Effects of epidermal growth factor, interleukin 1 and nitric oxide on prostaglandin production by guinea-pig uterus

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Initial experiments in the present study investigated the effects of epidermal growth factor (EGF), interleukin 1 β (IL-1 β) and sodium nitroprusside (a nitric oxide donor) on the output of prostaglandins from guinea-pig uterus on day 7 of the oestrous cycle. Superfusion of day 7 guinea-pig uterus *in vitro* with either EGF or sodium nitroprusside increased the output of PGF_{2 α} and 6-keto-PGF_{1 α} , but not of PGE₂. IL-1 β had no effect on the output of these three prostaglandins. EGF still increased the output of PGF_{2 α} , but did not increase the output of 6-keto-PGF_{1 α} in a calcium-depleted superfusate. Subsequent experiments investigated the effect of sodium nitroprusside on contractile activity of day 7 guinea-pig uterus. Basal spontaneous

activity of both the intact uterus and isolated myometrium superfused *in vitro* was low. Sodium nitroprusside increased the contractile activity of these tissues two- to fourfold. EGF did not affect the contractile activity of the uterus, indicating that sodium nitroprusside-induced contractions are not due to increased prostaglandin production. Overall, the findings indicate that EGF and nitric oxide may act as mediators in the mechanism by which oestradiol acting on a progesterone-primed uterus stimulates the increase in PGF_{2 α} production by the guinea-pig uterus necessary for luteolysis. Nitric oxide may increase the spontaneous activity of the uterus when this activity is low.

Introduction

PGF_{2 α} produced by the uterus towards the end of the oestrous cycle is responsible for causing luteolysis in a number of mammalian species, including guinea-pigs (Poyser, 1995). Oestradiol treatment of ovariectomized guinea-pigs maintained on progesterone results in an increase in PGF_{2 α} output from the uterus (Blatchley and Poyser, 1974; Poyser, 1983a). During the oestrous cycle in guinea-pigs, increased secretion of oestradiol from the ovary occurs after day 10 (Joshi *et al.*, 1973), which precedes by 1 day the increase in PGF_{2 α} secretion by the uterus (Blatchley *et al.*, 1972; Earthy *et al.*, 1975; Antonini *et al.*, 1976). Increased production of PGF_{2 α} by the uterus and the subsequent decrease in progesterone output from the ovary are prevented by treating guinea-pigs from day 11 to day 14 of the oestrous cycle with the oestrogen receptor antagonist, ICI 182780, or the progesterone receptor antagonist, onapristone (Poyser, 1993). Oestradiol acting on a progesterone-primed uterus is therefore the physiological stimulus for increased PGF_{2 α} production by the guinea-pig uterus (particularly the endometrium), particularly as oxytocin does not have a stimulatory effect on endometrial PGF_{2 α} synthesis in guinea-pigs or affect the

duration of the oestrous cycle when administered to this species (Donovan, 1961; Poyser and Brydon, 1983; Riley and Poyser, 1987a). The epithelial cells, rather than the stromal cells, are the main type of cell responsible for synthesizing PGF_{2 α} in guinea-pig endometrium (Naderali and Poyser, 1996a; Bracken *et al.*, 1997).

One of the main steps in the synthesis of PGF_{2 α} is the conversion of arachidonic acid to PGH₂ by prostaglandin H synthase (PGHS), of which there are two isoforms: PGHS-1 (constitutive form) and PGHS-2 (inducible form) (Smith *et al.*, 1996). However, PGHS-2 is the predominant form in guinea-pig endometrium during the oestrous cycle and very little PGHS-1 is present (Naderali and Poyser, 1994, 1996b; Bracken *et al.*, 1997). In ovariectomized guinea-pigs, oestradiol, but not progesterone, causes an increase in the activity of PGHS in the endometrium (Naylor and Poyser, 1975; Poyser, 1983b). This action of oestradiol is due solely to an increase in the expression of PGHS-2 mRNA (Bracken *et al.*, 1997). During the oestrous cycle of guinea-pigs, the concentration of PGHS in the endometrium increases from day 12 (which is after the increase in output of oestradiol from the ovary) and is prevented by treatment with ICI 182780 (Poyser, 1972, 1983b, 1993). However, there is no evidence that oestradiol stimulates PGHS-2 synthesis directly. Instead, PGHS-2 is induced by inflammatory mediators, such as growth factors and cytokines (Smith *et al.*, 1996), and various growth factors are produced by the uterus (Brigstock *et al.*, 1989). Epidermal growth factor (EGF) and interleukin 1 (IL-1) increase the concentrations of prostaglandins and PGHS-2 in endometrial cells from the

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uterus of both mice and rats primed for the decidual cell reaction (Paria *et al.*, 1991; Jacobs and Carson, 1993; Jacobs *et al.*, 1994; Bany and Kennedy, 1995a,b, 1997, 1999). In the mouse uterus IL-1 concentrations are highest on the day of implantation and oestradiol stimulates EGF production; therefore, EGF or IL-1 may mediate the action of oestradiol in stimulating increased endometrial prostaglandin production at the time of implantation in rodents (Huet-Hudson *et al.*, 1990; De *et al.*, 1993). EGF and IL-1 also stimulate prostaglandin production by human decidual cells, and IL-1 increases the expression of PGHS-2 mRNA (Mitchell, 1991; Kennard *et al.*, 1995). In addition to growth factors and cytokines, there is evidence that nitric oxide can stimulate prostaglandin synthesis (Di Rosa *et al.*, 1996; Maccarrone *et al.*, 1997; Goodwin *et al.*, 1999) and, in uterine tissues, this may be achieved by activating PGHS-2 (Swaigood *et al.*, 1997).

Although oestradiol increases the concentration of PGHS-2 in the guinea-pig endometrium, this increase alone is not sufficient to increase uterine $\text{PGF}_{2\alpha}$ output (Poyser, 1983a,b). Progesterone priming of the uterus is also required (Poyser, 1983a). The release of arachidonic acid from phospholipids by the action of phospholipase A_2 (PLA_2) is the rate-limiting step in $\text{PGF}_{2\alpha}$ production by the guinea-pig uterus (Poyser, 1985a, 1987; Johnson and Poyser, 1991; Norman and Poyser, 2000). Consequently, any local factor produced by oestradiol not only has to increase the uterine concentration of PGHS-2 in order to increase the amount of $\text{PGF}_{2\alpha}$ produced in the long term, but must also stimulate arachidonic acid release in the short term to provide the precursor for prostaglandin synthesis. Therefore, the aim of the present study was to investigate whether EGF, IL-1 and nitric oxide in the short term are able to stimulate prostaglandin production by the guinea-pig uterus superfused *in vitro*, and thereby become possible candidates for mediating the action of oestradiol in stimulating $\text{PGF}_{2\alpha}$ production *in vivo*.

Materials and Methods

Materials

Epidermal growth factor, sodium nitroprusside and IL-1 β were purchased from Sigma Chemical Co. (Poole).

Methods

Thirty virgin guinea-pigs (Dunkin Hartley) weighing 600–900 g were examined daily and, when the vagina was open, a vaginal smear was obtained and examined under a microscope. Day 1 of the oestrous cycle was taken as the day preceding the post-ovulatory influx of leucocytes when cornification is at a maximum. After at least two oestrous cycles of normal duration (16–18 days), the guinea-pigs were used on day 7 of the oestrous cycle. The animals were killed by stunning and an incision in the neck, and the uterus from each animal was removed. The uterus was divided into the two uterine horns, and each horn was

opened by longitudinal incision and superfused with Krebs' solution (5 ml min^{-1}) at 37°C as described by Poyser and Brydon (1983). In all studies, each uterine horn was superfused initially for a 'settling period' of 60 min before the experiments began. In the first series of experiments, samples of superfusate were collected for 10 min periods for the next 80 min (that is, eight samples from each uterine horn). During the superfusion of one uterine horn from each animal, EGF (100 ng ml^{-1}), sodium nitroprusside ($50 \mu\text{mol l}^{-1}$) or IL-1 β (10 or 20 ng ml^{-1}) was present in the Krebs' solution during the collection of samples four and five of the eight samples obtained. The other uterine horn from each animal was untreated. In an additional experiment, both uterine horns from each guinea-pig were superfused with Krebs' solution from which the calcium chloride had been omitted (that is calcium-depleted Krebs' solution). EGF (100 ng ml^{-1}) was present in this solution during the collection of samples four and five from one of the uterine horns. The amounts of $\text{PGF}_{2\alpha}$, PGE_2 and 6-keto- $\text{PGF}_{1\alpha}$ in each sample of superfusate were measured by radioimmunoassay.

In a second series of experiments, the contractile activity of one uterine horn from each animal was recorded isotonicity for 30 min before and after treatment, and for 20 min during treatment with sodium nitroprusside ($50 \mu\text{mol l}^{-1}$), using a Washington Isotonic Lever Transducer T2 connected to a Servogor 124 (Goerz) pen recorder. This procedure was also performed on the other uterine horn from which the endometrium had been removed by a technique described by Leaver and Poyser (1981), leaving just the intact myometrium. Contractile activity ($\text{cm}^2 \text{ min}^{-1}$) was calculated as the area between the contraction curves and baseline (that is area under the curve) before, during and after treatment divided by the period for which the contractions were recorded.

Assays

Immediately after collection, the pH of each sample of superfusate was reduced to 4.0 with HCl and the prostaglandins were extracted by shaking twice with 50 ml ethyl acetate. The two ethyl acetate fractions were combined and evaporated to dryness on a rotary evaporator at 50°C (Poyser, 1987). Each dried extract was dissolved in 10 ml ethyl acetate and stored at -20°C . The amounts of $\text{PGF}_{2\alpha}$, PGE_2 and 6-keto- $\text{PGF}_{1\alpha}$ in each sample were measured by radioimmunoassay using antibodies raised in this laboratory; crossreactivities of the antibodies have been reported elsewhere (Poyser, 1987). The inter- and intra-assay coefficients of variation were $< 12\%$. The detection limit was 0.5–0.8 pg prostaglandin per 100 mg tissue per min.

Statistical analysis

Changes in the outputs of prostaglandins with time were analysed by Duncan's multiple-range test. Other comparisons were made using the Student's *t* test.

Fig. 1. Mean ($\pm$ SEM, $n = 5$) outputs of (a) $\text{PGF}_{2\alpha}$, (b) PGE_2 and (c) 6-keto- $\text{PGF}_{1\alpha}$ from day 7 guinea-pig uterus superfused *in vitro* in the presence (●) and absence (■) of epidermal growth factor (EGF). *Significantly ($P < 0.05$) higher than corresponding values before treatment with EGF.

Results

Prostaglandin output

EGF significantly ($P < 0.05$) increased the outputs of $\text{PGF}_{2\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}$, but not of PGE_2 , from the day 7 guinea-pig uterus superfused *in vitro* (Fig. 1). After the omission of calcium from the superfusing solution to investigate whether extracellular calcium was necessary for these increases, EGF still significantly ($P < 0.05$) increased the output of $\text{PGF}_{2\alpha}$ but had no significant effect on the output of 6-keto- $\text{PGF}_{1\alpha}$ from the day 7 uterus. The output of PGE_2 was still not increased (Fig. 2). Sodium nitroprusside also significantly ($P < 0.05$) increased the outputs of $\text{PGF}_{2\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}$, but not of PGE_2 , from the day 7 guinea-pig uterus superfused in calcium-containing Krebs' solution (Fig. 3). The outputs ($\text{ng min}^{-1} (100 \text{ mg})^{-1} \text{ tissue}$) from the superfused guinea-pig uterus of $\text{PGF}_{2\alpha}$, PGE_2 and 6-keto- $\text{PGF}_{1\alpha}$ before treatment with $\text{IL-1}\beta$ (20 ng ml^{-1}) were 0.014 ± 0.002 , 0.007 ± 0.002 and 0.021 ± 0.005 , respectively, and during treatment were 0.012 ± 0.002 , 0.007 ± 0.001 and 0.016 ± 0.002 , respectively. Therefore, $\text{IL-1}\beta$ (20 ng ml^{-1}) had no significant effects on the outputs of

Fig. 2. Mean ($\pm$ SEM, $n = 5$) outputs of (a) $\text{PGF}_{2\alpha}$, (b) PGE_2 and (c) 6-keto- $\text{PGF}_{1\alpha}$ from day 7 guinea-pig uterus superfused in calcium-depleted Krebs' solution *in vitro* in the presence (●) and absence (■) of epidermal growth factor (EGF). *Significantly ($P < 0.05$) higher than corresponding values before treatment with EGF.

prostaglandins from the day 7 guinea-pig uterus. Similar findings were obtained with the lower dose (10 ng ml^{-1}) of $\text{IL-1}\beta$.

Contractile activity

The day 7 guinea-pig uterus showed low spontaneous activity when superfused *in vitro*. Sodium nitroprusside significantly ($P < 0.05$) increased this contractile activity by mainly increasing the frequency of the contractions. After cessation of the sodium nitroprusside treatment, the spontaneous activity of the uterus was reduced. Similar findings were obtained if the myometrium alone was used (Fig. 4).

Discussion

The basal outputs of prostaglandins from day 7 guinea-pig uterus superfused *in vitro* were similar to those obtained by Naderali and Poyser (1994). However, these outputs varied twofold among the experiments. Such a variation was also reported by Naderali and Poyser (1994) and is probably due to inter-experimental variation. In the present study, the

Fig. 3. Mean ($\pm$ SEM, $n = 5$) outputs of (a) $\text{PGF}_{2\alpha}$, (b) PGE_2 and (c) 6-keto- $\text{PGF}_{1\alpha}$ from day 7 guinea-pig uterus superfused *in vitro* in the presence (●) and absence (■) of sodium nitroprusside (SNP). *Significantly ($P < 0.05$) higher than corresponding values before treatment with SNP.

outputs of $\text{PGF}_{2\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}$ were increased 2.1- and 2.3-fold, respectively, by EGF treatment. The output of PGE_2 was unaffected by EGF. $\text{PGF}_{2\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}$ are the major prostaglandins synthesized by the endometrium and myometrium, respectively (Poyser, 1983b), although the endometrium also synthesizes and secretes 6-keto- $\text{PGF}_{1\alpha}$ (Riley and Poyser, 1987a,b). Calcium is necessary for the stimulation of prostaglandin production by the guinea-pig uterus (Poyser, 1985a,b; Riley and Poyser, 1987b). However, EGF increased the output of $\text{PGF}_{2\alpha}$ from the guinea-pig uterus 1.9-fold in the absence of extracellular calcium, indicating that the calcium involved in the action of EGF has to be of intracellular origin. Notwithstanding, EGF failed to stimulate the output of 6-keto- $\text{PGF}_{1\alpha}$ from the guinea-pig uterus in the absence of extracellular calcium, indicating that extracellular calcium is required for this increase in PGI_2 production and, hence, 6-keto- $\text{PGF}_{1\alpha}$ output. These findings are in agreement with the results of a study by Poyser (1984) in which the high output of $\text{PGF}_{2\alpha}$ from day 15 (compared with day 7) guinea-pig uterus

Fig. 4. Contractile activity (mean $\pm$ SEM, $n = 5$) of day 7 guinea-pig uterus (□) and myometrium (■) superfused *in vitro* before (0–30 min), during (30–50 min) and after (50–80 min) treatment with sodium nitroprusside (SNP). ^{ab}Significantly ($P < 0.05$) higher than corresponding values before and after treatment with SNP.

superfused *in vitro* was not reduced by the absence of extracellular calcium, whereas the output of 6-keto- $\text{PGF}_{1\alpha}$ was decreased by 50% in calcium-depleted medium. These findings also support the view that the production of $\text{PGF}_{2\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}$ by guinea-pig uterus is controlled by independent processes (Riley and Poyser, 1987a, 1989, 1990). As EGF is known to stimulate PLA_2 activity and release arachidonic acid for prostaglandin synthesis (Sato *et al.*, 1997; Bany *et al.*, 1999), that findings from the present study indicate that EGF could mediate the stimulatory action of oestradiol on uterine $\text{PGF}_{2\alpha}$ production in the guinea-pig.

The outputs of $\text{PGF}_{2\alpha}$, PGE_2 and 6-keto- $\text{PGF}_{1\alpha}$ from guinea-pig uterus were not affected by $\text{IL-1}\beta$. Therefore, it is unlikely that $\text{IL-1}\beta$ mediates the stimulation of uterine $\text{PGF}_{2\alpha}$ synthesis by oestradiol in guinea-pigs. Likewise, a study in cows indicated that IL-1 is not involved in increased $\text{PGF}_{2\alpha}$ production by the endometrium at the time of luteolysis (Leung *et al.*, 2001).

The outputs of $\text{PGF}_{2\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}$ from the day 7 uterus were increased 1.7- and 2.3-fold, respectively, by the nitric oxide donor, sodium nitroprusside. PGE_2 output was not affected. Therefore, nitric oxide should also be considered as a possible mediator in the stimulatory action of oestradiol on uterine $\text{PGF}_{2\alpha}$ production in guinea-pigs. This view is supported by reports that oestradiol stimulates the expression of nitric oxide synthase (NOS) types I and III in non-pregnant sheep uterus, and type III in late pregnant rat uterus (Zhang *et al.*, 1999; Yallampalli and Dong, 2000). However, oestradiol inhibits the expression of NOS-II and total nitric oxide production in late pregnant rat uterus by a

mechanism that may involve the action of $\text{PGF}_{2\alpha}$ (Dong *et al.*, 1997).

Somewhat surprisingly, sodium nitroprusside increased by almost fourfold the contractile activity of the superfused, day 7 guinea-pig uterus. Contractile activity was increased to a similar extent by sodium nitroprusside acting on the myometrium alone, indicating that the endometrium is not required for this increase in activity. The mechanism by which sodium nitroprusside increases contractile activity is not clear, although it is probably not as a result of increased prostaglandin production. EGF increased the output of prostaglandins from the guinea-pig uterus to a similar extent as sodium nitroprusside but had no effect on the contractile activity of the uterus. Another nitric oxide donor (s-nitroso-L-cysteine) failed to alter the spontaneous activity of non-pregnant and pregnant guinea-pig myometrium, although it reduced the contractions produced by acetylcholine or oxytocin (Kuenzli *et al.*, 1996). These contradictory results may be explained by differences in the methods used. In the study of Kuenzli *et al.* (1996), the tissue was suspended in an organ bath surrounded by fluid, which allows metabolites to build up and the spontaneous activity to increase. In the present study, the guinea-pig uterus was superfused with Krebs' solution, which continually washes away metabolites and allows only low spontaneous contractile activity. Thus, on the guinea-pig uterus, nitric oxide may increase contractile activity when there is little spontaneous activity but have no effect when the spontaneous activity is high. However, other species may be different in this respect as nitric oxide donors (glyceryl trinitrate and sodium nitroprusside) reduce, but do not abolish, the high spontaneous activity of pregnant human myometrial strips *in vitro* (Norman *et al.*, 1997; Wetzka *et al.*, 2001). It is assumed that the high NOS-II expression and nitric oxide production by rat uterus during pregnancy is necessary to maintain a quiescent uterus (Dong *et al.*, 1998). Similar studies need to be performed on other species to determine the role of nitric oxide in controlling uterine activity.

Overall, the results of the present study indicate that EGF or nitric oxide may be involved in the biochemical pathways by which oestradiol stimulates increased uterine $\text{PGF}_{2\alpha}$ in guinea-pigs. In fact, both compounds might be involved, as the stimulation of prostaglandin production by the oestrogenized rat uterus by EGF is apparently dependent on the production of NOS-II and nitric oxide (Ribeiro *et al.*, 1999). EGF activates the mitogen-activated protein (MAP) kinase pathway, and it is known that oestradiol can activate the MAP kinase pathway through mobilization of EGF (Filardo *et al.*, 2000). The activation of cytosolic PLA_2 is dependent on its phosphorylation by the MAP kinase pathway (Leslie, 1997), and the stimulation of $\text{PGF}_{2\alpha}$ production in the sheep uterus by oxytocin is dependent on the activation of MAP kinases (Burns *et al.*, 2001). Therefore, further study is required to establish the precise mediators and intracellular pathways by which oestradiol acting on a progesterone-primed uterus stimulates the increase in $\text{PGF}_{2\alpha}$ production necessary for luteolysis in the guinea-pig.

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