	$0.27+0.04^{abcde}$	$0.46+0.03^{ab}$	$0.06+0.01^{a}$	$0.19+0.02^{ab}$
10:00h met-oestrus	$0.30+0.04^{cde}$	$0.50+0.03^{abc}$	$0.06+0.01^{a}$	$0.20+0.02^{ab}$
14:00h met-oestrus	$0.32+0.06^{de}$	$0.55+0.05^{bc}$	$0.06+0.01^{a}$	$0.25+0.02^{abc}$
18:00h met-oestrus	$0.24+0.05^{abcd}$	$0.46+0.02^{ab}$	$0.04+0.01^{a}$	$0.20+0.02^{ab}$
22:00h met-oestrus	$0.36+0.07^{ef}$	$0.54+0.06^{bc}$	$0.06+0.01^{a}$	$0.35+0.05^{c}$
02:00h di-oestrus	$0.29+0.04^{bcde}$	$0.49+0.04^{abc}$	$0.05+0.01^{a}$	$0.29+0.04^{bc}$
06:00h di-oestrus	$0.35+0.10^{ef}$	$0.46+0.03^{ab}$	$0.07+0.01^{a}$	$0.20+0.04^{ab}$
10:00h di-oestrus	$0.30+0.03^{cde}$	$0.43+0.03^{a}$	$0.05+0.01^{a}$	$0.19+0.02^{ab}$
14:00h di-oestrus	$0.24+0.07^{abcd}$	$0.40+0.02^{a}$	$0.05+0.01^{a}$	$0.19+0.01^{ab}$
18:00h di-oestrus	$0.25+0.04^{abcde}$	$0.41+0.05^{a}$	$0.05+0.01^{a}$	$0.18+0.03^{a}$
22:00h di-oestrus	$0.46+0.05^{f}$	$0.54+0.10^{bc}$	$0.05+0.01^{a}$	$0.29+0.05^{bc}$
02:00h pro-oestrus	$0.39+0.08^{f}$	$0.59+0.05^{c}$	$0.06+0.01^{a}$	$0.23+0.04^{abc}$
06:00h pro-oestrus	$0.28+0.02^{bcde}$	$0.49+0.02^{abc}$	$0.05+0.01^{a}$	$0.18+0.05^{a}$
10:00h pro-oestrus	$0.32+0.06^{de}$	$0.43+0.05^{a}$	$0.05+0.01^{a}$	$0.20+0.03^{ab}$
14:00h pro-oestrus	$0.21+0.03^{abc}$	$0.56+0.08^{bc}$	$0.06+0.01^{a}$	$0.22+0.03^{ab}$
18:00h pro-oestrus	$0.25+0.02^{abcde}$	$0.58+0.06^{c}$	$0.06+0.01^{a}$	$0.21+0.06^{ab}$
22:00h pro-oestrus	$0.17+0.01^{a}$	$0.68+0.08^{c}$	$0.05+0.01^{a}$	$0.22+0.04^{ab}$
02:00 oestrus	$0.21+0.02^{abc}$	$0.48+0.11^{abc}$	$0.07+0.01^{a}$	$0.18+0.05^{a}$
06:00 oestrus	$0.18+0.15^{ab}$	$0.46+0.04^{ab}$	$0.05+0.01^{a}$	$0.25+0.04^{abc}$
10:00h oestrus	$0.20+0.03^{abc}$	$0.35+0.10^{a}$	$0.05+0.01^{a}$	$0.17+0.04^{a}$

An additional peak in PGE-2 production ocurred at 18:00h on pro-oestrus. Although showing the diurnal pattern, production of PGF-2α and TXB-2 did not increase in amplitude on pro-oestrus and oestrus. 6-keto-PGF-1α production was lower (and barely detectable on the day of oestrus) than on the other days of the cycle. At 06:00h on oestrus the ratio of PGE-2: PGF-2α production was approximately 5:1 whereas at the same time on other days of the cycle this ratio was only about 3:1.

Measurements of PG and TX production by the AH-POA are shown Fig. 16 and Table 14 shows their statistical analysis. PGF-2α was the major PG produced by the AH-POA with lesser quantities (in descending order) of PGE-2, TXA-2 (measured as TXB-2) and PGI-2 (measured as 6-keto-PGF-1α). PGE-2 and TXB-2 production tended to be greater at 22:00h on each day except pro-oestrus. PGF-2α production showed a similar pattern but showed an additional peak at 22:00h on pro-oestrus. At 22:00h on pro-oestrus, mean PGF-2α production by the AH-POA was highest although this value was not significantly different from that throughout most of the oestrous cycle. PGE-2 production tended to be lower on pro-oestrus than on other days of the cycle, the lowest value being recorded at 22:00h on pro-oestrus. This value was significantly lower than PGE-2 production at the corresponding time on oestrus, met-oestrus or di-oestrus. On pro-oestrus the PGF-2α: PGE-2 ratio was about 4 whereas at other times in the cycle, was only 2. There were no significant differences in 6-keto-PGF-1α production by the AH-POA during the oestrous cycle. On comparing Figs. 15 and 16, it is evident that PG and TX production by the ME was approximately ten times greater than that by the AH-POA, when expressed on a unit weight basis.

Conclusion

The pattern of PG and TX production by the ME and AH-POA differs greatly. Firstly, the ME has a far greater capacity to produce PGs and TX than the AH-POA. Secondly, PGE-2 is the major PG synthesized by the ME, whereas PGF-2α production predominates in the AH-POA. Both tissues showed evidence of differing diurnal rhythms in PG and TX production. In the ME, peak PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 production occurred at 06:00h whereas in the AH-POA, PGE-2, PGF-2α and TXB-2 production generally peaked at 22:00h. In the ME an extra peak in PGE-2 production occurred at 18:00h on pro-oestrus.

The ratio of PGE-2: PGF-2α production by the ME at 06:00h on oestrus was approximately 5:1 whereas at 22:00h on pro-oestrus in the AH-POA, this ratio was about 1:4. This may suggest that the relative production of these PGs is important at these times of the oestrous cycle.

After assaying the samples by RIA, there was insufficient samples for GC-MS analysis. However, other workers have confirmed the presence of PGE-2, PGF-2α, TXB-2 and 6-keto-PGF-1α in brain tissue by GC-MS (see discussion).

Section 2.2.b Measurement of i) PGE and PGF levels in the AH-POA and ii) the ability of this tissue to synthesize PGE and PGF in the presence of exogenous arachidonic acid

Introduction

To check that PG levels in the brain tissue were low and that arachidonic acid availability was not rate limiting for PG synthesis in the homogenates, PG levels in the AH-POA and PG production by homogenates of the AH-POA incubated in the presence of exogenous arachidonic acid have been measured.

Methods

i) 3 rats were killed at 10:00h on pro-oestrus and their AH-POA dissected out as described in Section 2.2.a. and after recording the tissue weights, each tissue was immediately transferred to a petri dish containing 5 ml ethanol. Tissues were homogenized and processed according to the method discussed in Section 2.1.b. Samples were stored in 1 ml ethyl acetate at -20°C until assayed for PGE-2 and PGF-2α by RIA as described in Sections 1.3a and 1.3b.

ii) 4 rats were killed at 10:00h on pro-oestrus and their AH-POA dissected out as described in Section 2.2.a. The tissues were homogenized and incubated for 90 mins as described in Section 2.2.a except that 0.1 ml arachidonic acid in ethanol was added to each homogenate to give a final concentration of $2 \mu g ml^{-1}$.

Each homogenate was extracted as described in Section 1.2.a. Samples were stored in 2 ml ethyl acetate at -20°C until they were assayed for PGE-2 and PGF-2α by RIA using the method described in Sections 1.3a and 1.3b. Results were compared with PG production by the AH-POA at 10:00h on pro-oestrus in the absence of arachidonic acid, by means of Student's t-test.

Results

i) Concentrations of PGE-2 and PGF-2α measured in samples from AH-POA tissue homogenized in ethanol were lower than concentrations of PGE-2 and PGF-2α measured in homogenates incubated for 90 min in Krebs' solution as shown in Fig. 17. There was insufficient sample remaining to measure 6-keto- PGF-1α and TXB-2 levels. ii) The addition of exogenous arachidonic acid did not significantly alter the PGE-2 or PGF-2α concentrations in incubated AH-POA homogenates (Fig. 17).

Conclusion

As the levels of PGE-2 and PGF-2α in the AH-POA were lower than production of these PGs after incubation of homogenates of this tissue, it can be concluded that fresh synthesis of PGE-2 and PGF-2α occurred in the homogenate during the incubation period. Moreover, the fact that addition of arachidonic acid did not significantly increase the ability of the homogenate to synthesize PGE-2 or PGF-2α confirms that PG synthesis was not limited to any extent by the availability of free arachidonic acid in the homogenate.

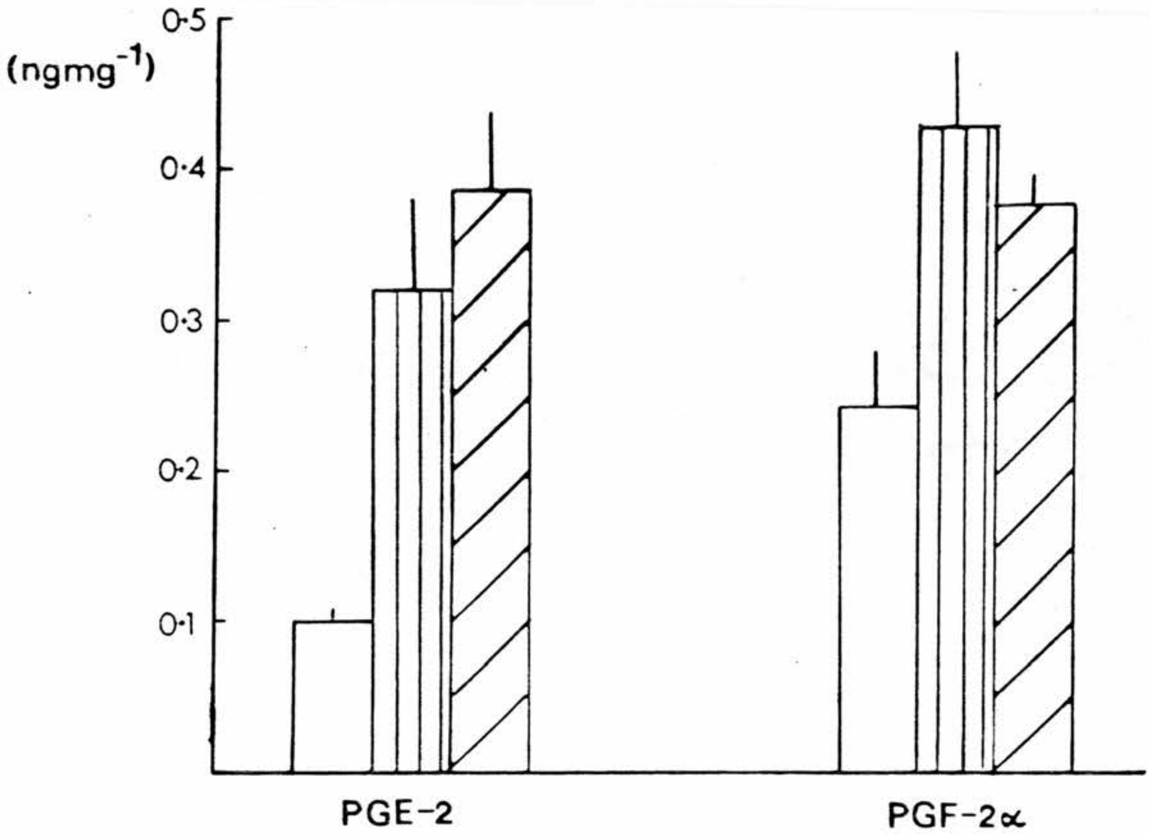


Figure 17

Concentrations ☐ (n=3) of PGE-2 and PGF-2α in ethanolic homogenates of AH-POA compared with production of PGE-2 and PGF-2α by AH-POA homogenates incubated in the absence ▥ (n=6) or presence ▨ (n=4) of 2μgml^{-1} arachidonic acid. Values are shown as mean ± s.e.m. for samples from rats killed at 10:00h on pro-oestrus.

Unfortunately, there was insufficient tissue to carry out similar experiments on the ME. However, in another study (Ojeda et al., 1978) PGE concentrations of about 0.1 $ngmg^{-1}$ in the ME has been reported which is much lower than PGE-2 production by the ME as reported here. It remains to be shown that arachidonic acid availability in brain homogenates is not rate limiting for PG synthesis.

Section 2.2.c <u>Measurement of the metabolism of PGE-2 and PGF-2α at 10:00h on pro-oestrus and 02:00h on oestrus by homogenates of ME and AH-POA</u>

Introduction

Metabolism of PGF-2α in rat brain has been shown to be negligible (Wolfe, Pappius and Marion, 1976; Gecse, Ottlecz and Telegdy, 1981). These workers measured metabolism of PGF-2α either in cortex or whole hypothalamus. No information is available on the ability of the ME to metabolize PGs. The following experiment was performed to study the metabolism of PGE-2 and PGF-2α by homogenates of ME and AH-POA at 10:00h on pro-oestrus and at 06:00h on oestrus.

Method

The ME and AH-POA of rats killed at 10:00h on pro-oestrus and 06:00h on oestrus were dissected out, homogenized in Krebs' solution as described in Section 2.2.a and incubated in the presence of 0.5 μCi ^{3}H-PGF-2α alone, or in combination with 2 μgml^{-1} PGF-2α and 2mM NAD+.

6 rats were killed for each time, providing 3 incubations in the presence of ^{3}H-PGF-2α alone and 3 incubations in the presence of ^{3}H-PGF-2α, PGF-2α and NAD+, for each of the ME and the AH-POA. This experiment was repeated for the study of PGE-2 metabolism by replacing ^{3}H-PGE-2 for ^{3}H-PGF-2α and adding PGE-2 instead of PGF-2α. The samples were processed according to the method previously described for the measurement of PG metabolism in tissue homogenates in Section 2.1.c. The results are expressed as percentage metabolism of PGE-2 or PGF-2α and were compared by means of Student's t test.

Results

Table 14 shows the percentage metabolism of ^{3}H-PGE-2 and ^{3}H-PGF-2α in homogenates of ME and AH-POA in the absence and presence of NAD+ at 10:00h on pro-oestrus and at 06:00h on oestrus.

The % metabolism results have not been corrected for tissue weight. However tissue weights did not change significantly between the two times (0.3 $\pm$ 0.03 mg at 10:00h on pro-oestrus and 0.3 $\pm$ 0.02 mg at 02:00h on oestrus for the ME, 17.6 $\pm$ 2.5 mg at 10:00h on pro-oestrus and 18.1 $\pm$ 2.9 mg at 02:00h on oestrus for the AH-POA).

Table 14. Percentage metabolism (mean ± s.e.m. of 3 observations) of ^{3}H-PGE-2 and ^{3}H-PGF-2α in homogenates of ME and AH-POA in the absence and presence of NAD+ at 10:00h on pro-oestrus and at 06:00h on oestrus.

Tissue	Time	PGE-2 metabolites		PGF-2α metabolites	
		no NAD+	with NAD+	No NAD+	with NAD+
ME	10:00h pro-oestrus	17.6±4.7	7.9±0.7	12.1±0.3	13.5±0.5
	06:00h oestrus	17.7±3.4	9.8±1.0	15.8±2.9	14.8±2.2
AH-POA	10:00h pro-oestrus	12.2±1.5	11.9±1.7	11.0±0.9	10.3±2.0
	06:00h oestrus	8.1±1.5	7.1±1.2	12.0±2.1	11.6±0.9

^{3}H-PGE-2 (Rf = 0.26) was metabolized by the ME and AH-POA into mainly its 15-keto-metabolite (Rf = 0.42) with little of the 13, 14-dihydro, 15-keto-metabolite (Rf = 0.43) in the absence or presence of NAD+. ^{3}H-PGF-2α (Rf = 0.14) was metabolized by the ME and AH-POA into approximately equal amounts of the 15-keto- (Rf = 0.25) and 13,14-dihydro,15-keto-metabolite (Rf = 0.35).

The percentage metabolism of PGE-2 and PGF-2α into these two metabolites is expressed as a single figure. Table 14 shows that the percentage metabolism of PGE-2 and PGF-2α by the ME and AH-POA is low in the absence and presence of NAD+ ranging from 7% to 18%.

There were no significant differences in PGE-2 or PGF-2α metabolism by these tissues between 10:00h on pro-oestrus and 06:00h on oestrus.

Conclusion

As metabolism of PGE-2 and PGF-2α in the homogenates of ME and AH-POA is low, the measurement of PG synthesis by the homogenates is unlikely to be altered by metabolism. It can also be concluded that as metabolism of PGE-2 or PGF-2α did not vary between the two times studied, it is unlikely that the changes in PGE-2 and PGF-2α production by homogenates of ME and AH-POA during the oestrus cycle are due to changes in PG metabolism.

Although PGI-2 and TXA-2 metabolism has not been studied here, it is likely that, as the same enzymes are involved (see Section 2.1.c) that metabolism of these compounds is also low.

Section 2.2.d. The effect of oestrogen and progesterone on PG and TX production by the ME and AH-POA

Introduction

The pre-ovulatory surge of LH depends upon the increase in plasma concentration of oestradiol that precedes the surge (Schwartz, 1969). The stimulatory effect of oestradiol is enhanced in the rat by ovarian progesterone secreted in response to LH (Mann and Barraclough, 1973) and this facilitatory effect of progesterone only occurs in oestradiol-primed rats (Brown-Grant and Naftolin, 1972).

It has been suggested (Craig, 1975) that when plasma oestrogen reaches a critical level, oestrogen-induced release of PGs occurs within the hypothalamus and the PGs stimulate the ovulatory surge of LH. This concept is supported by the observation that indomethacin blocks the progesterone-induced LH release in oestrogen pre-treated rats (Ojeda, Harms and McCann, 1975). Ovariectomy was shown to decrease the synthesis of PGF-2α by the hypothalamus and, while combined administration of oestrone and progesterone did not alter PGF-2α synthesis, it increased the synthesis of PGE-2 by the hypothalamus (Gecse, Ottlecz and Telegdy, 1981). In the latter study on the effects of ovarian steroid hormones on hypothalamic PG synthesis, the animals were treated with steroids either alone, or in combination, on every alternate day over a 14-day period. Thus the authors made no attempt to mimic the cyclical changes in circulating ovarian steroid concentrations in their experiments.

Experiments described in this section were performed to study the effect of pre-treating ovariectomized rats with oestrogen followed by progesterone on PG and TX synthesis by the ME and AH-POA.

One model which has been designed to simulate the changes in plasma steroid concentrations that occur before and during the LH surge, (Karla, Fawcett, Krulich and McCann, 1973) was used. Rats which have been ovariectomized on di-oestrus are given oestradiol benzoate on that day followed by progesterone on the following day. The other model which was used to study the stimulatory effect of progesterone (Caligaris, Astrada and Taleisnik, 1968) involves injecting progesterone 72 hr after injecting oestradiol benzoate into long-term ovariectomized rats. In both of these models, a surge in plasma LH occurs 5h after the injection of progesterone.

Methods

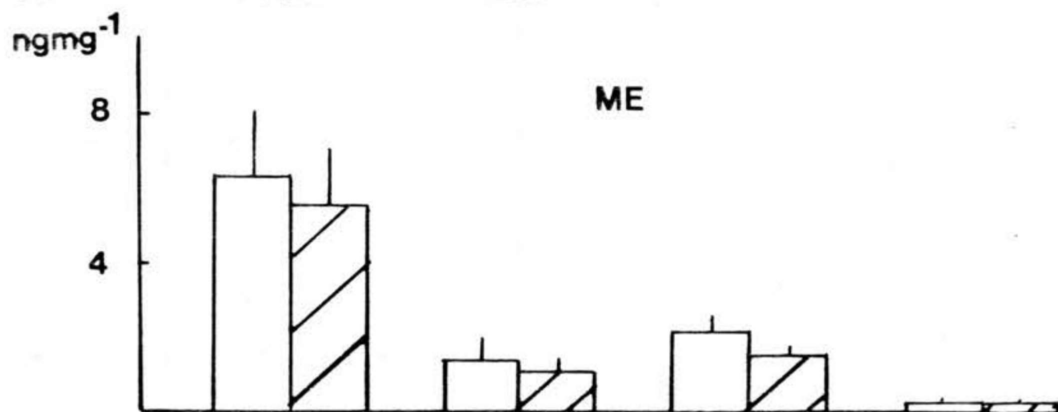
Mature female Albino Wistar rats weighing between 200 and 250g were maintained under controlled conditions as described in Section 2.1.a. These rats also provided tissue for use in experiments described in Section 2.3.d. Twelve rats were ovariectomized between 09:00h and 10:00h on di-oestrus (Group I). Ovariectomy was performed under Althesin (Glaxo, U.K.) anaesthesia (0.5 ml $100g^{-1}$ body weight i.p.). Six of these animals received 10μg oestradiol benzoate in 0.5 ml arachis oil as a subcutaneous (s.c.) injection. The other six animals were injected with the same volume of arachis oil alone.

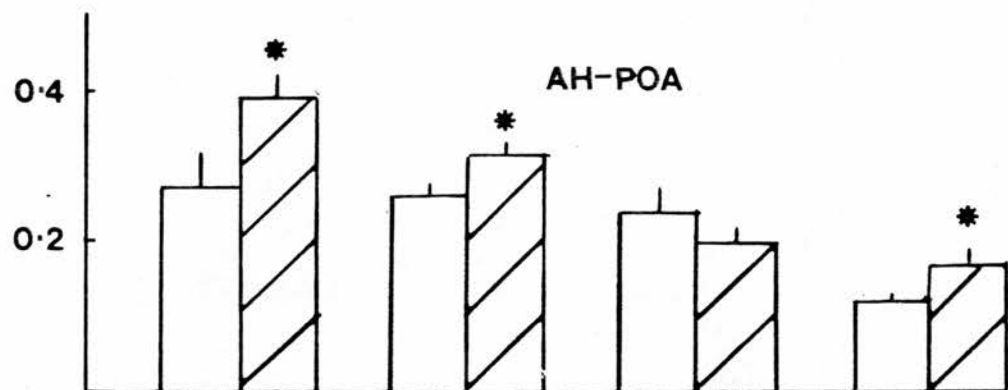
On the following day (the expected pro-oestrus) the animals were injected with 2 mg progesterone s.c. in 0.5 ml arachis oil at 12:00h.

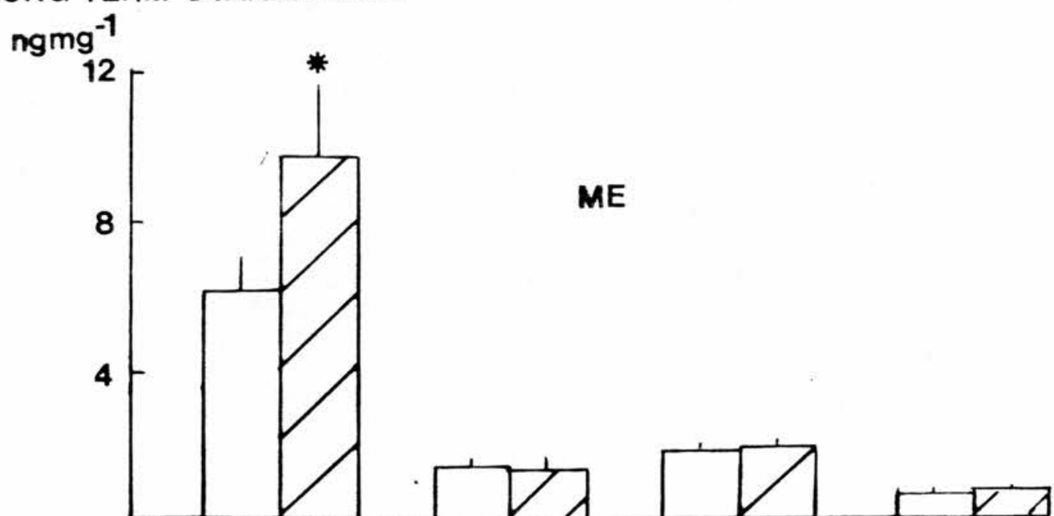
Another group of 22 rats (group II) were ovariectomized under Althesin anaesthesia. For these rats, the stage of the oestrous cycle at which ovariectomy was performed was unknown. Six weeks after ovariectomy, eleven of the rats received an s.c. injection of 10μg oestradiol benzoate in 0.5 ml arachis oil at 12:00h followed by 2 mg progesterone s.c. in 0.5 ml arachis oil 72h later. The eleven 'control' rats received s.c. injections of arachis oil alone at the above times. At 17:00h, five hours after the progesterone injection, the rats from both groups were killed and their ME and AH-POA dissected out, weighed, homogenized, incubated and extracted for PGs and TX as described in Section 2.2.a. The samples were stored in 2 ml ethyl acetate at -20°C until their content of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 was determined by RIA as described in Section 1.3.a, b, c and d. Results were compared by means of Student's t-test.

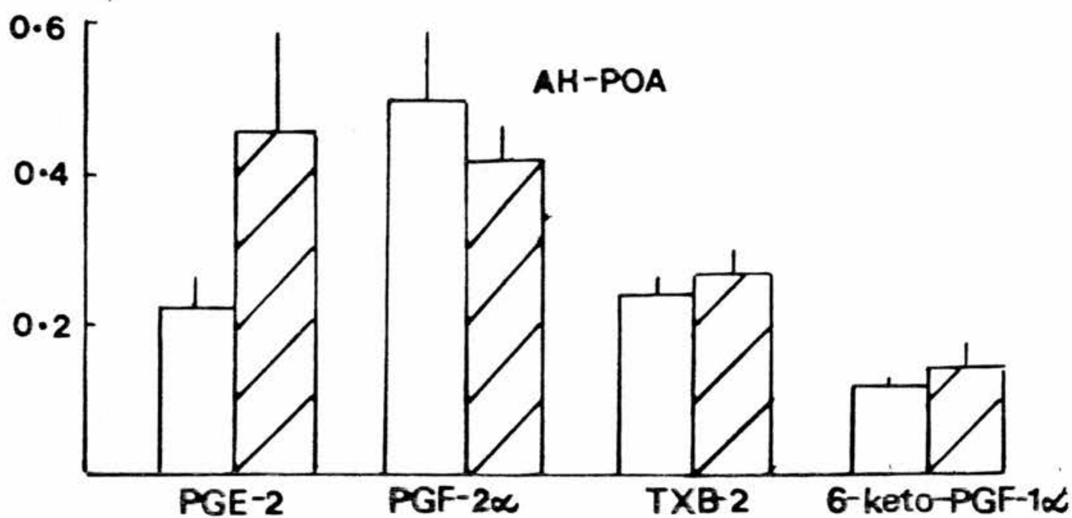
Results

Fig. 18 shows PG and TX production by homogenates of ME and AH-POA from either acute ovariectomized (Group I) or long-term ovariectomized (Group II) oestrogen and progesterone treated rats. Combined treatment with oestrogen and progesterone did not alter PG or TX production by the ME of acute ovariectomized rats.


GROUP I
ACUTE OVARIECTOMY
ngmg-1
8
4
ME


AH-POA
0·4
0·2


GROUP II
LONG TERM OVARIECTOMY
ngmg-1
12
8
4
ME


0·6
0·4
0·2
AH-POA
PGE-2
PGF-2α
TXB-2
6-keto-PGF-1α

Production of PGE-2, PGF-2α and 6-keto-PGF-1α was significantly ($p < 0.05$) increased in the AH-POA of acutely ovariectomized rats treated with oestrogen and progesterone, whereas TXB-2 synthesis was unaltered by treatment.

PGE-2 production by the ME of long-term ovariectomized rats was significantly increased by steroid treatment while production of PGF-2α, 6-keto-PGF-1α and TXB-2 was unaffected. Although the treatment of long-term ovariectomized rats did not affect PGF-2α, 6-keto-PGF-1α or TXB-2 production by the AH-POA, it did increase mean PGE-2 production by this tissue. However, this increase only approached significance $p < 0.1$.

Conclusion

Oestrogen and progesterone treatment of long-term ovariectomized rats increased PGE-2 production by the ME and AH-POA. Although the latter increase was not significant, it can be concluded that ovarian steroid treatment of the long-term ovariectomized rat can modulate PGE-2 synthesis by the hypothalamus and apparently in a selective manner.

In acute ovariectomized rats, PG and TX production by the ME was unaffected by treatment with oestrogen and progesterone. However, production of PGE-2, PGF-2α and 6-keto-PGF-1α by the AH-POA was significantly increased by the steroid treatment. These findings suggest that the stimulatory effect of ovarian steroids is in the AH-POA and not in the ME of acute ovariectomized rats.

It has not been established in these studies whether the stimulatory effects of the treatment on PG and TX production were due to decreased metabolism or increased arachidonic acid availability. However, other workers have shown that, after ovariectomy and hormone treatment, neither 15-hydroxy-PG dehydrogenase nor Δ-13-PG reductase activity could be detected (Gesce, Ottlecz and Telegdy, 1981) and that arachidonic acid availability in homogenates of hypothalamus is sufficient to completely saturate the brain cyclooxygenase enzyme (Wolfe, Pappius and Marion, 1976). Therefore the changes in PG and TX production observed are probably not due to changes in PG metabolism and/or arachidonic acid availability.

Section 2.2.e. Measurement of the in vitro effect of noradrenaline and catecholoestrogen on PG and TX production by the ME and AH-POA

Introduction

Although the ability of oestrogen to influence PG production by various tissues, including the uterus and hypothalamus, has been demonstrated in vivo (Ham, Cirillo, Zanette and Kuehl, 1975; Gecse, Ottlecz and Telegdy, 1981), this effect has rarely been observed in vitro. Naylor and Poyser, 1975 were able to show that in vitro oestradiol stimulated PGF-2α synthesis by homogenates of guinea pig uterus but this effect was small in comparison to the effect of in vivo oestradiol. These experiments suggest that a transformation of oestradiol to a more active metabolite is required in vivo for its action.

Metabolism of oestrogens into their catechol derivatives by hydroxylation at the C2 position is known to occur in the liver and brain (Fishman and Norton, 1974; Ball, Haupt and Knuppen, 1978). Kelly and Abel, (1981), have reported that the uterus can hydroxylate oestrogens at the 2 position and that catecholoestrogen stimulates PG production by rat uterine homogenates. Recently, catecholoestrogens have been proposed as mediators of the oestrogen-induced LH surge in the rat (Naftolin, Morishita, Davies, Todd and Ryan, 1975; Fishman, Norton and Krey, 1980; Rodriguez-Sierra and Blake, 1982). In a study of the distribution of 2-hydroxylase activity within the brain, the highest activity was observed in the hypothalamus (Fishman and Norton, 1975) and Fishman, Norton and Krey, 1980, have shown a sharp increase in 2-hydroxylase activity in brain between di-oestrus and pro-oestrus in cycling rats.

In view of these findings, it was decided to investigate the ability of the catecholoestrogen 2-hydroxyoestradiol (2-OH oestradiol) to stimulate PG and TX production by the ME and AH-POA *in vitro.* This was studied at two times during the oestrous cycle, i.e. 18:00h both on di-oestrus and on pro-oestrus. At these times the activity of the brain 2-hydroxylase enzyme is low and high respectively (Fishman, Norton and Krey, 1980). As the structure of the 2-OH oestradiol resembles noradrenaline in that they both have a catechol group present, it was decided to compare the effect of 2-OH oestradiol on PG and TX production by hypothalamic homogenates with that of noradrenaline.

Methods

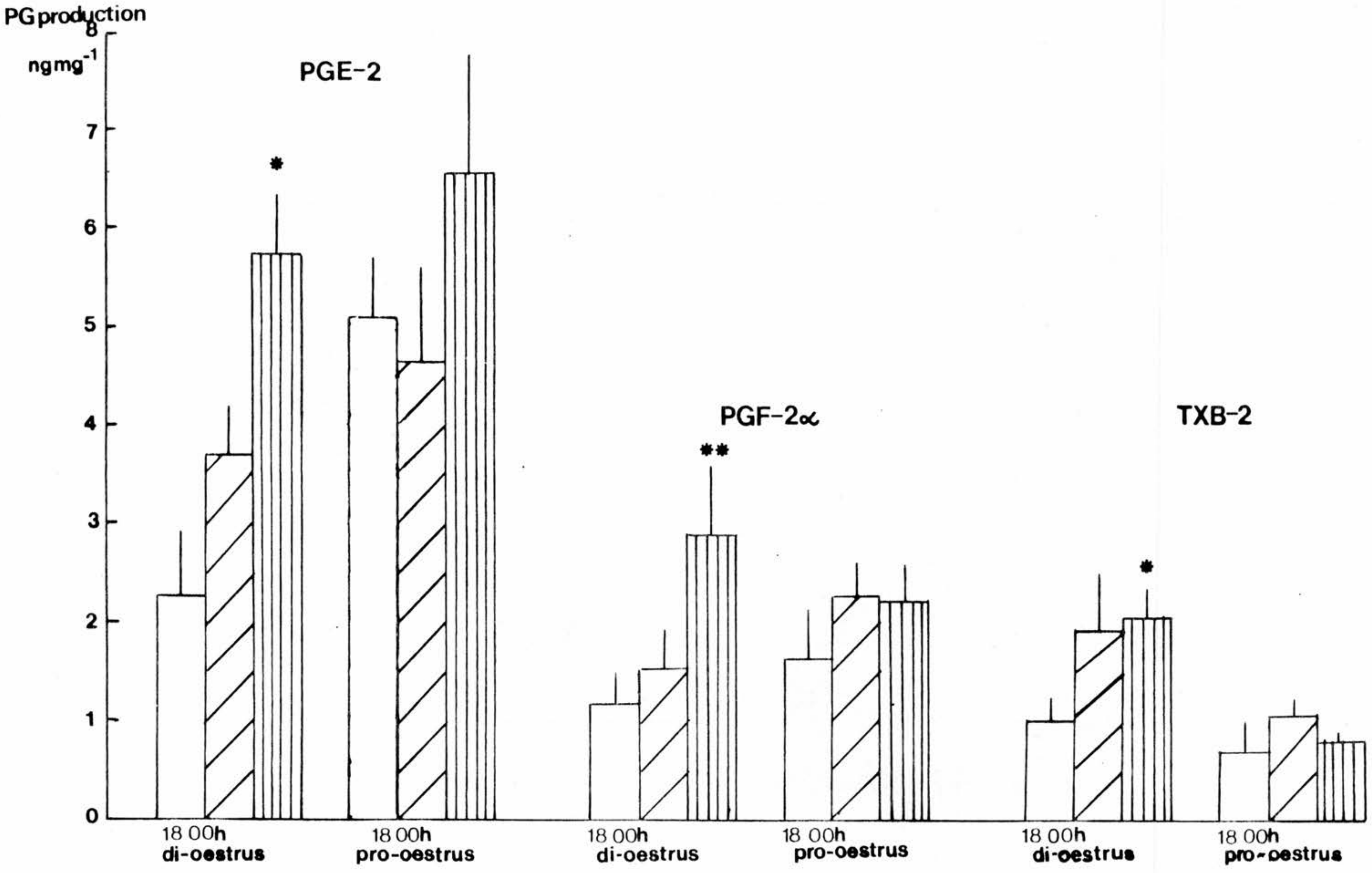
2-OH oestradiol was checked for purity by thin layer chromatography. The compound (10 μg) was spotted on to a silica gel plate in 50 μl of hexane. 10 μg of oestrogen was also applied to the plate. After allowing the spots to dry, the plate was developed in hexane: ethyl acetate (60:40). The spots were visualized by spraying with phosphomolybdic acid followed by incubation of the plate in an oven at 60°C.

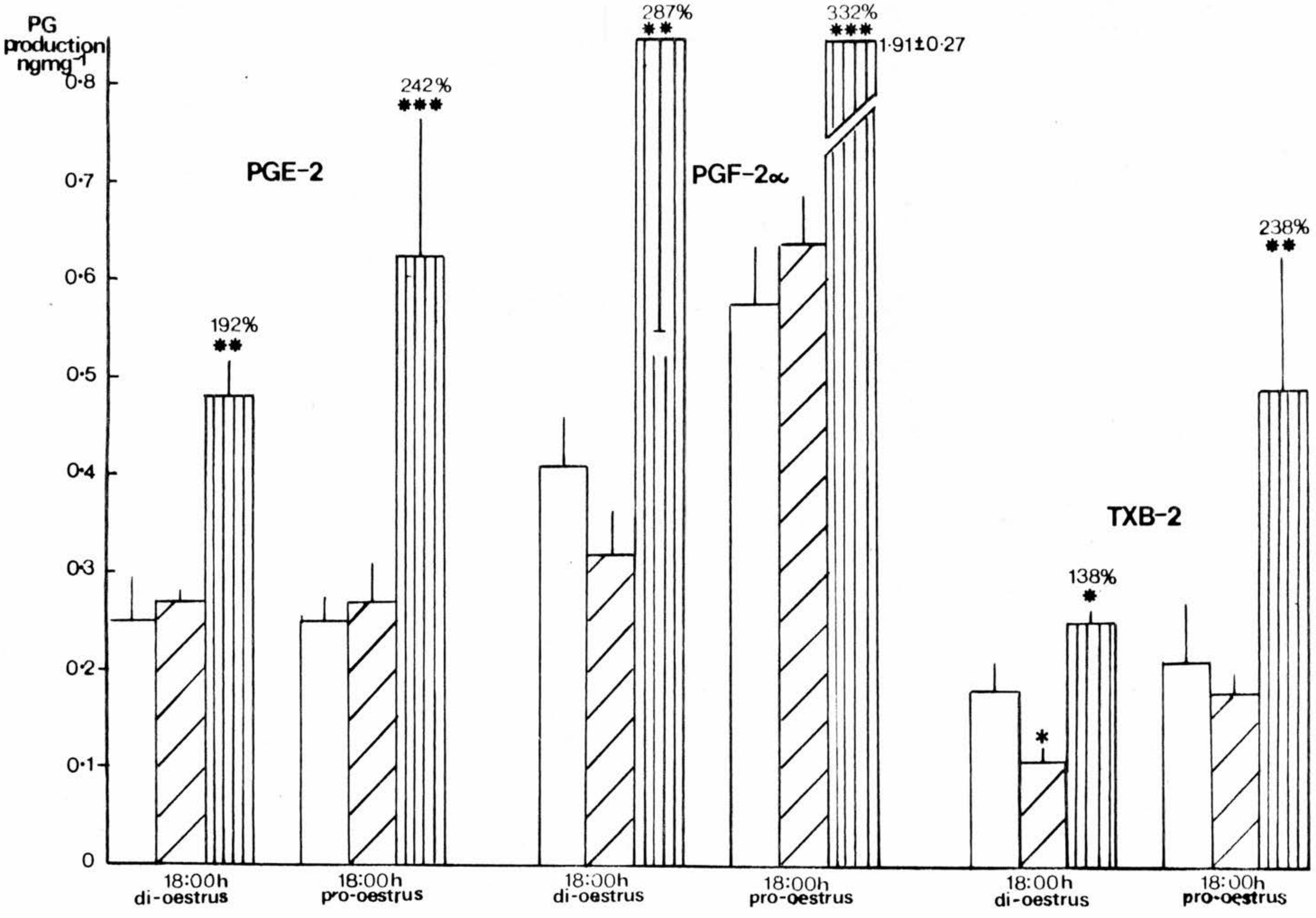
The ME and AH-POA from 10 rats which had been killed at 18:00h on di-oestrus and 10 rats killed at 18:00h on pro-oestrus were removed, weighed and homogenized as described in Section 2.2.a. Five homogenates of each type of tissue at each time were incubated for 90 min in the presence of 100μM 2-OH oestradiol. The other five homogenates of each type of tissue at each time were incubated in the presence of 100μM noradrenaline bitartrate and 0.06 mM ascorbic acid. Following incubation, the homogenates were extracted for PGs and TX using the method described in Section 1.2.a. Samples were stored in 2ml ethyl acetate at -20°C until they were assayed for PGE-2, PGF-2α and TXB-2 by RIA as described in Sections 1.3a, b and d. Results were compared by Student's t-test.

Results

Analysis by thin layer chromatography showed that the 2-OH oestadiol and oestradiol ran as single spots with differing Rf values (0.08 and 0.2 respectively). Thus the catechol oestrogen was not contaminated with oestrogen and was free from other impurities.

The effect of 2-OH oestradiol and noradrenaline on PGE-2, PGF-2α and TXB-2 production by homogenates of ME and AH-POA at 18:00h on di-oestrus and pro-oestrus is shown in Figs. 19 and 20.


PG production
ng mg^{-1}
8
7
6
5
4
3
2
1
0
PGE-2
*
PGF-2α
**
TXB-2
*
18 00h
di-oestrus
18 00h
pro-oestrus
18 00h
di-oestrus
18 00h
pro-oestrus
18 00h
di-oestrus
18 00h
pro-oestrus



PG production ngmg-1
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
PGE-2
PGF-2α
TXB-2
192%
**
242%
***
287%
**
332%
***
1·91±0·27
*
138%
*
238%
**
18:00h di-oestrus
18:00h pro-oestrus
18:00h di-oestrus
18:00h pro-oestrus
18:00h di-oestrus
18:00h pro-oestrus

The results have been compared with PG and TX production by these tissues as determined in experiments described in Section 2.2.a. There was no significant difference between PG or TX production by homogenates of ME incubated in the presence or absence of 2-OH oestradiol either at 18:00h on di-oestrus or pro-oestrus (Fig. 19). Noradrenaline significantly increased PGE-2 ($p<0.05$), PGF-2α ($p<0.02$) and TXB-2 ($p<0.05$) production by the ME at 18:00h on di-oestrus whereas at 18:00h on pro-oestrus, noradrenaline had no significant effect. As in the ME, 2-OH oestradiol had no effect on PGE-2 or PGF-2α production by AH-POA homogenates from rats killed at 18:00h on di-oestrus or pro-oestrus. (Fig. 20). There was an apparent decrease in TXB-2 production by AH-POA homogenates from rats killed at 18:00h on di-oestrus ($p<0.05$) when incubated in the presence of 2-OH oestradiol. However, this effect was not observed in homogenates from rats killed at 18:00h on pro-oestrus. In contrast, noradrenaline significantly increased PGE-2, PGF-2α and TXB-2 production at 18:00h on di-oestrus and at 18:00h on pro-oestrus. The percentage increase in production in the presence of noradrenaline is shown in Fig. 20. This shows that noradrenaline is more effective in stimulating PG and TXB-2 production by the AH-POA at 18:00h on pro-oestrus than at 18:00h on di-oestrus.

Conclusion

Whereas noradrenaline stimulates PG and TX production by the ME and AH-POA *in vitro*, 2-OH oestradiol has no effect. Noradrenaline was more effective in stimulating PG and TX production by the ME at 18:00h on di-oestrus than at 18:00h on pro-oestrus, this increase being between 200% and 250%. In contrast, noradrenaline was more effective in stimulating PG and TX production by the AH-POA at 18:00h on pro-oestrus than at 18:00h on di-oestrus. An effect of ascorbic acid on PG and TX production cannot be ruled out as no incubations with noradrenaline were carried out in the absence of ascorbic acid. However, ascorbic acid has been reported to have an inhibitory effect on PG production by the guinea pig uterus (Pugh, Sharma and Wilson, 1975). Consequently, the stimulatory effect is probably due to NA. The lack of effect of 2-OH oestradiol on PG and TX production cannot be due to contamination with oestrogen, or impurity of the substance, as this was checked before the experiment. It is possible that, as no antioxidant was added to the incubations with 2-OH oestradiol, the catechol oestrogen was inactivated by oxidation. However, Kelly and Abel, 1980 have reported that ascorbic acid reduces the effect of 2-OH oestradiol on PG synthesis and for this reason, ascorbic acid was not included in the 2-OH oestradiol incubations.

Section 2.2.f. The effect of Froben on the pro-oestrous LH surge

Introduction

If PGs are involved as mediators or modulators of gonadotrophin release from the hypothalamus, it should be possible to demonstrate that inhibition of hypothalamic PG synthesis results in altered gonadotrophin secretion. Although it has been demonstrated that indomethacin given daily for 5 days will depress serum LH levels in ovariectomized rats (Sato, Jyujo, Hirono and Iesaka, 1975) and that 5-, 8-, 11-, 14- eicosatetracynoic acid (TYA), a PG synthesis inhibitor, and indomethacin, administered intraventricularly also decrease the post-castration rise in plasma LH levels (Ojeda, Harms and McCann, 1975) it has yet to be shown that PG synthesis inhibitors will affect the pro-oestrous LH surge. Tsafriri, Koch and Lindner (1973) reported that indomethacin given as a single injection either at 08:30h or 14:30h failed to prevent the pro-oestrous LH surge, and likewise, indomethacin injected at 13:00h on pro-oestrus had no effect on serum LH levels in cycling rats (Sato, Taya, Jyujo and Igarashi, 1974). In the last study, however, rats treated with indomethacin on pro-oestrus ovulated and, after oestrus, di-oestrus persisted for 5 to 12 days suggesting that indomethacin may have had some effect on gonadotrophin release. The lack of effect of indomethacin in blocking the pro-oestrous LH surge may be due to the inability of the drug to penetrate the central nervous system (Hucker, Zacchei, Cox, Brodie and Cantwell, 1966; Goodman and Gillman, 1975).

The possibility also exists that a PG synthetase isoenzyme exists in the hypothalamus and that indomethacin is a relatively poor inhibitor of this form of the enzyme.

The effect of a new PG synthesis inhibitor 2-(2-fluoro-4-biphenylyl)proprionic acid (Froben) on the pro-oestrous LH surge has been the subject of the experiment described in this section. This inhibitor was used on the basis that it is more potent as an antipyretic agent than either aspirin, meclofenamic acid, paracetamol or phenylbutazone (Van Miert and Van Duin, 1977) and thus may be more effective in inhibiting PG synthesis in the hypothalamus.

Method

Twelve rats weighing between 200g and 250g were used in the experiment. The rats had been housed fed and smeared as described in Section 2.1.a. Between 08:00h and 10:00h on pro-oestrus, rats were anaesthetized with halothane. Each rat was prepared with a jugular cannula for collection of blood samples for the estimation of plasma LH. After exposing the right jugular vein, the wall of the vessel was cut with iris scissors and a cannula, filled with heparinized saline (50 I.U.ml^{-1}), passed along the inside until blood could be seen to well back into the cannula. This event marked the entry of the cannula into the right atrium. The cannula was secured with ligatures placed along a 0.4mm section of cannula which had been strengthened by placing a hollow metal tube inside.

The cannula was exteriorized at the back of the neck to a length of approximately 3 cm. After flushing the cannula with heparinized saline, the open end was stoppered with a brass pin. The rats recovered consciousness within 10 min of removal from the halothane. Six rats prepared in this way acted as controls and received 0.5 ml saline intra-peritoneally (i.p.) at 09:00h and again at 13:00h intravenously (i.v.). The remaining six animals were given Froben at 09:00h i.p. and at 13:00h i.v. Froben was injected in 0.5 ml saline at a dose of 2 $mgkg^{-1}$. Blood samples 0.4 ml were collected, via a 50cm extension cannula at 12:00h, 15:00h, 16:00h, 17:00h, 18:00h, 19:00h and 20:00h. The samples were stored on ice until they were centrifuged at 4°C for 20 min at 1300 x g on an MSE Coolspin centrifuge. The plasma was transferred to plastic tubes and stored at -20°C until assayed for LH. After collection of the last blood sample each rat was killed and the M E and AH-POA dissected out, weighed, homogenized in Krebs' solution, incubated and extracted for PGs and TX as described in Sections 1.2.a and 2.2.a. The samples were stored at -20°C until assayed by RIA for PGE-2 using the method described in 1.3.a.

Plasma was assayed for LH content by RIA according to Niswender, Midgley, Monroe and Riechart (1968). The assistance of Ms L. Nairn and Ms A. Horn of the MRC Brain Metabolism Unit, Edinburgh with this assay is acknowledged. Standard curves were based on triplicate estimates at 9 dose levels of ovine standard LH (NIH-LH-S13). Purified ovine LH was iodinated with I^{125} and an antibody (GDN#5) against ovine LH was used at a dilution of 1:60,000. All samples were assayed in duplicates of 20µl plasma per assay tube.

The lower limit of sensitivity of the assay was $1 ngml^{-1}$ in terms of the LH standard.

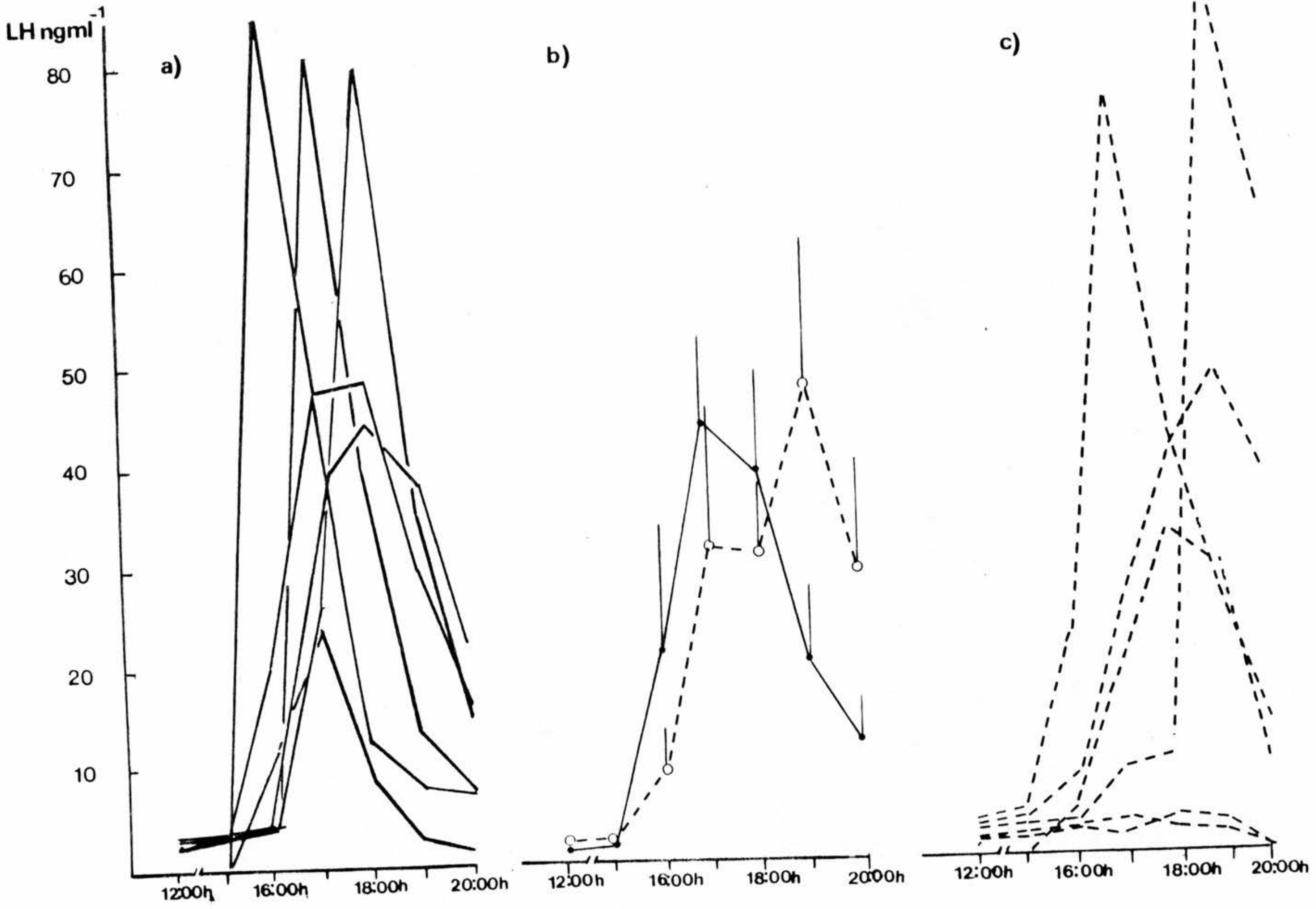
Results

Froben decreased the concentration of PGE-2 in samples of ME and AH-POA from a mean of 5.15 ± 0.6 $ngmg^{-1}$ and 0.25 ± 0.02 $ngmg^{-1}$ to 1.22 ± 0.35 $ngmg^{-1}$ and 0.08 ± 0.04 $ngmg^{-1}$ respectively.

All of the rats given saline alone showed a surge of LH which peaked between 16:00h and 18:00h (Fig. 21a). Of the rats treated with Froben, two showed no LH surge and the remaining 4 rats each showed a surge in LH which occurred between 17:00h and 19:00h (Fig. 21c). The mean values of plasma LH from control and from the treated rats which showed an LH surge are shown in Fig. 21b. Froben appears to delay the peak in plasma LH by 2 hours. However, the mean values of plasma LH at either 17:00h or 19:00h did not differ significantly between control and treated rats.

Conclusion

Froben treatment inhibited LH release in 2 out of the 6 rats. In the remaining 4 rats, the LH peak was delayed by up to 2 hours. Froben treatment therefore inhibited or delayed the appearance of the LH surge. As Froben significantly reduced PGE-2 levels in the ME and AH-POA, it is possible to speculate that inhibition of PG synthesis by Froben resulted in inhibition or delay of the LH peak.



LH ngml-1
80
70
60
50
40
30
20
10
a)
b)
c)
12:00h
16:00h
18:00h
20:00h

However, if PGE-2 produced by the brain is essential for LH release, Froben treatment should have prevented the LH surge in all 6 rats. The dose of Froben used, though, only reduced PGE-2 levels by 70%, therefore PG synthesis may not have been completely inhibited and sufficient synthesis of PGE-2 may have occurred in 4 of the 6 rats to cause the delayed release of LH. Further experiments are therefore required using a larger dose of Froben and/or an additional injection at 15:00h (i.e. 1-3h before the LH surge) in order to establish if PGs are essential for the LH surge.

Discussion

The results presented in this section confirm earlier observations on the presence of PGs in rat brain. Wolfe, Rostworowski and Pappius, (1975), have shown that rat brain synthesizes PGF-2α and PGE-2 from endogenous precursors in a ratio of approximately 3:1. Homogenates of rat cerebral cortex synthesize TXB-2 in similar amounts to PGF-2α (Wolfe, Rostworowski and Marion, 1976). 6-keto-PGF-1α could be identified in rat brain homogenates but was a minor component in relation to PGD-2, PGE-2 and PGF-2α (Saeed Abdel-Halim, Lunden, Cseh and Anggard, 1980). The major PG produced by rat brain homogenates is PGD-2 (Abdel-Halim, Hamberg, Sjoquist and Anggard, 1977). In a study on the distribution of PGD-2 in the rat brain, Narumiya, Ogorochi and Hayaishi (1982) have shown that the distribution is wide, with higher concentrations being found in the pituitary gland and hypothalamus.

PGD-2 synthesis has not been studied here due to the lack of an RIA for this compound. It would be interesting to study its production by the hypothalamus during the oestrous cycle. Although the samples were not analyzed for the presence of cross reacting substances, such as PGE-1 and PGF-1α, it has been reported that the rat brain lacks eicosatrienoic acid, the precursor of the I-series PGs (Baker and Thompson, 1972). Therefore the antibodies were probably measuring the PGs of the 2-series to which they had been raised.

The ratio of PGF to PGE concentrations in the medial hypothalamus measured by bioassay is approximately 2:1 whereas in the ME, this ratio is reversed (Cseh, Szabo, Lang and Palkovits, 1978). Furthermore, these workers report that the ME contains more than 10 times as much PGE-2 and 6 times as much PGF-2α as the medial hypothalamus. Ojeda, Naor and McCann (1978) have also found that PGE concentrations in the ME exceed those in the MBH by 4- to 5-fold. These results are in agreement with those presented in this section. The present results also show that the increased concentration of PGE and PGF in the ME is due to increased synthetic capacity of the tissues for them. PG and TX production by the ME showed a diurnal rhythm. PGE-2 production by the ME increased at the time of the LH surge (i.e. between 14:00h and 18:00h on pro-oestrus). This increase in PGE-2 production is unlikely to be due to increased arachidonic acid availability in the tissue as the addition of exogenous arachidonic acid to hypothalamic homogenates did not increase PGE-2 production.

Metabolism of PGE-2 by the ME and AH-POA did not change significantly betwen 10:00h on pro-oestrus and 06:00h on oestrus and so the increased PGE-2 production seen on these two days cannot be due to a decrease in its metabolism.

Peak PGE-2 production by the ME occurred at 06:00h on oestrus, a time when LH levels would have returned to basal. It cannot be ruled out therefore, that PGE-2 synthesis by the ME is related also to the release of other hypothalamic releasing factors. A surge in growth hormone (GH) occurs in the early hours of light (Martin, Tannenbaum, Willoughby, Renaud and Brazeau, 1975) and PGE-2 will stimulate GH release by acting within the hypothalamus (Ojeda, Negro-Vilar, Arimura and McCann, 1980). There are however, no reports of a diurnal variation in LHRH or LH release in the rat.

A diurnal rhythm exists in plasma progesterone concentrations (mainly of adrenal origin) during the oestrous cycle of the rat (Mann and Barraclough, 1973). Plasma levels of this steroid are at a nadir between 10:00h and 14:00h and peak during the early morning hours (01:00h to 05:00h) on each day of the cycle. Since the peak production of PGE-2 by the ME follows these peak levels of progesterone, a causal relation appears to exist between the diurnal rhythm in adrenal progesterone output and PGE-2 synthetic capacity of the ME. There is an extra peak in progesterone between 17:00h on pro-oestrus and 01:00h on oestrus due to increased ovarian secretion which may be responsible for the peak in ME PGE-2 production at 18:00h on pro-oestrus.

Further support for a stimulatory effect of progesterone on PGE-2 synthesis by the ME came from the observation that injecting long-term ovariectomized oestradiol-primed rats with progesterone increased PGE-2 production by the ME. This treatment is known to induce a surge in plasma LH within 5h of the progesterone injection (Caligaris, Astrada and Taleisnik, 1968). Unfortunately, no reports are available to show that the LH surge is coincident with a surge in LHRH. Nevertheless these results suggest that progesterone may facilitate an increase in LH release by firstly stimulating PGE-2 synthesis in the ME which would, in turn, cause LHRH release into the hypophyseal portal vessels (see Introduction). It is not clear, however, why treatment of short-term ovariectomized rats with oestrogen and progesterone had no effect on PGE-2 production by the ME.

Correlations of PG and TX synthesis in the AH-POA with plasma concentrations of ovarian steroids (as shown in Fig. 3) shows that a high ratio of PGF to the other PGs and TX occurs when plasma progesterone concentrations are high. However, treatment of either long-term or short-term ovariectomized rats with oestrogen and progesterone did not preferentially increase PGF production by the AH-POA. Indeed, it can be said that the steroid treatment which was designed to simulate the changes in plasma steroid concentrations that occur before and during the LH surge did not result in simulating the changes in PG and TX production by the AH-POA occurring at that time in cycling animals. The significance of these results is not clear.

In future experiments it would be interesting to study the effect of progesterone given separately to test whether priming with oestradiol affected the PG and TX synthesis by the brain in response to progesterone.

Although Kelly and Abel (1980) were able to show that 2-OH oestradiol stimulated PGE-2 and PGF-2α production by rat uterine homogenates, this effect was not seen in either the ME or AH-POA at a similar dose (i.e. 100μM) in the experiments described here. However, in order to conclude that catechol oestrogen does not affect PG synthesis by the hypothalamus a wider range of doses of this compound would require to be tested. The stimulatory effect of NA on PGE-2 and PGF-2α production by brain tissue confirms the results of Hillier and Roberts, 1976; Wolfe, Pappius and Rostworski, 1976 and Ojeda, Negro-Vilar and McCann, 1979. The mechanism of action of catecholamines in stimulating PG production is unknown but it is possible that they act as reducing agents in the conversion of PGH-2 to PGF-2α or act as free radical scavengers, thereby preventing cyclooxygenase inactivation during PG synthesis (To, Smith and Carpenter, 1980). Ojeda, Negro-Vilar and McCann (1982) have shown that the stimulatory effect of NA on PGE-2 and LHRH release from the ME *in vitro* is blocked by phentolamine and thus involves participation of an α-adrenergic receptor. Therefore the differing responses to NA with respect to PG production by the ME between 18:00h on di-oestrus and 18:00h on pro-oestrus observed in experiments described in this section, may reflect cyclical changes in the number of α-receptors in the ME.

Changes in PGE-2 release by the ME in response to NA throughout the oestrous cycle have also been reported by De Paolo, Ojeda, Negro-Vilar and McCann (1982). They observed a diurnal variation in responsiveness, being high at 13:00h and low at 16:00h. When progesterone was given several hours before the normal ovulatory increase, the onset of the LH surge as well as the increased response of the ME to PGE-2 were advanced (De Paolo et al., 1982). Therefore, as it has been suggested in this section that progesterone may increase the synthetic capacity of the ME for PGE-2, it would be interesting to speculate that progesterone increases the responsiveness of the ME to NA by increasing the ability of the ME to synthesize PGE-2.

The final experiment described in this section which was designed to study the role of hypothalamic PG production, showed that Froben prevented the release of LH in 2 out of 6 animals and appeared to delay LH release in the other 4. These results do not fully support the results of Tsafriri et al., (1973) or Sato et al., (1974) who showed that indomethacin did not affect the pro-oestrous surge of LH and suggested that hypothalamic PG synthesis is not essential for the LH surge. Because of the increase in pituitary responsiveness to LHRH on pro-oestrus, it is possible that only a very small increase in LHRH (and PG) release from the ME is required for a surge in LH. The effect of Froben on the pro-oestrus surge in LHRH requires studying.

Further experiments are required, therefore, in order to clarify the role of PGs in LHRH release.

As peak PGE-2 production by the ME occurred at the time of the ovulatory surge of progesterone, it would be interesting to study the effect of progesterone on PGE-2 synthesis by the ME. In particular the activity of the cyclooxygenase and isomerase enzymes involved in hypothalamic PGE-2 synthesis requires investigation.

Section 2.3 PROSTAGLANDIN PRODUCTION BY THE UTERUS

Introduction

PGs produced by the uterus have been implicated in the regulation of several processes in the female reproductive tract of the rat. It has been suggested that they mediate the vascular changes at blastocyst implantation sites (Kennedy, 1979b) as well as the uterine hyperaemia which occurs in response to oestrogen (Ryan, Clark, Van Orden, Farley, Edvinson, Sjoberg, Van Orden and Brody, 1974). A number of investigations suggest the involvement of endogenous PGs in uterine contractions (Vane and Williams, 1973; Phillips and Poyser, 1981a). PG production by the uterus fluctuates during the oestrous cycle (Ham, Cirillo, Zanetti and Kuehl 1975; Poyser and Scott, 1980; Van Orden, Goodale, Baker, Farley and Bhatnagar, 1980). However, the physiological significance of uterine PG production during the oestrus cycle is unknown at present.

The following experiments describe PG and TX production by the rat uterus throughout the oestrous cycle. PG and TX by the endometrium and myometrium and the effect of ovarian steroids on uterine production of PG and TX have also been studied.

Section 2.3.a Measurement of the ability of uterine tissue to produce PGs and TX during the oestrous cycle

Introduction

The rat uterus synthesizes 6-keto-PGF-1α in addition to PGE-2 and PGF-2α (Fenwick, Naylor, Poyser and Wilson 1977; Pace-Asciak and Rangaraj, 1977). In the same year, Williams and Downing showed that microsomes from the pregnant rat uterus can convert ^{3}H-AA into small amounts of TXB-2. Small quantities of TXB-2 (3 to 5 ng100mg^{-1}) were detected in the uterus of the cycling rat by GC-MS assay (Poyser and Scott, 1980) but, due to the relatively low sensitivity of this assay method, accurate measurements of TXB-2 production during the oestrous cycle were not possible in their study.

In the majority of studies on PG production by the uterus of the cycling rat (Ham et al., 1975; Poyser and Scott, 1980; Van Orden et al., 1980; Thaler-Dao, Saintot, Ramonatxo, Chavis and Crastes de Paulet, 1982) determinations were made only once or twice per day. The following experiments have examined uterine PG and TX production at 4-hourly intervals during the oestrous cycle.

Method

Rats weighing between 200g and 250g were killed at 02:00, 06:00h, 10:00h, 14:00h, 18:00h and 22:00h on each day of the cycle. These animals also provided tissue for use in experiments described in Sections 2.1a and 2.2a.

The uterus from each rat was dissected out and any cervical tissue removed. After weighing the uterus it was homogenized in 5 ml Krebs' solution in a Fisons glass homogenizer. The homogenate was transferred to a 50ml conical flask and the homogenizer was washed twice with 5ml Krebs' solution, the washings being added also to the flask. Each flask was incubated for 90 min at 37°C as described in section 1.2b and, following incubation, the PGs and TX were extracted according to the method given in Section 1.2.a. Samples were stored in 10ml ethyl acetate at -20°C until being assayed for PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 content by RIA as previously described in Sections 1.3a, b, c and d.

The results from the rats killed at 4-hourly intervals during the cycle were compared using Duncan's Multiple Range test for equal variances. Where comparisons were being made between means having differing variances, as determined by a F-ratio test, then significance was tested by the method of Fisher and Behrens. Significance was tested at the 5% level.

Identification of extracted PGs by GC-MS

Samples which had previously been assayed for PGs and TX by RIA were pooled, purified by silicic acid chromatography and the appropriate derivatives prepared according to the method described in Section 1.4. The samples were injected in 10μl BSTFA into the GC and full mass spectra taken at the retention times on the GC corresponding to the carbon values of PGA-2, PGB-2, PGD-2, PGE-1, PGE-2, PGF-1α, PGF-2α, 6-keto-PGF-1α and TXB-2.

Results

GC-MS analysis confirmed the presence of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 in the uterine samples as previously shown by RIA. PGD-2 was also present in these samples. Neither PGA-2, PGB-2, PGE-1 nor PGF-1α were detected in the samples.

Fig. 22 shows the amounts of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 formed from endogenous precursors by rat uterine homogenates at 4-hourly intervals throughout the oestrous cycle. A statistical analysis of these results is given in Table 15.

Homogenates of the whole uterus synthesized mainly 6-keto-PGF-1α with smaller amounts (in descending order) of PGF-2α, PGE-2 and TXB-2. Production of all 3 PGs and TXB-2 showed little variation on met-oestrus and di-oestrus but a comparison throughout the cycle shows production tending to be lowest on the day of di-oestrus. Between 06:00h and 14:00h on pro-oestrus PG and TX production started to increase. At 02:00h on oestrus, production of 6-keto-PGF-1α, PGF-2α and TXB-2 reached peak values. These values were significantly different from the values at 10:00h on pro-oestrus for the corresponding PG or TXB-2. Between 10:00h on pro-oestrus and 02:00h on oestrus TXB-2 production showed the greatest percentage increase, i.e. 225% compared to 50% for 6-keto-PGF-1α and 97% for PGF-2α. At 02:00h on oestrus, uterine TXB-2 production was significantly greater than at any other time in the cycle. By 14:00h on oestrus, production of PGF-2α and TXB-2 had declined to values which did not differ significantly from those on met-oestrus. 6-keto-PGF-1α production remained elevated at 14:00h on oestrus, but showed a gradual decline from then until 22:00h on di-oestrus.

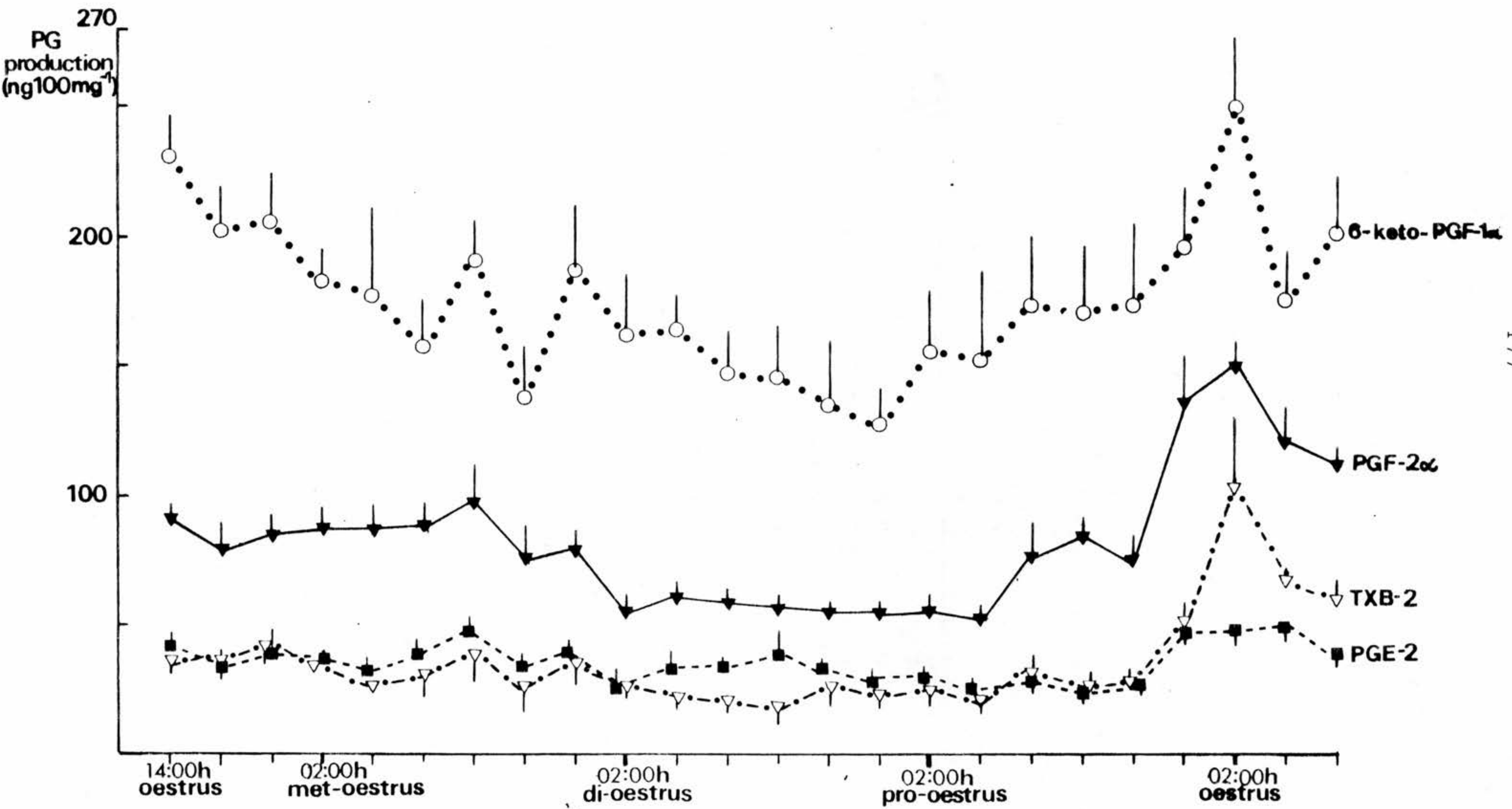

270
PG production (ng100mg^{-1})
200
100
6-keto-PGF-1α
PGF-2α
TXB-2
PGE-2
14:00h
oestrus
02:00h
met-oestrus
02:00h
di-oestrus
02:00h
pro-oestrus
02:00h
oestrus

Table 15. Statistical analysis of PG and TX production by rat uterine homogenates as shown in Fig. 22. Significance was tested by Duncan's multiple Range test or Fisher Behrens test*. Values with the same superscript within each column are not significantly different ($p > 0.05$). Values are expressed as mean ± s.e.m., n = 6 for each time.

PG production ng 100 mg^{-1} Time	PGE-2	PGF-2α	6-keto-PGF-1α	TXB-2
14.00h oestrus	42.2+4.8^{efgh}	91.4+6.3^{de}	230+17^{jk}	36.2+3.0^{bcdefg}
18:00h oestrus	35.1+7.0^{bcde}	79.7+10.0^{cd}	203+15^{hij}	36.4+3.1^{cdefg}
22:00h oestrus	39.6+4.0^{defg}	84.0+6.0^{cde}	207+17^{ijk}	40.8+3.6^{fgh}
02:00h met-oestrus	37.1+3.4^{cdef}	84.9+7.0^{cde}	183+11^{defghi}	34.1+6.0^{bcdef}
06:00h met-oestrus	32.1+4.8^{abcd}	86.5+8.1^{cde}	178+32^{cdefghi}	27.2+6.7^{abcde}
10:00h met-oestrus	38.2+6.0^{def}	88.0+7.9^{de}	158+19^{abcdef}	30.1+7.0^{abcdef}
14:00h met-oestrus	46.5+6.2^{fgh}	97.5+13.0^{ef}	192+14^{efghij}	39.8+6.7^{efgh}
18:00h met-oestrus	33.3+2.4^{abcde}	75.4+11.2^{bcd}	137+21^{abc}	26.6+5.1^{abcd}
22:00h met-oestrus	39.2+5.5^{defg}	79.9+6.9^{cd}	188+26^{efghij}	38.6+5.1^{defg}
02:00h di-oestrus	26.2+7.2^{ab}	57.0+5.0^{a}	161+23^{abcdefg}	27.3+1.9^{abcde}
06:00h di-oestrus	32.8+6.5^{abcde}	62.0+3.7^{ab}	164+13^{abcdefgh}	22.3+2.3^{a}
10:00h di-oestrus	32.9+5.4^{abcd}	58.7+6.0^{ab}	146+16^{abcd}	20.2+2.5^{a}
14:00h di-oestrus	29.4+8.1^{abc}	57.7+6.0^{a}	145+20^{abcd}	19.5+2.7^{a}
18:00h di-oestrus	26.0+3.0^{a}	55.4+3.2^{a}	135+27^{ab}	23.7+4.0^{ab}
22:00h di-oestrus	28.6+4.0^{abc}	55.5+3.5^{a}	127+13^{a}	22.3+2.9^{a}
02:00h pro-oestrus	29.2+3.5^{abc}	56.6+4.6^{a}	156+25^{abcde}	24.8+1.1^{abc}
06:00h pro-oestrus	24.3+5.0^{a}	53.3+5.2^{a}	153+33^{abcde}	20.4+3.3^{a}
10:00h pro-oestrus	28.8+2.4^{abc}	76.0+14.4^{bcd}	167+27^{abcdefgh}	31.8+7.2^{abcdef}
14:00h pro-oestrus	24.1+2.4^{a}	83.2+4.8^{cde}	170+25^{bcdefghi}	26.5+5.0^{abcd}
18:00h pro-oestrus	28.0+5.3^{abc}	74.3+9.9^{bc}	173 +29^{bcdefghi}	28.4+4.1^{abcdef}
22:00h pro-oestrus	47.7+4.0^{gh}	138.0+15.6^{gh}	197+24^{fghij}	51.7+8.8^{hi}
02:00h oestrus	47.9+7.5^{gh}	151.0+9.5^{h}	251+28^{k}	103.5+25.7^{j*}
06:00h oestrus	49.5+6.7^{h}	121.0+12.9^{g}	175+19^{bcdefghi}	47.6+5.0^{ghi}
10:00h oestrus	38.8+5.2^{defg}	113.0+4.9^{fg}	202+22^{ghij}	60.7+7.0^{i}

PGE-2 production by the uterus showed a slightly different trend. Like the other PGs, production of PGE-2 was lowest on the day of di-oestrus. However, production of PGE-2 increased significantly between 18:00h and 22:00h on pro-oestrus (by 77%) and remained constant at this elevated level between 22:00h on pro-oestrus and 06:00h on oestrus before showing a slight decline. There was no discernible peak at 02:00h on oestrus. Also, in contrast to production of 6-keto-PGF-1α, PGF-2α and TXB-2, PGE-2 production remained relatively high on met-oestrus before decreasing to its lowest value on the day of di-oestrus. The ratio of 6-keto-PGF-1α:PGF-2α:TXB-2:PGE-2 production changed from 4.4:1.8:0.6:1 at 02:00h on di-oestrus to 5.2:2.9:1.6:1 at 02:00h on oestrus.

Conclusion

Since the presence of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 in uterine samples was confirmed by GC-MS analysis and neither PGA-2, PGB-2, PGE-1 nor PGF-1α were detected, it can be concluded that the antibodies used in the RIA probably measured PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 in the samples.

There is an increase in the ability of the uterus to synthesize PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 on pro-oestrus. Peak PG and TXB-2 production occurred at 02:00h on oestrus, this being the time of ovulation and the time of maximum PG and TXB-2 production in the ovary (see Section 2.1). TXB-2 showed the greatest percentage increase between 10:00h on pro-oestrus and 02:00h on oestrus which may suggest that TXA-2 synthesis by the uterus is important at the time of ovulation.

Since the ratios of the production of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 changed during the oestrus cycle, this indicates that their synthesis may be controlled independently of each other.

Section 2.3.b Measurement of i) PG and TX levels in the uterus and ii) the ability of the uterus to synthesize PG and TX in the presence of exogenous arachidonic acid

Introduction

In order that production of PGs and TX by the uterine homogenates can be taken as a reflection of PG and TX synthesis for reasons described in Section 2.1.b, PG and TX levels in, and PG and TX production by uterine homogenates incubated in the presence of arachidonic acid has been measured.

Methods

i) 3 rats were killed at 10:00h on pro-oestrus and their uteri dissected out as described in Section 2.3.a. Tissues were weighed and homogenized in 5 ml ethanol and further processed according to the method given in Section 2.1.b. Samples were stored in 5 ml ethanol at -20°C before being assayed for PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 by RIA as described in sections 1.3a, b, c and d.

ii) The uteri from 4 rats killed at 10:00h on pro-oestrus were dissected out, homogenized and incubated for 90 min in Krebs' solution containing $2\mu g ml^{-1}$ arachidonic acid.

The homogenates were extracted for PGs and TX as described in Section 1.2.a. Samples were stored in 10ml ethyl acetate at -20°C until they were assayed for PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 by RIA using the method described in Sections 1.3a, b, c and d.

Results were compared with PG production at 10:00h on pro-oestrus in the absence of arachidonic acid, by means of Student's t-test.

Results

i) Uterine concentrations of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 were low in comparison to the amounts of PGs and TX formed during incubation of the homogenates, (Table 16).

Table 16. Concentrations of PGs and TX in ethanolic homogenates from rats killed at 10:00h on pro-oestrus and 02:00h on oestrus (mean ± s.e.m.)

PG levels ng 100mg^{-1} / Time	PGE-2	PGF-2α	6-keto-PGF-1α	TXB-2
10:00h pro-oestrus (n=3)	1.4±0.3	1.69±0.1	0.72±0.07	1.02±0.08
02:00h oestrus (n=3)	1.0±0.08	2.32±0.3*	2.66±0.39*	3.25±0.90*

* denotes values significantly different from those at 10:00h on pro-oestrus, ($p < 0.05$).

The major PG present in the uterus at 10:00h on pro-oestrus was PGF-2α whereas at 02:00h on oestrus, 6-keto-PGF-1α was the predominant one. At 02:00h on oestrus, uterine concentrations of PGF-2α, 6-keto-PGF-1α and TXB-2 were significantly increased from their corresponding values at 10:00h on pro-oestrus.

ii) the addition of arachidonic acid had no significant effect on PG or TX production by uterine homogenates from rats killed at 10:00h on pro-oestrus (Fig. 23), although 6-keto-PGF-1α production tended to be higher.

Conclusion

Levels of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 are low in comparison to the amounts produced after incubation of homogenates. Therefore synthesis must have occurred during the homogenization and incubation procedures. As the levels of PGF-2α, 6-keto-PGF-1α and TXB-2 were higher at 02:00h on oestrus than at 10:00h on pro-oestrus, and an increase in synthetic capacity was observed between these times, this suggests that the increased levels are the result of the increase in the ability of the uterus to synthesize PGF-2α,6-keto-PGF-1α and TXB-2 at 02:00h on oestrus. Addition of exogenous arachidonic acid to homogenates of uteri from rats killed at 10:00h on pro-oestrus did not increase the ability of the tissue to synthesize PGs or TX. It can therefore be concluded that the relatively low synthetic capacity of uterine homogenates for PGs and TX seen at 10:00h on pro-oestrus, was not due to a lack of free arachidonic acid in the homogenates.

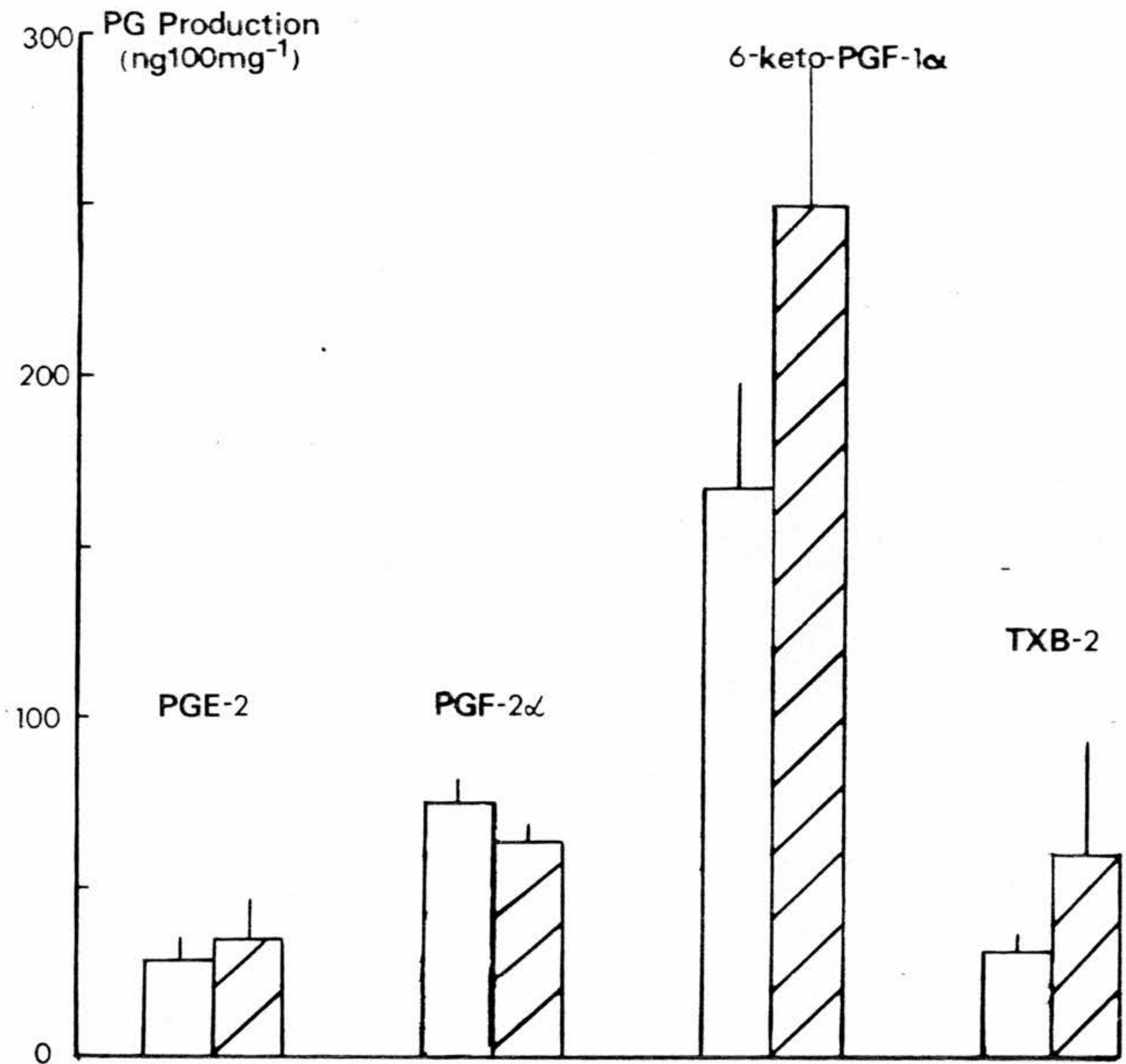


Figure 23

PG and TX production by uterine homogenates incubated in the absence ☐ (n=6) or presence ▨ (n=4) of 2μgml^{-1} arachidonic acid. Rats were killed at 10:00h on pro-oestrus.

This suggests that the increased synthetic capacity between pro-oestrus and oestrus is due to changes in the activity of the PG synthetase enzyme complex.

Section 2.3.c. Measurement of the ability of the uterus to metabolize PGE-2 and PGF-2α

Introduction

Existing studies show that the ability of the uterus of the swine (Anggard, 1971), human (Kierse, Williamson and Turnbull, 1975), guinea pig (Maule, Walker and Poyser, 1978) and rat (Poyser and Scott, 1980) to metabolize PGs is low. However, in the last study, significant changes in the metabolism of PGF-2α into 15-keto-PGF-2α and 13,14-dihydro-15-keto-PGF-2α were observed during the oestrous cycle. Metabolism was significantly higher on pro-oestrus and oestrus than on met-oestrus or di-oestrus. This suggests that PG metabolism may be modulated by ovarian steroid hormones as plasma oestradiol levels are increased on the days of pro-oestrus and oestrus (see Fig. 3). However, it has been reported that oestradiol decreases prostaglandin dehydrogenase activity in kidneys from ovariectomized rats (Blackwell and Flower, 1975).

The following experiments were performed to determine whether changes in PG and TX metabolism by the uterus could account for the increased production which occurred between 10:00h on pro-oestrus and 02:00h on oestrus.

Methods

The uterus was removed from rats killed at 10:00h on pro-oestrus (n=4) and 02:00h on oestrus (n=4). After separating the two uterine horns, 150mg of each horn was homogenized in 10ml Krebs' solution. NAD+ (2mM), and PGF-2α (2μgml^{-1}) were added to one uterine horn homogenate from each rat. All homogenates were incubated in the presence of 0.5 μCi ^{3}H-PGF-2α for 90 min. The extracts were further processed using the method described in Section 2.1.c. This experiment was repeated for the study of uterine PGE-2 metabolism by replacing ^{3}H-PGF-2α with ^{3}H-PGE-2 and adding PGE-2 in place of PGF-2α. The results are expressed as percentage metabolism of PGE-2 or PGF-2α and were compared using Student's t-test.

Results

The uterus metabolized ^{3}H-PGF-2α (Rf=0.14) and ^{3}H-PGE-2 (Rf=0.26) into a mixture of the corresponding 15-keto-metabolites (Rf=0.25 and 0.42 respectively) and 13,14-dihydro-15-keto-metabolites (Rf=0.35 and 0.43 respectively). Table 17 shows the percentage metabolism of PGE-2 and PGF-2α into these metabolites expressed as a single figure.

Table 17. Percentage metabolism (mean ± s.e.m. of 4 observations) of ^{3}H-PGE-2 and ^{3}H-PGF-2α in homogenates of uteri in the absence and presence of NAD+ from rats killed at 10:00h on pro-oestrus and 02:00h on oestrus

TIME	PGE-2 metabolites		PGF-2α metabolites	
	No NAD+	NAD+ present	No NAD+	NAD+ present
10:00h pro-oestrus	6.7±0.2	26.6±10.2	11.8±3.4	11.9±3.6
02:00h oestrus	16.5±3.5*	30.2±5.6	4.7±1.2*	12.8±4.2

In the absence of NAD+ the percentage metabolism of PGE-2 and PGF-2α is relatively low (from 7 to 20%). The presence of NAD+ increased the percentage metabolism to between 12 and 35%. In the absence of NAD+, PGE-2 metabolism was significantly ($p < 0.05$) greater at 02:00h on oestrus than at 10:00h on pro-oestrus. In contrast, PGF-2α metabolism was significantly lower at 02:00h on oestrus than at 10:00h on pro-oestrus.

Conclusion

In the absence of NAD+, metabolism of PGE-2 and PGF-2α in uterine homogenates is relatively low. Therefore the amounts of PGs produced during incubation of homogenates can be taken to reflect the synthetic capacity of the uterus. Although metabolism of PGF-2α did decrease at 02:00h on oestrus by approximately 7%, this is unlikely to account for the 97% increase in uterine PGF-2α production occurring between 10:00h pro-oestrus and 02:00h on oestrus. PGE-2 metabolism increased between these times and, therefore, the synthetic capacity at 02:00h on oestrus may be limited slightly by this change in metabolism.

It is assumed that, as the same enzymes are involved (see Section 2.1.c) that metabolism of PGI-2 and TXA-2 by the uterus is also low.

Section 2.3.d. Measurement of PG and TX production by the endometrium and myometrium at 10:00h on pro-oestrus and at 02:00h on oestrus

The uterus consists of two tissue types. The outer layer, or myometrium, contains smooth muscle cells arranged in distinctly oriented layers, while internally, the endometrium contains predominantly epithelial cells and glandular tissue. Evidence is accumulating which shows that PGs and TX synthesis is unevenly distributed between these two layers. In the pregnant rat, the endometrium is the major site of PGF-2α synthesis (Williams, Sneddon and Harney, 1974).

The same is true of the human endometrium, which also synthesizes PGE-2 and PGD-2 (Abel and Kelly, 1979). However, in the pregnant rat uterus (Williams, Dembinska, Zmuda and Gryglewski, 1978; Downing and Williams, 1980) and human uterus (Abel and Kelly, 1979), PGI-2 synthesis predominates in the myometrium.

Two reports exist which disagree on the main site of uterine TXA-2 synthesis. While Downing and Williams 1980, found that in the pregnant rat uterus only the endometrium converted arachidonic acid into TXB-2, Campos, Liggins and Seamark, (1980) found that in superfusates from uteri of oestrogen-treated ovariectomized rats, the major source of TXB-2 was the myometrium.

Since no information is available on PG and TX production by the endometrium and myometrium of the cycling rat, the following experiment was designed to study this. PG and TX production by these two tissues was studied at times of high (02:00h oestrus) and low (10:00h pro-oestrus) production by whole uterine homogenates.

Method

The uteri from 6 rats killed at 10:00h on pro-oestrus and from 7 rats killed at 02:00h on oestrus were removed and weighed. The endometrium was separated from the myometrium by scraping with a microscope slide (this was initially demonstrated by Dr. I. Downing). The efficiency of removal of endometrium by this method has been tested (Downing, 1979). The myometrium was weighed and thus the weight of the endometrium was calculated by difference.

Following separation, the two layers were homogenized in Krebs' solution (2 ml for the endometrium and 15ml for the myometrium) and the homogenates transferred to 25ml conical flasks. Each homogenate was incubated for 90 min at 37°C as previously described in section 1.2.b. and the PGs and TX extracted according to the method given in section 1.2.a. Samples were stored in 2 ml (endometrium) or 10ml (myometrium) ethyl acetate at -20°C until being assayed for PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 using the method described in Sections 1.3.a, b, c and d. The results were compared by Student's t-test.

Results

Table 18 shows the production of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 on a unit weight basis by endometrial homogenates and myometrial homogenates from rats killed at 10:00h on pro-oestrus and at 02:00h on oestrus. At both times, PGF-2α was the major product of the endometrium whereas 6-keto-PGF-1α predominated in the myometrium. Between 10:00h on pro-oestrus and 02:00h on oestrus, production of PGF-2α, TXB-2 and 6-keto-PGF-1α by the endometrium increased significantly ($p<0.001$) by 480%, 270% and 166% respectively. Although the mean production of PGE-2 had also increased, this was not significant. PGE-2 production by the myometrium showed a significant decrease between 10:00h on pro-oestrus and 02:00h on oestrus ($p<0.02$) while myometrial production of PGF-2α and 6-keto-PGF-1α significantly increased ($p<0.02$). Myometrial TXB-2 production showed no change between the two times.

Table 18. Production of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 (mean ± s.e.m.) by endometrial and myometrial homogenates from rats killed at 10:00h on pro-oestrus and at 02:00h on oestrus

TISSUE	PG PRODUCTION ($ng\ 100mg^{-1}$) TIME	PGE-2	PGF-2α	6-keto-PGF-1α	TXB-2
ENDOMETRIUM	10:00h pro-oestrus n = 6	170+61	504+127	126+27	183+72
	02:00h oestrus n = 7	291+45	2800+749 ***	315+49 ***	650+147 ***
MYOMETRIUM	10:00h pro-oestrus n = 6	18.0+3.1	24.2+5.0	45.0+5.8	16.0+1.9
	02:00h oestrus n = 7	11.1+1.0 **	37.3+3.1 **	78.2+8.9 **	17.3+1.0

Results are significantly different ** $p < 0.02$ and *** $p < 0.001$ from PG or TX production at 10:00h on pro-oestrus

Table 19. Total production of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 (mean ± s.e.m.) by endometrial and myometrial homogenates from rats killed at 10:00h on pro-oestrus and at 02:00h on oestrus

TISSUE	TOTAL PG (ng) TIME	PGE-2	PGF-2α	6-keto-PGF-1α	TXB-2
ENDOMETRIUM	10:00h pro-oestrus n = 6	39.3+8.4	141+24.5	29.7+5.6	43.4+8.4
	02:00h oestrus n = 7	32.4+3.1	316+48.6 ***	36.7+8.0	70.4+8.5 **
MYOMETRIUM	10:00h pro-oestrus n = 6	77.2+11.8	92.8+37.9	185+18.5	56.8+8.1
	02:00h oestrus n = 7	52.4+3.9 *	152+10.8 *	312+38.5 **	74.6+8.4

Results are significantly different * $p < 0.05$, ** $p < 0.02$ and *** $p < 0.001$ from PG or TX production at 10:00h on pro-oestrus

Between 10:00h on pro-oestrus and 02:00h on oestrus ratios of PGF-2α: TXB-2:PGE-2:6-keto-PGF-1α production by the endometrium had changed from 4.5: 1.6 : 1.5 : 1 to 10 : 2.1 : 0.9 : 1. Thus, endometrial PG production is directed towards PGF-2α and TXB-2 at 02:00h on oestrus. At 10:00h on pro-oestrus ratios of PGF-2α: TXB-2: PGE-2 : 6-keto-PGF-1α were 0.5 : 0.3 : 0.4 : : 1 in the myometrium, and by 02:00h on oestrus this had changed to 0.5 : 0.2 : 0.2 : 1.

Since the weights of the endometrium and myometrium vary considerably (1:20) the total PG and TX production by the whole endometrium and the whole myometrium is shown in Table 19. The myometrium produced more 6-keto-PGF-1α and PGE-2 in total than the endometrium. Total PGF-2α production was greatest in the endometrium whereas total TXB-2 production was equally distributed in the two tissues. Between 10:00h on pro-oestrus and 02:00h on oestrus, there were significant increases in total PGF-2α ($p<0.001$) and TXB-2 ($p<0.02$) production by the endometrium. In the myometrium, total PGE-2 and PGF-2α ($p<0.05$) and 6-keto-PGF-1α ($p<0.02$) production was significantly increased at 02:00h on oestrus.

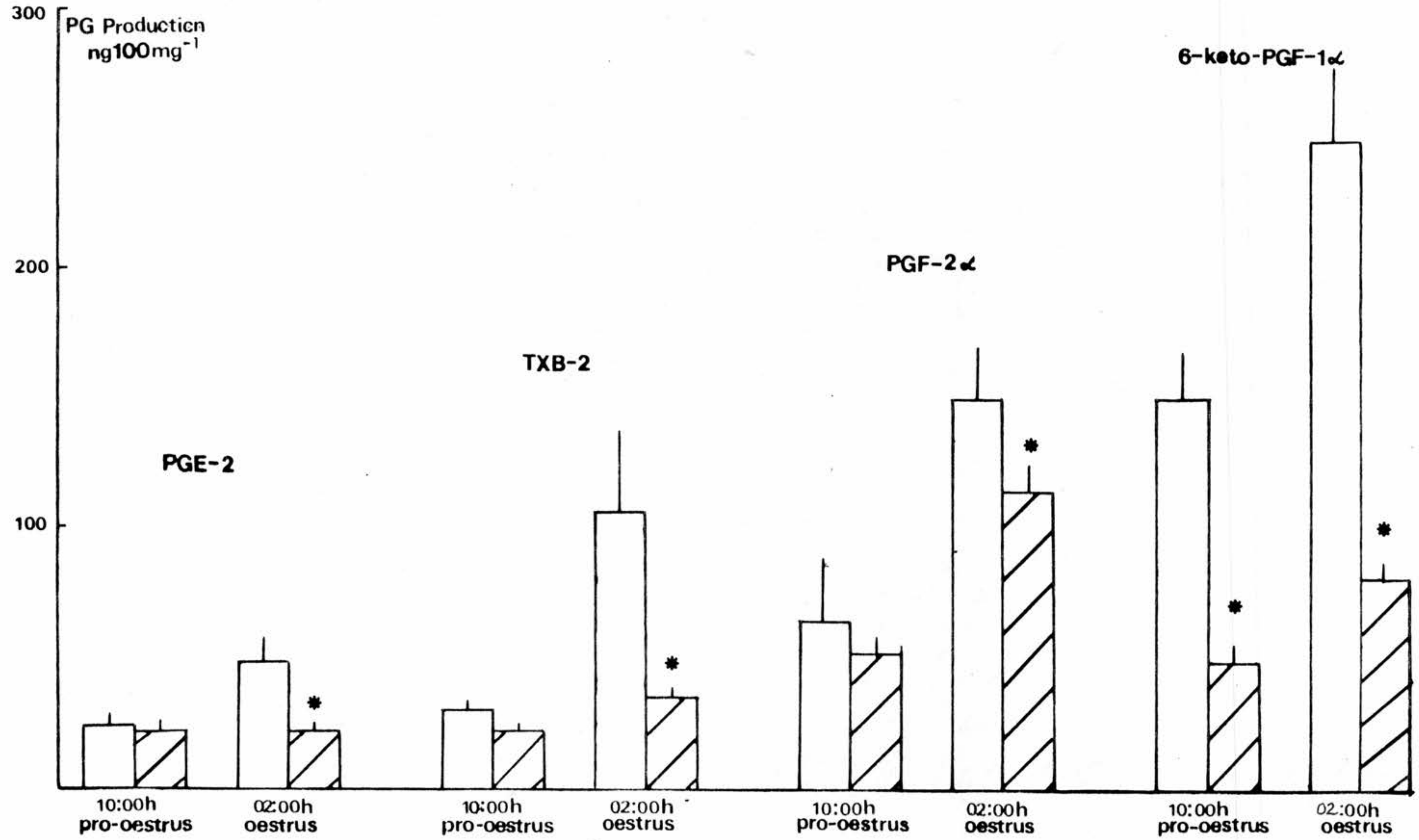
It was decided to examine whether co-incubation of the homogenized endometrium and myometrium, as opposed to incubating homogenates of these tissues separately, affected PG and TX production. The total production of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 produced by the endometrium and myometrium incubated separately has been divided by uterine weight and these values have been compared with the respective PG and TX production by whole uterine homogenates at 10:00h on pro-oestrus and at 02:00h on oestrus as determined in Section 2.3.a.

These results are shown in Fig. 24. Production of PGE-2, PGF-2α and TXB-2 was significantly greater ($p<0.02$, $p<0.001$, $p<0.05$ respectively) by homogenates of whole uterus than the total production by the endometrium and myometrium incubated separately at 02:00h on oestrus, but not at 10:00h on pro-oestrus. 6-keto-PGF-1α production by homogenates of whole uterus was significantly greater ($p<0.001$) than the total 6-keto-PGF-1α by the endometrium and myometrium incubated separately at both 10:00h on pro-oestrus and 02:00h on oestrus.

Conclusion

The ability of the uterus to synthesize PGE-2, PGF-2α, TXB-2 and 6-keto-PGF-1α, expressed on a unit weight basis, resides chiefly in the endometrium. However, because of the vastly differing weights of the endometrium and myometrium, expressing PG and TX production on a unit weight basis affects the pattern of results obtained. As the myometrium constitutes the greater part of the uterine mass, it can be concluded that this is the major site of 6-keto-PGF-1α production as this tissue produced greater amounts of 6-keto-PGF-1α in total than the endometrium. In contrast, the endometrium is the major site of uterine PGF-2α production as this tissue produced more PGF-2α in total than the myometrium.

It was noted in Section 2.3.a. that the ability of the uterus to synthesize PGs and TX increased between 10:00h on pro-oestrus and 02:00h on oestrus.



300
200
100
PG Production
ng100mg⁻¹
PGE-2
TXB-2
PGF-2α
6-keto-PGF-1α
10:00h pro-oestrus
02:00h oestrus
10:00h pro-oestrus
02:00h oestrus
10:00h pro-oestrus
02:00h oestrus
10:00h pro-oestrus
02:00h oestrus

In the case of PGE-2 and TXB-2 this increase is mainly due to increased endometrial synthesis as myometrial TXB-2 production did not change between these times, and myometrial PGE-2 production actually decreased. However, the increase in uterine PGF-2α and 6-keto-PGF-1α production is due to increases in both endometrial and myometrial production although PGF-2α production in the endometrium showed a far greater percentage increase between these two times.

There may be an interaction between the two uterine layers, as evidenced by a greater PG or TX production by whole uterine homogenates when compared to the sum of PG or TX production by the endometrium and myometrium incubated separately. Indeed these results suggest that the endometrium may provide enzymes or substrate for myometrial PG and TX synthesis or vice versa.

Section 2.3.e. The effect of oestrogen and progesterone on PG and TX production by the uterus

Introduction

The effects of ovarian steroids on uterine PG production have been reviewed (see Introduction). There is, however, considerable variation in experimental results. Oestradiol increased uterine PG production and PG release into the uterine vein (Ham et al., 1975; Castracane and Jordan, 1975). However, in the former study, PGF synthesis increased at the expense of PGE synthesis while the latter study showed a parallel increase in PGF and PGE in uterine venous plasma.

In contrast, other workers report that oestradiol decreased PGE-2 production while having no effect on PGF-2α production in ovariectomized rats (Thaler-Dao, Ramonaxto, Saintot, Chaintreuil and Crastes de Paulet, 1982b).

The influence of progesterone on uterine PG synthesis is also inconclusive. Castracane and Jordan (1975), reported that progesterone enhances the effect of oestradiol on uterine PGF and PGE output whereas Ham et al., (1975) found that progesterone had no effect on uterine PGF levels and actually depressed PGE levels.

An inhibitory effect of oestradiol on PGI-2 release (Gimeno, Borda, Lazzari and Gimeno, 1980) and 6-keto-PGF-1α production (Thaler-Dao et al., 1982b) by the rat uterus has been reported.

The variation in these experimental results can be ascribed to the different timing and dosage of steroids administered to the animals. Often these experiments did not mimic the *in vivo* situation (see General Introduction). The experiments described in this section have examined the effects of progesterone acting upon an oestrogen-primed uterus, on the production of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 in the ovariectomized rat.

Methods

Twelve rats were ovariectomized between 09:00h and 10:00h on di-oestrus (Group I), under Althesin anaesthesia (5mlkg^{-1}). At 12:00h on that day, six animals received 10ug oestradiol benzoate in 0.5ml arachis oil (s.c.). The other six animals were injected with 0.5 ml arachis oil.

On the following day (the expected day of pro-oestrus) the animals were injected s.c. with 2 mg progesterone in 0.5 ml arachis oil at 12:00h.

Twenty-two rats (Group II) which had been ovariectomized under Althesin anaesthesia 6 weeks previously, were divided into two groups. Eleven of the rats received an s.c. injection of 10μg oestradiol benzoate in 0.5 ml arachis oil at 12:00h followed by 2 mg progesterone s.c. in 0.5 ml arachis oil 72h later. The remaining eleven rats received s.c. injections of arachis oil alone at the above times.

The animals from both treatment groups were killed five hours after the progesterone injection and their uteri removed, weighed, homogenized, incubated and extracted for PGs and TX as described in Section 2.3.a. The results are expressed per unit wet weight of tissue. In addition protein determinations (using the method of Lowry et al., 1956) were made on 11 uterine homogenates from each of control and treated rats. In this way, PG production expressed per mg wet weight could be compared with that expressed per mg protein.

Results

Treatment with oestrogen and progesterone increased uterine protein content from 22.5 $\pm$ 2.9 mg (control) to 32.9 $\pm$ 5.9 mg. Table 20 shows that treatment of long-term ovariectomized rats with oestrogen and progesterone significantly ($p < 0.05$) increased PGF-2α production by the uterus, expressed on a unit weight basis or per mg protein.

The percentage increase in PGF-2α production calculated per mg wet weight was greater than when expressed as per mg protein, but the difference was not great.

Table 20. A comparison of PGF-2α production by uteri of oestrogen and progesterone treated rats with those of untreated rats when expressed on a unit weight basis or per mg of protein (mean ± s.e.m.)

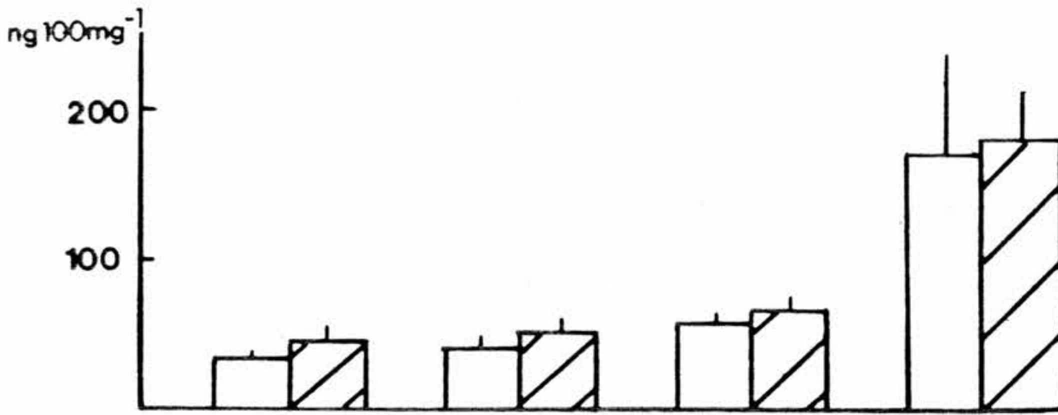
ng PGF-2α production	Control n = 11	Treated n = 11	% Increase
per mg wet weight	5.9±0.07	10.6±1.1*	79
per mg protein	65±7.4	110±8.2*	69

* significantly different from control $p < 0.05$.

Treatment of acute ovariectomized rats with oestrogen and progesterone had no significant effect on uterine production of PGE-2, PGF-2α, 6-keto-PGF-1α or TXB-2 on a unit weight basis (Fig. 25a). Production of PGF-2α and TXB-2 was significantly ($p < 0.05$) increased by treatment of long-term ovariectomized rats with oestrogen and progesterone whereas PGE-2 and 6-keto-PGF-1α production did not change (Fig. 25b).

GROUP I

a) ACUTE OVARIECTOMY



GROUP II

b) LONG-TERM OVARIECTOMY

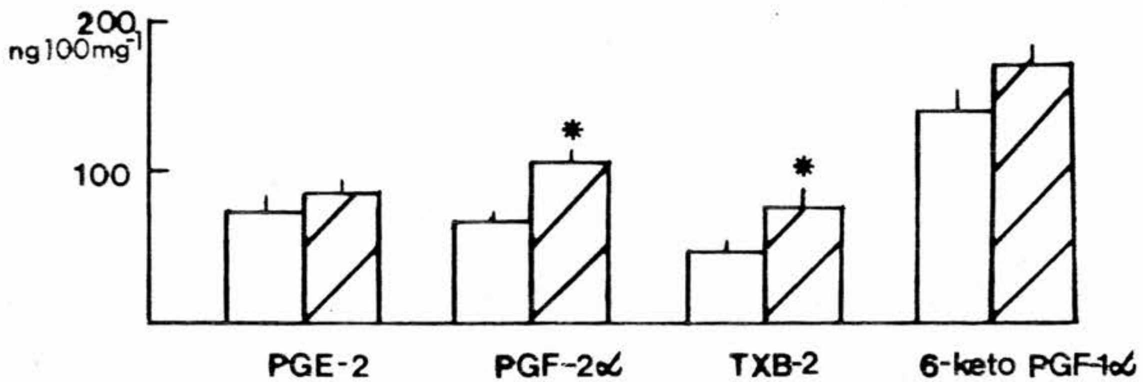


Figure 25

Effect of oestradiol and progesterone treatment of Group I) acute ovariectomized rats (n=6) and Group II) long-term ovariectomized rats (n=11) on PG and TX production by uterine homogenates expressed as ng100mg^{-1} tissue. Values are shown as mean ± s.e.m.

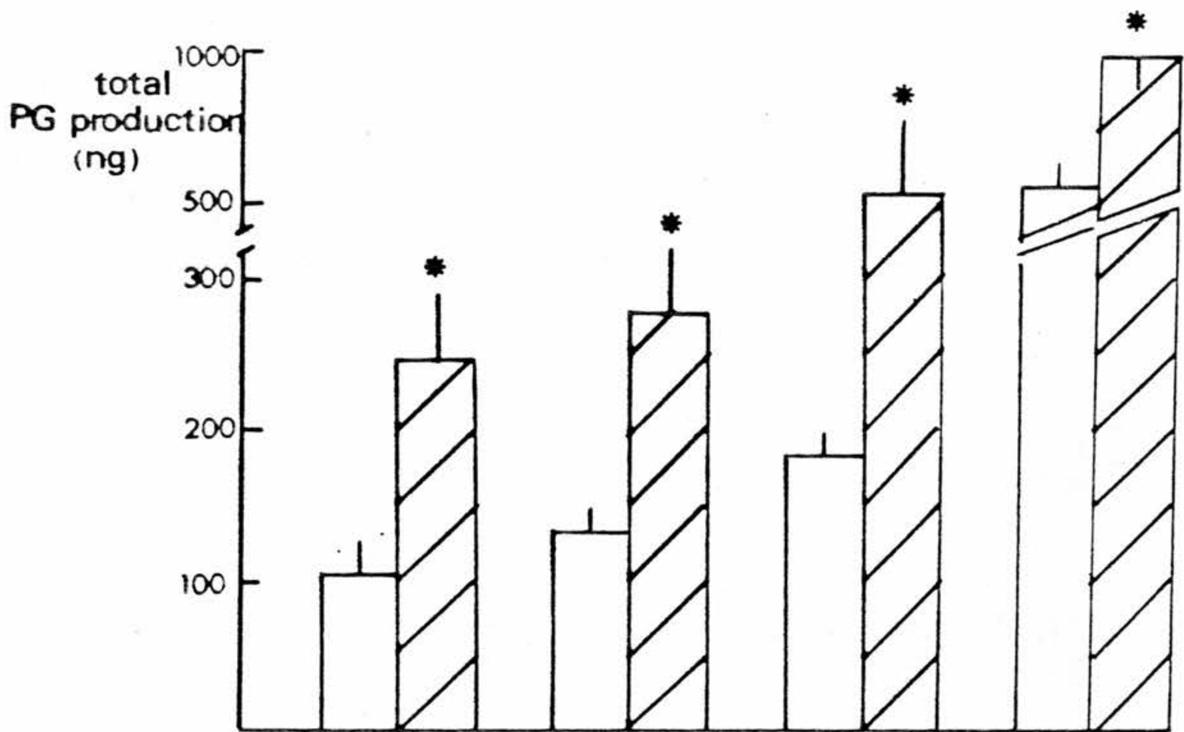
* denotes values significantly different from control ($p < 0.05$)

☐ control rats

▨ oestradiol-, and progesterone-treated rats.

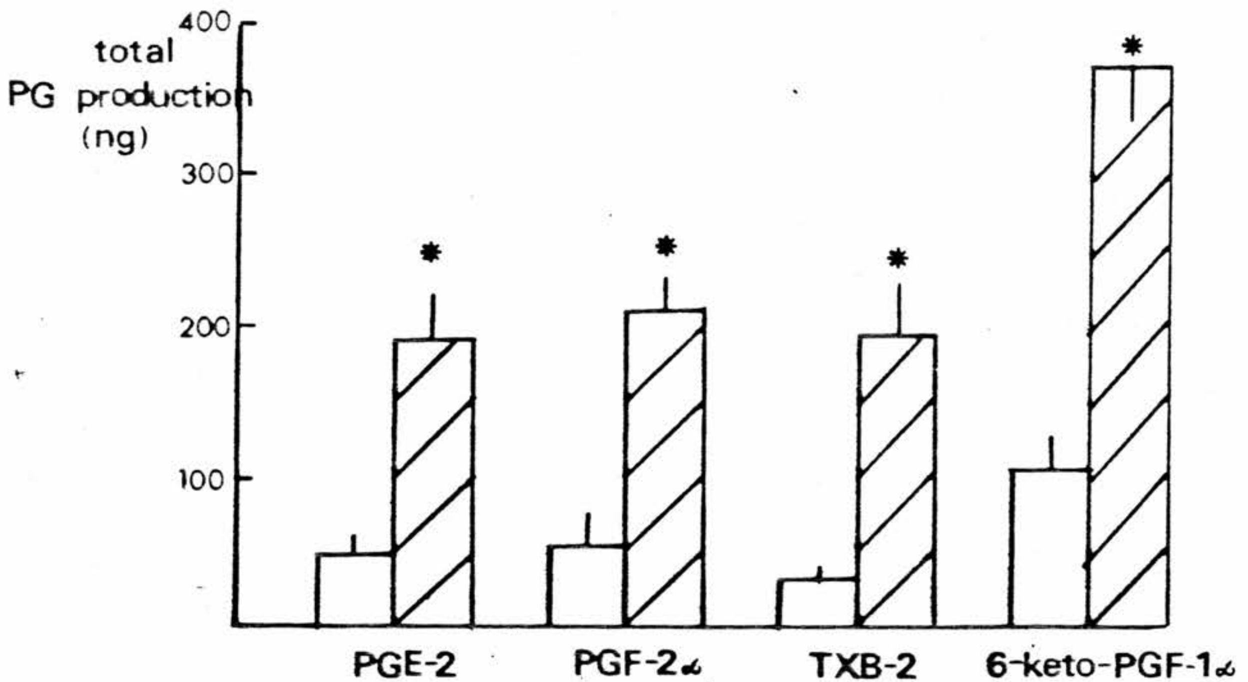
GROUP I

a) ACUTE OVARIECTOMY



GROUP II

b) LONG-TERM OVARIECTOMY



When total PGE-2, PGF-2α, 6-keto-PGF-1α or TXB-2 production by each uterus is considered, the treatment of both acute, and long-term ovariectomized rats with oestrogen and progesterone significantly ($p < 0.001$) increased production of all PGs and TXB-2 (Fig. 26a and b).

Conclusion

There is little difference between expressing uterine PGF-2α production on a unit weight basis or per mg protein with respect to the percentage increase in PGF-2α production following oestrogen and progesterone treatment of ovariectomized rats. Thus it would have been of no advantage to express the results according to uterine protein content. Due to the increase in uterine weight following oestrogen and progesterone treatment, PG and TXB-2 production expressed on a unit weight basis did not change (with the exception of PGF-2α and TXB-2 production in uteri of long-term ovariectomized rats) in contrast to total uterine production which was significantly increased by treatment.

The difference between the effects of oestrogen and progesterone treatment of acute and long-term ovariectomized rats may be due to the fact that in the former case, oestrogen was allowed to act for 24h before progesterone injection, whereas in the latter 72h, separated the oestrogen and progesterone injections. The experiment described in section 2.3.a. showed that between 10:00h on pro-oestrus and 02:00h on oestrus, PGF-2α and TXB-2 production increased by 100% and 200% respectively whereas 6-keto-PGF-1α and PGE-2 production showed much smaller percentage increases.

A similar response to oestrogen and progesterone treatment of long-term ovariectomized rats was observed, with the effect on uterine PGF-2α and TXB-2 production being much greater than on 6-keto-PGF-1α or PGE-2 production. Thus the sequential action of oestrogen and progesterone may be the stimulus for the increased ability of the uterus to synthesize PGs and TX which occurs towards the time of ovulation.

The effects of oestrogen and progesterone could be due to decreased PG and TX metabolism in the homogenates. However, as uterine metabolism of PGE-2 and PGF-2α (and presumably TXA-2 and PGI-2) was between 5 to 15%, even complete inhibition of this factor could not result in the increase in PGF-2α and TXB-2 production after treatment with oestrogen and progesterone.

Discussion

The endogenous concentrations of PGs and TX in the uterus, are low, and metabolism of PGE-2 and PGF-2α in the absence of NAD+ is also low. The addition of arachidonic acid to homogenates of uteri from cycling rats does not alter PG or TX production. Therefore the amounts of PGs and TX produced by uterine homogenates incubated in vitro reflect the relative abilities of the components of the PG synthetase complex to convert free endogenous arachidonic acid released during the homogenization and incubation, into PGs and TX.

Uterine production of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 increased on pro-oestrus and reached a peak at 02:00h on oestrus. The timing of the peak uterine PG and TX production coincides with ovulation. Other workers also report an increase in uterine PG production but there is disagreement on the timing of peak uterine PG levels and production. A pro-oestrous peak in uterine PGE-2 and PGF-2α levels has been reported by Ham et al., (1975), by Van Orden et al., (1980) and by Thaler-Dao et al., (1982), while Ishikawa and Fuchs (1978) and Poyser and Scott (1980) report highest PG levels and PG production on oestrus. These discrepancies can be attributed to the frequency of sampling employed in each study. The experiments described in this section and in section 2.1.a. show that the ability of the uterus and ovary to synthesize PGs and TX can change markedly in a 4-hour period and that peak production occurs around 02:00h in both tissues and this present study demonstrates the need for carrying out frequent measurements of PG levels or production by tissues.

An additional peak in PGE-2 production by the uterus was found on di-oestrus by Thaler-Dao et al., (1982). This finding was not confirmed in this study. They also did not find any significant differences in PGI-2 production during the oestrous cycle but a rather insensitive radiochemical assay was used by them to measure PGI-2 production.

There are no reports on uterine TXA-2 synthesis during the oestrous cycle although the uterus of the pregnant rat at term has been shown to release significant quantities of TXB-2 (Phillips and Poyser, 1981a, Dubin et al., 1982).

In the present study, the origin of uterine TXB-2 was not determined. It is possible that platelets trapped within the uterus is the source of TXB-2 and that the increased production reflects an increased blood volume at oestrus. It would be interesting therefore to see if the uterus retained the ability to synthesize TXB-2 after perfusion of its blood vessels with saline to remove any blood elements.

Evidence against the possibility that the platelets may be the source of uterine TXA-2 comes from the fact that total TXB-2 production by the endometrium and myometrium were similar. If the platelets were the only source of uterine TXA-2 it would be expected that the myometrium, due to its greater size, and therefore greater blood volume, would produce more TXA-2 but this was not found to be the case. Since TXB-2 production showed the greatest percentage increase between 10:00h on pro-oestrus and 02:00h on oestrus, TXA-2 synthesis by the uterus at the time of ovulation may be of physiological importance. In this regard, recent reports show that TXA-2 is highly potent in causing contractions of the non-pregnant human uterus in vitro (Wilhelmsson, Wikland and Wiqvist, 1981) and of the pregnant rat uterus (Dubin et al., 1982). While little is known of the uterine motility at the time of ovulation in the rat, increased uterine TXA-2 synthesis may increase uterine motility at that time. Sperm transport into the rat oviduct is most rapid around the time of ovulation (Shanghi and Kracier, 1978).

Moreover, gap junction formation, which is thought to be essential for synchronous uterine contractions (Garfield, Sims, Kannan and Daniel, 1978), is inhibited by indomethacin and restored by TXA-2 mimic 11,9-epoxymethano-PGH-2 but not by PGE-1, PGE-2, PGF-2α or TXB-2 (Garfield, Kannan and Daniel, 1980). The effect of a specific TXA-2 synthesis inhibitor or TXA-2 receptor antagonist on the uterine motility and the fertility of rats requires investigation.

In agreement with the present study, Downing and Williams (1980) report that the endometrium was the major source of TXB-2 in the uterus of the pregnant rat. However, Campos et al., 1981 find that the myometrium releases more TXB-2. Certain differences in experimental design account for these divergent results. In the latter study, the endometrium and myometrium were perifused by a method which enabled their anatomical relationship to be left intact. The authors claimed that their method gave a true indication of endometrial and myometrial PG and TX production by finding similar results when perfusing each of these tissues separately. However, their dissection of the uterus yielded two tissues of approximately equal weight. Hence, their calculation of endometrial weights must have been over-estimated considerably, since in the present study the endometrium accounted for only about 5% of the uterine weight. Also, Campos et al., (1980) used ovariectomized rats which had been given rather high doses (1 mg per day for 3 days) of oestradiol and this would also contribute further to the differences in results.

Previous reports on PGE-2, PGF-2α and 6-keto-PGF-1α production by the endometrium and myometrium agree with the results presented here, which show that the endometrium is the main source of uterine PGF-2α and that uterine 6-keto-PGF-1α production predominates in the myometrium (Williams, Sneddon and Harney, 1974, Williams et al., 1978; Abel and Kelly, 1979). The relatively large increases in uterine PGF-2α and TXB-2 production seen at the time of ovulation can be attributed to the relatively large increase in endometrial synthesis of these two compounds (480% and 270% respectively).

An important finding was that the separated endometrial and myometrial homogenates synthesized less PGs and TX than when homogenized together. This finding agrees with the results of Abel and Kelly, (1979) who found that the human endometrium was rich in cyclooxygenase and enzymes which convert PGH-2 into PGs and TX, but that the myometrium contained little cyclooxygenase. Thus, when the tissues are homogenized together, the endometrium provides cyclooxygenase for conversion of archidonic acid into PGH-2, and then the PGH-2 is acted upon by enzymes from both the endometrium and myometrium to produce the various PGs and TX. Although the authors extrapolated this to the in vivo situation by suggesting that the endometrium provides endoperoxides as substrate for the myometrium, this is unlikely to occur in vivo due to the instability of PGH-2.

Correlations of uterine PG and TX synthetase as determined here with plasma concentrations of ovarian steroids, show that peak PG and TX production occurs when plasma oestradiol and progesterone levels are maximal (Shaikh and Shaikh, 1975; Thaler-Dao et al., 1982).

Furthermore, oestradiol and progesterone treatment of long-term ovariectomized rats increased PGF-2α and TXB-2 production significantly. This suggests that the increased PGF-2α and TXB-2 production on pro-oestrus and oestrus occurs in response to oestrogen and progesterone. In contrast to the results of Ham et al., (1975) who found that oestradiol treatment increased uterine PGF-2α production at the expense of PGE, the results presented here show that the synthesis of one arachidonic acid metabolite did not occur at the expense of any other. However, Ham et al., (1975) used differing experimental conditions from those used here. They added exogenous arachidonic acid and the cofactors hydroqinone and reduced glutathione which promote PGE synthesis, whereas in this study neither arachidonic acid nor cofactors were added.

Unfortunately, time did not permit the study of either oestrogen and progesterone treatment alone. It is possible that oestradiol given alone is sufficient to increase uterine PG synthesis as Kogo, Yamada and Aizawa (1977) found this to be the case. However, uterine PG and TX production increased little between 10:00h on di-oestrus and 10:00h on pro-oestrus, when oestradiol levels are already high. Rather, they increased from the afternoon of pro-oestrus when the ovulatory surge of progesterone occurs (see Fig. 3). Other studies support the view that progesterone is necessary for maximum uterine PGF-2α production in the sheep (Barcikowski, Carlson, Wilson and McCracken, 1974; and in the guinea pig (Blatchley and Poyser, 1974).

Since oestrogen and progesterone increase the ability of the uterus to synthesize PGs and TX this is presumably due to increasing the amount of PG synthetase present in the uterus. However, Castracane and Jordan (1976) found that the increase in uterine PGF and PGE synthesis is unaffected by administration of an oestrogen antagonist or protein synthesis inhibitors. Thus, the action of the ovarian steroids on uterine PG and TX synthesis may be indirect. For example, the effect may be due to a catechol oestrogen (Kelly and Abel, 1981) or to catecholamines (Van Orden et al., 1980) which may act as co-factors during PG synthesis. A role for LH cannot be ruled out, as oestrogen and progesterone treatment causes LH release from the pituitary gland. Peak PG and TX production in the uterus coincided with peak ovarian PG production which is due to the action of LH on PG synthetase (see General Introduction). However, no studies concerning the effect of LH on uterine PG synthesis have been performed.

It was noticed that uterine PG and TX ratios changed during the oestrous cycle which may suggest that their synthesis is controlled independently. Therefore the synthesis of PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 by the endometrium and myometrium from PGH-2 requires investigation.

In conclusion, an increase in uterine PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 production occurs on pro-oestrus and reaches a peak at the time of ovulation. This increase occurs in the endometrium in the case of PGE-2, PGF-2α and TXB-2 and in the myometrium for 6-keto-PGF-1α. While oestrogen and progesterone treatment of ovariectomized rats was found to mimic these changes to some extent, their exact mechanism of action is unknown at present.

It is speculated that the peak in uterine PG and TX production at 02:00h on oestrus may be associated with changes in uterine motility at that time.

Section 2.4. THE EFFECT OF AGEING ON UTERINE PG AND TX SYNTHESIS

Introduction

The failure of the uterus to support pregnancy may be one of the factors to contribute to the decline in reproductive capacity which accompanies ageing. In support of this suggestion it has been demonstrated that transplantation of morulae and blastocysts from young animals into aged recipients resulted in their resorption, whereas a higher proportion survived and implanted when transplanted into young recipients (Blaha, 1964; Talbert and Krohn, 1966).

Since these initial studies, the impaired decidualization of uterine stromal cells in response to artificial stimuli has been documented in the aged mouse (Finn, 1966; Shapiro and Talbert, 1974), rat (Maibenco and Krehbiel, 1973) and hamster (Blaha, 1967). Differences in circulating levels of steroid hormones cannot account for these effects of ageing as these studies were generally carried out on ovariectomized, steroid-primed animals.

Decidualization (which can be brought about experimentally by chemical or mechanical irritation of the endometrium) can only be obtained when the uterus has previously been primed by oestrogen and progesterone (O'Grady and Bell, 1977). According to measurements of circulating progesterone in the ageing pregnant rat (Miller and Riegle, 1978), the hormonal milieu in these animals is adequate to support normal decidual development. It is possible then, that a reduced response to the hormonal stimulus occurs in the aged animal.

Uterine decidualization is preceded by and possibly dependent on, increased capillary permeability (Psychoyos, 1973) which has also been shown to be reduced in ageing pregnant hamsters (Parkening and Soderwall, 1973). Since there is evidence (see Introduction) that PGs are implicated in the control of endometrial vascular changes and in the decidual cell response, defective production of these compounds may explain the reduced decidual cell response in ageing uteri.

The experiments to be described in this Section were designed to study PG and TX production by the uterus of young and aged ovariectomized rats in response to treatment with oestrogen and progesterone, using a regimen designed to mimic pre-implantation endometrial development. Aged animals were used at 13-14 months. At this time a loss of fertility is evident but longevity exceeds 24 months on average. Three parameters of the response have been measured, i.e. PG and TX synthetic capacity in incubated uterine homogenates, PG and TX levels in ethanolic uterine homogenates and PG release from the superfused uterus.

Methods

Preparation and treatment of animals

Virgin Sprague-Dawley rats were housed under controlled lighting conditions (Lights on 05:00h, Lights off 19:00h) and allowed free access to food and water. Young rats were used at 4-5 months and aged rats at 13-14 months. They weighed 230-310g and 270-350g respectively.

Rats were bilaterally ovariectomized under ether anaesthesia, this being used to induce anaesthesia during all surgical procedures. The day of ovariectomy was designated as day 1 of treatment. On day 8 a silastic tubing implant (Dow & Corning, inner diameter 0.1550 cm, outer diameter 0.3125 cm, length 2 cm) either containing crystalline oestradiol-17β (treated rats) or empty (control rats) was inserted subcutaneously between the scapulae. All implants were preincubated overnight at 37°C in phosphate-buffered saline (pH 7.1) and after being filled with steroid, were sealed with silicone adhesive type A (Dow & Corning). On day 11, the oestradiol implants were removed and replaced by implants containing crystalline progesterone. A replacement empty implant was exchanged for the empty implant in control animals. At this stage, a blood sample was withdrawn by cardiac puncture from 4 animals from each of the groups, for estimation of plasma oestradiol by RIA. (The RIA for oestradiol was performed by Dr. H.M. Fraser, MRC Reproductive Biology Unit, Edinburgh). On day 14, animals in the treatment group received a subcutaneous injection of oestradiol-17β(0.5μgkg^{-1}) in a vehicle composed of benzyl benzoate and arachis oil (1:9, v:v) at 12:00h, while control animals received vehicle alone. Animals were killed on day 15, 21-23h after the oestradiol injection, and blood was withdrawn by cardiac puncture for estimation of plasma progesterone levels by RIA. (The RIA for progesterone was also performed as above).

Analysis of plasma oestrogen and progesterone levels showed them to be in the physiological range for early pregnancy (Oestradiol, 50-100 pgml^{-1} ; progesterone, 15-60ngml^{-1}). Preparation of animals in this way was carried out by Dr. R.G. Gosden of the Department of Physiology, University of Edinburgh.

Rats which had been sensitized in this way were then assigned to one of the experiments described in the following sub-sections. However, some animals provided tissue for use in more than one experiment.

Presentation of results

In each set of experiments uterine PG measurements are presented both as total per uterus, and also on a unit protein basis. The two ways of expressing the data are employed as part of the increase in uterine protein content in the aged animal can be ascribed to the accumulation of collagen in the uterus of the aged rat (Schaub, 1964). Since collagen is an extracellular protein, and PG synthetase is intracellular (Smith, Rollins and Dewitt, 1981), expressing the data on a unit protein basis alone may be misleading.

Section 2.4.a. Measurement of the effect of treatment on the capacity of the uterus of young and aged rats to produce PGs and TX

Method

A total of 20 young and 20 aged rats were used. From each group, 10 animals were treated with oestrogen and progesterone as described previously, while 10 animals served as controls. On day 15 of treatment the animals were killed and each uterine horn was dissected out and homogenized in 5 ml Krebs' solution.

A sample of the homogenate (100μl) was taken for protein estimation using the method of Lowry et al., (1956) and then each homogenate was added to a flask. Flasks were incubated as described in Section 2.1.a and extracted for PGs and TX as described in Section 1.3.a. Samples were stored at -20°C in ethyl acetate, until they were assayed for PGE-2, PGF-2α, 6-keto-PGF-1α and TXB-2 content by RIA using the methods described in Sections 1.3a, b, c and d.

The results have been expressed as mean ± s.e.m. and were examined by two-way analysis of variance (Anovar) and Student's t test, to provide information about the effects of treatment, age and their interaction.

Results

The major PG produced by young and aged animals was 6-keto-PGF-1α with lesser quantities of (in descending order) PGF-2α, PGE-2 and TXB-2. Uterine protein content (Table 21, Panel A) was significantly increased ($p < 0.001$) with age and by treatment with oestrogen and progesterone ($p < 0.05$).

On a unit protein basis (Table 21, Panel A) production of PGs and TXB-2 was significantly lower in ageing than in young uteri and was increased by steroid treatment, the sole exception was 6-keto-PGF-1α which just failed to reach an acceptable level of statistical significance ($p=0.05$). There was no effect of age or treatment on the proportions of PGs or TX produced with respect to one another.

Table 21 : Effects of age and steroid treatment on prostaglandin (PG) and thromboxane (Tx) production by homogenized rat uteri. Results are expressed per unit protein weight (panel A) and per uterus (panel B).

Age group	Treatment group	No. of animals	PGE_2	$PGF_{2\alpha}$	6-Keto-$PGF_{1\alpha}$	TxB_2	Total uterine protein (mg)
			Production: ng.mg protein^{-1} A				
Young	Control	10	4.6 ± 0.4	6.8 ± 1.1	14.0 ± 2.3	1.9 ± 0.3	24.1 ± 3.0
	Treated	10	6.7 ± 0.6	11.1 ± 0.9	20.4 ± 3.0	3.7 ± 0.2	31.7 ± 5.6
Middle-aged	Control	10	2.8 ± 0.2	5.3 ± 0.4	10.1 ± 1.5	1.7 ± 0.3	44.3 ± 4.6
	Treated	10	3.6 ± 0.3	5.5 ± 0.7	12.4 ± 2.0	2.0 ± 0.3	51.5 ± 2.5
			Production: ng.uterus^{-1} B				
Young	Control	10	104.6 ± 11.8	144.8 ± 20.9	310.3 ± 51.5	48.6 ± 12.0	
	Treated	10	208.0 ± 18.6	350.9 ± 36.9	659.0 ± 110.8	114.1 ± 6.1	
Middle-aged	Control	10	119.9 ± 9.9	236.7 ± 35.0	479.2 ± 112.3	78.3 ± 17.5	
	Treated	10	185.7 ± 18.5	279.2 ± 44.3	642.6 ± 120.2	99.8 ± 14.6	

Panel A - Significant effects of age and treatment for PGE_2 ($p<0.01$), $PGF_{2\alpha}$ ($p<0.01$), TxB_2 ($p<0.001$). Production of 6-keto-$PGF_{1\alpha}$ was reduced with age ($p<0.025$) but unaffected by treatment. Uterine protein was more abundant in aged uteri ($p<0.001$) and after steroid treatment ($p<0.05$)

Panel B - Significant effects of treatment on production of PGE_2 ($p<0.001$), $PGF_{2\alpha}$ ($p<0.01$), 6-Keto-$PGF_{1\alpha}$ ($p<0.025$) and TxB_2 ($p<0.01$).

Interactive effects of age and treatment revealed by Anovar were significant for PGF-2α ($p < 0.025$) and TXB-2 ($p < 0.01$) and was also significant when the data for all the PGs and TXB-2 were nested and transformed to logarithms ($p < 0.025$). Although steroid treatment significantly increased the production of these compounds in young animals ($p < 0.01$ by Student's t test) there was no such effect in the ageing groups.

In contrast to these results, production of PGs and TXB-2 expressed per uterus was unaffected by ageing (Table 21, Panel B) although there was a significant stimulation of production following steroid treatment ($p < 0.025$) and a significant interactive effect of age and treatment for PGE-2 ($p < 0.05$).

Conclusion

On a unit protein basis, PG production was diminished in the uteri of aged animals, but when expressed as total production per uterus, it was unaffected by age. The two ways of expressing the data were employed as there is evidence that a proportion of the increased uterine protein in aged animals is extracellular and presumably therefore is not associated with PG synthetase which is an intracellular protein. Accordingly, when expressed per mg of protein, the reduced PG and TX production in the aged animal may reflect a 'dilution' due to increased collagen. However, the significant interactive effect of age and treatment implies that the responsiveness of the aged uteri to treatment was diminished and this conclusion is not subject to the same limitations in interpretation as above.

Furthermore, a parallel increase in uterine protein content in response to treatment occurred in young and aged animals and yet PG and TX production increased only in young animals.

Section 2.4.b. Measurement of the effect of treatment on uterine PG levels in young and aged rats

Method

A total of 7 rats in each of the groups were used in this experiment. Rats were killed and their uteri dissected as before. The two uterine horns were divided and only one horn used in this experiment. Each uterine horn was homogenized in 5 ml ethanol and 100μl taken for estmation of protein content as before. After transferring the homogenate to a centrifuge tube, the homogenizer was washed twice with two 5 ml volumes of ethanol and the washings added to the centrifuge tube. The homogenate was centrifuged at 1000 x g for 5 min. Following centrifugation, the supernatant was transferred to a flask and evaporated to dryness under reduced pressure at 45°C. The residue was resuspended in 10ml Krebs' solution and the extraction of PGs proceeded as described in Section 2.1.a. The samples were stored at -20°C in ethyl acetate until they were assayed for PGE-2 and PGF-2α content by RIA using the method described in Sections 1.3a and b.

Results

Uterine PG levels were an order of magnitude lower than in the incubated homogenates above. The levels of PGE-2 were slightly higher than those of PGF-2α and, when expressed per mg protein, were found to be significantly greater in young compared to ageing uteri (Table 22, Panel A, $p < 0.001$). The results for PGF-2α were not significant but indicated a similar trend to that of PGE-2. As in Section 2.4.a, the effect of age was lost when results were expressed as total amounts per uterus (Table 22, Panel B). Steroid treatment did not affect the levels of PGs in either young or ageing animals.

Conclusion

Since uterine levels of PGE-2 and PGF-2α were an order of magnitude lower than PG production as determined in Section 2.4.a, it is likely that PG production by the incubated uterine homogenates reflects fresh synthesis. The reduced levels of PGE-2 in ageing uteri parallels the reduced PGE-2 production in ageing animals. In contrast, however, reduced PGF-2α production in aged animals was not accompanied by reduced PGF-2α levels in aged uteri.

Although treatment with oestrogen and progesterone increased synthetic capacity for PGE-2 and PGF-2α in young animals (Section 2.4.a) PG levels were unaffected by this treatment.

Table22 : Effects of age and steroid treatment on (1) levels and (2) release of prostaglandins (PG) in rat uteri. Results are expressed per unit protein weight (panel A) and per uterus (panel B).

Age group	Treatment group	No. of animals	PGE_2 levels	$PGF_{2\alpha}$ levels	No. of animals	PGE_2 release 20 min	PGE_2 release 40 min	$PGF_{2\alpha}$ release 20 min	$PGF_{2\alpha}$ release 40 min
			ng.mg protein^{-1} A			ng.mg protein^{-1} A			
Young	Control	7	0.459± 0.070	0.270± 0.051	4	0.433± 0.151	0.422± 0.134	0.212± 0.045	0.139± 0.046
	Treated	7	0.455± 0.067	0.296± 0.081	4	0.238± 0.036	0.242± 0.047	0.214± 0.100	0.195± 0.072
Middle-age	Control	7	0.258± 0.043	0.198± 0.055	4	0.215± 0.055	0.215± 0.060	0.217± 0.072	0.212± 0.015
	Treated	7	0.173± 0.016	0.169± 0.032	4	0.122± 0.017	0.089± 0.007	0.154± 0.041	0.183± 0.076
			ng.uterus^{-1} B			ng.uterus^{-1} B			
Young	Control	7	11.317 ±1.008	7.203± 1.670	4	5.040± 1.642	4.890± 1.360	2.5025 ±0.379	1.5925 ±0.384
	Treated	7	14.609 ±1.804	9.551± 2.659	4	3.8375 ±0.446	3.945± 0.833	3.1150 ±1.207	2.9475 ±0.839
Middle-aged	Control	7	11.294 ±2.114	8.687± 2.632	4	3.998± 1.156	4.0575 ±1.308	4.2075 ±1.548	2.1825 ±0.251
	Treated	7	8.484± 1.127	8.126± 1.621	4	2.770± 0.298	2.0675 ±0.219	3.7025 ±1.150	4.1650 ±1.557

Significant reduction in levels of PGE_2 ($p<0.001$) and release of PGE_2 ($p<0.05$) in aged uteri (panel A)

Section 2.4.c. Measurement of the effect of treatment on the capacity of the uterus of young and old rats to release PGs

Method

Uterine horns (n=4 for each group) were opened longitudinally and cotton threads were attached to either end. One end of the thread was secured to the base of a 20ml open ended chamber. The upper thread was attached to a pivoted arm which was calibrated to provide a tension of 1g. The tissues were superfused with Krebs' solution at 37°C at a rate of 5ml min^{-1}. After an initial equilibration period of 1h, the superfusate was collected for 10min, and, after another 20 min, a further 10 min collection was made. The superfusates were extracted for PGs as described in Section 1.2.a. and the samples stored at -20°C in ethyl acetate until they were assayed for PGE-2, PGF-2α as described in sections 1.3a and b. After the last collection of superfusate, each uterine horn was homogenized in 5ml Krebs' solution and 100μl of the homogenate taken for estimation of protein concentration.

Results

The amounts of PGE-2 and PGF-2α released by superfused uteri showed considerable variation between individuals but were approximately comparable.

Release of PGE-2 but not PGF-2α was significantly less in ageing compared to young uteri (Table 22, Panel A, $p < 0.05$). The effects of treatment appeared to be slight or possibly even negative in the case of PGE-2 where the statistics approached significance ($0.05 < p < 0.10$).

Conclusion

The small numbers employed in this experiment together with considerable variability in PG release limits the interpretation of the results. The reduced release of PGE-2 from superfused tissue in aged animals together with reduced PGE-2 levels in unincubated tissue, suggest that the reduced amounts of PGs produced by incubated homogenates reflect a physiological change. Although steroid treatment increased synthetic capacity for PGE-2 and PGF-2α in young animals (Section 2.4.a) the same treatment did not result in increased release of these compounds from superfused tissue.

Discussion

Production of PGs and TXB-2 per unit weight of protein was reduced in aged uteri when compared to young animals under comparable endocrine conditions. The pattern of results was generally similar for all 4 compounds measured although was perhaps more marked for PGE-2. Moreover, although steroid treatment significantly increased the production of PGs and TX in young animals there was no such effect in the ageing groups. However, steroid treatment did not affect PG levels or release by young or aged animals.

Providing that the availability of arachidonic acid within the homogenates was not limiting for PG and TX synthesis, these results provide evidence of a reduced capacity to synthesize PGs and TX in the aged animal.

Changes in the pattern of synthesis may reflect changes in the mass and in the cellular composition of the tissue, and this limits the interpretation of the data. The uterus continues to grow throughout life and accumulates collagen (Shaub, 1964-65) at a disproportionate rate. The increasing amount of extracellular protein would lead to values of PG production in aged animals for example, being lower than in young animals when expressed per mg protein.

Despite these limitations in interpretation of the reduced PG production, levels and release in aged animals, the interpretation of the reduced responsiveness to steroid treatment in these animals is not limited in this way. Expressing the data as total PG and TX production or PG levels and release per uterus highlighted the effect of treatment. These results imply that the major effect of treatment was to stimulate growth of the uterus rather than the amount of PGs and TX produced per unit protein mass.

The details of the growth promoting effects of steroids upon the various sub-populations of cells are likely to be very complex and presently there are no improvements available for the two methods of presentation of the data. Although there was no evidence that uterine protein production in response to treatment was reduced in aged compared with young animals, it is possible to explain the reduced PG production in aged animals as the result of a decreased growth response of some uterine cells.

Following steroid treatment of young rats, the amounts of PGE-2, PGF-2α and 6-keto-PGF-1α were similar to those previously reported for rats on day 5 of pseudopregnancy (Fenwick et al., 1977; Phillips and Poyser, 1981b). TXB-2 production by uteri of young rats was also increased. However, no reports on TXB-2 synthesis relating to the time of implantation are available.

At first sight it seems difficult to reconcile the fact that treatment of young rats increased synthetic capacity for PGs and TX while levels and release were unaffected by the same treatment. It is possible that the steroid treatment of young animals increases PG synthetic capacity but that PG synthesis has to be stimulated either artifically or by blastocyst implantation, in order to reveal this increased capacity as increased levels of PGs in the uterus. Several studies have demonstrated increased uterine PG levels in response to deciduogenic stimuli (Rankin et al., 1979; Kennedy, 1979, 1980; Kennedy, Barbe and Evans, 1980). These studies measured PG levels after sensitization of the uterus with oestrogen and progesterone, which is similar to the method used here. However, in contrast to the experiments reported here, PBSG or sesame oil was injected as an additional artificial decidualizing stimulus by these workers. Artificial deciduogenic stimuli cause tissue damage (Finn, 1977; Lundqvist, Ljunqkvist and Nilsson, 1977) and tissue damage is known to stimulate arachidonic acid release and PG synthesis (Ramwell and Shaw, 1970; Piper and Vane, 1971). In the physiological situation, the close contact of the blastocyst with the endometrial surface may cause a similar type of tissue damage (Psychoyos, 1973). It is proposed then, that steroid treatment

increases the activity of PG synthetase but that arachidonic acid release is required to express the increased enzyme activity as increased PG levels or release.

The diminished response to oestrogen and progesterone may be the result of decreasing concentrations of uterine cytosolic steroid receptors (Holinka, Nelson and Finch, 1975; Saiduddin and Zassenhaus, 1979; Gessell and Roth, 1981). However, the reduced amounts of PGE-2 in unincubated tissue and the reduced release of PGE-2 from superfused tissue in aged animals suggests that the reduced amounts of PGs produced in incubated homogenates reflect a physiological change in the PG synthetic pathway. In this regard it is interesting to note that the PG synthesizing capacity of the liver and its stimulation by phenobarbitol is reduced with ageing in rats (Murota and Morita, 1980). In their study, reduced PG synthesis was accompanied by increased uptake of arachidonic acid into cellular phospholipids. This suggests that there is a decline in phospholipase activity during the process of ageing. The study of phospholipase activity in the uterus of the aged animal merits investigation.

Reduced tissue concentrations of PGs (and possibly TXB-2) may have significant implications for fertility in ageing uteri. Decreased PGE-2 and PGI-2 concentrations may result in impaired hyperaemia and endometrial vascular permeability and perhaps together with reduced PGF-2α concentrations, may cause the impaired decidual response. It is of interest that Soderwall and Smith (1962) found that Vitamin E treatment improved the development of implantation sites in aged hamsters. Vitamin E, an antioxidant, is a potent inhibitor of soya bean lipoxygenase (Pangan amala, Miller, Gwebu, Sharma and Cornell, 1977). Fatty acid

hydroperoxides produced during the lipoxygenation reaction have been reported to inhibit PG synthesis (Gryglewski, Bunting, Moncada, Flower and Vane, 1976). It is also known that excessive lipid peroxidation secondary to free radical formation occurs during the process of ageing (Slater, 1972). Therefore the beneficial effect of Vitamin E on the development of implantation sites in aged animals may be by virtue of its protective effect against inhibition of PG synthesis by hydroperoxy fatty acids.

GENERAL DISCUSSION

The experiments described in this thesis have addressed the questions of the physiological significance of PG and TX synthesis in the ovary, hypothalamus and uterus and of how this synthesis is regulated.

Several of the multiple stages involved in the PG synthetic pathway have been examined viz., PG synthetase activity, PG metabolism and PGE-2-9-ketoreductase activity. PG and TX production by the ovary, hypothalamus and uterus varied throughout the oestrous cycle. Since in each case, these variations were not due to alterations in arachidonic acid availability or PG metabolism it was concluded that they were due to changes in the activity of PG synthetase. The regulation of PG synthetase activity therefore constitutes an important stage for regulation of PG and TX synthesis.

The dominant pathway for PG and TX synthesis varied among different tissues. This observation may reflect the presence of differing amounts of enzymes in different cell types.

In each type of tissue homogenate the ratios of PGs and TX produced were not constant during the oestrous cycle which suggests that there is specific control of the individual enzymes producing PGs and TX from PGH-2. However, despite this suggestion, it was concluded that in the ovary, LH controls PG synthesis by acting at the level of the cyclooxygenase enzyme.

It was proposed that LH could stimulate cyclooxygenase thereby increasing PGH-2 availability which would result in a preferential increase in PGE-2 synthesis. Several studies in other tissues support this proposal. Fig. 27 illustrates the effect of increasing substrate (PGH-2) concentrations on the formation of TXB-2 and 6-keto-PGF-1α by rabbit lung microsomes as reported by Sun, Chapman and McGuire (1977).

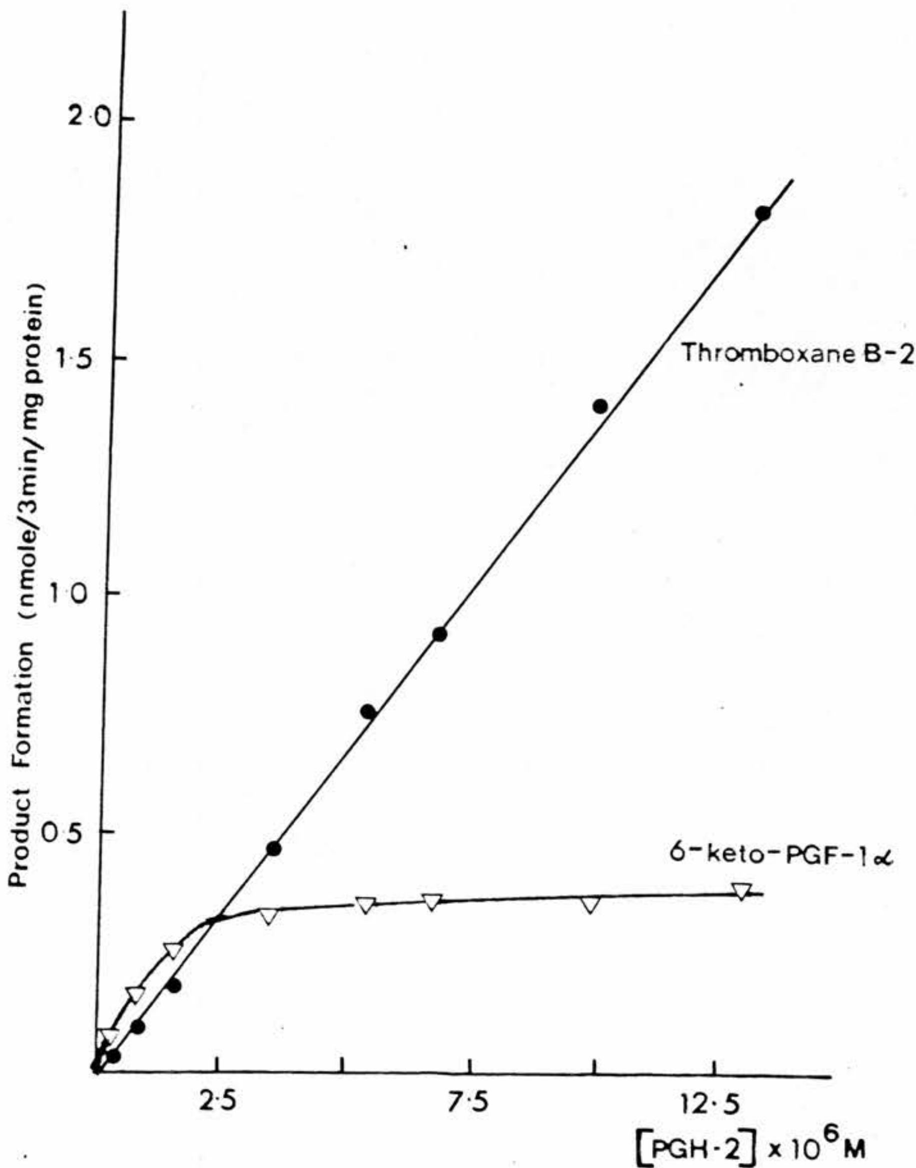


Figure 27. Effect of substrate concentrations on the formation of TXB-2 and PGI-2 by rabbit lung microsomes. Two ml of rabbit lung microsomes (2 mg/ml) were incubated with various concentrations of ^{14}C-PGH-2 for 2 minutes at 37°C. (From Sun, Chapman and McGuire, 1977).

Clearly low substrate concentrations favour 6-keto-PGF-1α synthesis while higher concentrations of PGH-2 favour TXB-2 synthesis. This study illustrates how a directional stimulation of one PG or TX could be brought about solely by increasing cyclooxygenase activity. It is proposed that a similar mechanism accounts for the preferential increase in PGE-2 production at the time of ovulation in the rat.

Smith and Lands (1972) have provided evidence of two separate types of cyclooxygenase - a 'slow' enzyme which provides basal levels of PGs and another 'rapid' enzyme which requires activation. In the ovary, the 'slow' enzyme may provide basal PG and TX levels, and, when activated by LH, the 'rapid' cyclooxygenase could provide elevated levels of PGH-2. Obviously, the effect of changing PGH-2 concentrations on ovarian PG and TX synthesis requires investigation.

In the studies on ovarian PG and TX synthesis, homogenates of whole ovaries were used. However, the granulosa cells within the ovarian follicles appear to be the main site of ovarian PG synthesis (Clark, Marsh and Le Maire, 1978 ; Erickson, Challis and Ryan, 1977). In future experiments on the control of ovarian PG synthesis, separation of ovarian follicular cell types will be required. Nevertheless, there may be an interaction between follicular cell types as LH will stimulate PG production by whole rabbit follicles in organ culture, (Moon, Zamecnik and Armstrong, 1974), whereas rabbit granulosa cells and thecal tissue grown separately, fail to respond consistently to LH (Erickson et al., 1977).
The physiological significance of ovarian PGE and PGF synthesis is well established in that they are essential for ovulation in many species, with the possible exception of humans although more studies are required in women.

The present study showed that in addition to PGE and PGF, 6-keto-PGF-1α synthesis was elevated at the time of ovulation implying that PGI-2 may have a role in the ovulatory process. Although the precise site of PGI-2 synthesis was not determined it is possible that the increased synthesis of PGI-2 is responsible for the vasodilation occurring in ovarian follicles just prior to ovulation (Jones, 1979). PGE-2 may be involved in causing vasodilation also.

An unexpected finding was that a peak in uterine PG and TX production occurred simultaneously with peak in ovarian PG production. The production of PGF-2α and TXB-2 increased between pro-oestrus and oestrus to a much greater extent than the production of PGE-2 and 6-keto-PGF-1α. This pattern of synthesis was mimicked by treatment of long-term ovariectomized rats with oestrogen and progesterone suggesting that ovarian steroids may directly stimulate uterine PG and TX synthesis. However, these steroids also cause an LH surge which may have affected uterine PG and TX synthesis. The fact that peak uterine PG and TX production coincided with peak ovarian production suggests that they may have a common stimulus. Since LH is the stimulus for ovarian PG synthetase, it would be interesting to examine the possibility that uterine PG synthetase is another 'target' enzyme for LH.

Separation of uterine tissue into the endometrium and myometrium again highlighted the fact that the dominating pathway in the synthesis of PGs varies between cell types. In the endometrium the major PG produced was PGF-2α, while in the myometrium 6-keto-PGF-1α predominated. However, an interaction

between the two tissues was apparent in that PG and TX production was greater when they were homogenized and incubated together. As suggested by Abel and Kelly (1979) it is possible that in vivo the endometrium supplies PGH-2 as substrate for conversion into PGs and TX by myometrial enzymes. While this interaction could occur at the interface between the two tissues, the instability of PGH-2 would indicate that diffusion of active PGH-2 between the tissues is unlikely.

The physiological significance of the cyclical variations in uterine PG synthesis is unknown at present. It is speculated, however, that the approximately 3-fold increase in uterine TXB-2 production (reflecting TXA-2 production) at the time of ovulation may be involved in the genesis of uterine contractions at that time. Increased uterine activity could account for the accelerated sperm transport into the oviduct close to, or just after ovulation in many species (see Hunter, 1981). Studies with specific TXA-2 receptor antagonists or TXA-2 synthesis inhibitors promise to elucidate further the physiological significance of uterine TXA-2 synthesis.

A disruption of uterine PG and TX synthesis was observed in the aged animal. PG and TX production was reduced in aged rats but this effect was most striking in the case of PGE-2. Since PGE-2, PGF-2α and PGI-2 are implicated in the vascular changes which precede uterine decidualization (see General Introduction) it was suggested that the reduced capacity to synthesize PGs (and TX) may explain the decline in the capacity of the aged uterus to support blastocyst implantaton.

It was demonstrated that the reduced PG and TX production was due to the reduced ability of the PG synthetase to convert arachidonic acid into PGs and TX. In agreement with a decreased PG synthetase activity, an age-related decrease in PGI-2 synthesis from PGH-2 occurs in aortic smooth muscle (Chang, Murota, Nakao and Orimo, 1980). However, decreased PG synthetase activity may occur in parallel with decreased phospholipase activity as this has been demonstrated in the liver of aged rats (Morita and Murota, 1980).

It was proposed that the accumulation of high levels of lipid hydroperoxides in ageing tissue (Slater, 1972) may account for the observed decrease in PG and TX synthesis. During PG synthesis the generation of high levels of lipid hydroperoxides and free radicals causes inactivation of cyclooxygenase. This emphasizes another control mechanism for PG synthesis in that cyclooxygenase is a self-limiting enzyme (Lands, 1981) and therefore regulation of the inactivation could alter the amounts of PGs and TX synthesized by tissues.

Antioxidants, which are capable of scavenging free radicals have the potential to modulate the activity of the cyclooxygenase enzyme. It was therefore proposed that this may explain the stimulatory effect of NA on PGE-2, PGF-2α and TXB-2 synthesis in the ME and AH-POA.

Stimulation of hypothalamic PG synthesis by NA may be of importance in the control of LHRH release as an increase in NA turnover occurs between 11:00h and 17:00h on pro-oestrus and this overlaps with a peak in PGE-2 synthesis in the ME (Barraclough and Wise, 1982).

Furthermore, it has been demonstrated that the ME releases PGE-2 in response to NA and that inhibition of PG synthesis suppresses the release of LHRH elicited by NA (Ojeda et al., 1979). Collectively, these results suggest that the stimulatory effect that NA exerts on LHRH release (Negro-Vilar et al., 1979) is mediated by increased PGE-2 synthesis. Whether PG synthesis is within or in the vicinity of the LHRH containing neurones, requires investigation. In addition to NA stimulating PGE-2 synthesis, PGE-2 may in turn stimulate NA release from hypothalamic neurones (Roberts and Hillier, 1976). Thus the stimulatory effect of PGE-2 on LH release may arise by potentiation of adrenergic neurotransmission in the hypothalamus.

A case has been put forward for regarding progesterone as a stimulus for the increased PG production in the ME. Plasma levels of this hormone (Mann and Barraclough, 1973) closely resemble the diurnal variation in PGE-2 production by the ME. Moreover, progesterone treatment of oestrogen-primed long-term ovariectomized rats increased PGE-2 production by the ME. This hypothesis is supported by the work of Labhsetwar and Zolovick (1973) who showed that aspirin, injected into the hypothalamus, blocked the stimulatory effect of progesterone on ovulation. Since this effect of progesterone on ovulation is transmitted through an α-adrenergic pathway in the hypothalamus, these workers suggested that PGs potentiate hypothalamic adrenergic neurotransmission. However, α-receptor antagonists do not block PGE-2-induced LH release (Harms et al., 1976). Therefore an alternative interpretation is that progesterone stimulates NA release and that NA is the stimulus for PG synthesis.

If progesterone and NA are physiological stimuli for an increase in hypothalamic PG synthesis, then a mechanism must exist which directs ME PG synthesis towards PGE-2 on pro-oestrus and oestrus. As was suggested for the ovary, the availability of PGH-2 may determine which PG is formed. The effect of changing PGH-2 concentrations on the ratios of PGE-2, PGF-2α, TXB-2 and 6-keto-PGF-1α production by the hypothalamus requires investigation.

The study of the effect of Froben treatment on the pro-oestrus LH surge was inconclusive, probably for two reasons. Firstly, the effects of the drug on the release of LH may be complicated by changes in pituitary responsiveness. If Froben increased pituitary responsiveness to LHRH it would be difficult to determine whether the drug could inhibit the LH surge. The effect of Froben on the pro-oestrous surge in LHRH would overcome this problem and this experiment requires investigation. Secondly, PG synthesis in the hypothalamus was not completely inhibited by Froben and sufficient levels of PGs may have remained which allowed a full LH surge. Conclusive evidence of a physiological role for PGs as mediators of LHRH release must await the demonstration that complete inhibition of hypothalamic PG synthesis blocks the pre-ovulatory LHRH surge.

In conclusion, these studies have provided further evidence that PGE-2 is involved in the processes of LHRH release and ovulation. It is proposed that LH controls ovarian PG synthesis by altering the availability of PGH-2 to the PG synthetase enzyme complex. The process of ovulation may be closely related to the increase in uterine PG synthesis at that time. Finally, reduced uterine PGE-2 synthesis may be a significant factor contributing to the reduced fertility in the aged animal.

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