Research in Veterinary Science 105 (2016) 180–187

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Relationships between leptin, KiSS-1/GPR54 expression and TSH secretion from pituitary cells of pubertal ewes *in vitro*

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ARTICLE INFO

Article history:
Received 28 May 2015
Received in revised form 8 February 2016
Accepted 21 February 2016

Keywords:
Kisspeptin
Leptin
Thyroid-stimulating hormone
Pubertal ewes
Pituitary cells *in vitro*

ABSTRACT

Kisspeptin and leptin play a crucial role in the puberty of sheep as they initiate the activity of hypothalamic-pituitary-ovarian axis. Also hormones of thyrotropic axis are probably involved in this process. The aim of study was to analyze the impact of leptin on kisspeptin-10 secretion as well as kisspeptin-1 and G protein-coupled receptor (GPR54) mRNA expression in pituitary cells of pubertal ewes *in vitro*. The influence of kisspeptin on TSH secretion was also examined. Cells were cultured in McCoy's 5A medium without hormones; with 10^{-10}–10^{-5} M of leptin; with 10^{-11}–10^{-5} M of kisspeptin-10; with peptide 234 (10^{-7} M, antagonist of GPR54) or 10^{-11}–10^{-5} M of kisspeptin-10 and peptide 234. Then, kisspeptin-10 and TSH secretion as well as KiSS-1 and GPR54 expression were analyzed. We found that leptin directly affected kisspeptin-10 secretion and kisspeptin-1/GPR54 expression in pituitary cells of pubertal ewes. Kisspeptin-10 did not change TSH secretion, except exerting a short-term influence after 2 h.

1. Introduction

Puberty is a complex biological process of initiation of reproductive activity, which is determined by a multi-hormonal effect as well as metabolic signals. It is known that the onset of sexual maturation depends on attaining sufficient energy stores, a critical body weight or a minimum percentage of body fat and a proper leptin level (Abadjieva et al., 2011; Castellano et al., 2005; Cheung et al., 1997; Mantzoros et al., 1997; Seminara et al., 2003). Leptin is an adipocyte-derived natural ligand of leptin receptors which provides a link between energy homeostasis and reproduction (Smith et al., 2002, 2006). Among other things, it plays a significant role in the process of puberty by initiating the activity of the hypothalamic-pituitary-ovarian axis (HPO). Leptin affects neurons of the hypothalamus and induces gonadotropin-releasing hormone (GnRH) secretion (Woller et al., 2001). Though it does not act on this process directly, because leptin receptors are not expressed in GnRH neurons. This effect is mediated, among others, through interneuronal pathways involving the kisspeptin/G protein-coupled receptors (KiSS-1/GPR54) system (Ahima, 2011; Hausman et al., 2012; Qiu et al., 2011). It is known that kisspeptin neurons are a direct target for leptin action and that they express leptin receptors (Backholer et al., 2010; Smith et al., 2006). It has been shown that treatment of ob/ob mice and food-restricted sheep with leptin increases cellular expression of KiSS-1 mRNA in kisspeptin neurons (Backholer et al., 2010; Smith et al., 2006). According to Backholer et al. (2010), *intracerebroventricular* (*icv*) infusion of leptin partially restores KiSS-1 expression in lean ewes. No reports are available, however, on the influence of leptin on kisspeptin secretion or expression at the level of the pituitary gland in pubertal sheep.

Kisspeptins, encoded by the *kiss-1* gene, are a group of hypothalamic neuropeptides and endogenous ligands of GPR54 receptors. The kisspeptin/GPR54 system is a key factor in the neuroendocrine control of reproduction as a positive upstream regulator of the HPO axis and plays a pivotal role in the timing of the onset of puberty (Castellano et al., 2009, 2010; Gutiérrez-Pascual et al., 2007; Lie et al., 2013). By binding to GPR54 in the arcuate nucleus and the preoptic area of the hypothalamus, kisspeptins cause an increase in pulsatile gonadotropin-releasing hormone secretion, which in turn induces the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland (Estrada et al., 2006; Pompolo et al., 2006; Smith et al., 2009). Moreover, a high level of expression of kisspeptins and GPR54 has been found in extracts of whole pituitary glands in rodents, sheep and humans, which points to the possible direct influence of kisspeptins on some tropic hormones at the pituitary level (Ramaswamy et al., 2009; Richard et al., 2008). It has been reported that treatment of ovine pituitary cells with kisspeptin results in elevation of LH concentration in culture media of ovine pituitary cells obtained from sheep during the follicular phase of the estrous cycle (Smith et al., 2008). Also, Gutiérrez-Pascual et al. (2007) have shown that kisspeptin stimulates LH secretion from pituitary cells of peripubertal female rats, whereas according to other authors kisspeptins do not affect gonadotropin release *in vitro* in rodents (Thompson et al., 2004). The literature provides no information about the influence of kisspeptin on the secretion of thyroid-stimulating hormone (TSH) at the level of the pituitary gland in sheep.

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http://dx.doi.org/10.1016/j.rvsc.2016.02.014
0034-5288/

There are some reports about the contribution of hormones of the thyrotropic axis to the regulation of reproductive processes and the onset of puberty in ruminants. According to Meza-Herrera et al. (2011), in goats, glutamate-induced acceleration of sexual maturation is dependent on triiodothyronine concentration. This suggests that thyroid hormones can exert an effect on the establishment of puberty in small ruminants (Meza-Herrera et al., 2011). Moreover, it is known that thyroid hormone receptors α (αTHR) are present in GnRH and A14 dopamine neurons in sheep brain and thyroid hormones play a pivotal role in the transition from the breeding season to the anestrus (Ebling, 2010; Jansen et al., 1997; Lehman et al., 2010). Furthermore, it has been shown that thyroxine and triiodothyronine are present in the ovarian follicular fluid in cattle and participate in the steroidogenesis and maturation of ovarian follicles (Błaszczyk et al., 2006; Mutinati et al., 2010; Spicer et al., 2001). Moreover, according to our previous study, thyroid-stimulating hormone secretion from the pituitary gland in pubertal ewes is regulated by leptin. It has been observed that 10^{-10} to 10^{-6} M of leptin increases TSH secretion from ovine pituitary cells *in vitro*, whereas a high concentration of leptin (10^{-5} M) reduces TSH secretion (Radwańska and Kosior-Korzecka, 2014). It is still not known, however, whether leptin affects the secretion of TSH in a direct way or *via* other factors, including kisspeptins. The available reports on the impact of kisspeptins on the secretion of TSH and the activity of the thyrotropic axis are ambiguous and do not relate to sheep. The only available data concern the influence of kisspeptins on the secretion of TSH in monkeys of the family *Cercopithecidae* and in women, in which no significant relationship between kisspeptin and TSH has been shown (Luque et al., 2011; Narayanaswamy et al., 2014; Ramaswamy et al., 2009).

Therefore, the aim of the present study was to analyze the impact of leptin on KiSS-10 secretion and KiSS-1 mRNA and GPR54 mRNA expression in ovine pituitary cells *in vitro*. Furthermore, taking into account the fact that hormones of the thyrotropic axis are involved in reproduction, the influence of kisspeptin on TSH secretion from pituitary cells of pubertal sheep *in vitro* was also examined.

2. Material and methods

2.1. Chemicals and laboratory materials

A large part of the chemicals used in the study were purchased from Sigma Chemicals Co. (St. Louis, MO, USA): Dulbecco's Modified Eagle's Medium (DMEM), Bovine Serum Albumin (BSA), D-(+)Glucose, HEPES, gentamicin solution, McCoy's 5A Modified Medium, MEM Non-essential Amino Acid Solution (100×), MEM Vitamin Solution (100×), metastin (45–54) amide, human, kisspeptin-234 trifluoroacetate salt, TRI Reagent, 1-bromo-3-chloropropane, 2-propanol, lauryl sulfate (sodium dodecyl sulfate), nitrotetrazolium blue chloride, water molecular biology reagent. Moreover, the following chemicals and materials were used in the study: *or* Leptin (ovine recombinant Leptin, a kind gift from Prof. H. E. Paczoska-Eliasiewicz, Department of Animal Physiology, University of Agriculture in Krakow, Poland and Prof. Arieh Gertler, Institute of Biochemistry, Food Science and Nutrition, Hebrew University of Jerusalem, Israel), 0.25% trypsin solution, fetal calf serum and horse serum (Biomed-Lublin, Poland), KiSS-1 (112−121) Amide/Kisspeptin-10/Metastin (45–54) Amide EIA KIT (Phoenix Pharmaceuticals Inc., CA, USA), Sheep Thyroid Stimulating Hormone ELISA KIT (Blue Gene, Shanghai, China), ethyl alcohol 99.8% (POCH, Gliwice, Poland), RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania), AmpliTaq Gold DNA polymerase (Taq Man Universal PCR Master Mix, Applied Biosystem, USA), forward and reverse primers, and fluorescently labelled probes for Real Time PCR (customized by Applied Biosystem, USA), 96-well plates with optical covers (Optical 96-Well Fast Thermal Cycling Plate, ABIPRISM Optical Adhesive Covers, Applied Biosystem, USA), 6-well, 24-well and 96-well culture plates (Nunclon Delta SI, Nunc A/S, Denmark).

2.2. Animals and isolation of pituitary glands

Crossbred, pubertal, non-cyclic ewe lambs of the SCP line (50% Suffolk + 25% Romanov + 25% Polish Lowland Sheep, n = 21), aged six months and housed at the Didactic-Experimental Station of Sheep and Goat Breeding in Bezek were used in this study. The experiment was carried out in July, at the beginning of the breading season, under natural light and temperature. Each ewe was fed with meadow hay (0.3 kg/day), green forage (4 kg/day), dried beet pulp (0.3 kg/day) and oats grain (0.15 kg/day) (1.36 kg of dry matter, gross energy: 5.67 MJ/kg of dry matter, 12.88% digestible protein on a dry matter basis) in two portions at 9.30 am and 7.30 pm. The animals had free access to fresh water. Ovarian inactivity was confirmed by laparoscopy. The ewe lambs were humanely euthanized by electric shock (ENZ 300, Metalowiec, Bydgoszcz, Poland) and exsanguinated. Pituitaries were dissected within 10 min of sacrifice and transported to the laboratory in cold DMEM (about 4 °C) supplemented with 0.1% BSA, 0.08% glucose, 0.59% HEPES and gentamicin (20 μg/mL) within 15 min. The present study was repeated on three independent cell cultures. Each cell culture was prepared using pituitaries isolated from seven ewe lambs. The protocol of the experimental design and all procedures were approved by the Second Local Ethics Committee for Animal Experimentation in Lublin (License No. 40/2013).

2.3. Isolation and culture of pituitary cells

2.3.1. Isolation of anterior pituitary cells

The anterior and posterior lobes of the pituitary were separated by blunt dissection. Anterior pituitary tissue was minced and trypsinized with 0.25% trypsin (12–14 times, 10 min, 37 °C). After each digestion, the cells were washed three times in DMEM (supplemented with 0.1% BSA, 0.08% glucose, 0.59% HEPES and gentamicin at a final concentration of 20 μg/mL) and centrifuged (1200 rpm for 10 min, 18 °C). After the last centrifugation, pituitary cells were filtered through a 60-μm nylon filter and counted in Bürker's chamber. Cell viability evaluated by 0.4% trypan blue dye exclusion was higher than 96%. Pituitary cells (250 000 cells/mL) were then resuspended in McCoy's 5A medium containing 10% horse serum, 2.5% fetal calf serum, a mixture of amino acids and vitamins, 0.59% HEPES and gentamicin (20 μg/mL) (adjusted to pH 7.4), and seeded in 24-well culture plates (1 mL/well, for kisspeptin and TSH concentration analysis), 96-well culture plates (0.1 mL/well, for proliferation index determination) or 6-well culture plates (4 mL/well, for KiSS-1 mRNA and GPR54 mRNA expression analysis). Cells were allowed to attach for 72 h (37 °C, 95% air/5% CO_2) (Bogacka et al., 2002; Kosior-Korzecka et al., 2012; Radwańska and Kosior-Korzecka, 2014) until the start of the experiments proper.

2.3.2. Experiment 1 — effect of leptin on kisspeptin secretion from ovine pituitary cells in vitro

After preculture, the medium was changed to McCoy's 5A medium (supplemented with a mixture of amino acids and vitamins, 0.59% HEPES and gentamicin (20 μg/mL)) without hormones (the control) or the same medium with 10^{-10} to 10^{-5} M of leptin, respectively. Each sample was prepared in duplicate. Next, cells were cultured for another 2–30 h. After 2, 6, 12, 18, 24 and 30 h of exposure to the aforementioned factor, the media were collected and stored at −20 °C to determine the cumulative concentration of kisspeptins by EIA. The intra- and inter-assay coefficients of variations of the assay for kisspeptin were 4.7% and 6.9%, respectively. The lower limit of detection was 30 pg/mL. At the same time, the proliferation indexes (PI) of control cells and cells treated with leptin were determined to calculate the secretion of kisspeptins. The assessment of cell proliferation was based on the reduction of tetrazolium salt (MTT) into a blue formazan. The control and experimental cultures were pulsed with 15 μL of MTT (for 3 h at 37 °C) and then solubilized overnight with a 10% solution of sodium dodecyl sulfate (SDS). The optical density (OD) of the blue formazan