Effects of inhibitors of arachidonic acid turnover on the production of prostaglandins by the guinea-pig uterus

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The supply of free arachidonic acid from phospholipids is generally regarded as the rate-limiting step for prostaglandin (PG) synthesis by tissues. Two enzymes involved in arachidonic acid uptake into, and release from, phospholipids are acyl-CoA:lysophospholipid acyltransferase (ACLAT) and phospholipase A₂ (PLA₂), respectively. PGF₂α produced by the endometrium induces luteolysis in several species including guinea-pigs. Thimerosal, an inhibitor of ACLAT, and aristolochic acid, an inhibitor of PLA₂, both reduced, in a concentration-dependent manner, the output of PGF₂α from guinea-pig endometrium cultured for 24 h on days 7 and 15 of the oestrous cycle. This study showed that the continual production of PGF₂α by guinea-pig endometrium is not only dependent upon the activity of PLA₂ for releasing free arachidonic acid for PGF₂α synthesis, but also on the incorporation of arachidonic acid into the phospholipid pool by the activity of ACLAT. The inhibitory effects of thimerosal and aristolochic acid on the outputs of PGE₂ and 6-keto-PGF₁α were less marked, particularly on day 7 when the low output of PGE₂ was unaffected and the output of 6-keto-PGF₁α was increased at the lower concentrations of thimerosal. This finding indicates that there are different pools of arachidonic acid bound as phospholipid for the syntheses of PGF₂α and 6-keto-PGF₁α by guinea-pig endometrium.

Introduction

Increased PGF₂α production by the uterus (particularly by the endometrium) after day 11 of the oestrous cycle is responsible for causing luteolysis in guinea-pigs (see Poyser, 1995). Prostaglandins are not stored in tissues (except for prostaglandins in seminal fluid), so their synthesis is immediately preceded by their release. The concentration of free arachidonic acid in tissues is extremely low (Irvine, 1982), so it is generally regarded that the rate-limiting step in prostaglandin synthesis is the release of arachidonic acid from a bound source (although changes in the concentration of prostaglandin H synthase are able to regulate the amounts of prostaglandins synthesized from the free arachidonic acid; Herschmann, 1996). There is an increase in the uptake of arachidonic acid, but not of oleic acid, into phospholipids of guinea-pig endometrium on day 15 (a day of high uterine PGF₂α production) compared with day 7 (a day of low uterine PGF₂α production) of the oestrous cycle (Ning et al., 1983). Arachidonic acid is not incorporated into phospholipids during their de novo synthesis, but rather by the sequential action of two enzymes, namely acyl-CoA synthetase (ACS) and acyl-CoA:lysophospholipid acyltransferase (ACLAT), acting on lysophospholipid (Irvine, 1982). In spite of the increased incorporation of arachidonic acid into phospholipids of guinea-pig endometrium on day 15, there is less arachidonic acid bound in phosphatidylethanolamine and phosphatidylcholine on day 15 than on day 7 of the oestrous cycle (Leaver and Poyser, 1981). This is due to arachidonic acid being released from phosphatidylethanolamine and phosphatidylcholine in guinea-pig endometrium at a faster rate on day 15 than on day 7 (Ning and Poyser, 1984). The enzyme responsible for the release of arachidonic acid from phosphatidylethanolamine and phosphatidylcholine is phospholipase A₂ (PLA₂). The enzymes ACS, ACLAT and PLA₂ are present in guinea-pig endometrium, and the activity of PLA₂ increases approximately twofold between days 7 and 15 of the cycle (Downing and Poyser, 1983; Norman and Poyser, 1998). The aim of the present work was to investigate the importance of arachidonic acid turnover in phospholipids for prostaglandin production by the guinea-pig endometrium by using inhibitors of ACLAT and PLA₂. Thimerosal is an inhibitor of ACLAT without affecting the activity of PLA₂ (ID₅₀ value approximately 75 µmol l⁻¹; Goppelt-Streube et al., 1986), while aristolochic acid is an inhibitor of PLA₂ (ID₅₀ value approximately 50 µmol l⁻¹; Rosenthal et al., 1989). The effects of these enzyme inhibitors on prostaglandin output from guinea-pig endometrium in culture were studied.
Materials and Methods

Materials

Medium 199 (plus Earle’s salts), amphotericin B, L-glutamine and kanamycin were purchased from Flow Laboratories, Irvine. Aristolochic acid and thimerosal were purchased from Sigma Chemical Co., Poole.

Methods

Virgin guinea-pigs (Dunkin Hartley) weighing 600–900 g were examined once a day, and a vaginal smear was taken and examined microscopically when the vagina was open. The first day of the oestrous cycle was taken as the day preceding the post-ovulatory influx of leucocytes when cornification was at a maximum. Animals were used on day 7 or day 15 of the oestrous cycle, but only after showing at least two cycles of normal duration (16–18 days). The animals were killed by stunning and incising the neck, and the uterus from each animal was removed. The uterus was divided into the two uterine horns, and each uterine horn was opened by cutting longitudinally. The endometrium was separated from the myometrium by cutting away 1–2 mm³ pieces of endometrium with a pair of fine scissors. This technique produces > 85% separation of the two tissues (Leaver and Poyser, 1981). Pieces of endometrium were placed on a raised platform in a Petri dish (20–40 mg tissue per dish) that contained 4 ml Medium 199 (plus Earle’s salts) supplemented with L-glutamine (1.6 mmol l⁻¹), amphotericin B (1.5 µg ml⁻¹) and kanamycin (30 µg ml⁻¹). The Petri dishes also contained thimerosal (20, 100 or 500 µmol l⁻¹) or aristolochic acid (10, 50 or 100 µmol l⁻¹). The Petri dishes, together with two further dishes containing tissue but no treatments (controls) were placed in modified Kilner jars, and the tissues were cultured for 24 h as described by Ning et al. (1983) and Ning and Poyser (1984). The culture medium was collected and replaced every 6 h. Each sample of culture medium was stored at –20°C, and the amounts of PGF₂α, PGE₂ and 6-keto-PGF₁α present were subsequently measured without extraction by radioimmunoassay. The results obtained for the control tissues were averaged. After culture, the tissue samples were dried at 37°C for 24 h and weighed.

PGF₂α, PGE₂ and 6-keto-PGF₁α were measured using antibodies raised in this laboratory; their crossreactivities were reported by Poyser (1987). The standard prostaglandin solutions used in the assay contained an amount of culture medium equivalent to that used for the samples. The inter- and intra-assay coefficients of variation were <9% for each assay. The detection limit was 10–30 pg prostaglandin per assay tube.

Statistical analysis

Changes in the outputs of prostaglandins with time were analysed by Duncan’s multiple-range test. Differences between groups were analysed by Student’s t test taking into account whether or not the variances of the two groups were unequal.

Results

Basal prostaglandin output

The basal outputs of PGF₂α, PGE₂ and 6-keto-PGF₁α from guinea-pig endometrium in culture generally decreased significantly (P < 0.05) with time over the culture period, except for the output of PGF₂α from day 7 tissue which increased significantly (P < 0.05) over the 24 h of culture (Fig. 1). The output of each prostaglandin during each 6 h period of culture was significantly (P < 0.05) higher on day 15 than on day 7 (Fig. 1).

Effects of thimerosal on prostaglandin output

Day 7. Thimerosal (20, 100 and 500 µmol l⁻¹) significantly (P < 0.05) inhibited the output of PGF₂α from day 7 guinea-pig endometrium cultured for 24 h. The basal outputs of PGF₂α, PGE₂ and 6-keto-PGF₁α from day 7 tissue were higher than those from day 15 (Fig. 1).

Fig. 1. Mean (± SEM, n = 5) outputs (ng per mg tissue per 6 h) of (a) PGF₂α, (b) PGE₂ and (c) 6-keto-PGF₁α from day 7 (□) and day 15 (□□) guinea-pig endometrium cultured for 24 h. For any one prostaglandin, bars with the same number (day 7) or same letter (day 15) are not significantly (P > 0.05) different. Asterisks indicate values that are significantly (P < 0.05) higher than the corresponding day 7 value.
pig endometrium after 12, 18 and 24 h of culture, except for 20 µmol thimerosal l⁻¹ after 12 h (Fig. 2). Thimerosal had no effect on PGF₂α output after 6 h of culture, nor on the output of PGE₂ during the 24 h culture period. The output of 6-keto-PGF₁α from day 7 guinea-pig endometrium was significantly (P < 0.05) increased by thimerosal (20 and 100, but not by 500 µmol l⁻¹) after 12, 18 and 24 h of culture, except for 20 µmol thimerosal l⁻¹ after 12 h. Thimerosal had no effect on the output of 6-keto-PGF₁α after 12 h (Fig. 2). Thimerosal had no effect on PGF₂α output after 6 h of culture, nor on the output of PGE₂ during the 24 h culture period. The output of 6-keto-PGF₁α from day 7 guinea-pig endometrium was significantly (P < 0.05) increased by thimerosal (20 and 100, but not by 500 µmol l⁻¹) after 12, 18 and 24 h of culture, except for 20 µmol thimerosal l⁻¹ after 12 h. Thimerosal had no effect on the output of 6-keto-PGF₁α after 12 h (Fig. 2).

**Day 15.** Thimerosal (20, 100 and 500 µmol l⁻¹) significantly (P < 0.05) inhibited the output of PGF₂α from day 15 guinea-pig endometrium throughout the 24 h culture period, except for 20 µmol thimerosal l⁻¹ after 6 h (Fig. 3). Thimerosal (500 µmol l⁻¹) significantly (P < 0.05) inhibited the output of PGE₂ during the 24 h culture period. The lower concentrations of thimerosal had no effect, except for 100 µmol l⁻¹ which significantly (P < 0.05) inhibited the output of PGE₂ after 24 h. Thimerosal (500 µmol l⁻¹) significantly (P < 0.05) inhibited the output of 6-keto-PGF₁α after 6 and 24 h of culture (Fig. 3).

**Effects of aristolochic acid on prostaglandin output**

**Day 7.** Aristolochic acid (10, 50 and 100 µmol l⁻¹) significantly (P < 0.05) inhibited the output of PGF₂α from day 7 guinea-pig endometrium after 12, 18 and 24 h, but not after 6 h of culture (Fig. 4). Aristolochic acid (10, 50 and 100 µmol l⁻¹) had no effect on the outputs of PGE₂ and 6-keto-PGF₁α from day 7 guinea-pig endometrium, except for 50 µmol aristolochic acid l⁻¹ which inhibited 6-keto-PGF₁α output after 18 h of culture (Fig. 4).

**Day 15.** Aristolochic acid (10, 50 and 100 µmol l⁻¹) significantly (P < 0.05) inhibited the output of PGF₂α from day 15 guinea-pig endometrium throughout the 24 h culture period.

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**Fig. 2.** Effect of thimerosal at concentrations of 0 (■; control), 20 (▲), 100 (□) and 500 (■) µmol l⁻¹ on mean (± SEM, n = 5) outputs (ng per mg tissue per 6 h) of (a) PGF₂α, (b) PGE₂ and (c) 6-keto-PGF₁α from day 7 guinea-pig endometrium cultured for 24 h. Asterisks indicate values that are significantly (P < 0.05) lower or higher than the corresponding control value.

**Fig. 3.** Effect of thimerosal at concentrations of 0 (■; control), 20 (▲), 100 (□) and 500 (■) µmol l⁻¹ on mean (± SEM, n = 5) outputs (ng per mg tissue per 6 h) of (a) PGF₂α, (b) PGE₂ and (c) 6-keto-PGF₁α from day 15 guinea-pig endometrium cultured for 24 h. Asterisks indicate values that are significantly (P < 0.05) lower than the corresponding control value.
period, except for 10 µmol aristolochic acid l−1 which had no significant effect after 12 h (Fig. 5). Aristolochic acid at 100 µmol l−1 significantly (P < 0.05) inhibited the output of PGE2 during the 24 h culture period. The lower concentrations of aristolochic acid had no effect, except for 10 µmol aristolochic acid l−1 which significantly (P < 0.05) inhibited PGE2 output after 18 h. The output of 6-keto-PGF1α from day 15 guinea-pig endometrium was significantly (P < 0.05) inhibited by 10 and 100 µmol aristolochic acid l−1 after 6 h, and by 50 µmol aristolochic acid l−1 after 12 and 18 h. Aristolochic acid (10, 50 and 100 µmol l−1) had no effect on 6-keto-PGF1α output after 24 h of culture (Fig. 5).

Discussion
The basal outputs of PGF2α, PGE2 and 6-keto-PGF1α from day 7 and day 15 endometrium significantly (P < 0.05) decreased or remained constant during the 24 h culture period, except for the output of PGF2α from day 7 endometrium which significantly (P < 0.05) increased. This increase may be due to the removal of the inhibitory influence of progesterone on endometrial PGF2α synthesis (Leaver and Seawright, 1982; Riley and Poyser, 1987) after taking the uterus out of the guinea-pig. The initial basal outputs (that is, during 0–6 h of culture) of PGF2α, PGE2 and 6-keto-PGF1α were 27.3-, 5.1- and 2.4-fold higher on day 15 than on day 7, reflecting the specific increase in uterine PGF2α release in vivo towards the end of the oestrous cycle (see Poyser, 1995). Oestriadiol acting on a progesterone-primed uterus is the physiological stimulus for increased PGF2α production by the guinea-pig uterus (Blatchley and Poyser, 1974; Poyser, 1983a, 1993). The biochemical processes by which the ovarian steroid hormones selectively increase PGF2α synthesis lead to the release of arachidonic acid, since the concentration of free arachidonic acid is rate limiting for the synthesis of
prostaglandins by the guinea-pig uterus (Poyser, 1991). The source of this free arachidonic acid is phospholipid (Leaver and Poyser, 1981). Therefore, in the present study, the importance of arachidonic acid turnover for prostaglandin synthesis in the guinea-pig endometrium was investigated by studying the effects of inhibitors on two enzymes involved in arachidonic acid uptake into, and release from, phospholipids.

Thimerosal increases the synthesis of prostaglandins and thromboxane by rat macrophages and human platelets by preventing the esterification of arachidonic acid into phospholipids (Goppelt-Strübe et al., 1986). Thus, thimerosal would be expected to increase prostaglandin output from guinea-pig endometrium. In the present study, thimerosal increased the output of 6-keto-PGF$_{1\alpha}$ from day 7 guinea-pig endometrium after 6 h of culture and maintained it at this amount. The effect was concentration dependent between 20 and 100 μmol l$^{-1}$, but a higher concentration (500 μmol l$^{-1}$) had no effect. In contrast to this stimulatory effect, thimerosal inhibited the output of PGF$_{2\alpha}$ from day 7 guinea-pig endometrium, whereas the very low output of PGE$_2$ was unaffected. On day 15, when prostaglandin production is higher, thimerosal inhibited the outputs of PGF$_{2\alpha}$, PGE$_2$, and 6-keto-PGF$_{1\alpha}$ in a concentration-dependent manner.

Goppelt-Strübe et al. (1986) reported that thimerosal had a stimulatory effect on prostaglandin synthesis, which is in contrast to the inhibitory effect observed in the present study. However, Goppelt-Strübe et al. (1986) used short incubation times of 10–60 min during which, by inhibiting arachidonic acid re-uptake into phospholipids, the concentration of free arachidonic acid in solution would increase and thus stimulate prostaglandin production. In the present study, the tissue was cultured for 24 h and the phospholipid arachidonic acid pool may have become depleted during this longer time period due to inhibiting arachidonic acid uptake, resulting in a reduction in the release of free arachidonic acid and a decrease in prostaglandin synthesis, particularly on day 15 when prostaglandin production is high. Since PGF$_{2\alpha}$ output is greater than the outputs of the other prostaglandins on day 15, PGF$_{2\alpha}$ production is affected to a larger extent. On day 7, thimerosal similarly decreased PGF$_{2\alpha}$ output while increasing 6-keto-PGF$_{1\alpha}$ output. This differential effect was unexpected; however, previous studies have shown that 6-keto-PGF$_{1\alpha}$ production by the guinea-pig endometrium is controlled by intracellular processes different from those that control PGF$_{2\alpha}$ production (Riley and Poyser, 1987, 1990).

Aristolochic acid, an inhibitor of PLA$_2$, significantly reduced (> 90%) the output of PGF$_{2\alpha}$ from both day 7 and day 15 guinea-pig endometrium. However, it had no effect on PGF$_{2\alpha}$ output from day 7 guinea-pig endometrium during the first 6 h of culture. However, the output of PGF$_{2\alpha}$ is very low during this period, which implies that there may be sufficient free arachidonic acid present in the tissue during this period such that any inhibition of PLA$_2$ has no significant effect on PGF$_{2\alpha}$ synthesis. Nevertheless, aristolochic acid inhibited the increase in PGF$_{2\alpha}$ output from day 7 guinea-pig endometrium that occurs after 6 h of culture. The inhibitory effects of aristolochic acid on the outputs of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ from guinea-pig endometrium in culture were less marked than the effect on PGF$_{2\alpha}$ output, particularly in day 7 tissue when prostaglandin production is generally lower. There is a twofold increase in PLA$_1$ activity in guinea-pig endometrium between day 7 and days 15 and 16 of the oestrous cycle (Downing and Poyser, 1983; Norman and Poyser, 1998), and PLA$_1$, and melittin (an activator of PLA$_2$) both stimulate prostaglandin output from the guinea-pig uterus (Poyser, 1987; Johnson and Poyser, 1991). These findings indicate that PLA$_1$ has an essential role in providing free arachidonic acid for PGF$_{2\alpha}$ synthesis by the guinea-pig endometrium. The main PGF$_{2\alpha}$-producing cells in the guinea-pig endometrium are the epithelial cells, and aristolochic acid inhibits PGF$_{2\alpha}$ output from these cells (Naderali and Poyser, 1996a). Aristolochic acid also inhibits oxytocin-induced PGF$_{2\alpha}$ release from ovine and bovine endometrium, and the increase in PGF$_{2\alpha}$ output from bovine endometrium induced by PLA$_1$ and melittin (Lee and Silvia, 1994; Burns et al., 1997). Therefore, PLA$_1$ has an essential role in the synthesis of PGF$_{2\alpha}$ by ovine and bovine as well as by guinea-pig endometrium.

Overall, the present study has reinforced the importance of PLA$_1$ in releasing arachidonic acid from phospholipids for the synthesis of PGF$_{2\alpha}$ by the guinea-pig endometrium. The significant new finding is that the continual production of PGF$_{2\alpha}$ by guinea-pig endometrium is also apparently dependent on the uptake of arachidonic acid into phospholipids. The rate of incorporation of arachidonic acid into phospholipids of the guinea-pig endometrium is two- to threefold higher on day 15 than on day 7 (Ning et al., 1983). Thus, the hormonal stimulus for increased uterine PGF$_{2\alpha}$ synthesis not only stimulates arachidonic acid release, but also increases the rate of arachidonic acid acylation into phospholipids. This increase in arachidonic acid turnover appears crucial for supplying adequate amounts of substrate for PGF$_{2\alpha}$ synthesis, especially as there is also a three- to fivefold increase in the concentration of prostaglandin H synthase-2 in guinea-pig endometrium after day 11 of the oestrous cycle (Poyser, 1983b; Naderali and Poyser, 1996b; Bracken et al., 1997).

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