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MECHANISMS SUBSERVING INSULIN ACTION IN THE GONAD: EVIDENCE THAT INSULIN INDUCES SPECIFIC PHOSPHORYLATION OF ITS IMMUNOPRECIPITABLE RECEPTOR ON OVARIAN CELLS

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To test the capacity of insulin to work through a classical insulin-receptor pathway in the ovary, cultured swine granulosa cells were treated with insulin and/or increasing concentrations of insulin-receptor antiserum. Insulin-receptor antiserum but not control serum significantly (> 85%) attenuated insulin's stimulation of progesterone biosynthesis. Moreover, in broken-cell preparations, insulin but not desoctapeptide insulin or somatomedins induced specific phosphorylation of the 95,000-dalton, immunoprecipitated beta subunit of the insulin receptor on ovarian cells. These observations provide the first evidence for discrete biochemical actions of insulin at the level of the cell-membrane receptor for insulin in gonadal cells.

Insulin has been recently recognized to exert significant trophic effects on gonadal cells in vitro (1-6). For example, in cultured swine ovarian cells, insulin augments the biosynthesis of steroid hormones by 3-20-fold, and induces a corresponding increase in the cellular content of cytochrome P_{450} associated with cholesterol side-chain cleavage (6). The magnitude of these differentiative actions of insulin in the ovary is as striking as that observed for classical hormonal effectors in this tissue, such as follicle stimulating hormone or estradiol. However, at present the exact biochemical mechanism(s) subserving such trophic effects of insulin on ovarian cells are not known. For example, there is as yet no direct evidence that insulin can work through an insulin-receptor pathway in the ovary. This question is particularly important, because concentrations of insulin that stimulate

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granulosa cells in vitro might act via receptors for insulin-like growth factors or somatomedins (6).

To determine whether insulin can exert specific biochemical actions through the classical insulin receptor, we have investigated: (1) the ability of insulin-receptor antibody to inhibit insulin's stimulation of progesterone biosynthesis by cultured swine granulosa cells , and (2) the capacity of insulin to induce specific phosphorylation of the immunoprecipitable 95,000-dalton beta subunit of the solubilized ovarian insulin receptor.

Methods

Cell cultures: Swine granulosa cells were harvested from 1-5 mm follicles of immature ovaries, as previously described (7). The washed cells were cultured for 48 hours in serum-free Eagles' Minimum Essential Medium (Gibco Laboratories, Grand Island, N.Y.) to assess the stimulatory effects of insulin (highly purified porcine insulin, Nova Laboratories, Wilton, CT), and the influence of insulin-receptor antiserum (B-9 of C.R. Kahn). After extraction in hexane, the progesterone content of cells combined with medium was measured by radioimmunoassay (7).

For phosphorylation studies, granulosa cells were initially cultured for 48 hours in the presence of 3% fetal calf serum, and then transferred to serum-free medium devoid of hormones for 24 hours before being mechanically harvested and washed in saline. The subsequent cell pellets were solubilized in 1% Triton X-100, exactly as described for the rat adipocyte system (8). The receptor content of this broken-cell preparation was partially purified by wheat-germ agglutinin chromatography (8). Before phosphorylation, the partially purified receptor preparation was preincubated for 30 minutes at 4°C with the desired hormones: insulin, multiplication stimulating activity (Collaborative Research, Inc., Waltham, MA), or human somatomedin C (isolated from Cohn fraction IV-1 as previously described (9) to yield a preparation that was > 80% pure). The subsequent phosphorylation reaction was begun by addition of 75 µl of 25 µM (γ ^{32}P) ATP (66.7 µCi/nmol) and 10 mM $MnCl_2$ and the reaction was continued for 20 min at 4°C. Phosphorylation was stopped by the addition of 150 µl of a solution containing 40 mM Hepes buffer, pH 7.6, and 0.4% Triton X-100, 20 mM EDTA, 200 mM KF, 100 mM ATP, 20 mM sodium pyrophosphate, and 40 mM sodium phosphate. Each fraction was incubated with human serum containing insulin-receptor antibody (from Dr. G. Bogen, 1:200 dilution) and with 5 µl of 0.2 M phenylmethylsulfonyl fluoride for 18 h at 4°C. 250 µl of Protein A (Pansorbin, 10% w/v) was added to each tube and tubes further incubated at 4°C for 2.0 hr. The precipitates were then sedimented by centrifugation at 3,000 X g for 5 min. at 4°C and washed twice wih 50 mM Hepes buffer, pH 7.6, containing 0.1% Triton X-100, and then once with 50 mM Hepes buffer, pH 7.6. The immunoprecipitates were solubilized by adding 100 µl of a solution that contained 40 mM Hepes buffer, pH 7.6, 2% $NaDodSO_4$, 5% 2-mercaptoethanol, 10% glycerol, 0.1% bromothenol blue, and 0.1 M dithiothretol, followed by boiling for 10 min.

Sodium dodecyl sulfate-gel electrophoesis was performed according to Laemmli using 4% stacking and 6% resolving gels (10). After electrophoresis, the slab gels were stained with Coomassie blue, destained, dried, and autoradiographed with Kodak XAR-5 film for 3 days at -70°C. Autoradiogram bands were also quantitated by densitometry (8).

Results

As shown in Figure 1, swine granulosa cells maintained for 48 hours under serum-free conditions in vitro were highly responsive to insulin. Moreover, the stimulatory effects of insulin on progresterone production were significantly antagonized by increasing concentrations of insulin receptor antibody. At dilutions of 1/150 and 1/50, this antiserum inhibited the stimulatory effect of insulin by approximately 85-90%. Dilutions of control female serum were ineffective, and neither control nor experimental antisera (1/10,000 - 1/50) significantly influenced basal progesterone production (not shown.)

In broken-cell preparations, the addition of concentrations of insulin (0.05 μM and 0.5 μM) that are respectively submaximally and maximally effective in stimulating progesterone biosynthesis by monolayer cultures of swine granulosa cells (6) significantly increased phosphorylation of an immunoprecipitable, 95,000-dalton polypeptide resolved on sodium dodecyl sulfate-gel electrophoresis (Fig. 2). By densitometry, the increases were 2.3 and 5.1-fold, respectively, for 0.05 μM and 0.5 μM insulin doses. The specificity of the immunoprecipitation reaction was attested by the absence of

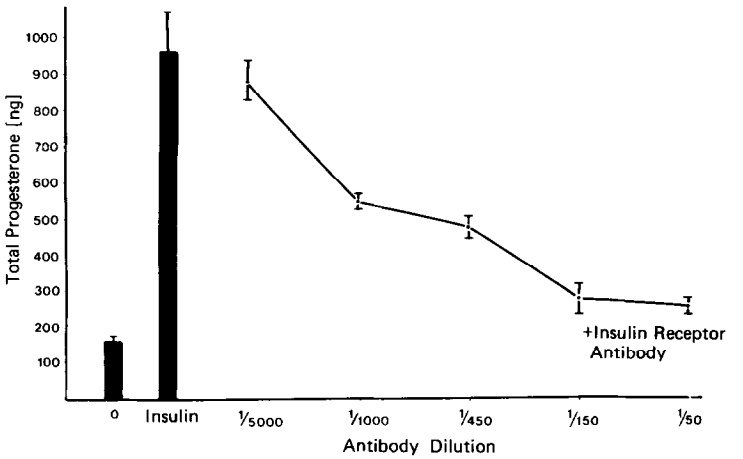

Figure 1. Capacity of insulin-receptor antibody to inhibit insulin's stimulation of progesterone production in serum-free cultures of swine ovarian cells.

Granulosa cells ($3x10^6$) were cultured for 48 hours without (denoted "0") or with insulin (0.05 μM) and increasing concentrations of insulin receptor antibody. Data are means ± SEM (N = 4 cultures) total progesterone in ng/48 hours measured in cells combined with medium.

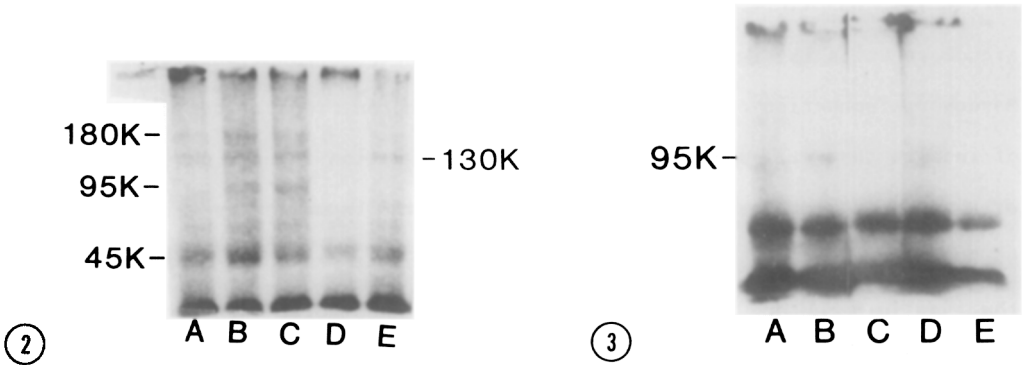

Figure 2. Specific phosphorylation of the insulin receptor by insulin but not multiplication stimulating activity (MSA) or desoctopeptide insulin in swine granulosa cells.

Autoradiograms of SDS-polyacrylamide gels of the immunoprecipitated insulin receptor were obtained after the following preincubations: Lane A: Control, Lane B: 0.05 μM insulin, Lane C: 0.5 μM insulin, Lane D: 1.0 μg/ml MSA, Lane E: 0.3 μg/ml desoctapeptide insulin. We observed a 95,000-dalton band in the presence of both doses of insulin (Lanes B and C), but not in the control (Lane A), MSA (Lane D), or desoctapeptide-insulin treated (Lane E) preparations.

Figure 3. Comparison of the capacity of insulin and somatomedin C (IGF-I) to phosphorylate the immunoprecipitated insulin receptor.

Solubilized receptor preparations for swine granulosa cells were preincubated at 4°C for 30 minutes with insulin or somatomedin C prior to initiating the phosphorylation reaction. The immunoprecipitated receptor extracts were then subjected to SDS-polyacrylamide gel electrophoresis. The subsequent autoradiograms are depicted above, as follows: Lane A, Control, Lane B: 0.05 μM insulin, Lane C: Control, Lane D: 0.03 μg/ml somatomedin C, Lane E: 0.1 μg/ml somatomedin C.

significant phosphorylation induced by desoctapeptide insulin or by multiplication stimulating activity. In addition, high concentrations of somatomedin C failed to stimulate detectable phosphorylation of the 95,000-dalton band resolved from the solubilized insulin-receptor preparation (Figure 3). In this experiment, the effect of insulin (0.05 μM) was approximately 3.6-fold above control by densitometric scanning, while no significant effects were observed for doses of somatomedin C that can significantly stimulate progesterone production by these cells in monolayer culture.

Discussion

The present study applies two biochemical probes of the classical insulin receptor to appraise the nature of insulin action in cultured ovarian cells. Our results indicate that insulin action in these gonadal cells conforms in certain important respects to that recognized in classical target tissues. In

particular, insulin's facilitation of progesterone biosynthesis by swine granulosa cells is significantly impeded by insulin receptor antiserum. Moreover, incubation of solubilized ovarian receptor preparations with doses of insulin that will stimulate progesterone production induces phosphorylation of a specific immunoprecipitable polypeptide having a molecular weight of 95,000-daltons. This polypeptide presumptively represents the beta subunit of the insulin receptor. The specificity of the immunoisolation procedure is indicated by the failure of high concentrations of desoctapeptide insulin, somatomedin C/IGF-I, or multiplication stimulating activity to induce similar phosphate transfer.

The present novel demonstration that insulin promotes phosphorylation of the beta subunit of its own receptor in granulosa cells provides direct evidence for a discrete biochemical action of insulin in the ovary. However, these studies do not exclude the possibility that non-insulin growth factors also act on ovarian cells, or that certain actions of insulin could be exerted on somatomedin receptors. In fact, we have recently observed that both multiplication stimulating activity and somatomedin C/IGF-I stimulate progesterone production at concentrations considerably lower than those required for insulin (6, and manuscript in preparation). Since our insulin-receptor antiserum does not block the actions of multiplication stimulating activity (11) but does effectively impede insulin's effects on progesterone production, we infer that insulin is not likely to stimulate steroidogenesis via the receptor for multiplication stimulating activity. In addition, this antiserum does not significantly inhibit somatomedin C/IGF-I binding to human placenta and lymphocytes (12). Thus, results using this antiserum are consistent with actions of insulin on the insulin receptor per se. Nonetheless, because of possible species difference between antisera and cells, we do not dismiss the additional possibility that insulin could enhance steroidogenesis by actions on a somatomedin receptor on porcine ovarian cells. Highly specific antiserum against the somatomedin C but not the insulin receptor would ultimately be required to test this separate possibility.

Available studies cannot directly ascertain how or whether phosphorylation of the insulin receptor is functionally coupled to steroidogenesis. On the other hand, our data indicate that insulin-receptor phosphorylation occurs at insulin concentrations that are biologically active in these cultures. Thus, the present observations make tenable - but do not prove - the hypothesis that insulin acts through a classical insulin-receptor pathway in ovarian cells.

In conclusion, the current results demonstrate that insulin-receptor antibody effectively antagonizes the trophic actions of insulin on cultured swine ovarian cells. In addition, insulin is capable of stimulating specific phosphorylation of the immuno-isolated beta subunit of the insulin receptor on granulosa cells. In contrast, desoctapeptide insulin, somatomedin C/IGF-I, and multiplication-stimulating activity do not share this property. Collectively, these results provide the first evidence for specific biochemical actions of insulin at the level of the cell-membrane receptor for insulin in the mammalian gonad.

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