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The Role of Insulin-Like Growth Factor-I (IGF-I) and Estradiol in Rabbit Corpus Luteum Progesterone Production

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To determine whether insulin-like growth factor-I (IGF-I) plays a role in rabbit corpus luteum (CL) physiology the authors examined: IGF-I expression, the effect of IGF-I on progesterone (P) production in vitro, and the interaction of IGF-I with estradiol, the primary luteotropin in the rabbit. Northern blot analysis revealed that IGF-I mRNA is present in the rabbit CL throughout pseudopregnancy. An intact ovarian in vitro perfusion model and dispersed luteal cell culture were used to determine the effects of IGF-I on P production and interactions with estradiol. IGF-I significantly stimulated P production compared to control medium by the isolated, intact perfused rabbit ovary and by dispersed, cultured luteal cells. Estradiol alone did not stimulate P production in vitro. Estradiol did augment IGF-I-stimulated P production in the intact perfused ovary and in luteal cell culture. These findings support a role for IGF-I in rabbit CL P production.

Key Words: Corpus luteum (CL); insulin-like growth factor-I (IGF-I); progesterone (P); estradiol; rabbit.

Introduction

Progesterone (P) secretion by the corpus luteum (CL) is essential for the maintenance of early pregnancy in mammals *(1)*. If pregnancy does not occur, the timely regression of the CL is essential for ovulation to occur again *(1)*. Despite the obvious importance of the CL in reproductive function, major deficits remain in our knowledge of the mechanisms that control P secretion, CL maintenance and regression.

Whereas luteotropins such as luteinizing hormone, chorionic gonadotropin, and estradiol are essential for CL maintenance, there is a growing body of evidence supporting paracrine and autocrine modulation of ovarian function by peptide growth factors such as insulin-like growth factor-I (IGF-I) *(2,3)*. Thus far, few studies have investigated the role of IGF-I in the regulation of CL function and its possible interactions with luteotropic hormones.

IGF-I is a 70 amino acid polypeptide hormone that affects cell growth, differentiation, and metabolism throughout the body *(4,5)*. Its structure is highly conserved among diverse mammalian species. Most studies of ovarian IGF-I have focused on its production, secretion, and action in follicular granulosa cells. Recent studies have shown that human, rabbit, and rat CL contain receptors for IGF-I *(6–10)*. Constantino et al. *(11)* demonstrated that IGF-I stimulates rabbit luteal cell P production in cell culture. Although estradiol is the primary luteotropin in the rabbit CL *(12)*, estradiol alone may not be sufficient for P secretion. It is possible that IGF-I may interact with estradiol to stimulate CL P production. Constantino et al. *(11)* did not demonstrate an interaction with estradiol and could not demonstrate the presence of estradiol receptors. In contrast, Miller et al. *(13)* found that IGF-I could stimulate P production by rabbit luteal cells only in the presence of estradiol. Thus, whereas IGF-I appears to play a role in CL function, the relation between IGF-I and the luteotropin estradiol is still unclear.

The objectives of this study were to determine if the rabbit CL expresses IGF-I and to investigate the interaction of IGF-I with estradiol in the regulation of CL function.

Results

IGF-I Gene Expression During Pseudopregnancy

To determine if the rabbit CL expresses IGF-I, total RNA was isolated from CL on days 4, 7, 11, and 18 of pseudopregnancy and analyzed using the Northern blot technique. Using a heterologous 400 bp cDNA probe against rat IGF-I mRNA *(14)*, a single band was identified at 7.5 kb on all days studied (Fig. 1), demonstrating that the rabbit CL expresses IGF-I mRNA throughout pseudopregnancy. Reprobing the blot under the same conditions for beta actin demonstrated equal loading between lanes and RNA

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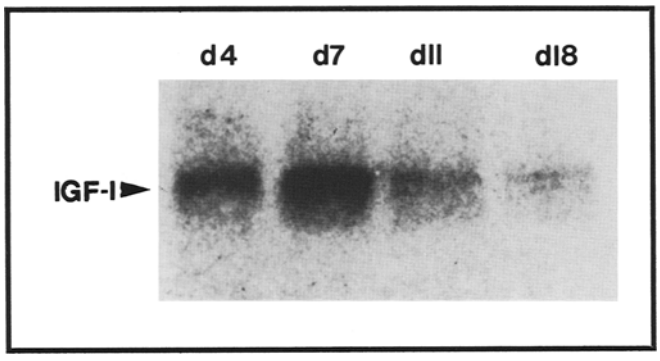

Fig. 1. Northern blot analysis of IGF-I mRNA expression by the rabbit CL throughout pseudopregnancy. Total RNA was isolated from corpora lutea on days 4, 7, 11, and 18 of pseudopregnancy. The blot was prepared as described in Materials and Methods.

integrity (not shown). Beta actin mRNA expression did not change during CL development and regression, whereas IGF-I mRNA expression is greater on days 4, 7, and 11 than in the regressed CL on day 18 (Fig. 1).

Effect of IGF-I and Estradiol on Progesterone Secretion

To determine if IGF-I stimulates P secretion, two different in vitro systems were used: the isolated, intact, perfused rabbit ovary and dispersed luteal cells in culture.

Ovaries were isolated on day 7 of pseudopregnancy and perfused for 6 h with tissue culture medium alone (control), estradiol, IGF-I, or estradiol and IGF-I together (Fig. 2A). Estradiol did not stimulate P secretion compared to control media. IGF-I alone did. IGF-I and estradiol together significantly stimulated P secretion over that seen with IGF-I alone.

Similar results were obtained when the experiment was repeated using dispersed, cultured luteal cells. CL were harvested on day 7 of pseudopregnancy. Luteal cells were dispersed as previously described *(15)* and plated for 24 h in 10% fetal calf serum. Media were then replaced with serum-free media containing estradiol, IGF-I, or estradiol and IGF-I together for 24 h. Results are shown in Fig. 2B. Estradiol did not stimulate P secretion compared to control media. IGF-I alone did. IGF-I and estradiol together significantly stimulated P secretion over that seen with IGF-I alone.

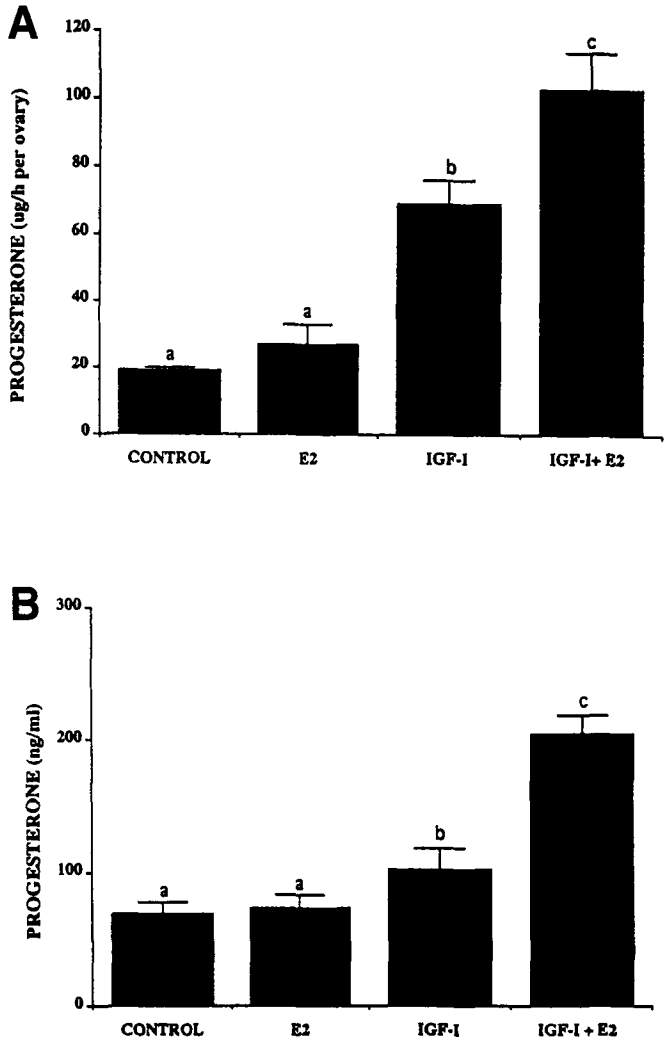

Fig. 2. (A) Effect of estradiol and IGF-I on P secretion rates by in vitro perfused rabbit ovaries. Rabbits underwent laparotomy on day 7 of pseudopregnancy. Ovaries were isolated and prepared as described in Materials and Methods. One ovary from each rabbit was perfused with either estradiol, IGF-I or estradiol and IGF-I together. The contralateral ovary, serving as the control, was perfused with medium alone. Values shown are mean calculated P secretion rates ±SEM. Different letters indicate significant differences between groups ($p < 0.05$). **(B)** Effect of estradiol and IGF-I on P accumulation in luteal cell-conditioned media. CL were harvested on day 7 of pseudopregnancy. Luteal cells were prepared as described in Materials and Methods. Cells were treated with serum-free media containing estradiol, IGF-I, or estradiol and IGF-I together for 24 h. Values shown are mean P levels in the conditioned media ±SEM. Different letters indicate significant differences ($p < 0.05$).

Discussion

CL are the primary source of P during pregnancy and pseudopregnancy in the rabbit. P production is essential for the initiation and maintenance of pregnancy in mammals. Estradiol produced by the ovarian follicles is the primary luteotropin in the rabbit CL and essential for the maintenance of P production *(12)*. In the rat, estradiol formed from ovarian and placental androgens is the primary luteotropin during midpregnancy *(16)*. How estradiol maintains CL steroidogenesis in these species is still unknown. The mechanism may involve autocrine or paracrine factors such as IGF-I. Although endocrine regulation of ovarian function has been well documented, it is only in recent years that evidence has emerged to support a role for autocrine factors such as IGF-I in the ovary *(3)*. Whereas the role of IGF-I in the regulation of preovulatory follicular development has been studied extensively *(2,3,5)*, the role of IGF-I in CL function has received relatively little attention. Recently, IGF-I and IGF-I receptors have been found in the CL of several mammalian species, including humans *(7–10,17)*.

This study demonstrates that the rabbit CL of pseudopregnancy expresses message for IGF-I throughout its lifespan. Although IGF-I mRNA expression was not quantitated, qualitative assessment demonstrates greater expression on days 4, 7, and 11 when the CL is actively secreting P than on day 18 when the CL has almost completely regressed. Only a single species of mRNA was found, in contrast to other studies which have demonstrated several bands on Northern analysis *(8)*. This may have occurred because a heterologous probe was used that could not identify variant mRNA species in the rabbit or because only one type of transcript is produced in the rabbit. Thus far, the rabbit IGF-I gene has not been cloned. In the chicken only one species of mRNA transcript has been identified *(18)*.

Previously, Dharmarajan et al. *(19)* have demonstrated that the isolated, intact, in vitro perfused rabbit ovary is a useful model for the study of P secretion by the rabbit CL. Using ovaries from pseudopregnant rabbits, P secretion by perfused rabbit ovaries steadily increases from day 0 to 11 of pseudopregnancy. Ovaries taken from rabbits on day 11 of pseudopregnancy secrete maximal amounts of P *(19)*. After day 11, perfused ovaries secrete decreasing amounts of P until baseline levels are reached by day 18. This pattern of secretion mirrors the pattern in vivo *(20–22)*. Day 7 of pseudopregnancy was chosen for these experiments in order to take advantage of high, but not maximal, P secretion rates. In addition, by day 7 the rabbit CL is dependent upon estradiol for its maintenance and the number of estradiol receptors are near maximal in vivo *(23)*, allowing us to study the interactions of estradiol and IGF-I.

This study also demonstrates that IGF-I stimulates P production by rabbit CL in intact in vitro perfused ovaries as well as dispersed luteal cells in culture. The authors have found that estradiol potentiates IGF-I-stimulated P synthesis by the CL, but estradiol alone cannot stimulate P production in vitro. Although estradiol is the ultimate luteotropin in the rabbit CL and is the only required factor to maintain CL function in vivo even in hypophysectomized rabbits *(24)*, it has been difficult to demonstrate a direct effect on P production in vitro *(25)*. It is only in an estrogen-deprived model that a direct effect has been demonstrated *(25)*. The requirement for autocrine or paracrine factors may explain the differences in the effects of estradiol between the in vivo and in vitro settings. It is possible that estradiol regulates steroid production via IGF-I, which may act as a mediator, or that IGF-I modulates the effects of estradiol upon the CL, perhaps by inducing estradiol receptors. This interaction between IGF-I and estradiol may parallel that seen in the uterus, where IGF-I is thought to act as an "estromedin" *(26)*. Although Constantino et al. *(11)* found that IGF-I could stimulate P production by dispersed rabbit luteal cells, they did not demonstrate any effects of estradiol alone or in combination with IGF-I, and could not demonstrate the presence of estradiol receptors in their rabbit luteal cell culture system. However, estradiol receptors have been demonstrated in the rabbit CL in vivo *(27)*. It is possible that prolonged culture (up to 7 d) of these cells caused a loss of estradiol receptors. Conditions in our studies may have maintained estradiol receptors. In the perfusion experiments, CL were left *in situ* in the ovary and perfused within 6 h of laparotomy. In the cell culture experiments, luteal cells were cultured for only 24 h in serum-free media, in contrast to Constantino's study in which cells were cultured for 4–7 d in serum-free media.

In this study, effects of IGF binding proteins were not looked at. IGF binding proteins were initially thought to maintain a storage pool for IGF-I. It is now known that this complex system of interacting proteins can modify and regulate IGF-I actions and are, in turn, regulated by many other factors *(3)*. Clearly, the role of binding proteins in the CL must be investigated.

In summary, rabbit CL express IGF-I mRNA throughout pseudopregnancy. IGF-I stimulates luteal P production in vitro in the intact, perfused ovary and by dispersed, cultured luteal cells. Estradiol, the primary luteotropin in this species, does not directly stimulate P production, but potentiates IGF-I-stimulated P production. These data support a role for IGF-I in the regulation of CL function and suggest an autocrine component to the previously demonstrated luteotropic effects of estradiol. Our data add to the growing body of literature supporting the crucial role of local regulation of ovarian function and demonstrate the need for further study.

Materials and Methods

Animals

Sexually mature New Zealand White rabbits weighing an average of 3.5 kg were housed individually under controlled temperature and light and given free access to food (Purina Rabbit Chow, Ralston Purina Co., St. Louis, MO) and water. Rabbits received 100 IU of human chorionic gonadotropin (hCG; Organon, West Orange, NJ) via the marginal ear vein to induce pseudopregnancy. The day of injection was defined as day 0 of pseudopregnancy. On the appropriate day of pseudopregnancy, rabbits were anesthetized with intravenous sodium pentobarbital (32 mg/kg), anticoagulated with heparin sulfate (120 U/kg) and then subjected to laparotomy. In the rabbit, pseudopregnancy lasts for 21 d with P secretion increasing from days 1 to 11 and declining to baseline levels by day 18 *(19–21)*. All protocols were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee.

Northern Hybridization

Rabbits were subjected to laparotomy on days 4, 7, 11, and 18 of pseudopregnancy. Corpora lutea were carefully separated from ovaries using microforceps, snap frozen, and stored at −70°C for later RNA extraction. Total RNA was isolated from CL using the method of Cathala et al.

(28). Ten micrograms of total RNA were loaded in each lane and resolved by agarose gel electrophoresis. RNA was then transferred to nitrocellulose paper and the blot was hybridized with a 400 bp rat IGF-I cDNA probe kindly provided by Dr. Charles Roberts of the NIH *(14)*. Autoradiography was performed by sealing membranes in plastic wrap and exposing to Kodak X-Omat films (Eastman Kodak, Rochester, NY) at –70°C. After probing for IGF-I mRNA, each blot was stripped and reprobed with a radiolabeled cDNA probe for beta actin under the same conditions to confirm equal loading between lanes and RNA integrity.

In Vitro Ovarian Perfusion

The cannulation procedure and perfusion apparatus have been described in detail previously *(19,29)*. On day 7 of pseudopregnancy rabbits underwent laparotomy as described above. After major anastomotic vessels were ligated, the ovarian artery and vein were cannulated *in situ*. The ovary with its cannulated vessels was then removed and immediately placed in the perfusion chamber. The perfusion apparatus consists of a chamber containing the ovary, an oxygenator, a reservoir, and a pump that maintains perfusate flow at 1.5 mL/min, which approximates the normal rate of blood flow to the rabbit ovary *(30)*. Ovaries were perfused at 37°C with 150 mL of medium 199 (Gibco, Grand Island, NY), pH 7.4, supplemented with 3% bovine serum albumin (BSA; Intergen, Purchase, NY), heparin sulfate (200 U/L), streptomycin sulfate (50 mg/L), and penicillin G (75 mg/L). Experimental ovaries were perfused with either 5 ng/mL estradiol (Sigma, St. Louis, MO) or 75 ng/mL IGF-I (Intergen) or IGF-I (75 ng/mL) and estradiol (5 ng/mL) together. The dose of estradiol used is the minimum dose required to elicit maximal P production in estrogen-suppressed ovaries *(25)*. The dose of IGF-I was chosen after examining the literature on in vitro studies *(8,9,11,13,31–33)*. Contralateral control ovaries were perfused with medium alone. One mL samples were obtained from the arterial and venous cannulae every half hour for the first 2 h and thereafter every hour for a total of 6 h. Samples were replaced with fresh medium to maintain perfusate volume. After collection, samples were stored at –20°C until assayed for P by radioimmunoassay (RIA). Ovarian P secretion was calculated by dividing the mean of the differences in concentration between perfusate venous and arterial samples by the perfusion time.

Luteal Cell Culture

On day 7 of pseudopregnancy, rabbits underwent laparotomy as described above. Luteal cells were dispersed as previously described *(15)*. Briefly, ovaries were removed aseptically. CL were dissected from each ovary and placed in medium 199 without phenol red, with Earles Balanced Salt Solution, 2.2 mg/mL sodium bicarbonate, 100 mg/mL L-glutamine, 25 m*M* HEPES, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate, hereafter referred to as medium 199. After weighing, the CL were cut into approx 1 cubic mm pieces and placed in a dissociation medium (1 mL/75 mg tissue) containing 250 U/mL collagenase (Worthington, Freehold, NJ) and 2% BSA in medium 199. Tissue was stirred at 37°C for 5 min and pipet-aspirated to facilitate dissociation. Dispersed cells were removed and centrifuged at 200*g* for 5 min and the pellet resuspended in medium 199 with 10% fetal calf serum. Collagenase treatment was repeated on the undigested tissue three to four times. Cells were pooled, counted, and cell viability assessed using trypan blue exclusion.

Dispersed luteal cells were plated in 24-well culture plates (250,000 cells/well) in 1 mL of medium 199 with 10% fetal calf serum (Gibco) for 24 h. Medium was then replaced with fresh serum-free medium 199 with 0.1% insulin-free BSA (Sigma). Cells were treated with estradiol (5 ng/mL), IGF-I (75 ng/mL), or estradiol (5 ng/mL) and IGF-I (75 ng/mL) together for 24 h. Cells were cultured at 37°C in an atmosphere of 95% air and 5% CO_2. Media were removed after 24 h and stored at –20°C until assayed for P by RIA.

Progesterone Radioimmunoassay

P levels in arterial and venous perfusate samples and cell culture-conditioned media were measured using a commercial RIA kit (Diagnostic Products, Los Angeles, CA) in which the P antibody is bound covalently to the inner surface of polypropylene assay tubes. All samples and P standards (100 μL) were assayed in duplicate. Samples from each experiment were assayed simultaneously to avoid error due to interassay variation. The intra-assay variation was 6.6%.

Statistical Analysis

Analysis of variance (ANOVA) was used to evaluate differences in means among the experimental groups. Multiple comparisons between groups was performed using the Student-Newman-Keuls test. A p value of <0.05 was considered significant.

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