77

BARD

FINAL REPORT
PROJECT NO. I-740-84

**Hormone Production and Maintenance of the
Corpus Luteum by the Bovine Blastocyst**

M. Shemesh, W. Hansel

1989

Standard BARD Cover Page for Scientific Reports

Date 20.3.89

BARD

P.O.Box 6

Bet Dagan, ISRAEL

BARD Project No. I-740-84 RC

Title of Research Project:

Hormone production and maintenance of the corpus luteum by the bovine blastocyst.

Names of Investigators:

Principal Investigator: M.Shemesh

Cooperating Investigator: W.Hansel

Name and Address of Affiliated Institutions:

Principal Institution: Kimron Veterinary Institute, P.O.Box 12, Bet Dagan, 50250

Cooperating Institution: Cornell University, Ithaca, NY 14853

Project's starting date: 1.12.85

Type of Report: 1st Annual ☐ 2nd Annual ☐ Final ☒

Signatures:

Principal Investigator

M. Shemesh

Institution's Authorizing Official

E. Pipano

MINISTRY OF AGRICULTURE
KIMRON VETERINARY
INSTITUTE
Prof. E. PIPANO DVM Ph.D.
DIRECTOR

Table of Contents

c. Abstract	
d. Objectives.....	1
e. Scientific reports	
A. Purification and characterization of a luteotropic substance from bovine placentome (bCG) and from the amniotic fluid..	1
B. Regulation of steroidogenesis in the bovine placenta,.....	7
C. Purification and characterization of an endogenous stimulator of prostaglandin synthesis.....	17
Figures and Tables.....	21
g. Evaluation of the research achievements with respect to the aims of the original research.....	28
h. List of publications.....	30
i. Appendix.....	31

Abstract

The objective of this project was to define the basic mechanisms which govern hormonal synthesis in the bovine trophoblast. The specific aims of the project were (1) to further purify and characterize a substance from the bovine placenta which has luteotrophic activity (bCG); (2) to define the factors that control progesterone synthesis in this tissue including a factor extracted from the placenta which inhibits steroidogenesis; and (3) to further purify and characterize an endogenous stimulator of prostaglandin synthesis extracted from the placenta. It was found that (1) there were both a high (60-70Kd) and low (20-30Kd) mol wt proteins which were secreted from bovine placental cell granules and which were active in both radioreceptor assay and bioassay. Trophoblastic tissue (as well as amniotic fluid) from 24-36 day was analysed and found to have similar high and low mol wt luteotrophic factors; (2) the Ca^{+2} second messenger regulation of steroid production was dependent on cholesterol availability and that both the 3 β -OH steroid dehydrogenase and side chain cleavage enzymes are regulated by this system. Furthermore, evidence for involvement of protein kinase C activation as well as calcium mobilization in the regulation of this steroidogenesis was found. Protein kinase C was specifically inhibited by the endogenous inhibitor of steroidogenesis extracted from the placentome; (3) that bovine placental granules contain a protein that is heat and acid stable, which stimulated both PGE and $\text{PGF}_{2\alpha}$ production by bovine seminal vesicle microsomal preparations and dispersed placental cells. In conclusion, the bovine placenta has a number of paracrine and autocrine factors which regulate both the steroidogenesis and production of prostaglandins; some of these factors are unique to this tissue. The data are consistent with the hypothesis that the bovine placenta is essentially autonomous as to its endocrine control.

Objectives:

The overall objective of this project was to define the basic biochemical mechanisms governing hormonal synthesis in bovine trophoblastic tissue. The specific aims were to (a) to further purify and characterize a substance extracted from the bovine placenta which has luteotrophic activity (bCG).; (b) to define the factors that control progesterone synthesis in this tissue including a factor extracted from the placenta which inhibits steroidogenesis; and (c) to further purify and characterize an inhibitor as well as a stimulator of prostaglandin synthesis extracted from the bovine placenta.

A. PURIFICATION AND CHARACTERIZATION OF A LUTEOTROPIC SUBSTANCE FROM THE BOVINE PLACENTOME (BOVINE CHORIONIC GONADOTROPIN, bCG) AND FROM THE ALLANOTIC FLUID.

Introduction

Chorionic gonadotropins are glycoprotein hormones synthesized by the chorionic cells. In humans and primates during early pregnancy stages they convey a signal from the trophoblast to the corpus luteum. This signal results in maintenance of the corpus luteum and progesterone production. This action of placental gonadotrophins is mediated by specific receptors located on the corpus luteum cell surface (Bahal, 1977).

Progesterone synthesis is essential for the normal development of the fetus during the first third of gestation in most domestic species. Corpus luteum removal in the first third of bovine pregnancy causes miscarriage which is prevented by progesterone administration (Deansesly, 1966).

Chorionic gonadotrophins have been characterized in humans (Saxena

et al., 1974), primates (Hodgen et al., 1974), mares (Cole & Gross, 1943) and partially purified from bovine placental tissues (Ailenberg & Shemesh, 1983; Hickey & Hansel, 1987).

Lunnen & Foote (1967) extracted a substance exhibiting LH-like activity from bovine cotyledons and maternal plasma. Ailenberg & Shemesh (1983) reported the presence of chorionic gonadotrophin like activity in bovine cotyledons from 50-100 days old fetuses. This protein, having a molecular weight of approximately 60 Kd, significantly increased progesterone and testosterone production by granulosa and Leydig cells in culture. Hickey et al., (1985) extracted a substance with LH-like luteotrophic activity from allantoic fluids of Day 25-37 bovine conceptuses. The substance was found to be a protein of Mr 50-70 Kd (Hickey and Hansel, 1987).

The importance of such substances found in many species, and known to maintain pregnancy, stimulated the present research. Since both the bovine chorionic gonadotrophin (Izhar & Shemesh, Israël) and the allantoic luteotropic substance (Hickey & Hansel, U.S.) have been only partly characterized, we conducted further studies to identify and purify these substances.

For Materials and methods see appendix:

Izhar & Shemesh (1989) Journal of Reproduction and Fertility
Suppl. 37:37-44.

Hickey, Walton & Hansel (1989) Journal of Reproduction and Fertility
Suppl. 37:29-35.

Results

Bovine chorionic gonadotropin:

Granule extracts

The total granule extract recovered from bovine fetal cotyledons was fractionated on a Sephacryl S-300 column, lyophilized and analysed for relative inhibitory binding of ^{125}I -labelled hCG in a radioreceptor assay. Fig. 1 shows the displacement of iodinated hCG by a substance found in the granule fraction according to a standard curve. There were two main peaks of activity for displacement of hCG: (a) Fraction 36-41 and (b) a lower molecular weight fraction, 65-73. The first peak was equivalent to 110 ng hCG/20 μg protein, as detected by RRA, and the low molecular weight substance was equivalent to 55 ng hCG/20 μg protein. Chorionic gonadotrophic activity of the major peak was not stable after 2 days at -20°C . On the other hand, the lower molecular weight peak was stable when frozen for at least 1 month.

To demonstrate biological activity, luteal cell cultures were incubated with Sephacryl S-300 fractions and progesterone synthesis was measured by RIA. As can be seen in Fig. 2, hCG (5ng/ml) increased progesterone synthesis by 65% above control. The low molecular weight fraction (67-78) increased progesterone synthesis by 85%, and the higher molecular weight peak (fraction 45-50) increased it by 57%.

Since low molecular weight substances were potent (RRA and bioassay), acidic extraction of granules was performed to enrich low molecular weight substances from fetal cotyledons. Fig. 3 shows an SDS polyacrylamide gel electrophoresis. Cytosolic extracts of bovine fetal cotyledons (a) were compared to the total granule extract (b) and to the acidic granule extract (c). Different protein profiles were obtained using these purification steps. The total and acidic extracts

both showed a larger variety of low molecular weight proteins than did cytosol. In the total extract (b) there was enrichment of proteins of Mr 29 Kd and 35 Kd, whereas the acid extract exhibited proteins of Mr 31 Kd and 32 Kd.

The acidic granule extract was further fractionated using HPLC C-18 columns, lyophilized and analyzed in the bioassay, i.e., the enhancement of progesterone production by dispersed bovine luteal cells. Fractions 17-20 enhanced progesterone production to 3.4 times more than the control (Fig.4). hCG by itself increased progesterone secretion 3 fold. None of these fractions displaced binding of ^{125}I labelled hCG to rat testicular receptors in RRA.

As shown in Table 1, the acidic granule extract was 10 times more active than the total granule extract. The C-18 peak was 470 times higher than that for the total granule extract.

When the HPLC active fractions were extracted by phase partition of methanol:chloroform (2:1 v/v) no biological activity as measured by progesterone production enhancement was found in the chloroform phase.

Since the Sephacryl fraction bound to hCG receptors, we decided to test if placental RNA would hybridize with cDNA probes for hCG. Total RNA extractions of bovine fetal and maternal placenta were analysed using dot blot techniques. RNAs were hybridized with ^{32}P -radiolabelled α and β cDNA for hCG. Preliminary results indicated that no hybridization signal was present (data not shown).

Luteotropic activity of allantoic fluid

It was found that 41% (28/65) of allantoic fluids from 24 to 37 day embryos had significant luteotropic activity over the control ($P < 0.05$). Twenty of 31 allantoic fluids collected before or on day 28 of pregnancy contained significant activity as opposed to 8 of the

allantoic fluids harvested between days 29-37 days.

Preliminary characterization indicated that the luteotropic factors present in allantoic fluid was proteinaceous since activity was ammonium sulfate precipitated and inactivated by trypsin and chymotrypsin digestion. The luteotropic activity also appeared to be heat labile. Gel filtration chromatography of allantoic fluid by Sephacryl S-300 or Sephadex G-100 identified one peak of activity in the eluate. The activity corresponded to a Mr 68 Kd protein as determined from column calibration. One dimensional SDS-PAGE of the pooled active fraction of the Sephacryl S-300 column identified three major protein bands between Mr= 55-75 Kd.

A Concanavalin A chromatography affinity column was used to isolate the glycoproteins of allantoic fluid that were Con-A reactive. One dimensional SDS-PAGE showed that the major allantoic fluid protein Mr=68 Kd was Con-A non-reactive while both the 55Kd and 72Kd proteins were Con A reactive. Significant luteotropic activity ($P < 0.01$) was partitioned predominately in the Con A reactive fractions.

Discussion

The data in this report suggest the existence of a protein of Mr 60-70Kd which can be extracted from granules obtained from bovine fetal cotyledons of 50-100 days of gestation. This protein was active in both the bioassay and radioreceptor assay. It may be identical with a luteotropic substance isolated from allantoic fluid 24-37 day old bovine fetuses. The similarity of this protein to the Mr 70 Kd pregnancy specific protein recently reported by Klima et al. (1987) remains to be determined. A second peak of lower molecular weight (35 Kd) which had activity in the radioreceptor and bioassay was also observed to elute from the Sephacryl S-300 column. This peak was

studied further by eliminating higher molecular weight substances by using acid extracts of the granules and analyzing the extract using reverse phase HPLC. It was found that the elution of this substance at 45% acetonitrile resulted in a 100-fold increase in luteotropic activity in the bioassay compared to the Sephadryl fraction. The small molecular weight substance was heat stable and not extracted to the organic phase when partitioned between ethanol:chloroform. However although it was luteotropic (as determined by bioassay), this compound did not displace hCG in the radioreceptor assay while the comparable Sephadryl fraction was both luteotropic and competed with hCG. It is therefore possible that the lower molecular weight bovine granule active substance eluted from the HPLC after acid treatment is a degradation product of a larger unstable molecule which does bind hCG. Alternatively, it may be a novel substance that does not bind the hCG receptors but enhances progesterone secretion by bovine luteal cells. Therefore it is possible that the bovine granules contain two different substances in the 30-35 Kd range with partial luteotropic activity: (1) a progestin production enhancer and (2) an hCG receptor binding competitor.

For References for section A see appendix:

Izhar & Shemesh (1989) Journal of Reproduction and Fertility
Suppl. 37:37-44.

Hickey, Walton & Hansel (1989) Journal of Reproduction and Fertility
Suppl. 37:29-35.

B. REGULATION OF STEROIDOGENESIS IN THE BOVINE PLACENTA

Introduction

The bovine placenta has been generally disregarded as a site of progesterone synthesis (Hoffman et al., 1979; Wendorf et al., 1983). Nevertheless, conversion of [3 H] pregnenolone to radioactive progesterone by bovine placental preparations has been demonstrated (Ainsworth & Ryan, 1967), and our previously reported studies indicated that enzyme-dispersed bovine placentomes are capable of producing progesterone in culture (Shemesh et al., 1983). The experiments described in this report were conducted to further define the endocrine activity of the bovine placentome with respect to progesterone production and to elucidate the mechanisms by which placental steroidogenesis is controlled.

Materials and Methods

See papers in appendix:

Shemesh, Hansel, Strauss & Shore. Journal of Reproduction and Fertility Suppl. 37:163-172, 1989

Shemesh, Strauss, Hansel, Shore & Izhar. Journal of Steroid Biochemistry 29:21-25, 1988

Shalem, Izhar, Shore, Shemesh & Hansel. Journal of Steroid Biochemistry 31:835-838, 1988.

Results

Progesterone synthesis by cultured fetal and maternal cells.

To define the pattern of progesterone synthesis by both fetal cotyledons and maternal caruncles, placental cells derived from cows at 182-283 days of gestation were cultured in medium 199 supplemented with 5% calf serum. Progesterone synthesis by both fetal and maternal cells was greatest during the first 4 days of culture and then declined (Fig.

5). The in vitro production of progesterone by maternal caruncle was considerably less, on a per cell basis, than by cotyledonary cells.

Effect of LH, cyclic nucleotides and MIX on placental cells steroidogenesis.

In view of this indication that placental cells produce progesterone, we decided to determine if the steroidogenetic activity of these cells could be modulated by trophic agents.

We examined the effect of addition of LH (100ng/ml), 8Br-cAMP (0.5 mM) and 3-isobutyl-1-methylxanthine (MIX, 0.5mM) on progesterone synthesis by fetal cotyledon and maternal caruncle cells. As indicated in Fig. 6, cells were exposed to these trophic agents for 3 hr., washed, retreated and incubated for another 12 hr. It was observed that progesterone synthesis in fetal cotyledon cells was not elevated by LH or 8Br-cAMP. However, addition of MIX during the first and second incubation resulted in progesterone accumulation 2-3 fold higher than was found in control cells at any given time. Addition of LH or 8Br-cAMP with MIX produced no greater progesterone synthesis than MIX alone (Fig.7). A similar response to MIX was observed when maternal caruncle cells harvested from cows at various stages of gestation were used. (Fig.8).

We next examined the possibility that the stimulatory effect of MIX is related to translocation of extra- and intracellular calcium by studying the effect of calcium ionophore A23187 (Weissman et al., 1980) on progesterone production by the placental cells. As can be seen in Fig. 9., A23187 and MIX each produced a 2 fold increase in progesterone production. Furthermore combination of A23187 and MIX resulted in an additive effect. The effect of A23187 was not potentiated by either 8Br-cAMP or 8Br-cGMP. To determine if the stimulatory effects of MIX

and A23187 on progesterone synthesis are on the conversion of pregnenolone to progesterone, caruncle cells were incubated with exogenous pregnenolone, at a concentration of 100 ng per tube (found to maximally increase progesterone formation) in the presence of MIX and A23187. As can be seen from Fig. 9, pregnenolone, MIX and A23187 each caused an almost 2 fold increase in progesterone production. An additive effect on progesterone synthesis was achieved by a combination of pregnenolone with either A23187 or MIX ($P < 0.01$), whereas a maximal stimulation (8-fold) was achieved by combination of all three additives.

Effect of cholesterol on placental cell steroidogenesis.

Addition of 20 $\mu\text{g/ml}$ 25-OH-C to dispersed fetal cotyledons was found to be optimal for augmenting progesterone formation yielding a 5 fold stimulation of steroidogenesis ($P < 0.01$) (Shemesh et al., 1988).

When the calcium concentration of the media was increased from 0 to 3 mM, an increase in progesterone production in the presence of 25-OH-C (20 $\mu\text{g/ml}$) was observed (Fig. 10). At higher calcium concentrations, basal and 25-OH-C stimulated progesterone production were reduced. MIX alone increased progesterone secretion and further increased the steroidogenic response to 25-OH-C at each calcium level in additive fashion ($P < 0.001$). MIX also increased pregnenolone secretion in the presence of a small amount of 25-OH-C (1 $\mu\text{g/ml}$). These effects were accentuated when an inhibitor of 3 β -hydroxysteroid dehydrogenase, cyanoketone, was included in the incubation medium (Fig. 11).

Effect of TPA on placental cell steroidogenesis.

TPA (10 ng/ml), a stimulator of C-Kinase, was tested for its ability to enhance progesterone production by fetal cotyledon cells. In most

experiments the phorbol ester alone had no significant effect on progesterone secretion. However, the addition of MIX (Fig.12) or the calcium ionophore A23187 (3 μ M) (data not shown) in the presence of TPA resulted in an augmented secretion of progesterone ($P < 0.01$). The effect of TPA on progesterone was consistent during the first two hr of incubation in that it stimulated progesterone production in 5/7 tests. However, less consistent results were obtained in longer term incubations. i.e., after 6 hr and 15 hr, only 3/7 and 4/7 of the tests were positive respectively.

A role for protein kinase C action in the regulation of P-450_{scc} enzyme.

- a. Effect of TPA: When TPA was incubated in the presence of submaximal concentrations of 25-OH-C (1 μ g/ml), it was found that TPA (10ng/ml) stimulated progesterone synthesis by the fetal cotyledon cells. (Fig. 13).
- b. Effect of TPA in the presence of cyanoketone: To determine if side chain cleavage enzyme was affected by TPA, cells with media containing 25-OH-C and MIX were incubated in the presence or absence of TPA (Fig 14). It was found that TPA potentiated the conversion of sterol to pregnenolone. Similar results were obtained when A23187 was added to the incubation in the presence or absence of TPA. It was found that both pregnenolone and progesterone synthesis were increased by the addition of TPA (Fig. 15). In some experiments cyanoketone was added to the incubation. The increase in pregnenolone synthesis evoked by the TPA indicated that TPA was active at the site of the cholesterol side chain cleavage reaction. To determine if TPA, MIX and A23187 could act synergistically to increase pregnenolone synthesis, cells were incubated in media containing submaximal concentrations of MIX and

A23187 as well as 25-OH-C in the absence or presence of TPA. Under these conditions TPA caused a 2-fold increase in the production of pregnenolone by the fetal cells. (Fig.16).

c. Effects of trifluoperazine on placental cell steroidogenesis.

Basal as well as MIX(0.5 mM) +A23187(3 μ M), 25-OH-C(20 μ g/ml), and 25-OH+MIX-stimulated progesterone synthesis were inhibited by trifluoperazine(20 μ M), an inhibitor of calmodulin and PKC mediated processes (Figs. 17 and 18). The effects of trifluoperazine on 25-OH-C+MIX stimulated steroidogenesis were dose dependent with concentrations of 10 μ M nearly abrogating the stimulatory effects of 25-OH-C+MIX and 20 μ M levels suppressing steroid production below control levels.

d. Determination of P-450scc: We also tested whether the cytochrome P-450 side chain cleavage was present in the placentome. Using Western blot analysis with a specific antibody, it was found that mitochondria from fetal cotyledons contained readily detectable amounts of cytochrome P-450scc(Fig.19) On the other hand maternal caruncle, up to 100 days of gestation did not contain detectable levels of the enzyme.

e. Determination of PKC activity: We determined the PKC activity in rabbit brain and fetal cotyledon and found that the activity in the cotyledon was 2.00 ± 2 nmol histone phosphorylated min/mg protein compared with 6.00 ± 0.03 in the rabbit brain which is known as a very rich source of this enzyme.

f. Effect of endogenous inhibitor: Previously we reported (Shemesh et al., 1983) the presence of an inhibitor of progesterone synthesis in both fetal and maternal bovine placental tissue. In these experiments, we examined the possibility that this inhibitor might act specifically on PKC. As can be seen in Fig.20, maximal activity of the enzyme was

achieved in the presence of phosphatidylserine (PS) , Ca^{+2} and diolein (DG). However in the presence of 10 μg of partially purified placental protein, there was more than a 50% inhibition in the enzyme activity. Further depression in the enzyme activity to undetectable values was achieved by 50 μg of this protein.

Discussion

Our studies demonstrate that dispersed bovine fetal cotyledon and maternal caruncle cells collected at 120-283 days of gestation produce progesterone in vitro. The ability of fetal cotyledons to secrete progesterone was evident in early as well as late pregnancy. However, appreciable progesterone synthesis by the maternal caruncles occurred only after 100-200 days of gestion. 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 corpus luteum of pregnancy is generally assumed to be the primary source of progesterone during pregnancy in the cow, but recent studies on the function of bovine luteal cells from corpora lutea of pregnancy in vitro argue against a key role for this gland in late pregnancy as these cells have a markedly diminished capacity to synthesize progesterone in the basal state and to respond to LH (Shemesh & Hansel, 1983, Fields et al., 1985). Moreover, the ovary can be removed late in gestation in the cow or the corpora lutea can be prematurely regressed by exogenous prostaglandins without induction of abortion (Hoffman et al., 1979). These findings point to a significant nonovarian source of progestin during gestation, which may well be the placenta.

The stimulatory effect of MIX on progesterone production by the bovine placental cells could be related to three basic actions of this compound: (i) the increased accumulation of cyclic nucleotides as a

result of inhibition of phosphodiesterases (Sutherland et al., 1968; (ii) the translocation of intracellular calcium (Blinks et al., 1972); and (iii) the blockage of receptors for adenosine (Sattin & Rall, 1970). Since neither LH, 8-Br-cGMP, cholera toxin nor isoproterenol alone had effects on progesterone synthesis and none of these agents potentiated the effects of LH, it is likely that MIX acted through translocation of intracellular calcium, or through its effects on adenosine binding. The lack of responsiveness of the placental cells to adenosine (Shemesh et al., 1984) supports the former concept. Furthermore, the calcium ionophore A23187 induced a 2 fold increase in progesterone production by the treated cells and had an additive effect when used in combination with MIX. Incubation of the cells in a calcium-free medium or in the presence of a calcium antagonist reduced progesterone formation.

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 testes, adrenal glands, and ovaries (Veldhuis & Kase, 1982). However in all of these tissues cAMP is thought to be the primary intracellular regulator of hormone production. cAMP levels rapidly increase when ACTH or gonadotropins act upon the adrenal cortex or gonads, respectively and cAMP analogues are potent stimulators of steroid hormone production by these tissues. 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 &

Catt, 1980).

Since basal progesterone synthesis is stimulated by optimal concentrations of 25-OH-C, we suggest that the steroidogenic activity of the fetal cotyledon cells is limited by the availability of sterol substrate to the side-chain cleavage enzyme. This could reflect a relative deficiency of cellular sterol, a lack of lipoprotein-carried cholesterol in the incubation medium or relative inefficiency of delivery of sterol to the cholesterol side-chain cleavage enzyme. The effects of 25-OH-C were dependent upon the extracellular calcium concentration, indicating a critical role for this ion in the steroidogenic processes. MIX and the ionophore, A23187, were both found to increase both pregnenolone and progesterone secretion by the dispersed fetal cotyledon cell. These findings suggest that both agents act at least in part by augmenting the conversion of cholesterol to pregnenolone. However, the effects of MIX appear to be independent of the action of 25-OH-C, as the combination of the two treatments produced an additive response in steroidogenesis.

Two distinct calcium messenger systems could be involved in the regulation of bovine placental steroidogenesis, calmodulin and the calcium activated, phospholipid-dependent protein kinase (PKC). These may function independently or in concert. Evidence for an involvement of calmodulin derives from the inhibition of 25-OH-C+MIX stimulation of progestin synthesis by trifluoperazine. However, trifluoperazine is not entirely specific for antagonism of calmodulin and it might influence PKC as well (Nishizuka, 1984). Our findings support a role for PKC, since TPA, which dramatically increases the affinity of the protein kinase C for Ca^{2+} (Ashendel, 1985), produced an enhancement of progesterone synthesis stimulated by calcium mobilization by MIX or

A23187. Similarly, reinforcement interactions between phorbol esters and calcium ionophores have been demonstrated in a variety of other systems (Berridge & Irvine, 1984). The recent finding that phorbol esters stimulate bovine luteal (Hansel et al., 1987) and adrenal steroidogenesis (Culty et al., 1984), and that bovine adrenal cortex cholesterol side chain cleavage enzyme is phosphorylated by PKC raise the possibility that the activation of PKC may increase flux through the steroidogenic pathway through phosphorylation of cytochrome P-450scc (Vilgrain et al., 1985).

In this study we have demonstrated both the presence of PKC and cytochrome P450scc in fetal cotyledons. In regards to the PKC, we have demonstrated here that the bovine fetal cotyledon contains appreciable amounts, being about one third of that present in the rabbit brain, a tissue believed to have the highest concentration of this enzyme. Furthermore, it was demonstrated that the endogenous placental steroidogenic inhibitor dramatically reduced the phosphorylation activity of this enzyme.

Since the synergistic effect of TPA with MIX or A23187 on progesterone synthesis in long term incubations in the absence of cholesterol was variable with time, TPA activation of PKC appears to be subject to a short feedback inhibition of receptors or down regulation. This is in contrast to MIX, A23187, or 25-OH-C which demonstrated increased progesterone production throughout the time course of the long term incubations. Addition of TPA to the media already containing MIX or A23187 caused a 2-fold further increase in pregnenolone production. This suggests that at least one locus of the action of TPA is the side chain cleavage reaction. These data indicate that both calcium mobilization and activation of the PKC are necessary for

maximal expression of side chain cleavage activity. It is possible that TPA also affects 3 β -OH steroid dehydrogenase in increasing progesterone production from cholesterol, but this enzyme was not evaluated in the present experiments.

The data presented clearly demonstrate that P-450scc cytochrome is present in the bovine cotyledon; however this enzyme is not expressed prior to 100 days of gestation in the maternal caruncle. Very low levels (if any) of P-450scc were found in this tissue. These data correlate with the failure of the early maternal caruncle to synthesize significant amounts of progesterone while the fetal cotyledon at least by 45 days of gestation can synthesize progesterone.

In conclusion: 1) Both Ca⁺² and PKC enhance progesterone synthesis by the bovine placentome. 2) This activation is at the locus of both the side chain cleavage enzyme and the 3 β -OH dehydrogenase. 3) This process is cyclic nucleotide independent 4) Ca⁺² is the second messenger of a putative hormone 5) Cytochrome P-450scc is present in the fetal cotyledon.

For references in section B see appendix:

Shemesh, Hansel, Strauss & Shore. Journal of Reproduction and Fertility Suppl. 37:163-172, 1989.

Shemesh, Strauss, Hansel, Shore & Izhar. Journal of Steroid Biochemistry 29:21-25, 1988.

Shalem, Izhar, Shore, Shemesh & Hansel. Journal of Steroid Biochemistry 31:835-838, 1988.

C. PURIFICATION AND CHARACTERIZATION OF AN ENDOGENOUS INHIBITOR AND A STIMULATOR OF PROSTAGLANDIN SYNTHESIS.

Introduction

Prostanoids are believed to play an important role in the control of placental function and uterine activity. Thus, there is considerable interest in defining the factors that regulate prostanoid synthesis in placental and uterine tissues. We have reported the presence of a cytosolic factor in extracts of bovine placentomes which inhibited the conversion of arachidonic acid to prostanoids (Shemesh et al., 1981, 1983, 1984). This mechanism was consistent with the rapid expression of the factor's activity. Exposure of bovine luteal cells to the placental extract for as little as 5 min was sufficient to suppress the synthesis of prostanoids. This factor suppressed prostanoid synthesis by dispersed fetal cotyledon cells as well as by bovine granulosa and luteal cells. In a study of the maternal caruncles (which might exert a direct influence on uterine prostanoid synthetic activity), it was determined that there was a temporal association between a fall in the caruncle content of inhibitor and an increase in the capacity of dispersed caruncle cells from the placentomes collected at term to secrete prostanoids in vitro, indicating that a change in the content of endogenous inhibitor may be an important event in the onset of parturition.

Additional evidence for the presence of an inhibitor of prostanoid synthesis in the placentome was derived from examination of prostanoid synthesis by dispersed placental cells in culture (Shemesh et al., 1984). Prostanoid production was low when cells were prepared from

placentae removed before term but there was an appreciable increase (3-20 fold) in prostanoid production when cells were prepared from placentae collected at or near term. When caruncle cells from 120-day placentomes were plated with an equal number of fetal cotyledon cells, prostanoid synthesis was markedly reduced. When similar co-culture experiments were performed with cells from term placentomes, inhibition was not observed. Indeed, co-culture of the two cell types obtained at this stage of gestation seemed to enhance prostanoid synthesis. This stimulatory activity to prostanoid synthesis was found in caruncle extracts after heat treatment so it is possible that this tissue also contains a separate factor which enhances prostanoid production. The activity of such a factor may be masked by the inhibitor until term when the inhibitor is lost. The nature of the stimulatory activity is unknown. However, Strickland et al.(1983) have recently proposed that an endogenous stimulator of PGH production, presumably elaborated by the fetus, plays a role in the onset of human labor.

Results

Extraction of prostaglandin stimulator (PGSt).

Preliminary studies show the presence of a stimulatory peptide in acid and heat treated bovine fetal placental extracts of granules. Isolation of placental granules was done according to Byatt et al. (1986) as described in Fig.21.

Conversion of arachidonic acid to prostaglandin metabolites by bovine seminal vesicles (BSV) was used to determine activity of the placental extract.

Previously, we used the cytosolic placental extract to demonstrate stimulatory or inhibitory activity. However, a better purification of certain proteins was achieved by the granule preparation, as shown in

the SDS-gel electrophoresis (12% acrylamide; Fig. 3). Prior to electrophoresis, the granule preparations were treated with heat (100 C for 5 min.) and with acid (4N acetic acid; 1:1; v/v) to removed high molecular weight proteins.

The acid and heat treated granule fraction was further purified on high performance liquid chromatography (HPLC), using a linear gradient of 0-80% acetonitrile, containing 0.1% trifluoroacetic acid on a C-18 reverse phase column at 210 nm absorbancy (Fig. 22).

Fractions of 1 ml were collected, lyophilized, resuspended in distilled water and aliquots applied to seminal vesicle microsomal preparations (1-4 mg protein/assay) to determine the effect on the conversion of arachidonic acid to prostaglandin metabolites, as described previously (Shemesh et al., 1981). Prostaglandins were extracted with 5 vol ethyl acetate and dried eluates were analysed for PGE₂ and PGF_{2α} production using specific radioimmunoassays.

A profile of PG production (A-PGE₂; B-PGF_{2α}) by the microsomal preparation in the presence of the HPLC fractions collected is shown in Fig. 23. Fraction 19-21 collected at about 50% acetonitrile showed 7-9 and 4-6 fold stimulation in PGE₂ and PGF_{2α} synthesis, respectively, over the control.

Discussion

We have previously reported the presence of cytosolic factors in extracts of bovine placentomes which stimulate or inhibit conversion of arachidonic acid to prostanoids (Shemesh et al., 1981, 1983, 1984). In the present experiments we have further purified the stimulator by use of granule preparations and HPLC analysis. The data suggest that it is a small peptide secreted by the granules and it is heat and acid stable. This finding is compatible with our previous finding that the

stimulator is present throughout gestation but its activity is masked by the inhibitor until term. Apparently, when the cytosolic extract is boiled or when the granule extract is treated with acid and heat, the inhibitor, which is heat sensitive, is destroyed, allowing the expression of the heat stable stimulatory factor.

Relevant bibliography for section C.

1. Byatt, J.C., Shimonura, K., Duello, T.M. & Bremel, P.D. (1986)
Endocrinology 119:1293-1350.
2. Raz, A., Wyche, A., Siegel, N. & Needleman, P. (1988)
J. Biol. Chem. 263:3022.
3. Shemesh, M., Ailenberg, M., Lavi, S. & Mileguir, F. (1981) In:
Schwartz, N.B., Hunzicker, -Dunn, M. (Eds) Dynamics of Ovarian
Function. Raven Press, NY, p.161.
4. Shemesh, M., Hansel, W. & Strauss, J.F. (1983)
Biol. Reprod. 29: 856-862.
5. Shemesh, M., Hansel, W., Strauss, J.F. III, Rafaeli, A., Lavi, S. &
Mileguir, F. (1984). Anim. Reprod Sci. 7:177-194
6. Strickland, D.M., Saaed, S.A., Casey, M.L. & Mitchell, M.D. (1983)
Science 220:521

Fig. 1. Relative inhibitory binding of ^{125}I -labelled hCG in a radioreceptor assay by the fractions obtained using Sephacryl S-300. Total granule proteins were fractionated on Sephacryl S-300 column and analysed for displacement of ^{125}I -labelled hCG. The data were calculated from a standard curve for hCG (not shown).

Fig. 2. Progesterone production by bovine luteal cells (2×10^5) treated with granule proteins fractionated on Sephacryl S-300 column. The amount of secreted progesterone was measured using radioimmunoassay. The control group ($4.7 \text{ ng} \pm 0.3 \text{ s.e.}$, $n = 10$) is represented as 100%. The stippled bar is the stimulation of progesterone synthesis by 5 ng hCG/ml .

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel (10%) electrophoresis followed by silver staining. Lane a, cytosol of fetal cotyledons; Lane b, total granule extract; Lane c, acidic granule extract. Number on the left side of the gel shows the migration of molecular weight marker proteins.

Fig. 4. Progesterone production by dispersed bovine luteal cells (2×10^4) treated with h.p.l.c.-derived fractions of the acidic granule extract. The C-18 reverse-phase column was eluted with a linear gradient of acetonitrile (0–80% in H_2O). Fractions of 1 ml were collected and lyophilized. The amount of secreted progesterone was measured by radioimmunoassay. The control group is represented by 100%.

Column	Progesterone
ng/ μ g granule protein/200000 cells/2h	
Total granule extract	0.8
Sephacryl S-300 high molecular weight peak	3.84
Acidic granule extract	10.2
C-18 h.p.l.c. (45% acetonitrile)	470

Table 1. Progesterone synthesis by bovine luteal cells in the presence of granular fractionated extracts.

FIG. 5. Progesterone formation by cultured dispersed cotyledon and caruncle cells. Medium was changed every 4 days of culture and progesterone secretion during the subsequent 4 days was then determined. Values are means $\pm$ SEM of net synthesis from 10 cows at 182-275 days of gestation.

FIG. 6. Effect of tropic agents on progesterone biosynthesis by incubated dispersed fetal cotyledon cells during 3 and 12 hr of incubation. Cells were washed by centrifugation and fresh medium was added after 3 hr of incubation. Values are means $\pm$ SEM for net synthesis by 9 animals from early and late stages of gestation.

FIG. 7. Effect of LH or 8-Br-cAMP with MIX on progesterone biosynthesis by incubated dispersed fetal cotyledon cells during 3 hr of incubation. Values are means $\pm$ SEM for net synthesis by 5 animals.

FIG. 8. Effect of MIX on progesterone biosynthesis by incubated dispersed maternal caruncle cells. Values are means $\pm$ SEM for net synthesis of five samples from 1 animal at each stage of gestation.

Fig. 9. Effect of pregnenolone with A23187 or MIX on progesterone biosynthesis by incubated dispersed maternal caruncle cells. Each point is the mean $\pm$ SEM of five replicates. These data are representative of 8 experiments.

Fig. 10. Effect of medium calcium concentration on progestin synthesis by dispersed fetal cotyledon cells incubated in the presence of 25-OH-C and MIX. Cells were incubated in the presence of various concentrations of calcium with or without MIX (0.5 mM), 25-OH-C (20 μ g/ml) or 25-OH-C and MIX (0.5 mM). Net progesterone synthesis was determined over a 15 h period. Values are the means $\pm$ SE from 4 separate experiments.

Fig. 11. Effects of MIX, A23187 and 25-OH-C on pregnenolone synthesis by dispersed fetal bovine cotyledon cells. Dispersed cells were incubated with or without MIX (0.5 mM), A23187 (3 μ M) or 25-OH-C (1 μ g/ml) in the absence or presence of cyanoketone (300 ng/ml) for 15 h. Secreted pregnenolone was measured as described in the text.

Fig. 12. Effects of TPA and TPA + MIX on progestagen synthesis by dispersed bovine fetal cotyledon cells. Dispersed cells were incubated for 1, 2, 6 or 15 h in the absence or presence of TPA (10 ng/ml) with or without MIX (0.5 mM). Values are the means $\pm$ s.e.m. from 5 replicate cultures from a representative experiment.

Fig. 12. Effect of TPA on conversion of 25-OH-C on progesterone synthesis by dispersed fetal bovine cotyledon cells. Dispersed cells were incubated with or without TPA (10 ng/ml) in media containing 1 μ g/ml of 25-OH-C for 10 h. Values are the means $\pm$ SE from 5 separate experiments. Each treatment resulted in a significantly higher production of progesterone ($P < 0.05$).

Fig. 14. Effects of MIX and TPA on pregnenolone synthesis by dispersed fetal bovine cotyledon cells. Dispersed cells were incubated with or without MIX (0.5 mM), TPA (10 ng/ml), 25-OH-C (1 μ g/ml) in the presence of cyanoketone (300 ng/ml) for 10 h. Values are the means $\pm$ SE from 5 separate experiments. Each treatment resulted in a significantly higher production of pregnenolone ($P < 0.05$).

Fig. 15. Effects of A23187 and TPA on pregnenolone synthesis by dispersed fetal bovine cotyledon cells. Dispersed cells were incubated with or without A23187 (3 μ M), TPA (10 ng/ml), or 25-OH-C (1 μ g/ml) in the presence of cyanoketone (300 ng/ml) for 10 h. Values are the means $\pm$ SE from 3 separate experiments. Each treatment resulted in a significantly higher production of pregnenolone ($P < 0.05$).

Fig. 16. Effects of MIX, A23187 and TPA on pregnenolone synthesis by dispersed fetal bovine cotyledon cells. Dispersed cells were incubated with or without MIX (0.5 mM), A23187 (3 μ M) or TPA (10 ng/ml) in the presence of cyanoketone (300 ng/ml) and 25-OH-C (1 μ g/ml) for 10 h. Values are the means $\pm$ SE from 3 separate experiments. The combination of MIX, A23187 and 25-OH-C resulted in a significant increase in pregnenolone synthesis ($P < 0.05$) and addition of TPA caused a further 2-fold increase in pregnenolone synthesis ($P < 0.005$).

Fig.17. Effects of trifluoperazine on A23187 and MIX-stimulated steroidogenesis by dispersed bovine fetal cotyledon cells. Dispersed cells were incubated for 15 h in the absence or presence of MIX (0.5 mM) and A23187 (3 μ M), with or without trifluoperazine (20 μ M). Data presented are means $\pm$ SE for net progesterone synthesis from 5 separate experiments.

Fig.18. Effects of trifluoperazine on 25-OH-C and 25-OH-C + MIX-stimulated progesterin synthesis by dispersed bovine fetal cotyledon cells. Dispersed fetal cotyledon cells were incubated in the absence or presence of 25-OH-C (20 μ g/ml) with or without MIX (0.5 mM) in the absence or presence of trifluoperazine (20 μ M). Net progesterone synthesis was determined during a 15 h incubation. Values presented are means $\pm$ SE from 5 separate experiments.

Fig.19 Cytochrome P-450scc (arrow) in bovine fetal placental (P) and luteal cell (CL) extracts as measured by immunoblotting to nitrocellulose paper and analysis by Western blot assay. A rabbit antibody against bovine P-450scc and a second antibody coupled to horse radish peroxidase were used as described in the text.

Fig. 20 Effect of maternal caruncle extract on protein Kinase-C activity

Figure 21. Preparation of bovine fetal cotyledon granular extract (according to Byatt et al., 1986).

Fig. 22. Elution profile of 100 μ l acid (100°C) treated bovine granule extract on C18 reverse phase column of high performance liquid chromatography (HPLC) with a linear gradient of 0-80% acetonitrile measured at 210 nm absorbancy.

Figure 23. Production of prostaglandins PGE_2 (A) and $\text{PGF}_{2\alpha}$ (B) by bovine seminal vesicle microsomes with addition of aliquots from HPLC column. Values are shown as % of control incubations.

f. Description of cooperation

Studies of luteotrophic substances were carried out in both countries using different approaches. The Cornell group studied trophoblastic tissue and amniotic fluid while in Israel we concentrated on fetal cotyledons from placentas from 50 - 100 days of gestation. The existence of luteotrophic substance secreted by the bovine placental tissue was therefore confirmed independently. In experiments currently underway are being tested in vivo at Cornell. Because of difficulties in importing the granule preparations into the U.S., it has been necessary to prepare them in the U.S.

In the framework of this grant, Dr. Shemesh visited Cornell in order to demonstrate the granule preparation and Dr. Hansel visited Israel to evaluate the results as well as take part in the International Workshop on the Maternal Recognition of Pregnancy. This workshop was sponsored by BARD and Dr. Hansel was the President of the workshop.

g. Evaluation of the research achievements with respect of the aims of the original research proposal.

The objective of this project was to define the basic mechanisms which govern hormonal synthesis in the bovine trophoblast. The specific aims of the project were (1) to further purify and characterize a substance from the bovine placenta which has luteotrophic activity (bCG); (2) to define the factors that control progesterone synthesis in this tissue, including a factor extracted from the placenta which inhibits steroidogenesis; and (3) to further purify and characterize an endogenous stimulator of prostaglandin synthesis extracted from the placenta.

It was found that (1) there is both a high (60-70Kd) and low (20-30Kd) mol wt protein which are secreted from bovine placental cell granules and which are active in both radioreceptor and bioassays. Similarly, trophoblastic tissue (as well as amniotic fluid) from 24-36 day embryos was analysed and found to have similar high and low mol wt luteotrophic factors; (2) that the Ca^{+2} second messenger regulation of steroid production is dependent on cholesterol availability and that both the 3 β -OH steroid dehydrogenase and side chain cleavage enzymes are regulated by this system. Furthermore, evidence for involvement of protein kinase C activation as well as calcium mobilization in the regulation of placental steroidogenesis was found. Protein kinase C was specifically inhibited by the endogenous inhibitor of steroidogenesis extracted from the placentome; (3) that bovine placental granules contain a protein that is heat and acid stable, which stimulates both PGE and PGF_{2 α} production by bovine seminal vesicle microsomal preparations and dispersed placental cells.

Although most of the objectives of the project were obtained, we were not able to complete the isolation of the bCG to sufficient homogeneity so that it could be used as a basis for an early pregnancy test. We were unable to do this as the protein lost its activity when additional purification steps beyond what we have already reported were undertaken. In addition, we did not study the endogenous inhibitor of prostaglandin synthesis in any greater detail as several publications by other laboratories on its nature and physiology have subsequently appeared. We therefore concentrated on the endogenous prostaglandin stimulator, a factor about which little is known but which may play an important role

in parturition.

List of publications:

Shalem,Z.,Izhar,M.,Shore,L.S,Shemesh,M. & Hansel,W. (1988)

Journal of Steroid Biochemistry 31:835-838.

Shemesh,M., Strauss,J.F.,Hansel,W.,Shore,L.S. & Izhar,M. (1988)

Journal of Steroid Biochemistry 29:21-25.

Hickey,G.J., Walton,J.S. & Hansel (1989)

Journal of Reproduction and Fertility Suppl. 37:29-35.

Izhar,M. & Shemesh,M. (1989)

Journal of Reproduction and Fertility Suppl.37:37-44.

Shemesh,M., Hansel,W., Strauss,J.F. & Shore,L.S.(1989)

Journal of Reproduction and Fertility 37:163-172.