Reproductive Biology 17 (2017) 19–24

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Original article

Metabolic state defines the response of rabbit ovarian cells to leptin

Abdel Halim Harrath[a], Olga Østrup[b], Jan Rafay[d,e], Iveta Koničková (Florkovičová)[d], Jozef Laurincik[c], Alexander V. Sirotkin[a,c,d,*]

[a] Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia
[b] Center for Genomic Medicine, Copenhagen University Hospital, Copenhagen, Denmark
[c] Department of Zoology and Anthropology, Constantine the Philosopher University, 949 74 Nitra, Slovakia
[d] Department of Genetics and Reproduction, Research Institute of Animal Production, 949 59 Lužianky, Slovakia
[e] Department of Biotechnology, University of SS. Cyril and Methodius, 917 01 Trnava, Slovakia

ARTICLE INFO

Article history:
Received 11 October 2016
Received in revised form 12 November 2016
Accepted 18 November 2016
Available online 25 November 2016

Keywords:
Caloric restriction
Leptin
Ovary
Proliferation
Apoptosis
Hormones
Rabbit

ABSTRACT

Leptin is a hormone that mediates the effect of the metabolic state on several biological functions, including reproduction. Leptin affects reproductive functions via alterations in the release of hormonal regulators. However, the extent to which caloric restriction (CR) can affect the complex processes of reproduction by other mechanisms, such as altering ovarian functions via direct binding/response to leptin, is unknown. Therefore, the aim of the present study was to show basic ovarian cell functions and CR on the response of ovarian cells to leptin. Female rabbits were subjected to 50% CR restriction for 10 days before ovulation. On the day of ovulation, both control and CR animals were sacrificed. Isolated granulosa cells were cultured for 2 days with and without leptin (100 ng/ml), and the accumulation of various markers was evaluated using immunocytochemistry; i.e., cell proliferation (PCNA and cyclin B1), apoptosis (bax), MAP/ERK1,2 kinase (MAPK), protein kinase A (PKA), and IGF-I. In addition, the release of IGF-I and estradiol (E_2) by cells cultured with and without leptin (1, 10, 100, 1000, or 10,000 ng/ml) was assessed by radioimmunoassay (RIA). In the granulosa cells of control animals, leptin promoted cyclin B1, MAPK, and PKA accumulation, but not that of PCNA, and reduced bax and IGF-I accumulation. These cells responded to leptin by increased IGF-I, but not E_2 release. In cells of CR animals, leptin increased cyclin B1 accumulation, but decreased PCNA, MAPK, and IGF-I expression. Bax and PKA were not affected. Leptin resulted in a decrease in IGF-I release. CR modulated the influence of leptin on E_2 release dose dependently, i.e., E_2 increased at 10 and decreased at 10,000 ng/ml. Therefore, CR modified the influence of leptin on PCNA, E_2, bax, PKA, MAPK, and IGF-I release, but it did not change the effect of leptin on cyclin B1 and IGF-I accumulation within the cells. Our data showed that leptin directly affected proliferation, apoptosis, and hormone release by ovarian cells, probably via PKA- and MAPK-dependent pathways. Furthermore, it was demonstrated that nutrition could influence reproduction by affecting the response of ovarian cells to leptin.

1. Introduction

Leptin (L) is a metabolic hormone that plays an important role in the control of reproduction. It can affect reproduction at different regulatory levels including the hypothalamus, pituitary, and reproductive organs [2,20,35]. Metabolic state affects reproduction and synchronizes reproduction (an energy demanding process) with optimal nutritional conditions, and defines livestock reproductive efficiency. Caloric restriction (CR) affects metabolism and modulates reproductive processes by altering the release of metabolic hormones including L, and subsequently suppresses the hypothalamo-hypophysial-gonadal axis and disturbs reproductive processes, such as puberty and fertility [2,17,23,26,8]. In rabbits, CR suppresses the release of reproductive hormones and embryo development [3,10,18,22], and promotes oocyte maturation, fertility, and kindling rates [11,9,25,1,6,7]. CR inhibits L, a promoter of reproductive processes in various mammalian species [2,20,23,26,8]. L was able to affect steroid and IGF-I release by cultured rabbit ovarian cells, whilst L injections to rabbit does reduced the level of these hormones in plasma and promoted

* Corresponding author at: Department of Zoology and Anthropology, Constantine the Philosopher University, 949 74 Nitra, Slovakia.
E-mail address: asirotkin@ukf.sk (A.V. Sirotkin).

http://dx.doi.org/10.1016/j.repbio.2016.11.002
1642-431X/

fecundity [30–32]. CR has been found to inhibit the levels of circulating L and ovarian steroid hormones in rabbits [3,10,6,7]. Similarly, several studies showed the stimulatory effect of L on rabbit ovarian cell functions [30,5,35,36]. Hence, CR can regulate reproductive processes in rabbits and other mammals via the down-regulation of L release. However, there are some indications that CR can affect not only hormone release, but also hormone reception and action. CR suppressed the expression of estrogen receptors in rat brains [12]; follicle stimulating hormone (FSH), luteinizing hormone (LH), and insulin-like growth factor I (IGF-I) receptors in bovine ovaries [38]; and ghrelin receptors in chicken hypothalami and ovaries [34]. Moreover, CR increased the sensitivity of chicken pituitaries to gonadotropin-releasing hormone (LH-RH) [4], and nutria ovaries to IGF-I and cyclic adenosine monophosphate (cAMP) analogs [27], suggesting that metabolism can affect the response of the hypothalamo-hypophysial-ovarian system to some hormonal regulators. However, it is unknown whether CR can affect reproduction not only via changes in L release, but also in the ovarian response to L. Therefore, the aim of the present study was to examine the effect of L on basic rabbit ovarian cell functions (proliferation, apoptosis, the secretory activity) and the effect of CR on the response of ovarian cells to L.

2. Materials and methods

2.1. Manipulation with animals

Female nulliparous non-cycling New Zealand White rabbit doe line M91 of 6 months of age, weight 4.0–4.9 kg, were bred and kept in individual cages under standard conditions (photoperiod 16:8 h, temperature $17 \pm 3\,^{\circ}C$, air humidity $75 \pm 10\%$) at the local rabbit farm of the Research Institute of Animal Production. All the animals were fed individually, once daily with a previously weighted standard granulated pelleted feed mixture (Polnohodspodarske Druzstvo Cataj, Cataj, Slovakia) containing 18% nitrogen substances, 12% fiber, 2.6% fat, metabolizable energy = 10.8 MJ. The experiments were approved by the State Veterinary and Food Administration of the Slovak Republic in agreement with the corresponding EU and Slovak regulations. From days 1 to 10, animals were randomly divided into two groups – a control group and a group subjected to 50% CR. All animals were injected on day 6 with pregnant mare serum gonadotropin (PMSG) (Sergon, Bioveta, Ivanovice Ivanovice na Hané, Czech Republic; 25 IU/animal in 1 ml of phosphate buffered saline, PBS), and three days thereafter with LH-RH (Supergstran, Ferring-Leciva, Pohori-Chotoun, Czech Republic; 0.25 IU/animal in 1 ml of PBS). On day 10 (*peri*-ovulatory period), all animals were weighted and killed by decapitation. Ovaries were collected into PBS and transported to the laboratory at ambient temperature within 0.5 h of slaughter.

2.2. Isolation and culture of ovarian cells

Ovaries were washed in PBS with 1% antibiotic-antimycotic solution (Sigma, St. Louis, USA), placed in 100 mm diameter culture dishes (Gama, České Budejovice, Czech Republic), and dissected using a multiblade knife. The tissue suspension was passed through a steel sieve to separate large pieces more than 0.1 mm in diameter. The cell filtrate was washed three times in PBS solution, and the granulosa cells were purified from cell debris and blood cells by centrifugation in a gradient of Percoll (Sigma) according to the manufacturer's instructions. Granulosa cells were aspirated from the Percoll fraction and rinsed twice in sterile incubation medium (DME/F12). After the final centrifugation ($200 \times g$ for 10 min), the cells were resuspended in incubation medium, supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution (all from Sigma). The cell concentration was determined with an hemocytometer and adjusted to 1×10^6 cells/ml by dilution with an incubation medium. Cell viability was determined by Trypan blue staining and found to be in the range of 75–80%. Two milliliter aliquots of granulosa cell suspension (1×10^6 cells/ml) intended for RIA were cultured in Falcon 24-well plates (Becton Dickinson, Lincoln Park, USA), and 300 ul aliquots intended for immunocytochemical analysis were incubated for 2 days in Lab-Tek chamber-slides (Nunc, Inc., Naperville, USA) at 37 °C and 5% CO_2 in humidified air with and without recombinant human L (Sigma; 0 or 100 ng/ml in chamber-slides intended for immunocytochemistry, and 0, 1, 10, or 100 ng/ml in plate wells intended for RIA). Frozen aliquots of L were dissolved in culture medium immediately before use. Control groups were represented by cell-free (blank) medium or cells cultured in medium with no exogenous hormones. After culture, the chamber slides were washed three times in ice-cold PBS, fixed for 20 min in 4% paraformaldehyde in PBS, washed in PBS (2×5 min), ethanol (70%: 5 min, 80%: 10 min, 96%: 2×10 min, 100%: 10 min), and kept in 100% ethanol at −22 °C until immunocytochemical analysis. The medium in the well plates was gently aspirated and frozen at −18 °C until RIA analysis. The cell concentration (1×10^6 cells/ml medium) and viability (between 95 and 99%) were determined using Trypan blue staining and an hemocytometer. No significant differences in these parameters were observed between the groups.

2.3. Immunocytochemical analysis

Intracellular PCNA (marker of the S-phase of mitosis; [21,37], cyclin B1 (marker of the G2 phase of the cell cycle and promoter of its transition to the S-phase; [19] bax (marker/promoter of cytoplasmic apoptosis [14], MAP/ERK1,2 kinase (MAPK), and protein kinase A (PKA) mediating the action of some hormones on ovarian functions and affecting ovarian cell functions [35] and IGF-I, were detected in granulosa cells plated on chamber-slides, using immunocytochemistry. The ImmunoCruz Staining System and primary mouse monoclonal antibodies against these molecules (all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA; dilution 1:100 in PBS) were used as directed by the manufacturer. For the visualization of primary antibodies, the corresponding secondary antibody from the ImmunoCruz Staining System or secondary polyclonal rabbit IgG labeled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:2000 in PBS) and DAB-reagent (Roche Diagnostics Corporation, IN, USA; 10%) were used. These cells were mounted in glycerol mounting medium (DAKO Corp., Carpinteria, CA, USA). The presence of molecules and percentage of cells containing these molecules were determined by light microscopy. Cells treated with secondary antibody but omitting the primary antibody were used as negative controls. The presence of some selected molecules within rabbit ovarian granulosa cells is illustrated in Fig. 1.

2.4. Immunoassay

Concentrations of IGF-I and estradiol (E_2) were determined in 25-ul incubation medium by RIA kits from DSL (Webster, Texas, USA) according to the manufacturer's instructions. RIA for IGF-I included an IGF-I extraction procedure. Antiserum against P_4 cross-reacted less than 0.001% to cortisol, corticosterone, cortisol, androstenediol, pregnenolone, oestradiol and testosterone. Sensitivity of the assay was 0,12 ng/ml, intra- and inter-assay coefficients of variation did not exceed 13,0 and 8,0%. The cross-reactivity of antiserum against IGF-I was less than 1.9% to IGF-II, less than 0.01% to insulin, proinsulin and less than 0.001% to EGF, oxytocin and P_4. Sewnsitivity of the assay was 0.3 ng/ml, the maximal intra- and inter-assay coefficients of variation were 3.4