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Leptin Affects Proliferation-, Apoptosis- and Protein Kinase A-Related Peptides in Human Ovarian Granulosa Cells

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Summary

The aim of our in vitro studies was to understand the role of leptin in controlling proliferation, apoptosis, and protein kinase A (PKA) in human ovarian cells. We analyzed the in vitro effects of leptin (0, 1, 10 or 100 ng/ml) on the accumulation of proliferation-related peptides (PCNA, cyclin B1), apoptosis-associated peptide (Bax) and the intracellular signaling molecule PKA in cultured human granulosa cells using immunocytochemistry and Western immunoblotting. It was observed that leptin stimulated in a dose-dependent manner the accumulation of PCNA (at doses 1-100 ng/ml), cyclin B1 (at doses 10 or 100 ng/ml), Bax (at doses 10 or 100 ng/ml) and PKA (at doses 1-100 ng/ml) in cultured human ovarian cells. These observations suggest the ability of leptin to control directly human ovarian cell functions: proliferation, apoptosis, and intracellular messenger PKA.

Key words

Leptin • PCNA • Cyclin B • Bax • Protein kinase A • Ovary

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Introduction

Metabolic state can affect reproductive processes and induce some reproductive dysfunctions *via* alterations in production, reception, or response to leptin. This product of adipose tissue can control reproductive processes through hypothalamic LH-RH (Smith *et al.* 2001), although direct effects of leptin on the ovary has been documented (Spicer 2001). Leptin can affect ovarian steroidogenesis: stimulatory and inhibitory effects on the secretion of progesterone (P), androgens and estradiol (E) by cultured rat, bovine, porcine and human ovarian cells have been reported (Kitawaki *et al.* 1999, Almog *et al.* 2001, Ruiz-Cortez *et al.* 2003, Sirotkin *et al.* 2005), although such changes have not been found consistently (Agarwal *et al.* 1999, Spicer *et al.* 2000, Spicer 2001, Smith *et al.* 2001, Huang *et al.* 2002, Duggal *et al.* 2002, Ruiz-Cortez *et al.* 2003). In addition to steroidogenesis, the inhibitory action of leptin on prostaglandin E_2 secretion by rat ovarian cells has been reported (Duggal *et al.* 2002). In human granulosa cells, leptin stimulated oxytocin, prostaglandin F and IGFBP-3 and suppressed IGF-I release (Sirotkin *et al.* 2005)

Effects of leptin on other ovarian functions (growth, proliferation and apoptosis) are uncertain. *In vitro*, leptin was able to inhibit the growth of ovarian follicles in mice (Kikuchi *et al.* 2001), although no effect on granulosa cell number in women (Huang *et al.* 2002), DNA synthesis in rats (Duggal *et al.* 2002) or cell death in pigs (Ruiz-Cortes *et al.* 2003) was observed. *In vivo*, leptin increased both pro-apoptotic (Bax) and anti-apoptotic (Bcl) peptides and the Bcl/Bax ratio, resulting in suppression of ovarian cell apoptosis and promotion of puberty (rat: Almog *et al.* 2001). Furthermore, leptin deficiency in mice is associated with suppression of ovarian folliculogenesis and increase in ovarian granulosa cell apoptosis, but not in PCNA, marker of DNA replication/repair and of S phase of cell cycle (Hamm *et al.* 2004). No influence of leptin on markers of other phases of cell cycle (e.g. on cyclins, markers of G phases)

was reported yet. Therefore, the available data demonstrate both inhibitory and stimulatory effect of leptin on ovarian folliculogenesis and apoptosis, but no influence of leptin on marker of mitotic S phase/DNA replication was detected, whilst effect of leptin on markers of other phases of cell cycle has not been studied yet. Thus, the action of leptin on these processes requires further studies.

The effects of some hormones and growth factors on ovarian functions may be mediated by protein kinase A (PKA) and PKA-regulated transcription factors (Richards 2001). The stimulatory effect of leptin on tyrosine phosphorylation of STAT3 in human granulosa cells (Ruiz-Cortes *et al.* 2003) and mouse oocytes (Matsuoka *et al.* 1999) has been reported, but the relationship between leptin and other intracellular messengers, in particular PKA, in the ovary remains unknown.

Thus, the available information concerning leptin action on ovarian folliculogenesis and apoptosis is inconsistent, effect of leptin on ovarian cell cycle and PKA in the ovary has not yet been demonstrated.

The general aim of our *in vitro* experiments was to examine the effect of leptin on ovarian apoptosis, different phases of cell cycle and PKA. For this purpose, we examined the action of leptin on the expression of PCNA (marker of S phase of cell cycle), cyclin B1 (marker of G phase), Bax (marker of cytoplasmic apoptosis), and PKA within cultured human ovarian cells.

Methods

Preparation, culture, and processing of granulosa cells

Isolation and processing of granulosa cells were performed as described previously (Sirotkin *et al.* 2005). Briefly, granulosa cells were harvested, 1-2 days after a spontaneous ovulation, from women 36-42 years of age with normal ovarian cycles and morphology who were undergoing ovariectomy because of non-metastatic cancer of the cervix uteri. The consent of the women was obtained in accordance with local ethical and medical regulations. Granulosa cells were aspirated from 2-6 mm diameter follicles, separated from follicular fluid by threefold centrifugation at 200 x g for 10 min and washing in DMEM/Ham's F-12 1:1 mixture supplemented with 10 % bovine fetal serum and 1 % antibiotic-antimycotic solution (all from Sigma, St. Louis, USA). Cells were cultured in 2 ml of this medium in Falcon 24-well plates (Becton Dickinson, Lincoln Park, USA) and Lab-Tek chamber slides (Nunc Inc., Naperville, USA) at 0.5×10^6 cells/ml (determined by hemocytometer), at 37 °C under 5 % CO_2 in humidified air. After 4 days of preculture, the medium was replaced with fresh medium of the same composition. Experimental groups were treated with biological grade recombinant human leptin (0, 1, 10, or 100 ng/ml). The efficiency and physiological relevancy of these doses of leptin were demonstrated previously on ovarian cells (Agarwal *et al.* 1999, Kitawaki *et al.* 1999, Almog *et al.* 2001, Spicer *et al.* 2000, Kikuchi *et al.* 2001, Spicer 2001, Smith *et al.* 2001, Huang *et al.* 2002, Duggal *et al.* 2002, Ruiz-Cortez *et al.* 2003). Leptin was dissolved in culture medium immediately before experiment. Control groups were represented by cell-free (blank) medium or by cells cultured in medium with no exogenous leptin. After 2-day culture, the cell monolayer formed in culture plates was lysed by 3 cycles of freezing-thawing and vigorous pipetting in ice-cold electrophoretic buffer (0.0625 M Tris-base, 2 % SDS, 10 % glycerol, 0.5 % 2-mercaptoethanol, 0.003 % bromophenol blue, all from Sigma, 25 µl/sample). The supernatant was separated from cellular membranes by centrifugation for 10 min at 200 *x* g and frozen at –18 °C to await electrophoresis and immunoblotting. Chamber slides were washed three times in ice-cold PBS, fixed 20 min in 4 % paraformaldehyde in PBS, washed in PBS (2 x 5 min), ethanol (70 %: 5 min, 80 %: 10 min, 96 %: 2 x 10 min, 100 %: 10 min) and kept in 100 % ethanol at –22 °C to await immunocytochemical analysis. After culture, before lysis and fixation, cell concentration and viability were determined by Trypan blue staining and counting on hemocytometer. No statistically significant differences in these indices were observed between the groups.

Immunocytochemical analysis

The intracellular proliferation-associated and signalling substances were detected in granulosa cells plated on chamber-slides, using immunocytochemistry (Osborn and Isenberg 1994). ImmunoCruz Staining System and primary rabbit polyclonal antibodies against the human PCNA (cross-reacts with mouse, rat, human, insect and yeast full-length PCNA), human PKA (cross-reacts with type I beta regulatory subunit and partially with I-α catalytic subunit of human, mouse, rat, bovine and porcine PKA), or mouse monoclonal antibodies against human cyclin B1 (cross-reacts with mouse, rat and human cyclin B1, all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA, dilution 1:100)

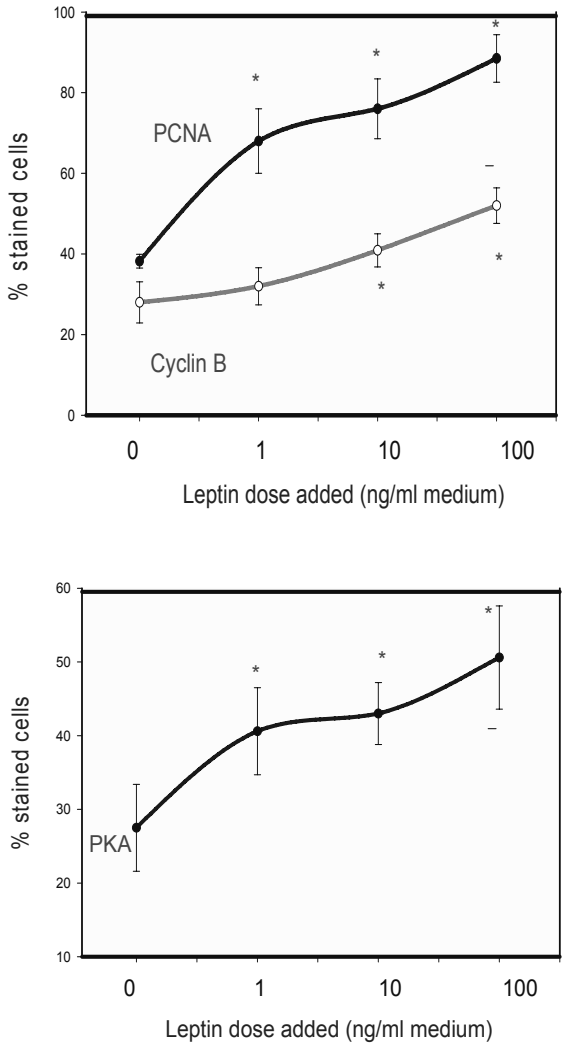

Fig. 1. Effect of leptin (0, 1, 10 or 100 ng/ml) on the percentage of cultured human ovarian granulosa cells containing (a) PCNA, cyclin B1 and (b) protein kinase A (data from immunocytochemistry). Values are mean±SEM, *significant difference ($P<0.05$) compared with control (medium without addition).

were used according to the instructions of the manufacturer. For the visualization of primary antibody, corresponding secondary antibody from ImmunoCruz Staining System or secondary polyclonal rabbit IgG labeled with horseradish peroxidase (Santa Cruz, dilution 1:1000 or Sevac, Prague, Czech Republic, dilution 1:2000) and DAB-reagent (Boehringer Mannheim GmbH, Mannheim, Germany, 10 %) were used. The specificity of primary antibodies and molecular weights of ligands were confirmed prior to experiment by Western blotting (not shown). Cells treated with secondary antibody and DAB but omitting the primary antibody were used as negative controls. The presence of specific immunoreactivity in cells was determined by light microscopy.

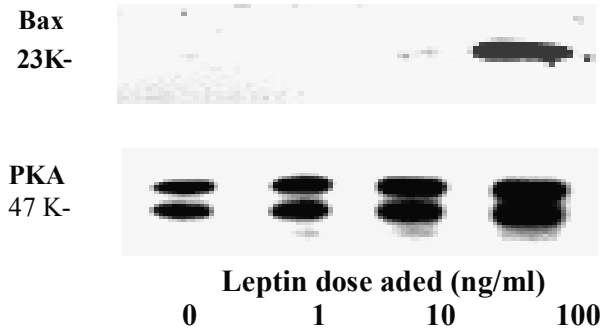

Fig. 2. SDS PAGE-Western immunoblots showing the effect of leptin (0, 1, 10 or 100 ng/ml) on the expression of Bax (top) and protein kinase A (bottom). The fractions/subtances and their molecular weights (kD) are marked to the left of the gel lanes.

Protein gel electrophoresis and immunoblotting

Lysates of granulosa cells were mixed 1:1 with electrophoretic buffer (0.0625 M Tris-base, 2 % SDS, 10 % glycerol, 0.5 % 2-mercaptoethanol, 0.003 % bromophenol blue, all from Sigma), boiled at 95 °C for 3 min and subjected to SDS-polyacrylamide gel electrophoresis in 4 % and 10 % stacking and resolving gels, respectively, at 25 mA constant current according to Laemmli (1970). The samples were then transferred to Porablot PVDF membranes (Macherey-Nagel, Duren, Germany) using a semi-dry transblotter (Bio-Rad Labs, Richmond, USA). Endogenous peroxidase in samples was quenched by incubation in 3 % H_2O_2 for 15 min. Non-specific binding of antiserum was prevented by incubation in 5 % blot-qualified BSA (Amersham) in TTBS (20 nM Tris-base, 137 nM NaCl, 0.1 % Tween-20). Blocked membranes were probed with rabbit polyclonal antibodies against the PKA (dilution 1:500) described above or against Bax (binds Bax of human, mouse and rat origin, dilution 1:250, both from Santa Cruz Biotechnology, Inc.). We failed to obtain the clear specific signal in Western blots using other antisera mentioned in chapter "Immunocytochemical analysis" of this manuscript. After treatment with primary antibody, membranes were incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (Sevac) and visualized using Super-Signal West-Pico luminescent substrate (Pierce, Rockford, Il, USA) and ECL Hyper-film (Amersham plc, Little Clafton, UK). Incubation medium without cells was used as a negative control, beta-actin was used as positive control and control for equail loading (not shown). Molecular weights of fractions were evaluated using a molecular weight calibration kit (18, 24, 45, and 67 kD, ICN Biomedicals Inc, Irvine, CA, USA).

Statistics

Each experimental group was represented by four culture wells or chambers. The proportions of cells containing specific immunoreactivity were calculated from inspection of at least 1000 cells per chamber. The data shown are mean values obtained in three separate experiments performed on separate days using separate pools of granulosa cells, each obtained from 4 women. The samples obtained in three experiments and intended for Western immunoblotting were pooled before electrophoresis Significant differences between groups in the proportion of cells containing specific immuno-reactivity were evaluated by the Chi-square test. Differences from control at $P<0.05$ level were considered as significant.

Results

Analysis of human granulosa cells and cell-conditioned medium revealed detectable levels of PCNA, cyclin B1, Bax and PKA within the cells. Immunocyto-chemical analysis (Fig. 1) showed that the addition of leptin alone increased the percentage of cells containing PCNA (at 10 or 100 ng/ml), cyclin (at 10 or 100 ng/ml) and PKA (at 1, 10 or 100 ng/ml). Western immunoblotting (Fig. 2) confirmed the concentration-dependent increase in accumulation of PKA in granulosa cells after leptin treatment. We also demonstrated the presence of visible Bax in the cells after leptin treatment (at 10 and 100 ng/ml). Leptin effect on signaling molecules detected using either immunocytochemistry and Western immunoblotting was dose-dependent because the accumulation of all substances rose with leptin dose added.

Discussion

The present observations confirm the production of cell cycle- and apoptosis-associated peptides by human granulosa cells. The two bands of PKA detected in our experiments represent probably the catalytic and regulatory subunits of PKA detected using present antiserum against PKA (see Materials and Methods). Furthermore, our data demonstrate that leptin affected all the ovarian substances examined.

This is the first evidence for an involvement of leptin in the control of ovarian cell cycle proteins (PCNA and cyclin). Expression of PCNA and cyclin B is associated with rodent and human ovarian cell proliferation (Kadakia *et al.* 2001, Chaffin *et al.* 2001) and with the transition of rodent ovarian cells through late G_1 and S phase of cell cycle (Naryzhny and Lee, 2001). Therefore, our data suggest a stimulatory effect of leptin on human ovarian cell proliferation *via* promotion of these phases of the cell cycle. This effect might induce ovarian follicular growth and development as described previously (Almog *et al.* 2001, Kikuchi *et al.* 2001) and/or promote granulosa cell luteinization, which is also associated with changes in cellular proliferation and cycle proteins (Murphy 2000).

The present stimulatory effect of leptin on Bax corresponds to the report of Almog *et al.* (2001) on leptin-induced expression of Bax in rat ovarian cells. This is the first demonstration of involvement of leptin in the control of human ovarian apoptosis.

The stimulatory effect of leptin on cell cycle proteins and apoptotic peptide suggests that leptin can control ovarian folliculogenesis and remodeling affecting both proliferation and apoptosis of ovarian cells and the equilibrium between these processes. The pattern of leptin influence on these processes suggests the gonadotropic role of leptin in human ovary. Although the data obtained during *in vitro* experiments should be extrapolated to *in vivo* conditions very carefully, our observations suggest the stimulatory role of leptin in human ovarian reproduction. These effects of leptin could explain the reported ovarian disturbances in animals and humans with altered leptin production or reception induced by obesity, malnutrition and genetic manipulations (Smith *et al.* 2001, Spicer 2001, Hamm *et al.* 2004).

An important role of PKA-dependent intracellular mechanisms in control of ovarian functions (Richards 2001), and the leptin-induced accumulation of PKA observed in our experiments provide the first evidence of the potential involvement of PKA in mediating leptin effects. The obtained results showed a potential new mechanism of leptin action on the ovary. This mechanism probably includes ovarian leptin receptors detected previously (Matsuoka *et al.* 1999, Duggal *et al.* 2002) and postreceptory intracellular PKA, the accumulation of which is induced by leptin, and which plays an important role in control of ovarian functions (Richards 2001, Murphy 2002). The interrelationship of PKA- and JAK/STAT-dependent intracellular mechanism of leptin action suggested previously (Matsuoka *et al.* 1999, Ruiz-Cortes *et al.* 2003) as well as the involvement of these mechanisms in

human ovarian proliferation/apoptosis/remodeling detected during present experiment, require further studies.

Nevertheless, our observations 1) confirm the importance of leptin in control of apoptosis, 2) provide the first evidence for the involvement of leptin in control of ovarian cell cycle (S- and G-phase) and 3) suggest the PKA as potential leptin target and mediator of action on human ovarian cell functions. A better understanding of the effects of leptin and its mechanisms could be helpful in the discovery of links between reproduction, growth, and metabolism. Moreover, studies of described mechanism(s) of leptin action can be potentially useful for the detection and treatment of leptin-dependent metabolic and reproductive diseases.

Conflict of Interest

There is no conflict of interest.

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