Secretory activity of bovine ovarian granulosa cells transfected with sense and antisense insulin-like growth factor (IGF) binding protein-3 and the response to IGF-I, GH, LH, oxytocin and oestradiol

A V Sirotkin, A V Makarevich, M R Corkins¹, J Kotwica², H B Kwon³, J Bulla and L Hetényi

Research Institute of Animal Production, 949 92 Nitra, Slovakia

¹Indiana University, Riley Hospital for Children, Indianapolis, Indiana 46202, USA

²Institute of Animal Production and Food Research, 10-718 Olsztyn-Kortowo, Poland

³Department of Biology, Chonnam National University, Kwangju 500-753, Korea

(Requests for offprints should be addressed to A V Sirotkin, Laboratory of Endocrinology, Research Institute of Animal Production, Hlohovská 2, 949 92 Nitra, Slovak Republic; Email: sirotkin@vuzv.sk)

ABSTRACT

The aim of our *in vitro* experiments was to examine if IGF binding protein (IGFBP)-3 is involved in control of bovine ovarian secretory activity. For this purpose we performed the transfection of bovine granulosa cells with cDNA sense and antisense constructs increasing or inhibiting IGFBP-3 synthesis. The release of IGFBP-3, progesterone, oxytocin, IGF-I and prostaglandins F (PGF) and E (PGE) by control and transfected cells was compared. The transfected ovarian cells were cultured with and without bLH (100 ng/ml), bGH (100 ng/ml), IGF-I (10 ng/ml), oxytocin (10 ng/ml) and oestradiol-17 β (100 ng/ml). The concentration of IGFBP-3 produced was assessed using ligand and western blotting and secretion of progesterone, oxytocin, IGF-I, PGF and PGE was evaluated using RIA/IRMA techniques.

Transfection of cells with the sense IGFBP-3 cDNA construct resulted in the expected increase in IGFBP-3 release, whereas the antisense IGFBP-3 construct induced the expected reduction in IGFBP-3 output. The granulosa cells transfected to overexpress IGFBP-3 had an increase in IGF-I, PGF and PGE release, and a decrease in basal and hormone- or growth factor-induced accumulation of progesterone and oxytocin. The granulosa cells transfected to have reduced IGFBP-3 expression gave primarily significant opposite findings.

The present results suggest the involvement of IGFBP-3 in control of bovine ovarian steroid, peptide hormone, growth factor and prostaglandin release. IGFBP-3 is a physiological stimulator of IGF-I and prostaglandin release and an inhibitor of steroid and peptide hormone output.

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INTRODUCTION

Gonadotrophins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)), growth hormone (GH) and oxytocin regulate ovarian functions by altering insulin-like growth factor I (IGF)-I and IGF binding protein (IGFBP) expression (Erickson 1995, Spicer & Echternkamp 1995, Yoshimura 1998, Schams *et al.* 1999). In non-ovarian cells, the action of IGF-I is modulated by binding to the IGFBPs that determine the bioavailability of IGF-I in a particular tissue. In addition to IGF-I binding

activity, the IGFBPs also have some IGF-I-independent effects. The most ubiquitous IGFBP present in the majority of tissues is IGFBP-3 (Rajaram *et al.* 1997, Rechler & Clemmons 1998). Mammalian ovarian cells have previously been shown to produce both IGF-I and the six classic IGFBPs (Sakal *et al.* 1992, Spicer & Echternkamp 1995, Monget *et al.* 1996, Mihm *et al.* 1997, Yuan *et al.* 1998, Schams *et al.* 1999, Sirotkin & Makarevich 1999). However, the role that a particular IGFBP exerts in control of ovarian functions remains to be elucidated.

There are several reports of the direct influence of IGFBPs on ovarian functions. IGFBPs-2, -3 and -5 have inhibitory effects on oocyte maturation, basal FSH and IGF-I-induced ovulation, and secretion of progesterone and oestradiol by human and rat ovaries (Spicer & Echternkamp 1995, Iwashita *et al.* 1996, Putowski *et al.* 1997, Yoshimura 1998). In cultured porcine granulosa cells, IGFBP-3 inhibited basal IGF-I and FSH-induced release of progesterone (Samaras & Hammond 1995). IGFBP-3 was able to inhibit IGF-I binding, in addition to IGF-I-induced (but not spontaneous) proliferation. IGFBP-3 also inhibited the secretion of progesterone and oestradiol by bovine theca (Spicer *et al.* 1997) and granulosa (Spicer & Chamberlain 1999) cells, but IGF-I was able to prevent these effects. Thus accumulation of IGFBPs in the ovarian structures can, probably primarily through binding of IGF-I, potentially inhibit ovarian proliferation, folliculogenesis, oogenesis and steroidogenesis. To understand the IGF-dependent and -independent effects of IGFBPs, a means of increasing and decreasing IGFBP secretion (without addition of IGF) is therefore necessary. Transfection of ovarian cells with sense and antisense cDNA constructs of an IGFBP can be utilized to examine the effect of increased or decreased IGFBP concentrations on cellular function. This approach has previously been used successfully with non-ovarian cells (Cohen *et al.* 1993, Corkins *et al.* 1995, Park *et al.* 1996).

Ovarian IGFBP production can be stimulated (pig: Grimes *et al.* 1994, cow: Echternkamp *et al.* 1994, rat: Putowski *et al.* 1997) or inhibited (Spicer & Echternkamp 1995, Mihm *et al.* 1997) by gonadotrophins. GH can be either a stimulator (cow: Sirotkin & Makarevich 1999) or an inhibitor (pig: Sirotkin *et al.* 1998) of IGFBP release by ovarian cells. There are reports on both a stimulatory influence (pig: Grimes *et al.* 1994) and a lack of effect (pig: Sirotkin *et al.* 1998) of IGF-I on ovarian IGFBPs. Both a stimulatory effect (cow: Manikkam & Rajamahendran 1997) and no effect (women: Hamori *et al.* 1991) of progesterone on this process have been reported. Prostaglandin F (PGF) treatment can either stimulate (cow: Spicer & Echternkamp 1995) or inhibit (cow: Echternkamp *et al.* 1994) ovarian IGFBP accumulation. Ovarian IGFBP accumulation can be induced by oestradiol (cow: Mihm *et al.* 1997) and dibutyryl cAMP (human: Hamori *et al.* 1991), but not by oxytocin (Sirotkin *et al.* 1998). Thus the ovarian IGFBPs can be both stimulated and inhibited by various substances (gonadotrophins, GH, IGF-I, steroids, PGF, cAMP etc.) that are known gonadotrophic substances. There are indirect indications that these regulators can affect ovarian functions through

alterations in the local IGFBP-3 concentration (Erickson 1995, Spicer & Echternkamp 1995, Schams *et al.* 1999, Sirotkin *et al.* 1998). Nevertheless, the hypothesis of involvement of IGFBPs in the control of ovarian functions and in the mediation of effects of hormones and other ovarian regulators requires direct confirmation.

The first aim of our *in vitro* experiments was to examine the effects of different gonadotrophic substances on ovarian steroid, peptide hormone and growth factor release, by studying the effects of LH, GH, IGF-I, oxytocin and oestradiol on the release of progesterone, oxytocin, IGF-I and PGF and prostaglandin E (PGE) by bovine granulosa cells. The second aim of our studies was to investigate whether IGFBP-3 is involved in the control of – and mediates the effects of – the gonadotrophic substances mentioned above, on ovarian secretory activity. For this purpose, we performed the transfection of bovine granulosa cells with cDNA constructs increasing or inhibiting IGFBP-3 synthesis. We then compared the release of IGFBP-3, progesterone, oxytocin, IGF-I, PGF and PGE between the control and transfected cells cultured with and without LH, GH, IGF-I, oxytocin and oestradiol.

MATERIALS AND METHODS

Gene constructs

The full-length cDNA for rat IGFBP-3 was a gift of Dr S Shimasaki (Whittier Institute for Diabetes and Endocrinology, La Jolla, CA, USA). The 2345-bp EcoRI fragment of the IGFBP-3 cDNA (Shimasaki *et al.* 1989) was ligated into EcoRI-digested pcDNA3 vector (Invitrogen, San Diego, CA, USA). After ligation, the DNA was used to transform DH5- α *Escherichia coli* (Gibco-BRL, Gaithersburg, MD, USA) and colonies were selected for ampicillin resistance. Plasmid minipreps were prepared to permit identification of the appropriate sense and antisense orientation of the cDNA insert (Corkins *et al.* 1995).

Preparation, culture and processing of granulosa cells

Granulosa cells were collected from the ovaries of Holstein cows (2–4 years of age), during the early and middle follicular phase of the oestrous cycle, after slaughter at a local abattoir. The content of 2–5 mm diameter follicles was aspirated and a suspension of granulosa cells isolated by repeated (three times) centrifugation (200 g, 10 min) and pipetting in fresh incubation medium (DMEM-F-12 1:1 mixture

(Sigma, St Louis, MO, USA)) supplemented with 10% bovine fetal serum (Institute of Veterinary Medicine, Brno, Czech Republic) and 1% antibiotic-antimycotic solution (Sigma). Afterwards, the cell population was divided into three groups; each group was transfected with gene constructs listed above using the lipofection reagent, DOTAP (Boehringer Mannheim GmbH, Mannheim, Germany), following the instructions of the manufacturer. A mixture of 15 µg cDNA, 90 µg DOTAP and 6×10^6 cells in 6 ml incubation medium was used for each transfection procedure. Control cells were incubated with DOTAP with 15 µg mixed bases (Gibco-BRL) instead of the cDNA constructs. Thereafter, granulosa cells were washed three times in culture medium and precultured at a concentration of 1×10^6 cells/ml in 2 ml culture medium in Falcon 24-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C under 5% CO₂ in humidified air. After 2 days of preculture, the medium was replaced with medium of the same composition including 10% bovine fetal serum from the Institute of Veterinary Medicine with bovine (b) GH (USDA-bGH-B-1, 100 ng/ml), bLH (USDA-bLH-B-6, 100 ng/ml; both 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 oestradiol (100 ng/ml) from Sigma. Control groups in each transfected and non-transfected group contained either no cells (blank control) or cells given no exogenous treatments. The GH, LH, IGF-I, oxytocin and oestradiol used were of immunological grade. Peptide hormones were dissolved in medium immediately before the experiment. Oestradiol was first dissolved in 50 µl absolute ethanol and then in incubation medium, so that the content of ethanol did not exceed 0.001% of the medium. After 2 days of culture, the medium from wells was gently aspirated and frozen at -18 °C until required for ligand blotting, western blotting and RIA. After culture, cells were gently scraped and pipetted to break the cell clumps. Afterwards, cell numbers and viability were determined by Trypan blue staining and counting in a haemocytometer. 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/IRMA. IGF-I and progesterone were assayed using RIA/IRMA kits from DSL (Webster, TX, USA) according to the instructions of the manufacturer. Oxytocin, PGF and PGE were measured using our RIA

described previously (Cetta *et al.* 1982, Kotwica & Skarzynski 1993, Chang *et al.* 1995). The characteristics of these assays are presented in Table 1.

Protein gel electrophoresis and immunoblotting

Samples of medium (20 µl) were mixed with 10 µl electrophoretic buffer (0.0625 M Tris base, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromophenol blue; all from Sigma) and boiled at 95 °C for 3 min and subjected to SDS-PAGE in 4% and 10% polyacrylamide in stacking and resolving gels respectively at 25 mA constant current, according to the method of Laemmli (1970). The samples were then transferred for 1 h to nitrocellulose membranes (enhanced chemiluminescence (ECL) Hybond, Amersham International plc, Little Chalfont, Bucks, UK) using a semi-dry trans-blotter (Bio-Rad Labs, Richmond, VA, USA). Endogenous peroxidase in samples was quenched by incubation in 3% H₂O₂ for 15 min. Non-specific binding of antiserum was prevented by blocking with 5% blot-qualified BSA (Amersham) in TTBS (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20). Blocked membranes were probed with rabbit polyclonal antibody against human recombinant IGFBP-3 (Upstate Biotechnology Inc., Lake Placid, NY, USA; dilution 1:2000). This antibody has high specificity for IGFBP-3 and only slight (0.1–0.5%) cross reactivity for IGFBPs-2, -4 and -5; cross reactivity with IGFBP-1 was 2%. Preliminary western blotting and immunocytochemical data showed the ability of this antiserum to detect IGFBP-3 not only in human, but also in rat, bovine and porcine biological fluids (data not shown, see also Fig. 2). Membranes were then incubated with a secondary horseradish peroxidase-conjugated antirabbit IgG antibody and visualized using ECL detection reagents and ECL Hyper-film (all from Amersham). Incubation medium without cells was used as a negative control. Rat serum samples (Transduction Laboratories, Lexington, CT, USA) and fluid aspirated from bovine ovarian follicles 2–5 cm in diameter and porcine ovarian follicles 2–3 cm in diameter (separated from cells by centrifugation as described above) were used as positive controls. The molecular weights of fractions were evaluated using a molecular weight calibration kit (14.4–94.0 kDa; Serva, Heidelberg, Germany).

Protein gel electrophoresis and ligand blotting

IGFBP-3 protein was compared by ligand blot analysis as described by Hossenlopp *et al.* (1986) and modified by McCusker *et al.* (1988). Briefly,

TABLE 1. Characteristics of immunoassays used in experiments

Assay	Specificity of assay (cross-reactivity of antiserum)	Sensitivity of assay (ng/ml)	Coefficient of variation (%)	
			Intra- assay	Inter- assay
IGF-I	<0.01% to IGF-II, insulin, proinsulin, EGF, bGH, oxytocin, progesterone, oestradiol	0.027	<3.4	<8.2
Progesterone	<0.001% to cortisol, corticosterone, cortisol, androstenediol, pregnenolone, oestradiol, testosterone	0.12	<13.1	<8.0
Oxytocin	<0.01% to arginine-vasopressin, lysine-vasopressin, arginine-vasotocin, somatostatin	0.003	<10.5	<5.6
PGF	<0.01% to PGA-1, PGA-2, PGB-1, PGB-2 <0.1% to PGE-1, PGE-2, 66% to PGF-1, 100% to PGF-2	0.005	<7.7	<4.3
PGE	<28.0% to PGA-1, <7.0% to PGA-2, <0.6% to PGB-1, <1.4% to PGB-2 <5.0% to PGF-1, <1.5% to PGF-2, 165% to PGE-1 100% to PGE-2	0.015	<7.5	<4.0

EGF, epidermal growth-factor; PGA, PGB, prostaglandins A and B.

80 µl conditioned culture medium or control serum (human and fetal bovine) were mixed with 80 µl electrophoretic buffer mentioned above, and this mixture was electrophoresed in 12.5% polyacrylamide gels in the presence of SDS under non-reducing conditions (Laemmli 1970). After electroblotting onto 0.05 µm BA-75 nitrocellulose (Schleicher and Schuell, Keene, NH, USA), the blots were probed with [¹²⁵I]IGF-II. Signal intensities for radioactive bands on each blot were quantified by use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) with the results reported as arbitrary units.

Statistics

Each experimental group was represented by four culture wells. Assays of hormone and growth factor concentrations in conditioned medium were performed in duplicate. The quantitative data shown are the means of three experiments on granulosa cells from a separate pool of ovaries harvested from 20 animals. The western and ligand blots shown

were obtained in separate experiments: one each, repeated twice. In the RIA/IRMA assays, the value of the blank control was subtracted from the value determined in the conditioned medium to exclude any non-specific background (0–26% of total values). The secretion rates were calculated per 1 × 10⁶ viable cells at the end of culturing/day. Significant differences between the experiments were evaluated by a two-way ANOVA. The treatment effects from the experimental and control groups were compared by Duncan’s multiple range test. Differences from controls with *P*<0.05 were considered significant.

RESULTS

The presence of IGFBPs in culture medium conditioned by bovine granulosa cells was demonstrated by ligand blotting (Fig. 1). The main IGF-binding protein expressed had a relative molecular weight of 45 kDa, characteristic of IGFBP-3. Lipofection of granulosa cells with the

FIGURE 1. Ligand blotting of culture medium conditioned by bovine granulosa cells. Lane 1: control cells (transfection with mixed bases); lane 2: cells transfected with sense IGFBP-3 cDNA construct; lane 3: cells transfected with antisense IGFBP-3 cDNA construct; lane 4: negative control (medium cultured without cells).

sense IGFBP-3 cDNA construct resulted in an increase in expression of IGFBP-3 in the conditioned medium. As presented in Fig. 1, the IGFBP-3 band increased from 172 611 arbitrary units in the control cells to 222 437 arbitrary units in the transfected cells. In contrast, transfection with an antisense IGFBP-3 construct markedly decreased the IGFBP-3 band to an expression of 131 842 arbitrary units.

The presence of IGFBP-3 in bovine granulosa cell conditioned medium was confirmed also by western blotting (Fig. 2). The 45 kDa band, which binds antiserum against IGFBP-3, was observed in rabbit serum and bovine (but not porcine) follicular fluid (positive control), and in conditioned medium after culture of control bovine granulosa cells (transfected with mixed bases), but not in cells transfected with the IGFBP-3 antisense construct or in medium cultured without cells (negative control).

Results of the RIA/IRMAs of the conditioned medium showed that bovine granulosa cells are able to secrete significant amounts of IGF-I, oxytocin, progesterone, PGF and PGE. The assays also showed that release of these substances is altered by transfection of cells with constructs altering IGFBP-3 production, by exogenous hormones and by IGF-I (Figs 3–7).

Progesterone release (Fig. 3) by control cells was significantly decreased by GH, oestradiol, LH and oxytocin treatments, whereas IGF-I dramatically increased it. Transfection of cells with the IGFBP-3 sense construct slightly decreased basal progesterone output and significantly reduced IGF-I-induced progesterone release, but not progesterone

FIGURE 2. Western immunoblotting for IGFBP-3 culture medium conditioned by bovine granulosa cells. Lane 1: negative control (medium cultured without cells); lane 2: cells transfected with antisense IGFBP-3 cDNA construct; lane 3: control cells (transfection with mixed bases); lane 4: positive control I (normal rabbit serum); lane 5: positive control II (bovine ovarian follicular fluid); lane 6: positive control III (porcine ovarian follicular fluid).

secretion under the influence of inhibitors (GH, oestradiol, LH, oxytocin). Introduction of the IGFBP-3 antisense construct resulted in significant increases in basal and oestradiol-inhibited progesterone release, but did not influence the stimulatory effect of peptide hormones on progesterone output.

IGF-I release (Fig. 4) in these experiments was not substantially affected by exogenous GH (its stimulatory influence was statistically insignificant), but oestradiol, LH and oxytocin significantly augmented it. Transfection of cells with the IGFBP-3 sense construct increased basal IGF-I release, but prevented the stimulatory effects of other hormones. Moreover, oestradiol and LH in these cells inhibited IGF-I output. Basal IGF-I release by cells transfected with the IGFBP-3 antisense construct did not significantly differ from the initial concentrations in control cells, but was significantly lower than in cells transfected with the IGFBP-3 sense construct. Moreover, transfection of cells with IGFBP-3 antisense construct significantly inhibited IGF-I release in the presence of GH and oestradiol, but not of other hormones: significant differences between respective groups of transfected and control cells (transfected with mixed bases) were found.

Oxytocin release (Fig. 5) in control cells was not affected by GH, IGF-I or oxytocin, but oestradiol significantly reduced it. Cells transfected with the IGFBP-3 sense construct produced significantly less oxytocin than control cells. Furthermore, in treated cells transfected with the IGFBP-3 sense construct, oxytocin release was reduced in the

FIGURE 3. Progesterone released by cultured bovine granulosa cells. Effect of hormones and growth factor on progesterone release by cultured bovine granulosa cells not transfected (transfection with mixed bases; Control) and transfected with sense (IGFBP-3 sense) and antisense (IGFBP-3 antisense) IGFBP-3 cDNA constructs. C, control (cells cultured without exogenous hormones or growth factor); 1, cells cultured with bGH (100 ng/ml medium); 2, cells cultured with IGF-I (10 ng/ml); 3, cells cultured with oestradiol (100 ng/ml); 4, cells cultured with bLH (100 ng/ml); 5, cells cultured with oxytocin (10 ng/ml). Values are means $\pm$ S.E.M. Significant differences ($P < 0.05$): ^abetween cells cultured with and without exogenous hormones or growth factors, ^bbetween respective groups of transfected and not transfected cells.

IGF-I-treated and increased in the LH-treated groups. No significant differences in basal oxytocin release between the IGFBP-3 antisense-transfected and control groups was detected, although in the control group oestradiol inhibited oxytocin, whereas in the IGFBP-3 antisense transfected cells oestradiol increased oxytocin release over both controls (without oestradiol and without transfection).

PGF release (Fig. 6) in control cells was only at the limit of sensitivity of the assays; however, it was increased several-fold by GH, oestradiol, LH and oxytocin, but not by IGF-I treatment. IGFBP-3 sense construct transfected cells had significantly stimulated basal, GH- and IGF-I-induced PGF output, but not PGF release, in the presence of other hormones. Transfection of cells with IGFBP-3 antisense construct did not significantly affect basal PGF accumulation, but it dramatically reduced hormone-induced PGF release: instead of stimulation under the

FIGURE 4. IGF-I released by cultured bovine granulosa cells. Effect of hormones on IGF-I release by cultured bovine granulosa cells not transfected (transfection with mixed bases; Control) and transfected with sense (IGFBP-3 sense) and antisense (IGFBP-3 antisense) IGFBP-3 cDNA constructs. C, control (cells cultured without exogenous hormones); 1, cells cultured with bGH (100 ng/ml medium); 2, cells cultured with oestradiol (100 ng/ml); 3, cells cultured with bLH (100 ng/ml); 4, cells cultured with oxytocin (10 ng/ml). Values are means $\pm$ S.E.M. Significant differences ($P < 0.05$): ^abetween cells cultured with and without exogenous hormones or growth factors, ^bbetween respective groups of transfected and not transfected cells.

influence of hormones (as in control and IGFBP-3 sense and treated cells), PGF accumulation was blocked at the minimal detectable level.

PGE release (Fig. 7) in control cells in the absence of hormone stimulation was low, near the limit of sensitivity of the assay. GH, oestradiol and oxytocin, but not IGF-I or LH, dramatically enhanced PGE expression. Transfection of cells with IGFBP-3 sense construct significantly increased the basal, IGF-I- and LH-induced PGE release, but not its accumulation, in the presence of other hormones. The IGFBP-3 antisense transfected cells were completely or partially blocked from the stimulatory influence of all hormones on PGE output.

DISCUSSION

The present data suggest that the culture of normal and transfected granulosa cells used in our experiments is suitable for maintenance of their viability and secretory activity, and for studying

FIGURE 5. Oxytocin released by cultured bovine granulosa cells. Effect of hormones and growth factor on oxytocin release by cultured bovine granulosa cells not transfected (transfection with mixed bases; Control) and transfected with sense (IGFBP-3 sense) and antisense (IGFBP-3 antisense) IGFBP-3 cDNA constructs. C, control (cells cultured without exogenous hormones and growth factor); 1, cells cultured with bGH (100 ng/ml medium); 2, cells cultured with IGF-I (10 ng/ml); 3, cells cultured with oestradiol (100 ng/ml); 4, cells cultured with bLH (100 ng/ml). Values are means $\pm$ S.E.M. Significant differences ($P < 0.05$):

^abetween cells cultured with and without exogenous hormones or growth factors, ^bbetween respective groups of transfected and not transfected cells.

the effect of transfection with cDNA constructs mentioned above. This confirms our previous observations (Makarevic & Sirotkin 1996, Sirotkin *et al.* 1998) of the usefulness of serum-supplemented medium for stimulation of basal ovarian secretion and response. Nevertheless, in interpreting the data obtained using serum-supplemented medium, it should be kept in mind that, despite the use of blank controls, serum (which can contain hormones, growth factors and IGFBPs) may affect basal secretory activity and responses of cells, and the metabolism of exogenous and endogenous substances.

Our observations confirm previous reports (Shemesh 1979, Sakal *et al.* 1992, Sirotkin & Nitray 1994, Spicer & Echternkamp 1995, Makarevich & Sirotkin 1996, Sirotkin 1996) of the production of IGFBP-3, IGF-I, oxytocin, progesterone and PGF by cultured bovine granulosa cells. The amounts of these substances produced by cells after the transfection procedures in our experiments are comparable to

FIGURE 6. PGF released by cultured bovine granulosa cells. Effect of hormones and growth factor on PGF release by cultured bovine granulosa cells not transfected (transfection with mixed bases; Control) and transfected with sense (IGFBP-3 sense) and antisense (IGFBP-3 antisense) IGFBP-3 cDNA constructs. C, control (cells cultured without exogenous hormones and growth factor); 1, cells cultured with bGH (100 ng/ml medium); 2, cells cultured with IGF-I (10 ng/ml); 3, cells cultured with oestradiol (100 ng/ml); 4, cells cultured with bLH (100 ng/ml); 5, cells cultured with oxytocin (10 ng/ml). Values are means $\pm$ S.E.M. Significant differences ($P < 0.05$):

^abetween cells cultured with and without exogenous hormones or growth factors, ^bbetween respective groups of transfected and not transfected cells.

those described in non-transfected bovine granulosa cells. Ovarian PGE production has been demonstrated previously in other species (Cetta *et al.* 1982, Chang *et al.* 1995, Sirotkin *et al.* 1998), but this seems to be the first demonstration of the release of this substance by bovine ovarian cells.

Our results support previous evidence of the involvement of GH (Sirotkin & Nitray 1994, Spicer & Echternkamp 1995, Makarevich & Sirotkin 1996, Sirotkin 1996, Sirotkin & Makarevich 1999), IGF-I (Erickson 1995, Spicer & Echternkamp 1995, Sirotkin *et al.* 1998), LH, oestradiol (Wathes 1989, Erickson 1995, Spicer & Echternkamp 1995, Sirotkin *et al.* 1998) and oxytocin (Wathes 1989, Sirotkin *et al.* 1998) in the control of the secretion of IGF-I, oxytocin, steroids and prostaglandins by mammalian ovarian cells. This is the first evidence of the involvement of oxytocin, IGF-I and steroids

FIGURE 7. PGE released by cultured bovine granulosa cells. Effect of hormones and growth factor on PGE release by cultured bovine granulosa cells not transfected (transfection with mixed bases; Control) and transfected with sense (IGFBP-3 sense) and antisense (IGFBP-3 antisense) IGFBP-3 cDNA constructs. C, control (cells cultured without exogenous hormones and growth factor); 1, cells cultured with bGH (100 ng/ml medium); 2, cells cultured with IGF-I (10 ng/ml); 3, cells cultured with oestradiol (100 ng/ml); 4, cells cultured with bLH (100 ng/ml); 5, cells cultured with oxytocin (10 ng/ml). Values are means $\pm$ S.E.M. Significant differences ($P < 0.05$): ^abetween cells cultured with and without exogenous hormones or growth factors, ^bbetween respective groups of transfected and not transfected cells.

in the control of prostaglandin release by bovine ovarian cells.

In bovine (Echternkamp *et al.* 1994, Spicer & Echternkamp 1995, Mihm *et al.* 1997, Khatir *et al.* 1997, Yuan *et al.* 1998), ovine (Spicer & Echternkamp 1995, Monget *et al.* 1996), porcine (Guthrie *et al.* 1995), murine (Wandji *et al.* 1998) and human (Yoshimura 1998) follicles there is a correlation between the IGFBPs-1, -2, -4 and -5 (but not IGFBP-3) concentrations in follicular fluid and follicular size, the expression of atresia, and the ability of follicular fluid to support oocyte maturation. This is an indirect indication of the involvement of the IGFBPs in these processes. However, IGFBP-3 concentrations in the follicular fluid during follicular growth and atresia change little or not at all. Therefore, these processes are associated with or regulated by (or both) changes in

the relative proportions of IGFBPs-2, -4 or -5 to that of IGFBP-3.

Results of our ligand (Fig. 1) and western (Fig. 2) blotting confirm the previous reports (Spicer & Echternkamp 1995, Mihm *et al.* 1997, Khatir *et al.* 1997, Schams *et al.* 1999, Sirotkin & Makarevich 1999) of the release of IGFBP-3 by bovine ovarian cells and do not corroborate other reports (Echternkamp *et al.* 1994, Yuan *et al.* 1998) of no or minimal IGFBP-3 production by these cells. Furthermore, identification of IGFBP-3 in bovine and rabbit, but not in porcine material using immunoblotting in our experiments (Fig. 2) suggests that substantial differences exist in the immunological properties of IGFBP-3 in different species. Our studies also confirmed that the transfection of cultured granulosa cells with an IGFBP-3 sense construct results in an increase in IGFBP-3 production, and that the related IGFBP-3 antisense construct inhibited IGFBP-3 production. These are the first data on the role of ovarian IGFBP-3 in mammals, utilizing a transfection approach to alter the normal levels of expression.

The ovarian cell transfection altered not only IGFBP-3 expression, but also basal IGF-I, oxytocin, progesterone, PGF and PGE concentrations, as observed in our experiments. These findings suggest the involvement of IGFBP-3 in control of the release of these substances. Sense IGFBP-3 cDNA transfection resulted in an increase in basal and hormone-induced IGF-I, PGF and PGE concentrations and a decrease in progesterone and oxytocin concentrations, in cell-conditioned medium. The IGFBP-3 antisense transfection had an opposite effect, at least in the case of progesterone and IGF-I release. Differences between IGFBP-3 sense and antisense cultures were more pronounced than those between transfected and control cells. These data suggest that IGFBP-3 can be a physiological stimulator of IGF-I and prostaglandin release, and an inhibitor of steroid and peptide hormone release, by bovine ovarian granulosa cells. A possible mechanism of action is the protective effect of IGFBP-3 for IGF-I: IGFBP-3 binds IGF-I to prevent its catabolism that has been observed in non-ovarian cells (Rajaram *et al.* 1997, Rechler & Clemmons 1998). The present observations of a change in basal progesterone release in the transfected cells are in agreement with a previous report by Samaras & Hammond (1995) of the antisteroidogenic action of exogenous IGFBP-3 treatment on porcine ovarian cells. This is the first evidence we know of the involvement of IGFBP-3 in mammalian ovarian peptide hormone, growth factor and prostaglandin release.

Our observations indicate that IGFBP-3 can affect not only basal secretory activity, but also the

response of ovarian cells to extra- and intraovarian regulators – gonadotrophin, GH, IGF-I, peptide and steroid hormones. The transfection-induced increase in IGFBP-3 was associated with inhibition of the effect of IGF-I on progesterone and oxytocin, and an augmentation of the influence of IGF-I on PGE, but not on PGF, release. In contrast, the transfection-induced decrease in IGFBP-3 production was associated with prevention of the effect of IGF-I on PGF and PGE, but not on other substances. The ability of IGFBP-3 both to inhibit and to promote IGF-I action may be explained by its ability to bind IGF-I, to reduce its bio-availability but also prevent its biodegradation. However, a possible IGF-I-independent effect of IGFBP-3 should not be excluded. For example, antisense IGFBP-3 transfection in our experiments did not affect basal IGF-I concentration, but prevented the effects of several hormones on the release of IGF-I, oxytocin, PGF and PGE. Moreover, an increase in basal IGF-I secretion induced by the transfection with the IGFBP-3 sense construct was not always associated with a change in the response of cells to hormonal treatments. Thus both an IGF-I-dependent and an IGF-I-independent action of IGFBP-3 can be hypothesised.

Reports on the influence of various hormones and IGF-I on the ovarian release of IGFBP-3 (Hamori *et al.* 1991, Echternkamp *et al.* 1994, Spicer & Echternkamp 1995, Manikkam & Rajamahendran 1997, Mihm *et al.* 1997, Putowski *et al.* 1997, Sirotkin *et al.* 1998, Sirotkin & Makarevich 1999) indirectly indicate that IGFBP-3 might be a potential mediator of the action of these substances on reproductive processes. Reduced expression of IGFBP-3, first used in our experiments, provides new evidence for this hypothesis: transfection-mediated inhibition of IGFBP-3 release almost completely blocked the effects of hormones and growth factors on PGF and PGE release by bovine ovarian cells. However, inhibition of IGFBP-3 failed to prevent fully the action of exogenous substances on IGF-I, oxytocin and progesterone release. This could be due to only a partial blockade of IGFBP-3 release by the transfected cells, so that the IGFBP-3 concentration was sufficiently decreased to block completely the responses of PGF and PGE, but not low enough to inhibit the responses of IGF-I, oxytocin and oestradiol. These observations suggest differences between the ovarian substances in response or sensitivity to IGFBP-3 action and, consequently, a different role for IGFBP-3 in their control. Nevertheless, they suggest that ovarian IGFBP-3 can be a potential mediator of hormonal and IGF-I actions, at least on ovarian prostaglandin production.

The significance and the mechanisms of IGFBP-3 action on the ovary require further detailed studies. Nevertheless, our approach strongly suggested the involvement of IGFBP-3 in the control of ovarian secretory activity – that is, of the release of steroid, peptide hormones, growth factor and prostaglandins. The precise mechanism of IGFBP-3 action probably varies, depending on the particular biological process and its regulation, but the role of IGFBP-3 as a regulator of ovarian secretory activity and as a mediator of the action of hormones and IGF-I on the ovary via IGF-I-dependent or IGF-I-independent (or both) mechanisms are suggested.

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