LH release by Cu and Ni salts and metal-GNRH complexes, *in vitro*

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Abstract

The present studies were undertaken to examine the effect of copper and nickel salts and their complexes with GnRH on LH release from the pig anterior pituitary cells *in vitro*. The potency of Cu-GnRH and Ni-GnRH binding to GnRH receptors with iodinated GnRH as a radioactive tracer was also verified. The incubation of pig pituitary cells with Cu and Ni acetate salts showed no effect of the studied ions on LH release at any concentration used. However, nickel salt at a lower dose (10^{-10} and 10^{-9} M) tended to decrease LH output. By contrast, the native GnRH as well as its metal complexes significantly stimulated LH release after three hours of treatment and Cu-GnRH was found to be the most effective. The results showed that Cu and Ni complexes with GnRH but not their acetate salts are effective in LH release from pig pituitary cells collected from adult female pigs.

Introduction

Copper and nickel are very important elements for the living organism, playing catalytic and structural roles in the activity of many proteins and other biomolecules. Based on results coming from systemic administration of copper salts inducing ovulation in rabbits, it was suggested that copper could be involved in the regulation of reproduction in mammals [6,15]. Copper was also shown to promote LH release from isolated hypothalamic granules [2].

GnRH plays an essential role in the regulation of biosynthesis and release of gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH is released from nerve terminals in the median eminence into the portal circulation in a pulsatile manner and binds to the specific receptors located at the surface of gonadotrope cells in the anterior pituitary [10,11]. Binding GnRH to its receptors induces a series of intracellular reactions involving multiple G protein-mediated signal transduction systems leading to the release of gonadotropins [9].

The biological activity of GnRH complexes [14] with cobalt [1], copper and nickel [7] was

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studied in cultured porcine pituitary cells. All these complexes increased LH release and were very effective in stimulating cAMP synthesis and accumulation. By contrast these complexes did not significantly stimulate inositol phosphate formation in comparison to the native GnRH [1, 7].

In this paper we examined the effect of metal salts: $Cu(CH_3COO)_2$ and $Ni(CH_3COO)_2$ on LH release from porcine pituitary cells *in vitro* and compared with the effect Cu-GnRH and Ni-GnRH. Additionally, we verified the potency of GnRH complexes in binding to GnRH receptors using the GnRH as an iodinated tracer.

Material and methods

Chemicals

The native GnRH (mammalian), Dulbecco's Modified Eagle's Medium (DMEM), McCoy's 5A Medium, BSA fraction V, nystatin, gentamycin, bacitracin were purchased from Sigma (St. Louis, MO, USA). Trypsin (0,25% solution), fetal calf serum (FCS) and horse serum were obtained from BIOMED Vaccine Laboratory (Lublin, Poland).

Isolation and culture of porcine pituitary cells

Pituitary glands were obtained from five crossbred (Large White x Polish Landrace) mature gilts at the pre-ovulatory phase of the oestrous cycle. Pituitary cells were dispersed aseptically according to methods described previously [1]. Briefly, the anterior lobes were dissected from each pituitary, minced into small pieces (1–2 mm) and washed several times with DMEM. A single cell suspension of anterior pituitaries was then prepared by sequential 0.25% trypsin digestions at 37 °C for 8–10 minutes. The pituitary cells were repeatedly centrifuged at 800 x g for 8 minutes, washed with DMEM, suspended and counted in a hemocytometer. Cell viability (97–98%) was determined by trypan blue dye exclusion. Finally, pituitary cells were resuspended in McCoy's 5A medium containing 10% horse serum, 2.5% FCS, 240 IU/ml nystatin and 20 μg/ml gentamycin at a density of 5 x 10^5 cells/ml. One ml of dispersed cells was transferred to each culture dish of 24-well plates and pre-incubated for 72 hours at 37 °C in a humidified atmosphere (95% air:5% CO_2) to allow completion of attachment.

The effect of copper and nickel salts and metal – GnRH complexes on LH release from pituitary cells

After reaching monolayer the cells were washed twice with fresh McCoy's 5A medium without serum. After the final wash, 1 ml of serum-free McCoy's medium containing bacitracin (2 x 10^{-5} M) was added and the pituitary cells were further incubated for three hours with the following doses of $Cu(CH_3COO)_2$ or $Ni(CH_3COO)_2$: 0 (control), 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M. Additionally, GnRH and its complexes with copper and nickel were added at the concentration of 10^{-7} M and incubated for also three hours. All incubations were performed in duplicates. At the end of incubation media were collected and stored in –20 °C for LH assay.

LH determination

LH concentration in incubation media was determined by EIA as described previously [1,12]. Porcine LH (USDA-pLH-I-1) was kindly provided by Prof. D.J. Bolt (USDA Hormone Program, Bethesda, USA). Standard curve (USDA-pLH-B-1) ranged from 3.12 to 50 ng/ml. All samples and standards were incubated for 18 hours at 4 °C with 100 μl of SZ/Z/89/370 [16] antiserum (1:200 000). The intra- and interassay coefficients of variation were 3.8 and 4.2%, respectively.

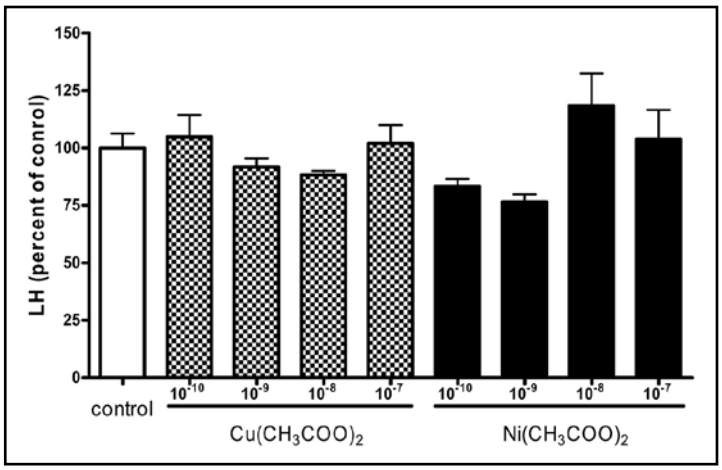

Fig. 1. Effect of different doses (10^{-10}-10^{-7} M) of copper and nickel acetates on LH release from cultured porcine pituitary cells. Incubation was performed for 3 hours. All values are expressed as mean ± SEM.

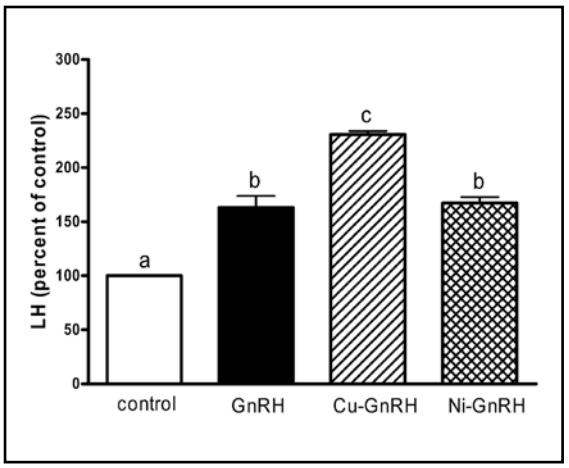

Fig. 2. Effect of GnRH, Cu-GnRH and Ni-GnRH on LH secretion from cultured porcine pituitary cells. Incubation was performed for 3 hours. All values are expressed as mean ± SEM. Values with different superscripts differ ($p<0.001$).

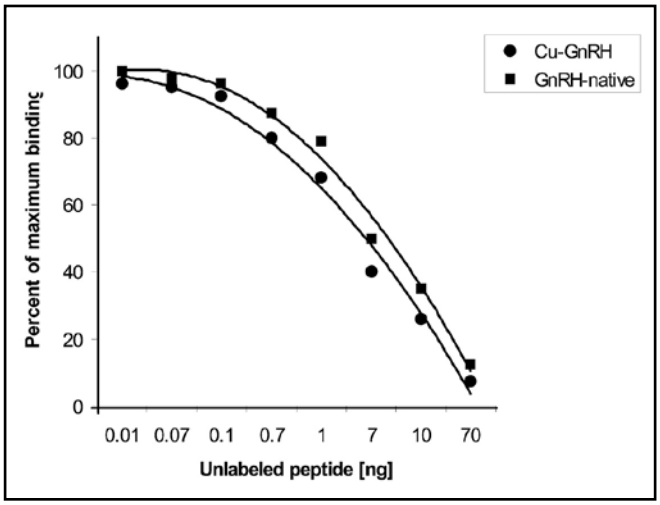

Fig. 3. Competitive binding of the native GnRH and Cu-GnRH with the GnRH receptor.

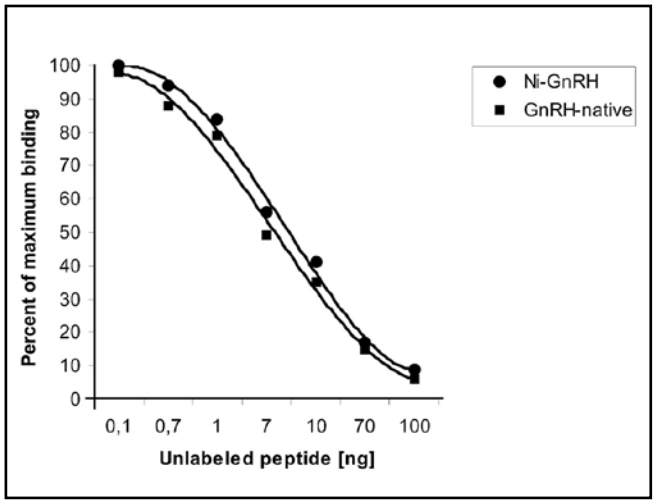

Fig. 4. Competitive binding of the native GnRH and Ni-GnRH with the GnRH receptor.

Assay of pituitary GnRH – R

The native mammalian GnRH native was used as a tracer and was iodinated to specific activity of 292 mCi/mg using a modification of the iodogen method.

GnRH-R were measured in an aliquot (100 μL) of pituitary homogenates by equilibration at 4 °C (100 μg of tissue per tube). Incubation was conducted for 16 hours at 4 °C in a total volume of 500 μL in phosphate-buffered saline (PBS; pH 7.4) containing 0.1% BSA. Nonspecific binding was measured in the presence of 1000-fold excess of unlabelled GnRH, and was less than 10% of total counts added.

Separation of bound ligand from free ligand was done by filtration, under vacuum, through a Whatman GFC glass fibre filters. At the end of the incubation, 50 μL of 0.3% bovine gamma-globulin (Sigma) was added to each tube, followed immediately by 1.0 mL of 25% polyethylene glycol (PEG 6000) dissolved in PBS, vortex mixed and the mixture was left standing for 15 minutes at 2–4 °C. After that 4.5 mL of 16% PEG was added. The mixture was then applied to GFC filters (Whatman, Clifton, NJ) presoaked in 2% bovine serum albumin and retained in multi-placed holder and filtered [13]. The filters were washed four times and the retained radioactivity was determined in a gamma-spectrometer with a counting efficiency of 60% for ^{125}I. The separation and washing procedure took less than 15 s/tube, and less than 10% of added counts was bound to blank filters.

Statistical analysis

All data are expressed as mean ± SEM. Differences between means were assessed by ANOVA, followed by Bonferonni test (GraphPad PRISM; GraphPad Software, Inc., San Diego, CA).

Results and Discussion

As shown on Figure 1 copper and nickel acetate salts had no significant effect on the release of LH from porcine pituitary cells after three hours of incubation at any concentration tested. Nevertheless, at the lowest concentration applied (10^{-10} and 10^{-9} M), Ni salt tended to decrease LH secretion from pituitary cells (however no statistical differences were observed) while at the higher concentrations (10^{-8} and 10^{-7} M), reverse dependence occurred. In contrast, three hour-long treatment with 10^{-7} M of either native GnRH as well as Cu-GnRH and Ni-GnRH complexes were very effective in the stimulation of LH output (Figure 2). The action of GnRH and Ni-GnRH was comparable (163 and 167% of control, respectively). The most potent stimulator of LH secretion was Cu-GnRH ($p<0.001$), when compared to the native GnRH and Ni-GnRH.

The receptor binding of GnRH, Cu-GnRH and Ni-GnRH in the presence of iodinated GnRH are shown on Figures 3 and 4. Cu-GnRH competed more effectively than the native GnRH for specific binding sites to pituitary GnRH receptor while binding potency of Ni-GnRH was slightly lower than GnRH but it was also well pronounced.

It is well known, that the native GnRH, after binding to specific receptors and coupling with G proteins, triggers the intracellular signaling involving the activation of protein kinase C and inositol phosphate formation. Metal complexes with GnRH are also able to bind to the GnRH receptors while copper ions were shown to modify the conformation of the GnRH receptor of rat pituitary cell surface [4,8].

In our previous studies, [1,7] we evidenced that Cu-GnRH and Ni-GnRH complexes efficiently activated the second messenger pathway in porcine gonadotropes *in vitro* by stimulating the synthesis and accumulation of cAMP. However, the intracellular signaling of these complexes did not significantly influence inositol

phosphate accumulation. Cu–GnRH and Ni–GnRH complexes increased LH release in the porcine pituitary cells although their intracellular signaling is different from that of the native GnRH.

The present studies compared the effect of inorganic copper and nickel salts on the LH release in relation to the concentration. We found that within the dose of 10^{-10}–10^{-7} M range both salts did not significantly affect LH secretion in studied cells. However, nickel at lower concentrations tended to decrease LH output.

Present results differ from that performed on immature female rats by Hazum who found that the copper ions stimulated LH release from pituitary cells *in vitro* and this effect was calcium–dependent [5]. Additionally, it was suggested that Cu^{2+} may modulate the effect of GnRH–stimulated LH release [3]. In contrast, in previous study we observed [8] that copper ions at the concentration of 20 ng/ml significantly inhibited the binding of GnRH to its receptor in rats whereas the Cu-GnRH complex was more efficient than the native GnRH and Ni-GnRH [4,8].

In summary, our results suggest that copper and nickel are able to enhance LH release from pituitary cells *in vitro* only when complexed with GnRH, whereas in their cationic from they are ineffective.

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