Regulation by epidermal growth factor of prostaglandin production and cyclooxygenase activity in sensitized rat endometrial stromal cells *in vitro*

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The effect of epidermal growth factor on prostaglandin production and cyclooxygenase activity in endometrial stromal cells isolated from the uteri of ovariectomized rats sensitized for the decidual cell reaction was examined. Treatment with epidermal growth factor (40 ng ml^{-1}) for 24 h caused approximately 6.9-, 3.4-, and 4.1-fold increases in prostaglandin E_2 , prostaglandin $F_{2\alpha}$ and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ accumulation in the media of cultured cells. The increase in prostaglandin E_2 accumulation induced by epidermal growth factor was inhibited by α -amanitin ($2 \mu\text{g ml}^{-1}$), cycloheximide ($0.5 \mu\text{g ml}^{-1}$) and dexamethasone ($5 \mu\text{mol l}^{-1}$). Epidermal growth factor increased cyclooxygenase activity in the stromal cells in a time-dependent fashion and this increase in activity was also inhibited by α -amanitin, cycloheximide and dexamethasone. These results provide evidence that epidermal growth factor stimulates prostaglandin production in rat endometrial stromal cells from uteri sensitized for the decidual cell reaction through a mechanism that involves an increase in cyclooxygenase activity.

Introduction

The paracrine and autocrine effects of growth factors in the uterus in rodents may be important during implantation (Pollard, 1990). There is indirect evidence in support of this notion for some ligands of the epidermal growth factor (EGF) receptor. EGF, transforming growth factor α (TGF- α) and heparin-binding EGF, all ligands of the EGF receptor (Prigent and Lemoine, 1992), are expressed in a cell- and time-specific fashion in the uterus during the peri-implantation period (Tamada *et al.*, 1991; Johnson and Chatterjee, 1993a; Das *et al.*, 1994a; Paria *et al.*, 1994). EGF receptors have been localized to all major uterine cell types in immature rats (Lin *et al.*, 1988) and mice (Bossert *et al.*, 1990). However, during the peri-implantation period, EGF receptors have been localized to all major uterine cell types except the luminal and glandular epithelia in mice (Das *et al.*, 1994b). Furthermore, the number of EGF receptors appears to increase in the uterus during implantation (Chakraborty *et al.*, 1988; Brown *et al.*, 1989). Thus, there is an increase in the number of EGF-receptors, and three of its ligands are present in the uterus during the peri-implantation period.

There is direct evidence that EGF may play a role in blastocyst implantation. EGF can initiate implantation of embryos transferred into progesterone-primed hypophysectomized rats (Johnson and Chatterjee, 1989a). In rats in which implantation has been delayed by hypophysectomy, EGF can replace oestrogen to induce implantation provided that uterine trauma occurs (Johnson and Chatterjee, 1989b). Therefore, it is

clear that, under the conditions of these studies, EGF can replace oestrogen in the induction of implantation. However, little is known about the effect of activation of the EGF receptor signalling pathway in the endometrium during implantation.

One possible role for ligands of the EGF receptor during implantation is to modulate prostaglandin (PG) concentrations. Such a role would be important because PGs play an obligatory role in the process of implantation and decidualization in many animals (Kennedy, 1990; Smith, 1991). In support of this possible role is the observation that the inhibitory effect of indomethacin on oestriol-induced implantation can be reversed by EGF in mice in which implantation has been delayed by ovariectomy (Paria *et al.*, 1991). Further, EGF stimulates prostaglandin E_2 (PGE $_2$) and prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$) secretion by endometrial stromal cells isolated from nonpregnant mice (Paria *et al.*, 1991). However, nothing is known about the effect of EGF receptor ligands on PG production by endometrial stromal cells from the peri-implantation uterus or from uteri sensitized for the decidual cell reaction. This study was undertaken to determine the effect of EGF on PG production and cyclooxygenase activity in endometrial stromal cells isolated from rat uteri that were sensitized for the decidual reaction.

Materials and Methods

Materials

The following reagents were purchased from GIBCO (Burlington, ON): Hank's balanced salt solution (HBSS) without

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Fig. 1. Schematic representation of the sequence of s.c. hormone administration to ovariectomized rats (oestrogen, E₂; progesterone, P). Dark areas, lights-off; open areas, lights-on. Animals were killed in the morning on the equivalent of day 5 of pseudopregnancy, at which time the uteri were sensitized for decidualization.

Mg²⁺ or Ca²⁺, heat-inactivated fetal calf serum, Hepes, pancreatin, penicillin-streptomycin, fungizone, Dulbecco's modified Eagle's medium:F-12 nutrient mixture (DMEM:F12), human recombinant EGF and Dulbecco's phosphate-buffered saline (DPBS). Dispase (neutral protease from *Bacillus polymyxa* Grade II) was purchased from Mannheim Boehringer Canada (Dorval, QU). Sesame oil, oestradiol, progesterone, cycloheximide, α -amanitin, indomethacin, dexamethasone, BSA, collagenase (type-II), SDS and EDTA were purchased from Sigma Chemical Company (St Louis, MO).

PGE₂, PGF_{2 α} arachidonic acid and 13,14-dihydro-15-keto-PGF_{2 α} (PGFM) were purchased from Cayman Chemical Company (Ann Arbor, MI). Antiserum against PGFM was provided by The Upjohn Company (Kalamazoo, MI) and [³H]PGE₂, [³H]PGF_{2 α} and [³H]PGFM were obtained from Amersham (Oakville, ON).

Animals

Female Harlan Sprague-Dawley rats (200–225 g), obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN), were housed under temperature- and light-controlled conditions (14 h light:10 h dark; 12:00 h as the midpoint of the light phase) with free access to food and water. The animals were ovariectomized under ether anaesthesia and allowed at least 6 days to recover. Oestradiol and progesterone in sesame oil were administered s.c. (as outlined in Fig. 1) to obtain rats with uteri sensitized for decidualization.

Stromal cell isolation and culture

On the equivalent of day 5 of pseudopregnancy, the rats were killed by decapitation. Uterine horns were obtained, dissected free from adherent tissue and split longitudinally. Endometrial stromal cells were then isolated by enzymatic dispersion using pancreatin and dispase followed by collagenase as described by Yee and Kennedy (1991). Stromal cells pooled from 5–10 uteri were suspended in DMEM:F12 containing heat-inactivated charcoal-stripped fetal calf serum (10%, v/v), penicillin (100 units ml⁻¹), streptomycin (100 µg ml⁻¹) and fungizone (1.25 µg ml⁻¹). The cell suspension was filtered through nylon mesh (70 µm) to remove glands and clumps of epithelial cells and plated at 5 × 10⁵ cells (in 0.5 ml culture medium) in 24-well plates (Becton-Dickinson, Lincoln Park, NJ). After an initial incubation period of 2 h at 37°C under 5%

CO₂:95% air to allow differential attachment of the stromal cells, the medium was removed and replaced with serum-free medium. This was defined as 0 h of day 1 of culture. The medium was changed every 24 h.

Measurement of PG accumulation

Stromal cells were incubated in serum-free DMEM:F12 with or without various treatments for 1.5–24 h on days 1–3 of culture. At the end of this incubation period, the medium was collected for measurement of PG concentrations. After washing with DPBS, the cells were solubilized in 1 ml 1% (w/v) SDS–1 mmol EDTA l⁻¹ and the amount of protein in each well was measured using the method of Lowry *et al.* (1951) with BSA as a standard. PG content, expressed as ng mg⁻¹ of protein, was used as a measure of PG accumulation.

Cyclooxygenase activity assay

Stromal cell cyclooxygenase activity was measured using a method similar to that of Lee *et al.* (1992). After a 1.5–24 h incubation with various treatments, the media were removed and replaced with fresh media containing saturating concentrations (100 µmol l⁻¹, data not shown) of arachidonic acid. After incubation for 10 min at 37°C under 5% CO₂:95% air, the media were removed for PGE₂ measurement and the cells were retained for protein determination. Under these conditions, the amount of PGE₂ accumulated in the media reflects its synthesis from the exogenous arachidonic acid, since no PG could be detected in the media of cells incubated without arachidonic acid (data not shown). Furthermore, the amount of PGE₂ accumulated in the media was not a consequence of non-enzymatic formation, since no PG could be detected in the media of cells incubated with arachidonic acid plus indomethacin (10⁻⁵ mol l⁻¹; data not shown). Therefore, as a measure of cyclooxygenase activity, the amount of PGE₂ accumulated per mg of cellular protein in 10 min was calculated.

Radioimmunoassay of prostaglandins

PGE₂, PGF_{2 α} and PGFM in the media were measured by radioimmunoassays using previously described antibodies (PGF_{2 α} : Kennedy, 1977; PGE₂: Olson *et al.*, 1984; PGFM: Guilbault *et al.*, 1994). The assays were carried out essentially as described by Kennedy (1979). However, because prostaglandins were measured directly in the media without extraction, the same volume of media as contained in the samples was added to the standards. Preliminary work showed that the concentrations of prostaglandins measured by this method were not significantly different from those determined after extraction. Intra- and interassay coefficients of variation were less than 8% and 12%, respectively. α -Amanitin, cycloheximide, arachidonic acid and dexamethasone, at the same concentrations present in the samples, did not affect the radioimmunoassay for PGE₂.

Statistical analyses

Heterogeneity of variance was removed by logarithmically transforming all data before statistical analysis. Analysis of

Fig. 2. The effect of exposure of rat endometrial stromal cells to various concentrations of epidermal growth factor (EGF, 0–160 ng ml⁻¹) for 24 h on prostaglandin E₂ (PGE₂) accumulation in the medium on days 1 (●), 2 (■) or 3 (▲) of culture. Each point represents the mean ± SEM (*n* = 4–5).

variance was used to determine treatment effects. When a significant ($P < 0.05$) interaction was detected, Duncan's multiple range test was used for group comparisons. All statistical analyses were carried out using SAS statistical software (Cary, NC).

The data are presented as means ± SEM (*n* = 4–6) from single experiments. Each experiment was performed at least twice on separate cell preparations. Since prostaglandin concentrations in treatment groups varied between experiments, the data from separate cell preparations were not pooled. However, in all experiments, treatment effects were similar.

Results

Concentration–response relationship of EGF

After the differential attachment period, cells were cultured for 0, 24 or 48 h before the addition of various concentrations of EGF for 24 h (day 1, 2 or 3, respectively). PGE₂ accumulation was significantly ($P < 0.005$) increased at all concentrations used (Fig. 2). During maximal stimulation (≥ 20 –40 ng ml⁻¹), EGF caused approximately 2-, 2.5-, and 5-fold increases in PGE₂ accumulation on days 1, 2 and 3 of culture, respectively. Because a greater response was consistently obtained on day 3 compared with days 1 or 2, the remaining experiments were performed on day 3 of culture.

In a separate experiment, basal accumulations of PGE₂, PGF_{2α} and PGFM on day 3 of culture were 60.7 ± 9.2 , 26.2 ± 0.6 and 14.4 ± 1.8 ng mg⁻¹ (24 h)⁻¹ (*n* = 5), respectively. Exposure to EGF (40 ng ml⁻¹) increased PGE₂, PGF_{2α} and PGFM accumulation to 418 ± 56 , 88.0 ± 7.4 and 59.8 ± 6.1 , respectively. Analysis of variance of the log-transformed data revealed a significant ($P < 0.01$) interaction between prostaglandin type and EGF treatment, brought about

Fig. 3. The effect of exposure of rat endometrial stromal cells to medium in the presence (■) or absence (●) of epidermal growth factor (EGF) (40 ng ml⁻¹) for various periods (1.5–24 h) on prostaglandin E₂ (PGE₂) accumulation in the medium on day 3 of culture. Each point represents the mean ± SEM (*n* = 4–5). *Significant differences between control and EGF-treated cells ($P < 0.05$).

because the increase in PGE₂ in response to EGF was greater than those of PGF_{2α} and PGFM, which were similar. PGE₂ accumulation was significantly greater ($P < 0.01$) than that of PGF_{2α} and PGFM for both control and EGF-treated cells.

Time-course of the effect of EGF on PGE₂ accumulation and cyclooxygenase activity

Stromal cells were incubated with or without EGF (40 ng ml⁻¹) for 1.5, 3, 6, 12, or 24 h on day 3 of culture, and PGE₂ accumulation in the media and cyclooxygenase activity in the cells were measured. There was a significant increase ($P < 0.001$) in PGE₂ accumulation in the media after 3 (2.7-fold), 6 (5-fold), 12 (5-fold) and 24 (4.6-fold) h exposure of the cells to EGF, compared with control cells, but not after 1.5 h exposure (Fig. 3). Similarly, there was a significant increase ($P < 0.001$) in cyclooxygenase activity in the cells after 3 (2.5-fold), 6 (3-fold), 12 (4.3-fold) and 24 (4.5-fold) h exposure to EGF, compared with control cells, but not after 1.5 h exposure (Fig. 4).

Effects of inhibitors of protein and mRNA synthesis on PGE₂ accumulation and cyclooxygenase activity

Cycloheximide (0.5 μg ml⁻¹) and α-amanitin (2 μg ml⁻¹) were used to determine whether the EGF-induced increases in PGE₂ accumulation and cyclooxygenase activity require protein synthesis and transcription, respectively. Cells were incubated with or without the inhibitor in the presence or absence of EGF (40 ng ml⁻¹) on day 3 of culture. After exposure for 24 h, PGE₂ accumulation in the media and cyclooxygenase activity in the cells were measured. For both PGE₂ accumulation and cyclooxygenase activity (Table 1), analysis of variance

Fig. 4. The effect of exposure of rat endometrial stromal cells to medium containing (■) and not containing (●) epidermal growth factor (EGF) (40 ng ml⁻¹) for various periods (1.5–24 h) on cyclooxygenase activity on day 3 of culture. Each point represents the mean $\pm$ SEM ($n = 4-5$). *Significant differences between control and EGF-treated cells ($P < 0.05$).

Table 1. The effects of exposure of rat endometrial stromal cells to cycloheximide (0.5 μ g ml⁻¹) or α -amanitin (2 μ g ml⁻¹) for 24 h in the presence or absence of epidermal growth factor (EGF; 40 ng ml⁻¹) on prostaglandin E₂ (PGE₂) accumulation in the medium and cyclooxygenase activity on day 3 of culture^a

Treatment	PGE ₂ (ng mg ⁻¹ protein (24 h) ⁻¹)	Cyclooxygenase activity (ng PGE ₂ mg ⁻¹ protein (10 min) ⁻¹)
Control	16.9 $\pm$ 0.9 ^b	41.4 $\pm$ 2.4 ^b
EGF	63.0 $\pm$ 6.9 ^c	329.0 $\pm$ 30.0 ^c
Cyclohexamide	9.6 $\pm$ 0.6 ^d	27.4 $\pm$ 4.9 ^d
EGF and cyclohexamide	16.8 $\pm$ 1.3 ^b	48.6 $\pm$ 4.2 ^b
α -Amanitin	11.5 $\pm$ 2.0 ^d	55.6 $\pm$ 5.9 ^b
EGF and α -amanitin	18.8 $\pm$ 2.2 ^b	89.0 $\pm$ 12.9 ^c

^aValues represent the means $\pm$ SEM (PGE₂ accumulation, $n = 4-5$; cyclooxygenase activity, $n = 5$).

^{bcd}Values with the same letters within the same column are not significantly different ($P > 0.05$).

revealed significant ($P < 0.02$) interactions between the effects of cycloheximide or α -amanitin and EGF. The stimulatory effect of EGF, when added alone, was greatly reduced in the presence of either cycloheximide or α -amanitin. In the absence of EGF, cycloheximide significantly decreased ($P < 0.05$) PGE₂ accumulation and cyclooxygenase activity to values below those of controls. In the presence of α -amanitin, basal PGE₂ accumulation was significantly reduced ($P < 0.05$) compared with control values.

Table 2. The effect of exposure of rat endometrial stromal cells to dexamethasone (5 μ mol l⁻¹) for 24 h in the presence or absence of epidermal growth factor (EGF; 40 ng ml⁻¹) on prostaglandin E₂ (PGE₂) accumulation in the medium and cyclooxygenase activity on day 3 of culture^a

Treatment	PGE ₂ (ng mg ⁻¹ protein (24 h) ⁻¹)	Cyclooxygenase activity (ng PGE ₂ mg ⁻¹ protein (10 min) ⁻¹)
Control	43.3 $\pm$ 4.5 ^b	62.1 $\pm$ 6.5 ^b
EGF	342.0 $\pm$ 43.0 ^c	266.0 $\pm$ 21.0 ^c
Dexamethasone	9.93 $\pm$ 1.4 ^d	38.2 $\pm$ 11.0 ^d
EGF and dexamethasone	19.1 $\pm$ 2.4 ^d	90.6 $\pm$ 9.5 ^b

^aValues represent the means $\pm$ SEM ($n = 5-6$).

^{bcd}Values with the same letters within a column are not significantly different ($P > 0.05$).

Effect of dexamethasone on PGE₂ accumulation and cyclooxygenase activity

Cells were incubated with or without dexamethasone (5 μ mol l⁻¹) in the presence or absence of EGF (40 ng ml⁻¹) for 24 h on day 3 of culture. For both PGE₂ accumulation and cyclooxygenase activity (Table 2), analyses of variance indicated significant interactions ($P < 0.0001$) between the effects of EGF and dexamethasone. Dexamethasone alone reduced PGE₂ accumulation and cyclooxygenase activity compared with controls, and greatly reduced the stimulatory effects of EGF on both responses.

Discussion

The study reported here showed that EGF stimulates the accumulation of PGE₂ and PGF_{2 α} in the media of cultured endometrial stromal cells isolated from rat uteri sensitized for the decidual cell reaction. In mammalian cells, prostaglandins of the 2-series are synthesized from arachidonic acid through the sequential actions of phospholipases, cyclooxygenase and several prostaglandin synthases (Smith *et al.*, 1991). Therefore, in the present study, EGF may have stimulated prostaglandin production in the stromal cells by increasing the activity of one or more of these enzymes. The mobilization of arachidonic acid from membrane lipids through the activation of phospholipases has been suggested to be the rate-limiting step of prostaglandin production in mammalian cells (Lapetina, 1982). Since the addition of exogenous arachidonic acid caused an increase in the accumulation of PGE₂ in the media of stimulated and nonstimulated cells, it is apparent that the mobilization of arachidonic acid is a rate-limiting step in the synthesis of prostaglandins by cultured endometrial stromal cells. However, the extent of net prostaglandin production in several non-uterine cell types depends on the level of expression of cyclooxygenase (DeWitt, 1991). The level of cyclooxygenase expression is important because it is inactivated during catalysis (Egan *et al.*, 1976) and thus must be replaced to sustain prostaglandin production. In this study, EGF caused a

time-dependent increase in cyclooxygenase activity, as indicated by an increase in PGE_2 accumulation in the media in the presence of saturating amounts of arachidonic acid in 10 min. It is notable that studies on other cell types have shown that EGF can also stimulate cyclooxygenase activity (Yokota *et al.*, 1986; Pash and Bailey, 1988). A time-dependent increase in PGE_2 accumulation in the media of the endometrial stromal cells was paralleled by an increase in cyclooxygenase activity. One interpretation of the results of the study reported here is that EGF may cause an increase in prostaglandin accumulation in the media of cultured endometrial stromal cells through mechanisms that include an increase in cyclooxygenase activity. Alternatively, the increase in prostaglandin accumulation brought about by EGF may be due to an increase in phospholipase activity, and a basal amount of cyclooxygenase activity may be sufficient.

Since cyclooxygenase is inactivated during catalysis, an increase in its activity in the endometrial stromal cells was probably the result of increased expression of the gene encoding cyclooxygenase. In this study, the EGF-induced increase in both PGE_2 accumulation and cyclooxygenase activity were both sensitive to α -amanitin and cycloheximide, thus showing their dependence on transcription and translation. Two isozymes of cyclooxygenase, cyclooxygenase-1 and cyclooxygenase-2, have been reported in rats (Feng *et al.*, 1993), mice (DeWitt *et al.*, 1991; Kujubu *et al.*, 1991) and humans (Hla and Neilson, 1992; Takahashi *et al.*, 1992). Although the abundance of mRNA of both cyclooxygenase-1 and cyclooxygenase-2 can be increased by agents such as serum, phorbol esters and cAMP, those of cyclooxygenase-1 are increased to a lesser extent, or not at all (DeWitt *et al.*, 1991; Simmons *et al.*, 1991; O'Banion *et al.*, 1992; Herschman *et al.*, 1993). In addition, expression of the gene encoding cyclooxygenase-2, but not cyclooxygenase-1, is negatively regulated by glucocorticoids (O'Banion *et al.*, 1992). It has been shown that EGF can cause a specific increase in the expression of cyclooxygenase-2 in 3T3 cells (Kujubu *et al.*, 1991). However, further studies are required to determine the effect of EGF on expression of genes encoding cyclooxygenase-1 and cyclooxygenase-2 in endometrial stromal cells.

PGE_2 and $\text{PGF}_{2\alpha}$ are synthesized from PGH_2 through the actions of synthases (Smith *et al.*, 1991). Therefore, the observed increases in the accumulation of these biologically active prostaglandins by the cultured endometrial stromal cells in response to EGF may involve the modulation of the activity of these synthases. Since EGF stimulated a greater increase in PGE_2 accumulation than $\text{PGF}_{2\alpha}$, it is possible that EGF preferentially stimulated PGE_2 synthase over $\text{PGF}_{2\alpha}$ synthase. However, further studies are required to determine whether EGF exerts such an effect.

The increases in PGE_2 and $\text{PGF}_{2\alpha}$ accumulation in the media of the cultured endometrial stromal cells may be brought about by an EGF-induced decrease in prostaglandin catabolism. PGFM and PGEM are formed from $\text{PGF}_{2\alpha}$ and PGE_2 by the sequential actions of two key enzymes of prostaglandin catabolism, prostaglandin dehydrogenase and $\Delta 13$ -14-reductase (Samuelsson *et al.*, 1971). Since PGFM is the more stable metabolite, the effect of EGF on PGFM accumulation in the media of cultured endometrial stromal cells was measured. The presence of PGFM in the media of the uterine stromal cells

detected in this study strongly suggests that the stromal cells contain these enzymes. Furthermore, an increase in $\text{PGF}_{2\alpha}$ accumulation in response to EGF was accompanied by a similar increase in PGFM accumulation, implying that EGF does not cause a decrease in prostaglandin catabolism in the endometrial stromal cells. If this is the case, then the amount of prostaglandin accumulation measured in the study reported here provides an appropriate index of prostaglandin production.

Regulation of endometrial stromal cell prostaglandin biosynthesis by factors other than EGF have been described. For example, interleukin 1 can induce prostaglandin secretion by endometrial stromal cells isolated from mice (Jacobs and Carson, 1993). In addition, oestrogen and progesterone increase prostaglandin production by the uteri of ovariectomized rats (Castracane and Jordan, 1975). The relationship between steroids, interleukin 1 and EGF-receptor ligands in the regulation of endometrial prostaglandin production during implantation is unknown; the action of steroids in the uterus may be mediated by locally produced endometrial growth factors (Huet-Hudson *et al.*, 1990a,b; Nelson *et al.*, 1992). Therefore, it is likely that a complex network of signals is responsible for modulating prostaglandin production in the endometrium during implantation.

In summary, the present study shows that EGF stimulates prostaglandin production and cyclooxygenase activity in endometrial stromal cells isolated from rat uteri sensitized for the decidual cell reaction. These effects were inhibited by dexamethasone and inhibitors of transcription and translation. Therefore, this study provides evidence that EGF causes an increase in the expression of cyclooxygenase in endometrial stromal cells.

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