

## Mechanisms subserving the trophic actions of insulin on ovarian cells. In vitro studies using swine granulosa cells

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### Research Article

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# Mechanisms Subserving the Trophic Actions of Insulin on Ovarian Cells

## IN VITRO STUDIES USING SWINE GRANULOSA CELLS

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**ABSTRACT** Direct actions of insulin on gonadal tissues have been difficult to demonstrate *in vivo*. We have developed an *in vitro* system in which swine ovarian cells remain highly responsive to trophic actions of insulin. Purified porcine insulin significantly augmented the biosynthesis and secretion of progesterone by cultured granulosa cells. These stimulatory actions of insulin were dose- and time-dependent and saturable. Under serum-restricted conditions, insulin also significantly amplified the capacity of estradiol and 8-bromo cyclic AMP to stimulate progesterone production. Inhibitors of protein and RNA synthesis (cycloheximide, actinomycin D, and alpha-amanatin) inhibited insulin action. The stimulation of progesterone production by insulin was attributable to increased biosynthesis of pregnenolone, rather than diminished catabolism of progesterone to its principal metabolite,

20 $\alpha$ -hydroxypregn-4-en-3-one. Insulin also enhanced progesterone production in the presence of a soluble sterol substrate, 5-cholesten-3 $\beta$ ,25-diol, which readily gains access to the mitochondrial cholesterol side-chain cleavage system. Moreover, exposure of granulosa cells to insulin produced a three- to sevenfold increase in mitochondrial content of cytochrome P-450 measured by difference spectroscopy, with a corresponding increase in mitochondrial cholesterol side-chain cleavage activity.

The capacity of insulin to facilitate progesterone biosynthesis by ovarian cells was mimicked by the insulinlike somatomedin, multiplication stimulating activity, but not by epidermal growth factor, fibroblast growth factor, or porcine relaxin. Insulin's augmentation of progesterone production reflected a selective action on progestin biosynthesis, since insulin significantly suppressed estrogen biosynthesis by granulosa cells.

Thus, our investigations indicate that insulin acts on ovarian cells selectively to stimulate pregnenolone (but not estrogen) biosynthesis. The actions of insulin are exerted by processes that require protein and RNA synthesis, and by mechanisms that augment mitochondrial cytochrome P-450 content and facilitate the utilization of cholesterol in the side-chain cleavage

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reaction. The striking mimicry of insulin effect by multiplication stimulating activity suggests that insulin action may be mediated through somatomedin receptors. Moreover, in view of the high concentrations of somatomedin in ovarian follicles in vivo, our in vitro observations suggest that specific trophic actions of insulin or insulinlike growth factors are likely to significantly regulate the differentiated function of the Graafian follicle in vivo.

## INTRODUCTION

Hypogonadism is a recognized sequela of insulinopenic diabetes mellitus in experimental animals and man (1-6). For example, men with diabetes mellitus exhibit reproductive impairment and impotence, children manifest delayed puberty, and women have an increased prevalence of anovulation, menstrual irregularity, and infertility (4-10). Presumptive abnormalities of both pituitary and gonadal function have been described in vivo (1-14). However, the interpretation of these observations is limited by confounding influences of weight loss, protein catabolism, hyperglycemia, gonadal vasculopathy, and homeostatic responses of the pituitary-gonadal and adrenal axes in intact animals (15-22). Moreover, precise cellular or biochemical alterations responsible for gonadal dysfunction in diabetes mellitus have not been delineated.

In an effort to characterize direct effects of insulin on gonadal tissues, investigators have recently studied the actions of this hormone on isolated pituitary and gonadal cells (23-26). Such studies have revealed specific insulin binding sites on both testicular and ovarian cells (27, 28). Moreover, gonadal insulin receptors seem to be functionally coupled to cellular responses, since insulin alters cellular morphology, and augments steroid hormone production by cultured rodent testicular cells, and rat or bovine ovarian cells (24, 25, 29, 30).

In the present work, we have investigated insulin action using a monolayer culture system of swine ovarian cells that remain highly responsive to insulin's trophic effects. This culture system has permitted us to define some of the biochemical mechanisms of insulin action on the mammalian ovary.

## METHODS

**Cell cultures.** Swine granulosa cells were aspirated aseptically from 1-3-mm follicles of immature porcine ovaries, as previously described (31, 32). Cells were separated from follicular fluid by centrifugation in Eagle's minimum essential medium, and enumerated in a hemocytometer using phase-contrast optics. Cell viability was determined by exclusion of trypan blue. Monolayer cultures were established with  $1 \times 10^6$  granulosa cells/cm<sup>2</sup> in medium consisting of 1 ml bicarbonate-buffered Eagle's minimum essential me-

dium (Gibco Laboratories, Grand Island, NY), with or without the designated concentration of fetal calf serum (31, 32). Cultures were maintained at 37°C in a humidified mixture of 95% air/5% CO<sub>2</sub> for varying intervals. Except in certain time course experiments, spent media were removed every 48 h and replenished with fresh media containing hormones. In some experiments, cells were harvested mechanically into 100% ethanol for the subsequent assay of progesterone (33). In other experiments, cells were enumerated on a culture-by-culture basis after harvesting by trypsinization (0.25% trypsin for 20 min at 23°C) to determine cell density by electronic (Coulter Electronics, Inc., Hialeah, FL) particle counting (32).

## Analytical methods

**Preparation of mitochondrial fractions.** Cell pellets from three (10 cm) culture plates were pooled after thawing and homogenized in 1 ml of the buffered medium described by Toaff et al. (34) using a small glass vessel and teflon pestle. The homogenates were centrifuged at 500 g for 10 min and the supernatants collected and centrifuged at 5,000 g for 10 min to prepare mitochondrial pellets. The pellets were suspended in a small volume of incubation medium consisting of 0.2 M sucrose, 0.2 mM EDTA, 20 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM KH<sub>2</sub>PO<sub>4</sub>, and 25 mM Tris-HCl, pH 7.4. Aliquots were then taken for quantitation of cytochrome P-450 or cholesterol side-chain cleavage activity.

**Quantitation of cytochrome P-450.** Cytochrome P-450 was quantitated in the mitochondrial suspensions by the method of Omura and Saito (35) from the carbon monoxide anaerobic minus the anaerobic difference spectrum using an Aminco DW-2A dual wave length, split beam spectrophotometer. An extinction coefficient of 91 cm<sup>-1</sup>·mm<sup>-1</sup> was used to calculate the cytochrome P-450 content from the absorbancy change between 450 and 490 nm.

**Assay of cholesterol side-chain cleavage activity.** Assays were carried out by a modification of the method of Toaff et al. (34). Aliquots of the mitochondrial preparations (~60 µg protein) were incubated in 1 ml of incubation medium containing 1 mM NADPH and 25 µg/ml 25-OH-cholesterol (Steraloids, Wilton, NH). Incubations were initiated after a zero-time sample was collected and were terminated after 10 min by addition of 0.3 NaOH as described by Toaff et al. (34, 36). Preliminary studies revealed that these incubation conditions were optimal for assay of cholesterol side-chain cleavage activity. Activity is expressed as nanomoles net steroid synthesis (pregnenolone plus progesterone) per milligram protein per minute.

**Other methods.** The concentration of progesterone in spent medium or cellular extracts was assayed after hexane extraction, using a specific radioimmunoassay with isotopic correction for recovery as previously reported (31-33). The cross-reactivity of this antibody with 17β-estradiol, insulin, cyanoketone, trilostane, or pregnenolone was <0.01%, and with 20α-hydroxypregn-4-en-3-one <3%. Concentrations of estrogen pregnenolone and 20α-hydroxypregn-4-en-3-one were measured by previously validated radioimmunoassays (37).

Protein concentrations were measured by the methods of Bradford (38), or Lowry et al. (39), and cellular DNA content by the method of Burton (40).

**Statistics.** Statistical analyses used one- or two-way analysis of variance to ascertain significance among multiple means, with the Newman-Keul's or Tukey procedures to interpret individually significant effects (41). All experiments

were repeated at least twice, to confirm the generality of our inferences in independent populations of ovarian cells.

**Materials.** Estradiol-17 $\beta$  and 8-bromo cyclic AMP (cAMP) were purchased from Sigma Chemical Co. (St. Louis, MO); highly purified monocomponent porcine insulin, from Nova Laboratories, Inc. (Wilton, CT); desoctapeptide porcine insulin, from Eli Lilly Co. (Indianapolis, IN); epidermal/fibroblast growth factors and multiplication stimulating activity (MSA),<sup>1</sup> from Collaborative Research, Inc. (Waltham, MA).

## RESULTS

Insulin stimulated progesterone secretion by primary cultures of swine granulosa cells. The influence of varying concentrations of serum in the culture fluid on insulin's stimulation of progesterone secretion is depicted in Fig. 1. In these experiments, granulosa cells were cultured either in the complete absence of serum, or in the presence of 1, 4, or 10% (vol/vol) serum supplementation for 2 or 4 d. At each serum concentration, or in the complete absence of serum, the addition of insulin (1  $\mu$ g/ml) markedly enhanced progesterone secretion ( $P < 0.01$  treatment effect). These stimulatory effects of insulin were demonstrable within 24–38 h (see early time course, Fig. 2). There was a  $\geq 5$ -fold stimulation of progesterone production corrected per microgram cellular protein after 38 h in culture in the presence of 1% serum. More striking responses to insulin were observed after 96 h of treatment, when insulin promoted an eightfold increase in progestin secretion above control levels in serum-free cultures, and 52-, 85-, and 40-fold stimulation in the presence of 1, 4, and 10% serum, respectively (day 4, Fig. 1). Because of the responsivity of granulosa cells to insulin in medium supplemented with low levels of serum, the remaining studies (unless noted otherwise) were performed in the presence of 1% serum.

To determine the dose-response of the insulin effect, cells were exposed to increasing concentrations of insulin for 40 h. Insulin elicited dose-dependent and saturable stimulation of total progesterone production (measured in cells combined with medium), as shown in Fig. 3. At concentrations  $> 30$  ng/ml ( $\sim 5$  nM), insulin increased total progesterone production by at least threefold. This action of insulin was specific in that it could not be mimicked by increasing concentrations of desoctapeptide porcine insulin (Fig. 3). Moreover, epidermal growth factor, fibroblast growth factor, and highly purified porcine relaxin were inactive (Fig. 4). Insulin increased both the cell content and the medium concentration of progesterone in a significant manner ( $P < 0.01$ ; Fig. 4). The latter ob-

servations suggest that the effect of insulin is to stimulate *de novo* progesterone production, rather than simply promote cellular release of previously synthesized steroid. This inference was confirmed by demonstrating that selective inhibitors of 3 $\beta$ -hydroxysteroid dehydrogenase, trilostane (150  $\mu$ M), or cyanoketone (5  $\mu$ M) blocked insulin's stimulatory action by 99.7 and 97%, respectively (total progesterone production measured in cells combined with medium in response to insulin declined from  $163 \pm 29$  ng/48 h to  $0.8 \pm 0.11$  and  $4.8 \pm 1.6$  ng/48 h, respectively).

The stimulatory actions of insulin could not be accounted for by alterations in cell number or protein content, because results from independent experiments (see Fig. 5) revealed that insulin stimulated a 24–29-fold increase in progesterone production, whether corrected for cell number, DNA content, or total cellular protein. These data also indicate that in the presence of 1% serum, basal progesterone secretion increased only slightly between days 2, 4, and 6 in culture. However, peak progesterone output in response to insulin increased markedly. Thus, insulin does not simply maintain progesterone production, but substantially augments steroid output.

The stimulatory effect of insulin on progesterone production was inhibited by concurrent or delayed treatment with cycloheximide, and concurrent treatment with actinomycin D or  $\alpha$ -amanatin (each at a concentration of 1  $\mu$ g/ml): Fig. 6. These concentrations of inhibitors of protein and RNA synthesis inhibited [<sup>14</sup>C]leucine and [<sup>3</sup>H]uridine incorporation into acid-insoluble material in these cells by  $>95$  and  $>85\%$ , respectively (42). Thus, we infer that continued protein synthesis and initial RNA synthesis are required for insulin's augmentation of progesterone production by ovarian cells.

Since estradiol and gonadotropic hormones (the latter presumably acting through the intracellular mediation of cAMP) are two major physiological regulators of progesterone biosynthesis, we assessed the capacity of insulin to augment the stimulatory actions of estradiol or a potent stable analogue of cAMP, 8-bromo cAMP. Combined treatment with estradiol and insulin synergistically increased progesterone production measured in cells combined with medium (Fig. 7 A). In this experiment, cultures were maintained for 4 d in order to demonstrate the stimulatory action of estradiol. In addition, insulin significantly amplified the dose-dependent stimulatory effects of 8-bromo cAMP assessed after 44 h (Fig. 7 B).

To examine further the mechanisms by which insulin stimulates the production of progesterone, we determined whether insulin treatment inhibits catabolism of progesterone to the principal progestin metabolite, 20 $\alpha$ -hydroxypregn-4-en-3-one. Insulin treat-

<sup>1</sup> Abbreviations used in this paper: csc, cholesterol side-chain cleavage; MSA, multiplication stimulating activity.

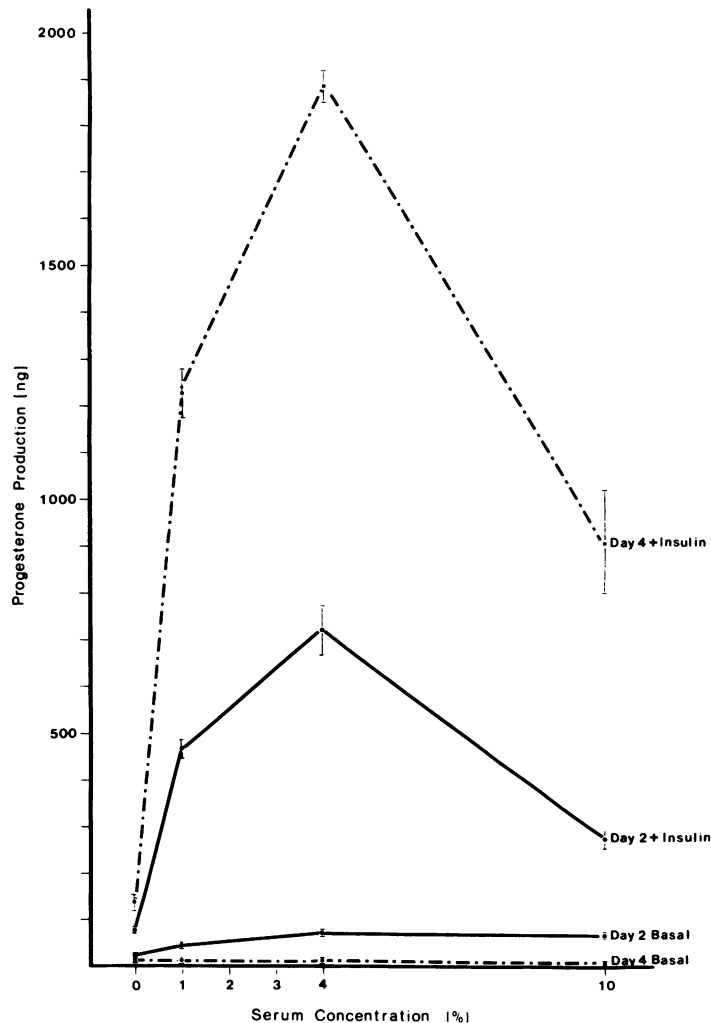


FIGURE 1 Influence of serum supplementation on insulin action in cultured swine granulosa cells. Ovarian cells were cultured in Eagle's minimum essential medium in the absence of serum supplementation (0% serum), or in the presence of increasing concentrations (vol/vol) of serum. Cultures were maintained for 48 h (day 2) or 96 h (day 4) with control solvent or a maximally stimulating concentration of insulin (1  $\mu\text{g}/\text{ml}$ ). Progesterone secretion (nanograms) measured in the media of replicate cultures is given as the mean  $\pm$  SEM ( $n = 4$  cultures) at each point.

ment increased both total progesterone and total  $20\alpha$ -hydroxypregn-4-en-3-one production (ng/ $3 \times 10^7$  cells  $\cdot$  48 h): basal progesterone production  $120 \pm 18$ , insulin-stimulated  $703 \pm 38$ ; and basal  $20\alpha$ -hydroxypregn-4-en-3-one  $3.4 \pm 0.6$ , insulin stimulated  $42 \pm 1.9$  ( $P < 0.01$  insulin-treatment effect). Thus, insulin does not augment progesterone production simply by blocking progesterone catabolism. To investigate the capacity of insulin to increase progesterone production by stimulating earlier steps in steroidogenesis, we tested insulin's capacity to increase pregnenolone production

from endogenous substrates. Insulin increased total pregnenolone production from  $8.5 \pm 0.76$  to  $26.7 \pm 1.8$  ng pregnenolone/48 h  $\times 10^6$  cells, when pregnenolone's metabolism to progesterone was blocked by 150  $\mu\text{M}$  trilostane (which inhibits progesterone production by  $>97\%$  under these conditions). In addition, insulin augmented progesterone production when cells were cultured in the presence of an exogenously supplied sterol substrate, provided in the form of 5-cholesten- $3\beta,25$ -diol (Fig. 8). This polar analogue of cholesterol has been shown to serve as effective substrate for mi-

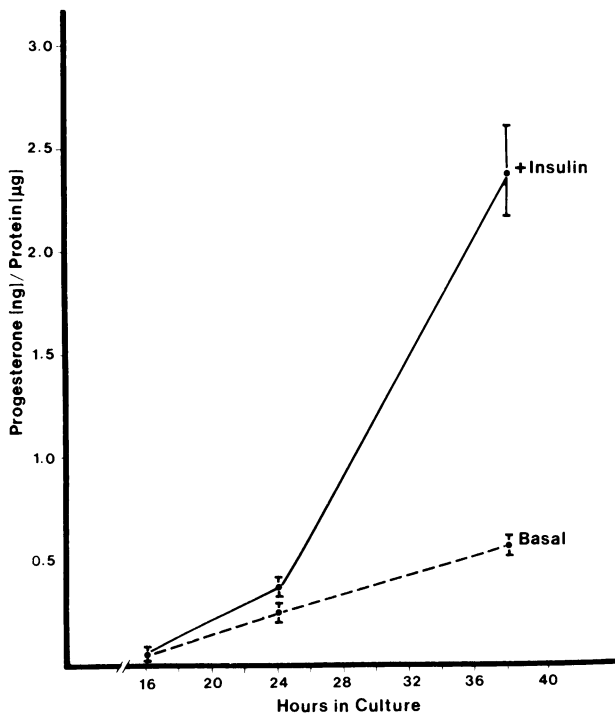


FIGURE 2 Time course of insulin's early stimulatory effects. Porcine granulosa cells were cultured in the presence of control solvent or a maximally stimulating concentration of insulin (1  $\mu\text{g}/\text{ml}$ ) for the intervals denoted. The total progesterone content (nanograms) of cells combined with medium is corrected per microgram cellular protein in each culture. Data are means  $\pm$  SEM ( $n = 4$  cultures). At time zero, the cultures contained  $<0.05$  ng progesterone/ $\mu\text{g}$  protein.

tochondrial side-chain cleavage in granulosa-luteal cells (36, 43). Thus, our findings indicate that insulin enhances steroidogenesis at least in part by stimulating a step (or steps) between cholesterol and pregnenolone.

To directly test the hypothesis that insulin increases cholesterol side-chain cleavage activity, we measured mitochondrial cytochrome P-450 content by difference spectroscopy and assayed mitochondrial cholesterol side-chain cleavage activity. The results from a typical experiment are given in Table I. Insulin increased cytochrome P-450 content sevenfold, and cholesterol side-chain cleavage activity fivefold. In a second experiment, insulin increased granulosa-cell cytochrome P-450 content from  $0.33 (\pm 0.12)$  to  $1.12 (\pm 0.18)$  nmol/mg protein (means  $\pm$  range for duplicate sets of cultures), and increased the rate of cholesterol side-chain cleavage (csc) 3.7-fold. These observations provide direct evidence that insulin increases the cytochrome P-450<sub>csc</sub> content of granulosa cells.

To assess the influence of increased glucose availability on progesterone production by granulosa cells,

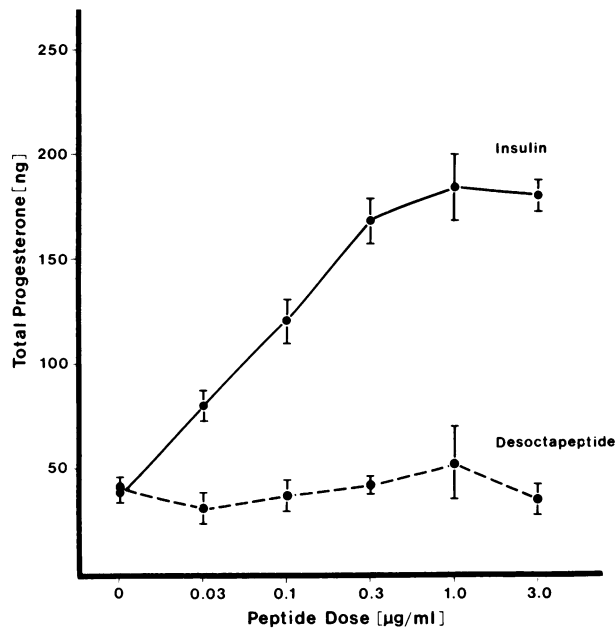


FIGURE 3 Dose-responsive stimulation of granulosa-cell progesterone production. Cultures of pig granulosa cells were treated with highly purified porcine insulin (upper solid curve) for 40 h. The total mass of progesterone produced in cells combined with medium is given in nanograms as mean  $\pm$  SEM ( $n = 4$  cultures) at each point. The lower interrupted curve depicts responses to the trypsin-degraded insulin species, desoctapeptide insulin.

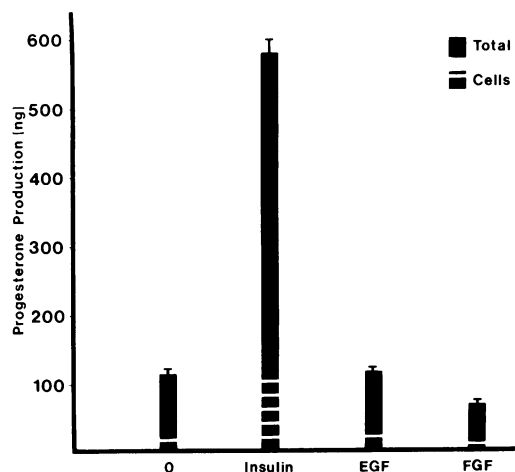


FIGURE 4 Specificity of insulin's stimulation of progesterone production by cultured swine granulosa cells. Cells were treated with insulin, epidermal growth factor (EGF), or fibroblast growth factor (FGF) (each at a concentration of 1  $\mu\text{g}/\text{ml}$ ) for 48 h. The medium and cellular content of progesterone were assayed separately. The mass of progesterone is depicted as the mean  $\pm$  SEM ( $n = 4$  cultures) nanograms steroid per culture. Highly purified porcine relaxin (1 and 3  $\mu\text{g}/\text{ml}$ ) was devoid of effect (not shown).

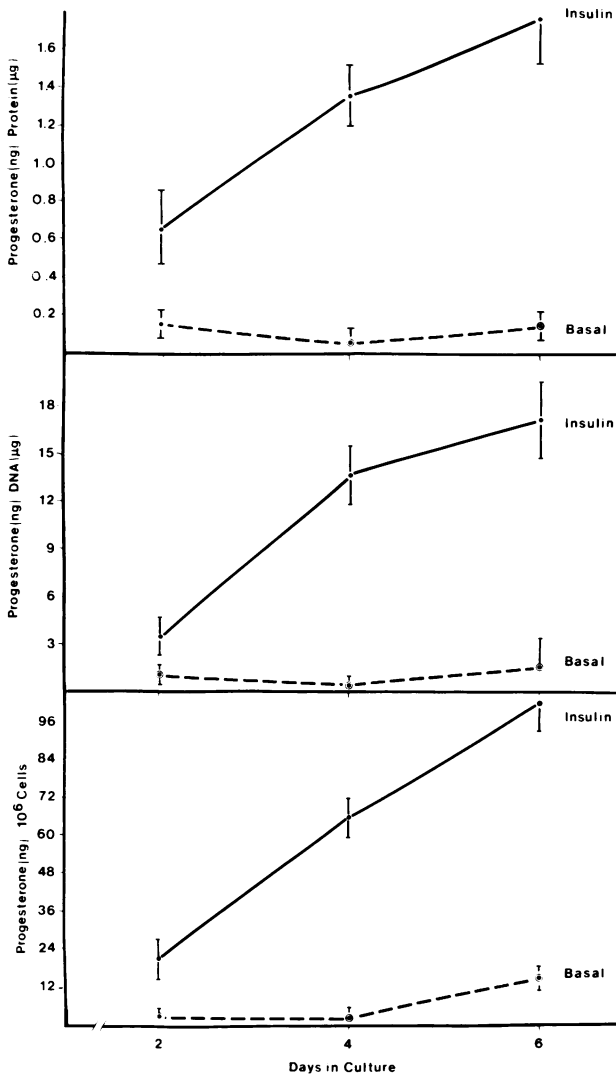


FIGURE 5 Effect of insulin on total progesterone production independently corrected per microgram total cellular protein (*upper panel*), per microgram DNA (*middle panel*), or per million granulosa cells (*lower panel*) on day 2, day 4, and day 6 of culture. Total progesterone content (nanograms) is given as the mean  $\pm$  SEM for four replicate cultures at each time point.

serum-free monolayer cultures were maintained in the presence or absence of a maximally stimulating concentration of insulin (1  $\mu$ g/ml), with increasing medium concentrations of glucose, as follows: 100, 200, 300, 400, and 500 mg/dl. As indicated in Table II, high medium concentrations of glucose could only partially mimic the facilitative effect of insulin on progesterone production. The maximal stimulatory effect of glucose alone was fourfold and occurred at glucose concentrations of 400 and 500 mg/dl ( $P < 0.01$  vs.

basal). In contrast, 500 mg/dl of a nonmetabolizable sugar, mannitol, was devoid of effect (not shown). The results further indicate that insulin possesses an even more pronounced effect than glucose per se. As Table II indicates, there is an eightfold stimulatory effect of insulin, which clearly cannot be accounted for solely by increased utilization of glucose per se.

The insulinlike growth factor, MSA, which is present in high concentrations in the ovarian follicle (see Discussion), was remarkably effective in stimulating progesterone production (Fig. 9). The stimulatory effects of MSA were observed at significantly ( $P < 0.001$ ) lower concentrations than that of insulin.

The selectivity of insulin's effect on the progesterin pathway is indicated by the suppressive effect of insulin on estrogen biosynthesis (Table III).

## DISCUSSION

These studies demonstrate that insulin selectively enhances the biosynthesis and secretion of progesterone by swine granulosa cells in vitro. Insulin augments progesterone production, whether corrected per granulosa cell, per microgram DNA, or per microgram cellular protein. These results are concordant with the recent description of insulin-binding sites on swine granulosa cells, and with prior reports that insulin can facilitate steroid production by gonadal cells (24, 25, 28-30). The more striking responsivity (>20-fold stimulation) to insulin that is attainable in the present swine granulosa-cell system may reflect, in part, our restriction of serum supplementation, and our use of tissue-culture medium devoid of cysteine. Supplementation with serum has recently been shown to impede the expression of differentiated function by cultured gonadal cells (44) and the presence of cysteine seems to accelerate insulin degradation in vitro (45).

Our results indicate that one facet of the mechanism of insulin's trophic effect in granulosa cells may be to increase glucose utilization, but that increased glucose utilization can account only partially for the stimulatory action of insulin on progesterone biosynthesis by granulosa cells. The time course of insulin action and the susceptibility of insulin's effects to inhibitors of protein and RNA synthesis, suggest that insulin exerts important facilitative or trophic effects on one or more steps in the steroidogenic pathway of ovarian cells. This inference is congruent with the capacity of insulin to augment granulosa-cell production of progesterone, as well as its precursor, pregnenolone, and progesterone's major metabolite, 20 $\alpha$ -hydroxypregn-4-en-3-one. Moreover, our studies indicate that insulin amplifies the stimulatory actions of two major effectors of ovarian steroidogenesis, namely, estradiol and 8-bromo cAMP. These two different effectors act via

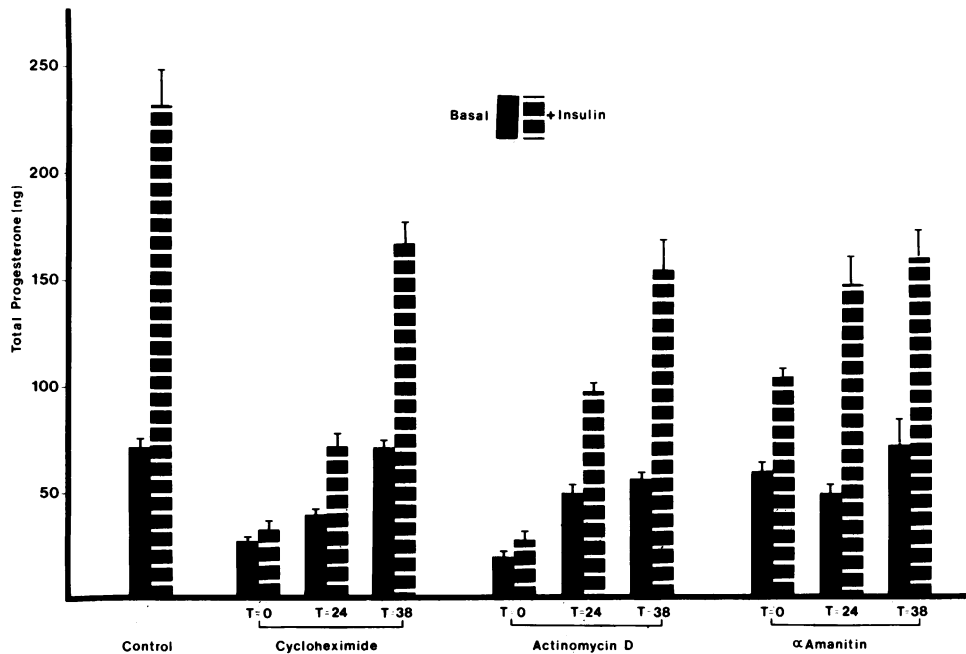


FIGURE 6 Dependence of insulin action on protein and RNA synthesis. Swine granulosa cells were cultured for 48 h in the presence or absence of a maximally stimulating concentration of insulin ( $1 \mu\text{g}/\text{ml}$ ). Certain cultures were also treated with cycloheximide, actinomycin D, or  $\alpha$ -amanatin (each at a concentration of  $1 \mu\text{g}/\text{ml}$ ) to suppress protein and RNA synthesis. Insulin was added at the outset of culture, and the inhibitors were added either simultaneously with insulin (denoted  $T = 0$ ), or after 24 ( $T = 24$ ) h or 38 ( $T = 38$ ) h of culture. Data are means  $\pm$  SEM nanograms progesterone per culture ( $n = 4$ ) measured in cells combined with medium.

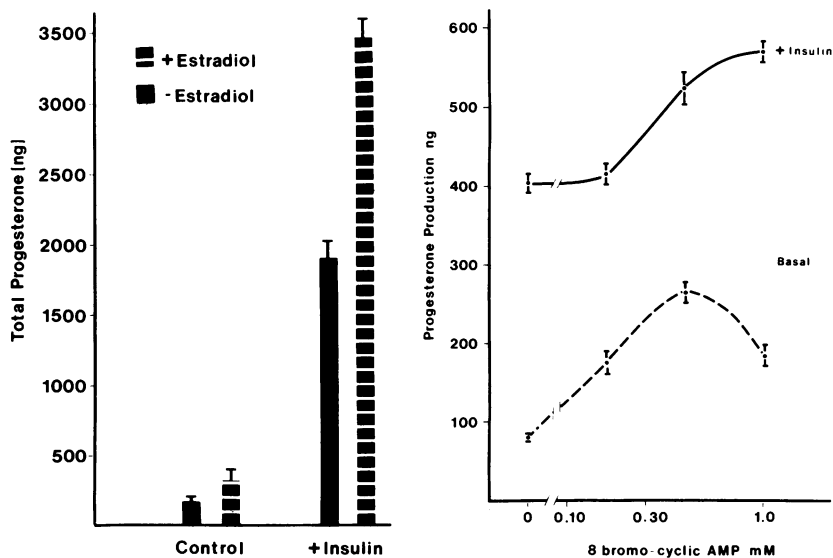


FIGURE 7 (A) Insulin amplifies the stimulatory action of estradiol. Swine granulosa cells were cultured in the presence or absence of a maximally stimulating concentration of insulin ( $1 \mu\text{g}/\text{ml}$ ) and/or estradiol- $17\beta$  ( $1 \mu\text{g}/\text{ml}$ ). The total progesterone content of cells combined with medium was measured. Data are given as mean  $\pm$  SEM ( $n = 4$  cultures) nanograms progesterone for the interval 43–96 h in culture. (B) Insulin augments the dose-dependent stimulation of progesterone production by the cAMP analog, 8-bromo cyclic AMP. Swine granulosa cells were cultured in the presence or absence of a maximally stimulating concentration of insulin ( $1 \mu\text{g}/\text{ml}$ ), and/or increasing concentrations of 8-bromo cyclic AMP. Data are means  $\pm$  SEM ( $n = 4$  cultures) total nanograms progesterone in cells combined with medium after 44 h of culture.



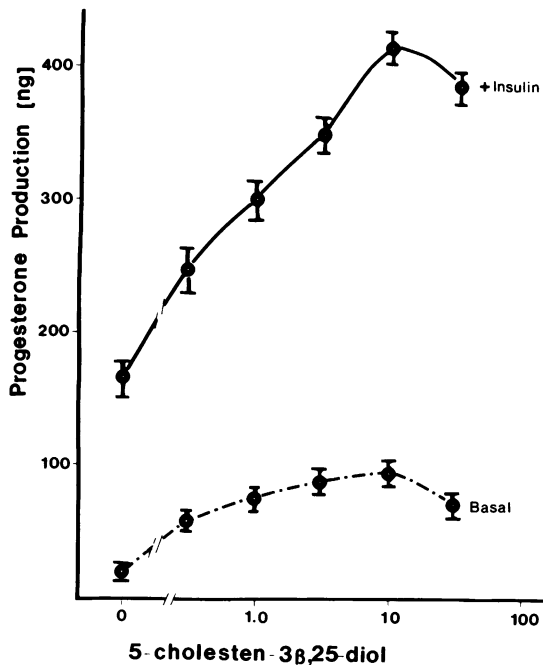


FIGURE 8 Insulin increases progesterone production from exogenously supplied sterol substrate. Swine granulosa cells were cultured for 40 h in the presence (*upper* solid curve) or absence (*lower* interrupted curve) of a maximally stimulating concentration of insulin (1  $\mu\text{g}/\text{ml}$ ), and/or increasing concentrations of 5-cholesten-3 $\beta$ ,25-diol (micrograms per milliliter<sup>-1</sup>). The total mass of progesterone (nanograms) contained in cells combined with medium is given as the mean $\pm$ SEM ( $n = 4$  cultures).

distinctly separable initial mechanisms (46, 47), but their distal intracellular effects seem to converge in the concerted stimulation of pregnenolone and progester-

TABLE I  
Influence of Insulin on Granulosa-Cell Secretion of Progesterone, Mitochondrial Cytochrome P-450 Content, and Cholesterol Side-Chain Cleavage Activity

Conditions	Progesterone secretion*	Cytochrome P-450 content†	Cholesterol side-chain cleavage activity‡
Control	90.7 $\pm$ 0.28	0.20	0.93
Insulin treatment (for 2 d)	586.0 $\pm$ 39.0	1.44	4.57

\* Nanograms of progesterone per  $4 \times 10^7$  cells  $\cdot$  48 h (mean $\pm$ SEM,  $n = 3$ ).

† Nanomoles per milligram protein (mean value from three pooled cultures).

‡ Nanomoles per milligram protein  $\cdot$  minute.

TABLE II  
Influence of Medium Glucose Concentrations on Progesterone Biosynthesis by Cultured Swine Granulosa Cells

Medium concentration of glucose mg/dl	Control*	Insulin*
100 (basal)	55 $\pm$ 2.6	434 $\pm$ 41
200	119 $\pm$ 12.2	459 $\pm$ 33
300	138 $\pm$ 5.8	386 $\pm$ 29
400	200 $\pm$ 13.1	400 $\pm$ 27
500	204 $\pm$ 4.8	357 $\pm$ 9.6

\* Total progesterone production in nanograms per  $3 \times 10^6$  cells  $\cdot$  48 h. Data are mean $\pm$ SEM ( $n = 4$ ). Cultures were maintained for 48 h under serum-free conditions with or without a maximally stimulating concentration of insulin.

one biosynthesis (33). Insulin's amplification of the stimulatory effects of estradiol and cAMP suggests that insulin may regulate one or more key steroidogenic steps in the final pathways of estrogen and cAMP action.

The present studies provide several significant and complementary lines of evidence that major actions of insulin are exerted at the level of the mitochondrial cytochrome P-450<sub>esc</sub>, the terminal oxidase of the cholesterol side-chain cleavage system that catalyzes the rate-determining step in progestin biosynthesis (48). First, insulin enhances pregnenolone production from endogenous substrate, when further metabolism of pregnenolone is blocked. Second, insulin increases progesterone production in the presence of an exogenous sterol substrate, 5-cholesten-3 $\beta$ ,25-diol. The access of this cholesterol analogue to the side-chain cleavage reaction is not limiting to steroidogenesis in intact or broken cells (36). Since insulin enhances progesterone production in the presence of maximally stimulating concentrations of 5-cholesten-3 $\beta$ ,25-diol, we infer that insulin must act at the level of the side-chain cleavage system. Third, treatment of granulosa cells with insulin significantly increases the mitochondrial content of cytochrome P-450 measured by difference spectroscopy. Fourth, this increase in cytochrome P-450 content is expressed functionally in an actual enhancement of mitochondrial cholesterol side-chain cleavage activity. The requirement for new RNA and protein synthesis for insulin action may well reflect the need to synthesize cytochrome P-450<sub>esc</sub> and other components of the side-chain cleavage system (i.e., adrenodoxin, and adrenodoxin reductase).

In the present studies, significant effects of insulin were observed at concentrations of 10 ng/ml ( $\sim$ 1.3 nM) or greater. However, the exact concentrations of insulin used in long-term (48 h) in vitro experiments

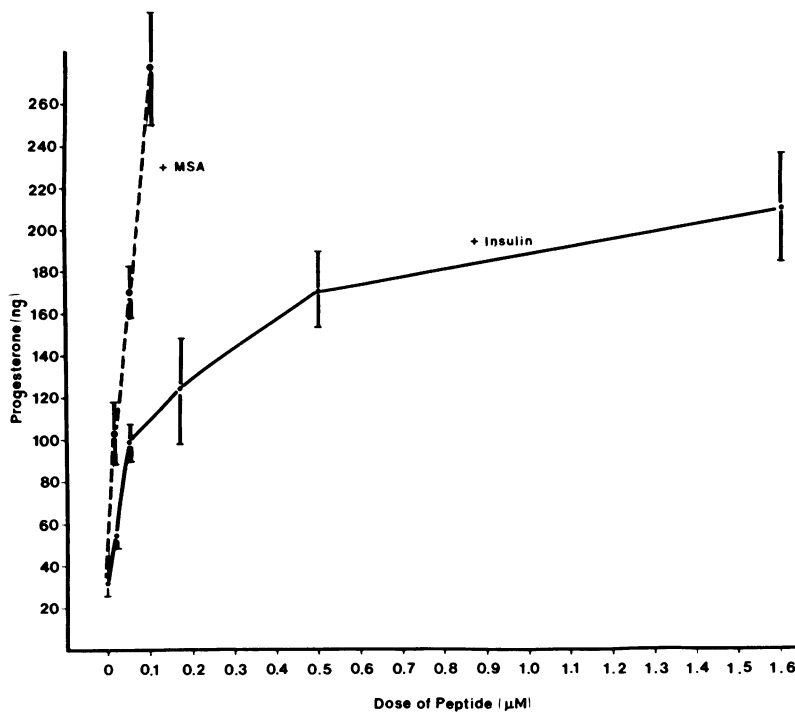


FIGURE 9 MSA, a somatomedin present in high concentrations in the ovary follicle, stimulates progesterone production by swine granulosa cells cultured in serum-free medium. The response to MSA was compared with that of highly purified porcine insulin during this 48-h incubation. Data give progesterone secretion in nanograms per  $2 \times 10^6$  cells  $\cdot$  48 h as means  $\pm$  SEM ( $n = 4$  cultures).

cannot be extrapolated facily to precisely equivalent concentrations in vivo, because significant degradation of insulin occurs under sustained culture conditions,

TABLE III  
*Insulin Suppresses the Aromatization of Testosterone to Estradiol by Swine Granulosa Cells*

Concentration of substrate*	Estradiol production	
	Control	Insulin-treated
$\mu\text{g/ml testosterone}$	$\text{ng}/5 \times 10^5 \text{ cells} \cdot 48 \text{ h}$	
0	$0.13 \pm 0.01$	$0.08 \pm 0.01$
0.3	$7.78 \pm 0.35$	$2.29 \pm 0.17^\circ$
1.0	$14.89 \pm 1.23$	$4.52 \pm 0.22^\circ$
3.0	$45.25 \pm 1.90$	$12.12 \pm 0.79^\circ$
10.0	$47.31 \pm 3.85$	$11.98 \pm 0.94^\circ$
30.0	$41.60 \pm 2.71$	$11.67 \pm 1.80^\circ$

\*  $P < 0.01$  vs. control.

Swine granulosa cells were incubated for 48 h in serum-free medium in the presence or absence of a maximally effective dose of insulin, and/or increasing concentrations of testosterone as substrate for estrogen biosynthesis. The medium content of  $17\text{-}\beta\text{-estradiol}$  was assayed (Methods) and data corrected per  $5 \times 10^5$  granulosa cells. Values are means  $\pm$  SEM ( $n = 3$  separate determinations).

and because other factors such as cell density and cytodifferentiation influence the apparent potency of insulin action in monolayer systems (45, and unpublished results). Since insulin was added discontinuously (every 48 h) to the culture system, but is present continuously in vivo, we cannot exclude significant responses to even lower, more physiological concentrations of the hormone in the intact Graafian follicle. Intact ovarian follicles are believed to be permeable to circulating peptides of considerably larger molecular weight than insulin (47). Furthermore, in maturing follicles, steroidogenesis is closely regulated by estrogen and gonadotropic hormones (46, 47), the latter presumably acting through the cAMP effector system (46). Our in vitro studies indicate that insulin effectively amplifies the stimulatory actions of estradiol and cyclic AMP. Thus, we suggest that insulin may play a critical role in maintaining and enhancing granulosa cell responsiveness to physiological stimulators of progesterone biosynthesis in maturing Graafian follicles. On the other hand, we have shown that insulin suppresses estrogen biosynthesis in vitro, which demonstrates the selectivity of insulin action on the progestin pathway.

The stimulatory actions of insulin could not be mimicked by desoctapeptide insulin, epidermal growth factor, fibroblast growth factor, or porcine relaxin. Although specific insulin binding sites have been reported in granulosa cells (28), the present studies do not prove that insulin works through the classical insulin receptor per se in ovarian cells. Studies by Veldhuis and Hammond (49) and Hammond et al. (50) indicate that granulosa-cell ornithine decarboxylase activity is responsive to certain insulinlike growth factors or somatomedins, such as MSA, which are present in high concentrations (~600 ng/ml [50]) in pig follicle fluid. Since insulin can bind to somatomedin receptors in various tissues (51), we would suggest the possibilities that: (a) insulin's trophic effects in granulosa cells are exerted via somatomedin receptors rather than insulin receptors, or (b) intraovarian somatomedins in vivo act in a manner similar to that observed for insulin in vitro. In this regard, May and Schomberg (52) have reported that in serum-replete cultures (containing other growth factors) MSA does not replace insulin in its facilitation of progesterone secretion. However, we have observed potent stimulatory effects of this growth factor in serum-free cultures, which suggests that insulin may act through somatomedin receptors. To test this hypothesis, we believe that more direct probes of the classical insulin receptor or its specific intracellular messengers will ultimately be required.

In summary, insulin selectively stimulates progesterone biosynthesis by granulosa cells. Insulin's actions are exerted via mechanisms that require protein and RNA synthesis to augment mitochondrial cytochrome P-450<sub>esc</sub> activity and, hence, the cell's ability to synthesize pregnenolone. These striking trophic effects of insulin indicate that the ovary must be considered a significant target organ for insulin action. Our in vitro studies may also shed light upon clinical observations in certain hyperinsulinemic states, in which high circulating concentrations of insulin are achieved chronically in association with a marked increase in ovarian steroid-hormone production (53, 54). In addition, the potent trophic actions of insulin on steroidogenesis permit us to suggest that profound insulinopenia may be linked plausibly to deficient ovarian function reported in poorly controlled diabetes mellitus (55-57).

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#### REFERENCES

1. Davis, M. E., N. W. Fugo, and K. G. Lawrence. 1947. Effect of alloxan diabetes on reproduction in the rat. *Proc. Soc. Exp. Biol. Med.* 66:638.
2. Shipley, E. G., and K. S. Danley. 1947. Pituitary and ovarian dysfunction in experimental diabetes. *Am. J. Physiol.* 150:84.
3. Lawrence, A. M., and A. V. Contopoulos. 1960. Reproductive performance in the alloxan diabetic female rat. *Acta Endocrinol.* 33:175.
4. Babbot, D., A. Rubin, and S. J. Ginsberg. 1958. The reproductive characteristics of diabetic men. *Diabetes.* 7:33-35.
5. Cicognani, A., F. Zappulla, F. Bernardi, M. Capelli, L. Mazzanti, S. Turchi, G. Radetti, P. Pirazzoli, and E. Cacciari. 1978. Hypophysio-gonadal function in the diabetic child. *Acta Paediatr. Scand.* 67:151-155.
6. Distiller, L. A., J. Sagel, J. E. Morley, B. I. Joffe, and H. C. Seftel. 1975. Pituitary responsiveness to luteinizing hormone-releasing hormone in insulin-dependent diabetes mellitus. *Diabetes.* 24:378.
7. Bergqvist, N. 1954. Gonadal function in female diabetics. *Acta Endocrinol.* 19:3-12.
8. Trzeciak, B. 1978. The effect of diabetes mellitus on the incidence of abnormal genital bleeding in women. *Ginekol. Pol.* 49:119-123.
9. Pinget, M., Y. Krummel, R. Gondar, and M. Dorner. 1979. Incidence de la maladie diabétique sur l'âge de survenue des premières règles. *Nouv. Presse Méd.* 44:3664-3668.
10. Djursing, H., H. C. Nyholm, C. Hagen, L. Carstensen, and L. M. Pedersen. 1982. Clinical and hormonal characteristics in women with anovulation and insulin-treated diabetes mellitus. *Am. J. Obstet. Gynecol.* 143:876.
11. Schoffing, K., K. Federlin, W. Schmitt, and E. F. Pfeiffer. 1967. Histometric investigations on the testicular tissue of rats with alloxan diabetes and Chinese hamsters with spontaneous diabetes. *Acta Endocrinol.* 54:335-346.
12. Kirchick, H. J., P. L. Keyes, and B. E. Frye. 1979. An explanation for anovulation in immature alloxan-diabetic rats treated with pregnant mare's serum gonadotropin: reduced pituitary response to gonadotropin-releasing hormone. *Endocrinology.* 105:1343-1349.
13. Cusan, L., A. Belanger, C. Seguin, and F. Labrie. 1980. Impairment of pituitary gonadal functions in alloxan-induced diabetic male rats. *Mol. Cell. Endocr.* 18:165-176.
14. Johnson, L. M., and R. L. Sidman. 1979. A reproductive endocrine profile in the diabetes (db) mutant mouse. *Biol. Reprod.* 20:552-559.
15. Platt, B. S., and R. J. C. Stewart. 1966. Experimental protein-calorie deficiency: histopathological changes in the endocrine glands of pigs. *Endocrinology.* 38:121-143.
16. Fraley, D. S., and R. S. Totten. 1968. An autopsy study of endocrine organ changes in diabetes mellitus. *Metab. Clin. Exp.* 17:896-900.
17. Stahler, E., H. O. Neumann, and K. M. Niethammer. 1975. Metabolism and ultrastructure in ovaries of al-

- loxan-diabetic juvenile rats. *Arch. Gynaekol.* 220:199-208.
18. Chanda, S., and C. Deb. 1977. Histochemical studies of  $\Delta^5$ - $3\beta$ -hydroxysteroid dehydrogenase in testis and adrenal cortex of alloxan diabetic rats. *Endokrinologie.* 69:361-364.
  19. Kirchick, H. J., P. L. Keyes, and B. E. Frye. 1982. Restoration of the LH surge and ovulation by insulin in alloxan-diabetic immature rats treated with pregnant mare's serum gonadotrophin. *Acta Endocrinol.* 100:266-273.
  20. Leaming, A. B., R. S. Mathur, and J. H. Levine. 1982. Increased plasma testosterone in streptozotocin-diabetic female rats. *Endocrinology.* 111:1329-1333.
  21. Djursing, H., H. C. Nyholm, C. Hagen, and L. Molsted-Pedersen. 1983. Decreased prolactin levels in diabetic women with anovulation. *Acta Obstet. Gynecol. Scand.* In press.
  22. Goodner, C. J., and N. Freinkel. 1961. Studies of anterior pituitary tissue in vitro. Effects of insulin and experimental diabetes mellitus upon carbohydrate metabolism. *J. Clin. Invest.* 40:261-272.
  23. Adashi, E. Y., A. J. W. Hsueh, and S. S. C. Yen. 1981. Insulin enhancement of luteinizing hormone and follicle-stimulating hormone release by cultured pituitary cells. *Endocrinology.* 108:1441-1449.
  24. Aidells, B. D., M. W. Konrad, and D. A. Glaser. 1979. Growth and morphology of colonies of Chinese hamster ovary cells growing on agar is affected by insulin. *Proc. Natl. Acad. Sci. USA.* 76:1863-1867.
  25. Johnson, C. C., W. E. Dawson, J. T. Turner, and J. H. Wyche. 1980. Regulation of rat ovarian cell growth and steroid secretion. *J. Cell Biol.* 86:483-489.
  26. Allen, W. R., M. Nilsen-Hamilton, and R. T. Hamilton. 1981. Insulin and growth factors stimulate rapid post-translational changes in glucose transport in ovarian granulosa cells. *J. Cell. Physiol.* 108:15-24.
  27. Saucier, J., J. Y. Dube, and R. R. Tremblay. 1981. Specific insulin binding sites in rat testis: characterization and variation. *Endocrinology.* 109:2220-2225.
  28. Rein, M. S., and D. W. Schomberg. 1982. Characterization of insulin receptors on porcine granulosa cells. *Biol. Reprod.* 26:113A. (Abstr.)
  29. Savion, N., G-M. Lui, R. Laherty, and D. Gospodarowicz. 1981. Factors controlling proliferation and progesterone production by bovine granulosa cells in serum-free medium. *Endocrinology.* 109:409-420.
  30. Adashi, E. Y., C. Fabrics, and A. J. W. Hsueh. 1982. Insulin augmentation of testosterone production in a primary culture of rat testicular cells. *Biol. Reprod.* 26:270-280.
  31. Veldhuis, J. D., P. A. Klase, and J. M. Hammond. 1980. Divergent effects of prolactin upon steroidogenesis by porcine granulosa cells in vitro: influence of cytodifferentiation. *Endocrinology.* 107:42-46.
  32. Veldhuis, J. D., P. A. Klase, and J. M. Hammond. 1981. Sex steroids modulate prolactin action in spontaneously luteinizing porcine granulosa cells in vitro. *Endocrinology.* 108:1463-1468.
  33. Veldhuis, J. D., P. A. Klase, and J. M. Hammond. 1981. Direct actions of  $17\beta$ -estradiol on progesterone production by highly differentiated porcine granulosa cells in vitro. II. Regulatory interactions of estradiol with luteinizing hormone and cyclic nucleotides. *Endocrinology.* 109:433-442.
  34. Toaff, M. E., J. F. Strauss III, G. L. Flickinger, and S. J. Shattil. 1979. Relationship of cholesterol supply to luteal mitochondrial steroid synthesis. *J. Biol. Chem.* 254:3777-3784.
  35. Omura, R., and R. Saito. 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its heme protein nature. *J. Biol. Chem.* 239:2370-2375.
  36. Toaff, M. E., H. Schleyer, and J. F. Strauss III. 1982. Metabolism of 25-hydroxy-cholesterol by rat luteal mitochondria and dispersed cells. *Endocrinology.* 111:1785-1790.
  37. Veldhuis, J. D., P. A. Klase, J. F. Strauss III, and J. M. Hammond. 1982. Facilitative interactions between estradiol and luteinizing hormone in the regulation of progesterone production by cultured swine granulosa cells: relation to cellular cholesterol metabolism. *Endocrinology.* 111:441-447.
  38. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-253.
  39. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-267.
  40. Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62:315-319.
  41. Winer, B. J. 1971. Statistical principles in experimental design. McGraw-Hill Book Co., New York. 196-210.
  42. Veldhuis, J. D., S. K. Sweinberg, P. A. Klase, and J. M. Hammond. 1981. Dissociated regulation of cellular progesterone secretion and cytosolic ornithine decarboxylase activity in isolated ovarian cells in vitro. *Endocrinology.* 109:1657-1662.
  43. Veldhuis, J. D., P. A. Klase, J. F. Strauss III, and J. M. Hammond. 1982. Mechanisms subserving estrogen's amplification of gonadotropin action. In *Factors Regulating Ovarian Function*. G. S. Greenwald and P. F. Terranova, editors. Raven Press, New York. 323-328.
  44. Mondschein, J. S., and D. W. Schomberg. 1981. Growth factors modulate gonadotropin receptor induction in granulosa cell cultures. *Science (Wash. DC).* 211:1179-1181.
  45. Mather, J. P., and G. H. Sato. 1979. Growth of mouse melanoma cells in hormone-supplemented, serum-free medium. *Exp. Cell Res.* 120:191-220.
  46. Catt, K. J., J. P. Harwood, R. N. Clayton, T. F. Davies, V. Chan, M. Katikineni, K. Dufau, and M. L. Dufau. 1980. Regulation of peptide hormone receptors and gonadal steroidogenesis. *Recent Prog. Horm. Res.* 36:557-622.
  47. Leung, P. C. K., and D. T. Armstrong. 1980. Interactions of steroids and gonadotropins in the control of steroidogenesis in the ovarian follicle. *Annu. Rev. Physiol.* 42:71-98.
  48. Strauss, J. F., III, L. A. Schuler, M. F. Rosenblum, and T. Tanaka. 1981. Cholesterol metabolism by ovarian tissue. *Adv. Lipid Res.* 18:99-127.
  49. Veldhuis, J. D., and J. M. Hammond. 1979. Multiplication stimulating activity regulates ornithine decarboxylase in isolated porcine granulosa cells in vitro. *Endocr. Res. Commun.* 6:229-309.
  50. Hammond, J. M., J. D. Veldhuis, T. W. Seale, and M. M. Rechler. 1982. Intraovarian regulation of granulosa-cell replication. *Adv. Exp. Med. Biol.* 147:341-356.

51. King, G. L., C. R. Kahn, M. M. Rechler, and S. P. Nissley. 1980. Direct demonstration of separate receptors for growth and metabolic activities of insulin and multiplication stimulating activity (an insulinlike growth factor) using antibodies to the insulin receptor. *J. Clin. Inv.* 66:130-140.
52. May, J. V., and D. W. Schomberg. 1981. Granulosa-cell differentiation in vitro: effect of insulin on growth and functional integrity. *Biol. Reprod.* 25:421-431.
53. Burghen, G. A., J. R. Givens, and A. E. Kitabachi. 1980. Correlation of hyperandrogenism with hyperinsulinism in polycystic ovarian disease. *J. Clin. Endocrinol. Metab.* 50:113-116.
54. Taylor, S. I., R. F. Dons, E. Hernandez, J. Roth, and P. Gorden. 1982. Insulin resistance associated with androgen excess in women with autoantibodies to the insulin receptor. *Ann. Int. Med.* 97:851-855.
55. Takeva, Z., and R. Kusheva. 1972. Histological and histochemical studies of ovaries of white rats treated with alloxan and those of women who died in diabetic coma. *Folia Histochem. Cytochem.* 10:325-328.
56. Amar, C. H., and C. Deb. 1966. Activity of corpora lutea in alloxan diabetic rats. A histochemical study. *Acta Anat.* 65:299-302.
57. Liu, F. T. Y., H. S. Lin, and D. C. Johnson. 1972. Serum follicle stimulating hormone, luteinizing hormone, and the ovarian response to exogenous gonadotropins in alloxan-diabetic immature female rats. *Endocrinology.* 91:1172-1179.