ORIGINAL ARTICLE

The evaluation of estradiol and leptin action on the activity of the somatotropic and gonadotropic axes in peripubertal female rats

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Submitted: 2012-01-15 Accepted: 2012-02-10 Published online: 2012-04-25

Key words: **estradiol; leptin; GH; IGF-I; LHRH; LH; FSH; estrogen receptor; puberty; female rat**

Neuroendocrinol Lett 2012; **33**(2):138–148 **PMID:** 22592194 NEL330212A03

Abstract

OBJECTIVE: Available data suggest that estrogens and leptin play a role in the control of the pubertal process. In humans and some mammal species the increase of the activity of gonadotropic axis accompanies the decrease in the rate of growth at puberty. The effect of 17β-estradiol and/or leptin administration on the somatotropic and gonadotropic axes was studied using prepubertal female rats as an animal model.

MATERIAL AND METHODS: Prepubertal female rats received estradiol/saline, estradiol/leptin, oil/leptin or oil/saline (vehicles) respectively. The changes of growth rate, and serum 17β-estradiol, leptin, GH, IGF-I and gonadotropins levels as well as LHRH and estrogen receptor (ER) concentrations in the medial basal hypothalamus (MBH) and the pituitary were determined. All hormones concentrations were measured by radioimmunoassay and ER by radioligand methods .

RESULTS: In estradiol and/or leptin treated animals noticeable reduction of rate of growth was found. The decrease of growth in response to estradiol treatment accompanied the increase GH level and the decrease of IGF-I concentration in the circulation. Both hormones operating together activated reproductive axis, what was manifested by a significant increase of LHRH abundant in the hypothalamus as well as elevated LH and FSH levels in the circulation. In these rats a significant decrease of the estrogen receptor concentrations in the pituitary was observed.

CONCLUSION: The role of estradiol and leptin in the control of growth and reproduction seems to overlap only partially. Estradiol plays a significant role in the activation of the reproductive axis, and leptin takes part as a permissive factor in pubertal process.

INTRODUCTION

Puberty is a very specific development period in mammals. The onset of the pubertal process is heralded by changes in the amplitude and the pattern pulses of the gonadotropin-releasing hormone (GnRH), known also as the luteinizing-releasing hormone (LHRH) and intensification of its secretion from hypothalamus. In consequence, the increase in the release of gonadotropins (LH and FSH) and gonadal steroids takes place. Ovarian steroids, specially 17β-estradiol via negative or positive feedback play a critical role in the synthesis and secretion of gonadotropins and are necessary to maintain the reproductive function. Estradiol also reduces food intake and increases energy expenditure through a hypothalamic mechanism (Dubuc 1985; Palmer & Gray 1986). A significant decrease of growth rate is frequently observed during this period. The growth rate may be regulated through complex interactions between gonadal steroids, growth hormone (GH) and insulin-like growth factor I (IGF-I). Some data indicate that exogenous estradiol is a potent anorectic agent which is involved in the reduction of food intake and body weight in ovariectomized and intact rats (Rivera & Eckel 2010). The growth reduction in response to exogenous estradiol administration accompanied an elevation of GH level and simultaneously suppression of IGF-I concentration in the circulation of female rats (Borski *et al.* 1996).

It has long been recognized that a minimum amount of stored energy is required for initiation and maintenance of reproductive function of rodents and humans (Kennedy 1969; Frisch & McArthur 1974). Identified by Zhang and colleagues (1994), the adipose-derived hormone, leptin, is a peptide conveying information about the nutritional status to the hypothalamic centers, which are involved among all in the control of food intake and energy balance. This hormone is also required for the onset of reproduction (Rosenbaum & Leibel 1998; Donato Jr. *et al.* 2011). In the circulation the leptin level is proportional to fat mass in rodents and humans and falls following weight loss (Maffei *et al.* 1995). It is well established that food restriction and high energetic exercise may delay the onset of puberty as well as disrupt fertility (Cunningham *et al.* 1999; Hill *et al.* 2008). In leptin-deficient ob/ob mice, the animals with mutation of the obese gene, delayed puberty and infertility was observed. Whereas, in transgenic mice, with overexpress of leptin, acceleration of puberty was found (Yura *et al.* 2000). In a peripubertal children with defect of leptin coding gene as well as in anorectic woman with hypoleptinaemia phenotype of hypogonadotropic and hypogonadism is present as the consequence. Leptin therapy in these hypoleptinaemic humans as well as mice induced puberty and restored their fertility. In addition, when juvenile wild-type mice were treated with exogenous leptin an acceleration in the onset of puberty was recorded (Ahima *et al.* 1997; Chehab *et al.* 1997; Farooqi at al. 1999; Welt *et al.* 2004). The role of leptin in the timing of puberty and fertility in the intact wild-type of rats remains ambiguous. Leptin administration can restore and maintain fertility in malnourished female rats (Nagatani *et al.* 1998). But, there is no clear evidence that leptin is a metabolic trigger for the onset of puberty in this species (Cheung *et al.* 2001).

Biological effects of estrogen and leptin are mediated via specific receptor for each ligand. The estrogen receptors (ERs) exist in two forms (ERα and ERβ), as the products of two separate genes. These two receptors belong to the nuclear receptor superfamily, which is binded to specific elements in the promoter region of estrogen-responsive genes, and in effect the activation or suppression of gene transcription in target cells takes place. Leptin receptor (ObR) is encoded by a single gene and exists in six isoforms. Only ObRb isoform with the longest intracellular domain is able to mediate various biological processes through JAK-STAT signaling (Tartaglia *et al.* 1995). It has been found that estrogen and leptin receptors are colocalized in the neurons within the hypothalamic nuclei which are involved in coordination of metabolism and reproductive function (Diano *et al.* 1998). This close coupling of both receptors in the same hypothalamic areas suggests that a functional cross-talk between estrogen and leptin signaling may exist (Goa & Horvath 2008). Estrogen and leptin receptors are also present in many peripheral organs, such as the pituitary gland, the ovary and the liver (Schreihofer *et al.* 2000; Ryan *et al.* 2003; Cohen *et al.* 2005).

The changes in ERs activity during different stages of juvenile period was observed. These changes were more or less significant depending on the methods used for their evaluation. Using a microdissection-based nuclear ER assay and in vivo autoradiographic studies a small increase in estrogen binding in the hypothalamic ventromedial and arcuate nuclei was found in the female rats at around the time of vaginal opening and the onset of reproductive cyclicity. However, when ER binding capacity was measured, it was remarkably similar in pre- and postpubertal rats (Brown *et al.* 1994). In different developmental stages the changes in the hypothalamic structures of leptin receptor level were also observed. In peripubertal female rats the increase of leptin receptor was significantly higher compared to its level in earlier phase of the prepubertal period (Smith & Waddell 2003).

Available data suggest that the increase, around the time of vaginal opening, of the activity of ERs and ObRs at the hypothalamic nuclei involved in the control of food intake, metabolism and reproductive processes may lead to alternations in the sensitivity of target tissues to circulating estrogens and leptin.

So, using peripubertal intact female rats as an animal model, the effect of estrogen (17β-estradiol) and/or leptin administration on the somatotropic and gonadotropic axis was studied.

The changes of the body weight rate as well as GH and IGF-I levels in the peripheral blood were monitored. For evaluation of the activity of reproductive axis, the age of vaginal opening, LHRH concentrations in the medial basal hypothalamus (MBH), estrogen receptors (ER) in the MBH and the anterior pituitary (PIT) as well as gonadotropins (LH, FSH) level in the circulation were determined.

MATERIALS AND METHODS

Reagents

Recombinant murine leptin (mleptin) was delivered from PeproTech EC LTD (London, England). 16α-[^{125}I]-Iodo-3,17β-Estradiol (2200 Ci/mmol specific activity) was obtained from PerkinElmer Life Sciences, Inc. USA. Diethylstilbestrol (DES) and 17β-estradiol (E2) were purchased from Sigma Chemical Co. (St. Louis, MO). Norit A-activated carbon was obtained from Merck, Darmstadt, Germany and dextran T500 from Amersham Pharmacia Biotech, Uppsala Sweden. The 96-wells microtiter plates were delivered from Corning Incorporated Corning, USA.

Animals

Immature, 28 days old, female Wistar rats were kept in a controlled environment (14 : 10h dark/ light cycle, light at 06.00h) and temperature (22°C), with free access to food and water. These rats at 30th day of life were randomly assigned to one of four experimental groups (15 animals per group). The experimental protocol is presented in Table 1. All animal procedures were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and were approved by the 1st Warsaw Ethic Committee for Experiments on Animals. All injections sc of estradiol and/or ip of leptin were given between 13.00–14.00 h. The body weight at the 30th, 33rd and 39th day of life and vaginal opening (VO) as an external index of puberty at the 30th, 33rd, 35th, 36th, 37th, 38th and 39th were recorded. However, vaginal smears from the rats with vaginal opening were not examined. The day after the final leptin administration, the animals were killed by decapitation. The PIT and the MBH were excised and weighted. The tissues were stored in liquid nitrogen until further processing. Trunk blood was collected, the serum separated and stored at –20 °C until assayed.

Procedure of homogenization and preparation of cytosolic fractions

The PITs (5 glands) and the MBHs (3 structures) were pulled together and homogenized (PIT in 1:25 and MBH 1:10; w/v) in ice cold 10 mM TEMG buffer [Tris-HCL (pH 7.4) containing 1.5 mM EDTA, 1 mM Na-thioglicolate and 10% (v/v) glycerol]. The homogenates were centrifuged at 100,000 g for 60 min at 4 °C for prepared soluble (cytosolic) fraction. In the pellets were measured DNA using the diphenylamine method for the colorimetric estimation of deoxyribonucleic acid.

Estrogen receptor assay

Estrogen receptor was determined by radioligand binding assay described by Fitzpatrick *et al.* (1999) and charcoal separation technique to trap radioligand unbound with receptor protein. For evaluation of estrogen receptor (ER) activity the measurement of the number of ligand binding sites (functional receptor) was performed. The functional receptor has physiological significance because it reflects the actual susceptibility of tissue to action of estrogen. Cytosolic fraction (50 µl) was added to each well and then 25 µl assay buffer for determining the total bound and the nonspecific binding was calculated in parallel incubation at the presence 500 fold excess of non-radioactive DES. Reactions were initiated by the addition of 25 µl of six consecutive concentrations (from 30 pM to 960 pM) of 16α- [^{125}I]-Iodo-3,17β-estradiol in assay buffer. The final reaction volume was of 100 µl. After 20h (4 °C) incubation, ice-cold buffer (100 µl) containing 1% (w/v) Norit A and 0.01% (w/v) dextran T500 was added to remove unbound radioligand. The microtiter plates were incubated in ice bath for 20 min and then centrifuged at 2,500 g for 10 min at 4 °C. Aliquot of supernatant (150 µl) was taken from each well. The radioactivity in each sample was counted using gamma counter. The amount of specifically bound radioactivity was calculated by subtracting nonspecific counts from total counts after correction the data for a total sample volume (200 µl).

Receptor data were processed according to Scatchard analysis, corrected for nonspecific binding according to Chamness & McGuire (1975). Using the Scatchard plot both dissociation constant (Kd) and the apparent maximum binding sites (Bmax) with 95% confidence limits was calculated. The results were expressed as Bmax per gram of tissue or expressed in relation to mg of DNA.

Tab. 1. Experimental protocol.

Experimental groups	Treatment procedures
estradiol/saline	The rats received sc injection of estradiol (37.5 µg/day) in 0.2 ml of oil for 3 consecutive days and following received ip daily injection of 0.3 ml of saline for 6 days
estradiol/leptin	The rats received sc injection of estradiol (37.5 µg/day) in 0.2 ml of oil for 3 consecutive days and following received ip daily injection of 100 µg of leptin in 0.3 ml of saline for 6 days
oil/leptin	The rats received sc daily injection of 0.2 ml of oil for 3 consecutive days and following received ip daily injection of 100 µg of leptin in 0.3 ml of saline for 6 days
oil/saline (vehicles)	The rats received sc daily injection of 0.2 ml of oil for 3 consecutive days and following received ip injection of 0.3 ml of saline for 6 days

The expression of binding data related to DNA unit is recommended by Snochowski (2002). The calculation of the numbers of estrogen binding sites per cell was also performed. In this case, the numbers of cells per mg of DNA were calculated on the basis of analysis of DNA and with assumption that 6.2 pg of DNA is an average content of mammal cell (Enesco & Leblond 1962). The number of ER molecules was calculated using Avogadro constant (6.02×10^{23} molecules per mole).

Determination of LH-RH in the MBH.

For LHRH determination the MBHs were prepared using the method described by Ponzo *et al.* (2001). Briefly, the MBH (6 structures per group) were homogenized in 0.1 N hydrochloride acid (0.5 ml) in ice bath using glass-glass homogenizer. The homogenate was centrifuged at 12,000 g and the supernatant obtained was diluted 1:4 (v/v) in 0.01 M PBS buffer (pH 7.4) containing 0.15% BSA (w/v) and was kept at –70 °C until LH-RH concentrations assayed. The results were expressed in nanograms per mg of tissue.

Radioimmunoassays

Serum LH and FSH concentrations were measured by RIA using antibodies and reference standards provided by Dr A.F. Parlow and the National Hormone & Pituitary Program, (Torrance, CA 90509, USA). Values were expressed in terms of rat LH (RP-3) and FSH (RP2), respectively. The sensitivity of these assays was 0.15 ng/ml for LH and 1.25 ng/ml for FSH. Serum estradiol level was measured by RIA methods using commercial veterinary kits (Immunotech, France). The detection limit of the assay was 10 pg/ml. LHRH concentrations were determined using commercial RIA kit (Phoenix Pharmaceuticals, Inc., USA). The sensitivity of the assay was 20 pg/ml of aliquot. Rat serum GH and leptin levels were assayed using the kits from Linco (USA). The sensitivity of both assays were 0.5 ng/ml. IGF-I concentration was measured using the IGF-I RIA kit with IGFBP blocked from Mediagnost (Germany). The limit of detection for IGF-I kit was 10 ng/ml. For each hormone all serum samples were analyzed in a single assay. In all hormonal analyses the intra-assay coefficients of variation (CV) were below 10%.

Statistical analysis

The data are presented as the means ± SEM. Only the values of the parameters for estrogen receptor were expressed as the means ± SD. Statistical analyses were done using Statsoft Statistica PL for Windows. Initially all groups of data were tested for normality by the Kolmogorov-Smirnov test and Shapiro-Wilk's test. Statistical differences between the groups were determined by one-way ANOVA. Duncan's multiple range test made post-hoc comparisons. However, when variances were found to be significantly heterogeneous, the comparisons between groups were done by nonparametric Kruskal-Wallis test followed by the Mann-Whitney U test. Differences in mean values were considered significant if $p \leq 0.05$.

RESULTS

Serum estradiol and leptin concentrations in peripubertal female rats treated with exogenous estradiol and/or leptin

Estradiol and leptin levels in the peripheral blood are showed in Figure 1.

In both groups of rats receiving sc estradiol (estradiol/saline and estradiol/leptin) for 3 consecutive days the total serum estradiol level in the circulation was significantly higher compared to the group receiving vehicles (oil/saline) at the same time ($p \leq 0.001$). In rats

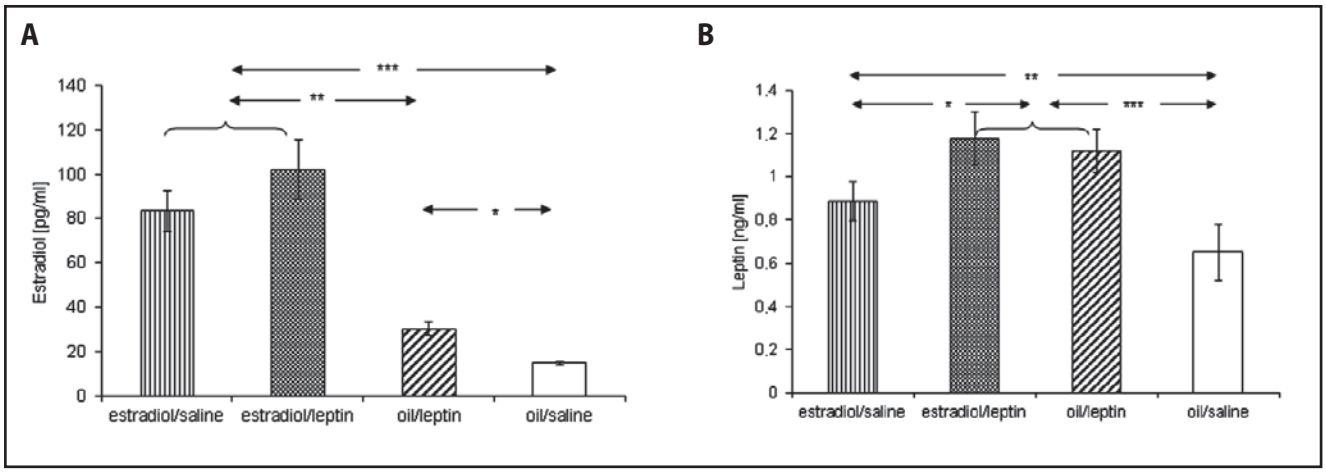

Fig. 1. Serum 17β-estradiol (a) and leptin (b) concentrations in the peripheral blood of peripubertal female rats after exogenous estradiol and/or leptin treatment.
The rats at 30th day of life were assigned to one of four experimental groups (15 animals per group). The rats were treated with estradiol or oil for 3 days and then for 6 consecutive days with leptin or saline. The next day after the last leptin or saline administration, the animals were killed, trunk blood was collected and the serum was separated. Serum estradiol and leptin concentrations were determined by radioimmunoassay method. Each bar represents mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

treated with leptin alone (oil/leptin group) the estradiol concentration was significantly higher compared to rats treated with vehicles ($p \leq 0.05$) but it was about 3 times lower compared to the estradiol level in both estrogenized groups ($p \leq 0.01$).

Leptin concentrations in the circulation were significantly higher in both leptin treated groups of rats (estradiol/leptin and oil/leptin) compared to vehicles (oil/saline) group ($p \leq 0.001$). In animals receiving only estradiol (estradiol/saline), circulating leptin level was significantly lower ($p \leq 0.05$) compared to both groups of animals treated with leptin (estradiol/leptin) and (oil/leptin) but significantly higher compared to vehicles group ($p \leq 0.01$).

Changes in the growth rate in peripubertal female rats treated with exogenous estradiol and/or leptin

Changes of growth rate are showed in Table 2. Only in female rats receiving estradiol and following leptin (estradiol/leptin group) the final body mass was significantly lower ($p \leq 0.01$) compared to the body mass of animals in other experimental groups. On the 33rd day of life, the mean of body mass of one group receiving oil was significantly higher compared to others groups ($p \leq 0.05$). These differences could be the result of diversity of the initial body weight. The effects of estradiol and leptin administration were more clear when the changes in the daily body weight gain were calculated. In animals treated with estradiol the mean body weight gain was significantly lower ($p \leq 0.01$) compared to the groups of rats receiving vehicle (oil). In the next days of the experiment, both groups of rats receiving leptin, the daily body weight gain was very significantly reduced ($p \leq 0.01$) compared to both groups of rats treated with saline.

Serum GH and IGF-I concentrations in peripubertal female rats treated with exogenous estradiol and/or leptin

The effect of estradiol and/or leptin on GH and IGF-I levels in the peripheral blood are shown in Figure 2.

Tab. 2. Body weight and body weight gain after estradiol and/or leptin treatment of peripubertal female rats.

Indices	Age [days]	Experimental groups				*p*-value
		estradiol/saline	estradiol/leptin	oil/leptin	oil/saline	
Body weight [g]						
Initial	30	86.8±1.64	84.2±2.45	86.9±2.46	80.4±1.89	NS
after estradiol/oil treatment	33	94.5±1.82	92.2±2.50	100.8±2.93[a]	93.1±2.11	≤0.05
after leptin/saline treatment	39	120.3±2.67	111.1±2.74[b]	119.8±3.21	118.2±2.63	≤0.01
Body weight gain [g/day]						
after estradiol /oil treatment	30–33	2.58±0.18[c]	2.67±0.27[c]	4.62±0.24	4.26±0.20	≤0.01
after leptin/saline treatment	33–39	3.69±0.20	2.71±0.23[d]	2.73±0.11[d]	3.58±0.14	≤0.01

The 30-days old female rats were assigned randomly to one of four group (15 rat/group). The body weight at 30th, 33rd and 39th was recorded. The body weight gain was calculated after 3 days of estradiol or oil treatment and then after 6 days of after leptin or saline administration. Means within rows with common letters are not significantly different from each other but are significantly different from others. NS - not significant. The values are expressed as mean ± SEM.

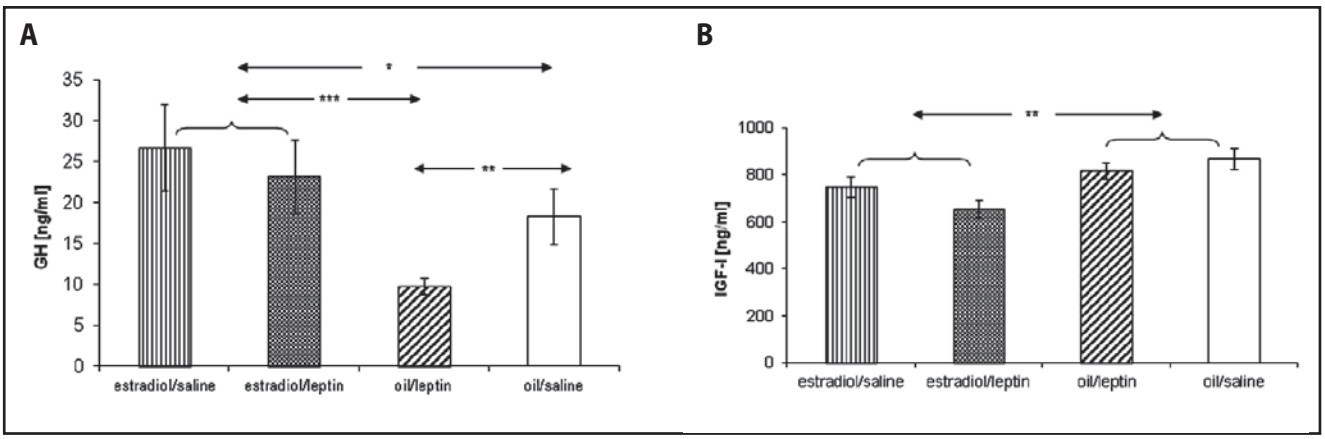

Fig. 2. Serum of GH (a) and IGF-I (b) concentrations of peripubertal female rats after exogenous estradiol and/or leptin treatment. The rats at 30th day of life were assigned to one of four experimental groups (15 animals per group). The rats were treated with estradiol or oil for 3 days and then for 6 consecutive days with leptin or saline. The next day after the last leptin or saline administration, the animals were killed, trunk blood was collected and the serum was separated. Serum GH and IGF-I concentrations were determined by radioimmunoassay method. Each bar represents mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

In both estradiol-treated groups of rats serum GH concentrations were significantly higher compared to non-estrogenized groups of animals ($p \leq 0.05$ vs. vehicles group and $p \leq 0.001$ vs. only leptin-treated group). In estrogenized rats no effect of leptin on GH level was observed. However, in the group of rats treated with leptin alone (oil/leptin group) a significant decrease of GH concentration ($p \leq 0.01$) compared with the vehicles (oil/saline) group was found. Serum concentrations of IGF-I were significantly lower in estrogenized female rats (estradiol/saline and estradiol/leptin groups) compared to groups of animals non-treated with estradiol ($p \leq 0.01$). No effect of leptin administration on IGF-I concentration was found.

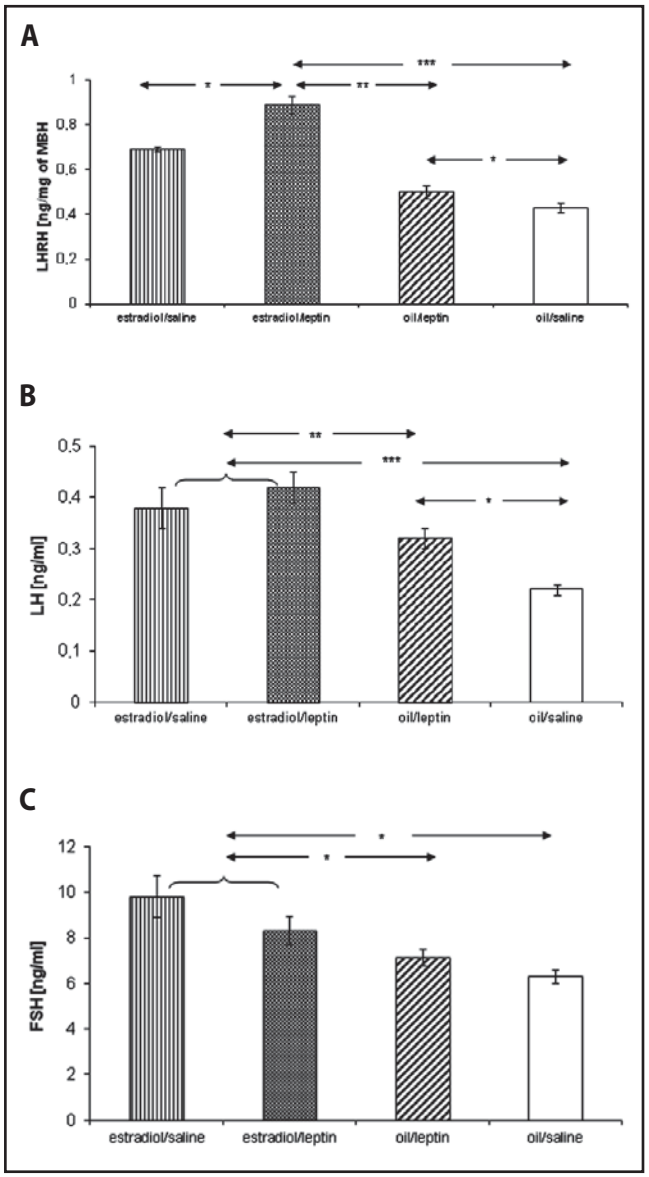

Fig. 3. LHRH in the MBH (A) as well as serum LH (B) and FSH (C) concentrations of peripubertal female rats after exogenous estradiol and/or leptin treatment. The rats at 30th day of life were assigned to one of four experimental groups (15 animals per group). The rats were treated with estradiol or oil for 3 days and then for 6 consecutive days with leptin or saline. The next day after the last leptin or saline administration, the animals were killed, trunk blood was collected and the serum was separated. All hormonal analysis were performed using radioimmunoassay method. Each bar represents mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

The age of vaginal opening in rats treated with exogenous estradiol and/or leptin.

The mean age of vaginal opening, external sign of the timing puberty, in estrogenized animals is advanced (estradiol/saline: 35 ± 0.5; estradiol/leptin: 34.2 ± 0.3 days of life) compared to the rats non-treated with estradiol (oil/leptin: 35.9 ± 0.4; oil/saline: 36.1 ± 0.4 days of life). It was found that the age of the vaginal opening in the group of rats treated with estradiol and following with leptin (estradiol/leptin) is significantly accelerated ($p \leq 0.01$) compared to the other experimental groups.

LHRH concentrations in the MBH of peripubertal female rats treated with exogenous estradiol and/or leptin

The effects of estradiol and/or leptin on LHRH concentrations in the MBH of rats are presented in Figure 3A. In estrogen pretreated and following leptin receiving (estradiol/leptin) rats the LHRH concentration in the MBH was significantly higher compared to the animals receiving only estradiol ($p \leq 0.05$), as well the animals given only leptin ($p \leq 0.01$), or vehicles ($p \leq 0.001$). In rats treated with leptin alone a significantly higher LHRH concentration was observed compared to the rats receiving vehicles ($p \leq 0.05$).

Gonadotropins (LH and FSH) concentrations in the peripheral blood of peripubertal female rats treated with exogenous estradiol and/or leptin

The estrogen and leptin effect on serum LH and FSH levels are presented in Figure 3B and 3C. A significant increase in serum LH concentration was noticeable in both estrogenized groups of rats compared to vehicles group of animals ($p \leq 0.001$) and leptin alone (oil/leptin) treated group ($p \leq 0.01$). In group of rats treated with leptin alone (oil/leptin) LH level was significantly higher ($p \leq 0.05$) compared to vehicles treated (oil/saline) group. In both groups of rats treated with estradiol the serum FSH levels were significantly higher ($p \leq 0.05$) compared to non-estrogenized groups of the animals. No effect of leptin on serum FSH levels was found.

Estrogen receptor in the medial basal hypothalamus (MBH) and the pituitary gland (PIT) of peripubertal female rats treated with exogenous estradiol and/or leptin

A single class of high-affinity (Kd: from 0.1 to 0.2 nM) and low capacity binding sites (Bmax) for estrogen receptor in MBH and PIT were detected by the Scatchard analysis in cytosolic fractions (Tables 3 and 4). No differences in numbers of the functional estrogen receptor in the MBH were found. A distinct decline of the number of ER in the pituitary cells of both groups of rat treated with estradiol compared with those groups non-treated with estradiol was observed. The lowest

Tab. 3. The estrogen receptor (ER) concentrations in the medial basal hypothalamus (MBH) of peripubertal female rats treated with exogenous estradiol and/or leptin.

Characteristic of ER	Experimental groups			
	estradiol/ saline	estradiol/ leptin	oil/ leptin	oil/ saline
DNA [mg/g]	1.44±0.07	1.48±0.14	1.30±0.03	1.30±0.15
Kd [nM]	0.23±0.01	0.15±0.01	0.15±0.02	0.23±0.01
Bmax [fmol/g of tissue]	373±28	345±24	276±40	332±21
Bmax [fmol/mg of DNA]	259±19	233±16	212±31	277±18
ER [number molecules/cell]	967±71	870±60	795±116	954±60

The ER analysis was performed using radioligand (16α-[^{125}I]-Iodo-3, 17β-estradiol) binding method. After ultracentrifugation, the cytosolic fraction of the MBH was used for measurement of ER. DNA concentration was determined in the pellet obtained after receptor fraction separation. Using the Scatchard equation, dissociation constant (Kd) and molar concentration of binding sites (Bmax) in the incubation mixture were calculated. The expression of binding data are presented its relation to weight tissue unit (Bmax/g of tissue). Binding data are also presented the relation to DNA unit (Bmax/mg of DNA). When binding data are expressed in DNA unit the mean cellular content of receptor molecules may be calculated by multiplication by the factor (F = 3.7338). This factor is calculated using Avogadro constant and assuming that 6.2 pg DNA is an average content of mammal cell (Enesco & Leblond, 1962). All values are expressed as mean ± SD.

Tab. 4. The estrogen receptor (ER) concentrations in the anterior pituitary gland (PIT) of peripubertal female rats after treatment with exogenous estradiol and/or leptin.

Characteristic of ER	Experimental groups			
	estradiol/ saline	estradiol/ leptin	oil/ leptin	oil/ saline
DNA [mg/g]	8.94±0.75	8.54±0.87	8.84±0.52	8.02±0.73
Kd [nM]	0.12±0.01	0.12±0.01	0.21±0.04	0.21±0.04
Bmax [fmol/g of tissue]	2647±314	1197±112	4125±1009	6009±1621
Bmax [fmol/mg of DNA]	296±35	140±13	467±114	749±202
ER [number molecules/cell]	1105±131	523±49	1744±426	2797±754

The analysis of estrogen receptor (ER) was performed using radioligand (16α-[^{125}I]-Iodo-3, 17β-estradiol) binding technique. The next day after last leptin or saline administration the anterior pituitary glands were excised and homogenized. After ultracentrifugation the cytosolic fraction of PIT for the assay of ER were used. Concentration of DNA was determined in the pellet obtained after receptor cytosolic fraction separation. Using the Scatchard equation, dissociation constant (Kd) and molar concentration of binding sites (Bmax) in the incubation solution were calculated. The binding data were expressed as the relation to weight of tissue (Bmax fmol/g of tissue). The complementary expression of data was related to DNA unit. When binding data are expressed in DNA unit (Bmax fmol/mg of DNA) the average cellular content of receptor molecules may be calculated by multiplication by the factor (F=3.7338). This factor was calculated using Avogadro constant and assuming that 6.2 pg DNA is an average content of mammal cell (Enesco & Leblond, 1962). All values are expressed as mean ± SD.

number of functional ER in the pituitary cell was in group of rats treated with both hormones (5 fold less than in vehicles group).

DISCUSSION

Our study provided evidence that estradiol and/or leptin can modify the growth rate, activity of GH/IGF-1 axis and LHRH/gonadotropins axis in peripubertal female rats. Applied exogenous doses of estradiol and route of its administration caused concentrations of estradiol in the peripheral blood of both estrogenized groups to increase to values similar to those in proestrus in the cycling female rats (Dohler & Wuttke 1975) and leptin level mimicked values which were observed in postpubertal period in intact female rats (Watanobe & Schioth 2002). Progesterone milieu in the circulation was similar in all experimental groups (data not shown).

In both estradiol-treated groups of rats higher circulating leptin levels than in non-treated with estradiol groups were found. In previous studies it was well established that estradiol stimulates the synthesis and secretion of leptin (Shimizu *et al.* 1997; Alonso *et al.* 2007). Our results are in accordance with the above reports. Data from literature showed that in ovariectomized estradiol-treated rats a significantly higher expression of leptin mRNA in the adipose tissue as well as elevated plasma leptin level was recorded. A direct action of estrogen on the leptin coding gene is probable, because the estrogen-responsive element in the promoter of leptin gene was identified. *In vitro* studies have shown that the expression of leptin mRNA is significantly higher, and leptin secretion also increases when adipocytes from ovariectomized rats are incubated with 17β-estradiol (Machinal *et al.* 1999; O'Neil *et al.* 2001).

Our results showed that only estrogenized and following leptin treated female rats significantly lowered (mean by 7%) their final body weight compared to the mean body mass of animals in other experimental groups. Detailed analysis of the dynamics of the growth rate during the experimental period showed that sc injection of estradiol for three consecutive days significantly reduced the body mass gain. After cessation of estradiol administration this effect of estradiol disappeared very fast in groups of animals non-treated with

leptin and compensatory effect on somatic growth rate was observed. Data from literature showed that exogenous estradiol administration attenuated food intake, body weight and body mass gain in ovariectomized rats (Richard 1986; Alonso *et al.* 2007). The presence of estrogen receptors in the hypothalamic structures which are involved in the control of food intake suggests that anorectic effect of estrogen acts via its specific receptor. Using two selective estrogen receptor agonists it was found that mainly ERα mediates the attenuating effects of estrogen on food intake and body weight gain. (Roesch 2006). The anorectic effect of estradiol was also found in intact female rats with peak in 2–3 days after sc implantation capsule containing estradiol but this effect of estradiol was transient and disappeared in 1 week (Rocha *et al.* 2004).

In our experiment, applied doses of leptin caused significant reduction in the daily gain of body mass (see Table 2). So, the final body mass reduction resulted in anorectic effects of both hormonal signals. We observed that lower growth rate in response to estradiol treatment accompanied both the increase of GH level and the decrease of IGF-I in the circulation (Figure 2).

The same stimulatory effect of estrogen on GH secretion was observed in humans, rodents and ruminants (Breier *et al.* 1988; Borski *et al* 1996; Leung *et al* 2004). It has been reported that estradiol administration led to visible suppressions of circulating IGF-I levels and liver IGF-I gene expression (Shulman *et al.* 1987; Borski *et al* 1996). Such dichotomous action of estrogen on individual elements of GH/IGF-I axis suggests that estradiol may antagonize GH-induced IGF-I production at sites distal to pituitary GH regulation.

In our study it was found that administration of leptin alone significantly reduced circulating GH levels but had no effect of leptin on IGF-I concentrations in the peripheral blood (Figure 2). Some results of *in vivo* research suggest that leptin administered centrally stimulates the release of GH in rats. This leptin-induced GH secretion may be suppressed by NPY. It is possible that NPY can overcome the stimulatory effect exerted by leptin on GH serum level (Carro *et al* 1998). Rettori and colleagues (1990) have found that neuropeptide Y (NPY) in rats suppressed GH release by stimulating somatostatin discharge. Recently, it has been described that the activity of the NPY neurons of the arcuate nucleus, closely related to somatostatin neurons in the periventricular nucleus, was lower in the peripubertal lambs compared to those in the prepubertal stage (Tillet *et al.* 2010). The withdrawal of this NPY effect may allow an elevation of somatostatin release, and in consequence inhibit GH secretion. Our results suggest that estradiol and leptin are involved in the transmission of signals leading to slowing down of the growth rate.

Data from our study showed that estradiol and leptin operating together play the most significant role in activation of reproductive processes at puberty. This activity was not only manifested by significant acceleration of the vaginal opening, but also by a considerable increase of LHRH abundance in the MBH as well as higher LH and FSH concentrations in the peripheral blood. In the peripubertal female rats treated only with estradiol or leptin alone significant increase of GnRH concentrations at the MBH as well as higher levels of LH secretion compared to vehicles treated animals were found. But, in these cases the acceleration of vaginal opening was not reported. Ponzo and colleagues (2001) demonstrated that a single ip injection of exogenous leptin at dose of 30 μg per kg of body weight elevated the GnRH concentrations at the MBH and LH levels in the peripheral blood in juvenile female rats. They found that leptin stimulated GnRH-LH axis more in younger (15 days of life) than older (30 days of life) prepubertal female rats. They suggest that changes in the sensitivity of hypothalamo-pituitary axis may be connected with the maturation of the neuroendocrine mechanisms involved in the onset of puberty. On the other hand, hypothalamic mRNA and protein of Ob-Rb in intact female was found to be significantly increase in late prepubertal than postpubertal period (Smith & Waddell 2003). It is possible that changes in hypothalamic response to leptin via increased Ob-Rb levels could enhance leptin action on neural centers involved in the control of reproduction.

A significant decrease of the functional (available for ligand) estrogen receptor in the pituitary cells in estrogen/leptin group of animals was observed but no significant changes of estrogen receptor in the MBH were found in experimental groups. It has been shown that ER-α is the predominant species in the hypothalamic neurons and pituitary cells (Kuiper *et al.* 1997; Mitchner *et al.* 1998). The radioligand 16α-[^{125}I]iodo-estradiol, which was used in our assay, was characterized by 4 times higher binding affinity for ER-α than for ER-β. The values dissociation constant (Kd) for ER-α in was 0.1 nM and for ER-β was 0.4 nM (Kuiper *et al.* 1997). The range of Kd from 0.1nM to 1.0 nM is generally reported for estradiol binding to ERs in various analytical systems (Clark *et al.* 1992). The values dissociation constant in our assay was within the range from 0.1 nM to 0.25 nM and these values of Kd suggested that in our assay mostly ER α was determined. According to Snochowski (2002), the expression of estrogen receptor data related to DNA unit is recommended because the size of cells may vary even within the same tissue depending on its actual activity and can be used for simple calculations of the average cellular content of receptor binding sites. It is established that the level of ER-α expression may be correlated to the magnitude of the ER-mediated response. A significant increase in gonadotropin mRNA levels was found in ER-α gene-disrupted female mice (Scully *et al.* 1997) and suppression of mRNA coding common α-subunit, β-LH and β-FSH subunits in response to administration of estradiol in ovariectomized rats was found (Corbani *et al.* 1990).

In our study a significant decrease of the functional ER level in pituitary cells of animals treated with estradiol and leptin was observed. In effect, the susceptibility of these cells to estradiol could be decreased and inhibitory action of estrogen on the expression mRNA coding gonadotropins units may be diminished. In our study ER levels in the MBH were similar in all studied groups. In such an ER analysis like ours of the whole MBH changes in the functional ER levels could be masked since they concern only a part of neurons of hypothalamic nuclei. Cumulative data suggest that distribution of ERs in different nuclei is heterogeneous. Particular abundance of ERs was found in the ARC nucleus, but in other hypothalamic structures the amount of ER was significantly lower (Brown *et al.* 1994).

No real evidence exists that ER-α and Ob-Rb are expressed by GnRH neurons at hypothalamus in rodents (Quennell *et al.* 2009). The most data suggest that the actions of estradiol as well as leptin on GnRH neuronal system occur indirectly through regulatory interneurons. Some evidence support the concept that leptin as well as estradiol may act suppressingly, inducingly and/or integratingly on neuronal pathways of neuropeptides which are involved in the hypothalamic control of food intake, energy expenditure and reproduction of female rats. Integration of leptin and estradiol peripheral signals, that convey information on metabolic state and gonadal function might occur in the same hypothalamic neurons. A number of peptidergic neurons at hypothalamic areas co-localize leptin and estrogen receptors. It was found that neuropeptide Y (NPY), Agouti-related peptide (AgRP), pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons in the arcuate nucleus, and melanin concentrating hormone (MCH) and orexin (Ox) neurons in the lateral hypothalamus are colocalized with leptin and estrogen receptors (Hakansson *et al.* 1998; Iqbal *et al.* 2001; Smith *et al.* 2006). The action of NPY which is synthesized in the arcuate nucleus of the hypothalamus is steroid-dependent. The intracerebroventricular administration of NPY stimulated LH secretion in intact rats but inhibited such secretion in castrated animals (Kalra 1993). Orexin A also stimulated GnRH release in hypothalamic explants harvested from female rats at proestrous stage but hypothalamic explants harvested at other stages of the estrous cycle were insensitive to orexin action (Lebrethon *et al.* 2000).

Recently, another possible link between metabolism and reproduction has been discovered. It is a new neuropeptide, kisspeptin , which is encoded by the KiSS1 gene and acts through the GPR54 receptor. The kisspeptin/GPR54 signaling system is necessary for normal reproduction. Mutations in GPR54 result in disruption of reproductive function in both humans and rodents (De Roux *et al.* 2003; Seminara *et al.* 2003). Same data indicate that ER participates in cross-talk between estrogen and leptin/Ob-R system. Estrogen receptor can activate STAT3 to stimulate a STAT-regulated promoter in target cells. Recently, a role for membrane ERs was discovered (Qiu *et al.* 2006). In vitro study indicated that estradiol is able to activate STAT3 through various signaling pathways in a nongenomic manner (Gao & Horvath 2008).

In conclusion, our results suggest that the functions of estrogen and leptin in the control of metabolism and reproduction seem to overlap only partially. Estrogen may mimic the action of leptin in reduction of growth but their effect on GH/IGF-I axis was completely different. Our results favour the view that estradiol plays a more substantial role in activation of gonadotropic axis than leptin during peripubertal period and that leptin plays a rather permissive role at puberty.

ACKNOWLEDGEMENTS

The work was supported by scientific grant of Medical Centre for Postgraduate Education. 501-2-2-25-70/01 and 501-1-31-22-11

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