Effect of a selective prostaglandin H synthase-2 inhibitor (NS-398) on prostaglandin production by the guinea-pig uterus

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Using guinea-pig uterine tissues, indomethacin (a non-selective inhibitor of prostaglandin H synthase) inhibited prostaglandin (PG) synthesis by homogenates of the endometrium, by cultured endometrium and myometrium, and by cultured epithelial glandular cells and stromal cells derived from the endometrium. NS-398, a selective inhibitor of prostaglandin H synthase-2 (PGHS-2), also inhibited PG synthesis by endometrial homogenates, by cultured endometrium and myometrium, and by cultured epithelial glandular cells and stromal cells. Indomethacin and NS-398 inhibited PG production to similar extents, except for 6-keto-PGF_{1α} production by the myometrium where indomethacin was more effective. In particular, indomethacin and NS-398 produced over 90% inhibition of PGF_{2α} output from the epithelial glandular cells, the main source of PGF_{2α} in the endometrium. These functional studies indicate that prostaglandin H synthase-2 is the predominant PG-forming enzyme in the guinea-pig uterus.

Introduction

Increased production of prostaglandin (PG) F_{2α} by the uterus towards the end of the oestrous cycle is responsible for luteolysis in several non-primate mammals including guinea-pigs (see Poyser, 1995). Prostaglandins are not stored in tissues, including the guinea-pig uterus (Poyser, 1972), so their release is immediately preceded by their synthesis. Prostaglandins of the 2-series are synthesized from arachidonic acid; the first step involves the conversion of arachidonic acid to PGH₂ by prostaglandin H synthase (PGHS; see Lands, 1979). This single enzyme has two separate actions, namely cyclooxygenase activity which converts arachidonic acid to PGG₂ and peroxidase activity which converts PGG₂ to PGH₂. Non-steroidal anti-inflammatory drugs (NSAID) inhibit the cyclooxygenase activity, but not the peroxidase activity, of PGHS (see Smith and Marnett, 1991). Indomethacin inhibits prostaglandin synthesis by the guinea-pig uterus (Poyser, 1972), and the intra-uterine administration of indomethacin to guinea-pigs prevents regression of the corpus luteum and increases the duration of the oestrous cycle (Horton and Poyser, 1973; Poyser and Horton, 1975).

Since these earlier studies, it has now been established that there are two isoenzyme forms of PGHS namely PGHS-1 and PGHS-2 (Fu *et al.*, 1990; Wong and Richards, 1991; Sirois and Richards, 1992). This raises the question as to which form is responsible for PGF_{2α} production by the uterus. Studies using western blotting techniques have shown that PGHS-2 is present in the guinea-pig endometrium on days 6 and 17 of the

oestrous cycle (Naderali and Poyser, 1994). Consequently, the present study has investigated the functional significance of PGHS-2 in the guinea-pig uterus by using the selective PGHS-2 inhibitor, NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide). This compound is of similar potency to indomethacin at inhibiting PGHS-2 but, in contrast to indomethacin, has no inhibitory effect on PGHS-1 (Futaki *et al.*, 1993, 1994). Therefore, the effects of NS-398 and indomethacin on PG production by the guinea-pig uterus were compared.

Materials and Methods

Virgin, Dunkin–Hartley guinea-pigs (600–900 g) were examined daily and a vaginal smear was taken when the vagina was open. Day 1 of the oestrous cycle was taken as the day preceding the post-ovulatory influx of leucocytes when cornification was at a maximum. All guinea-pigs had exhibited at least three cycles of normal duration before being killed (by stunning and incising the neck) on day 7 or day 15 of the cycle. Four guinea-pigs on day 7 were used in Expts 1–3, and four guinea-pigs on day 15 were used in Expt 1. The uterus from each guinea-pig was removed and used in one of the following experiments.

Experiment 1: effects of NS-398 and indomethacin on prostaglandin synthesis by homogenates of day-7 and day-15 endometrium

Each uterus was divided into its two horns, and each horn was 'opened' by cutting longitudinally. The endometrium was carefully cut away in small pieces from the myometrium by

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using a pair of fine scissors. This technique results in >85% separation of the two tissues (Leaver and Poyser, 1981). The endometrium from each animal was divided into five approximately equal amounts, blotted dry and weighed. Each sample of endometrium was homogenized in 10 ml Kreb's solution (for composition see Mitchell *et al.*, 1977) containing 100 μ l ethanol (control), indomethacin (14 and 28 μ mol l^{-1}) or NS-398 (16 and 32 μ mol l^{-1}) and then incubated for 60 min at 37°C. The pH of the incubates was lowered to pH 4 with 1 mol HCl l^{-1} , and the prostaglandins extracted by shaking twice with 20 ml ethyl acetate. The ethyl acetate fractions were combined and evaporated to dryness at 50°C on a rotary evaporator. Each dried extract was redissolved in 4 ml ethyl acetate and stored at -20°C. The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ in the extracts were measured by radioimmunoassay.

Experiment 2: effects of NS-398 and indomethacin on prostaglandin output from cultured endometrium and myometrium

The endometrium and myometrium in each uterine horn were separated as in Expt 1. Each tissue type was cut into small pieces (2 mm $\times$ 1 mm) and placed on a raised platform in a Petri dish which contained 4 ml Medium 199 plus Earle's salts and supplemented with L-glutamine (1.7 μ mol l^{-1}), amphotericin B (2.5 μ g ml^{-1}), penicillin (100 U ml^{-1}) and streptomycin (100 μ g ml^{-1}). Ten culture dishes were prepared from each tissue; four of the dishes contained 20 μ l ethanol (controls), and the remaining dishes were treated in duplicate with indomethacin (14 and 28 μ mol l^{-1}) or NS-398 (16 μ mol l^{-1}). The Petri dishes were placed in Kilner jars and incubated for 24 h as described by Ning *et al.* (1983). The cultured tissue remains viable for at least 72 h (Ning *et al.*, 1983). The culture medium was changed after 2, 8 and 24 h, and the samples of culture medium were stored at -20°C. The tissue in each Petri dish was blotted dry and weighed. The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ in the samples of culture medium were measured, without extraction, by radioimmunoassay.

Experiment 3: effects of NS-398 and indomethacin on prostaglandin output from cultured epithelial glandular cells and stromal cells

For each uterus, the endometrium was separated from the myometrium as in Expt 1. The endometrial cells were dissociated according to the method of Stayasawaroop *et al.* (1979) as modified by Chaminadas *et al.* (1986). In summary, pieces of endometrium were chopped into small fragments and were placed in 10 ml Nutrient Mixture F-10 Ham containing Hepes (20 mmol l^{-1}), 30% (v/v) heat-treated newborn calf serum, collagenase Type 1 (2 mg ml^{-1}), L-glutamine (10 mmol l^{-1}), amphotericin B (2.5 μ g ml^{-1}), penicillin (100 U ml^{-1}) and streptomycin (100 μ g ml^{-1}). The tissue was incubated with shaking at 37°C, during which the tissue fragments were further disrupted by gentle, repeated pipetting every 15 min. The degree of digestion was monitored periodically by decanting a small volume of the suspension into a sterile tissue culture dish and viewing the isolated cells under an inverted microscope. When adequate cell dissociation had been obtained, the remaining tissue fragments were collected by

sedimentation and discarded. This digestion process took approximately 2 h. The supernatant cell suspension obtained was centrifuged at 100 g for 5 min. The supernatant (containing stromal cells) was removed, and the pellet containing epithelial glandular cells was twice washed and centrifuged at 100 g for 5 min. The final pellet obtained contained approximately 95% epithelial glandular cells and 5% stromal cells. The supernatant containing the stromal cells was centrifuged at 400 g for 10 min, and a pellet of stromal cells was obtained.

The epithelial glandular cells and stromal cells were re-suspended each in 30 to 40 ml Nutrient Mixture F-10 Ham containing Hepes (20 mmol l^{-1}), L-glutamine (10 mmol l^{-1}), amphotericin B (2.5 μ g ml^{-1}), penicillin (100 U ml^{-1}) and streptomycin (100 μ g ml^{-1}). Samples (2.5 ml) of both cell suspensions were dispensed into each of 12 wells of a cell culture plate. The epithelial glandular cell suspensions contained 2.3×10^3 to 4.5×10^3 cells ml^{-1} , and the stromal cell suspensions contained 2.2×10^5 to 5.6×10^5 cells ml^{-1} . Cell culture plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. By 24 h after plating, the epithelial glandular cells had lost their shape and had become flattened to form a monolayer. Stromal cells were attached to the plastic surface, and by day 2 of culture a confluent layer of cells was formed. The medium in each well was changed every 72 h, and the cells were cultured for 6 days before being treated with 20 μ l ethanol (control), indomethacin (14 μ mol l^{-1}) or NS-398 (16 μ mol l^{-1}). The cells were cultured for a further 24 h, and the culture medium was changed after 2, 8 and 24 h. The samples of culture medium were stored at -20°C. The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ in each sample were measured, without extraction, by radioimmunoassay.

Cell viability at the time of plating and at the end of the culture was determined using a trypan blue exclusion method: 0.4 ml 0.4% (w/v) trypan blue solution was added to 0.1 ml of a cell suspension prepared in Hank's balanced salts solution. After standing for 10 min, the cells were washed and then counted in a haemocytometer to ascertain cell viability. Cell viability at the time of plating was >98%, and after culture was >95%.

Radioimmunoassays

The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ in each sample were measured by radioimmunoassay using antibodies raised in this laboratory; the cross-reactivities have been reported elsewhere (Poyser, 1987). When samples of culture medium were assayed directly, an equivalent amount of culture medium was added to the assay standards. The intra- and interassay coefficients of variation for the assays were <12%. The detection limit was 10–30 pg PG per assay tube.

Sources of material

NS-398 was kindly supplied by Taisho Pharmaceutical Co. Ltd, Tokyo and indomethacin by Merck, Sharpe and Dohme, Ltd, Hoddesdon, Herts. Medium 199 plus Earle's salts, L-glutamine and amphotericin B were purchased from Flow Laboratories, Irvine. Nutrient Mixture F-10 Ham, Hank's

Fig. 1. Mean ($\pm$ SEM, $n = 4$) amounts of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), PGE_2 and 6-keto- $PGF_{1\alpha}$ synthesized by homogenates of guinea-pig endometrium on (a) day 7 and (b) day 15 in the absence ($\square$) and presence of ($\blacksquare$) 14 μmol indomethacin l^{-1} , ($\square$) 28 μmol indomethacin l^{-1} and ($\square$) 16 μmol NS-398 l^{-1} and ($\square$) 32 μmol NS-398 l^{-1} . †Significantly ($P < 0.05$) lower than corresponding control value. *Significantly ($P < 0.05$) higher than corresponding value for indomethacin.

balanced salts medium, streptomycin and trypan blue were purchased from Sigma Chemical Co., Poole. Penicillin G potassium salt was purchased from ICN Pharmaceuticals Ltd, Thame.

The NS-398 and indomethacin were made up as concentrated solutions in ethanol and stored at -20°C . Appropriate aliquots were added to the Krebs' solution and culture media to produce the required concentration; the ethanol was diluted by at least 100-fold.

Statistical tests

The results were analysed by ANOVA, Student's t test, and the paired t test, as appropriate.

Results

Experiment 1: effects of NS-398 on prostaglandin synthesis by homogenates of day-7 and day-15 endometrium

The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ synthesized by homogenates of guinea-pig endometrium were 3.0-, 1.8- and 1.4-fold higher on day 15 than on day 7 of the cycle, respectively. These differences were statistically significant ($P < 0.05$) for $PGF_{2\alpha}$ and PGE_2 (Fig. 1). Indomethacin (14 and 28 μmol l^{-1}) significantly ($P < 0.05$) inhibited by 46–80% the synthesis of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ by guinea-pig endometrial homogenates on days 7 and 15 of the cycle. There

were no significant differences between the two concentrations used (Fig. 1). NS-398 (16 and 32 μmol l^{-1}) significantly ($P < 0.05$) inhibited by 42–65% the synthesis of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ by guinea-pig endometrial homogenates on both days of the cycle, and there were no significant differences between the two concentrations used (Fig. 1). However, at approximately similar concentrations, NS-398 was significantly ($P < 0.05$) less effective at inhibiting the synthesis of $PGF_{2\alpha}$ and PGE_2 (but not of 6-keto- $PGF_{1\alpha}$) by endometrial homogenates on day 7 but not on day 15 (Fig. 1).

Experiment 2: effects of NS-398 and indomethacin on prostaglandin output from cultured endometrium and myometrium

The control outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ from both the endometrium and myometrium significantly ($P < 0.05$) declined during culture, except for $PGF_{2\alpha}$ output from the endometrium which significantly ($P < 0.05$) increased between the second and third periods of culture ($P < 0.05$; Fig. 2). Indomethacin (14 and 28 μmol l^{-1}) and NS-398 (16 μmol l^{-1}) significantly ($P < 0.05$) inhibited by 25–95% the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ from cultured endometrium and myometrium, except for PGE_2 output from the myometrium during the second and third periods of culture. However, the basal output of PGE_2 from the myometrium during these periods was very low, and both drugs tended to decrease it further. The degree of inhibition by both drugs on the outputs of the three PGs increased with time. NS-398 in a similar concentration to indomethacin was as effective as indomethacin at inhibiting PG output, except for 6-keto- $PGF_{1\alpha}$ output from the myometrium during all three culture periods while NS-398 was significantly ($P < 0.05$) less effective than indomethacin (Fig. 2).

Experiment 3: effects of NS-398 and indomethacin on prostaglandin output from cultured epithelial glandular cells and stromal cells

On the experimental day (i.e. the seventh day) of culture, the respective initial outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ were significantly ($P < 0.05$) 460-, 116- and 140-fold higher from epithelial glandular cells than from stromal cells (Fig. 3). The outputs of the three PGs significantly ($P < 0.05$) decreased with time from both cell types over the 24 h culture period.

Indomethacin (14 μmol l^{-1}) and NS-398 (16 μmol l^{-1}) significantly ($P < 0.05$) inhibited by 20–95% the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ from both epithelial glandular cells and stromal cells, except for PGE_2 output from stromal cells during the third period of culture. Indomethacin and NS-398 were of similar potency at inhibiting PG output, and they were particularly effective at inhibiting (>90%) the output of $PGF_{2\alpha}$ from epithelial glandular cells during the second and third periods of culture (Fig. 3).

Discussion

The presence of the endometrium in the guinea-pig is essential for luteolysis to occur at the normal time (Butcher *et al.*, 1962).

This is consistent with the endometrium being the major site of PGF_{2α} synthesis in the guinea-pig (Poyser, 1983). The present study has shown that the epithelial glandular cells, and not the stromal cells, are the major cell type in the guinea-pig endometrium responsible for synthesizing PGF_{2α}. This agrees with studies in other species (see Poyser, 1995). Previous studies have shown that indomethacin inhibits PG synthesis by homogenates of guinea-pig uterus and by guinea-pig endometrium in culture (Poyser, 1972; Leckie and Poyser, 1990). In the study reported here on guinea-pig uterine tissues, indomethacin inhibited PG synthesis by homogenates of the endometrium, by cultured endometrium and myometrium, and by cultured epithelial glandular cells and stromal cells obtained from the endometrium. Indomethacin is a non-selective PGHS inhibitor, although there is evidence that it has a more potent action on PGHS-1 than on PGHS-2 (Mitchell *et al.*, 1993).

On guinea-pig uterine tissues, NS-398 inhibited PG synthesis by endometrial homogenates, by cultured endometrium and myometrium, and by epithelial glandular cells and stromal cells in culture. NS-398 is a selective inhibitor of PGHS-2 and has no significant inhibitory effect on PGHS-1 (Futaki *et al.*, 1993, 1994; Masferrer *et al.*, 1994). NS-398 was of similar potency to indomethacin at inhibiting PG synthesis by most uterine tissues. In particular, after 2 h of treatment, indomethacin and NS-398 both inhibited PGF_{2α} output from cultured epithelial glandular cells by over 90%. Since it has been reported that NS-398 and indomethacin have similar inhibitory potencies on PGHS-2 (Futaki *et al.*, 1994), the present study suggests that PGHS-2 is the main isoenzyme form of PGHS responsible for PGF_{2α} synthesis by the epithelial glandular cells in the endometrium. PGHS-2 also appears to be involved in PGE₂ and 6-keto-PGF_{1α} synthesis by the epithelial glandular cells and stromal cells.

Indomethacin and NS-398 were of similar potency at inhibiting the outputs of PGF_{2α} and PGE₂ from guinea-pig myometrium, suggesting that PGHS-2 is the main enzyme responsible for the synthesis of these two PGs by this tissue. Although indomethacin and NS-398 inhibited the output of 6-keto-PGF_{1α} from cultured myometrium, indomethacin was significantly more effective than NS-398 at similar concentrations. This suggests that both PGHS-1 and PGHS-2 may be controlling the synthesis of 6-keto-PGF_{1α} by the myometrium. A selective PGHS-1 inhibitor would be useful to investigate this suggestion.

Regarding other species, it was found that PGHS-2 is probably responsible for PG production by sheep endometrial cotyledons at term (Wimsatt *et al.*, 1993). PGHS-1, but not PGHS-2, is present in the rat uterus (Wong and Richards, 1991). This is true for the pregnant rat myometrium and,

Fig. 2. Mean ($\pm$ SEM, $n = 4$) outputs of (i) prostaglandin F_{2α} (PGF_{2α}), (ii) PGE₂ and (iii) 6-keto-PGF_{1α} from day-7 guinea-pig (a) endometrium and (b) myometrium cultured for consecutive periods of 2, 6 and 16 h in the absence ($\square$) and presence of ($\blacksquare$) 14 μ mol indomethacin l⁻¹, ($\boxtimes$) 28 μ mol indomethacin l⁻¹ and ($\boxdot$) 16 μ mol NS-398 l⁻¹. †Significantly ($P < 0.05$) lower than corresponding control value. *Significantly ($P < 0.05$) higher than corresponding value for indomethacin.

Fig. 3. Mean ($\pm$ SEM, $n = 4$) outputs of (i) prostaglandin F_{2α} (PGF_{2α}), (ii) PGE₂ and (iii) 6-keto-PGF_{1α} from (a) epithelial glandular cells and (b) stromal cells obtained from day-7 guinea-pig endometrium and cultured for consecutive periods of 2, 6 and 16 h in the absence (□) and presence of (■) 14 μ mol indomethacin l⁻¹ and (▨) 16 μ mol NS-398 l⁻¹. †Significantly ($P < 0.05$) lower than corresponding control value.

furthermore, the concentration of PGHS-1 increases from day 16 to day 22, the day of delivery (Myatt *et al.*, 1994). During the oestrous cycle of the rat, PGHS-1 is present in the endometrium throughout the cycle, but PGHS-2 is also present before and during oestrus. PGHS-2 is not detectable during most of dioestrus (Shoda *et al.*, 1995). In endometrial stromal cells obtained from ovariectomized rats treated with oestrogen and progesterone to sensitize the uterus for decidualization, interleukin-1 α (IL-1 α) increases the concentration of mRNA for PGHS-2 but not for PGHS-1 whereas epidermal growth factor increases the concentration of mRNA for both isoforms of PGHS (Bany and Kennedy, 1995). In mice, IL-1 α increases the concentration of PGHS-2 but not of PGHS-1 in endometrial stromal cells (Jacobs *et al.*, 1994). In addition, in mice, PGHS-2 is localized to the implantation sites in newly differentiated endometrial stromal cells, whereas PGHS-1 is found in the epithelial cells (Jacobs *et al.*, 1994). This suggests a functional role also for PGHS-2 in producing the endometrial prostaglandins necessary for implantation. Which functional form of PGHS is present in the human uterus requires investigating. If the human uterus is like the guinea-pig uterus, in which PGHS-2 is the main functional form in both the endometrium and myometrium, then the use of selective inhibitors of PGHS-2 for treating disorders of menstruation (for example, dysmenorrhoea, menorrhagia) should produce symptomatic relief without producing the gastric damage associated with non-selective inhibitors of PGHS (Futaki *et al.*, 1993).

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