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Hormone Production by the Bovine Blastocyst

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C. Abstract

The present project is a fundamental biochemical study of the basic mechanisms governing the hormonal synthesis found in the early blastocyst and to determine if this synthesis is modulated by a cyclic-nucleotide mediated-process. The ultimate objective of the research is to determine the signal for maternal recognition of pregnancy in the cow. Dispersed bovine trophoblast membrane as well as fetal cotyledon cells were shown to synthesize progesterone. None of the tropic agents (i.e. cAMP) significantly affected progesterone synthesis. In contrast, the phosphodiesterase inhibitor, methyl-isobutyl-xanthine (MIX; 0.2--0.5 mM), caused a marked increase in progesterone formation. In time course studies it was found that MIX produced a five-fold increase in progesterone production in 16 h with steroid production increasing linearly during this time. Since MIX is known to also affect intracellular calcium translocation, we examined the effect of the calcium inophore, A23187, on progesterone formation. The drug enhanced progesterone formation and augmented the stimulatory effects of MIX. The stimulatory action of A23187 and MIX was not affected by cyclic nucleotide analogues. Both MIX and A23187 also increased the conversion of exogenous pregnenolone to progesterone by trophoblast and fetal cotyledon cells. Our data suggest that progesterone synthesis in the bovine placentome is calcium-regulated and cyclic nucleotide-independent. During the course of these studies we also found a heat-stable inhibitor of steroidogenesis in placental extracts. However, no inhibitory activity could be demonstrated on similarly prepared extracts from term placenta. The inhibitory substance eluted as two distinct peaks from Sephadex G-100 columns, one with a molecular weight of about 60,000 daltons and the other about 30,000 daltons. Preliminary studies suggest that the substance may also be active in vivo. Extracts of placentomes were found to be effective in reducing plasma progesterone levels within 24 h when infused continuously into the uterine lumina or ovarian arteries of cyclic heifers beginning on Day 12 of the cycle. This inhibitory substance(s) may play a role in regulating placental steroidogenesis. In addition, an endogenous, heat-labile, inhibitor of prostanoid synthesis in maternal caruncle tissue of bovine placentomes was studied. Inhibitory activity was present in caruncle extracts from 120-250 of gestation, but was not detectable at term (260-280 days). Our findings indicate that an endogenous inhibitor modulates bovine placental prostaglandin synthesis. To determine the signal for maternal recognition of pregnancy tissue culture medium of 17-18th day trophoblast was infused into the uterine lumina of cows at day 12 of the estrous cycle; the life span of the corpus luteum was prolonged. It was found that the 3M ammonium sulfate precipitates of trophoblast and extracts of cotyledons, contained cG-like activity, as measured by RRA. It was found that 20 µg of protein eluted from Sephadex DEAE added to the culture medium significantly enhanced progesterone production by the bovine granulosa cells and testosterone production by the Leydig cells.

D. Introduction

A basic problem in reproductive biology is our failure to understand the mechanism by which the developing conceptus signals its presence to the mother. An essential requirement for establishment and maintenance of pregnancy is that the normal ovarian cycle be changed and the functional activity of the corpus luteum prolonged. In the cow, sheep and pig, the recognition signal has been transmitted even before the embryonic tissue becomes intimately attached to the uterine epithelium, and is therefore clearly distinct from implantation. In rodents, the site of implantation can

be visualized by means of dyes, such as Evan blue or Pontamine blue, before attachment of the blastocyst (Psychoyes, 1961; Orsini, 1963). Such findings have led to the concept that the blastocyst might participate in the events leading to its own implantation by signalling its presence to the uterus. In the cow, the early effect of the conceptus on plasma progesterone level was evident on the 19th day after ovulation (Shemesh et al., 1968) and in the sheep on day 16 (Shemesh et al., 1973). In species such as the pig, the blastocyst, while it remains free in the uterine lumen may produce some substance that is capable of diffusing into uterine "milk" and across the lumen, exerting a local effect on the uterine tissues (Perry et al., 1973).

There are at least three possible mechanisms by which the developing conceptus maintains the corpus luteum:

1) hCG-like activity produced by the blastocyst could act locally to stimulate progesterone secretion directly or indirectly through increasing blastocyst steroidogenesis;

2) Blastocysts may secrete an anti-prostaglandin synthetase;

3) Either progesterone and estrogen produced by the blastocysts may act directly or indirectly to suppress the uterine release of $\text{PGF}_2\alpha$

The present study was conducted to further define the endocrine activity of the bovine trophoblast with respect to progesterone production and to elucidate mechanisms by which trophoblast steroidogenesis are controlled. However, the ultimate objective of the proposed research is to determine the signal for maternal recognition of pregnancy in the cow and to characterize a chorionic gonadotropin-like substance produced by cultures of trophoblast cells.

Experimental

1. Tissue Culture

Cell dispersions for tissue culture were prepared from two sources:

(a) trophoblast tissue of early embryos and (b) cotyledonary tissue from first trimester embryos.

(a) Trophoblast tissue:

The tissues were removed from the uterus in a laminar flow hood using sterilized scissors and forceps and minced well. These were transferred into a 0.6 U/ml solution of dispase II (Boehringer Mannheim GmbH Chemicals) in PBS and stirred at 37° C for 30 min. Dissociated cells were removed and fresh dispase II solution was added for a further 30 min. The dissociated cells were subsequently centrifuged at 500 g and resuspended in Eagles medium containing 10% calf serum. The cells were counted and seeded into individual serum precoated culture plates (1×10^6 cells/5 ml). Culture plates were prepared by incubating them for one hour in calf serum. Subsequently the serum was discarded and the plates were rinsed twice in medium. This precoating of culture plates was found to facilitate cell settling.

(b) Cotyledon tissue:

Tissues were dispersed by using a collagenase solution (Sigma Chemical Co., USA) in Eagles medium in the absence of calf serum at a concentration of 0.8 mg/ml instead of the dispase II solution used for trophoblastic tissue. Cells were thus incubated in collagenase at 37° C for 30 min, when dissociated cells were removed and fresh collagenase solution was added for a further 30 min incubation. Subsequently, the cells were treated in the same manner as trophoblast cells.

2. Hormone extraction

The endogenous amount of hormone was determined in unincubated samples of dispersed cells in media. Incubation media were subsequently collected at various periods of the incubation. The samples were frozen until required for assay. Tritiated progesterone (sp. act. 80-110 Ci/mmol; New England Nuclear, Boston, Mass.) was added and samples were extracted with 10 volumes of ether, as previously described (Shemesh et al., 1975).

3. Assays

A series of preliminary experiments showed that the medium used contained undetectable (less than 50 pg/ml) amounts of each of the hormones measured. Progesterone, estrogen and testosterone were subsequently assayed by radioimmunoassays as described previously (Shemesh et al. (1979).

Purification of CG-like activity

Trophoblast tissue from day 16 to 50 embryos (N=17), as well as six bathes of 100-500 g of either maternal or fetal cotyledons, were homogenized in 3 vol/wt of cold (-20° C) absolute ethyl alcohol and centrifuged at 9000 g for 30 min. The dried precipitate (ppt) was then extracted with 10 vol NaHCO_3 (0.1M) containing 1 mM of phenyl methyl-sulfonyl fluoride and the pH was adjusted to 6.5 with concentrated HCl. Extracts were then made with 0.7 to 4.5 M ammonium sulfate solutions. After one h with constant stirring a ppt formed which was recovered by centrifugation at 9000 g for one h. Preliminary observations of the ppts indicated that the binding activity in RRA resided in the 1.5-3.0 M ammonium sulfate ppt. The ppt was then dialyzed against distilled water for 72 h and lyophilized.

The dry protein was then reconstituted in 1 ml of buffer and fractionated by means of column chromatography on Sephadex G-100 (Pharmacia, Uppsala, Sweden), using 0.01 M Tris HCl buffer, pH 9.0, containing 0.03 M NaCl. The absorbance of the column fractions was determined at 280 nm. The void volume of 150 ml as measured by dextran blue 2000 contained no measurable activity in the radioreceptor assay for hCG. Fractions 14-33 which showed maximum binding activity were combined and rechromatographed on DEAE-Sephadex (1.5x40 cm using graded molarities from 0.05 to 1.0 M NaCl and correlated with the absorbance at 280 nm. Skeletal muscle tissue (100 g), similarly processed, served as a control. All extractions and separation techniques were carried out at 0-5° C. The protein content of each tissue extract was determined by the method of Lowry et al. (1951).

Assays for CG-like Activity

The activity of the various fractions obtained throughout the purification procedure was determined by a radioreceptor assay for hCG, employing rat testicular tissue and [125 I]hCG as described by Catt and Dufau (1972). The hCG (mol wt 46,000) was labeled enzymatically with carrier free [125 I]Na, 13-17 mCi/pg, Amersham-Searle Corp., Arlington Heights, IL, according to the procedure described by Miyachi et al. (1972).

The biological potency of purified bovine chorionic gonadotropin (bCG) was determined in the following bioassays: (1) an in vitro LH assay in which progesterone production by cultured bovine granulosa cells is measured, performed as described previously (Shemesh, 1979); and (2) similar augmentation of testosterone production in cultured trypsinized-dispersed rat Leydig cells prepared as described by Ramachandran and Sairam (1975). The potency of the extract was compared to bovine luteinizing hormone, NIH-LH-S₁, which was equivalent to 1.06 NIH-LH-S₁ units/mg in the rat OAAD bioassay and 0.93 NIH-LH-S₁ units/mg by RRA (gift of NIAMDD, Bethesda, MD) and hCG, 1250 IU/mg (Pregnyl, Nobigonan, Boxmeer, Holland). The radioimmunoassays for testosterone and for progesterone were performed as previously described (Shemesh et al., 1979). The inter- and intra-assay coefficients of variation were 8.9 and 6.7% respectively for testosterone and 11 and 9.0% for progesterone.

Preparative Isoelectric Focusing

Preparative isoelectric focusing was carried out using a flat-bed LKB system (Multiphor). A circulating cooling bath maintained the cooling plate at a temperature of 4° C. A pH gradient of pH 3-11 was prepared using ampholytes purchased from Pharmacia (Uppsala, Sweden), on a support of Ultradex (LKB). Samples to be subjected to preparative isoelectric focusing (300 mg protein) were mixed with the ampholyte/Ultradex slurry and added to the flat-bed plate. Isoelectric focusing was then carried out as described in LKB Application Note 198. After isoelectric focusing, a grid was applied to the bed, dividing it into 15 fractions. Each section was removed, mixed in a tube with 1 ml of deionized water and poured into a 5 x 100 mm column (Econo-column, Bio-Rad, Richmond, CA.). The gel was then eluted with 5 ml of deionized water. The pH of each eluate was determined, and the eluates were then dialyzed against deionized water to remove ampholytes.

Assay of the inhibitory activity of placental extracts on progesterone synthesis was carried out as previously described by Shemesh, et al., 1983.

Assay of the inhibitor of prostanoid synthesis and measurement of prostaglandin synthetase activity has been described previously (Shemesh, et al., 1984).

Preparation of placental extracts and extracts of culture media has been described previously (Shemesh et al., 1983).

Results

During the course of these studies we found a heat-stable inhibitor of steroidogenesis in placental extracts. In the present study, we describe the activity and some of the properties of this inhibitory substance.

1. The effects of 20% ammonium sulfate precipitates from cytosolic extracts of whole placentomes collected between 100 and 150 days of gestation on progesterone secretion by bovine granulosa cells are shown in Table 1. These extracts produced a dose-dependent inhibition of progesterone secretion. However, no inhibitory activity could be demonstrated in similarly prepared extracts from term placentae. The marked inhibitory activity of the 100-150-day placental extracts was not due to generalized toxic effects, because the viability of the granulosa cells as assessed by trypan blue exclusion, was not affected by the extracts; viability ranged from 40-50% for all treatment groups. Subsequent studies revealed that the inhibitory activity could be extracted from both maternal caruncles and fetal cotyledons and that the maternal caruncles had the greater inhibitor activity per mg of 20% ammonium sulfate precipitate.

We next examined the effects of extracts from 100-150 day placentae on progesterone secretion by dispersed bovine luteal cells. Results from a representative experiment are shown in Table 2. In the presence of 2 mg/ml of caruncle extract, basal as well as bovine luteinizing hormone (bLH) and 8 bromocyclic AMP-stimulated progesterone secretion were dramatically reduced. In each of five additional experiments 2 mg/ml of caruncle extract produced >90% inhibition of basal progesterone secretion by the dispersed luteal cells. Fetal cotyledon extracts (2 mg/ml) from the same placentomes produced a 38% inhibition (data not shown).

TABLE 1. Inhibitor effects of 20% ammonium sulfate precipitates of bovine placentomes on progesterone secretion by bovine granulosa cells.

Treatment	Net progesterone secretion ^a (ng/4 x 10 ⁵ cells per 24 h)	Percent inhibition
Control (Medium 199 + BSA)	5.00 ± 0.17	
Placental age		
100-150 days		
1 mg/ml	3.62 ± 0.18	23
3 mg/ml	1.99 ± 0.12	61
5 mg/ml	0.37 ± 0.02	92
Term		
3-5 mg/ml	5.21 ± 0.17	0

^a Values presented are means ± SEM of progesterone secretion from 5 separate experiments, each utilizing a different placental preparation. In each experiment each dose of extract was tested on 8 replicates.

TABLE 2. Effects of extracts of maternal caruncles from a 150-day placentome on progesterone secretion by dispersed bovine luteal cells.

Treatment	Progesterone secretion ^a (ng/5.0 x 10 ⁵ cells per 2 h)	P value (Control vs. treatment)
Control (Medium 199)	240 ± 21	
bLH (10 ng/ml)	632 ± 30	<0.01
8 Br-cAMP (1 mM)	835 ± 30	<0.01
Maternal caruncle extract (2 mg/ml)	15 ± 4	<0.01
bLH (10 ng/ml) + maternal caruncle extract (2 mg/ml)	70 ± 5	<0.01
8-Br-cAMP (1 mM) + maternal caruncle extract (2 mg/ml)	75 ± 5	<0.01

^a Values presented are means ± SEM of 5 replicates from a single experiment.

To determine whether the inhibitory effects of the maternal caruncle extract could be reversed by removing the substance from the incubation medium, luteal cells were incubated for 2 h in the absence or presence of 2 mg/ml of the extract. The cells were then collected by centrifugation and resuspended in fresh medium without the placental extract and incubated for an additional 20 h. Progesterone secretion was determined during the initial 2 h incubation and the subsequent 20 h incubation. As can be seen in Table 3, in the presence of the maternal caruncle extract, progesterone secretion was markedly reduced, whereas the cells which had been incubated with the placental extract secreted essentially similar quantities of progesterone as controls after being reincubated in the absence of the placental factor.

Coincubation of dispersed fetal cotyledon or maternal caruncle cells from 145- to 265-day placentae with dispersed luteal cells produced reductions of 48% and 90%, respectively, in progesterone content of the medium (Table 4).

TABLE 3. Reinitiation of progesterone secretion after removal of maternal caruncle extract from dispersed luteal cells.

Treatment	Progesterone secretion ^a (ng/3 x 10 ⁵ cells)	
	Treatment incubation (2 h)	Post-treatment incubation (20 h)
Control	9.7 ± 1.0	26.7 ± 0.9
Maternal caruncle extract (2 mg/ml)	1.4 ± 0.2 (14%)	22.4 ± 0.2 (84%)

^a Dispersed cells from mid-gestation corpora lutea were first incubated in the absence or presence of 2 mg/ml maternal caruncle extract from a 150-day placentome. After an initial 2-h incubation, the cells were collected by centrifugation and resuspended in fresh Medium 199 without the placentome extracts and incubated for an additional 20 h. Progesterone secretion during each incubation is presented. Values are means $\pm$ SEM of 5 replicates in each treatment group from a single experiment. Values in parentheses are percent of control progesterone secretion.

TABLE 4. Effect of co-incubation of dispersed caruncle and cotyledon cells from 145- to 265-day placentae with dispersed bovine luteal cells on progesterone secretion.

Treatment	Progesterone secretion ^a		
	(ng/5 x 10 ⁵ cells per 2 h)	Percent inhibition	P
Luteal cells	164 $\pm$ 7	--	--
Luteal cells + caruncle cells	16 $\pm$ 2	90	<0.001
Luteal cells + cotyledon cells	86 $\pm$ 6	48	<0.001

^a Values presented are means $\pm$ SEM of 15 replicates from three experiments.

Dispersed fetal cotyledon cells from 250- to 260-day placentae were found to secrete small amounts of progesterone, compared to luteal cells, during short incubations (Table 5). Additions of 2 mg/ml of maternal caruncle or fetal cotyledon extracts from a 150-day placenta produced 100% and 54% inhibitions, respectively, of progesterone secretion by the dispersed cells. Two additional experiments with dispersed cells from 240- to 265-day placentae yielded similar results.

When extracts from 100- to 150-day placentomes were applied to a Sephadex G-100 column, two peaks of inhibitory activity, as indicated by testing in the bovine granulosa cell system, were eluted. Results from a representative experiment are shown in Fig. 1. One peak of inhibitory activity eluted with a molecular weight of 60,000 daltons (fractions 32-40) and another with a molecular weight of 30,000 daltons (fractions 40-50). Aliquots of 25-40 μ g protein/ml of fractions 43-48 produced >50% inhibition of basal progesterone secretion by the incubated granulosa cells. When the extracts of caruncles were subjected to preparative isoelectric focusing, several peaks of inhibitory activity were found. Results from a representative experiment, shown in Fig. 2, revealed one peak in the pI range of 3-5, and others with a pI of about 6.7, 7.7 and 8-9. One to 10 μ g of protein in these active fractions was capable of inhibiting progesterone synthesis to the same extent as 2 mg of the crude extracts. Extracts of fetal cotyledons or media of culture of cotyledon cells gave similar patterns of activity upon gel filtration and preparative isoelectric focusing (Fig. 3).

TABLE 5. Effect of extracts from caruncles and cotyledons from a 150-day placenta on progesterone secretion by dispersed cotyledon cells of a 250-day placenta.

Treatment	Progesterone secretion ^a (pg/10 ⁶ cells per 2 h)	Percent inhibition	P
Dispersed cotyledon cells	3880 ± 230	---	
Dispersed cotyledon cells + caruncle extract (2 mg/ml)	Nondetectable	100	<0.001
Dispersed cotyledon cells + fetal cotyledon extract (2 mg/ml)	1780 ± 120	54	<0.001

^a Values presented are means ± SEM of 5 replicates from a single experiment.

2. The cultures of 16-24 day old bovine trophoblast were found to consist of four morphologically distinct cell types: small round cells, mono or multinucleated giant cells, syncytial cells and fibroblast-like cells. During the first week of culture the majority of cells were of the small round type. These cells were randomly spread over the bottom of the flasks (Plate 1). After 14 days of culture the number of syncytial masses with multiple nuclei was significantly increased. The effect of cAMP on cell growth was examined. As shown in Plate 2, the addition of 1mM of 8Br-cAMP to the culture media enhanced growth of fibroblast-like cells, as well as syncytial cells at the expense of the other types.

3. As shown in Table 6, the trophoblast cells are capable of synthesizing prostaglandins of both E and F series, although PGE₂ was the predominant compound. However, a striking drop of prostaglandin synthesis occurs concomitant with the appearance of the syncytial and fibroblast cells after 144, or 288 h of culture, even though a 10-fold increase in the number of cells was noted at this time. This unexpected fall in prostaglandin synthesis occurred concomitant with the appearance of fibroblast-like and granulated cells. Indeed, cAMP stimulated the appearance of these cell types, which resulted in this dramatic drop in PGF₂α and PGE₂ synthesis.

TABLE 6. The effect of 8-bromo-cAMP on formation of PGF₂α and PGE₂ by cultured trophoblast cells.

Prostaglandin production ng/3 x 10 ⁵ cells/4 days			
PGF ₂ α		PGE ₂	
Control	8-bromo-cAMP	Control	8-bromo-cAMP
5 ± 0.2	0.5	25 ± 3	5 ± 0.4

Trophoblast from 16-18 day embryos was dispersed and placed in culture as described in the text. Medium was changed after 4 days of culture and 1 mM 8-bromo-cAMP was added to the medium. Prostaglandin secretion during the subsequent 4 days was then determined. Values presented are means ± SEM of net synthesis from a single experiment in which there were 17 replicate cultures in each group. 8-bromo-cyclic AMP reduced (p <0.001) secretion of both prostanoids.

4. To define the pattern of progesterone synthesis by both trophoblast membranes and fetal cotyledons, placental cells derived from 28-100 days of gestational age were cultured in medium 199 supplemented with 5% calf serum. Progesterone synthesis by both fetal cotyledons and trophoblast membrane cells was greatest during the first 4 days of culture and then declined (Fig. 4). The in vitro production of progesterone by trophoblast cells derived from 5 cows was considerably less, on a per cell basis, than by cotyledonary cells (N=11).

In order to determine if the steroidogenic activity of the placental cells could be modulated by tropic agents, we examined the effects of additions of LH (100 ng/ml), a hormone which enhances ovarian and testicular steroidogenesis, 8-bromo cyclic AMP (8-Br-cAMP; 0.5 mM) and isobutyl-1-methylxanthine (MIX; 0.5 mM) on progesterone synthesis by fetal cotyledon cells. As indicated in Fig. 5, cells were exposed to these tropic agents for 3 h, washed, re-treated and incubated for another 12 h. This protocol was used to assess both acute and longer term effects of the factors. Progesterone synthesis was not elevated by LH or 8-br-cAMP. However, addition of MIX during the first and second incubations resulted in progesterone accumulations 2-3 fold higher than was found in control cells at any given time. Addition of LH, 8 br-cAMP, with MIX produced no greater progesterone synthesis than MIX alone (Fig. 5).

The time course of progesterone production by dispersed fetal cotyledon cells in the presence of MIX was determined (Fig. 6). A concentration of 0.5 mM MIX, which caused maximal stimulation, produced a five-fold enhancement of progesterone secretion by the cells, with the steroid production being linear during the first 16 h of incubation. Basal progesterone secretion progressed at a low rate during this period.

We next examined the possibility that the stimulatory effect of MIX is related to translocation of extra- and intracellular calcium, by studying the effects of the calcium ionophore, A23187, on progesterone production by the placental cells. The time course of progesterone production by dispersed trophoblast cells derived from cows at 35 days of gestational age, in the presence of A23187 was determined (Fig. 7). A concentration of 3 μ M, which caused maximal stimulation, produced a 2-fold enhancement of progesterone net synthesis by the cells, with the steroid production being linear during the first 16 h of incubation. Basal progesterone secretion progressed at a low rate during this period. To determine if the stimulatory effect of A23187 on progesterone synthesis includes an action on the conversion of pregnenolone to progesterone, trophoblast cells were incubated with exogenous pregnenolone at a concentration (200 ng) found to maximally increase progesterone formation. An additive effect on progesterone synthesis was achieved by a combination of pregnenolone with A23187.

5. Partial purification of CG-like activity

The CG-like activity was measured by a radioreceptor assay (RRA) and the biological potency of the purified bovine chorionic gonadotropin (bCG) was determined in the following bioassays: (1) an in vitro LH assay, in which progesterone production by cultured bovine granulosa cells was measured (2) augmentation of testosterone production in cultured trypsinized dispersed rat Leydig cells. It was found that the 3M ammonium sulfate precipitate of

trophoblast, as extracts of cotyledons, contained CG-like activity as measured by RRA. The precipitate inhibited ^{125}I -hCG binding in a linear fashion. A nine-fold increase in specific activity was achieved by further purification on Sephadex G-100 and Sephadex-DEAE. In experiments with cell cultures, it was found that 20 μg of protein eluted from Sephadex-DEAE (equivalent to 16 mIU of hCG as determined by RRA) added to the culture medium significantly enhanced ($p < 0.05$) progesterone production by the granulosa cells and testosterone production by the Leydig cells. When the 3M precipitate was subjected to preparative isoelectric focusing two peaks of CG-like activity were found, one with a PI of 3-5 and another with an alkaline PI of 8-9.

6. Trophoblast CG-like activity

a. In vitro bioassay

Trophoblast tissues from embryos 16 to 45 days of age were extracted and 3M ammonium sulfate precipitations were prepared. It was found that the 3M ppt from embryos of 16 to 40 days contain a protein which inhibits the binding of labeled hCG to rat testicular membranes. Embryos older than days 40 (Table 7) do not contain the inhibitory activity.

TABLE 7. hCG-like activity as measured by RRA in 3M ammonium sulfate precipitates of aqueous extracts of trophoblastic tissue.

Age in Days	hCG-like Activity (mIU/mg protein)
16-17	425*
18	21
20	42
22	242
25	87,120
28	70 $\pm$ 14(6)
30	68 $\pm$ 24(4)
45-50	ND(2)

Number of embryos shown in parentheses.

* From a pool of 5 embryos following 24 hr incubation in medium 199.

b. In vivo bioassay (Effects of CG-like activity on bovine CL function). When tissue culture medium or its 3M ppt collected from tissue culture medium of 17-18 day trophoblast was infused into the uterine lumina of 5 cows at day 12 of the estrous cycle, prolongation of the life span of the CL and high peripheral plasma progesterone (4-8 ng/ml) resulted. Plasma progesterone declined when infusion was stopped at day 21-25.

7. Role of progesterone secretion by the early trophoblast

To study the possible role of progesterone secretion by the blastocyst on fertility and early embryo mortality, progestin-impregnated vaginal sponges (PIVS) were inserted 6-7 days post-insemination into 50 lactating dairy cows and removed on the 17th day. The fertility rate in the control group was 43% ($n=96$) and the PIVS treated group was 60% ($n=50$; $P < 0?$).

8. Presence of an inhibitor of postanoid synthesis in bovine placentomes

Extracts prepared from caruncle tissue collected during the last 120 days of gestation were tested for effects on $\text{PGF}_2\alpha$ and prostacyclin (PGI_2) synthesis by bovine luteal cells. Exposure of the luteal cells to the extracts for as little as 5 min resulted in a marked inhibition of

prostaglandin $F_2\alpha$ and PGI_2 formation during the subsequent 15 h of incubation (Fig. 8). In our routine analysis of inhibitory activity of extracts, cells were exposed to the material for 2 h, then washed by centrifugation and incubated for 15 h. Using this assay, inhibitory activity was found in extracts prepared from tissue collected between 150 to 250 days of gestation (Table 8). However, it was completely lost by term (260-280 days of gestation).

TABLE 8. $PGF_2\alpha$ production by cultured caruncle cells

Gestational age (days)	$PGF_2\alpha$ Production (ng/ 10^6 cells/24 days)
150	0.3 ± 0.07
182	0.7 ± 0.2
218	0.8 ± 0.2
246	0.9 ± 0.1
257	0.6 ± 0.1
260	1.1 ± 0.3
261	5.5 ± 0.5
272	7.5 ± 0.7
275	4.5 ± 0.5
282	14 ± 0.6 (ng/ 10^6 cells/ 3^h)

Dispersed caruncle cells were maintained in culture for 24 days, except where noted. Values presented are means $\pm$ S.E. of cumulative $PGF_2\alpha$ production of 5-8 replicates for each experiment.

Boiling of the extracts destroyed inhibitory activity. Indeed, the boiled extracts consistently enhanced prostanoid synthesis by dispersed luteal cells. Untreated extract (2 mg/ml) inhibited bovine luteal PGF_2 production by $89 \pm 1\%$ ($N=5$ experiments), while the boiled extracts increased PGF_2 secretion ($p < 0.001$) by the luteal cells by $61 \pm 15\%$ ($N=5$).

DISCUSSION

An inhibitor of progesterone secretion was found in extracts of both fetal and maternal bovine placental tissues collected at mid-gestation, and in incubation media obtained from cultures of dispersed fetal and maternal placental cells. This inhibitor reduced basal secretion of progesterone by incubated bovine granulosa cells, dispersed bovine luteal cells and dispersed bovine placental cells.

This inhibitor seems unrelated to the chorionic gonadotropin-like factor, which has been described in extracts of 50- to 100-day bovine placentae (Ailenberg and Shemesh, 1983), or to the antiluteolytic factor, trophoblastin, described by Martal (1981). This heat-labile substance is thought to stimulate steroidogenesis by the corpus luteum of pregnancy (Ailenberg and Shemesh, 1983). In contrast to these factors, the inhibitory substance is heat-stable and not extracted by ether. It elutes as two distinct peaks on Sephadex G-100 columns, one with a molecular weight of 60,000 daltons, the other with a molecular weight of 30,000 daltons. On preparative isoelectric focusing, several peaks of inhibitory activity are obtained, one with a pI 3-5, and others having pI's of 6-9. Thus, it is not known whether the inhibitory activity is due to a single compound. The relationships between the two peaks eluted from the Sephadex G-100 column and those identified by preparative isoelectric focusing have not been determined.

Preliminary studies suggest that the substance may also be active *in vivo*. Extracts of placentomes were found to be effective in reducing plasma progesterone levels within 24 h when infused continuously into the uterine lumina or ovarian arteries of cyclic heifers beginning on Day 12 of the cycle (Hickey, Shemesh and Hansel, unpublished data).

The mechanisms of action of the inhibitor is unknown. The onset of the inhibitory action is relatively rapid, as it is evident during a 2 h incubation. Moreover, its effect is not sustained as luteal cells recover from the action of the inhibitor during reincubation in inhibitor-free medium. Since the factor reduces steroid secretion stimulated by LH and a cyclic AMP analog, we must assume that it acts as a post-cAMP step. It is possible that the factor does not actually reduce progesterone synthesis but rather enhances progesterone metabolism or catabolism to other steroids (Eley et al. 1983).

The inhibitory factor(s) appears to be produced by cells of both the caruncles and cotyledons, and is presumably secreted since it can be recovered in the media of cultures of dispersed cells. While the physiological role of the factor is not known, it is of interest to note that the inhibitor disappears by term, when bovine placental steroidogenic activity increases (Hoffman et al., 1979).

Investigators searching for gonadotropic activity in bovine placental extracts should be aware of the presence of this factor, especially when assays based on steroidogenesis are utilized to assess biological activity. Our studies demonstrate that dispersed bovine trophoblast membranes and fetal cotyledon cells collected at days 30 to 45 and 120-283 respectively of gestation produce progesterone *in vitro*. The ability of fetal cotyledons to secrete progesterone was evident in early as well as late pregnancy. The production of progesterone by placental tissue may be important in the maintenance of gestation, as this steroid may act locally upon the uterus.

The stimulatory effect of MIX on progesterone production by the bovine placental cells could be related to two basic actions of this compound: (1) The increased accumulation of cyclic nucleotides as a result of inhibition of phosphodiesterase (16); (2) The translocation of intracellular calcium (17). Since neither LH, 8br-cAMP, cGMP, cholera toxin or isoproterenol had effects on progesterone synthesis by themselves, nor did they potentiate the effects of LH, it is likely that MIX acted through translocation of intracellular calcium. Furthermore, the calcium ionophore, A23187, induced a two-fold increase in progesterone production by the treated cells and had an additive effect when used in combination with MIX.

These findings indicate that translocations of intracellular calcium or influx of extracellular calcium stimulate progesterone production by placental cells. This action of calcium appears to be independent of cyclic nucleotides. Calcium ions have been implicated in the steroid biosynthetic processes in adrenal glands, testes and ovaries (Janszen et al., 1976; Podesta et al., 1980, Veldhuis and Klase, 1982). However, in all of these tissues cyclic AMP is thought to be the primary intracellular regulator of hormone production. Cyclic AMP levels rapidly increase when ACTH or gonadotropins act upon the adrenal cortex or gonads, respectively, and cyclic AMP analogs are potent stimulators of steroid hormone production by these tissues (Catt and Dufau, 1978). The bovine placenta appears to differ from these other steroidogenic tissues and may resemble the zona glomerulosa of the adrenal

cortex, a tissue in which calcium rather than cyclic nucleotides seems to be the primary intracellular signal increasing aldosterone synthesis (Fakunding, et al., 1979; Fakunding and Catt, 1982 and Foster et al., 1981).

It is not yet known whether a similar control process is used in placental steroidogenesis in other species. There have been reports that human placental progesterone synthesis is stimulated by hCG (van Leusden and Villee, 1966), presumably by a cyclic AMP-mediated mechanism (Menon and Jaffee, 1973). However, the extent of stimulated production is small, leaving open the possibility of a significant role for calcium.

Although the exact locus of calcium action in the steroidogenic process is not yet known, it is likely that it may influence the delivery, or utilization of cholesterol by the mitochondria (Hall et al., 1981 and Drosowsky et al., 1966). In addition, calcium may affect the conversion of pregnenolone to progesterone by Δ^5 -3-OH-SDH, as a combination of exogenous pregnenolone and MIX or A23187 increased progesterone secretion over that seen with exogenous pregnenolone alone. This step in the biosynthesis of steroids is generally not thought to be regulated, and our observations are, therefore, novel in this regard. The factors which regulate calcium metabolism in the placental cells are not known. Our findings would suggest, however, that some humoral factor acts upon placental tissue and enhances steroidogenesis through the intermediacy of calcium.

This study shows that bovine cotyledons contain a substance which inhibited binding of labeled hCG to membranes in the RRA used and enhanced steroidogenesis by bovine granulosa cells and rat Leydig cells. This substance was heat labile and had a molecular weight of approximately 60,000. These results taken with the earlier report of Lunnen and Foote (1967), who used the OAAD and rat ventral prostate bioassays, would seem to provide ample evidence that the bovine cotyledon contains a substance which broadly imitates LH bioactivity. Furthermore, when tissue culture medium of 17-18th day trophoblast was infused into the uterine lumina of cows at day 12 of the estrous cycle, the life span of the corpus luteum was prolonged. However, it is not clear whether the humoral factor acts upon placental tissue and enhances steroidogenesis through the intermediacy of calcium.

The present study also reveals that bovine caruncle tissue contains an endogenous factor which suppresses prostanoid synthesis. This factor is heat-labile and the inhibitory activity elutes with a Mr of about 60,000 on gel filtration. However, it is not yet known whether this is the molecular weight of the active factor, as it may be a lower molecular weight material associated with a higher molecular weight protein. Our earlier studies indicated that the inhibitor acts directly on the enzymatic system converting arachidonate to prostaglandins. This mechanism is consistent with the rapid expression of the factor's inhibitory activity. Our data raise the possibility that local regulation of prostanoid production by endogenous factors is important in determining the onset of parturition. The loss of a potent endogenous inhibitor of prostanoid synthesis at term may allow increased endometrial prostaglandin production which may be required for myometrial activation.

E. Description of Cooperation

Professor Hansel visited Dr. Shemesh to help plan the initial work. He was particularly active in setting up the synchronization and superovulation

techniques needed to obtain early embryos. Jointly, we have developed tissue culture of trophoblastic cells from days 16-18 at Cornell and infused the partially purified b-CG like substance into the uterine lumina of five cows. A similar experiment of the role of progesterone on the early embryo mortality was conducted by Hansel and Donaldson.

F. Benefit to Agriculture

Twenty percent of the ova fertilized in normal cattle perish before the 30th day of pregnancy (Bearden, Hansel and Bratton, Dairy Sci. 39: 312, 1956). This high early embryo mortality is the major cause of infertility in cattle and is probably the most important single cause of economic losses in the dairy cattle industry. Many factors contribute to early embryo mortality, but recent findings suggest that one cause may be a failure of the embryo to "activate" the corpus luteum of early pregnancy. Our recent findings also suggest that it may now be possible to isolate and identify the substance(s) of fetal origin responsible for "activating" the corpus luteum to cause the continued progesterone production necessary to maintain pregnancy. Isolation and identification of this substance(s) could lead to development of techniques for reducing early embryo losses and to development of a highly sensitive early pregnancy test for cattle. This is a possibility, as fetal antigens appear in maternal blood and can form the basis of a pregnancy test (Cerini et al., 1976).

G. List of Publications

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Legends

- Fig. 1. Inhibition of granulosa cell progesterone secretion by fractions of an ammonium sulfate precipitate of 100- to 150-day bovine placetomes eluted from a Sephadex G-100 column. One tenth of each fraction was tested for activity (V_o = void volume). Values are means $\pm$ SEM for 5 experiments.
- Fig. 2. Inhibition of granulosa cell progesterone secretion by 0.1 ml aliquots of fractions produced by isoelectric focusing of the extract of caruncles from a 200-day placentome.
- Fig. 3. Inhibition of granulosa cell progesterone secretion by 0.1 ml aliquots of fractions produced by isoelectric focusing of an extract of media collected during Days 8-16 of culture of cotyledon cells from a 150-day placentome.
- Fig. 4. Progesterone formation by cultured dispersed trophoblast and cotyledon cells. Medium was changed every 4 days of culture and progesterone secretion during the subsequent 4 days was then determined. Values presented are means $\pm$ S.E. of net synthesis for 5 cows at 28-35 and 11 cows at 60-100 days gestational age.
- Fig. 5. The effect of tropic agents on progesterone biosynthesis by incubated dispersed fetal cotyledon cells during 3 and 12 h incubation. Values presented are means $\pm$ S.E. for net synthesis by 9 animals.
- Fig. 6. The effect of MIX on progesterone biosynthesis by incubated dispersed cotyledone cells. Values presented are means $\pm$ S.E.M. for net synthesis of 5 triplicate samples from one animal at 100 days of gestational age.
- Fig. 7. The effect of pregnenolone with A23187 on progesterone biosynthesis by incubated dispersed trophoblast cells. Each point is the mean $\pm$ S.E. of 5 replicates from one animal at 35 days of gestational age.
- Fig. 8. Time course of inhibition of dispersed luteal cell prostanoid synthesis by placental extracts. Cells were incubated with (Δ) or without extracts (Δ) 2 mg/ml from 150 day caruncle tissue for the indicated times. The cells were then washed by centrifugation, resuspended in fresh medium and incubated for 15 h. Prostanoid production during this 15 h incubation was determined. Control cells were not exposed to placental extract, but were processed in an identical manner. Values presented are means $\pm$ S.E. for 5 replicates.

a

b

Plate 1. Tissue culture of trophoblastic cells during the two first weeks of incubation. In (a) the dominant cells during the first week are mono and bi-nucleated, while in (b) the syncytial, fibroblast-like cells are dominant after two weeks.

a

b

Plate 2. The effect of cyclic-AMP on cell types. In both (a) and (b) syncytial masses and fibroblast-like cells are apparent during the first two weeks. Multiple layers of syncytial cells are evident in (a).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8.