Regulation of steroid synthesis and metabolism in isolated binucleate cells of the placenta in sheep and goats

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Summary. Binucleate cells of sheep and goat fetal placentae comprise about one-fifth of the trophoctodermal layer at the feto–maternal interface. When isolated and incubated *in vitro* they produce the steroids that are synthesized by the placenta *in vivo* (progesterone in sheep, 5 β -pregnane-3 α ,20 α diol in goats). This study demonstrates that progesterone synthesis in binucleate cell preparations in sheep was increased by prostaglandin (PG) E-2, nordihydroguaiaracetic acid (NDGA) and methylisobutylxanthine, but reduced by indomethacin, whereas in goats only NDGA produced any effect (an increase). None of the other compounds tested (luteinizing hormone, follicle stimulating hormone, prolactin, dibutyryl cAMP, A23187 or phorbolmyristic acetate) had any effect. Sheep binucleate cells also produced PGE-2 from arachidonic acid. These results suggest that, in sheep, products of both the cyclooxygenase (producing PGE-2) and lipoxygenase (inhibited by NDGA) pathways of arachidonic acid metabolism have regulatory roles in placental steroid synthesis, but only the lipoxygenase pathway is relevant in goats.

Keywords: progesterone; steroids; placental cells; sheep; goat

Introduction

Although much evidence has been accumulated showing that the placentae of many mammalian species synthesize and secrete steroids, the mechanisms that regulate the process remain ambiguous (reviewed by Heap & Flint, 1984). Attempts to influence placental steroidogenesis using compounds known to affect steroid production in other endocrine tissues have either proved negative or produced conflicting results. This is in marked contrast to the findings in extraplacental steroidogenic tissues, such as the corpus luteum, in which luteinizing hormone (LH) is known to play a major role in most species (Niswender *et al.*, 1985; Hansel & Dowd, 1986).

Recent studies of the corpus luteum have stressed the importance of having two cell types within the gland, and current evidence suggests that biochemical differences between the two may account for the different ways in which each type responds to various test substances *in vitro*. The small luteal cells respond to LH stimulation by increased progesterone production, whereas the large cells do not respond to LH (O'Shea, 1987). These observations indicate that hormone production by specific cell types may be differentially regulated in steroidogenic tissues that contain heterogeneous cell populations. Such a concept is of particular relevance to the placenta, which exhibits considerable cellular heterogeneity. The study of purified luteal cell populations has therefore highlighted the importance of defining biochemical events in specific cell populations in a steroidogenic organ.

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In the studies presented here, purified binucleate cells from fetal placentae of sheep and goats which have been shown to be capable of synthesizing and transforming steroids (Hamon *et al.*, 1985; Wango *et al.*, 1991) were used to compare the control of placental steroid synthesis with the systems established for luteal steroidogenesis. The roles of the major luteotrophic hormones (LH, follicle-stimulating hormone (FSH) and prolactin) and of the intracellular messengers cAMP and protein kinase C on the endogenous production of progesterone and conversion of [^3H] pregnenolone to progesterone were investigated using enriched preparations of these placental cells. Dibutyryl cAMP, a potent analogue of cAMP, and the protein kinase C activator phorbol myristic acetate (TPA) were used to elucidate the roles of these compounds in steroid synthesis by binucleate cells. The importance of Ca^{++} was investigated using the calcium ionophore A23187, and a combination of the ionophore with TPA or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX). The latter experiment was prompted by the findings of Shemesh *et al.*, 1984 indicating that MIX or a combination of MIX and A23187 enhances progesterone production in minced bovine placentae. It was therefore of interest to determine whether such effects were common to placentae of domestic ruminants; Shemesh *et al.* (1984) used minced placentae as opposed to the purified binucleate cell preparations tested in this study.

Finally, the possibility that steroid synthesis by binucleate cells is regulated by local mechanisms was investigated using substances that inhibit the cyclooxygenase (indomethacin) and the lipoxygenase (NDGA) pathways of arachidonic acid metabolism. The effect of adding prostaglandins (PGE-2, I-2 or F-2 α) to the incubation was also assessed.

Materials and Methods

Binucleate cells were isolated by procedures described by Wango *et al.* (1991). Briefly, minced fetal cotyledons from sheep and goats 120–140 days pregnant were dispersed with the neutral protease Dispase (Sigma Chemical Co., Poole, Dorset, UK). Cells were separated on ficoll:trisil gradients, checked for purity (65–85%) viability (85–95%), counted and used for incubations within 6 h of death of the animal.

Endogenous progesterone production. Binucleate cells (3×10^6) were incubated in duplicate for each of three animals in 2 ml Ham's F10 medium containing ovine LH, ovine FSH or prolactin (25 ng/ml). In other experiments, dibutyryl cAMP (10 mmol/l), TPA (10 nmol/l), MIX (0.5 mmol/l) and A23187 (1 $\mu\text{mol/l}$) were added to the binucleate cells. The involvement of the products of arachidonic acid metabolism was tested by adding 0.1 $\mu\text{g/ml}$ of PGE-2, PGF-2 α , or PGI-2, or indomethacin (0.1 mmol/l).

The TPA and A23187 were prepared from stock solutions in dimethylsulphoxide (0.5 mmol/l and 1.0 mmol/l solutions respectively). Indomethacin, MIX and NDGA were prepared in ethanol. In all cases the stock was suitably diluted with medium before being added to the incubations and the highest concentration of solvent in the incubations was 0.5%. Appropriate amounts of solvent were added to control incubations. The samples were incubated in triplicate for 3 h and were analysed for progesterone concentration after extraction with organic solvent, using the radioimmunoassay described by (Wango *et al.*, 1991).

Metabolism of [^3H]pregnenolone. The experimental procedures and compounds tested were similar to those described for endogenous progesterone production, but [^3H]pregnenolone (5 μCi (Amersham International plc, UK)) was added to each incubation. The effect of NDGA, an inhibitor of the lipoxygenase pathway, was investigated.

Metabolism of [^{14}C]arachidonic acid by sheep binucleate cells. The cells (3×10^6) were incubated with labelled arachidonic acid (500 000 d.p.m. (Amersham International plc, UK)) in 2 ml of Ham's F10 medium in the presence or absence of indomethacin (0.025–10 mmol/l) or NDGA (10–120 $\mu\text{g/ml}$) for 3 h. At the end of the incubations, the reactions were stopped by acidifying the samples to pH 3 with 0.1 mol HCl/l. The cells and medium were extracted three times with 5 ml of diethyl ether, and the pooled ether extracts from each sample were dried under nitrogen and redissolved in 0.5 ml ethyl acetate. Prostaglandins were separated by thin-layer argention chromatography (Daniels, 1976) using ethylacetate:acetic acid:methanol:2,2,4 trimethyl pentane:water as the solvent system.

The plates were developed three times together with the unlabelled standards PGE-2, PGF-2 α , and PGI-2. Labelled areas containing prostaglandins were located by comparison with the standards developed with 10% phosphomolybdic acid in ethanol and use of a Panax radiochromatogram scanner. Areas of radioactivity were scraped from the plate and radioactivity was measured after precipitation by NaCl of the silver used in preparation of the thin-layer chromatography plate, which otherwise reduced the efficiency of measurement (Greenwald *et al.*, 1981).

Results

Endogenous progesterone production

Sheep binucleate cells produced substantial amounts of endogenous progesterone whereas those of goats produced very little (Fig. 1); neither LH, prolactin, FSH, dibutyryl cAMP, PGF-2 α , PGI-2, TPA, nor A23187 had a significant effect on production by sheep or goat binucleate cells. Indomethacin (0.1 mmol/l) suppressed progesterone production, MIX (0.5 mmol/l) produced a significant increase and PGE-2 (0.1 μ g/ml) a marked, though nonstatistically significant, increase (Fig. 1). Subsequent work demonstrated a significant increase in pregnenolone conversion to progesterone in the presence of PGE-2 (see Fig. 2b).

Fig. 1. Endogenous progesterone production by sheep ($\square$) and goat ($\blacksquare$) binucleate cells (3×10^6) incubated in 2 ml of Ham's F10 medium in the presence of luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, dibutyryl cAMP, prostaglandins (PG) F-2 α and E-2, methylisobutylxanthine (MIX), indomethacin, phorbolmyristate (TPA) or A23187 for 3 h. Values are means $\pm$ s.e.m. ($n = 3$). * $P < 0.05$; statistical analysis was by unpaired t test.

Metabolism of [3 H]pregnenolone

The conversion of labelled pregnenolone to progesterone and 5 β -pregnanediol in sheep or goat binucleate cells was not affected by different concentrations of dibutyryl cAMP, TPA (1, 10, 50, 100 and 200 nmol/l), A23187 (0.5 and 1 μ mol/l), MIX (0.1, 0.2 and 0.5 mmol/l), LH, prolactin (0.5 to 100 ng/ml), or a combination of A23187 (1.0 μ mol/l) and MIX (0.5 mmol/l) or TPA (10 nmol/l).

Fig. 2. Conversion of $[^3\text{H}]$ pregnenolone to progesterone in sheep binucleate cells (3×10^6) incubated for 3 h with (a) indomethacin and (b) prostaglandin (PG) E-2. Data are means from two animals. The slope of the regression line in (b) is 44.1 (s.e. 0.63), $P < 0.001$.

Effect of prostaglandins on progesterone synthesis

Indomethacin suppressed (Fig. 2a) whereas PGE-2 stimulated (Fig. 2b) the conversion of pregnenolone to progesterone in sheep binucleate cells in a dose-related manner; 0.1 mmol indomethacin/l was required to suppress the conversion to progesterone by 50%. The addition of stimulatory concentrations of PGE-2 ($0.1 \mu\text{g/ml}$) to sheep cells preincubated with 0.1 mmol indomethacin/l for 30 min failed to reverse the inhibitory effects of indomethacin (Fig. 3); PGF- 2α and PGI-2 had no effect (Fig. 3). Neither indomethacin nor any of the PGs affected the conversion of labelled pregnenolone to 5β -pregnanediol in sheep (Fig. 3). NDGA caused a dose-related increase in the conversion of labelled pregnenolone to progesterone; but, whereas $10 \mu\text{g}$ NDGA/ml stimulated the conversion of pregnenolone to 5β -pregnanediol and 20α -dihydroprogesterone, higher concentrations suppressed it (Fig. 4a).

In goat binucleate cells, only NDGA had an effect on steroid metabolism. NDGA decreased the conversion of labelled pregnenolone to 5β -pregnanediol, while increasing the production of 20α -dihydroprogesterone (Fig. 4b). The conversion to progesterone remained low. In contrast to the results in sheep, indomethacin and PGE-2 had no effect on steroid interconversion in goat binucleate cells (Fig. 3).

In studies of the production of PGE-2 in sheep binucleate cells, the largest peak of radioactivity was in a similar position to standard unlabelled PGE-2; a smaller peak corresponded to PGF- 2α (Fig. 5).

Fig. 3. Conversion of [^3H]pregnenolone to progesterone (▨) and 5 β -pregnanediol (□) in sheep binucleate cells (3×10^6) incubated for 3 h with indomethacin (0.1 mmol/l) or prostaglandins (PG) E-2, I-2 or F-2 α (0.1 $\mu\text{g}/\text{ml}$ in each case). Addition of PGE-2 to cells preincubated with indomethacin for 30 min fails to reverse the inhibitory effects of indomethacin. Values are means $\pm$ s.e.m. ($n = 3$ animals). * $P < 0.002$, analysis by unpaired t test.

The incorporation of labelled arachidonic acid into PGE-2 was suppressed by indomethacin in a dose-related manner (Fig. 6a) whereas up to 80 μg NDGA/ml increased the incorporation of labelled compound into PGE-2 (Fig. 6b); higher concentrations were suppressive.

Discussion

Binucleate cells of sheep and goat placentae differed markedly in their ability to synthesize steroids from endogenous or added precursors. As in the placenta *in vivo*, substantial amounts of progesterone were formed from pregnenolone by isolated binucleate cells in sheep, but in goats the major product was 5 β -pregnanediol. Progesterone synthesis in sheep binucleate cells was influenced by PGE-2, NDGA and MIX positively and by indomethacin negatively, whereas in goats only NDGA had an effect on the major product, 5 β -pregnanediol, and this effect was negative. None of the other compounds tested altered progesterone synthesis of pregnenolone metabolism in either species. These findings suggest that products of arachidonic acid metabolism may have a regulatory role in placental steroid synthesis in both species, but, whereas the cyclooxygenase and lipoxygenase pathways are implicated in sheep, only the lipoxygenase pathway seems to be involved in goats. In sheep the inhibition of the cyclooxygenase pathway by indomethacin may lead to an increase in the synthesis of the products of the lipoxygenase pathway, some of which may be inhibitory to the steroid synthetic processes. This may explain, in part, why the addition of PGE-2 failed to reverse the inhibitory effects of indomethacin. NDGA increased the conversion of pregnenolone to progesterone, probably because the compound also stimulated the production of PGE-2 in the binucleate cells, as indicated by increased incorporation of labelled arachidonic acid into PGE-2. Thus, the increase in progesterone production could have been due to raised concentrations of intracellular PGE-2. In addition to its effects on PGE-2, NDGA probably blocked the production of inhibitory lipoxygenase product(s) thereby enabling the conversion of pregnenolone to progesterone to proceed more rapidly. A similar suggestion that the products of the two pathways of arachidonic acid metabolism may have opposite effects on progesterone production by the bovine corpus luteum has been made by Milvae (1986), Hansel & Dowd (1986) and Alila *et al.*

Fig. 4. Conversion of $[^3\text{H}]$ pregnenolone to progesterone ($\text{—}\circ\text{—}$), 5 β -pregnanediol ($\text{--}\bullet\text{--}$) and 20 α -dihydroprogesterone ($\text{—}\bullet\text{—}$) in (a) goat and (b) sheep binucleate cells incubated with nordihydroguaiaretic acid (NDGA) for 3 h. Values are means $\pm$ s.e.m. ($n = 3$ animals).

(1988). It is possible that such a system operates in sheep binucleate cells, thereby contributing to the regulation of progesterone production by the placenta.

The production of 5 β -pregnanediol and 20 α -dihydroprogesterone by sheep binucleate cells treated with NDGA showed a biphasic dose-dependent pattern, low concentrations of NDGA causing an increase in the conversions to the two metabolites whereas higher concentrations caused a decrease. NDGA has been shown to produce a similar biphasic effect on the conversion of pregnenolone to testosterone isolated Leydig cells from the testis (Dix *et al.*, 1984). These responses were different from those in goat binucleate cells, in which NDGA had no effect on progesterone production, caused a dose-related increase in the conversion of pregnenolone to 20 α -dihydroprogesterone and decreased the conversion to 5 β -pregnanediol. With $> 10 \mu\text{g NDGA/ml}$, both sheep and goat binucleate cells exhibited a reduced capacity to convert pregnenolone to 5 β -pregnanediol, indicating some similarity in the action of NDGA in both species at high concentrations. These findings suggest a role for the products of the lipoxygenase pathway of arachidonic acid metabolism in the regulation of 20 α -hydroxysteroid dehydrogenase (responsible for the formation of 20 α -dihydroprogesterone) and $4\text{--}5\Delta$ reductase in both sheep and goats and it will be important to determine the specific lipoxygenase compounds involved in producing the reported effects in both species. The presence of an alternative pathway for pregnenolone metabolism that

Fig. 5. Metabolism of [^{14}C]arachidonic acid (500 000 d.p.m.) in sheep binucleate cells. Non-radioactive reference standards (—) (prostaglandins E-2 and F-2 α) were located using phosphomolybdic acid spray after co-chromatography with the samples. The main radioactive metabolite ran in a position corresponding to standard PGE-2; a smaller peak corresponded to PGF-2 α .

bypasses the conversion of pregnenolone to progesterone in goat binucleate cells would explain the lack of response to PGE-2 and indomethacin and strengthen the argument that the mechanisms that regulate steroid metabolism in the placentae of the two species differ.

The increase in endogenous progesterone production by sheep binucleate cells, caused by the addition of MIX, is more difficult to explain. Shemesh *et al.* (1984) and Tonkowicz & Poisner (1985) reported similar effects when MIX was added to minced bovine placenta and human chorionic cells, respectively. Shemesh *et al.* (1984) suggested that the Ca^{++} -mobilizing properties of MIX were mainly responsible for the effect and concluded that steroidogenic activity in the bovine placenta was not modulated by a process mediated by cyclic nucleotide. On the other hand, Tonkowicz & Poisner (1985) reported that cholera toxin, forskolin, dibutyryl cAMP and MIX increased the release of progesterone into the medium by cells from the human chorion and proposed that MIX acted by increasing concentrations of intracellular cAMP. In the studies reported here, neither A23187 nor dibutyryl cAMP influenced steroid metabolism in sheep and goat binucleate cells. Consequently, it is suggested that neither the ionophore properties of MIX nor its effects on cAMP accumulation were responsible for the increase in endogenous progesterone production in sheep binucleate cells. The inability of the phorbol ester TPA and the calcium ionophore A23187 to affect steroid metabolism in sheep and goat binucleate cells seems to indicate that the inositol lipid signal pathway is not involved in the regulation of placental steroid synthesis in these two species. This may represent species variation as it has been suggested that, in cows, protein kinase C is involved in the modulation of this process (Shemesh *et al.*, 1988, 1989).

In conclusion, the studies presented in this paper have pointed to the products of arachidonic acid metabolism as regulators of steroid synthesis in fetal binucleate cells in sheep and goats. The mode of regulation in the two species may differ as a result of the presence of alternative pathways for pregnenolone metabolism in the binucleate cells in the two species. Furthermore, evidence has been adduced showing that sheep binucleate cells actively synthesize PGE-2, a compound that consistently increased progesterone production by binucleate cells in this species. It is therefore

Fig. 6. Conversion of [¹⁴C]arachidonic acid to prostaglandin (PG) E-2 in sheep binucleate cells (3×10^6) incubated with (a) indomethacin and (b) nordihydroguaiaretic acid for 3 h. Production of PGE-2 by control samples taken as 100%. Values are means from two animals.

proposed that placental steroid synthesis in sheep and goats (as represented by steroid metabolism in binucleate cells in these studies) is regulated by local mechanisms, the products of arachidonic acid metabolism playing a significant role. The proportion of total placental steroid output by the binucleate cells is not known. Power & Challis (1987) have shown that, in sheep placenta, only cotyledonary tissue produces significant quantities of steroids; allantois, amnion and myometrium are inactive. In sheep and goat cotyledons, the syncytial plaques, probably at least equal in volume to (and largely formed from) the binucleate cell populations (Wooding, 1982; Wooding *et al.*, 1986), have many of the ultrastructural characteristics of a steroid-producing tissue. They are also

much closer to the maternal circulation for steroid delivery than the binucleate cells. The relative contributions to total placental steroid synthesis by syncytial plaques and binucleate cells are unknown.

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