Sn-1,2-diacylglycerols and phorbol ester stimulate the production of progesterone from the human placenta

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Abstract. Human term placental explants were used to investigate the possible role of phospholipid-sensitive and Ca²⁺ dependent protein kinase in the regulation of human placental progesterone production. Placental tissue was incubated with low density lipoprotein as a precursor of progesterone in the presence or the absence of phorbol 12-myristate-13-acetate, 1-oleoyl-2-acetylglycerol, and the calcium ionophore A23187. The rate of progesterone production by placental tissue was 21.7 ± 4.6 ng·(mg wet wt)⁻¹·(2 h)⁻¹ (mean ± SEM) with 500 mg low density lipoprotein/l (control). The rate of progesterone production was accelerated 2-fold by 1 nmol/l phorbol 12-myristate-13-acetate, 1.6-fold by 250 µmol/l 1-oleoyl-2-acetylglycerol and this increase was dose-related (25–250 µmol/l 1-oleoyl-2-acetylglycerol). A non-promoting derivative, 4α-phorbol-12,13-didecanoate had no effect. The phorbol 12-myristate-13-acetate or 1-oleoyl-2-acetylglycerol induced stimulation of progesterone production was not associated with a change in the intracellular cAMP level. Addition of 10 µmol/l A23187 further increased progesterone production with 125 µmol/l 1-oleoyl-2-acetylglycerol. The rate of progesterone production was accelerated 1.6-fold by 125 µmol/l 1-oleoyl-2-acetylglycerol and 10 µmol/l A23187 as compared with control. The effects of the phorbol ester and the diacylglycerol were completely blocked by the addition of the protein synthesis inhibitor cycloheximide. We conclude that these phorbol regents are able to stimulate human placental progesterone production. The possible roles of intracellular Ca²⁺ and protein kinase C in placental steroidogenesis are discussed.

The regulation of progesterone production in the human placenta is interesting in relation to the maintenance of pregnancy and the onset of labour. After the 10th week of gestation, progesterone is synthesized mainly by the placenta, and its synthesis is generally thought to be modulated by neurotransmitters, cyclic nucleotides, and/or other steroids (1, 2). Progesterone production is significantly increased by addition of terbutaline (β₂-stimulant) to human term placental tissue (3, 4). On the other hand, Ca²⁺ dependent protein kinase (protein kinase C) has been reported to be present almost universally in various phyla of the animal kingdom (5). Bovine luteal cells and rat placenta have been reported to possess protein kinase C (6). Protein kinase C has been reported to be one of the enzymes which phosphorylate cellular proteins, and is reported to be involved in transmembrane transmission of extracellular signals such as hormones, growth factors and tumour promoters. Phorbol-12-myristate-13-acetate (PMA) is the most potent tumour-promoting phorbol ester and has been shown to stimulate progesterone production in cultured choriocarcinoma cells (7), to stimulate choriogonadotropin secretion by cultured human choriocarcinoma cells (8), and also to modulate the human placental progesterone secretion (9). We have previously reported that human placental
progesterone production was regulated by Ca²⁺ and cAMP (4). In the present study, we have examined the role of protein kinase C in the regulation of progesterone production by human term placenta.

Materials and Methods

Materials

Blood was obtained from women 2 days after delivery. Whole serum was separated from erythrocytes by centrifugation, and the various lipoprotein fractions were prepared by ultracentrifugation employing methods reported by Goldstein & Brown (10). Low density lipoprotein (LDL) was collected at densities between 1.019–1.063 kg/l. Protein content of individual lipoprotein fractions was measured according to the method of Lowry et al. (11) using bovine serum albumin as the standard. The di-valent ionophore A23187, 1-oleoyl-2-acetyl-glycerol (OAG), the protein synthesis inhibitor cycloheximide, 1-methyl-3-isobutyl-xanthine (MIX), PMA, 4α-phorbol-12,13-didecanoate (4α-PDD), and cholera toxin were purchased from Sigma Chemical Co (St. Louis, MO); Eagle’s Minimum Essential Medium (MEM) was purchased from Nissui Pharmaceutical Co (Tokyo, Japan) and terbutaline from Fujisawa Pharmaceutical Co (Tokyo, Japan).

Preparation of human placental tissue

The method of human placental tissue preparation employed was similar to that described by Kasugai et al. (4). Full term placentas (N = 12) were obtained aseptically from women who had delivered transvaginally or by elective cesarean section. After perfusion of each placenta with 5 l of 0.15 mol/l NaCl to remove fetal blood, fragments of near-white fetal cotyledons were dissected, extending from the maternal surface to the chorionic plate. These were placed in a tube and rinsed twice with sterile 0.15 mol/l NaCl. The tissue was minced with scissors and washed with 50 ml of Eagle’s MEM to remove the remaining blood. Then, the minced tissue was pre-incubated for 24 h with 50 ml of MEM and 10% fetal calf serum at 37°C in an atmosphere of 5% CO₂ in air.

Incubation

After pre-incubation, the tissue explants were centrifuged at 900 × g for 15 min and the pellets were transferred to 15 ml Corning tubes (about 100 mg wet wt pellet/tube). The tissue was resuspended in 10 ml MEM containing 500 mg LDL/l as a progesterone precursor as well as protein kinase C activators, calcium ionophore and/or protein synthesis inhibitor, all performed in triplicate. The tubes were loosely capped and the explants were incubated in a humidified atmosphere of 5% CO₂ at 37°C for 30–180 min. After incubation, the incubation mixtures were centrifuged at 900 × g for 10 min, and the supernatants were frozen until analysis for progesterone. The medium progesterone concentration was measured in duplicate using a RIA Kit (Green Cross Corp, Tokyo, Japan). The placential tissue pellets were homogenized, and their protein content were measured by the Lowry method (11).

Placental tissue explants were incubated with 0.5 nmol/l MIX and several different combinations of protein kinase C activators for cAMP measurement. After 130 min of incubation the suspensions were centrifuged 900 × g for 10 min. The pellets were placed in liquid nitrogen to stop the reaction, and then weighed. After measurement of the wet weight, the pellets were homogenized with 1 ml of ice-cold 6% trichloroacetic acid (Katayama Chemical Co, Osaka, Japan) and the homogenate was centrifuged at 1500 × g at 4°C for 10 min. Trichloroacetic acid in the supernatant was removed by extraction with ethyl ether and cAMP in the supernatant was measured with Yamasa RIA Kits (Yamasa Shoyu Co, Choshi, Japan).

To prove that the tissues were viable, each incubated pellet was placed in MEM (5 ml/g tissue) containing 0.1% collagenase (type IA, Sigma; 420 U/mg), 0.1% hyaluronidase (Sigma type 1-S; 300 U/mg); 0.01% deoxyribonuclease (Sigma DNase-I; 2000 Kunitz U/mg), and 1% fetal bovine serum (Gibco, Grand Island, NY) and incubated at 37°C for 120 min in a shaking waterbath using a modification of the technique described by Wunsch et al. (12). The digest was then filtered twice through sterile gauze and centrifuged, the pellets were washed in MEM, and dispersed cells were resuspended in 1 ml MEM and counted with a standard hemocytometer using the trypan blue exclusion test. Cell viability always exceeded 90%.

The results of progesterone production were given as the mean ± SEM and subjected to regression analysis to examine the significance of dose-response and the effect of time. Differences between treatments were assessed by analysis of variance.

Results

The human placental tissue isolated by mechanical dispersion after 24 h pre-incubation responded to stimulation by PMA in a time-related manner. The amount of progesterone produced by unstimulated tissue and that produced by tissue incubated with PMA (1 nmol/l) containing 500 mg LDL/l was 21.7 ± 4.6 and 29.6 ± 2.8 ng · (mg wet wt)⁻¹ · (2 h)⁻¹ (triplicate determinations), respectively. Progesterone production also responded to stimulation by PMA (0–1 nmol/l) in a dose-related manner, the amount of progesterone produced by tissue with maximal steroidogenic dose of OAG
(250 μmol/l) containing 500 mg LDL/l was 34.5 ± 1.8 ng · (mg wet wt)⁻¹ · (2 h)⁻¹ (triplicate determinations). The effect of PMA on progesterone production by human placental tissue is shown in Fig. I. The data illustrate that progesterone production was stimulated by PMA in concentrations from 1 up to 100 nmol/l. On the other hand, 4α-PDD had no effect on placental progesterone production. Fig. 2 shows the time course of progesterone production by human placental tissue incubated with 10 nmol/l PMA for various periods of time up to 180 min. In the presence of PMA, the amount of progesterone accumulated in the medium was significantly greater (p < 0.01) than that produced in its absence (at 120 min and more).

Since PMA at nanomolar concentrations has been reported to activate protein kinase C in several tissues and diacylglycerol is known to be the intracellular activator of this enzyme (13,14), it was of interest to determine if diacylglycerol could mimic the steroidogenic action of PMA.

Human placental tissue was incubated in the presence of increasing concentrations of OAG and the progesterone produced was measured. The results presented in Fig. 3 show a dose-related stimu-

**Fig. 2.**
Time course of basal and phorbol-12-myristate-13-acetate (PMA)-stimulated progesterone production by isolated human placental tissue. The tissue was incubated in the absence (O—O) or in the presence of 10 nmol/l PMA (---) for various periods. The accumulated progesterone was measured (mean ± SEM, triplicate determinations). The vertical bars show SEM. * p < 0.01 compared with control.

**Fig. 3.**
Enhancement of progesterone production by human placental tissue in the presence of increasing concentrations of 1-oleoyl-2-acetyl-glycerol (OAG). Tissue was incubated for 2 h at 37°C with increasing concentrations of OAG and accumulated progesterone was measured by RIA. The data presented are mean ± SEM (triplicate determinations). * p < 0.05 compared with control.

**Fig. 1.**
Effect of phorbol esters on progesterone production in isolated human placental tissue, incubated for 2 h with 5 mg LDL/tube, in the presence of varying concentrations of phorbol-12-myristate-13-acetate (PMA), 10 nmol/l 4α-phorbol-12,13-didecanoate (4α-PDD). Data are mean ± SEM (triplicate determinations). The vertical bars show SEM. ** p < 0.01 compared with control.
Table 1.
Effect of PMA, OAG, cholera toxin, and terbutaline on cAMP production in isolated human placent al tissue.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Intracellular cAMP accumulation (pmol/g wet tissue; mean ± SEM)</th>
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<tbody>
<tr>
<td>Control</td>
<td>194.3 ± 22.3</td>
</tr>
<tr>
<td>OAG (50 µmol/l)</td>
<td>162.0 ± 14.5</td>
</tr>
<tr>
<td>OAG (250 µmol/l)</td>
<td>196.8 ± 26.9</td>
</tr>
<tr>
<td>PMA (1 nmol/l)</td>
<td>223.8 ± 28.1</td>
</tr>
<tr>
<td>PMA (10 nmol/l)</td>
<td>214.3 ± 11.2</td>
</tr>
<tr>
<td>Cholera toxin (1 nmol/l)</td>
<td>544.1 ± 18.6</td>
</tr>
<tr>
<td>Terbutaline (10 µmol/l)</td>
<td>379.2 ± 21.3</td>
</tr>
</tbody>
</table>

A suspension of human placental tissue was incubated in medium supplemented with 0.5 nmol/l MIX for 30 min in the absence of any additions (Control), with different concentrations of PMA and OAG. Amounts of total cAMP were determined as described in Materials and Methods. All data are mean ± SEM. * p < 0.01 compared with control.

Table 2.
Effect of cycloheximide on the PMA-stimulated progesterone production in human placental tissue.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Progesterone produced ng · (mg wet wt)^−1 · (2 h)^−1 (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.7 ± 4.6</td>
</tr>
<tr>
<td>10 nmol/l PMA</td>
<td>40.4 ± 1.5*</td>
</tr>
<tr>
<td>10 nmol/l PMA and</td>
<td>26.4 ± 0.7</td>
</tr>
<tr>
<td>4 mg/l cycloheximide</td>
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Tissues were incubated in triplicate for 2 h in the presence of PMA with or without cycloheximide and the progesterone produced was measured in the medium. All data are mean ± SEM. * p < 0.01 compared with control.

Fig 4.
Effect of the ionophore A23187 on 1-oleoyl-2-acetyl-glycerol (OAG)-stimulated steroidogenesis in human placental tissue. Isolated placental tissue was incubated with 125 µmol/l OAG and varying concentrations of A23187 and the progesterone content in the medium was measured (mean ± SEM, triplicate determinations). * p < 0.05 compared with control.

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Discussion

During human gestation, progesterone protects the human uterus from the labour-inducing potential of endogenous prostaglandins present in the myometrium, the placenta, and the decidua and from attack by hypophysial oxytocin. Increased progesterone production by stimulation of \( \beta \)-adrenergic receptors has been demonstrated in corpus luteum cells from sheep. This response to \( \beta \)-adrenergic stimulation is accompanied by cAMP synthesis and is inhibited by propranolol (16). Satoh & Ryan demonstrated that the catecholamines, epinephrine and norepinephrine increased adenylyl cyclase activity and cAMP in human placental tissue in a dose-related fashion (17). Also, human placental progesterone production can be modulated by stimulation of \( \beta \)-adrenergic, but not \( \alpha \)-adrenergic, receptors. This response may be mediated by increased intracellular cAMP.

These findings may be important in connection with other metabolic functions of the placenta for the treatment of preterm labour (3, 4). Shemesh et al. reported that progesterone production in the bovine placenta was stimulated by A23187 (18) and recently we reported that progesterone production by human placental tissue is increased by addition of A23187, as a result of increased intracellular Ca\(^{2+} \) concentration (4). Further, the placental progesterone production stimulated by \( \beta \)-agonists is significantly inhibited by addition of naphthalene sulfonamides such as N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7), a calmodulin antagonist (2, 19). These results show that placental progesterone production is regulated not only by the cAMP system, but also by other systems which are affected by intracellular Ca\(^{2+} \) (4).

Carr et al. found that LDL, but not HDL (high density lipoprotein: density, 1.25–1.215 kg/l), is the major source of cholesterol used by the human corpus luteum for progesterone biosynthesis (20). The human placenta uses lipoproteins as a source of cholesterol for steroidogenesis through receptor-mediated uptake and internalization, and the lipoproteins are intracellularly degraded (21). A very small conversion of acetate to cholesterol by the placenta has been observed in vitro (22). There is, however, sound evidence to show that the placenta is incapable of synthesizing the large amounts of cholesterol which would be required to account for placental progesterone production (23). Placental biosynthesis of progesterone is principally dependent upon cholesterol derived from maternal plasma LDL (24). In human term placenta in organ culture, the human lipoprotein has a carrier function for cholesterol and cholesteryl linoleate, and enhances placental progesterone formation (25).

Since the discovery in rat brain of a protein kinase activity stimulated by Ca\(^{2+} \) and phospholipid (26), the roles of this enzyme in the regulation of several cellular processes has elicited growing interest. The tumour promoter, PMA, is known to modulate the response of porcine adrenal glomerulosa cells (27), presumably by activating protein kinase C. Brunswig et al. reported that PMA stimulates basal progesterone production by bovine luteal cells in a dose- and time-related manner (28). Ascoli et al. and other researchers reported that mouse epidermal growth factors (e.g. epidermal growth factor) activates progesterone production in cultured Leydig tumour cells without affecting the levels of cAMP but activating phosphatidylinositol turnover (29–31). It is evident from our data that human placental progesterone production was stimulated by PMA in a dose-related manner. It is conceivable that some of the protein substrates are phosphorylated by PMA-activated protein kinase C in a steroiogenic response.

However, there exists no direct evidence that these phosphorylations play a role in cellular regulation, and the substrates themselves have not been identified. On the other hand, OAG causes dose-related stimulation of progesterone production and an additive effect with Ca\(^{2+} \). It appears that extracellular stimulation triggers the degradation of phosphatidylinositol, and its degraded product, diacylglycerol, activates protein kinase C. Further, another product, inositol 1,4,5-triphosphate, mobilizes Ca\(^{2+} \) from intracellular Ca\(^{2+} \) stores. Intracellular Ca\(^{2+} \) is necessary for phosphorylation of protein kinase C (32). In this study, our data demonstrated that protein kinase C also co-operates with Ca\(^{2+} \) in the regulation of progesterone production by the human placenta. It may be noted that the increase in the amount of progesterone produced in response to a combination of A23187 and submaximal doses of OAG was additive rather than synergistic. Furthermore, OAG had been shown to mimic the steroiogenic action of PMA, suggesting that the activation of protein kinase C is involved in the action of PMA.
From these points of view, human progesterone production is at least regulated by cAMP, intracellular Ca\(^{2+}\) and protein kinase C. It is not clear whether these systems stimulate progesterone production in the human placenta by singular or plural systems.

In conclusion, we have demonstrated that PMA and OAG could stimulate progesterone production by the human placenta and have observed that the effects of PMA and OAG are probably linked to their ability to activate protein kinase C.

References


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