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A ROLE FOR SOMATOMEDIN C AS A DIFFERENTIATING HORMONE AND AMPLIFIER OF HORMONE ACTION ON OVARIAN CELLS: Studies with synthetically pure human somatomedin C and swine granulosa cells

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Isolated swine granulosa cells incubated in chemically defined medium in vitro responded to synthetically pure human somatomedin C/IGF-I in a dose and time-dependent fashion with increased pregnenolone, progesterone and estradiol biosynthesis. These stimulatory actions were not mimicked by growth hormone, proinsulin, desoctapeptide insulin, epidermal growth factor, or fibroblast growth factor. Moreover, somatomedin C/IGF-I augmented the steroidogenic response of granulosa cells to exogenously supplied sterol substrate in the form of low-density lipoprotein, and amplified the stimulatory actions of the classical ovarian effector hormones, estradiol and follicle-stimulating hormone, in a synergistic fashion.

The ability of somatomedin C/IGF-I to stimulate estradiol production on the one hand, and to act synergistically with estradiol to stimulate progesterone biosynthesis on the other hand, suggests a unique intrafollicular mechanism for amplifying progestin biosynthetic capacity in granulosa cells.

Growth-promoting actions of somatomedin C (insulin-like growth factor I, IGF-I) on mesenchymal tissues are well recognized, but differentiating effects of this insulin-like peptide on epithelial cells have been investigated less extensively. Moreover, although IGF-I is structurally distinct from insulin and IGF-II (1,2), studies that have attempted to test direct actions of these insulin-like peptides on cytodifferentiation have been hampered to date by the lack of availabliliy of entirely pure preparations. In the present work, we

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have utilized pure human somatomedin C/IGF-I synthesized by recombinant DNA techniques to investigate actions of this important growth factor on the differentiated function of ovarian epithelial (granulosa) cells.

Methods

Granulosa cells were isolated from ovaries of immature (55-65 kg) swine by fine-needle aspiration of small and medium-sized follicles, as described earlier (3). Approximately $2X10^6$ viable cells, as determined by the exclusion of trypan blue, were plated in quadruplicate cultures (plastic multiwells, Falcon Plastics, Los Angeles, CA). Culture fluid consisted of Eagle's Minimul Essential Medium (Gibco, Grand Island, NY), buffered with bicarbonate in the absence of serum. Cultures were maintained for the indicated times at 37C in an atmosphere of 95% air and 5% CO_2 (3).

At designated intervals, culture medium and/or cells were removed for the subsequent extraction and RIA of progesterone, or pregnenolone, as described (3). Hormones or sterols were dissolved in saline or ethanol (<0.1% vol/vol).

Statistical Analyses

Data are presented as means $\pm$ SEM. Analysis of variance with Duncan's multiple range test was used to assess statistical significance. In addition, each experiment ws performed at least twice with a separate batch of 250-300 ovaries to test the reproducibility and generality of results.

Materials

Culture fluid was from Gibco (Grand Island, NY), and 25-hydroxycholesterol from Sigma. The somatomedin C used was prepared by recombinant DNA synthesis at Amgen Biologicals (Thousand Oaks, CA), and subsequent purity assessed by high-performance liquid chromatography, polyacrylamide gel electrophoresis, and N-terminal sequencing. This material differs from naturally occurring human somatomedin C/IGF-I only in the substitution of a methionine for threonine residue in position 59.

Results and Discussion

Addition of 1-100 ng/ml (0.13-13 nM) pure somatomedin C increased granulosa-cell progesterone secretion in a time and dose-dependent manner (Figure 1). Somatomedin C augmented progesterone production significantly after 48 hrs and even more prominently after 96 hrs of treatment. These effects were not attributable to increased cell proliferation, since the DNA content of the cultures was not altered significantly by pure somatomedin C (66 ± 3 µg DNA/control culture, and 56 ± 5 µg DNA/culture after treatment with 100 ng/ml somatomedin C). Moreover, somatomedin C (30 ng/ml) significantly enhanced the production of progesterone corrected per million cells: viz., from 18.5 ± 4.5 ng progesterone/10^6 cells · 48 hrs basally to 77 ± 7 ng progesterone/10^6 cells · 48 hrs ($P < 0.01$). These effects were not mimicked by growth hormone, proinsulin. desoctapeptide insulin, or epidermal or fibroblast growth factors (data not shown). Our results in pig granulosa

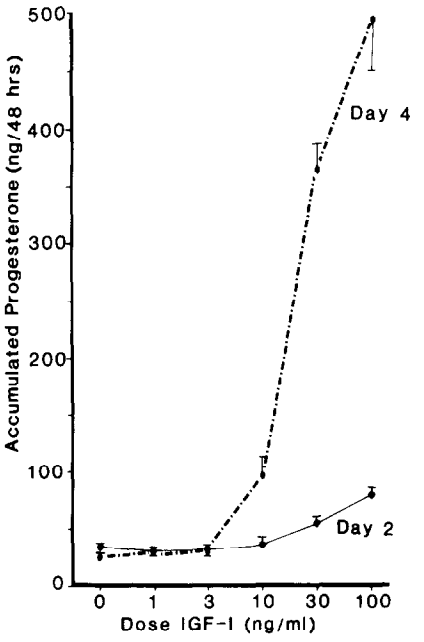

Figure 1: Swine granulosa cells were cultured under serum-free conditions (Eagle's minimum essential medium) for the indicated times in the presence or absence of increasing doses of somatomedin C/IGF-I (insulin-like growth factor, Type I). At 48 hrs the culture medium was removed for the subsequent assay of progesterone. Medium was replenished and the cultures harvested at 96 hours. At harvesting, the total content of progesterone in cells combined with medium was determined. Data represent means ± SEM (N = 4 cultures).

cells using synthetically pure somatomedin C differ from those in rat granulosa cells, where highly purified human somatomedin C failed to enhance basal steroid secretion (4). This may reflect species differences or properties of the somatomedin C preparation.

The stimulatory action of somatomedin C/IGF-I was antagonized > 97% by treatment with aminoglutethimide (1 mM), an inhibitor of cholesterol side-chain cleavage, indicating that the effect of somatomedin C was to promote de novo steroidogenesis rather than simply release previously synthesized steroid. This inference was confirmed by demonstrating that somatomedin C/IGF-I effectively amplified the biosynthesis of progesterone's precursor, pregnenolone, which was measured in the presence of trilostane to block further steroid metabolism. Enhanced pregnenolone synthesis occurred in a time-dependent fashion that parallelled increased progesterone production (Figure 2A, B).

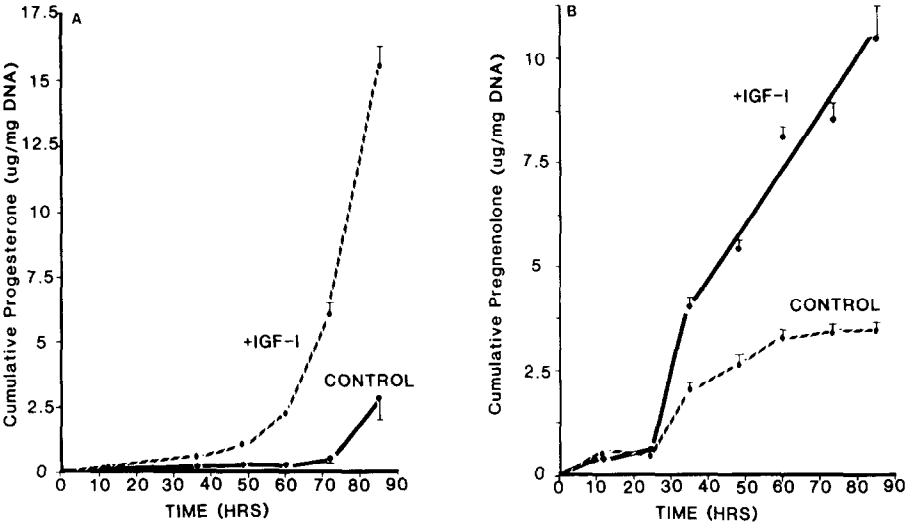

Figure 2: Swine granulosa cells were cultured with somatomedin C/IGF-I (30 ng/ml) in the absence (Panel A) or the presence of trilostane (Panel B, to inhibit pregnenolone metabolism). At the indicated times, medium was removed for the subsequent measurement of progesterone (Panel A) or pregnenolone (Panel B) concentrations by RIA. Data are given as presented in the legend of Figure 1, corrected for the DNA content of individual cultures.

Somatomedin C/IGF-I also significantly increased the aromatizing capacity of granulosa cells, i.e. augmented their ability to synthesize estradiol, a key steroidal product of the developing ovarian follicle. When granulosa cells were provided testosterone as substrate for the aromatization reaction, somatomedin C effectively stimulated estradiol synthesis at all substrate concentrations, including those maximally effective in enhancing estradiol production (Figure 3). The stimulatory effects of somatomedin C were specific to steroidogenesis in that this peptide did not increase the secretion of various prostaglandins (e.g. PGE_2 and $PGF_{2\alpha}$, data not shown).

Somatomedin C/IGF-I was also capable of markedly increasing the responsiveness of granulosa cells to low-density lipoprotein, to which granulosa cells become exposed after ovulatory rupture of the mature Graafian follicle (5,6). Treatment of granulosa cells with both somatomedin C and human LDL (10 μg/ml) augmented progesterone production by approximately 100-fold above the basal rate of steroidogenesis (Fig. 4A). This synergistic interaction between somatomedin C and LDL is similar to that observed in the

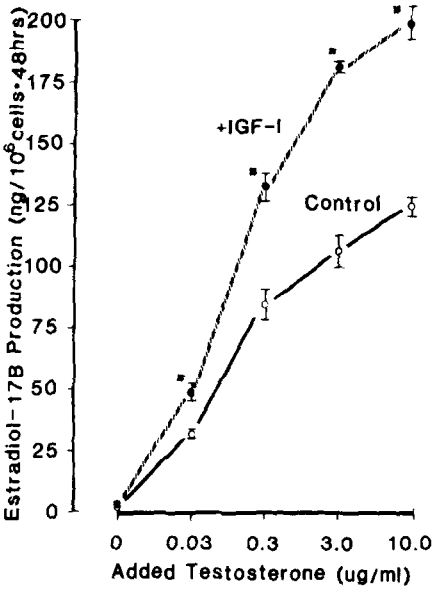

Figure 3: *Granulosa cells isolated from large pig follicles were exposed to control solvent or somatomedin C/IGF-I (50 ng/ml), with or without increasing concentrations of exogenously supplied testosterone as substrate for the aromatization reaction. The subsequent total (cells combined with medium) content of estradiol in the cultures was measured at 48 hrs. In these experiments, each culture contained one million granulosa cells. Data are means ± SEM (N = 4) from 2 experiments.*

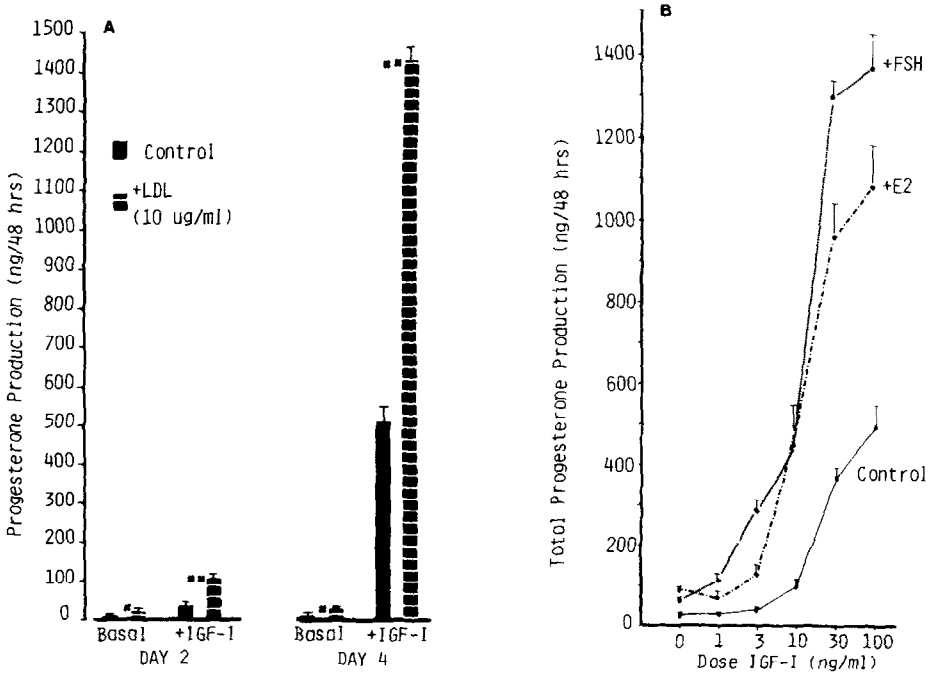

Figure 4, Panel A: Somatomedin C/IGF-I markedly amplifies the responsiveness of swine granulosa cells to low-density lipoprotein. Granulosa cells were cultured for 2 or 4 days in the presence or absence of human low-density

case of insulin, although approximately 50-fold higher concentrations of insulin are required (7).

The facilitative effects of somatomedin C on progesterone synthesis by swine granulosa cells were enhanced further by classical effectors of differentiated ovarian function, viz. estradiol and follicle stimulating hormone (8) (Figure 4B). Treatment with estradiol or follicle stimulating hormone amplified somatomedin C's dose-dependent stimulation of progesterone biosynthesis markedly ($P < 0.001$). The latter synergism between pure somatomedin C and FSH corroborates a similar observation in rat granulosa cells using highly purified human plasma-derived somatomedin C (3).

The physiological relevance of these observations is suggested by our recent observations that somatomedins are present in high concentrations within the swine ovarian follicle (9). Moreover, human somatomedin C can bind with high affinity (estimated Kd $\sim$0.69 nM) and specificity to pig granulosa cells (10). Accordingly, we suggest that this insulin-like growth factor may act in a pivotal and coordinate fashion with other ovarian hormones to effect differentiative changes in granulosa cells within developing Graafian follicles.

The present work suggests an interesting and novel intrafollicular role for somatomedin C. Thus, somatomedin C enhances the capacity of granulosa cells to synthesize estradiol, while estradiol in turn acts synergistically with somatomedin C to increase progesterone production. Moreover, estradiol itself may stimulate somatomedin production by granulosa cells (9). These

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lipoprotein (LDL, 10 µg/ml), with or without concomitant administration of somatomedin C/IGF-I (50 ng/ml). The subsequent total content of progesterone in cells combined with medium was assayed. These treatment conditions did not significantly alter the cell number or protein or DNA content of the cultures (N = 3 experiments).

Figure 4, Panel B: Somatomedin C/IGF-I interacts synergisticaly with estradiol (E_2) and follicle-stimulating hormone (FSH) in stimulating progesterone biosynthesis by granulosa cells. Granulosa cells were treated with the indicated doses of somatomedin C/IGF-I for 96 hours, in the presence or absence of a maximally effective concentration of estradiol (1 µg/ml) or follicle-stimulating hormone (200 ng/ml). The subsequent accumulation of progesterone was measured in cells combined with medium. The interaction among the hormonal treatments was highly significant by analysis of variance ($P < 0.001$).

interactions suggest unique intra-follicular mechanisms for amplifying maximal rates of progesterone biosynthesis in the later stages of follicle maturation, when intraovarian estradiol concentrations rise markedly (5). Finally, since somatomedin C enhances the responsiveness of ovarian cells to lipoprotein-carrried cholesterol substrate, this insulin-like growth factor would prepare granulosa cells to utilize the large quantities of sterol substrate that are needed (and become available) for progesterone biosynthesis after ovulation (5,6).

Acknowledgements

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