Original article

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LEPTIN INFLUENCES HISTIDINE DIPEPTIDES AND NITRIC OXIDE RELEASE FROM ANTERIOR PITUITARY CELLS OF SHEEP IN VITRO

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Our previous results show that leptin, as well as nitric oxide (NO) and some antioxidants (histidine dipeptides - HDP) change the secretion of gonadotrophins from ovine adenohypophysis cells in vitro. NO and HDP are produced by pituitary and can modulate gonadotropin secretion by autocrine action. It is possible that these compounds mediate leptin influence on gonadotropin secretion. Therefore, the objective of the present study was to analyse leptin effect on NO and HDP (3-metyl-L-histidine, carnosine and anserine) release from ovine pituitary in vitro.

Adenohypophysis cells were cultured in McCoy 5A medium with GnRH (4 × 10⁻⁹ M) and 10⁻¹⁰⁻¹⁰⁻⁵ M/l of leptin, respectively. Next, the media for analysis of NO (Griess method) and HDP (HPLC) were collected. Leptin in concentration of 10⁻⁸⁻¹⁰⁻⁶ M/l caused a significant augmentation in NO in the culture medium, whereas in the dose of 10⁻⁵ M/l reduced (P≤0.05) NO release. The level of 3-metyl-L-histidine and anserine, but not carnosine, was significantly lower in the culture with 10⁻⁸⁻¹⁰⁻⁷ M/l of leptin. Taking into account that 10⁻⁸⁻¹⁰⁻⁷ M/l leptin stimulates LH and FSH secretion, as show in our previous study, it is possible that this effect in ewes is mediated by augmented release of NO and reduction of HDP level.

Key words: leptin, histidine dipeptides, nitric oxide, pituitary, ewe

INTRODUCTION

Our previous results show that leptin, as well as nitric oxide (NO) and some antioxidants (histidine dipeptides - HDP: carnosine (β-alanyl-1-histidine) and its analogue anserine (β-alanyl-1-methyl-L-histidine)) change the secretion and release of gonadotropins from ovine anterior pituitary cells. In vitro, 10⁻⁸, 10⁻⁷ i
10^6 M/l of leptin stimulated GnRH-induced LH secretion, whereas under the influence of leptin in higher doses (10^{-5} M/l) the secretion of LH from ovine pituitary cells decreased (2, 3). FSH secretion was also significantly enhanced by leptin in concentration 10^{-8} and 10^{-7} M/l. However, contrary to LH, FSH secretion was suppressed already by 10^{-6} M/l of leptin (1). Additionally, both NO and HDP can modulate gonadotropin secretion by auto- and/or paracrine action. Nitric oxide influence LH and FSH secretion from the pituitary cells (1-3). As we have found before, the inhibition of NO synthesis by the treatment of ovine pituitary cells with L-NAME (N^ω-Nitro-L-arginine methyl ester), irrespective of the dose of leptin (10^{-10} - 10^{-5} M/l) used, disabled it from a stimulation of FSH secretion (1, 2). The concentration of histidine dipeptides, especially anserine and 3-methyl-histidine, instead, is in negative correlation with LH secretion from the pituitary cells of sheep in vitro (unpublished data). It is possible that NO and HDP, which are produced by pituitary, mediate leptin influence on gonadotropin secretion. Therefore, the objective of the present study was to analyse leptin effect on NO and HDP (3-metyl-L-histidine, carnosine and anserine) release from ovine pituitary in vitro.

MATERIAL AND METHODS

Pituitary glands were obtained from 3-4 year-old crossbreed ewes (50% Suffolk + 25% Romanov + 25% Polish Lowland Sheep) at slaughter. Isolation of cells was carried out through the digestion of the adenohypophysis with 0.25 % trypsin solution. Pituitary cells were finally cultured in McCoy 5A medium containing 2.5% fetal calf serum, 10% horse serum, mixture of amino acids and vitamins, gentamicin (20 g/ml), and adjusted to pH 7.4 (4). 1 ml of dispersed cell suspension at a concentration of 2.5 x 10^5/ml was transferred to each culture dish of 24-well (or 96-well, respectively) culture plates and incubated for 84 h at 37°C under the atmosphere of 5% CO_2. After attachment to the dishes, the cells growing as a monolayer were washed with McCoy 5A medium without serum, and finally incubated with McCoy 5A without hormones (negative control), with GnRH (4 x 10^{-9} M) (positive control) or with GnRH (4 x 10^{-9} M) and 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M/l of the recombinant ovine leptin (ro leptin), respectively. Each sample was performed in duplicate. After 2, 6, 12, 18, 24 and 30 h of incubation the media to histidine dipeptides analysis were collected and NO in the cultures of control cells and those treated with leptin was determined. 3-metyl-L-histidine, carnosine and anserine were analysed by HPLC (Jasco PU-158 and PU-158) with fluorescence detection (Jasco FP 920 with excitation and emission wavelengths: 340 and 445 nm, respectively) according to previously described modified protocol (5). Chromatography was performed on a column ion-exchange Xpertec: SP-SCX 5 µm (Cobbert Associates Inc.). Different mobile phases were composed from a mixture of hydrochloric acid 6mM and acetonitrile 95+5, 90+10, 85+15 and 80+20 (v/v). In each solution, sodium chloride was dissolved. Mobile phase flow was 1 ml/min. The derivatisation of solution with o-phthaldialdehyde (OPA) was prepared according to Aristoy et al. (6). The post column derivatisation was carried out at 50°C and at flow rate of 0.5 ml/min. Aliquots from the experiment described above were used for measuring concentrations of nitrite (NO_2^-) as an indicator of nitric oxide (NO) production. Equal amounts of sample and Griess reagent (sulfanilamide 2% (w/v), N-(1-naphthylethlenediamine 0.2% (w/v), phosphoric acid 4% (v/v)) were mixed. After 10 minutes of incubation at room temperature the
absorbance at 545 nm was measured. As a standard NaNO₂ was used (7). Each sample was performed in duplicate.

Statistical analysis

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

The study was approved by local Ethics Committee of the University of Life Sciences, Lublin.

RESULTS

The effect of leptin on NO release from ovine pituitary cells in vitro

The influence of leptin on NO release was dependent on the time and the dose of leptin used. Leptin in concentration of 10⁻⁸-10⁻⁶ M/l caused a significant

![Graph showing the effect of leptin on nitric oxide release from ovine pituitary cells in vitro.](image)

Fig. 1. Leptin effect on nitric oxide release from ovine pituitary cells in vitro in the presence of GnRH (4x10⁻⁹ M/l). * - significant (P ≤ 0.05) difference in comparison to the control.
Fig. 2. The relationship between leptin concentration in the culture medium and NO release from ovine pituitary cells *in vitro*.

Fig. 3. The changes in 3-methyl-histidine and anserine concentration under the influence of leptin.

* - significant (P ≤ 0.05) difference in comparison to the control.
(P ≤ 0.05) augmentation in NO in the culture medium, whereas the highest of the leptin concentrations (10^{-5} M/l) reduced (P ≤ 0.05) NO release (Fig. 1). The most effective concentration of leptin in stimulation of NO release was 10^{-7} M/l. 10^{-9}
M/l leptin increased NO release significantly, but only after 2, 6, 12 and 30 h of incubation. Leptin in the dose of 10^{-10} M/l, instead, did not affect NO release from ovine pituitary cells in vitro (Fig. 1). Very high positive linear correlation (r=0.85) between NO release and leptin concentration (0 - 10^{-7}M/l) in culture medium was found (Fig. 2).

The effect of leptin on histidine dipeptides release from anterior pituitary cells

The concentration of 3-metyl-L-histidine and anserine (Fig. 3), but not carnosine (Figs 4, 5), was significantly lower (P≤0.05) in the culture with 10^{-8}-10^{-5} M/l of ro leptin in comparison to the positive control. 10^{-10} - 10^{-9} M/l of leptin did not influence HDP level in the medium. There was found negative correlation between two histidine dipeptides and leptin dose. The correlation coefficient reached the value: r= -0.54 and -0.56, respectively for 3-metyl-L-histidine and anserine.

Influence of leptin on histidine dipeptides and NO release

Negative linear correlation between two histidine dipeptides concentration in culture medium and NO release under the influence of leptin in concentration 10^{-8}-10^{-6} M/l was found. The correlation coefficient reached the value: r= -0.73 and r= - 0.72, respectively for 3-metyl-L-histidine and anserine.

DISCUSSION

It is known that high level of histidine dipeptides: carnosine (β-alanyl-1-histidine) and its analogue anserine (β-alanyl-1-methyl-L-histidine) are present in skeletal muscles and brain of sheep (8, 9). These molecules are known to be an efficient intracellular pH buffer, modulator of enzymatic activities and hydrophilic antioxidants (10). They represent water-soluble counterparts to lipid-soluble antioxidant such as α-tocopherol and ubiquinone Q10 that protect the cell membrane from oxidative damage. Other roles ascribed to these dipeptides include actions such as neurotransmitters, modulation of enzymatic activities and chelation to heavy metals (11). Our unpublished data show that the rise in HDP concentrations is negatively correlated with LH secretion from the pituitary cells of ewes in vitro. Especially, the increase in 3-metyl-L-histidine concentration causes the drop in the quantity of secreted LH. The present results point out that leptin in the dose of 10^{-8} - 10^{-5} M/l decreases 3-metyl-L-histidine and anserine concentrations in culture medium. So, it is possible that these HDP, as the antioxidants, can influence negatively to NOS activity, reducing stimulatory effect of NO on gonadotropin secretion. However the negative correlation between NO and HDP was found only under the influence of 10^{-8} - 10^{-6} M/l of leptin. As show the presented results, leptin in the highest of the used
concentrations (\(10^{-5}\) M/l) decreases both HDP release and gonadotropin secretion from ovine pituitary cells in vitro. Therefore, the further studies in this subject are necessary.

The secretion of hormones from anterior pituitary cells can be modulated also by nitric oxide (NO) (12-16). The exact role of NO in modulating gonadotropin secretion from the pituitary, remains largely unexplored. According to some authors (7, 15, 17), NO mediates GnRH and leptin-induced gonadotropin release from the pituitary gland in rats. However, there are also contrary reports. According to Chatterjee (18), NO suppresses GnRH-induced gonadotropin release and the inhibition of NOS facilitates gonadotropin, secretion from rat pituitary. Our previous results show that the synthesis of NO by pituitary cells is necessary for the manifestation as well as maintenance of the positive effect of leptin on FSH secretion. Inhibition of NO synthesis by the treatment of ovine pituitary cells with NOS inhibitor - L-NAME, irrespective of the dose of leptin (\(10^{-10}-10^{-5}\) M/l) used, disabled it from a stimulation of FSH and LH secretion (1, 2). Contrary, the addition of L-arginine - substrate for nitric oxide synthesis, to the culture medium stimulates LH secretion.

The results of studies on ewes point out that leptin (in concentration \(10^{-8}\) and \(10^{-7}\) M/l) stimulates NO release from ovine pituitary cells in vitro in a way dependent on the used leptin dose. It is significant that there is a similar relationship between leptin dose and changes in LH and FSH secretion. There is also positive correlation between changes in NO release and gonadotropin secretion under the influence of the respective doses of leptin. This may be due to the fact that NO can activate guanylate cyclase and thus increase the synthesis of cGMP, which is responsible for the release of FSH (19-21). In contrast, the highest doses of leptin (\(10^{-5}\) M/l) suppresses both gonadotropin and NO release.

The possible reason for this occurrence may be the down-regulation of the leptin receptor in pituitary cells by leptin in this concentration and this way the lack of MAPK (mitogen-activated protein kinases) - mediated NOS stimulation by leptin (7).

The obtained results show that leptin in doses \(10^{-8}-10^{-7}\) M/l increases NO and decreases 3-metyl-L-histidine and anserine release from ovine pituitary cells. Taking into account that \(10^{-8}-10^{-7}\) M/l leptin stimulates LH and FSH secretion, as show our previous results (1-3), it is possible that this effect in ewes is mediated by augmented release of nitric oxide and reduction of the level of some antioxidant, like histidine dipeptides.

CONCLUSIONS

1. Leptin in concentration of \(10^{-8}-10^{-6}\) M/l caused a significant (\(P \leq 0.05\)) augmentation in NO in the culture medium, whereas the highest leptin concentrations (\(10^{-5}\) M/l) reduced (\(P \leq 0.05\)) NO release.
2. The concentration of 3-metyl-L-histidine and anserine, but not carnosine, was significantly (P ≤ 0.05) lower in the culture with 10^-8-10^-5 M/l of leptin.

Conflicts of interest statement: None declared.

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