Do GH, IGF-I and oxytocin interact by regulating the secretory activity of porcine ovarian cells?

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Abstract

The aims of this study on porcine ovarian granulosa cells were to examine the effect of GH on oxytocin (OT), IGF-I and IGF-I receptors, IGF-binding protein-3 (IGFBP-3), progesterone and prostaglandin E (PGE), as well as to determine whether IGF-I and/or OT may be mediators of GH action. The cells were cultured either with porcine GH (pGH) (1 ng/ml to 10 µg/ml or 100 ng/ml only), antiserum against IGF-I (0.1%), antiserum against OT (0.1%) or a combination of GH (10 ng/ml) with antiserum against IGF-I or antiserum against OT (0.1%). The secretion of IGF-I, OT, IGFBP-3, progesterone and PGE was determined using RIA/IRMA, whilst the IGF-I binding sites were measured using a radio-receptor assay.

It was observed that pGH increased the secretion of IGF-I and the abundance of IGF-I binding sites in granulosa cells. Furthermore, GH inhibited OT release, stimulated progesterone and PGE output, but had no

significant effect on IGFBP-3 secretion. Immunoneutralization of IGF-I by antiserum against IGF-I inhibited PGE secretion, but it did not influence progesterone or IGFBP-3 secretion. Binding of OT by antiserum suppressed IGFBP-3, PGE, but not progesterone secretion. Neither immunoneutralization of IGF-I nor OT substantially prevented the effects of GH on progesterone, IGFBP and PGE.

These observations demonstrate the involvement of GH, IGF-I and OT in the control of porcine ovarian secretory activity and the ability of GH to regulate IGF-I and OT production and IGF-I reception. Nevertheless, lack of correlation between the effects of GH, antiserum against IGF-I and antiserum against OT, as well as the inability of blockade of IGF-I or OT to prevent the effects of GH, suggests that IGF-I and OT, despite their dependence on GH, do not mediate GH action on ovarian cells.

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Introduction

Growth hormone (GH) appears to be a regulator of reproductive processes (for reviews see Franks 1998, Schams *et al.* 1999, Hull & Harvey 2000). The involvement of GH in the control of ovarian function is confirmed by the presence of GH receptors on ovarian cells (Kirby *et al.* 1996, Schams *et al.* 1999), and by their ability to respond to GH with changes in proliferation, apoptosis, oocyte maturation, secretion of hormones and growth factors, and production of gonadotrophin receptors (Spicer & Echternkamp 1995, Samaras *et al.* 1996, Franks 1998, Sirotkin *et al.* 1998, Lucy *et al.* 1999, Schams *et al.* 1999). Although these effects have been described in a range of species, pig granulosa are known only to exhibit the effects of GH on insulin-like growth factor-I (IGF-I) and steroid hormones (Spicer & Echternkamp 1995, Samaras *et al.*

1996). The actions of GH on other ovarian substances (IGF-binding proteins (IGFBPs), oxytocin (OT), receptors for IGF-I) have not been described. The first aim of this study was to confirm the action of GH on IGF-I and progesterone secretion, and to examine the effect of GH on receptors for IGF-I, and on OT, IGFBP-3 and prostaglandin E (PGE) release by porcine ovarian granulosa cells.

The local mediators of GH action on ovarian function are not completely understood. There is evidence that GH can influence the reproductive system through both IGF-I-dependent and IGF-I-independent mechanisms (Hull & Harvey 2000). The GH-induced increase in IGF-I and OT secretion, and the similarity of some effects of GH, IGF-I and OT on ovarian cells (women: Sirotkin & Schaeffer 1995; cow: Spicer & Echternkamp 1995, Sirotkin 1995, Sirotkin & Makarevich 1999, Sirotkin *et al.*

Table 1 Characteristics of immunoassays

Assay	Specificity (cross-reactivity)	Sensitivity (ng/ml)	Coefficient of variation (%)	
			Intra-assay	Interassay
IGF-I	<1.9% to IGF-II, <0.01% to insulin, proinsulin, <0.001% to EGF, pGH, OT, progesterone, oestradiol	0.34	<10.0	<18.0
Progesterone	<0.001% to cortisone, corticosterone, cortisol, androstenediol, pregnenolone, oestradiol, testosterone	0.12	<13.1	<8.0
OT	<0.005% to arg-vasopressin, <0.04% to lys-vasopressin, 17% to arg-vasotocin, 22% to des-OT	0.0035	<9.0	<5.0
IGFBP-3	<0.03% to IGFBP-1, -2, -4, -5, -6, IGF-I, -II, pGH, pLH, pFSH, OT	0.5	<3.9	<1.9
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; pLH=porcine luteinizing hormone; pFSH=porcine follicle-stimulating hormone.

2000; pig: Spicer & Echternkamp 1995, Samaras *et al.* 1996, Sirotkin *et al.* 1998, Lucy *et al.* 1999, Schams *et al.* 1999), provide indirect evidence that IGF-I and OT may be mediators of GH action on the ovary. On the other hand, the differences in the effects of GH, IGF-I and OT on ovarian secretory activity, apoptosis and oogenesis (sheep: Wathes *et al.* 1995; rodents: Apa *et al.* 1994, 1996, Chun *et al.* 1996, Hsueh *et al.* 1996; cow: Kirby *et al.* 1996, Izadyar *et al.* 1997; pig: Sirotkin *et al.* 1998), together with the absence of GH stimulation of IGF-I in sheep and bovine ovaries (Wathes *et al.* 1995, Kirby *et al.* 1996, Lucy *et al.* 1999) suggest that GH action is probably not mediated by IGF-I or OT. There is no direct evidence for the involvement of IGF-I and OT in the mediation of GH effects on ovarian cells. Such evidence would be the action of GH on IGF-I and OT and the prevention of GH action on substances, other than IGF-I and OT, after IGF-I and OT blockade. The second aim of our study was to determine, by immunoneutralization, whether IGF-I and/or OT may be mediators of GH action on progesterone, IGFBP-3 and PGE release by porcine ovarian cells.

Materials and Methods

Preparation, culture and processing of granulosa cells

Granulosa cells were aspirated from 3–6 mm diameter follicles from the ovaries of non-ovulated Slovakian white improved gilts (10 months of age, immediately before the start of sexual cyclicity), after slaughter at a local abattoir, and processed as described previously (Sirotkin 1995).

Granulosa cells (1×10^6 cells/ml) were cultured in 2 ml DMEM/Ham's F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic–antimycotic solution (all from Sigma Chemical Co., St Louis, MO, USA) in Falcon 24-well plates (Becton Dickinson, Lincoln Park, NJ, USA) at 37 °C under 5% CO₂ in humidified air. After

2 days of pre-culture the medium was replaced with medium of the same composition. Control groups contained either no cells (blank control) or cells but no exogenous hormones or drugs. Experimental groups received immunological grade porcine GH (pGH) (USDA-pGH-B-1; provided by Dr J A Proudman, USDA Animal Hormone Program, Beltsville, MD, USA).

The effects of GH on the secretion of IGF-I and OT was tested at 1, 10, 100, 1000 or 10 000 ng GH/ml. The effect of GH on other secretions was tested at 100 ng/ml, based on previous observations (Sirotkin *et al.* 1998, Sirotkin & Makarevich 1999). Other experimental groups were treated with rabbit antiserum against IGF-I, kindly provided by Dr A F Parlow (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA), rabbit antiserum against OT, kindly provided by Prof. A P F Flint (University of Nottingham, Sutton Bonington, Leics, UK), both as 0.1% (1:1000) solutions in culture medium, alone or in combination with pGH (100 ng/ml).

The immunological activity of GH and the two antisera at the concentrations used were previously evaluated by RIAs (partial data in Table 1). Solutions (0.1%) of antisera against IGF-I and OT were able to block 72–80% of respective ligand in the medium. All treatments were dissolved in culture medium immediately before experiment. After 2 days of culture, the medium was gently aspirated and frozen at –18 °C to await RIA. The cell monolayer was immediately subjected to radioreceptor assay (RRA). Cell number and viability were determined by Trypan Blue staining and haemocytometer counting. No statistically significant differences in these indices between control and experimental groups were observed.

Immunoassay

Concentrations of IGF-I, OT and PGE were determined in 25–100 µl incubation medium by RIA as described

Figure 1 Effect of pGH (0, 1, 10, 100, 1000 or 10 000 ng/ml) on (a) IGF-I and (b) OT secretion by cultured porcine granulosa cells (RIA/IRMA data). Values are means $\pm$ S.E.M. *Significant difference ($P < 0.05$) compared with control (medium without addition).

previously (Kotwica & Skarzynski 1993, Chang *et al.* 1995, Makarevich & Sirotkin 1999). Progesterone and IGFBP-3 were assayed using RIA/IRMA kits from DSL (Webster, TX, USA) according to the manufacturer's instructions. RIA for IGF-I and IGFBP-3 included an extraction procedure to break the IGF-I/IGFBP complex. All RIA/IRMAs were validated for use in samples of culture medium. Assay characteristics are presented in Table 1.

Figure 2 Effect of pGH (100 ng/ml) on expression of IGF-I binding sites in cultured porcine granulosa cells (RRA data). Values are means $\pm$ S.E.M. *Significant difference ($P < 0.001$) compared with control (medium without addition).

RRA

IGF-I binding sites in cell monolayers were determined by RRA (Linge & Green 1993). Immediately after culture, the cells were rinsed in 2 ml cold PBS and exposed for 3 h at 4 °C to ¹²⁵I-IGF-I (350 000 c.p.m./well), produced in our laboratory by radiolabelling of recombinant human (rh) IGF-I (Sigma) (Makarevich & Sirotkin 1999). They were rinsed again three times with cold PBS, lysed with 100 μ l 0.1 M NaOH, and the radioactivity of the lysate measured. Non-specific binding of ¹²⁵I was assessed by exposing the cells to ¹²⁵I-IGF-I together with excess (0.5 μ g/well) of rhIGF-II (Sigma). Non-specific binding did not usually exceed 12% of the specific binding.

Statistics

Each experimental group was represented by four culture wells. Assays of hormone and growth factor content in incubation medium were performed in duplicate. The data shown are means of values obtained in three separate experiments performed on separate days using separate pools of granulosa cells, each obtained from 20–40 animals. For RIA/IRMA, the values of blank controls (medium cultured without the cells) were subtracted from the values determined in cell-conditioned medium. In RRA, the values of non-specific binding were subtracted from specific binding values. Amounts of hormone secreted and numbers of IGF-I receptors were calculated per 10⁶ viable cells/day. Significant differences in RIA and RRA data between experiments were evaluated using two-way ANOVA. When effects of treatments were revealed, data

were compared by Duncan's multiple range test. The coefficients of variation of replicates within each group did not exceed 18%. Differences from control with $P < 0.05$ were considered as significant.

Results

Analysis of porcine granulosa cells and cell-conditioned medium showed that these cells secreted significant amounts of IGF-I, OT, progesterone, IGFBP-3 and PGE, and possessed IGF-I binding sites.

Addition of GH to the incubation medium at concentrations of 0.01–10 µg/ml induced a significant ($P < 0.05$ at 10 and 100 ng/ml, $P < 0.001$ at 1 and 10 µg/ml) increase in IGF-I secretion at all but the lowest concentration (Fig. 1a). Addition of GH at 100 ng/ml or more significantly reduced OT secretion ($P < 0.05$; Fig. 1b). Furthermore, this treatment significantly ($P < 0.001$) enhanced the number of IGF-I binding sites (Fig. 2). The secretion of progesterone was significantly stimulated by GH ($P < 0.001$) (Fig. 3a).

Antisera against IGF-I or OT did not affect basal or GH-stimulated progesterone secretion, although there was a slight reduction ($P < 0.05$) in GH-stimulated progesterone secretion in the presence of antiserum against OT (Fig. 3a). There was no significant effect of GH or antiserum against IGF-I on the concentration of IGFBP-3. Antiserum against OT significantly ($P < 0.05$) reduced IGFBP-3 secretion in both the presence and the absence of GH (Fig. 3b).

GH significantly ($P < 0.05$) enhanced PGE secretion. Antisera against IGF-I and OT both inhibited ($P < 0.001$ and $P < 0.05$ respectively) basal PGE secretion but did not affect GH-stimulated PGE secretion (Fig. 3c).

Discussion

The data presented in Figs 1 and 3 confirm previous reports (Spicer & Echternkamp 1995, Samaras *et al.* 1996, Sirotkin *et al.* 1998) on the production of IGF-I, OT, IGFBP-3, progesterone and PGE by porcine ovarian cells. However, this is probably the first demonstration of OT secretion by cultured porcine granulosa cells. Figure 2 represents the first evidence for the existence of receptors for IGF-I in porcine ovarian cells. The measured IGF-I binding sites are probably IGF-I receptors rather than

Figure 3 Effect of pGH (100 ng/ml), antiserum against IGF-I (ASI, 0.1%), antiserum against OT (ASO, 0.1%) and combination of pGH (10 ng/ml) with antiserum against IGF-I (0.1%) (GH+ASI) and against OT (0.1%) (GH+ASO) on (a) progesterone, (b) IGFBP-3 and (c) PGE release by cultured porcine granulosa cells (RIA/IRMA data). Values are means $\pm$ S.E.M. *Significant difference ($P < 0.05$) compared with control (medium without additions).

IGFBPs on the cell surface, because these and previous data show that cultured porcine ovarian cells produce relatively little IGFBP (Sirotkin *et al.* 1998), which is unable to bind significant amounts of IGF (Makarevich & Sirotkin 1999).

The actions of antisera against IGF-I and OT on ovarian secretory activity (Fig. 3), which in general are opposite to the effects of IGF-I and OT themselves, confirm previous reports on the involvement of IGF-I (Spicer & Echternkamp 1995, Franks 1998, Sirotkin *et al.* 1998, Lucy *et al.* 1999, Schams *et al.* 1999) and OT (Wathes 1989, Sirotkin *et al.* 1996, 1998) in controlling ovarian secretory activity. The differential effects of antisera against IGF-I and OT on IGFBP-3 (but not on progesterone or PGE) suggest that IGF-I and OT may in some cases influence the ovary independently of each other.

Our observations of GH effects on IGF-I (Fig. 1*a*) and progesterone (Fig. 3*a*) correspond to previous reports of GH action on growth factor and steroid secretion by porcine granulosa cells (Spicer & Echternkamp 1995, Samaras *et al.* 1996). This appears to be the first report of an action of GH on IGFBP-3, OT and PGE secretion, as well as on receptors to IGF-I, in porcine granulosa cells.

The actions of GH on IGF-I and IGF-I binding sites suggest that GH can activate the intraovarian IGF-I system at different sites including secretion and receptor availability. This observation suggests that in pigs as in other species, GH may affect the ovarian IGF-I/IGFBP system. However, in contrast to cattle (Sirotkin & Makarevich 1999) the mechanism appears not to involve IGFBP-3.

Our data suggest that GH is a potent regulator of secretion of other ovarian substances, for example OT, steroids and PGs. They agree with our previous observations of GH effects on OT, steroid and PGE secretion by isolated ovarian follicles (Sirotkin *et al.* 1998).

Analysis of the effects of GH on IGF-I and OT, as well as of the effects of GH and antisera against IGF-I and OT on progesterone, IGFBP-3 and PGE, provides evidence that IGF-I and OT do not mediate the actions of GH on ovarian substances other than IGF-I and OT. First, differences in the dose–response curves of IGF-I and OT, as well as the contrasting effects of GH on IGF-I and OT secretion, indirectly suggest the existence of multiple GH receptors and/or second messenger systems allowing independent control of IGF-I and OT. Secondly, the opposing effects of GH on IGF-I and OT secretion, the lack of correlation between effects of GH and antisera against IGF-I and OT (especially on IGFBP-3 and PGE), as well as the inability of antisera against IGF-I and OT to prevent GH action, strongly suggest that IGF-I and OT do not mediate the effects of GH on the porcine ovary. Thus, GH controls porcine ovarian progesterone, IGFBP-3 and PGE secretion but probably not through local IGF-I and OT. Other mechanisms for this action have been proposed (Kirby *et al.* 1996, Schams *et al.* 1999).

In our studies we attempted to examine whether GH affects IGF-I and OT, whether GH, IGF-I and OT are involved in control of other ovarian substances (progesterone, IGFBP-3, PGE), and whether GH affects these substances via IGF-I and OT. Taken together, our data demonstrate (i) the involvement of GH, IGF-I and OT in the control of porcine ovarian secretory activity, (ii) the ability of GH to influence IGF-I and OT production and reception, and (iii) that GH effects are not mediated by IGF-I or OT.

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