INTRODUCTION

Thyroid hormones as well as leptin are involved in regulation of energy metabolism, thermogenesis, food intake, glucose and lipid metabolism as well as oxidation of fatty acids (1-8). Moreover, leptin plays the significant role in neuroendocrine functions and initiates the activity of the hypothalamic-pituitary-ovarian axis (9). By means of Ob-Rb (long form) and Ob-Ra (short isoform) receptors, it affects the neurons in the arcuate nucleus of the hypothalamus (inducing the GnRH secretion) (10) and influences gonadotropic cells (increasing the FSH and LH secretion) (11). A few literature reports indicate that also leptin, depending on its dose and time of cell exposition, stimulates NO release from anterior pituitary cells of ewe lambs in vitro. The inhibition of NO synthesis with L-NAME annihilates the stimulating effect of leptin on thyroid-stimulating hormone secretion.

Taking into account that the relationship between leptin and thyroid-stimulating hormone (TSH) secretion in ewe lambs is not clear, the objective of the present study was to estimate the effect of leptin on basal TSH secretion from ovine pituitary cells in vitro. Moreover, the influence of leptin on nitric oxide (NO) release and its role in the leptin-modulated secretion of TSH was studied. Pituitary cells were cultured in the McCoy 5A medium without hormones (the control), with 10^{-10}-10^{-6} mol/L of leptin or with 10^{-10}-10^{-5} mol/L of leptin and L-NAME (N^O-nitro-L-arginine methyl ester, 3x10^{-4} mol/L, the inhibitor of NO synthesis). The secretion of thyroid-stimulating hormone as well as concentration of nitrite (as an indicator of nitric oxide release) were analysed after 2–72 hours of experiment. The obtained results show that TSH secretion from ovine pituitary cells in vitro is dependent on leptin concentration: 10^{-10}-10^{-6} mol/L of leptin causes an increase in TSH secretion, whereas the highest concentration of leptin (10^{-5} mol/L) suppresses thyroid-stimulating hormone release compared to the control. TSH secretion reaches the highest values under the influence of 10^{-4} mol/L of leptin. Moreover, leptin, depending on its dose and time of cell exposition, stimulates NO release from anterior pituitary cells of ewe lambs in vitro. The inhibition of NO synthesis with L-NAME annihilates the stimulating effect of leptin on thyroid-stimulating hormone secretion.

Key words: leptin, thyroid-stimulating hormone, nitric oxide, ewe lambs, pituitary cells, nitric oxide synthase, N^O-nitro-L-arginine methyl ester
MATERIAL AND METHODS

The protocol of the study concept and all procedures were approved by the Second Lublin Local Ethics Committee for Animal Experimentation.

Pituitary glands were obtained immediately after slaughter from the ewe lambs of the SCP line (50% Suffolk + 25% Romanov + 25% Polish Lowland Sheep), aged 10 months, which were selected on the basis of the ovarian activity (follicular phase, verified by laparoscopic method). The presented study was carried out on three independent cell cultures. Each cell culture was prepared using pituitaries isolated from 7 ewe lambs. Isolation of cells was carried out through the digestion of the pituitaries with 0.25% trypsin solution. Suspension of cells in trypsin combined with the preparatory medium (DMEM supplemented with 0.1% BSA, 0.08% glucose, 0.59 HEPES, and gentamycin with the final concentration of 20 µg/ml) was centrifuged (1200 rpm for 10 min). The sedimented cells were washed twice and suspended in the McCoy 5A medium containing 2.5% fetal calf serum, 10% horse serum, mixture of amino acids and vitamins, 0.59% HEPES, gentamycin (20 µg/ml), and adjusted to pH 7.4 (30-32). One millilitre of suspension containing 250,000/mL was transferred to each well of 24-well culture plates and incubated for 72 hours at 37°C under the atmosphere of 5% CO2. After attachment to the dishes, the cells were washed and finally cultured in the McCoy 5A medium without hormones (the control), with 10 –10–10–5 mol/L of leptin or with 10 –10–10–5 mol/L of leptin and L-NAME (Nω-nitro-L-arginine methyl ester, 3×10–4 mol/L, the inhibitor of NO synthesis), respectively. Each sample was prepared in duplicate. After 2, 24, 48 and 72 hours of incubation the media for thyroid-stimulating hormone and nitric oxide analysis were collected and the proliferation index (PI) of the control cells and those treated with leptin was determined. The results were used for calculation of TSH secretion. Assessment of cell proliferation was based on the reduction of tetrazolium salt (MTT) into a blue formazan. The control cultures and those incubated with leptin were pulsed with 15 µl of MTT (for 3 hours at 37°C) and then solubilized with 10% solution of sodium dodecyl sulphate (SDS) overnight. The optical density (OD) of the formed blue formazan was measured by ELISA microplate reader at the wavelength of 600 nm. The results were expressed as PI values. TSH concentration in the culture medium was determined using Sheep Thyroid Stimulating Hormone ELISA KIT (Blue Gene, Shanghai, China). TSH secretion was expressed as a concentration (µIU/mL) of hormone which was released into the culture medium by 250,000 cells for 2, 24, 48 or 72 hours, respectively. Concurrently, concentration of nitrite (NO2–) as an indicator of nitric oxide (NO) production was measured. Equal amounts of the sample and the Griess reagent (sulfanilamide 2% (w/v), N-(1-naphthyl)ethylenediamine 0.2% (w/v), phosphoric acid 4% (v/v)) were mixed. After 10 min of incubation at room temperature the absorbance at 545 nm was measured. As the standard NaNO2 was used (25, 26, 30).

Statistical analysis

The obtained results were calculated using Statistica 5.0 PL and expressed as mean and standard deviation, x ± S.D. Comparisons between the control and the experimental cultures were performed using the analysis of variance and the paired t-tests. Differences were considered as significant at P≤0.05.

RESULTS

Influence of leptin on thyroid-stimulating hormone secretion from ovine pituitary cells in vitro

The basal secretion of TSH amounted from 1.27 ± 0.02 to 1.31 ± 0.06 µIU/mL/250,000 cells during the whole time of the experiment (0–72 hours). The effect of leptin on TSH secretion

![Figure 1](image-url)
by ovine pituitary cells was dependent on time and the dose of leptin used. The introduction of leptin in the concentration 10^{-10} to 10^{-8} mol/L affected TSH secretion compared to the control after 24, 48, and 72 hours. After 2 hours, thyroid-stimulating hormone secretion did not change significantly. The addition of 10^{-10} mol/L of leptin to the culture medium resulted in slight increment in TSH secretion after 24, 48, and 72 hours (Fig. 1). Treatment of cells with 10^{-9} to 10^{-8} mol/L of leptin caused a significant ($P<0.05$) increase in TSH secretion compared to the control, starting with 72 or 48 hours of exposition, respectively. After 72 hours, TSH secretion reached the maximum value (1.64 ± 0.01 µIU/mL/250,000 cells/72 hours) under the influence of leptin in the concentration of 10^{-8} mol/L. It was significantly higher ($P<0.05$) compared to the control (1.31 ± 0.06 µIU/mL/250,000 cells/72 hours) (Fig. 2). The study of the relationship between 10^{-10}–10^{-8} mol/L of leptin and TSH secretion from ovine pituitary cells in vitro showed a positive correlation ($r=0.82$, $r=0.80$, and $r=0.75$ after 24, 48, and 72 hours, respectively). The addition of leptin in the concentration of 10^{-7} and 10^{-6} mol/L decreased TSH secretion compared to the culture with 10^{-8} mol/L of leptin, but at the same time caused the elevation compared to the control. However, the introduction of 10^{-5} mol/L of leptin reduced thyroid-stimulating hormone secretion, compared with both the control and other leptin treated cultures. The most significant suppressive effect was observed after 72 hours of exposure of cells to 10^{-5} mol/L of leptin (TSH: 1.09 ± 0.02 µIU/mL/250,000 cells/72 hours). The negative correlation between leptin in the concentration 10^{-7} to 10^{-5} mol/L and TSH secretion from ovine pituitary cells in vitro ($r=-0.56$, $r=-0.81$, and $r=-0.88$ after 24, 48, and 72 hours, respectively) was found.

![Fig. 2](image1.png)

**Fig. 2.** Changes in TSH secretion from ovine pituitary cells under the influence of 10^{-4} mol/L of leptin or 10^{-4} mol/L of leptin and L-NAME. a, b, c - the mean values obtained at the same time (24, 48 or 72 hours) and signed with various letters differ significantly ($P<0.05$).

![Fig. 3](image2.png)

**Fig. 3.** Changes in nitric oxide release from ovine pituitary cells under the influence of 10^{-4} mol/L of leptin or 10^{-4} mol/L of leptin and L-NAME. a, b, c - the mean values obtained at the same time (24, 48 or 72 hours) and signed with various letters differ significantly ($P<0.05$).
The effect of leptin on nitric oxide release by ovine pituitary cells in vitro

The effect of leptin on NO release depended on time and its dose. The addition of leptin in all used concentrations (10^{-10}–10^{-5} mol/L) increased NO release after 24, 48 or 72 hours compared to the control (Table 1, Fig. 3). The most stimulating effect was exerted by 10^{-7} mol/L of leptin with the maximum value (4.3±0.24 µM/mL/250,000 cells) after 72 hours. Therefore, leptin in the concentration 10^{-10}–10^{-8} mol/L and NO release were in positive correlation (r=0.33, r=0.66, and r=0.97 after 24, 48, and 72 hours, respectively). Inhibition of NO synthesis by the addition of L-NAME to the culture, irrespective of the dose of leptin and time, annihilated this stimulating effect (Table 2, Fig. 3). It was confirmed by the negative correlation between NO release from ovine pituitary cells and 10^{-10}–10^{-8} mol/L of leptin in the presence of L-NAME (r= –0.38, r= –0.74, and r= –0.62 after 24, 48, and 72 hours, respectively).

**DISCUSSION**

Leptin is an obligatory metabolic signal, which can influence the secretion of tropic hormones e.g. TSH. However, these data are not unequivocal and do not relate to sheep. It has been demonstrated that leptin exerts a stimulating effect on TSH secretion in rats. Food deprivation, and the associated low leptin levels, resulted in decrease of TSH synthesis in the pituitary and TRH in the hypothalamus (15, 33). In contrast, Nowak et al. (34) noted that leptin-induced elevation in the thyroid hormones level, probably by negative feedback, suppresses TSH release in

**Table 1.** Nitric oxide (NO) release by ovine pituitary cells in vitro under the influence of leptin after 24, 48 or 72 hours. * - significant difference compared to the control (P≤0.05).

<table>
<thead>
<tr>
<th>Leptin concentration in culture medium (mol/L)</th>
<th>NO release by ovine pituitary cells in vitro (µmoles/mL/250,000 cells)</th>
<th>Time of exposition of cells to leptin (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.4±0.08</td>
<td>1.7±0.11</td>
</tr>
<tr>
<td>10^{-10}</td>
<td>1.9±0.11*</td>
<td>2.2±0.16*</td>
</tr>
<tr>
<td>10^{-9}</td>
<td>1.7±0.11*</td>
<td>1.9±0.08</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>1.8±0.11*</td>
<td>2.3±0.15*</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>2.3±0.12*</td>
<td>2.4±0.14*</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>2.2±0.12*</td>
<td>2.3±0.12*</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>1.7±0.11*</td>
<td>2.3±0.14*</td>
</tr>
</tbody>
</table>

**Table 2.** Nitric oxide (NO) release by ovine pituitary cells in vitro under the influence of leptin and L-NAME after 24, 48 or 72 hours. *- significant difference compared to the control (P≤0.05).

<table>
<thead>
<tr>
<th>Leptin and L-NAME concentration in culture medium (mol/L)</th>
<th>NO release by ovine pituitary cells in vitro (µmoles/mL/250,000 cells)</th>
<th>Time of exposition of cells to leptin and L-NAME (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.4±0.09</td>
<td>1.7±0.11</td>
</tr>
<tr>
<td>10^{-10}</td>
<td>1.2±0.07*</td>
<td>1.2±0.07*</td>
</tr>
<tr>
<td>10^{-9}</td>
<td>1.2±0.07*</td>
<td>1.2±0.07*</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>1.2±0.07*</td>
<td>0.9±0.05*</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>0.7±0.04*</td>
<td>1.2±0.07*</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>1.2±0.07*</td>
<td>1.4±0.09*</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>1.2±0.07*</td>
<td>1.6±0.12</td>
</tr>
</tbody>
</table>

The effect of leptin on nitric oxide release by ovine pituitary cells in vitro

The effect of leptin on NO release depended on time and its dose. The addition of leptin in all used concentrations (10^{-10}–10^{-5} mol/L) increased NO release after 24, 48 or 72 hours compared to the control (Table 1, Fig. 3). The most stimulating effect was exerted by 10^{-5} mol/L of leptin with the maximum value (4.3±0.24 µM/mL/250,000 cells) after 72 hours. Therefore, leptin in the concentration 10^{-10}–10^{-5} mol/L and NO release were in positive correlation (r=0.33, r=0.66, and r=0.97 after 24, 48, and 72 hours, respectively). Inhibition of NO synthesis by the addition of L-NAME to the culture, irrespective of the dose of leptin and time, annihilated this stimulating effect (Table 2, Fig. 3). It was confirmed by the negative correlation between NO release from ovine pituitary cells and 10^{-10}–10^{-5} mol/L of leptin in the presence of L-NAME (r= –0.38, r= –0.74, and r= –0.62 after 24, 48, and 72 hours, respectively).

**Changes in thyroid-stimulating hormone secretion from ovine pituitary cells under the influence of leptin and L-NAME**

Inhibition of NO synthesis by the treatment of ovine pituitary cells with L-NAME, significantly (P≤0.05) reduced leptin-modulated secretion of TSH after 24, 48 and 72 hours (Fig. 4). The marked (P≤0.05) difference between TSH secretion under the influence of leptin and leptin with L-NAME was found. There was observed the significant (P≤0.05) decrease in thyroid-stimulating hormone secretion under the condition of inhibited NO synthesis compared to the culture with leptin only (Fig. 2). The most suppressive action was observed after 24 and 48 hours of exposure of cells to 10^{-5} mol/L of leptin and L-NAME (TSH: 0.57±0.03 µIU/mL/250,000 cells/24 hours and 0.55±0.01 µIU/mL/250,000 cells/48 hours, respectively). It was significantly (P≤0.05) lower compared to the culture with leptin (TSH: 1.25±0.02 µIU/mL/250,000 cells/24 hours and 1.14±0.02 µIU/mL/250,000 cells/48 hours, respectively), and to the control (TSH: 1.27±0.02 µIU/mL/250,000 cells/24 hours and 1.28±0.04 µIU/mL/250,000 cells/48 hours, respectively). This effect was confirmed by the negative correlation between leptin in the concentration 10^{-5} to 10^{-1} mol/L and TSH secretion from ovine pituitary cells in vitro, modulated by leptin and L-NAME (r= –0.45, r= –0.49, and r= –0.58 after 24, 48, and 72 hours, respectively). In the case of leptin and L-NAME treated cultures, negative correlation between 10^{-10}–10^{-8} mol/L of leptin and TSH secretion (r= –0.20, r= –0.26, and r= –0.18 after 24, 48, and 72 hours, respectively) was also found.
female rats. However, Ortiga-Carvalho et al. (16) showed that leptin presents a stimulating effect on the secretion of TSH in vivo, but decreases it in vitro in male Wistar rats. The obtained results indicated that thyroid-stimulating hormone secretion from pituitary cells isolated from ewe lambs is dependent on leptin concentration. It was noted that $10^{-10}$–$10^{-6}$ mol/L of leptin increases TSH secretion. The most stimulating effect of leptin on TSH secretion was observed after cells exposure to $10^{-4}$ mol/L of leptin and TSH secretion from ovine pituitary cells confirmed this stimulating action. Whereas, the highest concentration of leptin ($10^{-3}$ mol/L) decreased TSH secretion compared to the control. Positive correlation between $10^{-8}$–$10^{-5}$ mol/L of leptin and TSH secretion from ovine pituitary cells confirmed this stimulating action. Therefore, the negative correlation between $10^{-7}$–$10^{-5}$ mol/L of leptin and TSH secretion was found. This suppressive effect may be caused by down-regulation of the leptin receptors in pituitary cells due to the highest leptin concentration. According to Kosior-Korzecka (35), $10^{-5}$ mol/L of leptin significantly reduces mRNA expression of OB-Ra and OB-Rb in ovine pituitary cells in vitro. Moreover, Oliveira et al. (36) reported that leptin can exert stimulating as well as inhibiting action on thyroid-stimulating hormone secretion. Acute treatment of leptin resulted in higher TSH level in rats in vivo. However, chronic addition of leptin did not change TSH secretion. According to our previous studies on immature seven-month old ewes, TSH secretion in pituitary cells in vitro was also dependent on leptin concentration. However, the most stimulating effect was observed after the culture cells exposure to $10^{-7}$ mol/L of leptin (unpublished data). Taking into account that leptin receptor expression increases by around fivefold from fetal to postpubertal life, the reason of this discrepancy may be the lower sensitivity of pituitary cells of pubertal ewe lambs to leptin (37). However, it is difficult to confront the results of our study with the literature data, because no other reports on the effect of leptin on TSH secretion by ovine pituitary cells have appeared so far.

The secretion of tropic hormones from pituitary cells can be modulated by nitric oxide. However, the contribution of NO to regulation of TSH secretion is not well established. It was reported that NO may participate in the modulation of the hypothalamic-pituitary-thyroid axis activity. It was noted that treatment of rats with L-NAME elevates TRH levels, while decreasing serum TSH concentration, probably by a direct effect on anterior pituitary cells in vivo. Whereas thyroid-stimulating hormone secretion was not changed under condition of inhibited nitric oxide synthesis in vitro (38). According to Coiro et al. (28), treatment with L-NAME did not modify basal release of thyroid-stimulating hormone, but significantly reduced TRH-induced TSH secretion in humans. Therefore, it suggests that TRH stimulates thyroid-stimulating hormone release via nitric oxide. However, there are also contradictory reports. Kasperska-Zajac et al. (29) noted that in female Wistar rats NO may play an inhibitory role in TSH secretion. The addition of L-NAME did not change TSH concentration in plasma, while treatment with L-arginine (the substrate for NO synthesis) resulted in decrease in thyroid-stimulating hormone secretion. The present results show that the leptin effect on TSH secretion in ewe lambs is mediated by nitric oxide. Inhibition of NO synthesis with L-NAME significantly reduced leptin-modulated secretion of TSH and annihilated a stimulating effect of leptin on thyroid-stimulating hormone secretion. The marked difference was observed between TSH secretion under the influence of leptin and leptin with L-NAME. This effect was confirmed by negative correlation between leptin in the presence of L-NAME and TSH secretion from ovine pituitary cells in vitro. Moreover, the results of studies on ewe lambs pointed out that leptin in all concentrations increases NO release in the dose-dependent way. Leptin in the concentration $10^{-7}$ mol/L exerted the most stimulating effect. However, the higher doses decreased NO release from ovine pituitary cells compared to $10^{-7}$ mol/L of leptin. Also according to other author, addition of $10^{-8}$–$10^{-6}$ mol/L of leptin resulted in elevation, whereas $10^{-5}$ mol/L caused a drop in NO release in vitro (39). Inhibition of NO synthesis with L-NAME significantly annihilated this stimulating effect.
influence. However, in our previous studies, which was carried out on pituitary cells isolated from younger ewes (seven-month old), TSH secretion was suppressed under the condition of L-arginine-stimulated NO synthesis, whereas inhibition of NO production by L-NAME caused elevation in the TSH level (unpublished data). The explanation of these differences in the response of pituitary cells from pubertal and mature ewes to leptin and NO requires the further investigation. However, it is known that regulation of TSH secretion is dependent on ovarian steroid hormones and the phase of the oestrous cycle. There was reported, that estrogens might stimulate thyroid-stimulating hormone release by increasing proliferation of thyrotrophs as well as modulate the level of receptors for the neurohormones in thyrotropic cells (40, 41). Therefore, the reason for this discrepancy may be the lack of the reproductive cyclicity, low level of female steroid hormones, and then the different capacity of thyrotropes to respond to leptin and nitric oxide in pubertal seven-month old ewes. The obtained results show that leptin in the doses 10^{-10}–10^{-6} mol/L elevates TSH secretion from ovine pituitary cells in vitro and this effect is mediated by nitric oxide in ten-month old lambs. Moreover, it was observed that the highest concentration of leptin (10^{-4} mol/L) decreases thyroid-stimulating hormone secretion. It suggests that high body mass, associated with high level of leptin can result in some disturbances in TSH release. Taking into account that hormones of thyrotropic axis participate in the regulation of reproductive processes in ruminants, the influence of leptin on pituitary cells isolated from younger ewes (seven-month old), TSH secretion was suppressed under the condition of L-NAME caused elevation in the TSH level. However, it is known that regulation of TSH secretion is dependent on ovarian steroid hormones and the phase of the oestrous cycle. There was reported, that estrogens might stimulate thyroid-stimulating hormone release by increasing proliferation of thyrotrophs as well as modulate the level of receptors for the neurohormones in thyrotropic cells (40, 41). Therefore, the reason for this discrepancy may be the lack of the reproductive cyclicity, low level of female steroid hormones, and then the different capacity of thyrotropes to respond to leptin and nitric oxide in pubertal seven-month old ewes.

The obtained results show that leptin in the doses 10^{-10}–10^{-6} mol/L elevates TSH secretion from ovine pituitary cells in vitro and this effect is mediated by nitric oxide in ten-month old lambs. Moreover, it was observed that the highest concentration of leptin (10^{-4} mol/L) decreases thyroid-stimulating hormone secretion. It suggests that high body mass, associated with high level of leptin can result in some disturbances in TSH release. Taking into account that hormones of thyrotropic axis participate in the regulation of reproductive processes in ruminants, the influence of leptin on pituitary cells isolated from younger ewes (seven-month old), TSH secretion was suppressed under the condition of L-NAME caused elevation in the TSH level.

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Author’s address: Dr. Paulina Radwanska, Department of Pathophysiology, Chair of Preclinical Veterinary Sciences, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 12 Akademicka Str., 20-033 Lublin, Poland.

E-mail: radwanek@autograf.pl