Localization of binding sites for IGF-I, insulin and GH in the sow ovary

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Abstract

Binding sites for IGF-I, insulin, and GH were localized by *in situ* binding of ^{125}I -labelled hormones to the different compartments of the sow ovary. Binding sites for IGF-I were detected in oocytes, granulosa and thecal cells of healthy and atretic follicles as well as in the antrum and the stroma. Competition of ^{125}I -labelled IGF-I with IGF-I, insulin and an analogue of IGF-I (Long R³IGF-I), which allowed discrimination between binding to binding proteins from binding to type-I receptors, suggested that type-I receptors were present in granulosa cells of healthy follicles, whilst binding in other compartments was mainly due to binding proteins. Binding of insulin was revealed in oocytes, granulosa and theca interna cells of healthy preantral and antral follicles, and, to a lesser extent, in

theca externa and stromal cells, and was still observed in granulosa cells of atretic follicles. Labelling with ^{125}I -labelled bovine GH was demonstrated in oocytes, granulosa cells, theca interna cells, and, although less intense, in theca externa and stromal cells. It disappeared in granulosa cells during atresia. Binding sites for GH were detected at all follicular stages, from preantral to preovulatory stages, but the intensity of labelling in granulosa cells was more intense in preantral than in large follicles. These data support the participation of insulin, GH and IGF-I in oocyte maturation, follicular growth and stromal cell function in swine.

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Introduction

Ovarian follicular growth and differentiation is controlled by pituitary gonadotrophins, luteinizing hormone and follicle-stimulating hormone, and by local factors, such as steroids and growth factors. In numerous species, evidence has been provided that nutrition may influence ovarian function, and in pigs some observations suggested that nutritional effects alter folliculogenesis, not only through gonadotrophin-mediated effects, but also through direct effects at the ovarian level (reviewed by Cosgrove & Foxcroft 1996, Cox 1997). Insulin, insulin-like growth factor-I (IGF-I) and growth hormone (GH) have been proposed as metabolic mediators of these latter effects for several reasons. First, their systemic concentrations are closely dependent upon nutrition. Secondly, they may stimulate or inhibit ovulation and increase the ovulation rate when administered to prepubertal or cyclic gilts or to reproductive sows (insulin: Cox *et al.* 1987, Ramirez *et al.* 1997, IGF-I: Okere *et al.* 1996, GH: Kirkwood *et al.* 1988). Thirdly, *in vitro* studies in various species provided evidence that these hormones can alter the activity of follicular cells, either in synergy with gonadotrophins, or alone. Thus, GH accelerates *in vitro* differentiation of cultured granulosa cells, mainly by amplification of gonadotrophin action (Jia *et al.* 1986, Hsu & Hammond 1987), but GH alone may also stimulate steroid production

(Hsu & Hammond 1987, Manson *et al.* 1990). Insulin and IGF-I stimulate granulosa cell mitogenesis and steroidogenesis of follicular cells (Adashi *et al.* 1985). These effects are mediated by specific receptors and these receptors, or their mRNAs, have been detected in the ovaries of numerous species, including the pig (Giudice 1992). However, in this latter species, localization of these receptors in the different ovarian compartments has never been fully described. Thus, the aim of the present study was to localize the binding sites for GH, insulin and IGF-I in follicles and corpora lutea in the female pig.

Materials and Methods

Materials

Large White gilts (INRA, Saint-Gilles, France) had their oestrous cycles synchronized with a progesterone agonist, Regumate (Roussel Uclaf, Romanville, France). Ovaries were removed by laparotomy conducted under general anaesthesia at days 4 (late follicular phase, three sows) or 10 (early luteal phase, two sows) after the last day of treatment. Ovaries of two gestating gilts were removed at slaughter at day 104 of gestation. They were coated with cryoprotectant embedding medium (Tissue-Tek, Miles Laboratories, Elkhart, IN, USA), frozen in liquid nitrogen and stored at -80°C .

Purified bovine GH (bGH) (USDA-bGH-B1; USDA Animal Hormone Program, Beltsville, MD, USA) was iodinated by the chloramine-T method to a specific activity of 40–50 $\mu\text{Ci}/\mu\text{g}$. This radioligand was used rather than porcine GH (pGH) because it has been previously shown that specific binding in porcine tissue was higher with ^{125}I -labelled bGH than with homologous ^{125}I -labelled pGH (Breier *et al.* 1989). bGH was also used as unlabelled ligand. Recombinant human (rh) IGF-I and IGF-II, and Long R³IGF-I (LR³IGF-I), a potent analogue of rhIGF-I, were supplied by GroPep Pty Ltd (Adelaide, SA, Australia). rhIGF-I was iodinated by the chloramine-T method to a specific activity of 150–160 $\mu\text{Ci}/\mu\text{g}$. Iodinated porcine insulin (INSI-TRA, approximate specific activity 300 $\mu\text{Ci}/\mu\text{g}$) was purchased from CIS Bio International (Gif-sur-Yvette, France) and unlabelled insulin from porcine pancreas from Sigma (St Louis, MO, USA).

Binding assays on histological sections

Binding of iodinated hormones was detected by an autoradiographic method adapted from Monget *et al.* (1989). Frozen ovaries were cut into 8 μm thick serial sections with a cryomicrotome. After fixation for 10 min at 4 °C in 4% paraformaldehyde-PBS (0.01 M, pH 7.4) and three subsequent washes in cold PBS, sections were stored at 4 °C for a maximum of 48 h and then circled with a special pen (PAP pen, Labonord, Templemars, France). They were incubated for 30 min at 4 °C in 200 μl incubation buffer, then overnight at 4 °C in 200 μl incubation buffer containing the ^{125}I -labelled ligand (10^5 c.p.m./100 μl). The incubation buffers were Tris 25 mM, CaCl_2 10 mM, BSA 0.5%, NaN_3 0.02%, pH 7.4 for bGH and Tris 50 mM, MgCl_2 5 mM, BSA 0.5%, NaN_3 0.02%, pH 7.4 for IGF-I and insulin. To determine nonspecific binding, for each ligand tested, an adjacent serial section of ovary was incubated with an excess of unlabelled ligand (400, 300 and 200 ng/100 μl for bGH, IGF-I and insulin respectively). To discriminate IGF-I binding to IGF-binding proteins (IGFBPs) from binding to type-I receptor ('IGF-I receptor'), displacements were performed with various ligands. Indeed, it has been demonstrated in several species that IGF-I binding on receptors may be completely displaced by an excess of IGF-I, IGF-II or LR³IGF-I, or partially displaced by a large amount of insulin, whereas IGF-I binding to IGFBPs could not be removed by LR³IGF-I (Francis *et al.* 1992) or insulin, even in large excess (reviewed by Giudice 1992). After incubation, sections were washed three times in PBS, postfixed in 3% glutaraldehyde-PBS, washed in PBS and air dried for 2 h. For autoradiography, they were dipped into Kodak NTB2 emulsion diluted 1:1 with distilled water, air dried and exposed at 4 °C for 4 or 10 days. The emulsion was developed and fixed according to the manufacturer's instructions, then the sections were counterstained with

Harris' haematoxylin solution and mounted. Sections were observed using a Leitz Orthoplan microscope (Leica, Rueil Malmaison, France) in darkfield to see silver grains or in brightfield to see cells. The intensity of staining for each compartment was compared within sections or within experiments.

To assess the quality of the follicles, some sections were fixed in methanol-formaldehyde-acetic acid (80:15:5) and stained with Feulgen reagent. Follicles were classified as early atretic and atretic when few (at least four) or numerous pycnotic granulosa cells were found on the section respectively. When almost all granulosa cells were pycnotic, follicles were classified as late atretic.

Hormone binding to follicles was examined in ovaries taken 4 days after Regumate treatment (during the follicular phase) and binding to corpora lutea in ovaries removed 10 days after the treatment (during the early luteal phase) and during late gestation.

Results

Binding sites for IGF-I

Binding sites for IGF-I were widespread in the ovary. They were detected in all the oocytes examined ($n=20$). They were also observed in granulosa cells and theca interna and externa cells of healthy and atretic follicles as well as in the antrum, in corpora lutea and in the stroma (Figs 1, 2 and 3; Table 1).

Binding of ^{125}I -labelled IGF-I in granulosa cells of healthy follicles was partially displaced by LR³IGF-I (Fig. 2). Binding in thecal cells (interna and externa) of healthy and atretic follicles and in granulosa cells of atretic follicles was not displaced, or only poorly displaced, by LR³IGF-I. In corpora lutea of gestation, IGF-I labelling in large luteal cells was displaced by IGF-I (completely) and IGF-II (partially), and was poorly displaced by LR³IGF-I or by insulin in excess (Fig. 3).

Specific binding sites for insulin

A slight labelling was observed in six out of nine oocytes examined. Labelling was more intense in granulosa and theca interna cells of healthy follicles, and less marked in theca externa and stroma (Fig. 4; Table 1). In atretic follicles, binding was observed in both granulosa cells and thecal cells (Fig. 4). Labelling was present in luteal cells of cyclic or gestating corpora lutea (Fig. 5). No labelling was found in the antrum.

Specific binding sites for GH

Specific labelling was found in granulosa, thecal and stromal cells (Fig. 6, Table 1). All the oocytes observed

Figure 1 Binding of radiolabelled IGF-I to different compartments of the swine ovary. The hormone may bind the type-I receptor and IGF-BPs. (A) Preantral follicles; (B) small antral follicle (2 mm); (C) large antral follicle (7 mm); (D) atretic follicle. (a, b) Total binding; (c) nonspecific binding to these same follicles (these sections were incubated with ^{125}I -labelled IGF-I and an excess of IGF-I (300 ng/100 μl) simultaneously). (a) brightfield; (b, c) darkfield ($\times 74$). An: antrum, G: granulosa cells, T: theca interna cells.

were also labelled ($n=30$, Fig. 6). In healthy follicles, both granulosa and thecal cells presented binding sites for GH (Fig. 6). In early atretic follicles, granulosa cells still presented a clear labelling, which disappeared in late atretic

follicles (Fig. 6). Binding sites were detected in all follicular stages, from preantral to preovulatory stages. However, the intensity of labelling in granulosa cells was more intense in preantral follicles than in large follicles.

Table 1 Binding sites for ¹²⁵I-labelled insulin, IGF-I and bGH in preantral, small (1–3 mm) and large (>5 mm) healthy follicles, in atretic follicles and in corpora lutea during mid-luteal phase or late gestation. Binding sites for IGF-I consist of type-I receptors and IGF-BPs

	IGF-I				Insulin				GH			
	n	Granulosa cells	Theca interna cells	Theca externa cells	n	Granulosa cells	Theca interna cells	Theca externa cells	n	Granulosa cells	Theca interna cells	Theca externa cells
Follicular phase												
Preantral follicle	26	+	+	±	13	+	+	±	73	+	+	–
Small follicle	17	+	+	±	20	+	+	±	30	+	+	±
Large follicle	13	+	+	±	6	+	+	±	11	±	±	±
Early atretic follicle	4	+	+	±	8	+	+	±	8	+	–	–
Atretic follicle	7	+	+	±	14	+	+	±	16	–	–	–
			Luteal cells ^a				Luteal cells				Luteal cells	
Luteal phase	6		+		6		+				Nd	
Gestation	6		+		6		+				Nd	

^aMixed large and small luteal cells.
Intensity of staining: + intense; ± weak; – undetected.

Figure 2 Binding of radiolabelled IGF-I to a healthy medium-sized follicle (3 mm). (a, b) Total binding; (c, d) nonspecific binding to the same follicle (these sections were incubated with ¹²⁵I-labelled IGF-I and an excess of IGF-I (300 ng/100 µl, (c)) or an excess of LR³IGF-I (400 ng/100 µl, (d)) simultaneously). Labelling in granulosa cells was partially displaced by LR³IGF-I. (a) Brightfield; (b–d) darkfield (× 74). An: antrum, G: granulosa cells, T: theca interna cells.

Discussion

To our knowledge, no attempt to localize binding sites for insulin and GH in the different compartments of the swine ovary has been previously reported. Therefore, we have localized simultaneously binding sites for IGF-I, insulin and GH by *in situ* binding on frozen ovarian sections. Our results show the presence of binding sites for IGF-I in

oocytes, granulosa and thecal cells (interna and externa) of preantral follicles, antral healthy follicles and atretic follicles, as well as in the stroma. Since IGF-I binding on granulosa cells of healthy follicles was partially, but clearly, displaced by the IGF-I analogue LR³IGF-I, these binding sites are likely to be, at least in part, type-I receptors. In pigs, previous studies have revealed two classes of specific IGF-I-binding sites in granulosa cells from small, medium

Figure 3 Binding of radiolabelled IGF-I to corpora lutea from late gestation. (a, f) Total binding; (b–e) nonspecific binding (these sections were incubated with ^{125}I -labelled IGF-I and an excess of IGF-I (300 ng/100 μl , (b)), IGF-II (300 ng/100 μl , (c)), LR³IGF-I (400 ng/100 μl , (d)), or insulin (10 μg /100 μl , (e)) simultaneously). (a–e) Darkfield ($\times 77$); (f) brightfield ($\times 144$).

and large follicles (Maruo *et al.* 1988) and the expression of IGF-I receptor mRNAs in granulosa cells of developing and dominant healthy follicles (Zhou *et al.* 1996). In other cell types (thecal cells of healthy and atretic follicles and granulosa cells of atretic follicles) and in the antrum, our data show that IGF-I binding was not displaced by LR³IGF-I, suggesting the presence of binding proteins, rather than of receptors. This result is consistent with the localization of IGF-I receptor mRNAs exclusively in granulosa cells of healthy follicles (Zhou *et al.* 1996). However, it contrasts with the data of Caubo *et al.* (1989), who demonstrated the presence of specific IGF-I receptors in thecal cells from large follicles. Moreover, IGF-I has been demonstrated to modulate *in vitro* steroidogenesis of porcine thecal cells (Caubo *et al.* 1989, Engelhardt *et al.* 1991). Using our method, we cannot exclude that a minor population of IGF-I binding in thecal cells and in apoptotic granulosa cells was due to specific receptors. Concerning

IGFBPs in swine ovaries, the expression of IGFBP-2 and -4 mRNAs has been reported in granulosa and thecal cells throughout the follicular development (Zhou *et al.* 1996), and the presence of IGFBP-2, -3 and -4 has been demonstrated in follicular fluid, IGFBP-2 being most related to atresia and IGFBP-3 to preovulatory maturation (Mondschein *et al.* 1991, Grimes *et al.* 1994).

The labelling with IGF-I observed in corpora lutea, poorly displaced by LR³IGF-I or insulin, indicates the presence of large amounts of binding proteins and low levels of receptors in corpora lutea. Similarly, low expression of IGF-I receptor mRNAs and high expression of IGFBPs have been reported in porcine corpora lutea from the luteal phase (Zhou *et al.* 1996).

We demonstrated the presence of binding sites for insulin in the ovaries of pigs. Since insulin is known to bind insulin receptors with high affinity and type-I receptors with low affinity, and to have no affinity for type-II

Figure 4 Binding of radiolabelled insulin to different compartments of the swine ovary. (A) Preantral follicle; (B) small antral follicle (2 mm); (C) large antral follicle (5 mm); (D) atretic follicle. (a, b) Total binding; (c) nonspecific binding on the same follicle (these sections were incubated with ^{125}I -labelled insulin and an excess of insulin (200 ng/100 μl) simultaneously). (a) Brightfield; (b, c) darkfield ($\times 76$). An: antrum, G: granulosa cells, T: theca interna cells.

receptors and IGFBPs (reviewed by Giudice 1992), and since unlabelled insulin completely competed for the labelled ligand (present data), these binding sites represent

mainly specific receptors for insulin. They were localized in stroma and in oocytes, granulosa and thecal cells (interna and externa) of preantral, small antral and large follicles and

Figure 5 Binding of radiolabelled insulin to corpora lutea. (A) During the mid-luteal phase; (B) during late gestation. (a, b) Total binding; (c) nonspecific binding (these sections were incubated with ^{125}I -labelled insulin and an excess of insulin (200 ng/100 μl) simultaneously). (a) Brightfield; (b, c) darkfield ($\times 84$). S: stromal cells, L: luteal cells.

in luteal cells. Similarly, insulin receptors or corresponding mRNAs have been reported in human ovary in stroma, granulosa and theca cells and oocytes of growing follicles (Poretsky *et al.* 1985, El-Roeiy *et al.* 1993, Samoto *et al.* 1993), as well as in rat and human luteal cells (Ladenheim *et al.* 1984, Samoto *et al.* 1993). In pigs, the presence of insulin receptors has been previously investigated only in granulosa cells and their presence has been detected (Rein & Schomberg 1982, Otani *et al.* 1985). A role for insulin has been described in follicular and luteal steroidogenesis (Veldhuis *et al.* 1983, Otani *et al.* 1985, Caubo *et al.* 1989, Morley *et al.* 1989), in oocyte maturation (Tsafiri & Channing 1975) and in glucose metabolism of granulosa cells (Otani *et al.* 1985, Weber & LaBarbera 1988). In our study, atretic follicles also presented a clear labelling in granulosa and thecal cells. In women, insulin receptor

mRNAs have been detected in pycnotic granulosa cells (El-Roeiy *et al.* 1993), whereas no immunoreactivity for insulin receptor has been revealed in atretic follicles (Samoto *et al.* 1993). The presence of insulin receptor in granulosa and theca interna of atretic and healthy follicles, as well as in corpora lutea of both stages, may suggest that this receptor is constitutively expressed in pigs.

We demonstrated that bGH binding sites presented a widespread distribution in the swine ovary. The use of bGH for *in situ* binding avoids confusion between somatotrophic and lactogenic sites, since bGH binds only GH sites without affinity for prolactin binding sites (Posner *et al.* 1974, Ranke *et al.* 1976). The method, however, did not allow unequivocal distinction between binding to GH receptor and binding to GH-binding proteins (GHBP). The observation of a single mRNA transcript encoding for

Figure 6 Binding of radiolabelled bGH to different compartments of the swine ovary. (A) Preantral follicle; (B) small antral (2 mm)+large antral follicles (5 mm); (C) preantral+antral healthy (3–4 mm, lower)+atretic (5 mm, upper) follicles. (a, b) Total binding; (c) nonspecific binding on the same follicle (these sections were incubated with ^{125}I -labelled bGH and an excess of bGH (400 ng/100 μl) simultaneously). (a) Brightfield; (b, c) darkfield ($\times 77$). An: antrum, G: granulosa cells.

GH receptor in porcine liver and muscle supports the hypothesis that GHBP arise from GH receptors by proteolysis (Schnoebelen-Combes *et al.* 1996). This suggests that the presence of GHBP is related to the presence of GH receptors. Moreover, ^{125}I -labelled bGH specific binding has been observed in microsomal membrane proteins of whole ovaries and Scatchard analysis has revealed the presence of a single binding site of high affinity ($K_a = 2.8\text{--}3.4 \times 10^9$ l/mol, data not shown). We may therefore argue that binding sites detected in the present study are, at least in part, GH receptors.

Our data show binding sites for bGH in follicular cells, as well as in oocytes of preantral and small antral follicles. The presence of GH receptor mRNAs has been recently reported in oocytes of preantral and small antral follicles in the ewe (Eckery *et al.* 1997) and the cow (Kölle *et al.* 1998). While receptors for GH or mRNAs have been previously localized in the corpus luteum of all species tested (in rats, Lobie *et al.* 1990; in heifers, Lucy *et al.* 1993a; in humans, Sharara & Nieman 1994; in pigs, Yuan & Lucy 1996), various data have been reported concerning follicular localization. In the present study, GH binding sites were detected in granulosa and thecal cells of follicles from the preantral to the preovulatory stage. In heifers, Lucy *et al.* (1993a) observed neither immunoreactivity nor mRNAs for GH receptors in follicles of any size, whereas Kölle *et al.* (1998) reported the expression of GH receptor mRNAs and protein in oocyte and granulosa cells of primary and secondary follicles and in the cells of the cumulus oophorus in tertiary follicles. In humans, mRNAs coding for GH receptor were detected in granulosa cells of antral and dominant follicles, but not in preantral follicles, theca interna and externa cells, oocytes or stroma (Sharara & Nieman 1994). In adult rats, only moderate immunoreactivity for GH receptors appeared in follicles (Lobie *et al.* 1990). In pigs, Yuan & Lucy (1996) failed to detect any GH receptor mRNAs in small antral follicles in porcine ovary on day 10 of the oestrous cycle, whilst in sheep Eckery *et al.* (1997) reported mRNAs for GH receptors in granulosa cells of small antral follicles.

In cattle, Webb *et al.* (1994) have suggested that the increase in the number of small- or medium-sized follicles following administration of GH (Gong *et al.* 1991, Lucy *et al.* 1993b) was more likely due to the increase in either IGF-I, insulin or both than to a direct effect of GH on follicles. Administration of GH to cyclic gilts has been demonstrated to increase ovulation rate (Kirkwood *et al.* 1988) and the number of medium-sized, but not of small-sized, antral follicles (Echternkamp *et al.* 1994). The presence of GH binding sites in follicles suggests that, in gilts, GH effects may be mediated by GH receptors and not necessarily by IGF-I action at the ovarian level. Cattle and pigs may thus differ with respect to the action of GH on follicular growth. In the present study, the intensity of GH binding on granulosa cells seemed to be inversely related to the follicular size, being strong in preantral and

small antral follicles and barely detectable in preovulatory follicles. Moreover, these binding sites are undetected in atretic follicles. This may imply that GH has a direct role, particularly important in early stages of folliculogenesis, when follicular growth does not require the action of gonadotrophins (Driancourt *et al.* 1995).

In conclusion, present data support the participation of IGF-I, insulin and GH in the process of follicular growth and suggest that their action is, at least in part, mediated through their specific receptors.

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