PTGER1 and PTGER2 receptors mediate regulation of progesterone synthesis and type 1 11β-hydroxysteroid dehydrogenase activity by prostaglandin E_2 in human granulosa–lutein cells

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Abstract

In luteinizing granulosa cells, prostaglandin E_2 (PGE_2) can exert luteotrophic actions, apparently via the cAMP signalling pathway. In addition to stimulating progesterone synthesis, PGE_2 can also stimulate oxidation of the physiological glucocorticoid, cortisol, to its inactive metabolite, cortisone, by the type 1 11β-hydroxysteroid dehydrogenase (11βHSD1) enzyme in human granulosa–lutein cells. Having previously shown these human ovarian cells to express functional G-protein coupled, E-series prostaglandin (PTGER)1, PTGER2 and PTGER4 receptors, the aim of this study was to delineate the roles of PTGER1 and PTGER2 receptors in mediating the effects of PGE_2 on steroidogenesis and cortisol metabolism in human granulosa–lutein cells. PGE_2-stimulated concentration-dependent increases in both progesterone production and cAMP accumulation (by 1·9±0·1- and 18·7±6·8-fold respectively at 3000 nM PGE_2). While a selective PTGER1 antagonist, SC19220, could partially inhibit the steroidogenic response to PGE_2 (by 55·9±4·1% at 1000 nM PGE_2), co-treatment with AH6809, a mixed PTGER1/PTGER2 receptor antagonist, completely abolished the stimulation of progesterone synthesis at all tested concentrations of PGE_2 and suppressed the stimulation of cAMP accumulation. Both PGE_2 and butaprost (a preferential PTGER2 receptor agonist) stimulated concentration-dependent increases in cortisol oxidation by 11βHSD1 (by 42·5±3·1 and 40·0±3·0% respectively, at PGE_2 and butaprost concentrations of 1000 nM). Co-treatment with SC19220 enhanced the ability of both PGE_2 and butaprost to stimulate 11βHSD1 activity (by 30·2±0·2 and 30·5±0·6% respectively), whereas co-treatment with AH6809 completely abolished the 11βHSD1 responses to PGE_2 and butaprost. These findings implicate the PTGER2 receptor–cAMP signalling pathway in the stimulation of progesterone production and 11βHSD1 activity by PGE_2 in human granulosa–lutein cells.

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Introduction

Prostaglandins (PGs) have been implicated in the co-ordination of ovarian function, particularly in ovulation and in regulating the functional lifespan of the corpus luteum. For example, $PGF_{2\alpha}$ has luteolytic effects and can trigger functional luteal regression in a range of species, including humans and non-human primates (reviewed by Michael *et al.* 1994, Olofsson & Leung 1994), whereas selected PGs, like PGE_2, can exert luteotrophic actions in human and non-human primate ovarian cells, stimulating progesterone synthesis apparently via the cAMP signalling pathway (Richardson 1986, Michael *et al.* 1993, 1994, Olofsson & Leung 1994). Although PGs are established as major paracrine agents in ovarian physiology, their exact modes of action still remain to be elucidated.

Glucocorticoid steroids are also known to be important for gonadal function and have been implicated in the control of oocyte maturation (Greeley *et al.* 1986, Harlow *et al.* 1997, Yang *et al.* 1999, Chen *et al.* 2000). In potential target tissues, the physiological glucocorticoids, cortisol and corticosterone, are oxidised to inert metabolites (cortisone and 11-dehydrocorticosterone respectively) by 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes (Seckl & Walker 2001, Seckl 2004, Draper & Stewart 2005). These enzymes are expressed and appear to play significant physiological roles in

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reproductive tissues (reviewed by Michael *et al.* (2003)). For example, in the uterine–placental complex, both the relatively low-affinity NADP(H)-dependent type 1 11βHSD enzyme (11βHSD1) and the high-affinity NAD^+-dependent type 2 11βHSD enzyme (11βHSD2) have recently been implicated in the physiological mechanism of labour (Challis *et al.* 2000, Whittle *et al.* 2001), although prior studies of human placenta and decidua had found no significant changes in cortisol metabolism by 11βHSD at parturition (López Bernal *et al.* 1982*a*,*b*).

Previously, we have established that luteinizing human granulosa cells, recovered from the ovarian follicular aspirates of women undergoing oocyte retrieval for *in vitro* fertilisation, exclusively express 11βHSD1 with no detectable expression of 11βHSD2 at either the mRNA or the protein level (Michael *et al.* 1997, Thurston *et al.* 2003*a*). Although 11βHSD1 generally acts as a 11-ketosteroid reductase to regenerate cortisol from cortisone (Seckl & Walker 2001, Seckl 2004, Draper & Stewart 2005), we have previously reported that in intact human granulosa–lutein cells in primary culture, 11βHSD1 acts predominantly as a 11β-dehydrogenase enzyme to catalyse the oxidative inactivation of cortisol (Michael *et al.* 1997, Thurston *et al.* 2003*a*). Since the utero-placental feedback loop for the induction of parturition (at least in sheep) appears to require stimulation of 11βHSD1 by PGs (Alfaidy *et al.* 2001, Challis *et al.* 1997), we have investigated the possible role for local PGs in control of 11βHSD1 in human granulosa–lutein cells *in vitro*. We recently reported that inhibition of local PG synthesis (using four structurally distinct inhibitors of PGH synthase) could suppress cortisol oxidation by 11βHSD1 in these human cells, but that this enzyme activity could be stimulated by PGE_2 (Jonas *et al.* 2006).

At the cellular level, PGE_2 actions are mediated through G protein-coupled, E-series PG (PTGER) receptors. To date, four subtypes of PTGER receptors have been cloned, designated PTGER1, PTEGER2, PTGER3 and PTGER4 (Hirata *et al.* 1991, Sugimoto *et al.* 1992, Funk *et al.* 1993, Honda *et al.* 1993, Watabe *et al.* 1993), with multiple isoforms of the PTGER3 receptor subtype (reviewed by Coleman *et al.* (1994)). While PTGER1 and PTGER3 receptors mediate increases in intracellular calcium concentrations, PTGER2 and PTGER4 receptors mediate stimulation of intracellular cAMP accumulation (Narumiya *et al.* 1999). Since PTGER receptors have different affinities for synthetic PGE_2 analogues, agonists and antagonists with differing degrees of preference or selectivity for each PTGER receptor subtype can be used to delineate the role for particular PTGER receptors in a given cellular response (Coleman *et al.* 1994). Using RT-PCR and preferential receptor agonists/antagonists, we and other researchers have established that human granulosa–lutein cells express PTGER1, PTGER2 and PTGER4 receptors, and that the PTGER1 and PTGER2 receptors are able to elicit increases in intracellular calcium and cAMP respectively (Harris *et al.* 2001, Narko *et al.* 2001). However, the exact PG receptors through which PGE_2 exerts its effects on progesterone synthesis and cortisol–cortisone interconversion by 11βHSD1 have not been previously examined in ovarian cells. Hence, the aim of the current study was to examine the potential roles for PTGER1 and PTGER2 receptors in mediating the effects of PGE_2 on steroidogenesis and cortisol metabolism using human granulosa–lutein cells as our *in vitro* ovarian model.

Materials and Methods

Isolation and culture of human granulosa cells

Human granulosa cells were isolated from follicular aspirates of women undergoing controlled ovarian hyperstimulation for assisted conception at the Lister Private Hospital (Chelsea, London, UK) as previously described by Webley *et al.* (1988). Follicular aspirates were collected with informed patient consent in accordance with the Declaration of Helsinki and as approved by the local ethics committee.

Following isolation on 60% (v/v) Percoll, human granulosa–lutein cells were cultured for 48 h in 1:1 Dulbecco's modified Eagle's medium (DMEM):Ham's F12 medium supplemented with 10% (v/v) fetal calf serum (Invitrogen Life Technologies), 2 mM L-glutamine (Life Technologies), penicillin (87 000 IU/l; Sigma–Aldrich) and streptomycin (87 mg/l; Sigma–Aldrich) in an atmosphere of 5% (v/v) CO_2 in air. Cells were cultured either in 250 μl volumes at a density of 1×10^5 viable cells/well for assessing regulation of progesterone production and cAMP accumulation or in 1 ml volumes at a density of 5×10^4 viable cells/well in 24-well plates for measurement of 11βHSD1 activities. For all experiments, cell viability, assessed by exclusion of 0·4% (v/v) trypan blue dye, was consistently $>85\%$.

Concentration-dependent effects of PGE_2 on progesterone production and cAMP accumulation in the absence or presence of SC19220 and AH6809

In order to examine whether PTGER2 receptors were involved in mediating the steroidogenic response to PGE_2, cells were transferred to serum-free 1:1 DMEM:Ham's F_{12} medium supplemented with 1 μM meclofenamic acid (Sigma–Aldrich) to suppress intrinsic PG synthesis (Jonas *et al.* 2006). Cells were challenged for 24 h with a range of PGE_2 concentrations (0–3000 nM; Cayman Chemical Company, MI, USA) in the absence and presence of either 10 μM SC19220 or 10 μM AH6809 (Alexis Biochemicals, Nottingham, UK). SC19220 and AH6809 were prepared to a stock concentration of 10 mM in dimethylsulphoxide (DMSO), the final concentration of which was diluted in all wells to 0·1% by volume.

After the 24-h treatment period, culture plates were frozen at -20 °C. Samples were subsequently thawed and progesterone and cAMP concentrations were measured using RIAs previously described by Pallikaros *et al.* (1995) and Steiner *et al.* (1972) respectively. The progesterone RIA had a working range of 0·5–8·0 nM with inter- and intra-assay coefficients of variation of 9 and 14% respectively. The cAMP

RIA included the acetylation step proposed by Harper & Brooker (1975), which gave the assay a working range of 0·2–1·3 nM with inter- and intra-assay coefficients of variation of 9 and 5% respectively.

Effects of SC19220 and AH6809 on 11βHSD1 activities in response to PGE_2 and butaprost in human granulosa–lutein cells

As in the assessment of the effects on progesterone production and cAMP accumulation, cells were pre-incubated for 48 h in serum-supplemented 1:1 DMEM:Ham's F12 medium. Following this, the cells were transferred to serum-free 1:1 DMEM:Ham's F12 medium, supplemented with 1 μM meclofenamic acid and containing PGE_2 or butaprost (Cayman Chemical Company; 0–1000 nM of each), each in the absence or presence of 10 μM SC19220 or AH6809 for 4 h. To overcome potential confounding effects of progesterone on 11βHSD activities (Thurston *et al.* 2003*b*), these experiments were performed in the presence of 100 μM aminoglutethimide, having confirmed that this effectively suppresses progesterone output from human granulosa–lutein cells (Fowkes *et al.* 2001) without affecting cAMP accumulation. During the 4 h over which cells were exposed to PGE_2/butaprost ± SC19220/AH6809, 11βHSD1 activities were assessed using the radiometric conversion assay as previously described by Michael *et al.* (1995). In brief, intact cells in primary culture were co-incubated with 100 nM [1,2,6,7-^{3}H]-cortisol (Amersham Biosciences), diluted to a specific activity of 0·5 μCi/100 pmol using non-radiolabelled cortisol (Sigma–Aldrich). At the end of the 4-h treatment period, spent culture medium was transferred to screw cap borosilicate tubes in which steroids were extracted by the addition of two volumes of chloroform (Merck). After evaporation to dryness under nitrogen gas at 45 °C, the steroid residues were suspended in 25 μl ethyl acetate containing 1 mM cortisol and 1 mM cortisone (Sigma–Aldrich) before being resolved by thin layer chromatography (TLC) on silica 60 TLC plates (Merck), developed in an atmosphere of 92:8 chloroform:95% (v/v) ethanol. [^{3}H]-cortisol and [^{3}H]-cortisone were quantified using a Bioscan 200 TLC radiochromatogramme scanner (Lablogic, Sheffield, UK) and the oxidative activities of 11βHSD1 were calculated as net pmoles cortisol oxidised to cortisone over 4 h (Michael *et al.* 1995).

Statistical analyses

All experimental data are presented as the mean ± S.E.M. for up to six independent experiments, where each experimental replicate was performed using cells from individual patients and each experimental condition was repeated either in quadruplicate or in triplicate within an experiment. Due to differences in absolute levels of progesterone production, cAMP accumulation and enzyme activities between patients, the results for each experiment were standardised and presented as a percentage of basal control. However, all statistical analyses were performed using absolute data.

In all experiments, a one-way ANOVA with repeated measures was performed, followed by Bonferroni's multiple comparison as the *post hoc* test. All statistical evaluations were performed using GraphPad Prism version 3.02 (GraphPad Software Inc., San Diego, CA, USA) and significance was assessed in all experiments as a probability value of $P<0·05$.

Results

Effects of SC19220 and AH6809 on progesterone production and 11βHSD1 activity

Before use, both PTGER receptor antagonists were tested for possible direct effects on basal progesterone production and 11βHSD1 activity in human granulosa–lutein cells. Under conditions in which intrinsic PG synthesis had been suppressed with 1 μM meclofenamic acid, incubation for 24 h with the preferential PTGER1 receptor antagonist SC19220 (10 μM) and the PTGER1/PTGER2 receptor antagonist AH6809 (10 μM) had no significant effect on basal progesterone production (over 24 h) or the oxidative activity of 11βHSD1 (over 4 h; Table 1).

Concentration-dependent effects of PGE_2 on progesterone production in the absence or presence of SC19220 and AH6809

Treatment with PGE_2 for 24 h resulted in a concentration-dependent increase in progesterone synthesis (Fig. 1). At PGE_2 concentrations ≥30 nM, progesterone production increased progressively by up to 91 ± 6% (at 3000 nM PGE_2) relative to the control rate of progesterone output in the absence of PGE_2 ($P<0·01$). However, co-treatment with SC19220 attenuated the responses to both 1000 and 3000 nM PGE_2 by 56 ± 4% ($P<0·001$) and 35 ± 3% ($P<0·001$) respectively, while co-treatment with AH6809 completely

Table 1 Effects of SC19220 and AH6809 on basal progesterone production and 11β-hydroxysteroid dehydrogenase (11βHSD1) activity. Values are the mean ± S.E.M. for three independent experiments with quadruplicate determinations in each experiment (one-way ANOVA)

	Progesterone (pmol/10^5 cells)	**11βHSD1 activity** (pmol cortisone/4 h .5 $\times 10^4$cells)
Control	2351·1 ± 1028·3	3·7 ± 1·2
+SC19220	2041·6 ± 913·6	3·8 ± 1·1
+AH6809	2712·5 ± 1318·1	2·5 ± 0·4
ANOVA	$P=0·161$	$P=0·158$

Progesterone concentrations in culture medium and 11βHSD1 activities in human granulosa–lutein cells were each assessed following a 24- or 4-h incubation respectively with 10 μM SC19220 or 10 μM AH6809.

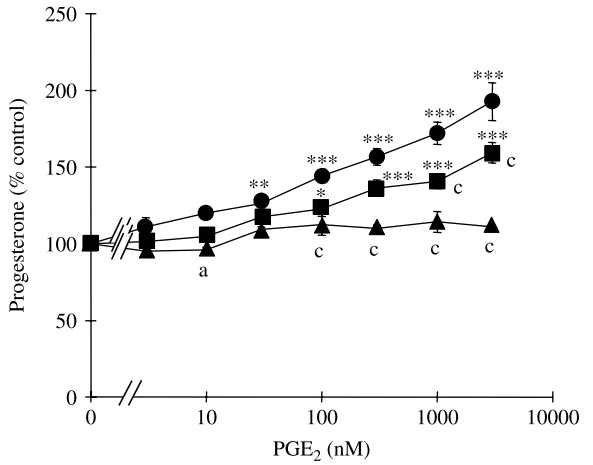

Figure 1 Effects of SC19220 and AH6809 on the progesterone responses to PGE_2 by human granulosa–lutein cells. Progesterone concentrations in medium collected after a 24-h incubation in the presence of 0–3000 nM PGE_2, either in the absence (circle) or in the presence of 10 μM SC19220 (square) or 10 μM AH6809 (triangle). Values are the mean ± S.E.M. for three independent experiments with quadruplicate determinations in each experiment. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$ relative to 0 nM PGE_2 in the absence or presence of the PTGER receptor antagonist, as appropriate. $^aP<0{\cdot}05$, $^cP<0{\cdot}001$ relative to cells treated with the same PGE_2 concentration in the absence of PTGER receptor antagonists (one-way ANOVA plus Bonferroni's *post hoc* multiple comparison test).

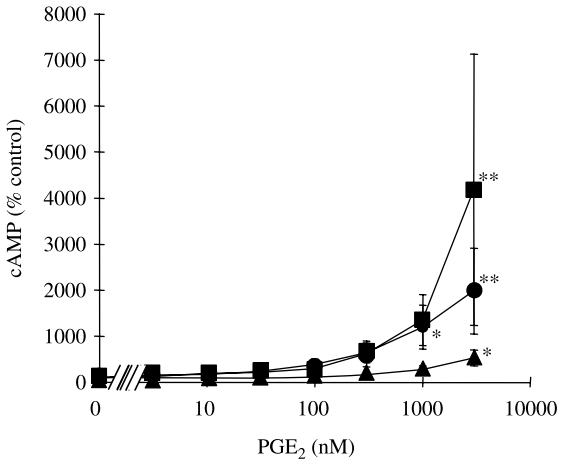

Figure 2 Effects of SC19220 and AH6809 on the cAMP responses to PGE_2 by human granulosa–lutein cells. Concentrations of cAMP after a 24-h incubation in the presence of 0–3000 nM PGE_2, either in the absence (circle) or in the presence of 10 μM SC19220 (square) or 10 μM AH6809 (triangle). Values are the mean ± S.E.M. for three independent experiments with quadruplicate determinations in each experiment. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$ relative to 0 nM PGE_2 in the absence or presence of the PTGER receptor antagonists, as appropriate (one-way ANOVA plus Bonferroni's *post hoc* multiple comparison test).

abolished the ability of all concentrations of PGE_2 to stimulate progesterone output.

Incubation for 24 h with PGE_2 evoked a concentration-dependent increase in cAMP accumulation which only achieved significance at the supraphysiological concentrations of 1000 and 3000 nM PGE_2 (Fig. 2). At the highest tested concentration of 3000 nM, PGE_2 increased cAMP accumulation by 18·7 ± 6·8-fold ($P<0{\cdot}01$) in the absence of PTGER receptor antagonists. In cells co-treated with SC19220, this maximal concentration of PGE_2 increased cAMP accumulation by 40·3 ± 27·8-fold ($P<0{\cdot}01$), whereas co-treatment with AH6809 suppressed the cAMP response to 3000 nM PGE_2 to just 4·1 ± 1·2-fold ($P<0{\cdot}05$; Fig. 2).

Effects of SC19220 and AH6809 on 11βHSD1 activities in response to PGE_2 and butaprost in human granulosa–lutein cells

Consistent with our recent report (Jonas *et al.* 2006), PGE_2 significantly increased 11βHSD1 activity in a concentration-dependent manner; net oxidation of cortisol to cortisone was increased by up to 43 ± 3% at the highest tested PGE_2 concentration of 1000 nM ($P<0{\cdot}001$; Fig. 3). Co-treatment with SC19220 further enhanced 11βHSD1 activities at each of the tested concentrations of PGE_2. At the highest tested concentration of 1000 nM PGE_2, co-treatment with SC19220 increased the 11βHSD1 activity by a further 32·7 ± 10·2% ($P<0{\cdot}05$) relative to the cells treated with PGE_2 in the absence of PTGER receptor antagonists. In contrast, co-treatment with AH6809 completely abolished the 11βHSD1 response to all tested concentrations of PGE_2 (Fig. 3).

Incubation for 4 h with the preferential PTGER2 receptor agonist butaprost also elicited a concentration-dependent increase in 11βHSD1 activities (Fig. 4). At the highest tested concentration of 1000 nM, butaprost increased net cortisol oxidation by 40 ± 3% ($P<0{\cdot}01$). Co-treatment with SC19220 further increased 11βHSD1 activity at each tested concentration of butaprost by up to 31 ± 1% ($P<0{\cdot}05$) relative to cells treated with butaprost alone. In contrast, as for PGE_2, co-treatment with AH6809 completely abolished the stimulation of 11βHSD1 activities by each concentration of butaprost.

Discussion

This study has investigated the potential participation of PTGER1 and PTGER2 receptors in mediating the effects of PGE_2 on progesterone production and glucocorticoid metabolism in human granulosa–lutein cells. Using preferential pharmacological agonists and antagonists for the PTGER1 and PTGER2 receptor subtypes, the combined data implicate PTGER2 receptors as mediators of the stimulation of progesterone synthesis and 11βHSD1 activity by PGE_2 in this human ovarian cell model *in vitro*.

The ability of PGE_2 to stimulate progesterone synthesis in human and non-human primate luteal cells, and in the widely used model of human granulosa–lutein cells, is well documented (Hahlin *et al.* 1988, Fehrenbach *et al.* 1999,

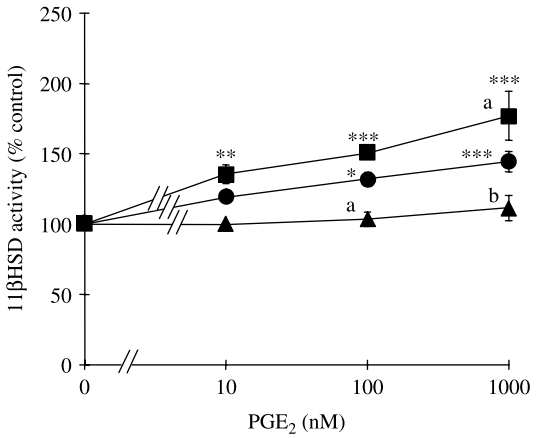

Figure 3 Effects of PGE_2 on 11βHSD1 activities human granulosa–lutein cells in the absence and presence of SC19220 and AH6809. 11βHSD1 activities following treatment of cells with 0–1000 nM PGE_2 either in the absence (circle) or in the presence of 10 μM SC19220 (square) or 10 μM AH6809 (triangle). Values are the mean±S.E.M. for three independent experiments with triplicate determinations in each experiment. *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ relative to 0 nM PGE_2 in absence or presence of the PTGER receptor antagonists, as appropriate. [a]$P<0{\cdot}05$, [b]$P<0{\cdot}01$ relative to cells treated with the same PGE_2 concentration in the absence of PTGER receptor antagonists (one-way ANOVA plus Bonferroni's *post hoc* multiple comparison test).

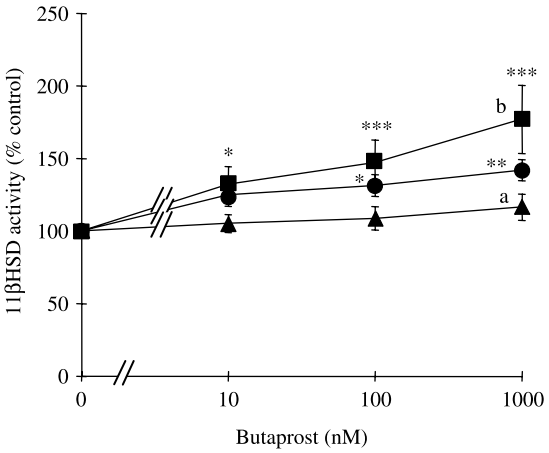

Figure 4 Effects of butaprost on 11βHSD1 activities in human granulosa–lutein cells in the absence and presence of SC19220 and AH6809. 11βHSD1 activities following treatment of cells with 0–1000 nM butaprost either in the absence (circle) or in the presence of 10 μM SC19220 (square) or 10 μM AH6809 (triangle). Values are the mean±S.E.M. for three independent experiments with triplicate determinations in each experiment. *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ relative to 0 nM butaprost in the absence or presence of the PTGER receptor antagonists, as appropriate. [a]$P<0{\cdot}05$, [b]$P<0{\cdot}01$ relative to cells treated with the same butaprost concentration in the absence of PTGER receptor antagonists (one-way ANOVA plus Bonferroni's *post hoc* multiple comparison test).

Vaananen *et al.* 2001). Given the reported ability of PGE_2 to increase intracellular cAMP concentrations in these cells (Michael *et al.* 1993) and the established role for cAMP as a second messenger that stimulates steroidogenesis (Cooke 1999), it has widely been assumed that the progesterone response to PGE_2 is mediated via this cyclic nucleotide. We have previously demonstrated that both progesterone production and cAMP accumulation in human granulosa–lutein cells can be stimulated by the mixed PTGER1/PTGER2 receptor agonist, dimethyl-PGE_2 and by the preferential PTGER2 receptor agonist, butaprost (Harris *et al.* 2001). The cAMP responses to dimethyl-PGE_2 and butaprost were completely abolished by co-treatment with the PTGER1/PTGER2 receptor antagonist AH6809, but not the selective PTGER1 receptor antagonist SC19220, implicating the PTGER2 receptor in the cAMP response to these pharmacological agonists (Harris *et al.* 2001). Interestingly, the cAMP response to PGE_2 was only partially suppressed (by around 60%) with AH6809, and we did not assess the ability of these compounds to interfere with the steroidogenic responses to PGE_2 or its functional analogues.

In the present study, when cells were co-treated with SC19220, the progesterone response to PGE_2 was partially inhibited but not completely abolished. This suggests that the PTGER1 receptors participate in the stimulation of steroidogenesis by PGE_2, but do not mediate the full steroidogenic response. We have now confirmed that co-treatment with AH6809 could completely prevent the steroidogenic response to PGE_2 at all tested concentrations, implicating an AH6809-sensitive receptor in the steroidogenic response to PGE_2.

The concentrations of PGE_2 (and butaprost) used for the studies reported herein were selected based on previous investigations of human granulosa–lutein cells, which routinely use PGs at supraphysiological concentrations of 500–5000 nM. It is noteworthy that, at the lower tested concentrations of 3 and 10 nM, PGE_2 failed to stimulate steroidogenesis, even though Abramovitz *et al.* (2000) have previously reported that PGE_2 concentrations of <30 nM should be sufficient to stimulate all four PTGER receptor subtypes. Moreover, although PGE_2 and butaprost were able to stimulate progesterone synthesis (and cortisol metabolism) at concentrations of 30 nM and above, at these concentrations, PGE_2 would be expected to saturate all four classes of PTGER receptor and possibly to activate non-PTGER receptors (i.e. PTGFR and D series prostaglandin (PTGDR) receptors), particularly at the conventional concentrations of 300, 1000 and 3000 nM (Abramovitz *et al.* 2000). Since PTGFR receptors are known to inhibit (rather than stimulate) progesterone production (Sugimoto *et al.* 1997, Tsai *et al.* 1998, 2001), it seems highly unlikely that the steroidogenic response to PGE_2 (even at concentrations $>$ 30 nM) is mediated via the PTGFR receptor. However, we cannot yet exclude participation of the PTGDR receptor in the stimulation of steroidogenesis at high concentrations of PGE_2, given that AH6809 has also been reported to function

as a weak PTGDR antagonist when used at a concentration of 10 μM (Keery & Lumley 1988, Woodward *et al.* 1995).

In terms of effects on cAMP accumulation, we found that although PGE_2 increased cAMP accumulation in a concentration-dependent manner, this response only achieved statistical significance at the higher concentrations of 1000 and 3000 nM PGE_2, which would be expected to bind all PTGER maximally, and possibly to activate PTGDR and PTGFR receptors as well (Abramovitz *et al.* 2000). Co-treatment of cells with AH6809 suppressed the PGE_2-induced increase in the cAMP accumulation, but did not completely abolish the cAMP response to PGE_2, indicating that the residual effect of high PGE_2 concentrations on cAMP may be mediated through an AH6809-insensitive receptor. SC19220, on the other hand, did not suppress the responsiveness of the cells to PGE_2. If anything, co-treatment with the PTGER1 antagonist SC19220 enhanced the increase of cAMP in response to PGE_2. These observations raise two possibilities. First, inhibition of PTGER1 receptor binding by SC19220 may have facilitated increased binding of PGE_2 to other prostanoid receptors which are capable of stimulating cAMP accumulation (e.g. PTGER2, PTGER4 and/or PTGDR receptors). Secondly, co-stimulation of the PTGER1 receptor would be expected to activate the calcium–protein kinase C signalling pathway (Narumiya *et al.* 1999), which can inhibit the stimulation of cAMP production (Abayasekara *et al.* 1993*a*,*b*). Based on these observations, prostanoid receptors which are sensitive to AH6809 but insensitive to SC19220 (e.g. PTGER2 and/or PTGDR) are implicated in the ability of PGE_2 to stimulate cAMP accumulation in human granulosa–lutein cells. In light of our previous report (Harris *et al.* 2001), we would suggest that while the ability of dimethyl-PGE_2 and butaprost to elevate intracellular cAMP concentrations is mediated solely via a PG receptor that can be antagonised with AH6809, consistent with a role for the PTGER2 receptor in eliciting a cAMP response, at least some of the second messenger response to PGE_2 is mediated via AH6809-resistant receptors, which may include PTGER4 receptors and possibly even PTGDR receptors. We also note that, in the present study, the residual cAMP responses to the higher concentrations of PGE_2 in cells co-treated with AH6809 were not accompanied by any significant increases in progesterone synthesis, showing that second messengers other than cAMP may be required for PGE_2 to stimulate steroidogenesis.

Turning to the regulation of cortisol–cortisone metabolism by PGE_2, we have previously established that luteinizing human granulosa cells only express 11βHSD1 with no detectable expression of 11βHSD2 protein or mRNA, even when the PCR is used to increase the sensitivity of detection (Michael *et al.* 1997, Thurston *et al.* 2003*a*). Although the 11βHSD1 enzyme is intrinsically bidirectional, in most tissues this enzyme acts predominantly as an 11-ketosteroid reductase to regenerate cortisol from cortisone (Seckl & Walker 2001, Seckl 2004, Draper & Stewart 2005). However, we have previously found that 11βHSD1 acts predominantly, if not exclusively, to catalyse the oxidative inactivation of cortisol in human granulosa–lutein cells (Michael *et al.* 1997, Thurston *et al.* 2003*a*), bovine granulosa and luteal cells (Thurston *et al.* 2007) and porcine granulosa cells (Sunak *et al.* 2007). 11βHSD1 has also been reported to act predominantly as a 11β-dehydrogenase in rat testis Leydig cells (Gao *et al.* 1997, Ge & Hardy 2000) suggesting that the major action of the 11βHSD1 enzyme may be fundamentally different in the steroidogenic cells of the ovary and testis (Michael *et al.* 2003, Ge *et al.* 2005). Recent studies have established that the predominant direction of 11βHSD1 is dependent on the ratio of the reduced form of nicotinamide adenine dinucleotide phosphate to its oxidised form (NADPH: $NADP^+$) within the lumen of the smooth endoplasmic reticulum (Draper *et al.* 2003, Atanasov *et al.* 2004, Banhegyi *et al.* 2004, Bujalska *et al.* 2005, McCormick *et al.* 2006) such that changes in this ratio in steroidogenic gonadal cells may favour the oxidative activity of 11βHSD1 in these cells (Michael *et al.* 2003, Ge *et al.* 2005).

In the present study, the stimulation of the net oxidation of cortisol in human granulosa–lutein cells by PGE_2 showed good agreement with our recently published data (Jonas *et al.* 2006). The fact that this stimulation could be reproduced using the preferential PTGER2 receptor agonist butaprost, combined with the fact that AH6809 could completely suppress the effects of PGE_2 and butaprost on cortisol oxidation, suggests that the stimulation of the oxidative activity of 11βHSD1 is mediated via the PTGER2 receptor. In contrast, co-treatment with SC19220 enhanced the 11βHSD1 response at each of the tested concentrations of PGE_2, suggesting that the co-activation of PTGER1 receptors limits the stimulation of cortisol–cortisone metabolism by PGE_2. PTGER1 receptors are known to act via calcium as a second messenger, and we have confirmed that in human granulosa–lutein cells, SC19220 can antagonise the ability of PGE_2 and its dimethyl derivative to increase the intracellular calcium concentration (Harris *et al.* 2001). Given that studies of placental biochemistry have shown that calcium can mediate the inhibition of 11βHSD1 activity in response to PGs and leukotriene B_4 (Hardy *et al.* 1999, 2001), we would propose that activation of the PTGER1 receptor–calcium signalling pathway by PGE_2 limits the ability of this PG to stimulate 11βHSD1 in human granulosa–lutein cells via the PTGER2–cAMP pathway.

In comparing the three responses considered in this study, we note that at the lower concentrations tested (<1000 nM), which are likely to fall within the physiological range and selectively to activate only the PTGER receptor subtypes, PGE_2 can stimulate both progesterone synthesis and cortisol metabolism without exerting a significant effect on the intracellular cAMP concentration. This suggests that either the effects of 10–300 nM PGE_2 on steroid synthesis and metabolism are mediated via local increases in cAMP within specific subcellular microenvironments (not reflected by changes in the total intracellular cAMP concentration) or

these effects are mediated via a second messenger other than cAMP.

In summary, we have presented data which implicate PTGER2 receptors in the stimulation of progesterone synthesis, cAMP accumulation and cortisol metabolism via 11βHSD by PGE_2 in human granulosa–lutein cells. Further studies are necessary to elucidate potential roles for other prostanoid receptors, such as PTGDR receptors, in the responses at the upper concentrations of PGE_2.

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