Involvement of the transcription factor STAT1 in the regulation of porcine ovarian granulosa cell functions treated and not treated with ghrelin

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Abstract

The aim of our *in vitro* experiments was to study the role of the transcription factor STAT1 and the hormone ghrelin in controlling porcine ovarian function. The effects of treatment with ghrelin (0, 1, 10, 100 ng/ml), transfection-induced overexpression of transcription factor STAT1, and their combination on apoptosis (expression of apoptosis-related peptides caspase-3, BAX and anti-apoptotic peptide BCL2), proliferation (expression of proliferating cell nuclear antigen PCNA, proliferation-associated protein kinase MAPK/ERK1,2) and release of the hormones progesterone (P₄), prostaglandin F (PGF) and oxytocin (OXT) in cultured porcine ovarian granulosa cells was evaluated using RIA, immunocytochemistry and SDS-PAGE–western immunoblotting. It was found that ghrelin, when given alone, increased the expression of proliferation-associated PCNA and MAPK/ERK1,2, decreased the accumulation of apoptosis-related substances caspase-3, BAX, BCL2, decreased P₄, and increased PGF and OXT release. Ghrelin tended to promote accumulation of STAT1 in both control and transfected cells, although in transfected cells ghrelin at 1 ng/ml decreased STAT1 accumulation. Transfection of porcine granulosa cells by a gene construct encoding STAT1 promoted the expression of STAT1 and apoptosis-related-BAX but the expression of BCL2 did not, and decreased the accumulation of proliferation-associated MAPK/ERK1,2 but not that of PCNA. It also promoted PGF and OXT but not P₄ release. Overexpression of STAT1 reversed the effect of ghrelin on STAT1, PCNA, PGF, OXT (from stimulatory to inhibitory), BCL2, P₄ (from inhibitory to stimulatory), prevented ghrelin effect on caspase-3 and BAX, but did not affect ghrelin's effect on MAPK/ERK1,2 expression. These results suggest that ghrelin directly affects porcine ovarian cells function – stimulates proliferation, inhibits apoptosis and affects secretory activity. Furthermore, they demonstrated the involvement of the transcription factor STAT1 in controlling these functions, the promotion of some markers of apoptosis (BAX), inhibition of some markers of proliferation (MAPK/ERK1,2) and stimulation of PGF release. Finally, the obtained data failed to demonstrate that STAT1 is involved in mediating the action of ghrelin on ovarian cell functions. *Reproduction* (2009) **138** 553–560

Introduction

Searching for new extra- and intracellular regulators of reproductive processes is important for both theoretical and practical reasons. One such extracellular regulator could be the recently described hormone ghrelin, produced by stomach and other tissues. Ghrelin can inhibit secretory activity (Barreiro & Tena-Sempere 2004, Tena-Sempere 2005, 2008) of rat testicular cells and proliferation of rat prostatic cells (Yeh *et al.* 2005), and may be involved in the control of cell proliferation in ovarian and testicular tumours. Recently it was demonstrated that it can directly control the function of

ovarian cells by stimulating proliferation; inhibiting apoptosis; promoting steroid hormone, insulin-like growth factor-I (IGF1) and vasotocin secretion (chicken, Sirotkin & Grossmann 2006; pig, Rak & Gregoraszczyk 2008); and stimulating prostaglandins F (PGF) and PGE (pig, Sirotkin *et al.* 2008a), progesterone (P₄) and IGF1 (rabbit, Sirotkin *et al.* 2008b) release.

Intracellular signalling substances can be involved in controlling ovarian function and in mediating the effects of hormones. Among these substances, the role of some protein kinases, transcription factors and their targets (apoptosis and proliferation-related molecules) have been elucidated (Machino *et al.* 2003, Mittler *et al.* 2004).

The transcription factor STAT1 can affect the proliferation and apoptosis of both ovarian and non-ovarian cells. It can increase apoptosis in human cardiomyocytes (Stephanou 2004) and granulosa cells (Benifla *et al.* 2002), and stimulate the proliferation of human T leukaemia lymphoma cells (Takemoto *et al.* 1997) and hamster ovarian cells (Kaszukska 2000). The involvement of STAT in controlling secretory activity in ovarian and non-ovarian cells has not yet been demonstrated.

Furthermore, activation of STAT1 phosphorylation after addition of leptin to human keratinocytes (Groschl *et al.* 2005), and after LH and insulin treatment of rat ovary (Carvalho *et al.* 2003), indicates that STAT1 can potentially mediate the effects of some hormones on ovarian cells. Nevertheless, there is no direct evidence for an involvement of STAT in mediating hormone action. Such evidence could take the form of a modification of hormone action by changes in a transcription factor. Interrelationships between STAT1 and other hormones (e.g. ghrelin) have not been studied. It remains unknown whether STAT can mediate ghrelin action.

The role of STAT1 in regulating ovarian cell function and in mediating the effects of hormones (including ghrelin) in the ovary remains insufficiently studied. The aim of our *in vitro* studies was to examine 1) the action of ghrelin on proliferation, apoptosis and secretory activity of mammalian (porcine) ovarian cells, 2) the role of transcription factor STAT1 in control of these functions, and 3) the interrelationships between ghrelin and STAT1. For this purpose we have studied the effect of ghrelin treatment, transfection of ovarian cells with a gene construct encoding STAT1, and their combination on the expression of STAT1, markers of apoptosis (BAX, caspase), the antiapoptotic peptide BCL2, markers of proliferation (MAPK/ERK1,2 and proliferating cell nuclear antigen (PCNA)) and hormone secretion (P_4 , PGF and oxytocin (OXT)) in cultured porcine ovarian granulosa cells.

Results

Ovarian cells contained markers of apoptosis and proliferation, STAT1 and they secreted the three assayed hormones. In addition, after transfection, granulosa cells produced EGFP, indicative of successful cell transgenesis (Fig. 1).

SDS-PAGE–western immunoblotting: effect of ghrelin and transfection with gene construct for STAT1 on the presence of STAT1 within ovarian granulosa cells

Presence of STAT1 in ovarian granulosa cells was demonstrated. Transfection of cells with gene construct for STAT1 induced overexpression of the *STAT1* gene, shown as a dramatically enhanced accumulation of STAT1 in the lysate of cells cultured either with or without ghrelin. Ghrelin tended to promote

Figure 1 Presence of green fluorescent protein EGFP (green fluorescence) as a marker of successful transfection in cultured porcine ovarian granulosa cells. Magnification $\times 400$.

accumulation of STAT1 in both control and transfected cells, although in control cells these changes were poorly expressed, and in transfected cells ghrelin at 1 ng/ml decreased STAT1 accumulation (Fig. 2).

Immunocytochemical analysis: effect of ghrelin and transfection with STAT1 gene construct on the expression of apoptosis- and proliferation-related substances within ovarian granulosa cells

Immunocytochemical analysis demonstrated the presence of apoptosis-related (caspase-3, BAX, BCL2) and proliferation-related (PCNA, MAPK/ERK1,2) substances.

Ghrelin at 1 and 100 ng/ml, but not 10 ng/ml, significantly decreased the percentage of cells containing caspase-3. Transfection of cells with a gene construct for STAT1 stimulated expression of caspase-3. Furthermore, it was able to prevent the inhibitory action of ghrelin on this parameter (Fig. 3).

Ghrelin at 1 and 10 ng/ml significantly decreased the proportion of BAX-positive cells, but at a higher concentration (100 ng/ml) stimulated BAX expression. Transfection with *STAT1* gene increased the proportion of BAX-positive cells. Overexpression of STAT1 did not modify the inhibitory influence of ghrelin on BAX, but prevented the stimulatory effect of ghrelin (100 ng/ml) on this parameter (Fig. 4).

Ghrelin at 1 and 10 ng/ml, but not 100 ng/ml, decreased BCL2 expression. Transfection with gene construct encoding STAT1 did not affect BCL2 expression but it did prevent the inhibitory effect of ghrelin on BCL2 (Fig. 5).

Figure 2 Effect of ghrelin (0, 1, 10, 100 ng/ml medium) on accumulation of STAT1 in porcine granulosa cells subjected and not subjected to transfection with gene construct encoding STAT1 (SDS-PAGE–western immunoblotting). Samples from the corresponding groups obtained in different experiments were pooled before electrophoresis. Housekeeping protein is not shown. Molecular weight of fractions is indicated on the left.

Figure 3 Influence of ghrelin on expression of caspase-3 in cultured porcine granulosa cells subjected (Overexpression of STAT1) and not subjected (Control) to transfection with gene construct encoding STAT1. Data from immunocytochemistry: a, shows a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells; b, shows significant ($P < 0.05$) differences between ghrelin (1, 10 or 100 ng/ml) treated and control (0 ng/ml) cells.

Ghrelin stimulated PCNA expression at all concentrations used. Transfection that induced overexpression of STAT1 did not affect PCNA, but it reversed the effect of ghrelin on PCNA from stimulatory to inhibitory (Fig. 6).

Ghrelin had a stimulatory effect on expression of MAPK/ERK1,2 at all concentrations used. Transfection with STAT1 inhibited expression of MAPK/ERK1,2. Overexpression of STAT1 did not modify the effect of ghrelin on MAPK/ERK1,2 (Fig. 7).

RIA: effect of ghrelin and transfection with STAT1 gene construct on hormones secretion

Analysis of the culture medium after cell culture showed that granulosa cells secreted hormones P_4 , PGF and OXT. Ghrelin at 1 and 10 ng/ml, but not 100 ng/ml, significantly decreased P_4 secretion. Transfection with the *STAT1* gene construct did not affect P_4

Figure 4 Influence of ghrelin on expression of BAX in cultured porcine granulosa cells subjected (Overexpression of STAT1) and not subjected (Control) to transfection with gene construct encoding STAT1. Data from immunocytochemistry: a, shows a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells; b, shows significant ($P < 0.05$) differences between ghrelin (1, 10 or 100 ng/ml) treated and control (0 ng/ml) cells.

secretion, but it reversed the effect of ghrelin on P_4 from inhibition to stimulation (Fig. 8).

Ghrelin at all concentrations markedly stimulated PGF and OXT secretion. Transfection with the gene construct encoding STAT1 increased both secretions and reversed the effect of ghrelin from stimulation to inhibition (Figs 9 and 10).

Discussion

The role of ghrelin in control of apoptosis, proliferation and secretory activity in porcine ovarian cells

We have shown that ghrelin inhibits expression of the apoptosis-related markers, caspase-3 and BAX. Our observations correspond with the ability of ghrelin to inhibit apoptosis in chicken ovarian cells (Sirotkin & Grossmann 2006). These observations suggest that ghrelin can suppress apoptosis in ovarian tissues through these pro-apoptotic markers in different species. On the other hand, the inhibitory effect of ghrelin on the expression of anti-apoptotic BCL2 (which binds BAX) suggests that ghrelin may have some indirect pro-apoptotic effect. The net effect of ghrelin is probably just anti-apoptotic because not only BAX (which could be neutralised by BCL2), but also caspase accumulation was reduced after ghrelin treatment.

The clear ghrelin-induced stimulation of PCNA and MAPK/ERK1,2 suggests that ghrelin can promote proliferation through stimulation of PCNA and MAPK/ERK1,2 accumulations. These data are in line with related data concerning the proliferation-stimulating effect of ghrelin on testicular tumours (Garcia *et al.* 2007), some non-reproductive (prostatic cancer) cells (Yeh *et al.* 2005), avian (Sirotkin & Grossmann 2006) and porcine (Rak & Gregoraszczuk 2008) ovarian cells, but not with a report on the inhibitory action of ghrelin on proliferation of cultured Leydig cells (Tena-Sempere 2005).

Figure 5 Influence of ghrelin on expression of BCL2 in cultured porcine granulosa cells subjected (Overexpression of STAT1) and not subjected (Control) to transfection with gene construct encoding STAT1. Data from immunocytochemistry: a, shows a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells; b, shows significant ($P < 0.05$) differences between ghrelin (1, 10 or 100 ng/ml) treated and control (0 ng/ml) cells.

Figure 6 Influence of ghrelin on expression of PCNA in cultured porcine granulosa cells subjected (Overexpression of STAT1) and not subjected (Control) to transfection with gene construct encoding STAT1. Data from immunocytochemistry: a, shows a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells; b, shows significant ($P < 0.05$) differences between ghrelin (1, 10 or 100 ng/ml) treated and control (0 ng/ml) cells.

The anti-apoptotic and pro-proliferative action of ghrelin observed in our experiments suggest that this hormone can be a physiological stimulator of ovarian folliculogenesis and/or inhibitor of ovarian follicle atresia. The inhibitory effect of ghrelin on onset of rat puberty previously reported (Tena-Sempere 2008) indicates that the net effect on the hypothalamo-hypophysial-gonadal system and the biological role of this hormone can change during sexual maturation. Furthermore, the inhibitory influence of calorific intake on ghrelin production and the influence of ghrelin on reproductive functions confirm the hypothesis (Barreiro & Tena-Sempere 2004, Tena-Sempere 2005, 2008, Garcia *et al.* 2007) that ghrelin can be a mediator of the effects of nutrition on reproductive processes.

The stimulatory action on PGF and nonapeptide hormone secretion correspond generally with data obtained using cultured chicken (Sirotkin & Grossmann

Figure 7 Influence of ghrelin on expression of MAPK/ERK1,2 in cultured porcine granulosa cells subjected (Overexpression of STAT1) and not subjected (Control) to transfection with gene construct encoding STAT1. Data from immunocytochemistry: a, shows a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells; b, shows significant ($P < 0.05$) differences between ghrelin (1, 10 or 100 ng/ml) treated and control (0 ng/ml) cells.

2006) and porcine (Sirotkin *et al.* 2008a) ovarian cells, but not with previous reports of the stimulatory action of ghrelin on P_4 and IGF1 release in rabbit ovarian cells (Sirotkin *et al.* 2008b) and on lack of effect of ghrelin on P_4 release in porcine granulosa cells (Sirotkin *et al.* 2008a). Therefore, ghrelin controls ovarian functions in a species-specific manner. Furthermore, comparison results of our previous (Sirotkin *et al.* 2008a) and present studies suggest that some effects of ghrelin on porcine granulosa cells in a different series of experiments can be different. Such alterations in ghrelin action could be due to the initial state of ovarian cells affected by season, nutrition and other environmental factors.

P_4 , OXT and PGF are known to control of ovarian luteogenesis, uterine contractions and the immune response (Mitchell & Schmid 2001, Lewis 2004), while steroid hormones and IGF1 are known promoters of ovarian folliculogenesis (Hillier 2000, Berisha & Schams 2005). It is possible that ghrelin acting through P_4 , OXT, IGF1, steroids and PGF mediates effect of nutrition on these processes.

Understanding ghrelin action on the ovary can be potentially useful for promotion of animal and human reproductive processes and for treatment of some metabolic and reproductive disorders. For example, in women, some indicators of polycystic ovary syndrome (plasma testosterone, leptin, insulin resistance etc.) negatively correlate with plasma ghrelin levels (Mitkov *et al.* 2008), while many of these indicators decreased after ghrelin treatment (Fusco *et al.* 2007).

The role of STAT1 in control of apoptosis, proliferation and secretory activity in porcine ovarian cells

The increased accumulation of STAT1 observed in cells transfected with the gene construct encoding STAT1 indicates the overexpression of this transcription factor

Figure 8 Influence of ghrelin on progesterone release by cultured porcine granulosa cells subjected (Overexpression of STAT1) and not subjected (Control) to transfection with gene construct encoding STAT1. RIA data: a, shows a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells; b, shows significant ($P < 0.05$) differences between ghrelin (1, 10 or 100 ng/ml) treated and control (0 ng/ml) cells.

Figure 9 Influence of ghrelin on prostaglandin F release by cultured porcine granulosa cells subjected (Overexpression of STAT1) and not subjected (Control) to transfection with gene construct encoding STAT1. RIA data: a, shows a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells; b, shows significant ($P < 0.05$) differences between ghrelin (1, 10 or 100 ng/ml) treated and control (0 ng/ml) cells.

in these cells. Further observations demonstrated the usefulness of such cells as a new model for understanding the role of transcription factors in reproduction. The comparison of transfected and non-transfected cells cultured without ghrelin (0 ng/ml) suggests that overexpression of STAT1 is associated with increased apoptosis (expression of caspase-3, BAX, but not of anti-apoptotic BCL2). Our data corresponds with that of Scarabelli *et al.* (2006) on the ability of STAT1 to promote apoptosis in human myocardiocytes. This is the first report of an involvement of STAT1 in controlling apoptosis in ovarian cells.

Our experiments demonstrated the ability of STAT1 to inhibit ovarian cell proliferation. Transfection with STAT1 decreased the expression of two markers of proliferation – PCNA and MAPK/ERK1,2. Previously, only indirect indications of an involvement of STAT1 in controlling proliferation in hamster ovarian (Kaszubska

Figure 10 Influence of ghrelin on oxytocin release by cultured porcine granulosa cells subjected (Overexpression of STAT1) and not subjected (Control) to transfection with gene construct encoding STAT1. RIA data: a, shows a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells; b, shows significant ($P < 0.05$) differences between ghrelin (1, 10 or 100 ng/ml) treated and control (0 ng/ml) cells.

2000) and human non-ovarian cells (Takemoto *et al.* 1997), based on activation of JAK/STAT systems in these cells after treatment with mitogens, were reported. Our observations provide the first direct evidence for an anti-proliferative action of STAT1 in ovarian cells.

Our data on the ability of STAT1 to control P_4 , PGF and OXT secretion in porcine granulosa cells represent the first demonstration of an involvement of this transcription factor in controlling ovarian secretory activity. Previously, only indirect evidence (activation of STAT1 during increased secretion of pro-opiomelanocortin by hypophysial corticotrophs) was available for the involvement of STAT1 in controlling hormone secretion.

Taken together, our observations that STAT1 can influence both intracellular substances (caspase-3, BAX, MAPK/ERK1,2, PCNA) and the secretion of PGF and OXT is the first direct demonstration of the importance of this transcription factor in controlling ovarian cell proliferation, apoptosis and hormone secretion. These observations demonstrate that STAT1 can inhibit ovarian functions, acting as a suppressor of ovarian follicular cell proliferation and, perhaps, of resulting follicular growth, and as a promoter of ovarian cell apoptosis and resulting follicular atresia. Furthermore, STAT1 can stimulate ovarian PGF and OXT, anti-luteal and anti-conception factors (Wathes 1989, Mitchell & Schmid 2001, Lewis 2004). Therefore, STAT1 can be considered to be a negative regulator of reproductive processes and mediator of unknown extracellular factors inhibiting these processes.

Is STAT1 involved in mediating the effects of ghrelin on porcine ovarian cells?

Activation of the JAK/STAT system in some non-ovarian cells in response to leptin, LH and insulin (Carvalho *et al.* 2003) provides indirect evidence that STAT may mediate the effect on some hormones from the ovary. Direct evidence, especially in relation to ovarian cells, was absent. We postulated 1) that a putative mediator of hormone action should be influenced by the hormone, 2) that changes in this mediator should mimic the effect of the hormone, and 3) that changes in this mediator should modify the effect of the hormone.

First, some slight stimulatory influence of ghrelin on the accumulation of STAT1 was demonstrated in our experiments, although it remains unknown whether ghrelin influenced not only total, but also phosphorylated/activated STAT1. Secondly, both ghrelin and cell transfection with the STAT1 gene construct had a similar influence on release of hormones (but not on other parameters). Thirdly, transfection-induced overexpression of STAT1 was able not only to affect basal ovarian cell functions, but also to modify the effect of ghrelin on some markers of apoptosis (BCL2, caspase-3, BAX), proliferation (PCNA) and hormone secretion (P_4 , PGF, OXT). This can be viewed as the first evidence

Figure 11 Hypothetical targets of ghrelin and STAT1 action in porcine ovary. +, stimulatory influence; −, inhibitory influence; ?, possible but not demonstrated influence.

that STAT1 can be involved in mediating ghrelin action at least on ovarian secretory activity. Nevertheless, over-expression of STAT1 did not mimic the effects of ghrelin on cell proliferation and apoptosis, and it did not promote, but rather prevented or reversed the effect of ghrelin on ovarian cells. It is not to be excluded that STAT may not (or not only) mediate ghrelin’s effect, but may affect common intracellular signal transduction pathways modifying the endocrine response of ovarian cells to ghrelin. It remains unknown whether STAT1 is required for ghrelin to function in the ovary. Such evidence could be provided by experiments with down-regulation of STAT1. Therefore, the hypothesis that STAT mediates ghrelin action, in contrast to evidence for the involvement of ghrelin and STAT1 *per se* in reproduction, requires further confirmation.

Taken together, our results suggest 1) that ghrelin can be a regulator of the key porcine ovarian functions of apoptosis, proliferation and secretion; 2) that the transcription factor STAT1 can be involved in regulation of apoptosis, proliferation and secretion; and finally 3) the obtained data failed to demonstrate that STAT1 is involved in mediating the action of ghrelin on ovarian cell functions. Possible interrelationships between ghrelin and STAT1 in the control of ovarian processes are shown in Fig. 11.

Materials and Methods

Preparation, culture and processing of ovarian cells

Ovaries of non-cycling prepubertal gilts, 180 days of age, were obtained after slaughter at a local abattoir. Ovaries were washed several times (5 s each) in sterile 0.9% NaCl and in 95% alcohol. Granulosa cells were aspirated by syringe and sterile needle from follicles 3–5 mm in diameter, suspended in DMEM/F-12 1:1+2% FCS (all from Sigma), and washed twice by centrifugation for 10 min at 200 *g* and resuspension in DMEM/F-12+2% FCS. Two kinds of plasmids were multiplied and used for transfection of granulosa cells. Expression vector for STAT1 pRC/CMV-derived construct containing an insertion for resistance to ampicillin was kindly provided by Dr S Shapiro (Rockefeller University, New York, NY, USA). Reporter plasmid pEGFP-N1 for enhanced green fluorescent protein (EGFP) and resistance to kanamycin was provided by Clontech. Plasmids were multiplied as previously described (Sirotkin *et al.* 2008a, 2008b).

An experimental group of cells was transfected with the EGFP plasmid and STAT1 plasmid. A control group of granulosa cells was transfected by a gene constructs encoding EGFP and the same plasmid vector without STAT1. Transfection was preformed by using transfection reagent Roti Fect (Carl Roth, Karlsruhe, Germany) according to the manufacturer’s instructions.

After transfection, granulosa cells (1×10⁶ cells/ml) were cultured in DMEM/F-12 supplemented with 15% FCS and 1% antibiotic–antimycotic solution (all from Sigma) in Falcon 24-well plates (Becton Dickinson, Lincoln Park, NJ, USA), 2 ml medium per well, and in Chamber slides (Nunc Inc., Naperville, TN, USA), 200 µl medium per well, at 38 °C and 5% CO₂ in humidified air. After 2 days pre-culture, the medium was replaced with medium of the same composition. In addition, cells transfected either with gene construct encoding EGFP construct (control) or with that encoding EGFP+STAT1 were treated with 0, 1, 10 or 100 ng/ml of ghrelin (biological grade; Peptides International Int., Louisville, KY, USA). Ghrelin was dissolved in culture medium immediately before experiment. After 2 days of culture, the medium from 24-well plates was aspirated and frozen at −18 °C to await RIA. Cells in 24-well plates were multiple frozen at −78 °C to await western blotting. Cells in chamber slides were washed in 700 µl well ice-cold PBS (5 min), fixed in paraformaldehyde (4% in PBS, pH-7.2–7.4), and held at 4 °C to await immunocytochemistry.

Table 1 Characteristics of immunoassays used in experiments.

Substance assayed	Specificity of assay (cross-reactivity of antiserum)	Sensitivity of assay (ng/ml)	Coefficient of variation	
			Intraassay	Interassay
Progesterone	<0.001% to cortisol, corticosterone, cortisol, androstenediol, pregnenolone, oestradiol, testosterone	0.12	13.1	8.0
PGF	<0.01% to PGA1, PGA2, PGB1, PGB2, <0.1% to PGE1, PGE2, 66% to PGF1, 100% to PGF2	0.003	10.5	5.6
Oxytocin	<0.01% to arginine–vasopressin, lysine–vasopressin, arginine–vasotocin, somatostatin	0.12	13.1	8.0

At this time, cell numbers and viability were determined by Trypan blue staining and counting in a haemocytometer. Viability was 70–80%. No statistically significant differences in these indices between control and experimental groups were observed.

Immunoassays

Concentrations of P₄, PGF and OXT were determined by RIA in 25–100 µl samples of incubation medium. P₄, PGF and OXT were assayed by our own RIA/EIA systems (Kotwica & Skarzynski 1993, Skarzynski *et al.* 1999, Duras *et al.* 2005). Determination of PGFM in medium reliably reflects PGF_{2α} secretion ($r=0.92$; $P<0.001$) from cultured cells (Skarzynski *et al.* 1999).

The characteristics of these assays are presented in Table 1.

Immunocytochemical analysis

The presence of BAX, caspase-3, BCL2, MAPK/ERK1,2 and PCNA was demonstrated by immunocytochemistry (Osborn & Isenberg 1994) using primary rabbit or mouse antibodies against total BAX (Chemicon, San Diego, CA, USA; dilution 1:1000), cleaved caspase-3 (BD Trans Lab, Eagle Point, OR, USA; dilution 1:500), BCL2 (BD Trans Lab; dilution 1:500) MAPK/ERK1,2 (BD Trans Lab; dilution 1:500) and PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:250). Secondary porcine polyclonal antibodies against rabbit or mouse IgGs, labelled with HRP (Sevac, Prague, Czech Republic; dilution 1:1000) and 3,3'-diaminobenzidine reagent (Boehringer Mannheim GmbH, Mannheim, Germany) were used for the visualization of primary antibody. The presence of immunoreactivity in the cells was determined by one observer by using light microscopy. The percentage of cells containing visible antigen was counted. Cells processed without the primary antibody were used as negative control.

Presence of cells containing EGFP was detected using a fluorescence microscope.

SDS-PAGE–western immunoblotting

Expression of transcription factor STAT1 was detected by western blotting according to Laemmli (1970) in the presence of SDS under non-reducing conditions by using first rabbit polyclonal antibodies against total STAT1 (BD Trans Lab; 1:500), GAPDH (loading control, not shown; Santa Cruz Biotechnology; 1:500) and secondary porcine peroxidase-conjugated polyclonal antibody against rabbit IgG (DAKO, Carpinteria, CA, USA; 1:1000). Positive signals were visualised using ECL detection reagents and ECL Hyper-film (all from Amersham) and quantified by densitometry. Groups of cells without ghrelin addition (0 ng/ml) and without STAT1 transfection (transfected with EGFP) were used as a negative control. As housekeeping protein GAPDH and corresponding antibody (BD Trans Lab, 1:500) was used (not shown). MW was determined by kit (14.4–94.0 kDa; Serva, Heidelberg, Germany).

Statistical analysis

The data shown are the means of values obtained in three separate experiments performed on separate days with separate groups of granulosa cells, each obtained from 15 to 17 animals.

RIA

Each experimental group was represented by four culture wells. Assays of hormone content in the incubation medium were performed in duplicate. The rates of substance secretion were calculated per 10⁶ viable cells/day.

Immunocytochemistry

In each chamber (3 per group), 1000 cells were scored. The percentage of cells containing antigens in different groups of cells was calculated.

SDS-PAGE–western immunoblotting

Samples intended for SDS-PAGE–western immunoblotting (8 samples × 3 experiments = 24 samples per group) were pooled before processing.

Significant differences between the experiments were evaluated by using two-way ANOVA. When effects of treatments were revealed, data from the experimental and control groups were compared by Wilcoxon–Mann–Whitney multiple range test (Systat Software, GmbH, Erkrhart, Germany). Differences from control at $P<0.05$ were considered as significant.

Declaration of interest

No conflict of interest that would prejudice the impartiality of this research.

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