Mitogen-activated protein kinase activation and regulation of cyclooxygenase 2 expression by platelet-activating factor and hCG in human endometrial adenocarcinoma cell line HEC-1B

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The activation of mitogen-activated protein kinase (MAP kinase) and the regulation of cyclooxygenase 2 (COX-2) were investigated in the human endometrial adenocarcinoma cell line HEC-1B by treatment with platelet-activating factor (PAF) and hCG. Pre-treatment of the cells with both oestradiol and medroxyprogesterone acetate was required for MAP kinase activation and COX-2 expression to respond to PAF and hCG. PAF-induced MAP kinase activation was sensitive to MAP kinase kinase (MEK) inhibitor, PD098059, and phosphatidylinositol-3-OH kinase (PI3K) inhibitor, wortmannin. In contrast, hCG-induced MAP kinase activation was sensitive to PD098059 and protein kinase A inhibitor, H-89, but not to wortmannin. PAF-induced COX-2 expression was insensitive to PD098059 but sensitive to wortmannin, whereas hCG-induced COX-2 expression was sensitive to PD098059 and H-89 but insensitive to wortmannin. 8-(4-chlorophenylthio)-cAMP, a potent cAMP analogue, induced activation of MAP kinase and expression of COX-2. These results indicate that MAP kinase is activated with PAF and hCG in HEC-1B cells. In addition, COX-2 expression is stimulated through the MAP kinase activation pathway with hCG and the wortmannin sensitive pathway with PAF in HEC-1B cells. These results also imply that protein kinase A remains upstream of hCG-induced activation of MAP kinase in HEC-1B cells.

Introduction

Decidualization of the endometrium involves extensive morphological and physiological changes in the endometrial tissue in preparation for implantation of the blastocyst. There is strong evidence for an obligatory role for prostaglandins in the initiation and maintenance of the decidual cell response. The role of prostaglandins was demonstrated in experiments in which several prostaglandins, including PGE₂ and PGF_{2α}, induced decidualization in hormonally primed animals (Kennedy, 1985). The inflammatory response is a characteristic feature during decidualization (McRae and Heap, 1988) and prostaglandins play a role in modulating this response. There is increasing evidence that prostaglandin production is necessary for the normal intercellular homeostatic mechanism, particularly in cells related to the immune system (Kelly, 1994). PGE₂ inhibits the function of many cells of the immune system (Kelly, 1994). Monocytes produce significant amounts of PGE and thus a negative feedback control may operate during inflammation to limit cell activation and cytokine secretion (Kelly, 1994).

The discovery of cyclooxygenase 2 (COX-2) contributed significantly to the current understanding of prostaglandin

synthesis. COX-2 is a 70 kDa protein induced by chorionic gonadotrophin, cytokines and tumour promoters (Smith and Dewitt, 1996); COX-1 is the non-inducible counterpart. Arachidonic acid is the principal substrate of the COX protein, which is a rate-limiting enzyme in prostaglandin biosynthesis (Smith and Dewitt, 1996). Prostaglandin receptors are expressed in the peri-implantation endometrium (Yang *et al.*, 1997) and COX-2 is expressed during implantation in the mouse endometrium (Chakraborty *et al.*, 1996).

Platelet-activating factor (PAF), a potent lipid mediator, is produced by the embryo (Collier *et al.*, 1988) and uterus and is thought to be an important mediator of maternal recognition of pregnancy (O'Neill *et al.*, 1987). In humans, PAF is present in the luteal phase endometrium (Alecozay *et al.*, 1989). PAF has been correlated with generation of inositol phosphate in endometrial tissue (Battye *et al.*, 1992), which implies it is capable of activating phospholipase C. In guinea-pigs, PAF treatment significantly increased the output of prostaglandins in the late luteal phase *in vitro* (Norman and Poyser, 1992), and this was dependent upon the mobilization of intracellular calcium. There is no evidence for PAF-induced COX-2 expression in endometrial cells.

hCG is a glycoprotein hormone synthesized by the placental trophoblast. Although the physiological relevance of the action of hCG in the endometrium is not well

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established, there is evidence that gonadotrophins, including hCG, enhance stromal decidualization (Han *et al.*, 1995a). hCG also upregulates COX-2 expression during differentiation of human endometrial stromal cells into decidua (Han *et al.*, 1995b). hCG is present in plasma after initiation of implantation of the blastocyst and hCG receptors are present in endometrial cells (Reshef *et al.*, 1990). Therefore, it is possible that hCG plays an important role in the endometrium in early pregnancy, in addition to its role in maintaining the corpus luteum steroidogenesis of the ovary.

Most of the prostaglandin in the endometrium is produced by glandular epithelial cells (Kelly, 1994). In the human endometrium, complex interactions occur within a network of epithelial, stromal, endothelial and lymphoid cells. Since uterine luminal epithelial cells appear to mediate a decidualizing signal through their basal cell surface in response to the blastocyst, it has been hypothesized that prostaglandins released by the epithelial cells may act on the underlying stromal cells. In the present study, PAF and hCG-induced COX-2 expression was examined in the HEC-1B cell line to investigate the potential role of prostaglandins of epithelial origin. This cell line retains many of the characteristics of endometrial epithelial cells and is a substrain of the HEC-1A cell line. The original cell line (HEC-1A) was derived from endometrial adenocarcinoma obtained from human uterus. Oestradiol receptors are present in HEC-1B cells (Satyaswaroop *et al.*, 1978) and exposure of the cells to 10 or 100 nmol oestradiol l⁻¹ for 48 h modulates PAF-evoked phospholipase D activity in this cell line (Ahmed *et al.*, 1995). Progesterone receptors are also present in HEC-1 cells (Oyama and Izutsu, 1992). The presence of mRNA encoding the PAF receptor was demonstrated using RT-PCR in the HEC-1B cell line (Ahmed *et al.*, 1994).

In the present study, the PAF- and hCG-mediated signalling pathway of COX-2 expression was investigated using an *in vitro* model in which HEC-1B cells were exposed to oestradiol and medroxyprogesterone acetate. Of particular interest was the role of MAP kinase in COX-2 expression.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: fetal bovine serum (FBS), JRH Biosciences (Lenexa, KS); [³²P]ATP and ¹²⁵I-labelled protein A, DuPont-New England Nuclear (Wilmington, DE); PAF-16, Calbiochem (La Jolla, CA); hCG, Mochida Pharmaceutical Co. Ltd (Tokyo, Japan); oestradiol, medroxyprogesterone acetate and 8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate (CPT-cAMP), Sigma Chemical Co. (St Louis, MO); minimal essential medium (MEM) and sodium pyruvate, GIBCO BRL (Gaithersburg, MD); PD098059, RBI (Natick, MA); wortmannin, Wako Pure Chemical Industries (Osaka); H-89, Seikagaku Corporation (Tokyo, Japan); anti-cyclooxygenase-2 antibody, anti-pan extracellular signal-regulated kinase (ERK) monoclonal antibody and anti-ERK-2 monoclonal antibody, Transduction Laboratories (Lexington, KY); rabbit anti-mouse IgG1, Zymed Laboratories (South San

Francisco, CA). Myelin basic protein (MBP) was purified from bovine brain (Deibler *et al.*, 1972).

Cell culture

HEC-1B cell line was obtained from ATCC (Bethesda, MD). Cells were cultured in MEM supplemented with 10% FBS, 1 mmol sodium pyruvate l⁻¹ and 60 mg kanamycin l⁻¹. Cell cultures were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The medium was replaced every 3–4 days and the cells were split every 4–5 days using trypsin (0.05% v/v) in Mg²⁺- and Ca²⁺-free PBS. Cells were plated in dishes of 35 mm diameter for the MAP kinase assay, and in dishes of 100 mm diameter for the immunoblot analysis, and were cultured for 3–5 days to reach around 60% confluency. Oestradiol, medroxyprogesterone acetate or both were added to the medium for 3–5 days as indicated.

Assay for MAP kinase

The cultured cells were washed once with Krebs–Ringer Hepes buffer (KRH buffer) containing 128 mmol NaCl l⁻¹, 5 mmol KCl l⁻¹, 1 mmol sodium phosphate l⁻¹, 1.2 mmol MgSO₄ l⁻¹, 10 mmol glucose l⁻¹, 2.7 mmol CaCl₂ l⁻¹ and 20 mmol Hepes l⁻¹ (pH 7.4) and preincubated at 37°C for 1 h in the same buffer. The cells were incubated at 37°C for the specified time without (control) or with the specified test agents in KRH buffer. After incubation, the medium was removed quickly by aspiration, and the cells were frozen in liquid nitrogen and stored at –80°C until the assay was performed.

The frozen cells were scraped off the dishes and solubilized in 0.15 ml of 50 mmol Hepes l⁻¹ (pH 7.4), 0.1% (v/v) Triton-X100, 4 mmol EGTA l⁻¹, 10 mmol EDTA l⁻¹, 15 mmol Na₄P₂O₇ l⁻¹, 100 mmol β-glycerophosphate l⁻¹, 25 mmol NaF l⁻¹, 0.1 mmol leupeptin l⁻¹, 75 μmol pepstatin A l⁻¹, 1 mmol dithiothreitol l⁻¹, 1 mmol (*p*-amidinophenyl)-methanesulfonyl fluoride hydrochloride l⁻¹, 1 mmol Na₃VO₄ l⁻¹ and 100 nmol calyculin A l⁻¹ (solubilization solution). The procedures for the treatment of cells were carried out at 0–4°C. After sonication (Sonifier 250; Branson), the insoluble material was removed by centrifugation at 15 000 g for 5 min. The extracts were treated with SDS sample buffer (Laemmli, 1970) and boiled for 3 min. Samples containing the same amount of protein (10–15 μg of protein) were assayed for MAP kinase by SDS-PAGE with MBP as a substrate as described by Kurino *et al.* (1995). After the gel was dried, the amount of ³²P incorporation into MBP at the position of MAP kinase in the gel was quantified with a Bio-imaging analyser (BA 100; Fujifilm, Tokyo).

For immunoblot analysis of MAP kinase, a similar procedure as described below for COX-2 was used, with the exception that anti-pan ERK and anti-ERK-2 antibodies (1:400 dilution) were used and the anti-mouse IgG1 antibody was omitted.

Assay for COX-2 expression

HEC-1B cells were cultured for 3–5 days in medium without hormones or with 10 nmol oestradiol l⁻¹, 1 μmol

medroxyprogesterone acetate 1^{-1} or a combination of both hormones. Cells were incubated for 12 h in the absence or presence of $1 \mu\text{mol PAF } 1^{-1}$ or $0.15 \text{ iu hCG ml}^{-1}$. After incubation, the medium was removed quickly by aspiration and the cells were frozen in liquid nitrogen and stored at -80°C until assayed.

The frozen cells were scraped from the dishes and solubilized in 0.3 ml solubilization solution, as described for the MAP kinase assay with the exception that Na_3VO_4 and calyculin A were omitted. The cells were sonicated and centrifuged as described earlier and the solubilized protein prepared in this way was designated as the Triton-X100 soluble fraction. The insoluble pellet fraction remaining after centrifugation was homogenized in 0.1 ml solubilization solution and centrifuged, and the supernatant obtained was designated as the Triton-X100 insoluble fraction. Samples ($50 \mu\text{g}$) of these proteins were then subjected to SDS-PAGE in 10% (w/v) acrylamide and transferred electrophoretically at 75 V for 2 h to a nitrocellulose membrane. After incubating the membrane in 4.5% (w/v) skimmed milk-Tris buffered saline (TBS; 10 mmol Tris-buffered isotonic saline 1^{-1} , pH 7.4) for 2 h to block non-specific binding sites, the membrane was incubated at 4°C overnight with the monoclonal anti-COX-2 antibody at a dilution of 1:125. After washing, the membrane was incubated with the anti-mouse IgG1 antibody at a dilution of 1:200 for 2 h and finally with ^{125}I -labelled protein A at room temperature for 1 h. After the membrane was dried, incorporation of ^{125}I into COX-2 was quantified using a Bio-imaging analyser (BA100; Fujifilm, Tokyo).

Other procedures

Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard.

Statistical analyses

Values are expressed as mean $\pm$ SE. For multiple comparisons, the significance of the difference was calculated by one-way ANOVA plus Duncan's multiple-range test and comparisons between two experimental groups were made by the unpaired Student's *t* test. *P* values < 0.05 were considered significant.

Results

MAP kinase in HEC-1B cells

MAP kinase represents a family of proteins that includes 44 kDa ERK-1 and 42 kDa ERK-2 in various types of cell. HEC-1B cells contained only one band of 42 kDa by SDS-PAGE, which was recognized with both the anti-pan ERK and the anti-ERK-2 antibodies (Fig. 1a). This band also migrated at the same position as ERK-2 from the positive control brain extract (Fig. 1a). Therefore, only ERK-2 of 42 kDa was examined in the subsequent experiments.

Requirement of oestrogen and progesterone for PAF- and hCG-induced activation of MAP kinase and stimulation of COX-2 expression

HEC-1B cells were cultured for 3–5 days in the absence of hormones or in the presence of $10 \text{ nmol oestradiol } 1^{-1}$ and $1 \mu\text{mol medroxyprogesterone acetate } 1^{-1}$ alone or in combination to examine the effects of oestrogen and progesterone on MAP kinase activation. After culture, cells were stimulated for 10 min with $1 \mu\text{mol PAF } 1^{-1}$ or $0.15 \text{ iu hCG ml}^{-1}$. Addition of both oestradiol and medroxyprogesterone acetate was required for activation of MAP kinase in HEC-1B cells with PAF and hCG (Fig. 1b,c), although a small and significant increase was obtained with PAF alone in the cells pretreated with medroxyprogesterone acetate alone (Fig. 1c). In the non-stimulated cells, oestradiol or medroxyprogesterone acetate alone or in combination did not result in an increase in MAP kinase activity (data not shown).

The effects of oestrogen and progesterone on COX-2 expression were examined under the same conditions as for MAP kinase activation (Fig. 2). The Triton-X100 soluble fraction was used for all COX-2 immunoblot analyses. A small amount of COX-2 was present in the Triton-X100 insoluble fraction by centrifugation. The percentage of COX-2 in the Triton-X100 insoluble fraction was about 14% of the Triton-X100 soluble fraction by treatment with hCG (data not shown). Addition of both oestradiol and medroxyprogesterone acetate was required for stimulation of COX-2 expression with PAF and hCG, although a small increase in COX-2 expression was observed in the cells pretreated with oestradiol alone (Fig. 2b). In non-stimulated cells, oestradiol or medroxyprogesterone alone caused a small increase in COX-2 expression, but in combination the hormones caused a significant increase of 60% (data not shown).

Therefore, the subsequent experiments were performed using cells pretreated for 3–5 days with both oestradiol and medroxyprogesterone acetate.

Time course and dose dependence of PAF-stimulated MAP kinase activation

The time course of PAF-induced MAP kinase activation is shown (Fig. 3a,b). HEC-1B cells were incubated with $1 \mu\text{mol PAF } 1^{-1}$ for up to 30 min. MAP kinase activation was measured from 3 min to 30 min after stimulation with PAF (Fig. 3a). The maximum activity of MAP kinase was obtained at 10 min after stimulation with PAF and then decreased gradually (Fig. 3b).

HEC-1B cells were treated for 10 min with PAF at concentrations ranging from $10 \text{ nmol } 1^{-1}$ to $10 \mu\text{mol } 1^{-1}$ to determine whether the effect of PAF on MAP kinase activation was dose-dependent. Maximum MAP kinase activation was obtained with $1 \mu\text{mol PAF } 1^{-1}$. A higher concentration of PAF ($10 \mu\text{mol } 1^{-1}$) inhibited MAP kinase activity (Fig. 3c).

Time course and dose dependence of hCG-stimulated MAP kinase activation

A rapid but transient activation of MAP kinase was also observed in response to hCG during incubation of the HEC-1B cells for up to 30 min. Maximum activation of MAP kinase

Fig. 1. Occurrence of mitogen-activated protein kinase (MAP kinase) in HEC-1B cells and the requirement of oestradiol and medroxyprogesterone acetate for the activation of MAP kinase with platelet-activating factor (PAF) and hCG. HEC-1B cells were grown for 3–5 days in culture medium without hormones or with 10 nmol oestradiol l^{-1} or 1 μ mol medroxyprogesterone acetate l^{-1} alone or in combination. The cells were incubated for 10 min in the absence (control) or the presence of 1 μ mol PAF l^{-1} or 0.15 iu hCG ml^{-1} . (a) Immunoblot analysis of the cell extracts from HEC-1B cells and the homogenate of rat hippocampus (brain) with the anti-pan extracellular signal-regulated kinase (ERK) and anti-ERK-2 antibodies. (b) A representative autoradiogram of MAP kinase activation. (c) Effects of oestradiol and medroxyprogesterone acetate pretreatment on MAP kinase activation. □, control; ▨, PAF; ■, hCG. Intensity values of control samples of cells treated with oestradiol, medroxyprogesterone acetate, or oestradiol plus medroxyprogesterone acetate relative to no hormones (none) (arbitrarily set at 100) were 105 ± 6 , 107 ± 5 , and 110 ± 10 , respectively. The data are expressed as a percentage of the control value (Krebs–Ringer Hepes buffer only at 10 min) which was designated as 100%. Values are mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus control.

Fig. 2. Requirement of oestradiol and medroxyprogesterone acetate for stimulation of expression of cyclooxygenase 2 (COX-2) protein with platelet-activating factor (PAF) and hCG. HEC-1B cells were grown for 3–5 days in culture medium without hormones or with 10 nmol oestradiol l⁻¹ or 1 µmol medroxyprogesterone acetate l⁻¹ alone or in combination. Cells were incubated for 12 h in the absence (control) or presence of 1 µmol PAF l⁻¹ or 0.15 iu hCG ml⁻¹. (a) A representative autoradiogram of the COX-2 immunoblot. (b) PAF and hCG-induced COX-2 expression by pretreatment with oestradiol and medroxyprogesterone acetate. □, control; ▨, PAF; ■, hCG. Intensity values of control samples of cells treated with oestradiol, medroxyprogesterone acetate, or oestradiol plus medroxyprogesterone acetate relative to no hormones (none) (arbitrarily set at 100) were 121 ± 3.1, 115 ± 5, and 160 ± 3, respectively. The data are expressed as a percentage of the control value which was designated as 100%. Values are mean ± SE (n = 3). *P < 0.05, **P < 0.01 versus control.

Fig. 3. Time course and concentration dependence of mitogen-activated protein kinase (MAP kinase) activation with platelet-activating factor (PAF). (a) A representative autoradiogram of the time course of MAP kinase activation induced by $1 \mu\text{mol PAF l}^{-1}$. (b) MAP kinase activation quantified by a Bio-imaging analyser. Values are expressed as a percentage of the control value (without PAF for zero time) which was designated as 100%. (c) Cells were incubated without (control) or with various concentrations of PAF for 10 min. MAP kinase activation is expressed as a percentage of the control value which was designated as 100%. Values are mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus control.

with $0.15 \text{ iu hCG ml}^{-1}$ was obtained at 10 min after stimulation with hCG, and then decreased gradually to the control value by 30 min (Fig. 4a,b).

The dose-response curve of MAP kinase activation with hCG was examined at concentrations from 0.015 to 1.5 iu ml^{-1} . Maximum MAP kinase activation was obtained with $0.15 \text{ iu hCG ml}^{-1}$ (Fig. 4c). A higher concentration of hCG (1.5 iu ml^{-1}) inhibited MAP kinase activation (Fig. 4c).

Fig. 4. Time course and concentration dependence of mitogen-activated protein kinase (MAP kinase) activation with hCG. (a) A representative autoradiogram of the time course of MAP kinase activation induced by $0.15 \text{ iu hCG ml}^{-1}$. (b) MAP kinase activation quantified by a Bio-imaging analyser. Values are expressed as a percentage of the control value which was designated as 100%. (c) Cells were incubated without (control) or with various concentrations of hCG for 10 min. MAP kinase activation is expressed as a percentage of the control value which was designated as 100%. Values are mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus control.

Effects of PD098059 and wortmannin on PAF-mediated MAP kinase activation and COX-2 expression

PD098059 is a selective inhibitor of the phosphorylation and kinase activity of MAP kinase kinase 1 (MEK-1) and MEK-2 (Alessi *et al.*, 1995), the upstream kinases responsible for the phosphorylation of both tyrosine and threonine residues of ERK-1 and ERK-2. Pretreatment of the cells with

Fig. 5. Effects of PD098059 and wortmannin on platelet-activating factor (PAF)-induced mitogen-activated protein kinase (MAP kinase) activation and COX-2 expression. (a) Effects of the inhibitors on PAF-induced MAP kinase activation. Cells were preincubated for 1 h in Krebs-Ringer Hepes (KRH) buffer and inhibitor (75 μmol PD098059 l^{-1} or 200 nmol wortmannin l^{-1}) was added for the last 30 min of preincubation. The cells were then incubated for 10 min without (control) or with 1 μmol PAF l^{-1} in the presence of the inhibitor in KRH buffer. The data are expressed as a percentage of the control value which was designated as 100%. Values are mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus control. (b) A representative autoradiogram showing the effects of the inhibitors on PAF-stimulated COX-2 expression. The cells were incubated for 12 h without (control) or with 1 μmol PAF l^{-1} in the presence or absence of the inhibitors. (c) Effects of the inhibitors on PAF-induced COX-2 expression. The data are expressed as a percentage of the control value which was designated as 100%. Values are mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus control.

75 μmol PD098059 l^{-1} resulted in complete inhibition of MAP kinase activation (Fig. 5a), but had no effect on COX-2 expression (Fig. 5b,c). Wortmannin is a fungal metabolite that has been characterized as an inhibitor of phos-

phatidylinositol-3-OH kinase (PI3K) at nanomolar concentrations (Grammer and Blenis, 1997). Pretreatment of HEC-1B cells with 200 nmol wortmannin l^{-1} resulted in complete inhibition of PAF-mediated MAP kinase activation

Fig. 6. Effects of PD098059, wortmannin and H-89 on hCG-induced mitogen-activated protein kinase (MAP kinase) activation and cyclooxygenase 2 (COX-2) expression and the effect of 8-(4-chlorophenylthio)-cAMP (CPT-cAMP). (a) Effects of the inhibitors on hCG-induced MAP kinase activation and effect of CPT-cAMP on MAP kinase activity. The cells were preincubated for 1 h in Krebs–Ringer Hepes buffer and inhibitor ($75 \mu\text{mol PD098059 l}^{-1}$, $200 \text{ nmol wortmannin l}^{-1}$ or $10 \mu\text{mol H-89 l}^{-1}$) was added for the last 30 min of preincubation. The cells were then incubated for 10 min without (control) or with $0.15 \text{ iu hCG ml}^{-1}$ in the presence of the inhibitor. In the case of CPT-cAMP, after preincubation in KRH buffer, cells were incubated with $1 \text{ mmol CPT-cAMP l}^{-1}$ for 10 min. The data are expressed as a percentage of the control value which was designated as 100%. Values are mean $\pm$ SE ($n = 3$). $*P < 0.05$, $**P < 0.01$ versus control. (b) A representative autoradiogram showing the effects of PD098059 and wortmannin on hCG-stimulated COX-2 expression. The cells were incubated for 12 h without (control) or with $0.15 \text{ iu hCG ml}^{-1}$ in the presence or absence of inhibitor. (c) Statistical analyses of the data shown in (b) and the effects of H-89 and CPT-cAMP. The effect of H-89 on hCG-induced COX-2 expression was examined in the same way as for the other inhibitors. Cells were also incubated with $1 \text{ mmol CPT-cAMP l}^{-1}$ for 12 h. The data are expressed as a percentage of the control value which was designated as 100%. Values are mean $\pm$ SE ($n = 3$). $*P < 0.05$, $**P < 0.01$ versus control.

(Fig. 5a). Addition of 200 nmol wortmannin l^{-1} and 1 μ mol PAF l^{-1} inhibited both PAF-mediated MAP kinase activation and COX-2 expression (Fig. 5a,c). These results indicate that activation of MAP kinase and stimulation of COX-2 expression are regulated by at least two different pathways.

Effects of PD098059, wortmannin and H-89 on hCG-mediated MAP kinase activation and COX-2 expression

The effects of PD098059, wortmannin and H-89 on hCG-induced MAP kinase activation and COX-2 expression were examined (Fig. 6). H-89 is a specific inhibitor of protein kinase A (Chijiwa *et al.*, 1990). hCG-induced MAP kinase activation was inhibited by addition of 75 μ mol PD098059 l^{-1} or 10 μ mol H-89 l^{-1} , but was not inhibited with 200 nmol wortmannin l^{-1} . Similarly, both PD098059 and H-89 inhibited hCG-induced COX-2 expression, but wortmannin did not (Fig. 6b,c). These results indicate that MAP kinase activation is downstream of protein kinase A activity during stimulation with hCG, and that PAF (Fig. 5) and hCG (Fig. 6) have different effects on MAP kinase activation and COX-2 expression.

Effect of CPT-cAMP on MAP kinase activation and COX-2 expression

cAMP is thought to be the principal mediator of the actions of LH and hCG in gonadal cells (Segaloff and Ascoli, 1993). CPT-cAMP is a potent cAMP analogue and at a concentration of 1 mmol l^{-1} it increased the activation of MAP kinase (Fig. 6a). CPT-cAMP at a concentration of 1 mmol l^{-1} also increased expression of COX-2 (Fig. 6c).

Discussion

Endometrial cells are exposed to oestrogen and progesterone in a cyclical manner *in vivo*. In the present study, addition of both oestradiol and medroxyprogesterone acetate for 3–5 days increased MAP kinase activation and COX-2 expression induced by PAF and hCG in HEC-1B cells. Oestrogen and progesterone can induce modulation of RNA and protein synthesis within this time frame. Sato *et al.* (1996) reported that pretreatment with oestradiol or oestradiol plus progesterone for a period of 48 h induced accumulation of PAF receptor transcript in human endometrial epithelial cells *in vitro*, although how these steroids modulate the PAF and hCG response in HEC-1B cells remains to be elucidated.

PAF activates MAP kinase in Chinese hamster ovary cells expressing PAF receptor (Honda *et al.*, 1994), as well as in blood cells (Ferby *et al.*, 1994; Coffey *et al.*, 1998). Interaction of PAF with the PAF receptor activates heterotrimeric G proteins (Hwang *et al.*, 1986), which in turn activates phospholipase C and increases intracellular calcium concentrations (Shukla, 1991). In Chinese hamster ovary cells, stimulation of the cloned PAF receptor activated MAP kinase through the activation of the upstream MEK in a G protein-dependent manner (Honda *et al.*, 1994). Stimulation of PAF

receptors is capable of activating PI3K (Coffey *et al.*, 1998) and MAP kinase activation with PAF is sensitive to wortmannin (Ferby *et al.*, 1994), thus linking PI3K upstream to MAP kinase.

Segaloff and Ascoli (1993) suggested that the increase in intracellular cAMP elicited by binding of hCG to the LH and hCG receptor is the principal mediator of the actions of LH and hCG in gonadal cells. Cameron *et al.* (1996) reported for the first time that FSH and LH can activate MAP kinase in pig granulosa cells through a cAMP-dependent mechanism. However, there is insufficient evidence to explain the actual mechanism of gonadotrophin-stimulated MAP kinase activation.

In the present study, PD098059, a selective inhibitor of MEK-1 and MEK-2 (Alessi *et al.*, 1995), completely inhibited PAF-induced MAP kinase activation. This result is consistent with similar observations made in HEC-1A cells after stimulation with PAF (Bonaccorsi *et al.*, 1997). These findings indicate that MAP kinase activation with PAF is a result of p21 Ras-mediated signalling. PAF-induced activation of MAP kinase was also inhibited completely by wortmannin in the present study. However, other studies showed that PAF-induced MAP kinase activation was only partially inhibited by wortmannin (Ferby *et al.*, 1994). Wortmannin at nanomolar concentrations is a specific inhibitor of PI3K (Grammer and Blenis, 1997). In a study using the HEC-1B cell line, Ahmed *et al.* (1995) reported that oestradiol upregulates PAF-induced phospholipase D activity. In addition, activation of phospholipase D was suppressed with PI3K inhibitors (Cissel *et al.*, 1998). These observations imply that PI3K lies upstream to phospholipase D in HEC-1B cells.

In the present study, use of PD098059 resulted in complete inhibition of hCG-induced MAP kinase activation, providing evidence that MEK lies upstream of hCG-mediated MAP kinase activation. Unlike PAF, activation of MAP kinase by hCG was unchanged by wortmannin treatment. On the other hand, a cAMP-induced increase in MAP kinase activation and the ability of H-89 to inhibit hCG-induced MAP kinase activity supports the view that cAMP lies upstream of the hCG-induced MAP kinase activation pathway.

Many studies have focused on regulation of COX-2 by MAP kinase. Use of promoter deletion and site-directed mutagenesis of COX-2 luciferase chimeric reporter genes showed that MAP kinase is partially involved in v-src- (Xie and Herschman, 1995), serum-, and PDGF-induced COX-2 induction in NIH 3T3 cells (Xie and Herschman, 1996). PD098059 also blocked stimulation of lysophosphatidic acid-mediated COX-2 expression in rat mesangial cells (Reiser *et al.*, 1998) and lipopolysaccharide-induced COX-2 expression in a murine macrophage cell line (Hwang *et al.*, 1997). Contrary to these reports, PAF-induced COX-2 expression was not inhibited by addition of PD098059 in the present study. However, wortmannin was effective at blocking the PAF-induced COX-2 expression. In view of the low concentration of wortmannin used (200 nmol l^{-1}), its effects would be expected to be exerted through specific inhibition of PI3K. PI3K has been implicated in the activation of jun N-terminal kinase (JNK) (Klippel *et al.*, 1996), nuclear factor kappa B (NF kappa B), and activated protein 1 (AP-1) (Reddy

et al., 1997), all of which are associated with stimulation of COX-2 expression. Furthermore, protein kinase B (PKB/c-Akt) has been identified as a downstream target of PI3K (Burgering and Coffey, 1995). Pedersen *et al.* (1997) reported that wortmannin inhibited protein synthesis by affecting translation. It is concluded that PAF-induced expression of COX-2 is probably mediated through the PI3K signalling pathway, although the reactions downstream to the PI3K pathway remain to be determined.

Consistent with other studies, in the present study, PD098059 inhibited hCG-mediated COX-2 expression. This implicates a role for MAP kinase in hCG-mediated COX-2 expression. In addition, CPT-cAMP stimulated COX-2 expression directly and hCG-induced COX-2 expression was inhibited by H-89. H-89 also inhibited hCG-induced MAP kinase activation, so it appears that MAP kinase is the ultimate effector in this kinase cascade. Borsch-Haubold *et al.* (1998) reported that the activity of COX-1 and COX-2 was inhibited by PD098059 at micromolar concentrations. Since, in the present study, the activity of COX-2 was not determined but the expression of COX-2 was measured by inhibition of MAP kinase, it is considered that the use of PD098059 did not influence the results.

Studies in knockout mice showed that COX-2 is a critical protein for implantation and decidualization (Lim *et al.*, 1997). Because PAF is involved in implantation and hCG is involved in decidualization, and PAF (Alecozay *et al.*, 1989) and hCG are present in the luteal phase endometrium, the regulation of PAF- and hCG-induced COX-2 expression may be important in these events.

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