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Regulation of prostaglandin endoperoxide H synthase by glucocorticoids and activators of protein kinase C in the human amnion

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Since glucocorticoids decrease and protein kinase C (PKC) activators increase amniotic PGE_2 production, the possibility that they regulate the activity of prostaglandin endoperoxide H synthase (PGHS), the rate-limiting enzyme of prostaglandin synthesis from arachidonate, was investigated. Glucocorticoids inhibited the production of PGE_2 from exogenous arachidonate specifically and in a concentration dependent fashion. Furthermore, cortisol decreased PGHS activity and the amount of PGHS protein in amnion microsomes, and reduced the rate of recovery of PGHS after acetylsalicylic acid (ASA) pretreatment. Actinomycin D blocked the inhibition of PGHS recovery by cortisol, but did not suppress the spontaneous recovery of the enzyme, indicating that the glucocorticoid induced a post-transcriptional inhibitor of PGHS synthesis. PKC-activating phorbol esters, such as 12-tetradecanoyl phorbol 13-acetate (TPA) increased the synthesis of PGE_2 from exogenous arachidonate, also in a specific and concentration dependent manner. PGHS recovery after ASA treatment was enhanced by TPA. PGHS activity and protein concentrations were increased by phorbol ester treatment; however, this was apparent only in tissues in which the concentrations of PGHS were initially low. These results show that the synthesis of PGHS is positively and negatively regulated in the human amnion by PKC and glucocorticoids, respectively, and suggest that effectors using these pathways may regulate the enzyme *in vivo*.

Introduction

Prostanoids synthesized by the intrauterine tissues play important roles in the initiation or maintenance of labour in a number of species (Challis and Olson, 1988). In pregnant women, the amnionic membrane is a major intrauterine source of prostaglandins (Mitchell, 1986). The amnion produces increased amounts of PGE_2 at labour (Mitchell, 1986; Casey and MacDonald, 1986), which suggests that the stimulation of amniotic PGE_2 synthesis is an essential part of the physiological process leading to birth.

Prostaglandin endoperoxide H synthase (PGHS, EC 1.14.99.1) catalyses the first, committed step of prostaglandin synthesis from arachidonic acid (Smith *et al.*, 1991). PGHS activity increases in the amnion at parturition (Okazaki *et al.*, 1981; Olson *et al.*, 1991), and the findings of Gaffney *et al.* (1990) indicate that the rate of PGHS synthesis in the chorio-amnion is higher following labour than before the spontaneous onset of labour. The increase of amnion PGE_2 production at term labour may therefore be the consequence, at least in part, of stimulated synthesis and high concentration of PGHS in this tissue.

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PGHS activity is controlled in many cells by the regulation of enzyme mass. As reviewed by DeWitt (1991) and Smith *et al.* (1991), the expression of PGHS is affected by a variety of agonists including growth factors, cytokines, steroids and tumour promoters in fibroblasts, vascular cells, monocyte- and macrophage-like cells, ovarian, uterine and renal cells. Confluent cultured amnion cells also respond to a number of effectors with increased PGE_2 output (see Olson *et al.*, 1990 for review). Several of these, such as epidermal growth factor (EGF), interleukin 1 (IL-1), dexamethasone and the tumour promoter phorbol esters were demonstrated to stimulate the *de novo* synthesis of PGHS (Olson *et al.*, 1990; DeWitt, 1991; Smith *et al.*, 1991). Recent data indicate, however, that fresh amnion tissue or freshly dispersed amnion cells respond differently to certain agonists compared with confluent cultured amnion cells. Contrary to their effect on confluent cell cultures, glucocorticoids inhibit the prostaglandin production in fresh amnion (Gibb and Lavoie, 1990), and EGF has no effect on the PGE_2 output of freshly dispersed amnion cells (Gibb and Lavoie, 1990). Since the responses of freshly isolated amnion may reflect the responsiveness of amnion tissue *in vivo* more closely than the responses of confluent cultured amnion cells, we developed a tissue incubation system to study the mechanisms

regulating amniotic prostaglandin production. Using this model, we showed that the activation of protein kinase C (PKC) by phorbol esters increases the PGE_2 production of the amnion (Zakar and Olson, 1992).

In the present experiments, we determined the effects of glucocorticoids and phorbol esters on the rate of synthesis, enzyme activity and immunoreactive protein concentrations of PGHS in amnion tissue. We hypothesized that glucocorticoids inhibit and PKC activators stimulate amniotic prostaglandin production by affecting PGHS activity in the amnion membranes. After demonstrating that amniotic PGHS may be positively and negatively regulated, we suggest that one of the factors that controls intrauterine prostaglandin synthesis at term is the activity of PGHS in fetal membranes.

Materials and Methods

Materials

[5,6,8,11,12,14,15-N-^{3}H]PGE_2 (specific activity 140 Ci $mmol^{-1}$) was obtained from Amersham Canada (Oakville, ON). Phorbol esters, 4β-phorbol, calf thymus DNA, bis-benzimidazole (Hoechst 33258), fatty acid free BSA, cycloheximide, actinomycin D, acetylsalicylic acid (ASA), indomethacin, leupeptin, phenylmethylsulfonyl fluoride (PMSF) and diethyldithiocarbamic acid (DDC), were purchased from Sigma Chemical Co. (St Louis, MO). All steroids except for RU486 (17β-hydroxy-11β-(4-dimethylaminophenol)-17α-(prop-1-ynyl)estra-4,9-diene-3-one), which was the product of Roussel UCLAF (Romainville), were also obtained from Sigma. Prostaglandin E_2, PGHS purified from sheep seminal vesicles, and rabbit polyclonal antibody raised against sheep seminal vesicle PGHS were from Cayman Chemical (Ann Arbor, MI). Arachidonic acid and reduced glutathione (GSH) were from Nu-Chek Preparations (Elysian, MN) and Boehringer Mannheim Canada (Laval, PQ), respectively. The Sep-Pak C18 cartridges were the products of Waters (Milford, MA). Anti-rabbit IgG (Fc)-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), and nitro blue tetrazolium (NBT) were supplied by Promega Corp. (Madison, WI). Reagents for SDS-polyacrylamide gel electrophoresis and for the electrophoretic transfer of proteins from polyacrylamide gels to membranes (MagnaGraph Nylon, MSI, Westboro, MA) were from Fisher Scientific (Ottawa, ON). Prestained SDS-polyacrylamide gel electrophoresis standards were obtained from Bio-Rad Canada (Mississauga, ON). All other chemicals were of analytical purity.

Methods

Tissues. Reflected amnion membranes were isolated from placentas delivered spontaneously at term after uncomplicated pregnancies. The patients received no drugs during labour other than analgesics. The pregnancies were singleton fetuses with normal outcomes. The use of these tissues was approved by the University of Alberta Ethics Review Committee.

Preparation and incubation of minced amnion tissue. The procedures used have been described in detail by Zakar and Olson (1992). Briefly, the amnion membranes were placed in synthetic ('pseudo-') amniotic fluid (PsAF, for composition and properties see Zakar and Olson, 1992 and Schwartz *et al.*, 1977), cleaned, minced, washed three times and preincubated in PsAF for 3 h with one change of medium at 1.5 h. Fresh medium with agonists, dissolved in dimethyl sulfoxide or ethanol, was then added for 14–16 h at 37°C. The concentration of the solvents was less than 0.05% v/v in the incubation media, which had no effect on the PGE_2 production of the tissues. After the agonist treatments, the tissue samples (each representing 1–5 μg DNA) were incubated for 2 h in 1 ml of fresh PsAF supplemented with 10 μmol arachidonic acid l^{-1}, and PGE_2 output was determined by radioimmunoassay.

In experiments involving ASA treatment, the drug was added to the tissues at 0.4 mmol l^{-1} final concentration for the second 1.5 h of the preincubation period. The tissues were then washed twice with PsAF and incubated with agonists for 16 h at 37°C. Fresh medium with 10 μmol arachidonic acid l^{-1} was then added for 1 h, and PGE_2 output was determined. The efficiency of the ASA treatment protocol to inhibit PGE_2 synthesis was determined in control incubations, in which tissues were treated with ASA in PsAF for 30 min, followed by fresh medium with ASA and arachidonate for 1 h. PGE_2 output during the arachidonate plus ASA treatment period was determined, and compared with the PGE_2 output of tissue treated with arachidonate but not with ASA.

The steady state values of PGHS enzyme activity and PGHS immunoreactive protein were determined by cutting the amnion membranes into 1–4 cm^2 pieces, and 3–7 g tissue (wet weight) was incubated in 50 ml of medium with agonists. An aliquot of the incubation medium was saved for PGE_2 determination, and the tissue pieces were blotted on filter paper (Whatman 3MM), rinsed with PsAF, blotted again, and frozen in liquid N_2. The frozen tissues were stored at −74°C until further processing.

PGE_2 determination. PGE_2 content was determined directly from the incubation media with a specific radioimmunoassay (Evans *et al.*, 1981) characterized previously by Olson *et al.* (1984). The intra-assay and interassay coefficients of variation of this procedure were 6.0 and 6.9%, respectively (at 2000 pg PGE_2 ml^{-1}, calculated using the recovery equation $y = 1.10x - 30$; $r = 0.993$; $P < 0.001$; Student's t test) (Olson *et al.*, 1984). Blanks containing incubation media with arachidonic acid were included in each assay, and experiments with detectable background values were excluded from the study.

DNA extraction and determination. DNA was extracted from the tissues, and determined by a fluorimetric method using procedures of Zakar and Olson (1992). In brief, the samples were homogenized in ice-cold 1 mol NH_4OH l^{-1} and 10 mmol EDTA l^{-1}, and incubated over 5 mol H_2SO_4 l^{-1} at room temperature overnight. An aliquot of the supernatant was used to measure DNA content with a *bis*-benzimidazole fluorescence enhancement assay (Downs and Wilfinger, 1983). Calf thymus DNA was used as standard.

Determination of PGHS activity. The method was as described by Smieja *et al.* (1993) with minor modifications. The frozen tissue samples were pulverized in liquid N_2, and

homogenized in a solution containing 0.25 mol sucrose l^{-1}, 50 mmol Tris–HCl l^{-1} (pH 8.0), 2.5 mmol tryptophan l^{-1}, 2 mmol EDTA l^{-1}, 1 mmol DDC l^{-1}, 1 mmol PMSF l^{-1} and 10 μg leupeptin ml^{-1}. The homogenate was centrifuged at 2000 *g* at 2°C for 10 min, and the floating layer of fat was removed. The aqueous supernatant was ultracentrifuged at 105 000 *g* at 2°C for 60 min, and the particulate fraction, homogenized in 50 mmol Tris–HCl l^{-1} (pH 8.0), 2.5 mmol tryptophan l^{-1}, 2 mmol EDTA l^{-1} and 1 mmol DDC l^{-1}, was used as enzyme preparation.

The PGHS enzyme reaction mixtures contained 50 mmol Tris–HCl l^{-1} (pH 8.0), 2 mmol EDTA l^{-1}, 4 mmol tryptophan l^{-1}, 1 mmol GSH l^{-1}, 10 μmol arachidonic acid l^{-1} and 50 μl of enzyme preparation (3–25 μg protein) in a final volume of 250 μl. The cofactor and the arachidonate concentrations were shown to be optimal and saturating, respectively, by Smieja *et al.* (1993). Reactions were started with enyzme and allowed to proceed at 37°C for 4 min, representing initial velocity. Reactions were stopped by adding 2.5 ml 50 mmol sodium citrate l^{-1} (pH 3) containing 15% (v/v) ethanol, and prostaglandins were extracted using Sep-Pak C18 cartridges as described by Smieja *et al.* (1993). PGE_2, recovered with 85–95% efficiency, was determined in the extracts by radioimmunoassay. Each enzyme preparation was assayed in triplicate at two protein concentrations. Results were normalized to protein content and expressed as ng PGE_2 produced μg^{-1} protein in 4 min.

SDS-polyacrylamide gel electrophoresis, membrane transfer and immunodetection of PGHS. Low speed (2000 *g*, 10 min) supernatants of the tissue homogenates were subjected to ultracentrifugation (105 000 *g*, 60 min). The pellets were dissolved in electrophoresis sample buffer (Laemmli, 1970) by heating at 90°C for 10 min. The proteins (2–4 μg per lane) were separated by electrophoresis in 7.5% SDS-polyacrylamide gels according to Laemmli (1970), and transferred electrophoretically (100 V, 60 min at 4°C) to MagnaGraph Nylon membranes using the method described by Towbin *et al.* (1979). The membranes were blocked by incubating in 5% nonfat dry milk dissolved in TBS (25 mmol Tris–HCl l^{-1}, pH 7.5; 0.5 mol NaCl l^{-1}) at room temperature for 1 h. Rabbit polyclonal anti-PGHS antiserum, diluted 1:1000 in TBS, 5% nonfat dry milk, 0.02% sodium azide, was then added to the membranes for 14 h at room temperature. The primary antibody was removed by washing three times for 20 min with TBS, 0.3% Tween 20, and the membranes were incubated for 2 h with an alkaline phosphatase conjugated anti-rabbit (IgG antibody, diluted 10 000 fold with TBS and 0.3% Tween 20. After three 20 min washes with TBS, 0.3% Tween 20, alkaline phosphatase buffer (100 mmol NaCl l^{-1}; 50 mmol Tris–HCl l^{-1}, pH 10; 2 mmol $MgCl_2$ l^{-1}) containing a chromogenic alkaline phosphatase detection system (BCIP/NBT) was added to the membranes. The colour was developed at room temperature for 15–30 min. The blots were scanned with an LKB Ultroscan laser densitometer, and the peaks corresponding to immunoreactive PGHS were integrated manually.

Protein determination. Proteins were solubilized by incubating the particulate fractions in 0.2 mol NaOH l^{-1} at 37°C overnight. The NaOH was then neutralized with an equivalent amount of HCl and the protein concentrations were determined according to Bradford's dye binding method as described by Zakar and Olson (1988). BSA was used as standard.

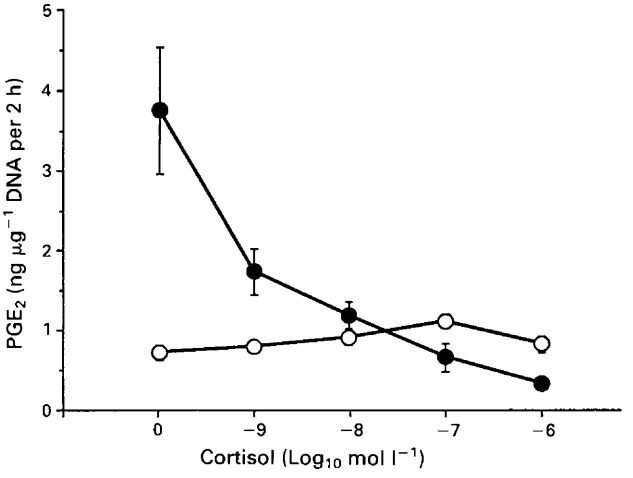

Fig. 1. The effects of cortisol and RU486 on the arachidonate stimulated PGE_2 output of the human amnion. Minced amnion tissue was incubated with increasing concentrations of cortisol in the presence (○) or absence (●) of the glucocorticoid antagonist RU486 (0.1 μmol l^{-1}) for 16 h. PGE_2 output was determined after steroid treatment by adding fresh medium with 10 μmol arachidonic acid l^{-1} for 2 h, and measuring PGE_2 by radioimmunoassay. Each point is the mean of four parallel incubations ± SEM. Cortisol and RU486 significantly ($P < 0.05$) inhibited the prostaglandin output.

Statistical analysis and presentation of data. PGE_2 values on all figures were normalized individually to the DNA content of the corresponding tissue sample. The data were then subjected to analysis of variance using a computer program (Super ANOVA, Abacus Concepts, Inc., Berkeley, CA), and where a significant *F* value was obtained, treatment means were separated using the Newman–Keuls post hoc test. $P < 0.05$ was considered significant.

Results

The possibility that a decrease of PGHS activity is involved in the inhibition of amniotic PGE_2 production by glucocorticoids was investigated (Gibb and Lavoie, 1990) (Fig. 1). Cortisol inhibited the arachidonate-stimulated PGE_2 output with an IC_{50} value of approximately 10^{-9} mol l^{-1}. A parallel set of tissue samples was incubated with cortisol in the presence of 10^{-7} mol RU486 l^{-1}, a glucocorticoid receptor antagonist (Jung-Testas and Baulieu, 1983), to determine the involvement of the glucocorticoid hormone receptor in the action of the steroid. Unexpectedly, RU486 also blocked PGE_2 output, with a tendency to antagonize the effect of higher concentrations (10^{-7}–10^{-6} mol l^{-1}) of cortisol.

Cortisol, RU486 and dexamethasone, added at 10^{-7} mol l^{-1}, caused a significant ($P < 0.05$) decrease in arachidonate-stimulated prostaglandin output, whereas 10^{-7} mol l^{-1} of oestradiol, progesterone, dehydroepiandrosterone sulfate, testosterone and 17-hydroxyprogesterone did not influence PGE_2 production relative to the vehicle-treated controls (Fig. 2).

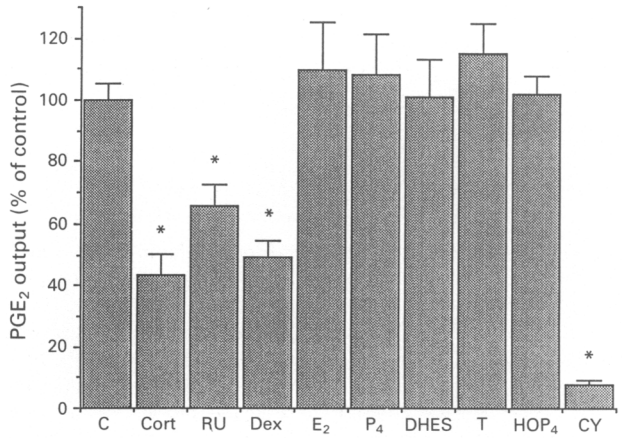

Fig. 2. The effects of various steroids on the arachidonate-promoted PGE_2 production of the human amnion. Amnion tissue was incubated for 16 h with 0.1 μmol l^{-1} of each of the following steroids: cortisol (Cort); RU486 (RU); dexamethasone (Dex); oestradiol (E_2); progesterone (P_4); dehydroepiandrosterone sulfate (DHES); testosterone (T); 17-hydroxyprogesterone (HOP_4). Additional samples were treated with 100 μg cycloheximide ml^{-1} (CY). Controls (C) were incubated with vehicle. After these treatments, fresh medium with 10 μmol arachidonic acid l^{-1} was added for 2 h, and PGE_2 output was determined by radioimmunoassay. PGE_2 output values, normalized to tissue DNA content, were expressed relative to the control. Each bar is the mean of results from 3–6 patients ± SEM. *$P < 0.05$ versus control (ANOVA, followed by Newman–Keuls test).

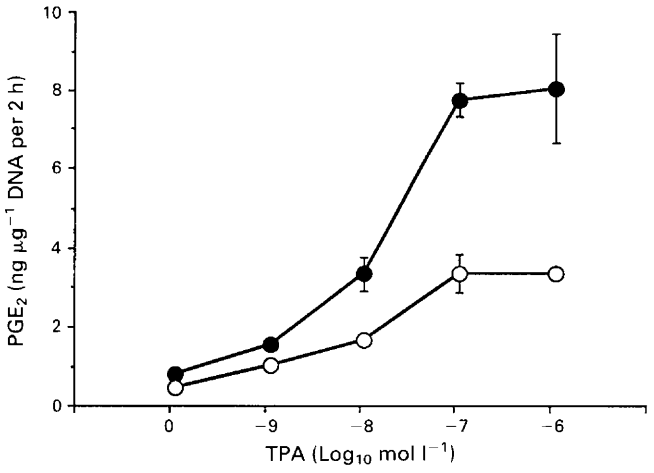

Fig. 3. The effect of TPA and cortisol on the arachidonate-stimulated PGE_2 production of the human amnion. Minced amnion tissue was treated for 16 h with increasing concentrations of the tumour promoter phorbol ester 12-tetradecaonylphorbol 13-acetate (TPA) in the presence (○) or absence (●) of 100 nmol cortisol l^{-1}. PGE_2 output was determined in subsequent 2 h incubations in fresh medium supplemented with 10 μmol arachidonic acid l^{-1}. Each point is the average of four incubations ± SEM. Cortisol significantly ($P < 0.05$) decreased the TPA-stimulated PGE_2 production.

Thus,the inhibition involved a decrease in PGHS activity, and was specific to steroids that bind to the glucocorticoid receptor.

Cycloheximide (100 μg ml^{-1}), a protein synthesis inhibitor, blocked PGE_2 synthesis by more than 90% (Fig. 2). Amino acid incorporation into acid precipitated material was blocked by this concentration of cycloheximide to a similar extent (> 95%, data not shown), confirming previous observations that protein synthesis was required for prostaglandin production by the amnion (Gibb and Lavoie, 1990; Zakar and Olson, 1992), and suggesting that the maintenance of PGHS activity depends on continuous protein synthesis.

The effect of 12-tetradecanoyl phorbol 13-acetate (TPA), a PKC activating phorbol ester (Castagna *et al.*, 1982) and a strong stimulant of amnion PGE_2 production (Zakar and Olson, 1992), on the arachidonate-promoted PGE_2 output is shown (Fig. 3). TPA increased the conversion of arachidonic acid to PGE_2 in a concentration dependent fashion. Maximal stimulation was reached at 10^{-7} mol TPA l^{-1}; half maximal increase was achieved with approximately 15 nmol TPA l^{-1}. When the tissues were co-incubated with TPA and 10^{-6} mol cortisol l^{-1}, the basal, as well as the stimulated, prostaglandin production was reduced by 40–60% without affecting the EC_{50} value of the phorbol ester.

Treatment with TPA or phorbol-12,13-dibutyrate (PBD), which are strong activators of PKC (Castagna *et al.*, 1982), significantly ($P < 0.05$) increased the prostaglandin production during subsequent stimulation with arachidonic acid (Fig. 4). Moreover, their efficacy (TPA > PBD) corresponded to their relative potency to activate PKC. 4-Methyl TPA or 4β-phorbol, which are weak tumour promoters and do not activate PKC, were ineffective.

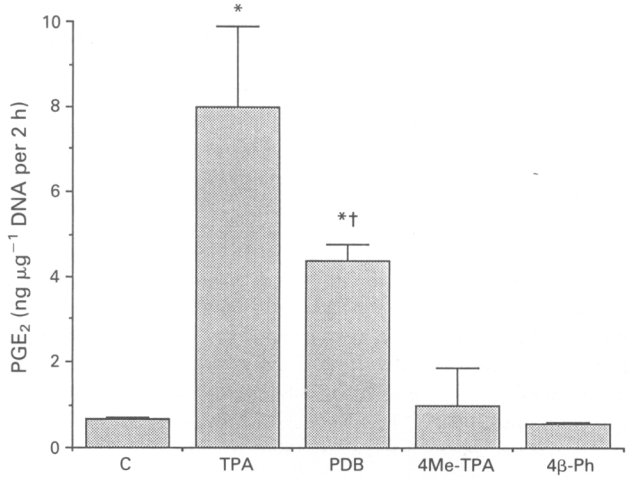

Fig. 4. The effect of phorbol derivatives on the arachidonic acid-promoted PGE_2 production of human amnion tissue. Amnion tissue samples were incubated with 100 nmol l^{-1} of 12-tetradecanoylphorbol 13-acetate (TPA), or phorbol-12,13-dibutyrate (PDB), or 4-methyl-TPA (4Me-TPA), or 4β-phorbol (4β-Ph), or vehicle (C) for 16 h. PGE_2 output was measured during the subsequent 2 h incubations in fresh medium containing 10 μmol arachidonic acid l^{-1}. *Significantly ($P < 0.05$) different from the control; †significantly ($P < 0.05$) less than after treatment with TPA.

Since PGHS irreversibly self-inactivates while performing its catalytic function (Smith and Lands, 1972), the PGHS activity probably reflects the rate of *de novo* enzyme synthesis. We studied the effect of TPA and cortisol on the *de novo* synthesis of amniotic PGHS using tissues pretreated with ASA (Fig. 5). ASA irreversibly inactivates PGHS (Smith and Marnett, 1991), and prostaglandin production after ASA pretreatment requires

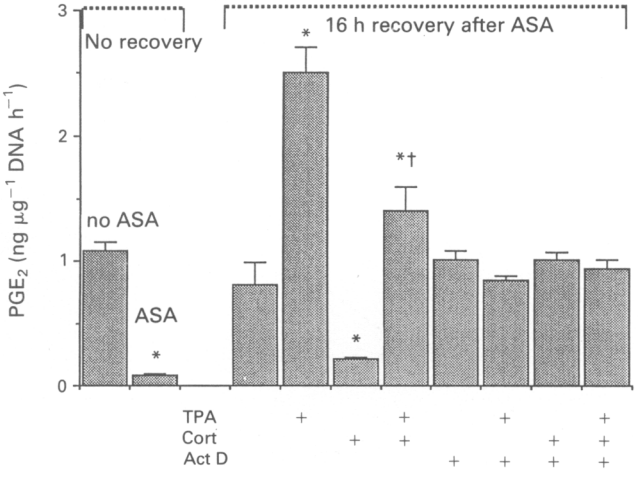

Fig. 5. The effect of TPA, cortisol and actinomycin D on the recovery of PGE_2 synthesis by the human amnion after acetylsalicylic acid treatment. Amnion tissue was incubated with 0.4 mmol acetylsalicylic acid l^{-1} (ASA) for 90 min. ASA was then removed, and the tissue samples were allowed to recover for 16 h in fresh medium with different combinations of TPA (0.1 μmol l^{-1}) cortisol (Cort, 0.1 μmol l^{-1}), and actinomycin D (Act D, 2 μg ml^{-1}). After recovery, new medium with 10 μmol arachidonic acid l^{-1} was added for 1 h, and PGE_2 output was determined. The ability of ASA treatment to inhibit PGE_2 production was tested by adding arachidonic acid for 1 h to tissue samples not treated with ASA (no recovery, no ASA), and comparing the prostaglandin output of these samples to the prostaglandin production of tissues incubated with arachidonic acid during the last 1 h of the ASA treatment period (no recovery, ASA). Treatment combinations during the recovery interval are shown (+) at the bottom of the columns. Each column is the mean of four parallel incubations ± SEM. *$P < 0.05$ versus spontaneous recovery; †significantly ($P < 0.05$) less than after recovery in the presence of TPA.

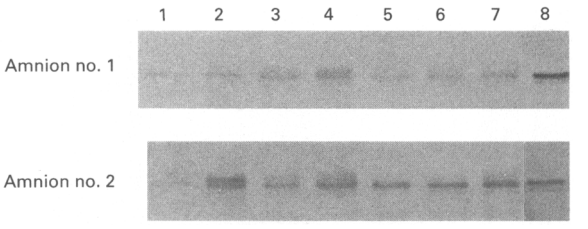

Fig. 6. The effects of TPA, cortisol and indomethacin on immunoreactive PGHS in the human amnion. Amnion tissue samples were incubated with TPA (0.1 μmol l^{-1}) or cortisol (0.1 μmol l^{-1}) or vehicle (Control) in the presence or absence of indomethacin (5 μmol l^{-1}) for 16 h. Particulate (microsomal) fractions were prepared and analysed with SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to nylon membrane, and the immunoreactive PGHS protein was detected with a rabbit polyclonal antibody raised against ovine seminal vesicle PGHS, and an alkaline phosphatase-conjugated second antibody against rabbit IgG. Ovine PGHS standard (70 kDa), 2 and 20 fmol on lanes 1 and 8, respectively, was also run on each gel. Band intensities were determined by laser densitometry and manual integration of the PGHS peaks. (Band intensities are presented in Table 1.) Lane 2: control; lane 3: TPA; lane 4: TPA + indomethacin; lane 5; cortisol; lane 6: cortisol + indomethacin; lane 7: indomethacin. The amount of protein loaded on each lane was 4 μg and 2 μg from Amnion no. 1 and Amnion no. 2, respectively.

the synthesis of new PGHS protein. Control incubations, in which arachidonate was added for the last 60 min of the ASA treatment, showed that ASA inhibited PGE_2 output by approximately 90% (compare the two bars on the left side of Fig. 5). However, the ability of the tissue to produce PGE_2 from arachidonate returned to the pretreatment value almost completely after 16 h. TPA (10^{-7} mol l^{-1}) significantly increased, and cortisol (10^{-7} mol l^{-1}) significantly decreased ($P < 0.05$), the recovery compared with tissue incubated without agonists. Combined treatment with TPA and cortisol resulted in significantly lower recovery than with TPA alone, but in higher recovery than in the absence of agonists. Furthermore, actinomycin D (2 μg ml^{-1}), an RNA synthesis inhibitor, blocked the phorbol ester stimulation as well as the glucocorticoid inhibition of PGHS synthesis, but did not significantly affect the spontaneous recovery of the enzyme. These results indicate that the phorbol ester stimulated and the glucocorticoid inhibited the *de novo* synthesis of PGHS in an RNA synthesis dependent manner. At the same time, the spontaneous recovery of PGHS did not require continuous RNA synthesis.

Alterations of the PGHS synthesis rate may lead to changes in the steady state enzyme activity. To test this possibility and to obtain direct evidence that glucocorticoids and PKC activators influence the amount of PGHS in the amnion, we determined the effects of cortisol and TPA on the specific activity of PGHS in amnion microsomes. The PGHS specific activity of the microsomal preparations from untreated tissues was 40.08 ± 14.88 ng PGE_2 mg^{-1} protein per 4 min (mean ± SEM, $n = 6$ patients). Cortisol decreased the PGHS activity to 36.81 ± 11.88 ng PGE_2 mg^{-1} protein per 4 min ($P < 0.05$; ANOVA). Enzyme activity in microsomes from TPA-treated tissues was 39.92 ± 8.07 ng PGE_2 mg^{-1} protein per 4 min and was not different from the control value. However, using logarithmically transformed data, a significant stimulation by TPA was seen (control: 3.17 ± 0.48; TPA: 3.52 ± 0.27; mean ± SEM; $P < 0.05$, ANOVA). Although these results are in general in agreement with the results of experiments in which the arachidonic acid promoted PGE_2 output was measured, they also suggested that there is a complex relationship between PGHS synthesis rates and steady state PGHS activity.

Changes in the rate of synthesis and the steady state activity of PGHS may accompany corresponding changes in the amount of enzyme protein. To determine the effects of TPA and cortisol on the PGHS mass in the amnion, we measured the amount of immunoreactive PGHS in amnion membranes. Representative PGHS immunoblots obtained with tissues from two patients are shown (Fig. 6), and Table 1 contains the results of the densitometric quantification of PGHS bands. PGE_2 output during the agonist treatment periods was also determined (Table 1). In amnion no. 1 (Fig. 6), a single immunoreactive band co-migrating with the ovine PGHS standard (70 kDa) was detected (control, lane 2). This band was not seen when the blots were exposed to the PGHS antiserum in the presence of excess PGHS, verifying the specificity of the immunodetection (not shown). The amount of PGHS in this preparation was between 0.5 and 5 fmol μg^{-1} protein, as judged from the intensity of bands generated by known

Table 1. The effect of the cortisol, 12-tetradecanoylphorbol 13-acetate and indomethacin on prostaglandin endoperoxide H synthase and on PGE_2 output by the human amnion

Parameter	Amnion number	Treatment[a] Control (2)	TPA (3)	Cort (5)	Ind (7)	TPA + ind (4)	Cort + ind (6)	Standard PGHS 2 fmol (1)	20 fmol (8)
Band intensity	1	1.0	1.5	0.83	1.1	2.2	0.89	0.45	1.4
PGE_2 output	1	136	1142	67	16	15	9	N/A	N/A
Band intensity	2	1.0	0.50	0.56	0.94	0.69	0.56	0.21	0.60
PGE_2 output	2	309	690	104	19	20	18	N/A	N/A

Values in parentheses are lane numbers in Fig. 6.

[a]Amnion tissue samples were treated with 12-tetradecanoylphorbol 13-acetate (TPA, 100 nmol l^{-1}), or cortisol (Cort, 100 nmol l^{-1}), or indomethacin (Ind, 5 μmol l^{-1}), or vehicle (control), or the combination of 12-tetradecanoylphorbol 13-acetate and indomethacin (TPA + ind) or cortisol and indomethacin (Cort + ind) for 16 h. Proteins in the particulate (microsomal) fractions were analysed by polyacrylamide gel electrophoresis, membrane transfer and prostaglandin endoperoxide H synthase (PGHS) immunodetection using a rabbit polyclonal antibody against ovine seminal vesicle PGHS, and an alkaline phosphatase-conjugated second antibody against rabbit IgG. PGHS (70 kDa) purified from sheep seminal vesicles was used as standard. PGHS band intensities were determined by laser densitometry and manual integration, and were expressed relative to the controls. A photograph of the immunoblots corresponding to the region of PGHS is presented (Fig. 6). PGE_2 production during the agonist treatment periods was determined by radioimmunoassay and expressed as ng g^{-1} wet weight per 16 h. N/A: not applicable.

amounts of purified PGHS on the same blot (lanes 1 and 8; Fig. 6, Table 1). TPA treatment increased, and cortisol decreased, the immunoreactive PGHS content of the particulate fraction (lanes 3 and 5, respectively). Moreover, the phorbol ester caused the expansion of the band identified as PGHS, which indicates the formation of immunoreactive material with a molecular weight higher than that of the standard. In agreement with the changes of enzyme protein content, the PGE_2 output of the tissue increased over eightfold in the presence of the phorbol ester, and decreased by more than 50% during cortisol treatment (Table 1).

In addition, tissue samples from the same amnion were treated with agonists in the presence of indomethacin. Indomethacin inhibits cyclooxygenase activity and stabilizes the enzyme against proteolytic degradation (Mizuno *et al.*, 1982; Kulmacz, 1989). Accordingly, in amnion no. 1, indomethacin increased the immunoreactive PGHS in the absence and in the presence of cortisol or TPA (Fig. 6, Table 1). The maximal increase was seen with 5 μmol indomethacin l^{-1}, whereas 1 μmol or 10 μmol indomethacin l^{-1} had little or no effect (not shown). Furthermore, cortisol inhibited, and TPA stimulated, PGHS protein accumulation in indomethacin-treated tissues; and the effect of the phorbol ester was particularly enhanced by the enzyme inhibitor (lanes 5 and 4). The PGE_2 output of the tissues was blocked by more than 85% with 5 μmol indomethacin l^{-1} under all experimental conditions (Table 1). Responses such as those of amnion no. 1 were seen with three of the five amnion membranes included in the study.

Amnion no. 2, a representative of two of the five tissues studied, exhibited another type of response to the above treatments (Fig. 6, lower panel). This tissue contained more PGHS than did amnion no. 1 (> 10 fmol μg^{-1} protein) in the particulate fraction, and the PGHS band (lane 2) was wider than the band generated by the PGHS standard. This finding suggests an increased heterogeneity of the PGHS protein in this amnion compared with the ovine enzyme, including the possible presence of a protein doublet. The intensity of the PGHS band decreased after incubating the tissue with cortisol as well as with TPA, and a single band co-migrating with the ovine PGHS was seen (lanes 5 and 3, respectively). The PGE_2 output of amnion no. 2 was greater than that of amnion no. 1, which was in agreement with the relative amount of immunoreactive PGHS (Table 1). Cortisol treatment decreased the prostaglandin production of amnion no. 2 by 66%, but TPA caused a more than twofold increase of PGE_2 output, despite its inhibitory effect on PGHS protein accumulation. Indomethacin caused an increase in PGHS protein in this tissue only in the presence of TPA (compare lanes 3 and 4), and did not influence immunoreactive enzyme levels in the cortisol treated tissue. After incubation with indomethacin alone, a single PGHS band co-migrating with the ovine standard was detected in amnion no. 2 (lane 7). The amount of immunoreactive material in this band, determined by densitometry, was slightly diminished compared with the control (lane 2).

In summary, cortisol consistently decreased the amount of immunoreactive PGHS and the production of PGE_2 by the amnion tissues. Phorbol ester stimulated the PGE_2 output of all tissues studied, which was accompanied by an increase of PGHS protein content in some tissues, and a decrease in immunoreactive PGHS in others. Indomethacin consistently increased the amount of PGHS protein only in TPA-treated tissues.

Discussion

The rate-limiting step in the conversion of arachidonate to prostaglandins is the oxygenation of arachidonic acid to prostaglandin endoperoxides by PGHS (Needleman *et al.*, 1986; Smith *et al.*, 1991). In agreement with this, the incubation of amnion tissue with 10 μmol arachidonic acid l^{-1}, a concentration that saturates amniotic PGHS in a cell-free assay system (Smieja *et al.*, 1993), increases PGE_2 output maximally (López

Bernal *et al.*, 1988). The inclusion of 10 μmol arachidonate l^{-1} in the medium of our tissue mince incubation system also caused a significant stimulation of PGE_2 production (0.437 ± 0.053 versus 0.662 ± 0.039 ng μg^{-1} DNA per 2 h; mean ± SEM, $n = 8$ patients, $P < 0.01$, Student's t test). We therefore assessed PGHS activity in the amnion by determining the capacity of the tissue to convert arachidonate to PGE_2.

In many tissues and cells, glucocorticoids inhibit prostaglandin production (Russo-Marie, 1990; Smith *et al.*, 1991; Smith and Marnett, 1991). However, the intracellular mechanisms mediating this physiologically important action of corticosteroids are unclear. In the present investigation, we provided evidence showing that glucocorticoids diminish amniotic PGE_2 production by inhibiting the activity of PGHS, the enzyme catalysing the rate-limiting step of prostaglandin synthesis from arachidonic acid. We demonstrated that cortisol, dexamethasone and RU486, which bind to the glucocorticoid receptor, specifically inhibited the production of PGE_2 from exogenous arachidonate. Furthermore, cortisol treatment resulted in a decrease in PGHS activity and of immunoreactive PGHS protein in the particulate (microsomal) fraction. The recovery of arachidonate-stimulated PGE_2 synthesis after irreversible inactivation of PGHS by ASA treatment was also reduced by cortisol, suggesting that the steroid inhibited the synthesis of new enzyme protein. Interestingly, RU486, an established glucocorticoid and progesterone receptor antagonist, mimicked the effect of the glucocorticoids on arachidonate promoted prostaglandin output. Agonistic effects of this steroid were reported in the human pituitary (Laue *et al.*, 1988) and endometrium (Gravanis *et al.*, 1985), and in monkey endometrium *in vivo* (Wolf *et al.*, 1989) and in human peripheral blood mononuclear cells *in vitro* (Van Voorhis *et al.*, 1989). Clearly, the human amnion is another tissue in which RU486 may act as a steroid hormone agonist.

Experiments with protein and RNA synthesis inhibitors provided information about the mechanism of glucocorticoid action. Inhibition of protein synthesis with cycloheximide blocked the arachidonate-stimulated PGE_2 production by 90%, indicating that continuous protein synthesis was required for the conversion of arachidonic acid to PGE_2. PGHS may be one of the proteins that are continuously replenished, since it self-inactivates while performing its normal catalytic function, and has to be synthesized continuously to maintain a steady state activity. In agreement with this, PGHS activity in amnion tissue recovered almost completely during 16 h following ASA pretreatment. The recovery was independent of RNA synthesis, since it was not inhibited by actinomycin D. This finding suggests that pre-existing RNA molecules were used for the synthesis of new PGHS. Cortisol inhibited this process in an RNA synthesis dependent fashion. On the basis of these observations, it is reasonable to suggest that the glucocorticoid inhibited the synthesis of PGHS at a post-transcriptional step by inducing a regulatory protein (or RNA species). Post-transcriptional control of PGHS synthesis by glucocorticoids was described in cells such as human dermal fibroblasts (Raz *et al.*, 1989) and rat vascular cells (Pash and Bailey, 1988), and may represent one of the universal mechanisms of PGHS regulation.

Studies using tumour promoting phorbol esters revealed a complex pattern of PGHS regulation by protein kinase C. TPA and phorbol-12,13-dibutyrate consistently stimulated the PGE_2 output in the presence and in the absence of exogenous arachidonate, and TPA promoted the recovery of PGHS after ASA pretreatment. Logarithmically transformed data indicated a significant stimulation of microsomal PGHS activity by TPA. These results show that the activation of PKC resulted in an increase in *de novo* PGHS synthesis.

Experiments in which the effect of TPA on immunoreactive PGHS was measured suggested that PKC activation facilitated the degradation as well as the synthesis of PGHS enzyme protein. Apparently, the TPA-induced PGHS degradation may lead to a decrease in the steady state PGHS protein, in tissues where the basal rate of PGHS breakdown is low. In tissues in which PGHS degradation rates are higher, phorbol ester stimulation may result in the accumulation of more enzyme protein. This interpretation is in agreement with the finding that indomethacin increased the amount of PGHS protein consistently only in the presence of the phorbol ester, that is where the breakdown of the enzyme was facilitated. Poor correlations between prostaglandin output, PGHS activity and protein were reported in PDGF stimulated mouse fibroblasts (Lin *et al.*, 1989; DeWitt, 1991), and were attributed to an increase of PGHS turnover induced by PDGF. The increased turnover resulted in high prostaglandin output, while the amount of enzyme protein remained constant. Protein kinase C activators may regulate the PGE_2 synthesis of the human amnion by a similar mechanism.

A PGHS-immunoreactive band with a molecular weight slightly higher than that of the ovine standard was detected in one amnion. The relationship of this material to PGHS co-migrating with the ovine standard (70 kDa), and the requirements of its production are unclear. However, in rat ovaries, a second form of PGHS induced by LH was described, which had a molecular mass of 70–72 kDa (Sirois and Richards, 1992). PGHS isoforms induced by mitogens, such as PDGF, EGF, TPA, serum and Rous sarcoma virus oncogen product, were reported in several cell lines (reviewed by Xie *et al.*, 1992), and the predicted molecular masses of these proteins were all close to 70 kDa. In amnions in which TPA increased the amount of PGHS protein, TPA induced PGHS-immunoreactive material with higher molecular mass than the ovine standard. Together, these observations suggest that alternative form(s) of PGHS may appear in the amnion membrane following stimulation by phorbol esters or other factors *in vivo*.

In conclusion, the above experiments demonstrate that PGHS in the human amnion is positively and negatively regulated by PKC activators and glucocorticoids, respectively. Treatment of amnion tissue with these agonists changes PGHS activity and enzyme synthesis rates, parameters shown to change *in vivo* at labour. However, the involvement of PKC or corticosteroids in the *in vivo* regulation of amniotic PGHS at parturition still remains to be established.

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