REGULATION OF STEROIDOGENESIS IN THE OVINE CORPUS LUTEUM

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ABSTRACT

To examine the factor affecting LH-induced progesterone production in vitro in ovine luteal slices, an experimental procedure was employed wherein each slice served as its own control. The role of microfilaments in steroidogenesis was studied in luteal slices treated with cytochalasin B (an inhibitor of microfilament function). Cytochalasin B treatment resulted in significant reduction of progesterone production by luteal slices in response to LH and the addition of serum to the medium did not alter this effect. The ability of luteal slices to respond to LH with increased progesterone secretion was restored when cytochalasin B was removed from the medium. Further studies indicated that inhibition of LH-induced progesterone production by treatment with cytochalasin B was not a result of a change in: 1) cyclic adenosine 3'-5'-monophosphate production in response to LH; 2) mitochondrial membrane permeability to cholesterol; or 3) activity of 3β-hydroxysteroid dehydrogenase, Δ^5,Δ^4-isomerase enzyme complex.

The possibility existed that microfilaments were necessary for cholesterol transport to mitochondria in response to LH stimulation. However, mitochondrial cholesterol content was unchanged in response to LH in the presence or absence of aminoglutethimide (an inhibitor of cholesterol side-chain cleavage enzyme activity) as determined by uptake of ^{3}H-cholesterol or total content determined by gas-liquid chromatography. Further, treatment with cytochalasin B had no effect on mitochondrial cholesterol content. These results suggest a role for microfilaments in LH-induced progesterone production at a point prior to the conversion of cholesterol to pregnenolone.

INTRODUCTION

Microfilaments have been implicated as important components of the contractile and mitotic systems of many cell types (1) and it has been suggested that these cytoskeletal elements play some role in steroidogenesis (2). Cytochalasin B, a drug that alters microfilament morphology,

blocked ACTH and cAMP-stimulated steroid production in mouse Y-1 adrenal tumor cells but had no effect on basal steroid output (2). Further, ACTH-stimulated accumulation of ^{3}H-cholesterol by mitochondria in the presence of aminoglutethimide (which blocks cholesterol side chain cleavage) was inhibited by cytochalasin B, but uptake of ^{3}H-cholesterol by the adrenal cell was unaffected (3). Cytochalasin B was shown to have no direct effect on cholesterol conversion to pregnenolone in isolated mitochondria or in a purified, reconstituted cholesterol side-chain cleavage enzyme system (3). Likewise, there was no effect on protein synthesis or cellular ATP levels (3). Conflicting data indicate that in the presence of serum or high density lipoproteins (HDL), treatment with cytochalasin B resulted in increased steroidogenesis in Y-1 adrenal cells (4). This later observation suggested that microfilaments restrict or regulate availability of cholesterol to mitochondria and that disruption of microfilament function may result in increased cholesterol transport to mitochondria when lipoproteins are present.

The following experiments were designed to determine the role microfilaments play in steroidogenesis in the ovine corpus luteum.

MATERIALS AND METHODS

Corpora lutea were removed surgically from superovulated Western range ewes 8-11 days after ovulation (5). After removal of the capsule, slices of 0.5 mm thickness were made on a Stadie-Riggs tissue slicer. To reduce variability one slice from each corpus luteum was included in each treatment group. Slices were blotted and weighed, placed into 15 ml plastic beakers containing 2 ml Hank's balanced salts solution (pH 7.4) and incubated in a Dubnoff Metabolic Shaking Incubator at 37° C with gentle shaking. Media were changed every 15 min using 12 ml disposable syringes with blunted 16 gauge needles. Progesterone was quantified in media by radioimmunoassay (6) to establish baseline progesterone secretion. Thus, each slice served as its own control. Subsequent treatments were employed as described for individual experiments. One group of slices (at least 3) in each experiment received 1 µg/ml LH (NIH-LH-S21) or 1 µg/ml hCG (Organon, West Orange, NJ) to determine whether the tissue was responsive to tropic

hormone. In all cases LH or hCG stimulated progesterone production.

Mitochondrial fractions were prepared from slices by homogenizing each slice in 1 ml 0.25 M sucrose for 2 sec in a Brinkman Polytron (Brinkman Instruments, Westbury, NY). Sucrose (0.25 M) was added to a final concentration of 20 mg/ml and the homogenate subjected to several strokes in a Dounce homogenizer. One to 2 ml of the homogenate were centrifuged for 10 min at 650 x g, the pellet was discarded and the supernatant centrifuged at 10,000 x g for 15 min. The resultant mitochondrial pellet was washed 3 times in 1 ml 0.25 M sucrose and centrifuged at 10,000 x g for 15 min. The protein concentration of crude homogenates and mitochondrial pellets was determined using a Bio-Rad protein assay kit (Bio-Rad Labs, Richmond, CA) with bovine serum albumin as standard. For quantification of cholesterol in various fractions by gas chromatography samples were prepared by addition of approximately 10,000 dpm of ^{3}H-cholesterol (53 Ci/mmol, New England Nuclear, Boston, MA), extraction in 7 ml chloroform:methanol (2:1, v/v) and thin-layer chromatographic purification of the cholesterol on alumina sheets (Eastman Kodak) developed twice in hexane:ethyl acetate (3:2, v/v). Areas on the thin-layer chromatogram that corresponded to cholesterol were cut out and cholesterol eluted with 2 ml chloroform. The eluate was taken to dryness under nitrogen. Each value was corrected for the recovery of ^{3}H-cholesterol, which was approximately 40%, after all purification procedures. A Beckman GC-45 gas chromatograph with a flame ionization detector and a 6 foot glass column packed with 3% OV-17, 120/140 mesh on Gas Chrom Q (Applied Science Laboratories, State College, PA) was used for quantitation of cholesterol. The carrier gas was nitrogen (38 ml/min) with air and hydrogen flow rates of 270 ml/min and 52 ml/min, respectively. The column, injection port and detector temperatures were 270° C, 285° C and 310° C, respectively.

The specificity of the gas-liquid chromatographic procedures was evaluated three ways: 1) Pregnenolone (3 β-hydroxy-5-pregnen-20-one), progesterone, testosterone, prednisone (17,21-dihydroxy-1,4-pregnadiene-3,11-20-trione) and androstenedione (4-androstene-3,17-dione) were shown to elute from the gas chromatographic (GC) column before cholesterol. 2) Samples containing added ^{3}H-cholesterol were subjected to four sequential thin-layer purifications and specific activity calculated following each analysis. 3) After the final GC analysis, samples and cholesterol standards were derivatized and rechromatographed.

Effect of Cytochalasin B on Steroidogenesis in Luteal Slices. To determine the effect of cytochalasin B (purified from Helminthosporium dermatioideum, Sigma Chemical Co., St. Louis, MO) on luteal steroidogenesis and to establish an effective, non-toxic dose of the drug, slices were preincubated for 15 min with cytochalasin B (5, 10, 20, or 30 µg/ml) in 0.5% DMSO final concentration. Following preincubation, slices were incubated with cytochalasin B for an additional 30 min with media changes every 10 min, after which hCG (1 µg/ml) was added to each slice for an additional 30 min with media changes at 10 min intervals. Subsequently, all slices were washed six times at 5-min intervals followed by changes of media at 15 min intervals for 30 min. Finally, the slices were incubated in media containing hCG (1 µg/ml) for a 30 min period. All media were analyzed for their progesterone content.

To determine if serum influenced the effect of cytochalasin B on luteal steroidogenesis, slices were preincubated for 15 min in media containing serum from a hypophysectomized ewe (10% final concentration) with or without 20 µg/ml cytochalasin B, followed by a 1 hr incubation in the presence of absence of 1 µg/ml hCG. All slices were then washed in medium 5 times over a 30 min period and subsequently incubated for 30 min with 2 changes of medium, each of which was assayed for progesterone.

The possibility that cytochalasin B (or the DMSO, dimethylsulfoxide, Fischer Scientific Co., Fair Lawn, NJ, used to solubilize the drug) might affect directly mitochondrial membrane permeability was also examined. A mitochondrial fraction was prepared as described previously and distributed equally among 3 treatment groups. Two groups were incubated in 1 ml medium for 30 min with 3.75×10^5 cpm ^{3}H-cholesterol and the third group was preincubated for 15 min in 1 ml medium containing 20 µg/ml cytochalasin B in 0.5% DMSO final concentration, followed by the addition of ^{3}H-cholesterol for a 30 min period. All mitochondrial fractions were centrifuged at 10,000 x g for 15 min and the supernatants counted for radioactivity. Mitochondria were then incubated for 30 min in the presence of DMSO (0.5% final concentration) or 20 µg/ml cytochalsin B in 0.5% DMSO and then washed 3 times in 1 ml medium and the pellets and washes were counted for radioactivity.

The effect of cytochalasin B on LH-stimulated cAMP production was examined by incubating slices in the presence of 1 µg/ml LH, 20 µg/ml cytochalasin B or LH and cytochalasin B. Following a 60 min treatment, slices were homogenized in 5% TCA, centrifuged at 3,000 rpm for 30 min and the supernatant neutralized with 1 M K_2HPO_4, diluted to 10 ml in 0.1% PBS-gel and analyzed for cAMP by radioimmunoassay (7).

To determine the possible effect of cytochalasin B on pregnenolone conversion to progesterone, slices were preincubated for 15 min in the presence or absence of 20 µg/ml cytochalasin B, followed by incubation for 1 hr with 5 µg/ml pregnenolone in 0.5% DMSO final concentration. Media were analyzed for progesterone.

Effect of Aminoglutethimide and Cytochalasin B on Cholesterol Uptake. Based on the findings of Mrotek and Hall (3), the uptake of ^{3}H-cholesterol by mitochondria in the presence of aminoglutethimide was evaluated by preincubating slices for 30 min with one of the following: medium, aminoglutethimide (10^{-3} M), LH (1 µg/ml) or LH and aminoglutethimide. Following the preincubation, media were changed and ^{3}H-cholesterol (6×10^5 cpm) was added for 30 min. Media were changed and treatment continued for 30 min with 2 changes of media. Slices were rinsed twice in 0.25 M sucrose with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) to aid removal of non-specifically bound ^{3}H-cholesterol and homogenized. Mitochondrial pellets were prepared, resuspended in 500 µl 0.25 M sucrose and a 200 µl aliquot was diluted in Triton X-100 scintillation solution and counted.

In an attempt to eliminate the possibility that ^{3}H-cholesterol did not equilibrate with intracellular cholesterol pools, the above experiment was repeated and the cholesterol content of the mitochondria

was quantified by gas-liquid chromatography.

Finally, the effects of hCG and cytochalasin B on mitochondria cholesterol content were studied. Slices were preincubated for 15 min in medium and one of the following: cytochalasin B (20 μg/ml) or cytochalasin B plus aminoglutethimide (10^{-3} M) followed by a 30 min incubation in the presence or absence of hCG (1 μg/ml). Subsequent to the preincubation periods, slices were subjected to the various treatments for 1 hr with media changes every 15 min. Endogenous cholesterol content of the mitochondrial fraction was determined by gas chromatography.

Statistical Analyses: Differences between means were tested for significance using Students' t test (8).

RESULTS

The Effects of Cytochalasin B on Luteal Steroidogenesis. Treatment of slices with cytochalasin B (5, 10, 20 or 30 μg/ml) resulted in a significant inhibition ($P < 0.05$) of hCG-stimulated progesterone output (Table 1). Following a 30 min wash with media without cytochalasin B, progesterone production of slices increased in response to hCG. These data suggest a role for microfilaments in the tropic hormone stimulation of luteal steroidogenesis. However, contrary to the data of Cortese and Wolff (4) ovine luteal slices treated with cytochalasin B in the presence of serum resulted in no increase in progesterone production (0.79 $\pm$ 0.09 ng/min/mg tissue) over progesterone production from slices incubated in cytochalasin B alone (0.85 $\pm$ 0.04 ng/min/ mg tissue). Further, cytochalasin B inhibition of LH-stimulated progesterone production in the ovine corpus luteum does not appear to result from a decreased ability of LH to stimulate cAMP production. There was no significant difference in cAMP production in response to hCG in the presence or absence of cytochalasin B (Table 2).

The effects of cytochalasin B on steroidogenesis in the ovine corpus luteum can not be explained by direct effects on mitochondrial membrane permeability to cholesterol. As seen in Table 3, when the mitochondrial

TABLE 1

Effect of Cytochalasin B on Progesterone Output in hCG Stimulated Ovine Luteal Slices

Cytochalasin B (μg/ml)	Progesterone Output % of Baseline*	
	During Treatment	After 30 min Wash
0	214 ± 7[1]	216 ± 7
5	120 ± 13	166 ± 16*
10	120 ± 11	171 ± 4*
20	110 ± 10	179 ± 19*
30	92 ± 12	144 ± 15*

* Post-treatment values are different ($P < 0.05$) from treatment values.

[1] mean ± SEM; n=6

TABLE 2

Effect of Cytochalasin B on LH-Stimulated cAMP Production

Treatment	cAMP (pg/mg Tissue)*
LH (1 μg/ml)	6.9 ± 1.0
LH and Cytochalasin B (20 μg/ml)	9.4 ± 1.0

* mean ± SEM; n=3

TABLE 3

Effect of Cytochalasin B on Mitochondrial Permeability to ^{3}H-Cholesterol

Treatment		% Uptake of ^{3}H-Cholesterol*
1st Incubation	2nd Incubation	
^{3}H-Cholesterol	DMSO	15.11 ± 0.70
^{3}H-Cholesterol	Cytochalasin B (20 μg/ml)	15.14 ± 0.90
Cytochalasin B + ^{3}H-Cholesterol	Cytochalasin B (20 μg/ml)	16.04 ± 0.96

* mean ± SEM; n=3

fraction was incubated with ^{3}H-cholesterol in the presence or absence of cytochalasin B there was no difference in the quantity of ^{3}H-cholesterol remaining in the mitochondria after 30 min. Additionally, cytochalasin B had no effect on the conversion of exogenous pregnenolone to progesterone (Table 4). These data indicate that cytochalasin B does not have a direct effect on 3β-hydroxysteroid dehydrogenase-Δ^{5},Δ^{4}-isomerase complex, the enzyme responsible for the conversion of pregnenolone to progesterone.

TABLE 4

Effect of Cytochalasin B on the Conversion of Pregnenolone to Progesterone

Treatment	Progesterone (ng/min/mg Tissue)*
Pregnenolone	0.29 ± 0.05
Pregnenolone + Cytochalasin B	0.26 ± 0.01

* mean ± SEM; n=3

Effects of Aminoglutethimide, LH, and Cytochalasin B on Uptake of Cholesterol by Luteal Mitochondria. The addition of 10^{-3}M aminoglutethimide to the incubation medium inhibited basal progesterone secretion to approximately half of that in untreated controls and did not have a general toxic effect since it could be washed out and the ability of the tissue to respond to LH was restored (Table 5). The addition of LH to ovine luteal slices did not result in accumulation of ^{3}H-cholesterol in mitochondria in the presence or absence of aminoglutethimide (Table 6). Further, the mitochondrial content of endogenous cholesterol as determined by gas-liquid chromatography was unaffected by aminoglutethimide or LH treatment (Table 6).

Measurements of mitochondrial cholesterol content by gas chromatographic analysis indicate that cytochalasin B treatment does not result

TABLE 5

Effect of Aminoglutethimide on Progesterone Output in hCG-Stimulated Ovine Luteal Slices

Aminoglutethimide	Progesterone Output, % of Baseline*	
	During Treatment	After 30 min Wash
0	178 ± 30[1]	167 ± 30
3×10^{-3} M	52 ± 4	128 ± 4*
10^{-3} M	40 ± 5	130 ± 7*
10^{-4} M	65 ± 5	116 ± 22*

* Treatment values are different ($P < 0.05$) than post-treatment values
[1] mean ± SEM; n=3

TABLE 6

Effect of LH or hCG on Cholesterol Content of Mitochondria From Luteal Slices as Determined by Two Methods

Treatment	% Uptake ^{3}H-Cholesterol[b]	Cholesterol[a] (mg/mg protein[b])
Control	11.61 ± 1.11	19.2 ± 1.4
LH or hCG	12.01 ± 1.46	18.2 ± 2.4
Aminoglutethimide	11.84 ± 0.63	16.5 ± 1.2
Aminoglutethimide + hCG	12.61 ± 0.90	20.5 ± 2.4

[a] determined by gas chromatographic analysis
[b] mean ± SEM; n=6

TABLE 7

Effect of Cytochalasin B on Cholesterol Content of Luteal Mitochondria as Determined by Gas Chromatographic Analysis

Treatment	Cholesterol (mg/mg protein*)
Control	19.2 ± 1.4
hCG	18.2 ± 2.4
Cytochalasin B	24.7 ± 1.7
Cytochalasin B + hCG	20.2 ± 2.6
Cytochalasin B + aminoglutethimide	22.4 ± 2.2
Cytochalasin B + aminoglutethimide + hCG	28.2 ± 4.7

* mean ± SEM, n=6

in decreased mitochondrial content (Table 7). These data suggest that the stimulation of luteal progesterone secretion by LH or hCG is a result of an increased rate of cholesterol transport through mitochondria and that cytochalasin B does not inhibit hCG-stimulated steroidogenesis by causing a decreased cholesterol content of mitochondria.

DISCUSSION

In these experiments each slice served as its own control for monitoring progesterone secretion. That the corpus luteum is not a homogeneous tissue is indicated by studies on the bovine (9) and ovine corpus luteum (10). Therefore, in any one luteal slice, there may be areas of high and low progesterone production, hence, the establishment of a baseline progesterone production for each slice permits the collection of more accurate information concerning the effect of treatment on that slice. Due to the variablity of progesterone responses between slices expressed as ng progesterone/min/mg tissue, results were presented as a percent of baseline progesterone.

It has been shown that cytochalasin B blocks the steroidogenic effect of ACTH in mouse Y-1 adrenal tumor cells and that cytochalasin B blocked the ACTH-stimulated accumulation of mitochondrial cholesterol in the presence of aminoglutethimide suggesting that microfilaments play a role in cholesterol transport in steroidogenic tissue (3). Further, highly purified anti-actin (incorporated into liposomes to aid entry into cells) also blocks the ACTH-stimulated accumulation of cholesterol by mitochondria (11). Although cytochalasin B inhibited LH-stimulated progesterone production in ovine corpus luteum, there was no effect of cytochalasin B in the presence or absence of aminoglutethimide on mitochondrial cholesterol content as determined by gas-liquid chromatographic analysis.

Further experimentation showed that inhibition of LH-stimulated progesterone production was not a result of decreased cAMP production in response to LH. These data indicate that cytochalasin B had no effect on LH-receptor-adenylate cyclase coupling and are compatible with those of Azhar and Menon (12) who demonstrated that cytochalasin B at 10, 50 or 100 μM, higher concentrations than those used in these experiments, blocked progesterone production in response to hCG but had no effect on ^{125}I-hCG binding to rat luteal cells. These data conflict with those of Zor et al. (13) who demonstrated that low levels of cytochalasin B (1-3 μg/ml) or anti-actin did not affect ^{125}I-hCG binding to follicular cell membranes, but did inhibit the cAMP response to LH. These authors suggested that microfilaments play a role in the biochemical coupling of the LH receptor-adenylate cyclase system. Cytochalasin B was present in their follicular culture for 24 hr before the binding experiments were performed. Thus, a more detailed study of the short term versus long term response of microfilaments in steroidogenesis is warranted.

The conversion of pregnenolone to progesterone in ovine corpora lutea is unaffected by cytochalasin B. These results support the observation that cytochalasin B treatment of mouse Y-1 adrenal tumor cells had no effect on the conversion of pregnenolone to 20α-hydroxy-4-pregnen-3-one (4). These data suggest that microfilaments are necessary for tropic hormone-stimulated steroidogenesis at a point before the conversion of cholesterol to pregnenolone. However, in these studies pregnenolone that is added to the tissue does not have to be processed through mitochondria before being converted to progesterone; therefore, microfilaments may be involved in pregnenolone egress from mitochondria and this possibility warrants study.

Luteal mitochondrial membrane permeability to cholesterol is unaffected by cytochalasin B treatment. Thus, the inhibition of LH-stimulated steroidogenesis by cytochalasin B is not a result of a direct effect of the drug on the mitochondrial membrane. Data presented in this communication indicate that incubation of ovine luteal slices in the presence of cytochalasin B and serum does not result in increased steroidogenesis, contrary to previous reports (4). Thus, it appears that microfilaments are necessary for LH-stimulated steroidogenesis in the ovine corpus luteum and that maintenance of microfilament function is necessary for increased progesterone production in the presence or absence of serum.

It is known that cytochalasin B blocks hexose transport (14) and although this effect of cytochalasin B was not directly tested on the ovine corpus luteum, several lines of evidence suggest that inhibition of glucose transport is not responsible for the actions of cytochalasin B on steroidogenic tissue. In mouse Y-1 adrenal tumor cells, cytochalasin B treatment did not result in measurable changes in cellular ATP levels suggesting that the inhibition of glucose transport by cytochalasin B had no effect on energy production (3). Further, LH administration to rat luteal tissue stimulated progesterone production without a corresponding increase in glucose uptake or metabolism (15). Cortese and Wolff (4) demonstrated that inhibition of glucose transport by 2-deoxy-glucose had no effect on steroidogenesis in Y-1 adrenal tumor cells. Thus, it appears that glucose uptake is not important, at least for the short term response of steroidogenic tissue to tropic hormones.

Contrary to the findings of Mrotek and Hall (3), luteal mitochondria fail to exhibit an increased cholesterol content in response to

steroidogenic stimulation by LH or hCG as determined by uptake of ^{3}H-cholesterol or gas-liquid chromatography. These results may be explained in several ways. First, it is possible that in the corpus luteum, LH stimulates progesterone production by evoking an increased rate of cholesterol transport into mitochondria and therefore an increased rate of cholesterol cleavage. Thus, per unit time, the cholesterol content of luteal mitochondria would remain the same. The hypothesis that tropic hormones regulate the rate of substrate flow into the side chain cleavage system is supported by Bell et al. (16). Second, the ovine corpus luteum contains many non-steroidogenic cells. Mitochondria from the non-steroidogenic cells may contribute enough cholesterol to mask any effect of LH on cholesterol accumulation in mitochondria of steroidogenic cells. This problem could best be overcome by performing the same experiments in a purified luteal cell system.

It should also be noted that there is no way to distinguish between cholesterol that has been extracted from the membrane and that which is part of a steroidogenic pool. Thus, differences in cholesterol made available for steroidogenesis in response to LH may have been hidden by the cholesterol contribution from mitochondrial and other contaminating membranes.

While no changes in mitochondrial cholesterol content have been detected as a result of treatment, disruption of microfilament function by cytochalasin B significantly blocks the LH-stimulated progesterone production in ovine luteal slices. However, treatment of luteal slices with cytochalasin B does not affect LH-induced cAMP production or pregnenolone conversion to progesterone indicating that microfilaments are involved in LH-stimulated steroidogenesis at a point prior to the

conversion of cholesterol to pregnenolone. Moreover, protein synthesis is also required for both basal and LH-induced production in ovine (17) and rat (18) corpora lutea; thus, a labile protein may interact with the microfilament network for the transport of cholesterol to or within mitochondria.

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