—Full Paper—

Action of IGF-I on Expression of the Long Form of the Leptin Receptor (ObRb) in the Prepubertal Period and Throughout the Estrous Cycle in the Mature Pig Ovary

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Abstract. Leptin is an important satiety hormone and reproductive regulator found, along with its receptors, throughout the ovary. To date, the changes in ovarian expression of leptin receptor during the prepubertal period and throughout the estrous cycle in the pig have not been studied. In this study, the long form of the leptin receptor was a detectable level in immature pig ovarian follicles when assayed using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Moreover, its level was increased in growing follicles during follicular phase of the estrous cycle (6-fold in early antral [EAF] and antral [AF] follicles) and was highest in newly formed corpora lutea. Its changes paralleled those in steroid secretion and especially progesterone (P₄) secretion. Additionally, we showed that insulin-like growth factor (IGF)-I had stimulatory effect on leptin receptor expression using a tissue culture model of follicular and luteal cells, with a 12-fold increase in prepubertal ovaries and a 1.5- to 2-fold increase in growing follicles and newly formed corpora lutea (CL). These results suggest that ovarian expression of leptin receptor is regulated throughout the estrous cycle by ovarian steroids, with peak expression at ovulation, indicating a possible involvement in follicular development and corpus luteum formation. Moreover, this data points to an important role of IGF-I in leptin receptor expression during the entire estrous cycle, with a special role during the prepubertal period.

Key words: Corpus luteum, Insulin-like growth factor (IGF)-I, Leptin receptor (ObRb), Ovarian follicles, Pig

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Leptin, a product of the obese (*ob*) gene, is an important satiety and reproductive hormone classically secreted by adipose tissue. It has been discovered that it is synthesized in many other organs, including the stomach, placenta, and ovary [1–4]. Gene expression of leptin receptor has been

demonstrated in the porcine ovary and the complementary DNA of porcine leptin receptor have been cloned and sequenced. Moreover, it has been shown that leptin receptor mRNA is present in freshly isolated granulosa cells and that its level increased along with the process of luteinization during cell culture [1].

Leptin has significant direct effects on ovarian granulosa and theca cells. Our previously published data shows that leptin directly affects

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ovarian function in prepubertal gilts by the action on estradiol (E_2) secretion and cell apoptosis. We showed that growth hormone (GH) and insulin-like growth factor (IGF-I) modulate the action of leptin on steroid secretion by ovarian follicles and the stimulatory action of IGF-I on leptin receptor expression in prepubertal animals [2]. Moreover, leptin has maturation-dependent action on GH- and IGF-I-stimulated follicular steroidogenesis. During follicular growth, leptin acts synergistically with GH and IGF-I to influence estradiol production, while in preovulatory follicles, they acted with both hormones in the luteinization process, which starts before follicular disruption [3]. During luteal phase, leptin action is restricted to the stage of corpus luteum (CL) formation. It acts as an antiapoptotic factor and at the same time, it reverses the antiapoptotic action of IGF-I, thereby protecting cells from excessive apoptosis and supporting retention of appropriate cell numbers, which is necessary for maintenance of homeostasis in the developing CL [4]. It has been suggested that expression of the leptin receptor gene varies throughout the estrous cycle, thereby modulating the sensitivity of the ovary to leptin. More recently, Duggal *et al.* [5] reported that the gene expression of ovarian leptin receptors for both OB-RB and OB-RA vary throughout the estrous cycle in response to the changing environment in the ovary. In our previously published data [2], we clearly showed that IGF-I modulates the action of leptin in prepubertal animals. Moreover, we showed that IGF-I has stimulatory action on leptin expression.

The aims of this study were to describe the pattern of leptin receptor expression in the ovaries of immature pigs and throughout the estrous cycle, and to describe the role of IGF-I in leptin receptor expression during the natural estrous cycle.

Materials and Methods

Reagents

Parker medium M199, trypsin, and calf serum (CS) were purchased from the Laboratory of Sera and Vaccines (Lublin, Poland). Antibiotic-antimycotic solution (100 $\times$), HEPES, CHAPS, glycerol, DTT, and caspase-3 colorimetric substrate Ac-DEVD-pNA were obtained from Sigma Chemical (St. Louis, MO, USA). Trizol LS reagent for RNA isolation was purchased from Invitrogen

Live Technologies (Paisley, UK) and all reagents for RT-PCR reaction, including Taq DNA Polymerase, were obtained from Promega (Madison, WI, USA). Ovine GH was prepared in the lab of Professor Gertler at Institute of Biochemistry, Food Science and Nutrition (Rehovot, Israel). Its biological activity was equal to that of human GH as documented using FDC-P1 cells stably transfected with the rabbit growth hormone receptor (GHR) [6]. IGF-I was prepared in the lab of Dr J. Byatt (Monsanto, St. Louis, MO, USA), and its biological activity was documented using undifferentiated mammary epithelial cells cultured in collagen, serum-free medium [7], and bovine mammary cell line MME-L1 [8]. Ovine leptin (oLEP) was prepared at the Institute of Biochemistry, Food Science and Nutrition and was the gift of Professor Gertler. All data concerning the source of leptin is described previously by Gertler *et al.* [9].

Isolation and culture of ovarian follicles

Ovarian follicles (approximately 5 mm in diameter, collected from prepubertal pigs; <5 mm in diameter, classified as early antral; 5–8 mm in diameter, classified as antral follicles) and corpora lutea (1–3 days after ovulation) were pressed down, separated from the connective tissue, placed into Eppendorf tubes, and snap-frozen in liquid nitrogen. A specific type of follicles or corpus luteum was used in our experiment to examine the influence of IGF-I on the expression of leptin receptor Ob-Rb (long form). The follicles or corpora lutea were cut with small scissors, placed individually in 24-well plates (Nunc) with 2 ml of Parker medium supplemented with 5% calf serum for the controls or 50 ng/ml IGF-I for experimental group, and were then maintained at 37 C in humidified atmosphere containing 5% CO₂ for 24 h. The dose of IGF-I was chosen according to our preliminary results [10–12]. The expression of the functional leptin receptor (Ob-Rb) mRNA was examined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Five or six follicles or corpora lutea were used in each extraction, and each experiment was repeated 5 times.

RNA isolation and semiquantitative RT-PCR

Total RNA was extracted using Trizol reagent according to the manufacture's procedure. Reverse transcription reaction was performed using an

ImProm-IITM Reverse Transcription System (Promega). One microgram of total RNA was denatured with oligo(dT)₁₅ (0.5 µg/reaction) and random primers (0.5 µg/reaction) at 94 C for 5 min and chilled on ice. Then, 5 µl of 5 × ImProm-IITM reaction buffer, 1 µl of dNTP mixture (10 mM), MgCl₂ (25 mM), 20 U of Recombinant Rnasin® Ribonuclease Inhibitor, 1 µl of ImProm-IITM Reverse Transcriptase, and RNase-free water were added to a final reaction volume of 20 µl. The tubes were incubated at 25 C for 5 min, to 42 C for 60 min, and then 15 min at 70 C in a thermocycler (Mastercycler, Eppendorf). A 383 bp region of porcine long form leptin receptor cDNA was PCR-amplified using the following primers designed by Lin *et al.* [13]: sense primer 5'-TCGGAAGATATCAGTGTGA-3' and antisense primer 5'-TTGGGATGCTGATCTGATAA-3'. The reaction was performed using 5 µl of the first strand cDNA, 15 pmol of each primer, 2.5 µl of 10 × reaction buffer, 5 pmol dNTP mixture, MgCl₂ (25 mM), and 0.75 U Taq DNA polymerase. The samples were amplified for the indicated number of cycles by denaturing at 94 C for 30 sec, annealing at 50.8 C for 45 sec and extension at 72 C for 1 min. The reactions were completed by a final extension at 72 C for 7 min. Reactions were normalized by evaluating the level of amplification of the 18S transcript using commercial primers (classic 18S primers; Ambion). PCR reactions were performed within the linear range of the reaction for leptin receptor (granulosa cells, 34 cycles) and 18S rRNA (granulosa cells 14 cycles). PCR-amplified DNA was visualized by 2% agarose gel electrophoresis in TAE buffer and in the presence of ethidium bromide. Product yield was determined using the Kodak 1D v.3.6.1 software. The data was expressed in arbitrary units of the bound intensity from densitometric analysis of PCR products separated in 2% agarose gel and visualized in UV light.

Steroid hormone analysis

The concentration of 17β-estradiol (E₂) in the media was determined using EIA kits (IBL, Germany).

Progesterone (P₄) in the culture medium was assayed using a direct enzyme immunoassay (EIA) as described by Szafranska *et al.* [14]. The standard curve range was from 0.39 to 100 ng/ml, and the effective dose for 50% inhibition (ID₅₀) of the assay was 4.5 ng/ml. The intra- and inter-assay

Fig. 1. Progesterone (a) and estradiol (b) secretion by follicles collected from prepubertal pig ovaries (PPF), early antral follicles (EAF), antral (AF) follicles, and corpora lutea (CL). Statistically significant differences between points in each graph are indicated with different letters ($P < 0.05$). The same letters indicate no significant difference ($n = 12$).

coefficients of variation were 5.4% and 8.6%, respectively.

Statistical analysis

Statistical analysis was performed using Statistica 6.0. The data was analyzed by one-way analysis of variance (ANOVA) followed by the Tukey honestly significant difference (HSD) multiple range test. The data shown in graphs are means ± SEM from at least 5 different experiments.

Results

Progesterone and estradiol secretion by ovarian follicles and newly formed corpora lutea

Progesterone secretion was very small in prepubertal follicles and increased throughout follicular development with the highest amount secreted by corpora lutea (Fig. 1a). Estradiol

Fig. 2. Expression of leptin receptor (Ob-Rb) in porcine follicles and corpora lutea using semiquantitative RT-PCR assay. Data are shown as the ratio of leptin receptor expression relative to 18S rRNA (Ob-Rb/18S rRNA) in arbitrary units (n=5). ** P<0.01.

Fig. 3. Estradiol (open bars) and progesterone (black bars) secretion alone or in combination with insulin-like growth factor I (IGF-I) by follicles collected from prepubertal pig ovaries (PPF), early antral follicles (EAF), antral (AF) follicles, and corpora lutea (CL). * P<0.05, ** P<0.01 (n=12).

secretion was the highest in preovulatory follicles and decreased after ovulation (Fig. 1b).

Leptin receptor expression

The ratio of the active form of leptin receptor expression relative to 18S (Ob-Rb/18S) in the prepubertal follicle control cells was 0.143 ± 0.039 and they was 0.614 ± 0.092 , 0.625 ± 0.12 and 0.742 ± 0.11 in the EAF, AF and CL of mature porcine ovaries, respectively (Fig. 2).

Fig. 4. Expression of leptin receptor (Ob-Rb) in porcine follicles and corpora lutea using semiquantitative RT-PCR assay. Data are shown as the ratio of leptin receptor expression relative to 18S rRNA (Ob-Rb/18S rRNA) in arbitrary units (n=5). * P<0.05, ** P<0.001.

The action of IGF-I on estradiol secretion by ovarian follicles and progesterone secretion by newly formed corpora lutea

At all stages of follicular development, IGF-I increased estradiol secretion in a statistically significant manner and had a stimulatory action on progesterone secretion by newly formed corpora lutea (Fig. 3).

The action of IGF-I in relation to leptin receptor expression

In comparison with no-treated control cells, there were changes in the Ob-Rb mRNA level in all types of follicle and in corpora lutea cultured for 24 h in the presence of IGF-I. The ratio of the active form of leptin receptor relative to 18S (Ob-Rb/18S) increased by 12-, 1.4-, 1.8- and 1.8-fold in the prepubertal follicles, EAF, AF and CL, respectively (Fig. 4).

Discussion

Several authors have reported the relevance of

leptin in the regulation of reproductive processes in the ovary. Leptin receptor gene expression has been demonstrated in the porcine ovary, and the complementary DNA of porcine leptin receptor has been cloned and sequenced. Moreover, it has been shown that leptin receptor mRNA is present in freshly isolated granulosa cells and that its level increases along with the process of luteinization during cell culture [1].

Hormonal status in the ovary and expression of the long form of leptin receptor (ObRb) in ovarian follicles and newly formed corpora lutea

In swine, the recruitment of follicles occurs between day 14 and 16 of the estrous cycle under the influence of gonadotropins as a result of a decline in P_4 secretion and withdrawal of its negative feedback effect. Adequately regulated production of estrogens by the ovary is essential for follicular development, ovulation, and luteal function.

In the present study, we investigated the expression of the functional leptin receptor (Ob-Rb) mRNA in the ovary was investigated at different stages of the porcine estrous cycle. Ovaries were collected and mRNA was examined by RT-PCR. This study demonstrated that the expression of OB-R varied in response to the changing environment of the ovary. OB-Rb expression was low in prepubertal ovaries and increased during follicular development and in corpora lutea. Estradiol secretion increased from the prepubertal period to the preovulatory stage and dropped during the luteal phase. Just after the LH surge, follicles began to secrete progesterone, and the progesterone secretion continued after ovulation and was presented during corpus luteum formation. In summary, the fluctuations in leptin receptor mRNA content may constitute a response to the concentrations of follicular steroid hormones and to the changing environment of the ovary.

In regard to granulosa cells, leptin protein (determined by immunohistochemistry; IHC) and mRNA (evaluated by RT-PCR) have been detected in human cultured cells [15] and intact ovaries [16], although the follicular stage at which they are still controversial. In a previous report, Ryan *et al.* [17] found slight reactivity for leptin in the granulosa cells of murine ovaries did not specify the follicular stage. Ruiz-Cortes *et al.* [1] showed that the abundance of leptin receptor transcripts and

protein varies during luteinization of granulosa cells *in vitro* and in the CL during the porcine luteal phase. In the postovulatory CL, both the mRNA and protein of leptin receptors stay low; however, detectable, maximal expression was observed in the midcycle CL, and the lowest abundance occurred in the regressed CL. Leptin receptor mRNA was present in granulosa cells at isolation and increased in abundance as the cells luteinized over 96 h of culture. Leptin receptor protein was detectable after 12 h of *in vitro* luteinization.

The detailed description of the leptin receptor expression in follicles developing during natural estrous cycle in pigs, and not in *in vitro* luteinizing granulosa cells collected from prepubertal animals as was done by Ruiz-Cortes *et al.* [1], is novel finding of the present study. Archanco *et al.* [18] detected the production of leptin in postovulatory corpora lutea by IHC and in situ hybridization (ISH) and found that the number of luteal cells containing leptin protein increased during maturation and decreased during regression of the corpora lutea. Löffler *et al.* [16] also reported variations in leptin mRNA and protein in the developing corpus luteum. However, contrary to the findings of Archanco *et al.* [18], these authors reported that the highest amounts of leptin were found in the developing corpus luteum, whereas lesser amounts were detected during the secretory phase. This finding is in agreement with our observations. Cella *et al.* [19] showed that the serum leptin concentration increased during the luteal phase of the estrous cycle after a periovulatory peak in normal-weight women. They suggested a permissive role of leptin with regard to corpus luteum function. It is possible that leptin exerts an inhibitory effect in preovulatory follicles on the P450arom expression and E_2 synthesis known to occur during luteinization *in vitro* [3]; this has been also suggested by Ruiz-Cortes *et al.* [1].

IGF-I and leptin receptors

The porcine ovarian IGF system is particularly interesting because it appears that porcine ovarian cells produce IGF-I [20]. IGF-peptide as well as its mRNA can be also detected in the porcine ovary [21]. Kolodziejczyk *et al.* [12] showed that porcine theca and granulosa cells were sites of IGF-I production and action on steroidogenesis and proliferation. The stimulatory action of IGF-I on

steroidogenesis increases with follicular development, whereas its mitotic action decrease with follicular phase progression.

The presented results confirm previous data (Fig. 3) and show that IGF-I has its highest stimulatory effect on estradiol secretion in antral follicles.

Previously, we reported the maturation-dependent action of leptin on IGF-I-stimulated steroid secretion by porcine ovarian follicles. During follicular growth, leptin acts synergistically with IGF-I on estradiol secretion. In preovulatory follicles, leptin acts synergistically with IGF-I on the luteinization process, which starts just before ovulation [3]. During CL development, leptin given alone acts as an antiapoptotic factor, and when combined with IGF-I, a known antiapoptotic factor, it protects against excessive apoptosis and supports the appropriate cell numbers necessary for maintenance of homeostasis in the developing CL [4]. Studies performed mainly in rats and pigs have suggested that intraovarian IGF-I synthesis is of major importance in promoting follicular development, primarily by amplifying the actions of pituitary gonadotrophins and GH [18, 19]. Taking into consideration the results of the presented study and those of previously published studies, we hypothesis that in addition to the direct action of IGF-I on ovarian cells steroidogenesis and proliferation, IGF-I may also have stimulatory action on leptin receptor expression that constitutes another mechanism of IGF-I action in the ovary.

The presented data clearly shows that the ratio of the active form of the leptin receptor relative to 18S (Ob-Rb/18S) increased 12-, 1.4-, 1.8- and 1.8-fold in prepubertal follicles, EAF, AF and CL, respectively, in the presence of IGF-I. The important role of leptin in puberty was confirmed by the 12 fold increase of leptin receptor expression in prepubertal animals under the influence of IGF-I.

Moreover, it suggests that the action of IGF-I as a regulator of ovarian function is partly due to stimulation of leptin receptor expression, which promotes the availability of leptin. Ghanzzoni *et al.* [22] suggested that the relationship between GH and leptin could be a direct and characterized prevalently by a positive effect of GH on leptin secretion. Synchronicity has also been shown by Kautkia *et al.* [23] between GH and leptin (lag -39 min), cortisol and leptin (lag -211 min), and leptin and insulin, with leptin following insulin by 275 min.

However, additional data is requires that shows the action of IGF-I or GH on leptin secretion by ovarian follicles. This type of data is surprisingly scarce.

In conclusion, we suggest that (1) ovarian expression of leptin receptor is regulated throughout the estrous cycle by ovarian steroids, with peak expression at ovulation, indicating a possible involvement in follicular development and corpus luteum formation and (2) IGF-I may play an important role in communicating the metabolic status of the animal to the reproductive system directly by increasing expression of leptin receptor in follicles and the newly forming CL.

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