The transfection-induced overexpression of IGF-binding protein-4 affects the secretory activity of porcine ovarian granulosa cells and their response to hormones and IGF-I

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ABSTRACT

The aim of our studies was to examine whether IGF-binding protein (IGFBP)-4 is involved in the control of the secretion of various ovarian substances and also the mediation of the effects of several hormones and growth factors on this secretion. For this purpose, we carried out the transfection of porcine granulosa cells with a cDNA sense construct, increasing IGFBP-4 synthesis. We then compared the release of IGFBP-3, progesterone, oxytocin and IGF-I by control and transfected cells cultured with and without porcine LH (100 ng/ml), porcine GH (100 ng/ml), oxytocin (10 ng/ml) and estradiol-17β (100 ng/ml). The concentration of IGFBP-4 produced was assessed using ligand blotting, and the release of progesterone, oxytocin, IGF-I and IGFBP-3 was evaluated using RIA/IRMA techniques.

It was observed that GH, IGF-I, estradiol, LH and oxytocin alter the progesterone, oxytocin, IGF-I and IGFBP-3 release by porcine ovarian granulosa cells. Transfection of these cells with an IBFBP-4 cDNA expression construct significantly increased the IGFBP-4 accumulation in cell-conditioned medium. Furthermore, this transfection significantly reduced progesterone, oxytocin and IGFBP-3 release, and increased IGF-I output in cells cultured in the absence or presence of GH, IGF-I, estradiol and LH. The addition of oxytocin, but not of other tested substances, fully or partially prevented the effects of IGFBP-4 overexpression on IGFBP-3, IGF-I, but not on progesterone release. The present results suggested that IGFBP-4, as well as GH, IGF-I, estradiol, LH and oxytocin, is a potent regulator of porcine ovarian steroid (progesterone), nonapeptide hormone (oxytocin), growth factor (IGF-I) and growth factor-binding protein (IGFBP-3) release. IGFBP-4 is an inhibitor of basal progesterone, oxytocin and IGFBP-3 release and a stimulator of IGF-I output by porcine ovarian cells. The action of IGFBP-4 on the ovary can be mediated by (1) inhibition of oxytocin release, (2) suppression of receptor/postreceptor events induced by other hormones and IGF-I and (3) stimulation of IGF-I release.

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INTRODUCTION

Insulin-like growth factor I (IGF-I) and IGF-binding proteins (IGFBPs) can be mediators of hormone action on the ovary (Erickson 1995, Spicer & Echternkamp 1995, Yoshimura 1998, Schams et al. 1999). IGFBPs can affect target tissues by regulating the availability of free (bioactive) IGF-I or via IGF-I-independent mechanisms (Rajaram et al. 1997, Rechler & Clemmons 1998). Production of both IGF-I and the six classic IGFBPs by mammalian ovarian cells has been shown previously (Grimes et al. 1994, Spicer & Echternkamp 1995, Armstrong et al. 1996, Mihm et al. 1997, Schams et al. 1999), but the role that a particular IGFBP exerts in the control of ovarian functions remains to be elucidated. There are several reports of the direct influence of the IGFBPs on ovarian functions. IGFBP-2, -3, -4 and -5 treatment, probably through the blockade of IGF-I and its receptors, can inhibit basal follicle-stimulating hormone (FSH)- and IGF-I-induced

In different species the ovarian production of various IGFBPs can be inhibited by gonadotropic substances (luteinizing hormone (LH), FSH, growth hormone (GH), IGF-I, oxytocin and estradiol; Grimes et al. 1994, Spicer & Echternkamp 1995, Armstrong et al. 1996, Mihm et al. 1997, Putowski et al. 1997, Sirotkin et al. 1998, Sirotkin & Makarevich 1999), although a stimulatory effect of LH on the production of IGFBP-4 (Armstrong et al. 1996) and its mRNA (Armstrong et al. 1998) in ruminant theca cells has also reported. These data indirectly suggest that these gonadotropic substances can affect ovarian cells through the regulation of both IGF-I and IGFBP production. This hypothesis was partially confirmed by direct evidence of the inhibition of the effects of FSH and IGF-I by IGFBP additions (see above). It remains unknown whether IGFBPs can modify and mediate the effects of other gonadotropic substances – LH, GH, oxytocin and estradiol.

To understand the role of IGFBPs in the control of ovarian functions, only one approach to increase IGFBP levels – treatment with exogenous IGFBP – has been used. An alternative way, overproduction of an endogenous IGFBP by cell transfection with a sense cDNA construct of an IGFBP, has previously been used only on non-ovarian cells (Cohen et al. 1993, Corkins et al. 1995, Park et al. 1996), but not on ovarian cells.

The first aim of our in vitro experiments was to examine the effects of different gonadotropic substances on ovarian steroid, nonapeptide hormone, growth factor and growth factor-binding protein release by studying the effects of LH, GH, IGF-I, oxytocin and estradiol on the release of progesterone, oxytocin, IGF-I and IGFBP-3 by porcine granulosa cells. The second aim was to find out whether IGFBP-4 mediates the release of IGFBP-3 and other ovarian secretions and modifies the effects of the gonadotropic substances mentioned above. For this purpose, we carried out transfection of porcine granulosa cells with cDNA constructs increasing IGFBP-4 synthesis, and compared the release of IGFBP-3, progesterone, oxytocin and IGF-I by the control and transfected cells cultured with and without LH, GH, IGF-I, oxytocin and estradiol.

MATERIALS AND METHODS

Gene constructs used

The full-length cDNA for rat IGFBP-4 and IGFBP-3 was a gift from Dr S Shimasaki (The Whittier Institute for Diabetes and Endocrinology, La Jolla, CA, USA). The EcoRI fragments of the IGFBP-3 and -4 cDNA (Shimasaki et al. 1989) were ligated into EcoRI-digested pcDNA3 vector (Invitrogen, San Diego, CA, USA). After ligation, the DNA was used to transform DH5-α E. coli (GIBCO-BRL, Gaithersburg, MD, USA) and colonies were selected for ampicillin resistance. Plasmid minipreps were prepared to permit identification of the appropriate orientation of the cDNA insert (Corkins et al. 1995).

Preparation, culture and processing of granulosa cells

Granulosa cells were collected from the 2–5 mm diameter follicles from ovaries of Slovakian white gilts at 180 days of age, slaughtered at a local abattoir and processed as described previously (Sirotkin 1996, Sirotkin & Makarevich 1999). After isolation and washing, the cell population was divided into three groups. One group was transfected with the sense IGFBP-4 cDNA construct listed above using lipofection reagent DOTAP (Boehringer Mannheim GmbH, Mannheim, Germany) following the manufacturer’s instructions. A mixture of 15 µg cDNA, 90 µg DOTAP and 6 000 000 cells in 6 ml incubation medium was used for each transfection procedure. Control cells were incubated with DOTAP with 15 µg IGFBP-3 sense and antisense gene constructs or mixed bases (GIBCO-BRL) instead of the IGFBP-4 sense cDNA construct. Thereafter the granulosa cells were washed three times in culture medium and precultured at a concentration of 1 000 000 cells/ml in 2 ml culture medium (Dulbecco’s modified Eagle’s medium/F-12; 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic-antimicrobial from Sigma, St Louis, MO, USA) in Falcon 24-well plates (Becton Dickinson, Lincoln Park, CA, USA) at 37 ºC under 5% CO2 in humidified air. After 2 days of preculture the medium was replaced with medium of the same composition with porcine GH (pGH; USDA-pGH-B-1, 100 ng/ml), porcine LH (pLH; USDA-pLH-B-6, 100 ng/ml) which were kindly provided by Dr J A Proudman (USDA Animal Hormone Program,
Beltsville, MD, USA), or recombinant IGF-I (10 ng/ml), synthetic oxytocin (10 ng/ml) or estradiol-17β (100 ng/ml) from Sigma. Control groups in each transfected and non-transfected group contained either no cells (blank control) or cells with no exogenous treatment. The experimental substances were of immunological grade. They were dissolved in medium immediately before each experiment. After 2 days in culture, the conditioned medium from each well was gently aspirated and frozen at −18°C to await ligand blotting and radioimmunoassay (RIA). After culture, cell number and viability were determined by Trypan blue staining and hemocytometry. No statistically significant differences in these indices between control and experimental groups were observed.

### Immunoassay

Concentrations of hormones in 25–100 µl incubation medium were determined by RIA/immunoradiometric (IRMA) assays with extraction of IGF-I and oxytocin, but not of IGFBP-3 and progesterone prior to assay. IGF-I, IGFBP-3 and progesterone were determined using RIA/IRMA kits from DSL (Webster, TX, USA) according to the manufacturer’s instructions. Oxytocin was measured using our RIA described previously (Kotwica & Skarzynski 1993). The characteristics of these assays are presented in Table 1.

### Protein gel electrophoresis and ligand blotting

Production of IGFBP-4 and other IGFBPs was estimated by ligand blot analysis as described by Hossenlop et al. (1986) and modified by McCusker et al. (1988). Briefly, the proteins in conditioned medium and control serum (human and fetal bovine) samples were electrophoresed in 12.5% polyacrylamide gels in the presence of SDS under non-reducing conditions (Laemmli 1970). Following electroblotting onto 0.05 µm BA 75 nitrocellulose (Schleicher and Schuell, Keene, NH, USA), the blots were probed with 125I-IGF-II (Amersham, Little Chalfont, Bucks, UK). Signal intensities for radioactive bands on each blot were quantified by the use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) with the results reported in arbitrary units.

### Statistics

Each experimental group was represented by four culture wells. Assays of hormone and growth factor content in the conditioned medium were performed in duplicate. The data shown are means of values obtained on granulosa cells harvested from at least 20 animals. In ligand blotting, expression of the IGFBP-4 band in medium conditioned by cells transfected with IGFBP-4 sense cDNA construct (specific transfection) was compared with this band produced by cells treated with mixed bases (control

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**Table 1. Characteristics of immunoassays used in experiments**

<table>
<thead>
<tr>
<th>Assay (cross-reactivity of antiserum)</th>
<th>Specificity of assay</th>
<th>Sensitivity of assay (ng/ml)</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intra-assay</td>
<td>Interassay</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt;0.001% to cortisol,</td>
<td>0.12</td>
<td>&lt;13.1</td>
</tr>
<tr>
<td></td>
<td>corticosterone, cortisol, androstenediol, pregnenolone, estradiol, testosterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxytocin</td>
<td>&lt;0.01% to arg-vasopressin,</td>
<td>0.003</td>
<td>&lt;10.5</td>
</tr>
<tr>
<td></td>
<td>lys-vasopressin, arg-vasotocin, somatostatin, IGF-I, IGFBP-3, pGH, pLH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td>&lt;0.1% to IGF-II, insulin, proinsulin, pGH, oxytocin, progesterone</td>
<td>0.027</td>
<td>&lt;3.4</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>&lt;0.03% to IGFBP-1, -2, -4, -5, -6, IGF-I, pGH, pLH, oxytocin</td>
<td>0.5</td>
<td>&lt;3.9</td>
</tr>
</tbody>
</table>

**Protein gel electrophoresis and ligand blotting**

Production of IGFBP-4 and other IGFBPs was estimated by ligand blot analysis as described by Hossenlop et al. (1986) and modified by McCusker et al. (1988). Briefly, the proteins in conditioned medium and control serum (human and fetal bovine) samples were electrophoresed in 12.5% polyacrylamide gels in the presence of SDS under non-reducing conditions (Laemmli 1970). Following electroblotting onto 0.05 µm BA 75 nitrocellulose (Schleicher and Schuell, Keene, NH, USA), the blots were probed with 125I-IGF-II (Amersham, Little Chalfont, Bucks, UK). Signal intensities for radioactive bands on each blot were quantified by the use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) with the results reported in arbitrary units.
transfection), with IGFBP-3 sense and antisense constructs (non-specific transfection) and by intact cells (intact control). In RIA/IRMA assays, the values of substances released by cells transfected with IGFBP-4 sense cDNA construct were compared only with control cells transfected with mixed bases. The values of blank control (medium cultured without cells) were subtracted from the value determined in cell-conditioned medium to exclude any non-specific background. It did not usually exceed 10% of the total values measured in cell-conditioned medium. The rates of experimental substance secretion were calculated per 10^6 viable cells/day. Significant differences between the experiments were evaluated using two-way ANOVA. When effects of treatments were revealed, data from the experimental and control groups were compared by Duncan’s multiple range test. Differences from controls with a P value of <0·05 were considered significant.

RESULTS

Accumulation of IGFBP-4 in culture medium conditioned by porcine granulosa cells was demonstrated by ligand blotting (Fig. 1). The radiolabeled band with a relative molecular weight of 30 kDa, characteristic for IGFBP-4, was located under the IGFBP-3 band (45 kDa). No significant visible bands corresponding to any of the other IGFBPs or IGFBP-3 and -4 fragments were detected in cell-conditioned medium. Lipofection of granulosa cells with the sense IGFBP-4 (but not with sense and antisense IGFBP-3) cDNA construct resulted in a significant increase in expression of IGFBP-4 in the culture medium: the radiolabeled band intensity increased from 586 068 arbitrary units in control (transfected with mixed bases) cells to 1 086 239 arbitrary units in the experimental group. The transfection by either of the cDNA constructs had no effect on the IGFBP-3 band or on the position and structure of the IGFBP-4 bands.

The results of the RIA/IRMA assays of the conditioned medium showed that porcine granulosa cells are able to secrete significant amounts of IGF-I, IGFBP-3, oxytocin and progesterone, and that release of these substances is altered by transfection of cells with an IGFBP-4 sense cDNA construct, by exogenous hormones and by IGF-I (Figs 2–5).

Progesterone release (Fig. 2) in control cells was significantly increased by GH, IGF-I, LH and oxytocin, but not by estradiol treatment. Transfection of cells with the IGFBP-4 sense construct
significantly decreased basal, hormonal- and growth factor-induced progesterone release.

Oxytocin release (Fig. 3) in control cells was significantly suppressed by GH, estradiol and LH, while IGF-I significantly increased oxytocin release. Cells transfected with the IGFBP-4 sense construct had a significant inhibition of both basal, hormonal- and growth factor-induced oxytocin release.

IGF-I release (Fig. 4) was not substantially affected by the exogenous hormones, but transfection of the cells with the IGFBP-4 sense construct significantly increased the basal IGF-I release. Furthermore, IGFBP-4-transfected cells cultured with hormones produced significantly more IGF-I than control cells and the inhibitory influence of LH and oxytocin on IGF-I release was still expressed in these transfected cells.

IGFBP-3 release (Fig. 5) was stimulated by IGF-I and LH, and inhibited by GH and oxytocin, but not by estradiol. Transfection of cells with the IGFBP-4 sense construct reduced the IGFBP-3 release in cells cultured with and without hormones and growth factors. In the transfected cells, GH, LH and oxytocin stimulated IGFBP-3 release, while IGF-I and estradiol suppressed it; however, in these cells, both the absolute IGFBP-3 secretion rate and the response to each treatment was reduced.

DISCUSSION

The results of the RIAs confirmed previous reports (Wathes 1989, Spicer & Echternkamp 1995, Sirotkin 1996, Sirotkin et al. 1998) of the production of IGFBP-3, IGF-I, oxytocin and progesterone by cultured porcine ovarian cells, and of the involvement of GH, IGF-I, estradiol, LH and oxytocin in the control of this process. On the other hand, failure of GH, estradiol, LH and
transfection of cultured granulosa cells with an IGFBP-4 sense construct (but not with IGFBP-3 sense and antisense construct or mixed bases) produced an increase in IGFBP-4 release, providing validation of the efficiency and specificity of our transfection procedure.

This is the first reported study on the role of ovarian IGFBP-4 in mammalian reproduction utilizing a recombinant transfection to alter native secretion of this growth factor. These data suggest that IGFBP-4 is involved in the control of the release of various ovarian products—a steroid hormone (progesterone), a nonapeptide hormone (oxytocin), IGF-I and IGFBP-3.


The observation of an inhibitory effect of IGFBP-4 overexpression on the basal, hormonal- and IGF-I-regulated oxytocin and IGFBP-3 release is, to our knowledge, the first demonstration of the involvement of IGFBP-4 in the control of ovarian nonapeptide hormones and of other IGFBPs. Both oxytocin and IGFBP-3 are important regulators of the reproductive processes (Wathes 1989, Erickson 1995, Spicer & Echternkamp 1995, Sirotkin et al. 1998, Schams et al. 1999). Therefore, IGFBP-4 can potentially control ovarian functions through its influence on local oxytoxin and/or IGFBP-3. This hypothesis is supported by the present observation that addition of oxytocin fully or partially prevented the effects of IGFBP-4 overexpression on IGFBP-3 and IGF-I release. The effect of IGFBP-4 on progesterone release is probably not mediated by oxytocin because exogenous oxytocin was unable to eliminate the action of IGFBP-4 on progesterone. The potential mediator of IGFBP-4 action on ovarian functions (including basal and hormone-stimulated progesterone and oxytocin release) might be IGF-I: IGFBPs are able to bind endogenous IGF-I, the presence of which could be required for the maintenance of ovarian secretory activity and ovarian response to stimulators. Our observations...
that an IGFBP-4 transfection-induced inhibition of the IGF-I effects on progesterone, oxytocin and IGFBP-3 release support the previous evidence of an inhibitory influence of IGFBP-4 on ovarian IGF-I action (formation of inactive IGF/IGFBP complex, prevention of IGF-I binding and action on ovarian receptors; Spicer & Echternkamp 1995, Iwashita et al. 1996, Spicer & Chamberlain 1999). On the other hand, our data suggest that IGFBP-4 can inhibit ovarian function via the blockade of IGF-I action but not by the blockade of IGF-I expression because, in our experiments, the addition of IGF-I itself was not able to overcome the inhibitory effects of IGFBP-4 on progesterone, oxytocin and IGFBP-3. Furthermore, our observation of a transfection-induced increase in IGF-I release by cells cultured with and without various hormones demonstrates the stimulatory action of IGFBP-4 on ovarian IGF-I release. The reasons for and physiological significance of IGFBP-4-stimulated IGF-I release remain to be studied, but the protective role of IGFBP-4 against IGF-I degradation or the feedback effect of an IGFBP-4-induced blockade of the effects of IGF-I, which could induce an additional IGF-I surge, could be proposed.

An additional mechanism whereby IGFBPs may be involved in the control of ovarian function includes changes in IGFBP proteolysis: IGF-I (Myers et al. 1993, Grimes & Hammond 1994, Iwashita et al. 1998) and FSH (Iwashita et al. 1998, Resnik et al. 1998) can alter the proteolysis of IGFBPs and, therefore, the binding of IGFBPs to IGF with a resultant change in the local level of bioavailable IGF.

The complex inter-relationships between IGFBP-4 and IGF-I require further elucidation, but our present observations suggest that IGFBP-4 can inhibit the effects of IGF-I but, on the other hand, stimulate IGF-I release. The significance and mechanisms of IGFBP-4 action on various ovarian functions require further detailed studies. Nevertheless, our approach suggests that IGFBP-4 can be a mediator of some hormonal actions on the ovary and a potent regulator of ovarian secretory activity, specifically steroid, nonapeptide hormone, growth factor and growth factor-binding protein release.

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