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Involvement of transcription factor p53 and leptin in control of porcine ovarian granulosa cell functions

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Abstract

The aim of our *in vitro* experiments was to examine the role of transcription factor p53 and the metabolic hormone leptin, in controlling basic functions (proliferation, apoptosis and secretory activity) of ovarian cells, as well as involvement of p53 in mediating or modulating actions of leptin, on ovarian cells. Porcine ovarian granulosa cells, transfected and non-transfected with a gene construct encoding p53, were cultured with leptin (at concentrations of 0, 1, 10 or 100 ng/ml). Accumulation of p53 and of apoptosis-related (bax) and proliferation-related (PCNA, cyclin B1) substances was evaluated by SDS–PAGE-western blotting. Secretion of progesterone (P4) was measured by RIA. Transfection with the *p53* gene construct promoted accumulation of this transcription factor within cells. It also stimulated expression of bax (which can be thought of as a marker of apoptosis), and reduced accumulation of proliferation-related substances PCNA and cyclin B1. Overexpression of p53 resulted in reduced P4 secretion. Leptin, when added alone, increased accumulation of p53, bax and PCNA, decreased accumulation of cyclin B1 and had no effect on P4 secretion. Transfection of cells with *p53* gene construct reversed effects of leptin on cyclin B1 and induced stimulatory effects of leptin on P4 release, but did not modify leptin action on p53, bax and PCNA. These multiple effects of the *p53* gene construct on granulosa cells, cultured with and without leptin, (i) demonstrate that leptin can be involved in control of porcine ovarian cell proliferation, apoptosis and expression of p53, but not on P4 release; and (ii) confirm involvement of p53 in promoting apoptosis and suppression of proliferation and P4 secretion in these cells. (iii) The similarity of p53 and leptin's actions on bax and cyclin B1, and inability of p53 to further promote leptin action on this parameter suggest that p53 can be a mediator of leptin's action on ovarian cell apoptosis. (iv) On the other hand, p53 can modulate, but probably not mediate the effects of leptin on ovarian cell proliferation and P4 release.

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Introduction

The well-known transcription factor p53 has been shown to be an important inducer of apoptosis and blocker of proliferation in different cell types (1,2). Under conditions of stress, it induces G_1/S cell-cycle arrest (through transcription factors p21 and c-MYC) and G_2/M arrests [through cyclin B and CDC2 kinase (3)]. It can activate mitochondrial caspases, which cleave MAP kinases [activators of CDC2 kinases and cell cycle at G_2/M (4)] and induce apoptosis-related events (2,5). Regarding the role of p53 in controlling secretory activity, in non-ovarian cells, p53 is able to suppress growth factor secretion [VEGF (6)] and insulin-like growth factor binding proteins (7), and promote vasopressin and catecholamine secretion (8).

Presence of p53 has been demonstrated in mammalian (9–12) and avian (13,14) ovarian cells. In those of pig (11) and chicken (14) its expression has been shown to be associated with apoptosis and atresia of ovarian follicles, indicating involvement of p53 in control of these processes. Indeed, in rat and macaque ovarian granulosa cells, it has been shown to promote apoptosis and basal progesterone (P4) and pregnenolone secretion (1,15), but not cell proliferation (15). Transfection of porcine granulosa cells with a *p53* gene construct promoted their apoptosis, P4 release and inhibition of proliferation. It also affected prostaglandin and oxytocin secretion, and response of the

cells to FSH (16). However, evidence of the role of p53 in controlling ovarian function is limited and requires further validation.

Interrelationships between p53 and hormonal regulators of ovarian functions remain to be investigated. There is evidence that p53 not only regulates basal ovarian function directly but also mediates the effects of hormones. Accumulation of p53in ovarian granulosa cells can be either promoted [chicken: (13)], suppressed [pig: (12)] or not affected [pig: (16)] by ghrelin. Expression of p53 can be induced by gonadotropin [women: (9,10); pig: (16)] and leptin [pig: (12,17)], although in some cases, leptin reduced p53 accumulation in ovarian cells [chicken: (14)]. Moreover, blockade of p53 prevented hCG-induced progestagen, but not oestradiol, secretion by human granulosa cells (15). Although p53 is controlled by FSH, and p53 can modify FSH and ghrelin's effects on porcine ovarian cells, it has been demonstrated that p53 is not a mediator of FSH and ghrelin's actions (16). It remains unknown whether other hormones, including leptin, affect ovarian function by increase in p53 accumulation. Such a mediating role of p53 would be demonstrated by stimulation of p53 by hormone/s and similar, but not cumulative, action of hormone/s and p53.

The aim of the present study was to examine the role of p53 in controlling apoptosis, proliferation and secretory activity of porcine ovarian cells, cultured with and without leptin. We also investigated whether p53 would either mediate or modulate action of leptin on the ovary. For these purposes, we studied (i) influence of transfection with a gene construct encoding p53, on markers of apoptosis (accumulation of bax), proliferation (expression of PCNA, cyclin B1) and secretory activity (secretion of P4), and (ii) effects of the hormone leptin on p53 accumulation, and on selected parameters as listed above and (iii) ability of the *p53* gene construct to mimic or to modify effects of leptin on porcine ovarian granulose cells. For this purpose, we examined effects of *p53* transfection, leptin treatment and their combination on accumulation of p53, bax, PCNA, cyclin B1 (analysed by SDS–PAGE-western blotting) and P4 release (measured by RIA).

Materials and methods

Preparation, culture and processing of ovarian cells

Ovaries of non-cycling pubertal gilts, 180 days of age, were obtained after slaughter, from a local abattoir and were subjected to transfection and culture as described previously (16). 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 Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 + 2% foetal calf serum (all from Sigma, St Louis, MO, USA), and washed twice by centrifugation for 10 min at 200 ***g***, and re-suspension in DMEM/F-12 + 2% FCS. Three kinds of plasmid were subcloned from plasmid pcDNA3/pc53-SN3 and used for transfection of granulosa cells: an expression vector for p53 construct containing an insertion for resistance to ampicillin (University of Dundee, Dundee, UK), a 'scramble' control plasmid vector without p53 insertion (University of Dundee) and a marker reporter plasmid pEGFP-N1 for green fluorescent protein EGFP, and resistance to kanamycin (Clontech, Mountain View, CA, USA). The wild type pc53-SN3 construct containing 1.8 Kb insert of 13 copies of p53 RE 5′-CCTGCCTGGACTTGCCTGG-3′ has been described previously (18). These plasmids were multiplied by the following procedures: bacteria *Escherichia coli*, stem DH5α were cultured in LB medium consisting of 1l H_2O, 10 g tryptone, 5 g yeast autolysate and 10 g NaCl; pH was brought to 7.0 with NaOH. One millilitre of LB medium was inoculated into one colony of *E. coli* stem DH5α and incubated at 37 °C, × 100 g. Ten millilitres of LB medium was inoculated into 100 μl night bacterial culture and incubated for 3 h at 37 °C, 220 rpm. Cell suspensions were cooled for 10 min and centrifuged at 4000 rpm for 10 min at 4 °C. Cells were then re-suspended in 10 ml ice-cold 100 mM $CaCl_2$. The cell suspension was centrifuged at × 200 g for 10 min at 4 °C. Sedimented cells were re-suspended in 2 ml ice-cold 100 mM $CaCl_2$. Aliquots (200 μl) of competent cells were re-suspended in cold Eppendorf tubes (Greiner Bio-One, Longwood, FL, USA) and were placed in a refrigerator at 4 °C.

To 10 aliquots (200 μl) of competent cells in each tube, 50 ng DNA (10 μl) was added to be cloned, mixed and incubated on ice for 30 min. Tubes were preheated in a water bath at 42 °C for 90 s and quickly cooled on ice for 1–2 min. Thereafter, 0. 8 ml SOC medium was added to each tube and incubated at 37 °C for 45 min. A proportionate quantity of bacteria were inoculated on selective LB medium with agar (15 g of agar in 1L LB medium) in a Petri dish (Gama a. s. České Budějovice, Czech Republic) and incubated at 37 °C for 16 h. Five millilitres of LB medium with ampicillin (Gibco BRL,. Paisley, UK) for p53- related plasmids, and kanamycin (Gibco) for EGFP-N1, were inoculated into one colony of transformed cells and incubated overnight at 37 °C and × 100 g. Cell suspensions were centrifuged at × 2000 g. for 10 min at 4 °C and recombinant plasmids were isolated using a Nucleo-Spin Plasmid kit (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions.

Each experimental group of cells was transfected with the gene construct encoding transcription factor p53.

Control groups of granulosa cells were transfected either by the same vector, but with no plasmid vector insertion, or by a gene construct encoding EGFP (to validate efficiency of cell transfection). Transfection was performed using transfection reagent Roti Fect (Carl Roth, Karlsruhe, Germany), according to the manufacturer's instructions.

After transfection, granulosa cells (1×10^6 cells/ml) were cultured in DMEM/F-supplemented with 10% calf foetal serum and 1% antibiotic-anti-mycotic solution (all from Sigma) in Falcon 24-well plates (Becton Dickinson, Lincoln Park, NJ, USA), 2 ml medium per well, at 38 °C and 5% CO_2, in humidified air. After 2-days pre-culture, medium was replaced with fresh medium of the same composition. In addition, cells transfected either with gene construct encoding EGFP + construct without p53 (control) and those encoding EGFP + p53, were treated with 0, 1, 10 or 100 ng/ml of human recombinant leptin (NHPP, Torance, CA, USA), biological grade. The hormone was dissolved in culture medium immediately before experimentation commenced. After 2 days culture, media from 24-well plates was aspirated and frozen at −18 °C to await RIA. Cells were frozen at −78 °C, to await western blotting. Cell numbers and viability were then determined by trypan blue staining and were counted using a haemocytometer. Viability was 70–80%. No statistically significant differences in these indices between control and experimental groups were observed.

SDS–PAGE – western immunoblotting

Expressions of p53, bax, PCNA and cyclin B1 were detected by western blotting, as according to Laemmli (19), in the presence of SDS under non-reducing conditions, by using mouse monoclonal antibodies against p53 (1:500; Chemicon, Temecula, CA, USA), bax, PCNA (1:500; BD Trans Lab, Eagle Point, OR, USA), cyclin B1 (1:500; Chemicon) and GAPDH (loading control, not shown, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). These antibodies cross-reacted with corresponding antigens of human, rat, mouse and yeast origin, respectively. Secondary polyclonal porcine peroxidase conjugated polyclonal antibody against mouse immunoglobulin was purchased (1:1000; Dako, Carpinteria, CA, USA). Positive signals were visualized using ECL detection reagents and ECL Hyper-film (all from Amersham) and quantified by fraction densitometry. Groups of cells without hormone addition (0 ng/ml) and without p53 transfection (transfected with EGFP and plasmids without p53) were used as negative controls. As housekeeping protein, GAPDH and corresponding antibody (1:500; BD Trans Lab) were used (not shown). MW was determined using the appropriate kit (14.4–94.0 kDa; Serva, Heidelberg, Germany).

Radioimmunoassay

Concentrations of progesterone (P4) were determined in 50 µl incubation medium using RIA kit from DSL (Webster, TX, USA) according to the manufacturer's instructions. Assay sensitivity was 0.12 ng/ml and cross-reactivities of antiserum to pregnenolone, and rostenediol, testosterone, oestradiol and cortisol, were <0.001%. Intra- and inter-assay coefficients of variation did not exceed 8% and 13% respectively.

Statistical analyses

Each series of experiments was performed three times. Data shown are means of values obtained in these three separate experiments performed on separate days with separate groups of granulosa cells, each obtained from 15 to 20 animals.

SDS–PAGE-western immunoblotting. Samples intended for SDS–PAGE-western immunoblotting (4 samples × 3 experiments = 12 samples per group), were pooled before processing.

RIA. Each experimental group was represented by four culture wells, that is, each value represents means of 4 wells × 3 experiments = 12 replicates. Assays of hormone concentration in incubation medium were performed in duplicate. Values of blank controls (serum-supplemented medium incubated without cells) were subtracted from specific values determined by RIA, in cell-conditioned medium, to exclude non-specific background (<10% of total values). Rates of secretion were calculated per 10^6 viable cells/day.

Differences between experiments were evaluated using two-way ANOVA. When effects of treatments were revealed, data from experimental and control groups were compared using Student's *t*-test with Sigma Plot 9. 0 software (Systat Software, GmbH, Erkhart, Germany). Differences from control at $P < 0.05$ were considered significant.

Results

The cells after culture expressed markers of apoptosis, proliferation and p53 and secreted the assayed P4. Presence of intracellular p53, bax, PCNA and cyclin B1 was demonstrated by SDS–PAGE-western blotting (Fig. 2). Furthermore, RIA showed secretion of P4 by these cells (Fig. 3).

Control groups of cells transfected with a plasmid DNA vector containing the reporter gene for EGFP, synthetized EGFP, more than 50% of cells had positive signal, revealed using fluorescent microscopy; this indicated

successful transfection with reporter plasmid encoding EGFP (Fig. 1). In addition, SDS–PAGE-western immunoblotting revealed that transfection of cells with p53 cDNA construct significantly increased accumulation of p53 within these cells (Fig. 2, see below), indicating efficient transfection of cells with plasmid encoding p53, too.

Transfection of cells with the gene construct for p53 induced over-expression of *p53* gene, shown as enhanced accumulation of p53 in lysate of cells cultured either with or without leptin. Additions of leptin like *p53* gene, promoted accumulation of p53 in both control and transfected cells (Fig. 2). Transfection-induced over-expression of p53 was associated with increased accumulation of bax. Leptin, added alone, also increased bax. Transfection of cells with *p53* gene constructs did not substantially modify the effect of leptin on bax (Fig. 2).

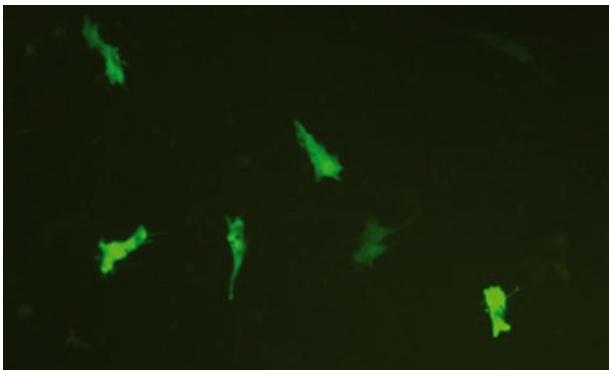

Figure 1. Presence of green fluorescent protein EGFP (green fluorescence) as a marker of successful transfection in cultured porcine ovarian granulosa cells. Magnification ×800. Fluorescence microscopy.

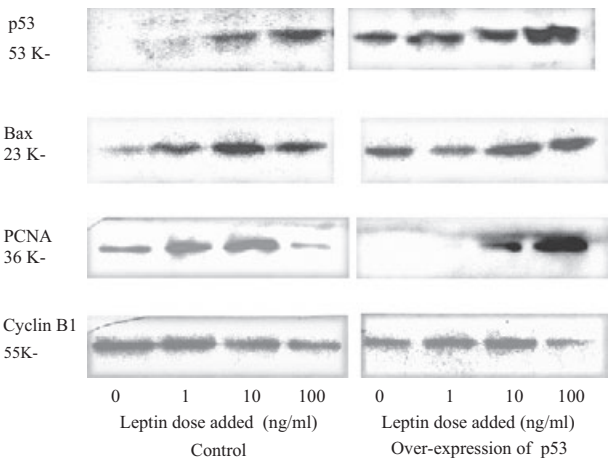

Figure 2. Influence of leptin (0, 1, 10, 100 ng/ml medium) on accumulation of p53, bax, PCNA and cyclin B1 in porcine granulosa cells, transfected or non-transfected with gene construct encoding p53 (SDS–PAGE – western immunoblotting). Molecules and their MWs are indicated on the left.

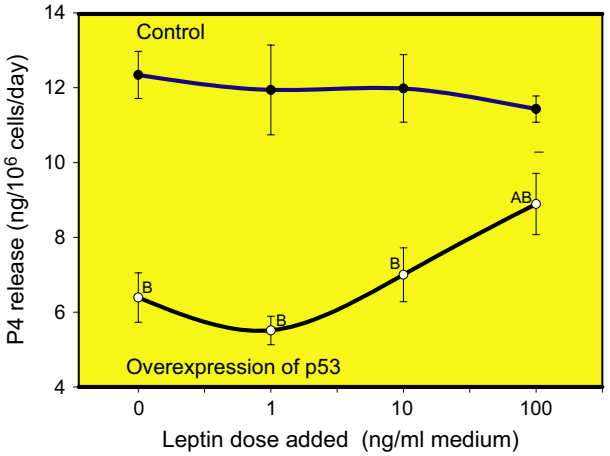

Figure 3. Influence of leptin on progesterone secretion by cultured porcine granulosa cells, transfected or non-transfected with the gene construct encoding p53. RIA data. 'A' effect of leptin, significance ($P < 0.05$), difference between hormone-treated (100 ng/ml) and control (0 ng/ml) cells; 'B' effect of the p53 construct, significance ($P < 0.05$), difference between corresponding groups of transfected and non-transfected cells.

Transfection with p53 decreased accumulation of PCNA. Leptin increased PCNA accumulation in control cells when added at 1 and 10 ng/ml, but decreased it when added at 100 ng/ml. In transfected cells, it increased PCNA accumulation when added at concentrations 10 or 100 ng/ml (Fig. 2). Transfection with *p53* decreased accumulation of cyclin B1. Leptin decreased it in control cells when added at concentrations of 10 or 100 ng/ml. In transfected cells, leptin increased cyclin accumulation at 10 ng/ml, but decreased it at 100 ng/ml (Fig. 2).

Over-expression of p53 induced decrease in P4 secretion. Leptin treatment of control cells did not affect this parameter, but at 100 ng/ml, it increased P4 secretion from transfected cells (Fig. 3).

Discussion

Western immunoblotting demonstrated a dramatic increase in accumulation of p53 in cultured ovarian granulosa cells transfected with the gene construct encoding p53, indicating over-expression of this transcription factor in these cells. Comparison of transfected and non-transfected cells cultured without hormone suggests that over-expression of p53 is associated with increased apoptosis (expression of the apoptosis-related protein, bax). Our data confirm the role of p53 as promoter of apoptosis, previously shown in non-ovarian (2,5) and ovarian (1,11,15,16) cells. These data suggest involvement of p53

in promotion of ovarian cell apoptosis and apoptosis-related atresia and selection of ovarian follicles.

The inhibitory influence of p53 over-expression on accumulation of PCNA and cyclin B1 observed in our experiments is the first demonstration of anti-proliferative activity of this transcription factor in the ovary. Previously, anti-proliferative action of p53 has been described only in non-ovarian cells (3,4) and in porcine ovarian granulose cells (16), but not in macaque ovarian cells (15). In our previous experiments, p53 overexpression suppressed accumulation cyclin B1, a crucial proliferation protein (16). Present studies have demonstrated the inhibitory influence of p53 overexpression on two proliferation proteins – cyclin B1 and PCNA. Ability of p53 to suppress both PCNA (promoter of S phase of the cell cycle) and cyclin B1 (promoter of G2 phase of mitosis) suggests that p53 blocks the ovarian cell cycle at these two checkpoints. These observations suggest involvement of p53 in promotion of ovarian cell apoptosis, in inhibition of ovarian cell proliferation and therefore in suppression of resulting ovarian follicle growth.

The inhibitory effect of p53 on P4 secretion, observed in the present experiments, demonstrates that p53 can be a potent regulator of ovarian secretory activity. It confirms previous reports on inhibitory action of p53 ovrexpression on rat (1) and porcine (16) ovarian cell steroidogenesis. Action of p53 on ovarian P4 suggests its involvement in control of ovarian cell luteinization, as P4 is considered to be an important regulator of this process (9,20,21).

The present observations of leptin's effects confirm previous reports (12,22–24) on pro-apoptotic and pro-proliferation action of this hormone, and on absence of leptin's effects on P4 secretion from cultured rat, human and porcine granulosa cells. The ability of leptin to promote accumulation of both proteins related to apoptosis and cyclin B1, to proliferation, has been reported previously (16). This however, is the first evidence that leptin can stimulate expression of other proliferation-related protein, PCNA. Ability of leptin to induce accumulation of both PCNA (S-phase of the cell cycle) and of cyclin B1 (G_2 phase) suggests that leptin can be a promoter of transition of these two main phases of mitosis. Furthermore, the stimulatory effect of leptin on both apoptosis and proliferation observed here and previously (16) suggests that leptin can promote ovarian cell and follicle turnover. Absence of any leptin effect on P4 release in normal porcine granulose cells confirms our previous observations (16) and suggests that leptin is probably not involved in control of ovarian cell luteinization. On the other hand, ability of leptin to inhibit P4 secretion from p53-transfected cells suggests that leptin can prevent ovarian cell luteinization and that this effect requires cooperation between leptin and p53. Ability of p53 to modify leptin's effect suggests that p53 can affect local production or reception of leptin, its post-receptor mediators of action, or that p53 can be even one of such post-receptor mediators.

It remains unknown whether p53 can mediate these effects of leptin on ovarian cells. As postulated above and in our previous studies (16), p53 could be considered to be a mediator of hormone action if (i) the hormone affects p53, and (ii) p53 mimics or modifies effects of the hormone. Regarding the first requirement, previous studies have demonstrated stimulatory [pig (12,17)] or inhibitory [chicken (14)] action of leptin on p53 accumulation in ovarian cells in different species. A stimulatory effect of leptin has also been observed in our experiments. Concerning the second requirement, effects of p53 and hormone have not previously been studied in the same experiment, although ability of a p53 blocker to prevent gonadotropin function has been described (15). In our experiments, p53 had some similar effects to those of leptin (on bax and cyclin B1, but not on PCNA and P4). Furthermore, p53 was able to promote effects of leptin on bax, suppress its action on PCNA, induce leptin action on P4 and not affect leptin's action on cyclin B1. Evaluation of these results according to the two criteria listed above shows that leptin does not meet the requirements for p53 as mediator of leptin action on *all* ovarian functions. Nevertheless, it is possible that p53 mediates effects of leptin on *some* ovarian parameters, especially on accumulation of apoptosis-related bax. Studies of consequences of p53 deficiency *in vivo* or *in vitro* might provide additional evidence concerning the mediatory role of p53, nevertheless, the ability of p53 to modify leptin's action, as observed in our experiments, suggests functional interrelationships between this transcription factor and this metabolic hormone in control of ovarian cell apoptosis, proliferation, secretory activity and related events (follicular growth, differentiation, selection, luteinization and atresia).

Taken together, these observations of effects of the *p53* gene construct on five parameters of granulosa cell activity, in the presence and absence of leptin (i), demonstrate that leptin can be involved in control of porcine ovarian cell proliferation, apoptosis, secretory activity and expression of p53, (ii) confirm involvement of p53 in promoting apoptosis and in suppressing cell proliferation and P4 secretion from these cells and (iii) suggest that p53 can be a mediator of leptin action on ovarian cell apoptosis. Moreover, (iv), p53 can modulate, but probably not mediate, effects of leptin on ovarian cell proliferation and P4 release.

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