ESTRUS CYCLE-DEPENDENT ACTION OF LEPTIN ON BASAL AND GH OR IGF-I STIMULATED STEROID SECRETION BY WHOLE PORCINE FOLLICLES

Ewa L. Gregoraszczuk[1], Anna Ptak[1], Anna K.Wojtowicz[1], Tatiana Gorska[2], Krzysztof W. Nowak[2]

[1]Laboratory of Physiology and Toxicology of Reproduction, Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Krakow, Poland; [2] Department of Animal Physiology and Biochemistry, August Cieszkowski University of Agriculture, Poznan, Poland.
E-mail: greg@zuk.iz.uj.edu.pl

Objective. To determine the levels of leptin in the follicular fluid and using culture of whole ovarian follicles, to test the hypothesis that leptin may directly influence GH and IGF-I stimulated ovarian function.

Methods. Porcine follicles were recovered from ovaries during early, middle, and preovulatory stage of the follicular phase of the estrus cycle. They were cultured in the presence of the recombinant ovine leptin (oLEP) added either alone or with oGH or hIGF-I. Steroid concentrations in the media were determined after 48 h of culture

Results. The respective values for leptin in follicular fluid from small, medium and large follicles were 1.98, 2.18 and 1.96 ng/ml, respectively. Leptin added alone at a dose of 2 ng/ml had no effect on basal steroid secretion by small and medium follicles. However, in small follicles a synergic action of GH and IGF-I was noted. Leptin did not influence the secretion of progesterone by follicles collected during the early and middle follicular phases. In preovulatory follicles, leptin added alone to the culture media caused a decrease in basal estradiol secretion with a concomitant increase in progesterone secretion. Moreover, it acted synergistically with IGF-I and GH causing further stimulation of progesterone secretion.

Conclusions. The presented data show a direct, maturation dependent action of leptin on GH and IGF-I stimulated follicular steroidogenesis. During follicular growth they acted synergistically with GH and IGF-I in estradiol production, while in preovulatory follicles, they acted with both investigated hormones in luteinization process, which starts before follicular disruption.

Key words: Leptin – Estrus cycle – GH – IGF-I – Steroid secretion – Porcine follicles

Leptin, the obese (ob) gene product, is secreted by adipocytes and regulates appetite through interaction with hypothalamic leptin receptors (Considine and Caro 1997). Leptin mediates in transmitting information about the nutritional status to the centres, which control the function of reproductive system. Leptin was also found in follicular fluid and could induce a biological response in ovarian cells, suggesting that it may have a direct effect on the ovary (Karlsson et al. 1997). The porcine leptin receptor complementary DNA was cloned and sequenced, and the gene expression was evaluated in the porcine ovary (Ruiz-Cortes et al. 2000). These authors showed the presence of leptin mRNA in porcine corpus luteum, theca, and granulosa cells. In the recent paper Ruiz-Cortes et al. (2003) showed a biphasic effect of leptin. They also measured progesterone production by cultured porcine granulosa cells isolated from ovaries of prepubertal gilts and showed that leptin at 10 ng/ml increased progesterone production, while such production was decrease at a dose of 1000 ng/ml.

Most reports suggest that direct effect of leptin on ovarian cells is inhibitory and can be reversed by go-

nadotropin, insulin and insulin like growth factor-I (Duggal et al. 2000; Spicer et al. 2000). As follows from the literature, the mechanism of exogenous leptin action on the follicular steroidogenesis in pig has not been fully elucidated and the results achieved so far are controversial.

In both female and male mammals endocrine functions of gonads are regulated primarily by two pituitary gonadotropins, e.g. luteinizing hormone (LH) and follicle-stimulating hormone (FSH). However, there is considerable evidence that, in addition to these regulatory inputs, ovarian function can be influenced by growth hormone (GH) either directly or via stimulation of systemic and/or local production of the insulin-like growth factors (Apa et al. 1996). Elimam et al. (1999) suggested a direct effect of GH on leptin production, metabolism or clearance. Suter et al. (2000) were the first to show an increase in nocturnal leptin and GH-induced IGF-I secretion prior to the onset of puberty in the gonadal male monkey. The majority of data on the action of leptin in ovarian follicles were found in human granulosa cells obtained at the occasions of in vitro fertilization (Brannian et al. 1999; Kitawaki et al. 1999; Ghizzoni et al. 2001), in bovine granulosa and theca cells cultured alone as a monolayer (Spicer and Francisco 1997; Spicer et al. 2000) or, recently, in porcine granulosa cells aspirated from medium-size follicles of ovaries from prepubertal gills (Ruiz-Cortes et al. 2003). Surprisingly, there are no data showing any interaction of leptin with GH and IGF-I which would be dependent on the stage of follicular development.

In this study, we measured the levels of leptin in the follicular fluid and, by using culture of whole ovarian follicles, we tested the hypothesis that leptin may directly influence GH and IGF-I stimulated ovarian function.

Material and Methods

Reagents. Parker medium (M199), PBS was obtained from Biomed (Lublin, Poland). Antibiotic-antimycotic solution was purchased from Sigma (St. Louis, MO, USA). Ovine GH was prepared in the Institute of Biochemistry, Food Science and Nutrition (Rehovot, Israel) in the laboratory of professor Gertler. Its biological activity was equal to that of human GH as documented by using FDC-P1 cells stable transfected with the rabbit GHR (Herman et al. 1999). IGF-I was obtained from D. John Byatt (Monsanto Co., St. Louis, MO, USA) and its biological activity was documented using undifferentiated mammary epithelial cells cultured in collagen in serum free medium (Peri et al. 1992) and human mammary cell line MME-L1 (Fine et al. 1997). Ovine leptin (oLEP), were prepared (Gertler et al. 1998).

Isolation and culture of ovarian follicles and follicular fluid collection. Pig ovaries were obtained from a slaughterhouse. Follicles were classified as small (<5 mm in diameter), medium (5-8 mm in diameter) and large (10-12 mm in diameter) preovulatory follicles. These groups of follicles correspond to estrus cycle days 15, 18 and 21-day in which Day 0 is estrus (Liu et al. 1998). Whole follicles were isolated from ovaries. Follicular fluids aspirated from particular type of follicles were frozen for analysis of leptin levels. Isolated follicles were put to Erlenmeyer flask containing 5 ml of M199 medium according to Gregoraszczuk (1990). The flasks were continuously shaking at 70 rpm, for 48 hrs. Such model of culture permits the examination of hormone action on the whole follicles and has been used also in our previous study (Gregoraszczuk et al. 2003). There was 81.2 % of proliferating cells as measured using the MIB-I labelling index in 6 days of culture, which indicated that this model is applicable. Follicles were maintained at 37 °C in humidified atmosphere of 5 % CO_2 for 48 h either in control medium or in the presence of oLEP (2 ng/ml) alone, oGH (100 ng/ml) alone, IGF-I (30 ng/ml) alone or of a combination of oLEP with GH or IGF-I in the same concentrations as indicated above for each hormone added alone. The dose of leptin was chosen by taking into consideration the amount of leptin in the follicular fluid and also according to Kiwataki et al. (1999). The dose of GH was selected according to our preliminary results (Gregoraszczuk et al. 2000) while the dose of IGF-I was chosen according to Kolodziejczyk et al. (2003). After 48 hours the experiment was terminated and the conditioned media were collected and stored at –20 °C. The level of steroid production was calculated per follicle per 48 h. Each treatment was conducted in 5 wells and each experiment was repeated 3 times.

Steroid analysis. Progesterone and estradiol were determined by radioimmunoassay using Spectra kits (Orion, Diagnica, Finland) as supplied by Polatom (Świerk, Poland).

In the progesterone assay the lowest level sensitivity was 94 pg/ml and the coefficients of variation between and within assays were 5.8 % and 2.9 %, respec-

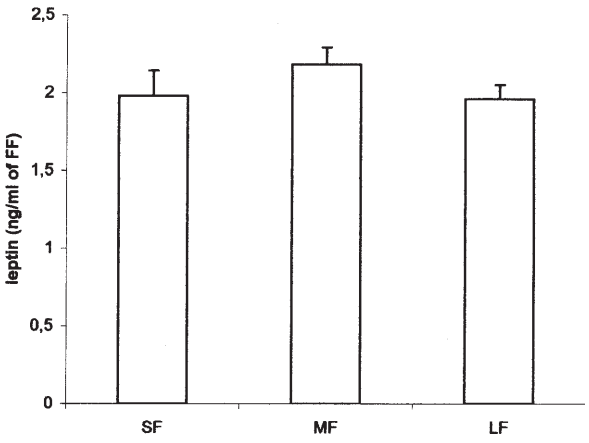

Fig. 1 The leptin levels in follicular fluid collected from small (SF), medium (MF) and large size (LF) follicles.

tively. The mean recoveries were 95.1-103.7 %. The cross-reaction with pregnenolone was 2.9 %, whereas other tested steroids such as (5β-dihydroprogesterone, 20β-hydroxyprogesterone, corticosterone, testosterone, and estrone) showed less then 1 % cross-reaction.

The detection limit of estradiol assay was 5 pg and the coefficients of variation between and within assays were 10.28 % and 2.9 % respectively. The mean recoveries were 85.6-108.9 %. The cross-reaction with ethinyl estradiol was 1.4 %. All other tested steroids (estrone, estriol, progesterone, testosterone, and corticosterone) showed less then 1 % cross-reaction.

Leptin determination. Leptin was determined by radioimmunoassay using Multi-Species Leptin RIA Kit (Linco Research, St. Charles, MO, USA). The lowest leptin level detected by this assay was 1.0 ng/ml of human leptin equivalent and the intra- and interassay variations were 3,4 % and 8,7 %, respectively. The mean recoveries of leptin in human serum were 93-104 % and crossreactivity of used antibody was: human leptin – 100 %, porcine leptin – 67 %, rat leptin – 61 % mouse leptin – 73 % and canine leptin – 3 %. There were not detectable cross reactions with human insulin, human proinsulin, human C-peptide, rat insulin, glucagons and IGF-I.

Statistical analysis. The number of replicates per treatment was 12. Since the variations between the experiments were small, these 12 results were analysed by ANOVA followed by Duncan's new multiple range test. Each average (n=12) was expressed as means and S.E.M.

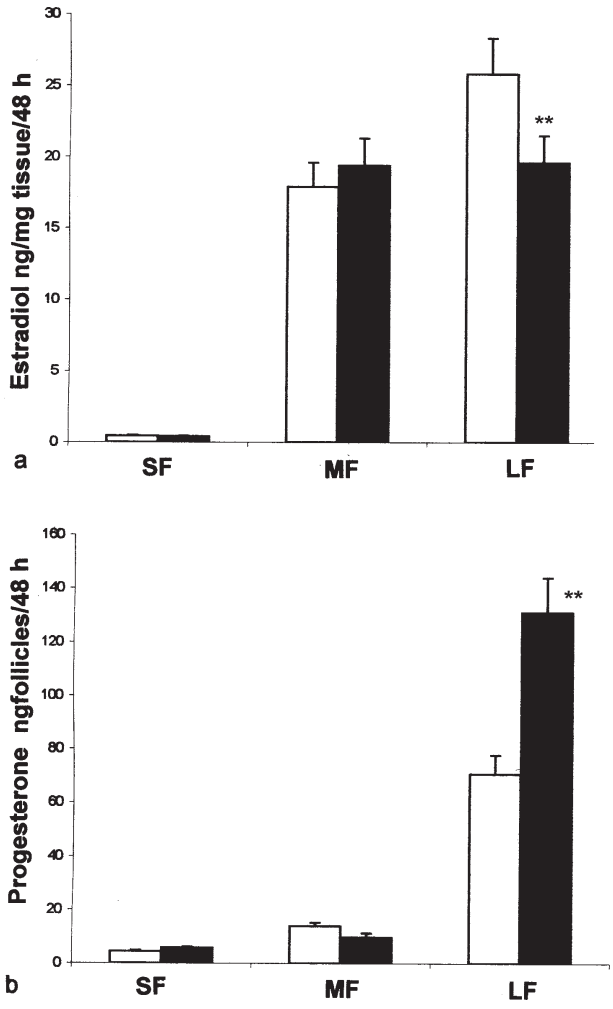

Fig. 2 Effect of leptin alone (LEPT – black columns) on basal (C – white columns) a) estradiol (upper panel) and b) progesterone secretion (lower panel) by small (SF), medium (MF) and large size (LF) follicles. **p<0.01

Results

Leptin levels in the follicular fluid. The respective values for leptin in follicular fluid from small, medium and large follicles were 1.98 ± 0.16, 2.18 ± 0.11 and 1.96 ± 0.09 ng/ml (Fig. 1).

Effect of leptin on estradiol and progesterone secretion. Leptin alone added in a dose of 2 ng/ml had no effect on basal estradiol and progesterone secretion by small and medium follicles. (Fig. 2a,b). However, after the addition leptin to the culture medium of large follicles a decrease of estradiol secretion ($p<0.01$) but doubled progesterone secretion ($p<0.01$) was fund (Fig. 3a,b).

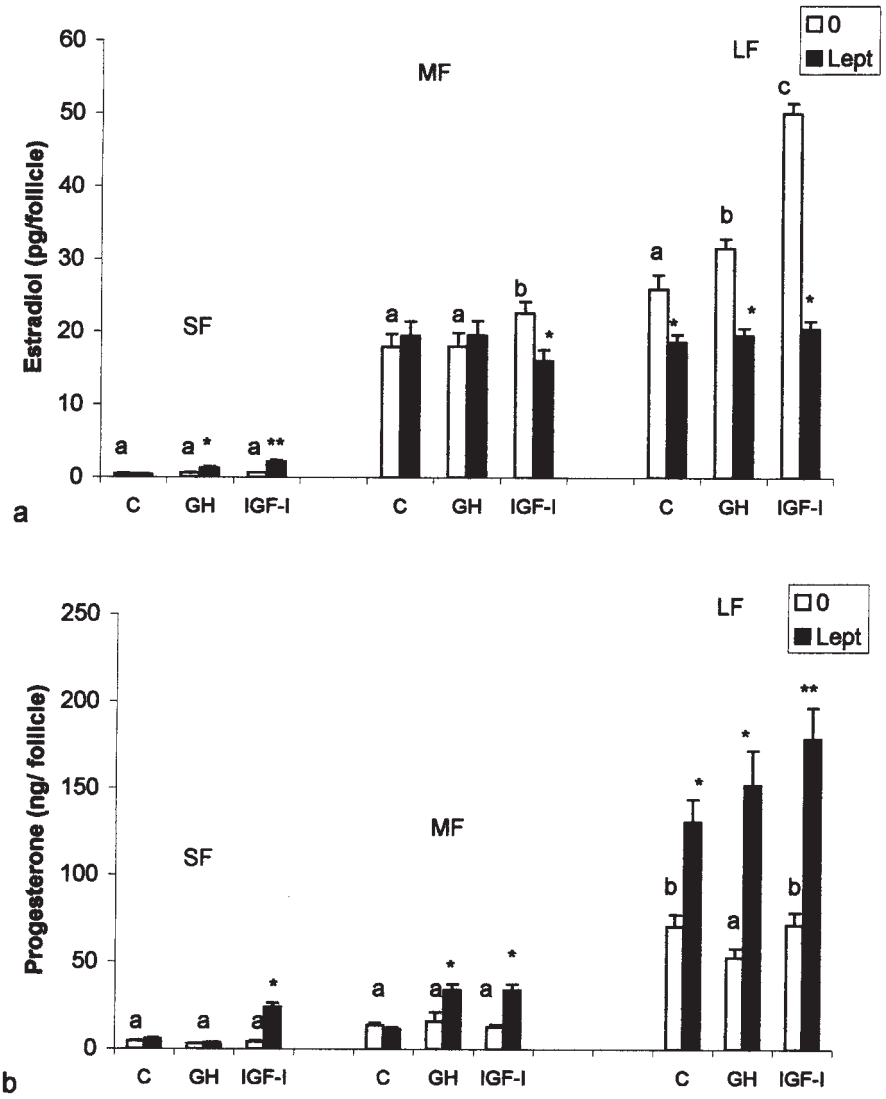

Fig. 3 Estradiol (a) and progesterone (b) secretion in leptin alone or in combination with growth hormone (GH) and insulin-like growth factor-I (IGF-I) treated follicles collected during early (SF), middle (MF) and late (LF) follicular phase. Bars headed by different letters differ significantly ($p<0.05$) from their respective controls.

Effect of leptin on GH or IGF-I-stimulated estradiol secretion. GH and IGF-I alone had no effect on estradiol secretion by small follicles. However, simultaneous addition of leptin with GH doubled estradiol secretion by this type of follicles ($p< 0.05$), while in the case of IGF a fivefold increase of estradiol secretion was noted ($p<0.01$) (Fig. 3a). In medium follicles, GH had no effect on estradiol secretion, while 2-fold increase of estradiol secretion was noted under the influence of IGF-I. The simultaneous addition of leptin with GH was without any effect on the estradiol secretion by this type of follicles, while in the case of IGF a twofold decrease of estradiol secretion ($p<0.05$) was noted in simultaneous treatment with leptin (Fig. 3a).

In large follicles both GH ($p<0.05$) and IGF-I ($p<0.01$) increased estradiol secretion. In addition, 1.6 fold decrease of estradiol secretion was noted after simultaneous addition of leptin with GH ($p<0.05$) and 2.4 fold decrease was found after simultaneous addition of leptin with IGF-I ($p<0.01$) (Fig. 3a).

Effect of leptin on GH or IGF-I-stimulated progesterone secretion. In small follicles GH showed no effect on progesterone secretion when added alone or in combination with leptin. IGF-I added with leptin

showed synergistic action resulting in a fivefold increase in progesterone secretion (Fig. 3b). In medium follicles GH significantly stimulated progesterone secretion and this effect was intensified by leptin ($p<0.05$). IGF-I showed no effect on progesterone secretion when added alone, while 2 fold increase of progesterone secretion was observed after combined treatment with leptin ($p<0.05$). In large follicles IGF-I alone had no effect on progesterone secretion, while this that was decreased after GH ($p<0.05$). However, the simultaneous addition of leptin with IGF-I doubled progesterone secretion by this type of follicles ($p<0.05$), while in the case of GH a fourfold, statistically significant ($p<0.01$) synergistic increase with leptin was noted (Fig. 3b).

Discussion

Presented data showed no changes in follicular fluid leptin levels during follicular phase after incubation of isolated pig ovarian follicles. This is the first data obtained in pigs which are in accordance with these by Bennett et al. (1999) who showed unchanged serum leptin levels during the estrous cycle together with the correlation between the expression of leptin receptor and neuropeptide Y in rat. Despite of the mentioned unchanged follicular fluid leptin levels, the presented data showed that the action of leptin on steroidogenesis depends on the stage of follicular development. Thus, we noted no effect of leptin on small and medium size follicles, while a decrease of estradiol secretion with parallel increase of progesterone secretion by large preovulatory follicles was found. Zachow and Magoffin (2001) showed that leptin alone had no effect on estradiol production by rat granulosa cells thus confirming a part of our observation concerning estradiol secretion. In the last paper Ruiz-Cortes et al. (2003) showed that leptin at 10 ng/ml increased progesterone production by porcine granulosa cells collected from medium follicles of ovaries from prepubertal gilts.

In the presented data we noted a stimulatory action of leptin on large preovulatory follicles. This discrepancy could be due to different culture model, e.g. whole follicles in our experiments and only granulosa cells in these of Ruiz-Cortes et al. (2003) and, moreover, we used cycling pigs while they used prepubertal gilts.

Additionally, in the presented paper we showed that leptin acted synergistically with GH and IGF-I by increasing estradiol secretion only by small follicles, while decreased IGF-I stimulated estradiol secretion by medium size follicles and decreased both GH- and IGF-I stimulated estradiol secretion by preovulatory follicles. This observation could explain the findings by Hardie et al. (1998) who showed that, when normal prepuberty mice are given recombinant leptin, their reproductive system matures earlier and they also reproduce earlier than mice not given leptin. So it is possible that observed earlier reproductive maturation of mice is due to leptin action on early antral follicles.

Taking into consideration that leptin receptor varies during pig ovarian cell differentiation (Ruiz-Cortes et al. 2000), we assume that leptin may accelerate the onset of puberty through its action with GH and IGF-I. It remains possible that leptin exerts an inhibitory effect on estradiol secretion known to occur during luteinization in vitro (Gregoraszczuk 1994, Pescador et al. 1999).

Leptin dependent increase of progesterone secretion by large preovulatory follicles as observed in our experiments and also synergistic action of leptin with GH and IGF-I on inhibition of estradiol secretion with concomitant stimulation of progesterone secretion could be explained by the data of Hardie et al. (1998) who showed the increase in leptin levels during transformation from the follicular to the preovulatory phase when the values peaked levels 1.5 times higher than these in follicular phase. In the preovulatory follicles we observed increase of progesterone secretion and parallel decrease of estradiol secretion. It is well known that progesterone is an inhibitor of aromatase (Gregoraszczuk 1994) and thus shortly prior to the ovulation, after LH surge, estradiol secretion decreased and follicular cells luteinized secreted larger amount of progesterone. It is possible that leptin in this stage of follicle development acted together with other hormones in changing follicles to corpus luteum. This hypothesis is supported by our previous results showing synergistic action of leptin with FSH on estradiol secretion by small and medium size follicles and leptin with LH on progesterone secretion by large size follicles (Gregoraszczuk et al. 2003) as well as by the observation of Ruiz-Cortes et al. (2000) who showed that leptin receptor expression in porcine granulosa cells increased at the time of with luteinization *in vivo* and *in vitro*.

We conclude, that during follicular growth leptin acted synergistically with IGF-I and GH thus supporting estradiol production. In preovulatory follicles, the finding of decreased estradiol secretion with the simultaneously increased progesterone secretion under the influence of leptin showed its action on the process of luteinization which starts immediately before the ovulation and after that on the transformation of follicles to corpus luteum as suggested by Ruiz-Cortes et al. (2003).

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Corresponding author: Dr. Ewa L. Gregoraszczuk
Department of Animal Physiology
Institute of Zoology
Jagiellonian University
Ingardena 6
30-060 Krakow, Poland
e-mail: greg@zuk.iz.uj.eolu.pl