Endocrine, *vol. 13, no. 1, 103–110, August 2000* 0969–711X/00/13:103–110/$12.00

Insulin-like Growth Factor (IGF)-I Stimulates IGF-I and Type 1 IGF Receptor Expression in Cultured Rat Granulosa Cells

*Autocrine Regulation of the Intrafollicular IGF-I System**

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A growing body of information documents the existence of a complete rat intrafollicular insulin-like growth factor (IGF)-I system replete with a ligand (IGF-I), a receptor (type 1 IGF receptor) IGF binding proteins (4 and 5), and IGFBP-directed endopeptidases (4 and 5). Previous studies have established the ability of IGF-I to promote the elaboration of granulosa cell-derived IGFBP-5 and to suppress the activity of granulosa cell-derived IGFBP-5-directed endopeptidase. It was the purpose of this article to examine the effects of treatment with IGF-I on the other components of the intrafollicular IGF system, i.e., IGF-I itself and the type 1 IGF-receptor. Granulosa cells, obtained by follicular puncture from 25-d-old estrogen-primed rats were cultured in polystyrene tubes for 72 h under serum-free conditions, in the absence or presence of the indicated agents. At the conclusion of each experiment, media were discarded, and RNA was extracted and subjected to an RNase protection assay. Treatment of cultured rat granulosa cells with IGF-I resulted in a significant 1.8-fold increase in the steady-state levels of IGF-I mRNA. No effect was noted on the total cellular DNA content thereby arguing against the possibility that the relative increase in IGF-I transcripts can be ascribed to a possible treatment-induced increase in cell number in culture. The IGF-I effect was apparent ($p < 0.05$) at IGF-I doses as low as 1 ng/mL, minimal additional increments being noted thereafter. Treatment with insulin and des *(1–3)* IGF-I proved equally effective, producing 2.0- and 2.6-fold increases, respectively, thereby suggesting that the IGF-I effect may be mediated via the type 1 IGF receptor. Treatment with IGF-I also resulted in a significant ($p < 0.005$) increase in type 1 IGF receptor expression (2.3-fold increase), the first significant effect being noted at the 30 ng/mL dose level. Similar results obtained for insulin and des *(1–3)* IGF-I thereby suggest that the ability of IGF-I to upregulate the expression of its own receptor is probably type 1 IGF receptor-mediated. Taken together, these findings indicate that treatment of estrogen-primed granulosa cells with IGF-I will result in upregulation of the steady-state levels of transcripts corresponding to IGF-I itself and to its type 1 IGF receptor. These observations emphasize the importance of positive autoregulatory phenomena as determinants of the intrafollicular content of IGF-I and its receptor.

Key Words: IGF-I; receptor; granulosa.

Introduction

A growing body of evidence relevant to the rat ovary supports the existence of a complete intrafollicular IGF-I system replete with a ligand (IGF-I) *(1–7)*, a receptor (type I IGF receptor) *(8–15)*, IGF binding proteins (4 and 5) *(16–35)*, as well as corresponding proteases *(36–39)*. Although the endocrine and paracrine regulation of components of the intrafollicular IGF-I system has been the subject of intense investigation, limited information is available relative to autocrine regulatory loops. It was thus the purpose of this communication to examine the ability of IGF-I to regulate the steady-state levels of transcripts corresponding to itself and to its receptor. To this end, we set out to develop a responsive granulosa cell culture system. Such was necessary, given that previous approaches proved incapable of sustaining IGF-I gene expression, the expression of the type 1 IGF receptor proving constituitive and nonrespon-

Received March 3, 2000; Revised May 23, 2000; Accepted May 23, 2000.
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sive to conventional hormonal manipulation *(3)*. Using this approach we hereby demonstrate a series of significant autocrine regulatory phenomena, the potential impact of which on the determination of the intrafollicular IGF-I content is self-evident. Such form of positive autoregulation could serve a valuable role in amplifying the overall impact of the intrafollicular IGF-I system.

Results

IGF-I Gene Expression in Cultured Rat Granulosa Cells: Effect of Treatment with IGF-I

To determine the effect of treatment with IGF-I on IGF-I gene expression, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to an RNase protection assay with an antisense riboprobe corresponding to rat IGF-I. As shown (Fig. 1), treatment with IGF-I produced a significant ($p < 0.05$) 1.8-fold increase in the steady-state levels of IGF-I transcripts. Data represent the mean ± SE of 22 replicates, an effort designed to ensure reproducibility in the absence of a normalizing probe.

Search for a Normalizing Probe: Effect of Treatment with IGF-I on RPL19 Gene Expression

In search of an adequate normalizing probe, initial use was made of large ribosomal protein 19 or RPL19, the utility of which has previously been established in related but not identical experimental settings *(40)*. To determine the effect of treatment with IGF-I on RPL19 gene expression, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to an RNase protection assay using an antisense riboprobe corresponding to rat RPL19. Careful evaluation revealed RPL19 to be highly dependent on IGF-I in the current experimental setting and therefore of little value as a normalizing gene. Indeed, as shown in Fig. 2, treatment of cultured granulosa cells with IGF-I resulted in a significant ($p < 0.01$) 2.8-fold increase in the steady-state levels of RPL19 transcripts. Evaluation of other probes including cyclophillin, CHOB, and β actin proved equally unrewarding, each being increased by IGF-I treatment (not shown). Because of the inability to identify IGF-I-independent transcripts, strict quantitative normalization of the data was not possible. Instead, accuracy of RNA loading relied on the estimation of the concentration of 18S and 28S RNAs using UV-illuminated ethidium bromide-stained gels. Moreover, each experiment was carried out at least three times in an effort to minimize possible errors introduced by a given individual experiment.

Effect of Treatment with IGF-I on Total Cellular DNA Content

Given the fact that IGF-I is a growth factor, one may attribute the preceding observations to an increase in cell

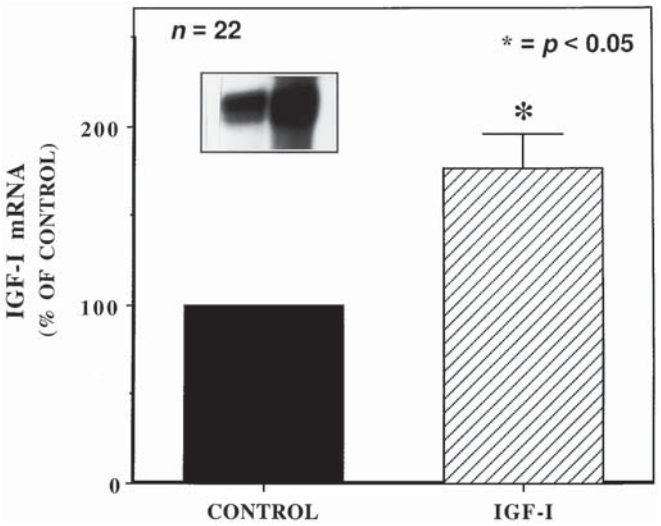

Fig. 1. IGF-I gene expression in cultured rat granulosa cells: effect of treatment with IGF-I. Granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to a RNase protection assay using a [^{32}P]UTP-labeled antisense IGF-I riboprobe as described. The intensity of the signals was quantified as described. The bar graphs depict the mean ± SE of 22 independent experiments. In each individual experiment, data were normalized to control values. The inset reflects a single representative autoradiograph.

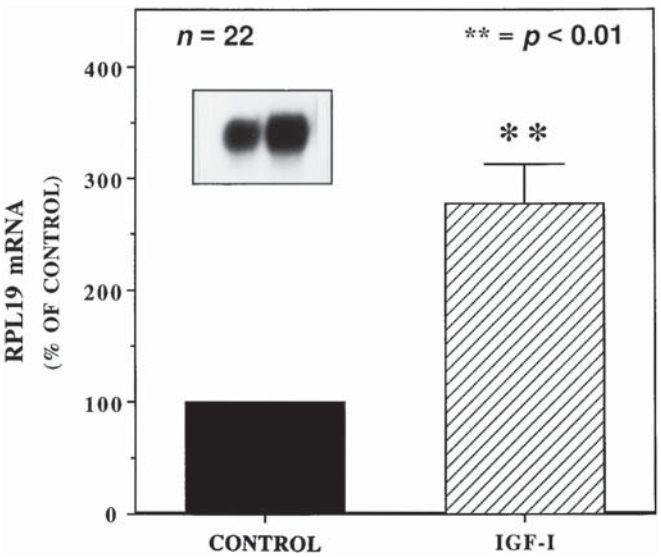

Fig. 2. RPL19 gene expression in cultured rat granulosa cells: effect of treatment with IGF-I. To determine the effect of in vitro treatment with IGF-I on RPL19 gene expression, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to an RNase protection assay using a [^{32}P]UTP-labeled RPL19 antisense riboprobe as described. The intensity of the signals was quantified as described. The bar graphs depict the mean ± SE of 22 independent experiments. In each individual experiment, data were normalized to control values. The inset reflects a single representative autoradiograph.

number in culture. To assess this possibility, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL). At the conclusion of this period, media were removed and the DNA content was evaluated by a fluorimetric assay. As shown in Fig. 3, treatment with IGF-I was without effect on the total cellular DNA content, thereby arguing against the possibility of an increase in cell number in culture. These observations also suggest that the IGF-I-induced increase in IGF-I transcripts represents an increase in transcript content/cell.

IGF-I Gene Expression in Cultured Rat Granulosa Cells: IGF-I Dose Dependence

To determine the dose requirements of IGF-I, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of increasing concentrations (1–50 ng/mL) of IGF-I. At the conclusion of this period, media were removed, and total RNA was extracted and subjected to an RNase protection assay using an antisense riboprobe corresponding to rat IGF-I. As shown (Fig. 4), the ability of IGF-I to stimulate its own transcripts proved dose dependent, the first significant increase being noted at the 1 ng/mL dose level (1.7-fold increase) with minimal additional increments being noted thereafter.

IGF-I Gene Expression in Cultured Rat Granulosa Cells: Effect of Treatment with IGF-I Analogs

To assess the possibility that the IGF-I effect is mediated via the type 1 IGF receptor, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL), insulin (1 μg/mL), or des *(1–3)* IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to an RNase protection assay using an antisense riboprobe corresponding to rat IGF-I. As shown (Fig. 5), the IGF-I effect was reproduced by pharmacologic concentrations of insulin, acting in all likelihood as an IGF-I surrogate (2.0-fold increase). Treatment with des *(1–3)* IGF-I, a potent analog immune from sequestration by IGF binding proteins *(25)*, proved equally effective, producing a 2.6-fold increase in IGF-I transcripts. These data suggest, but do not prove, that IGF-I action is mediated via the type 1 IGF receptor.

Effect of Treatment with IGF-I on the Steady-State Levels of Type 1 IGF Receptor Transcripts

To assess the ability of IGF-I to regulate the steady-state levels of type 1 IGF receptor transcripts, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to an RNase protection assay using an antisense riboprobe corresponding to the rat type 1 IGF receptor. As shown (Fig. 6), treatment with IGF-I produced a significant ($p < 0.05$) 1.6-fold increase in type 1 IGF receptor transcripts.

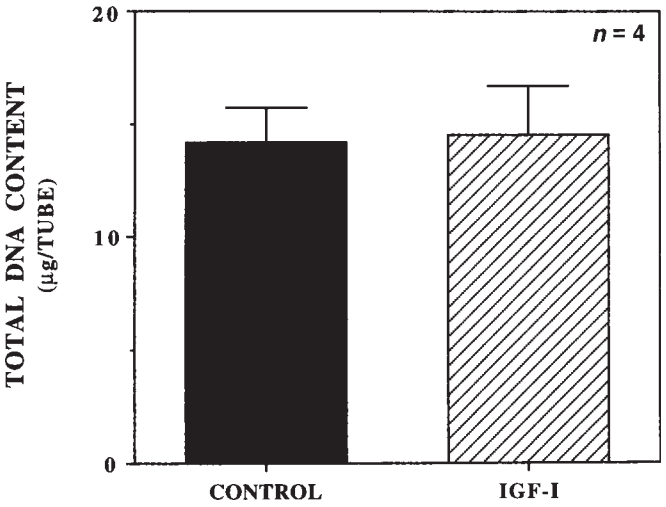

Fig. 3. Effect of treatment with IGF-I on the total cellular DNA content. To assess the possibility of an increase in cell number in culture, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL). At the conclusion of this period, media were removed and the DNA content was evaluated by a fluorimetric assay. The bar graphs depict the mean ± SE of four independent experiments.

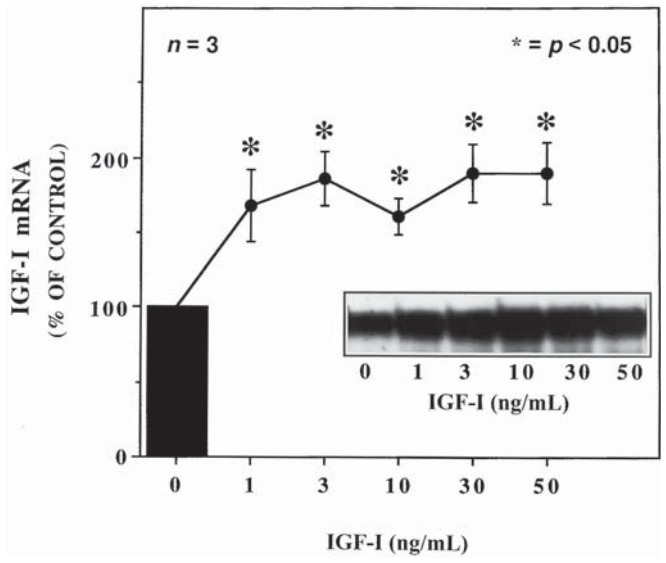

Fig. 4. IGF-I gene expression in cultured rat granulosa cells: IGF-I dose dependence. To determine the dose dependence of in vitro treatment with IGF-I on IGF-I gene expression, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (1–50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to a RNase protection assay with a [^{32}P]UTP-labeled IGF-I antisense riboprobe as described. The intensity of the signals was quantified as described. The bar graph depicts the mean ± SE of three independent experiments. In each individual experiment, data were normalized to control values. The inset reflects a single representative autoradiograph.

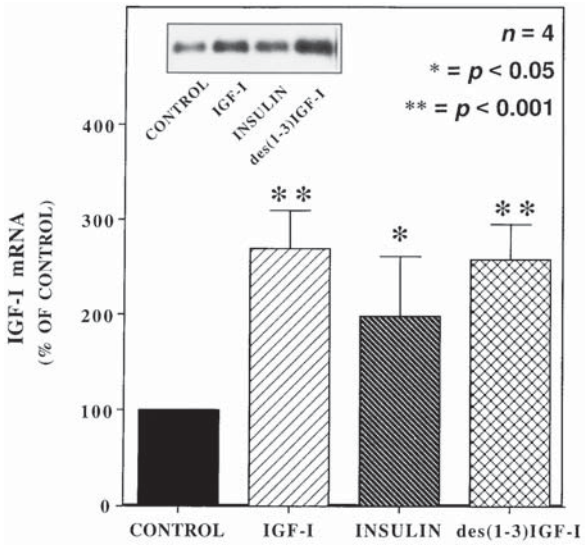

Fig. 5. IGF-I gene expression in cultured rat granulosa cells: effect of treatment with IGF-I analogs. To determine the effect of in vitro treatment with IGF-I analogs on IGF-I gene expression, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (1–50 ng/mL), Insulin (1 μg/mL), or des (1–3) IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to a RNase protection assay with a [^{32}P]UTP-labeled IGF-I antisense riboprobe as described. The intensity of the signals was quantified as described. The bar graph depicts the mean ± SE of four independent experiments. In each individual experiment, data were normalized to control values. The inset reflects a single representative autoradiograph.

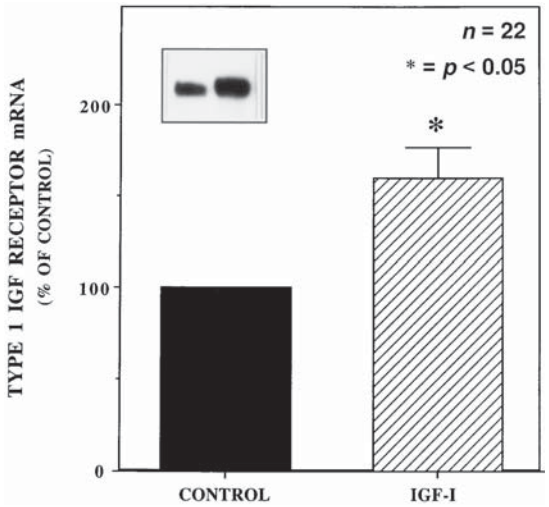

Fig. 6. Effect of treatment with IGF-I on the steady-state levels of type 1 IGF receptor transcripts. Granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (1–50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to a RNase protection assay with a [^{32}P]UTP-labeled antisense type 1 IGF receptor riboprobe as described. The intensity of the signals was quantified as described. The bar graphs depict the mean ± SE of 22 independent experiments. In each individual experiment, data were normalized to control values. The inset reflects a single representative autoradiograph.

Type 1 IGF Receptor Gene Expression in Cultured Rat Granulosa Cells: IGF-I Dose Dependence

To determine the dose requirements of IGF-I, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of increasing concentrations (1–50 ng/mL) of IGF-I. At the conclusion of this period, media were removed, and total RNA was extracted and subjected to an RNase protection assay using an antisense riboprobe corresponding to the type 1 IGF receptor. As shown (Fig. 7), the ability of IGF-I to upregulate type 1 IGF receptor transcripts proved dose dependent, the first significant effect ($p < 0.05$) being noted at the 30 ng/mL dose level. The maximal stimulatory response (evident at the 50 ng/mL dose level) constituted a 2.3-fold increase relative to untreated controls.

Type 1 IGF Receptor Gene Expression in Cultured Rat Granulosa Cells: Effect of Treatment with IGF-I Analogs

To assess the possibility that the IGF-I effect is mediated via the type 1 IGF receptor, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (50 ng/mL), insulin (1μg/mL), or des *(1–3)* IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to an

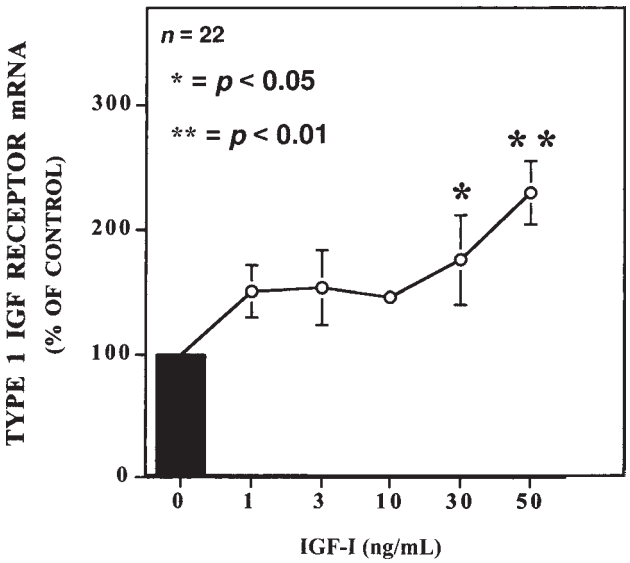

Fig. 7. Type 1 IGF receptor gene expression in cultured rat granulosa cells: IGF-I dose dependence. To determine the dose dependence of in vitro treatment with IGF-I on type 1 IGF receptor gene expression, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (1–50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to a RNase protection assay with a [^{32}P]UTP-labeled antisense type 1 IGF receptor riboprobe as described. The bar graphs depict the mean ± SE of 22 independent experiments. In each individual experiment, data were normalized to control values. The inset reflects a single representative autoradiograph.

RNase protection assay using an antisense riboprobe corresponding to the type 1 IGF receptor. As shown (Fig. 8), the IGF-I effect was reproduced by insulin (2.0-fold increase), herein applied at concentrations required to activate the type 1 IGF receptor. The IGF-I analog des *(1–3)* IGF-I proved equally effective (1.6-fold increase). These findings suggest that the ability of IGF-I to upregulate the expression of its own receptor is receptor-mediated involving most likely the type 1 IGF receptor itself.

Discussion

The present article is concerned with the ability of IGF-I to regulate the expression of itself and of its type 1 receptor in cultured estrogen-primed rat granulosa cells. This work was made possible by a newly constructed in vitro cell culture system responsive to IGF-I. The current system was arrived at by way of a systematic evaluation of multiple culture variables. This search was prompted by prior experience with a related granulosa cell culture system that proved incapable of sustaining IGF-I gene expression and wherein the expression of the type 1 IGF receptor proved constituitive and nonresponsive to conventional hormonal manipulation *(3)*. Although the precise reason(s) underlying the improved functionality of the current system remain uncertain, mention is made of the use of estrogen-primed granulosa cells, of polystyrene tubes (as distinct from dishes), and the employment of predetermined cellular plating densities.

The current work was complicated by the apparent inability to identify an IGF-I-independent normalizing probe. Indeed, preliminary experiments have clearly established the ability of IGF-I to upregulate the steady-state levels of granulosa cell RPL19 transcripts (Fig. 2). Similar observations were made for "housekeeping" genes including β actin, cyclophilin, and CHOB (not shown). Fortunately, treatment with IGF-I was not associated with an increase in total cell number as assessed by the total cellular DNA content (Fig. 3). Under those circumstances, increments in the steady-state levels of relevant transcripts cannot be attributed to an increase in cell number. Although the absence of a normalizing probe compromises the ability to compensate for variability in RNA loads, the use of multiple replicates is designed to minimize such occurrence.

The current observations document the ability of IGF-I to meaningfully alter the relative representation of several components of the intrafollicular IGF-I system, a new end point, complementing numerous earlier end points *(41–53)*. Specifically, our observations establish the ability of IGF-I to (1) upregulate its own transcripts and (2) upregulate transcripts corresponding to its type 1 receptor. On the surface, one could argue that IGF-I acts to amplify its own impact by virtue of upregulating transcripts corresponding to itself

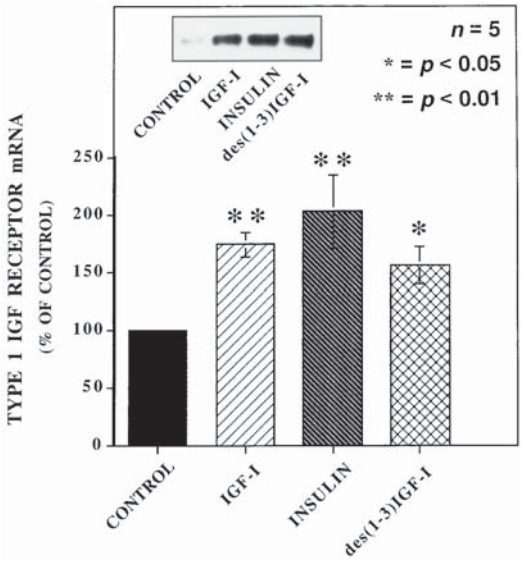

Fig. 8. Type 1 IGF receptor gene expression in cultured rat granulosa cells: effect of treatment with IGF-I analogs. To determine the dose-dependence of in vitro treatment with IGF-I analogs on type 1 IGF receptor gene expression, granulosa cells (2.0×10^6 cells/tube) were cultured for 72 h in the absence or presence of IGF-I (1–50 ng/mL), insulin (1 μg/mL) or des *(1-3)* IGF-I (50 ng/mL). At the conclusion of this period, media were removed, and total RNA was extracted and subjected to a RNase protection assay with a [^{32}P]UTP-labeled antisense type 1 IGF receptor riboprobe as described. The bar graphs depict the mean ± SE of five independent experiments. In each individual experiment, data were normalized to control, values. The inset reflects a single representative autoradiograph.

and to its receptor. On the other hand, IGF-I would appear to attenuate its own impact by upregulating transcripts corresponding to IGFBP-5 *(32)*. However, more careful evaluation of the data reveals disparate sensitivities for these IGF-I end points. Indeed, the minimal effective strength of IGF-I required to upregulate IGF-I transcripts proved to be 1 ng/mL. The corresponding value for the type 1 IGF receptors was 30 ng/mL. Both could lead to increased IGF-I impact. Virtually pharmacologic concentrations of IGF-I (100 ng/mL) would be required to upregulate IGFBP-5 transcripts thereby resulting in a reduction in the IGF-I impact *(32)*.

In light of the above, it is proposed that IGF-I concentrations in the physiologic range are in fact engaged in autocrine self-amplification. In contrast, IGF-I concentrations in the pharmacologic range may activate corrective phenomena designed to buffer the IGF-I impact when present in excess. In this respect, the high-dose IGF-I effect may be viewed as a defensive mechanism activated under circumstances wherein pharmacologic levels of the hormone run the risk of perturbing a delicate physiologic balance. We speculate that, by orchestrating a series of diverse actions, IGF-I may exert some control over its own destiny.

Materials and Methods

Animals

Immature (25–28-d-old) Sprague-Dawley female rats, purchased from Zivic-Miller Laboratories (Zelienople, PA), were sacrificed by CO_2 asphyxiation. Diethylstilbestrol (DES) priming was achieved by the subcutaneous placement of silastic DES-containing capsules by the vendor.

Reagents

McCoy's 5a medium (modified; without serum), penicillin-streptomycin solution, L-glutamine, Trypsin-ethylenediamine tetraacetic acid, and Trypan blue stain were obtained by GIBCO-BRL Life Sciences (Grand Island, NY). Ovine FSH (NIH-oFSH-S17; FSH potency = 20 NIH FSH-S1 units/mg; LH activity = 0.04 NIH LH-S1 units/mg; PRL activity <0.1% by weight) was generously provided by the National Pituitary Agency, Pituitary Hormone Distribution Program, NIADDK, NIH (Bethesda, MD). Recombinant IGF-I was from Bachem Laboratories (Torrance, CA). Bovine insulin was from Sigma Chemical Company (St. Louis, MO). des *(1–3)* IGF-I was generously provided by Dr. Ron G. Rosenfeld, University of Oregon Health Sciences Center , Portland, Oregon.

In Vitro Studies

Granulosa cells from DES-primed immature rats, obtained by follicular puncture as previously described ***(54)***, were inoculated into loosely capped 12 × 75 mm sterile polystyrene tubes (Falcon Plastics, Oxnard, California) containing 1 mL McCoy's 5a medium (modified; without serum) supplemented with L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin sulfate (100 μg/mL). Viability of cells was assessed by Trypan blue exclusion. Cell cultures were maintained for 72 h at 37°C under a water-saturated atmosphere of 95% air and 5% CO_2. All agents were dissolved in sterile culture medium and applied in 50 μL aliquots. At the end of each experiment, the media (and/or cells) were collected before further processing as described below.

RNA Extraction

Total RNA was phenol/chloroform-extracted and ethanol-precipitated using RNAZOL-B as recommended by the manufacturer, Tel Test, Inc. (Friendswood, TX). Precipitated RNA was resuspended in 1 mL of 75% ethanol, stored at –70°C, and quantified by absorbance at A_{260} before use.

Preparation of cDNA Probes

The rat IGF-I 3' cDNA *(55)* was generously provided by Dr. Derek LeRoith of the Diabetes Branch, NIADDK, NIH (Bethesda, MD). For the purpose of RNase protection assays, a Sau3A-Eco RI fragment was excised and subcloned into Bam HI-Eco RI sites of the pGEM3 Promega vector (Madison, WI). This latter construct was then linearized with Hind III and transcribed with T7 RNA polymerase in the presence of [α-^{32}P]UTP to yield a 415 riboprobe, which, upon hybridization, was projected to generate a 224 nt protected fragment.

The rat type 1 IGF receptor cDNA *(56)*, kindly provided by Dr. Derek LeRoith of the Diabetes Branch, NIADDK, NIH, was inserted into Eco RI-Sma I sites of a pGEM3 vector. For the purpose of RNase protection assays, this construct was linearized with Eco RI and transcribed with SP6 in the presence of [α-^{32}P]UTP to yield a 310 nt riboprobe, which upon hybridization was projected to generate a 265 nt protected fragment.

The ribosomal protein large (RPL)19 probe was generated by reverse transcription of 1 μg of RNA followed by amplification via the polymerase chain reaction using primers CTGAAGGTCAAAGGGAATGTG and GGA CAGAGTCTTGATGATCTC, which were designed to amplify the rat gene *(40)*. The PCR product was then cloned into an Invitrogen pCR1000 vector, verified by DNA sequencing, digested with Eco RI or Fok I, and transcribed with T7 RNA polymerase to generate a 234 nt probe capable of protecting a 253 nt segment.

RNase Protection Assay

Linearized DNA templates were transcribed with T7 or SP6 RNA polymerases to specific activities of 800 Ci/mmol UTP (IGF-I and type 1 IGF receptor) or 80 Ci/mmol UTP (RPL19). The DNA templates were digested with 10 μg of DNAse I and the riboprobe products purified by phenol/chloroform extraction followed by ethanol precipitation in the presence of 15 μg yeast tRNA carrier. The riboprobes were gel-purified as described *(57)* in order to eliminate transcribed products that are shorter than the full-length probe. RNA samples (about 20 μg) were hybridized with excess probe in 20 m*M* Tris pH 7.6, 1 m*M* EDTA, 0.4 *M* NaCI, 0.1% SDS, and 75% formamide. Positive and negative controls (i.e., 20 μg yeast tRNA) were included in each experiment. Reactions were denatured at 95°C before overnight hybridization at 45°C. After hybridization, 9 vol of 10 m*M* Tris-HcI pH 7.6, 5 m*M* EDTA, 0.3 *M* NaCI containing 11 μg RNase A and 27 μg of RNase T1 were added to each sample for a 1 h incubation at 30°C. The negative control was incubated in the same buffer without RNases. RNases were eliminated by digestion with 50 μg Proteinase K in 1.5% SDS at 37°C for 15 min followed by phenol/chloroform extraction and ethanol precipitation in the presence of 20 μg yeast tRNA carrier. RNA pellets were reconstituted in 95% formamide, 1 m*M* EDTA, 0.1% xylene cyanol/bromophenol blue, and denatured at 95°C prior to electrophoresis on 8% acrylamide gels containing 8.3 *M* urea in 0.1 *M* Tris/0.1 *M* borate (pH 8.3) 2 m*M* EDTA. Gels were exposed to X-ray film (Kodak, Rochester, NY) for varying lengths of time with intensifying screens. Gels were

also quantified by exposure to phosphor screens, which were analyzed with ImageQuant software from Molecular Dynamics (Sunnyvale, CA).

Statistical Analysis

Except as noted, each experiment was replicated a minimum of three times. Data points are presented as mean ± SE and statistical significance (Fisher's protected least significance difference) determined by ANOVA and the Student's *t*-test. Statistical computations were carried out using Statview 512 + for Macintosh (Brain Power, Inc., Calabasas, CA).

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