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Evidence suggesting that ghrelin O-acyl transferase inhibitor acts at the hypothalamus to inhibit hypothalamo-pituitary-adrenocortical axis function in the rat

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ABSTRACT

Production of n-octanoyl-modified ghrelin (GHREL), an active form of the peptide requires prohormone processing protease and GHREL O-acyltransferase (GOAT), as well as n-octanoic acid. Recently a selective GOAT antagonist (GO-CoA-Tat) was invented and this tool was used to study the possible role of endogenous GHREL in regulating HPA axis function in the rat. Administration of GOAT inhibitor (GOATi) resulted in a notable decrease in plasma ACTH, aldosterone and corticosterone concentrations at min 60 of experiment. Octanoic acid (OA) administration had no effect on levels of studied hormones. Plasma levels of unacylated and acylated GHREL remained unchanged for 60 min after either GOATi or OA administration. Under experimental conditions applied, no significant changes were observed in the levels of GOAT mRNA in hypothalamus, pituitary, adrenal and stomach fundus. After GOATi injection hypothalamic CRH mRNA levels were elevated at 30 min and pituitary POMC mRNA levels at 60 min. Both GOATi and OA lowered basal, but not K^+-stimulated CRH release by hypothalamic explants and had no effect on basal or CRH-stimulated ACTH release by pituitary slices. Neither GOATi nor OA affected corticosterone secretion by freshly isolated or cultured rat adrenocortical cells. Thus, results of our study suggest that in the rat endogenous GHREL exerts tonic stimulating effect on hypothalamic CRH release. This effect could be demonstrated by administering rats with selected inhibitor of ghrelin O-acyltransferase, the enzyme responsible for GHREL acylation, a process which is absolutely required for both GHSR-1a binding and its central endocrine activities.

1. Introduction

Ghrelin (GHREL), a peptide consisting of 28 amino acids, is an endogenous ligand of growth hormone secretagogue receptor, GHS-R1a [25]. It exerts potent GH-secretagogue and orexigenic effects. Both prepro-ghrelin (ppGHREL) and its receptor genes are expressed widely in different organs, among others in the hypothalamo-pituitary-adrenal (HPA) axis [13,14,27]. It is well documented that in humans exogenous GHREL exerts stimulating effects on CRH, ACTH and cortisol secretion [13,14,27]. Also in rats and mice GHREL administration stimulates CRH, ACTH and corticosterone secretion [3,37,54,60].

The above presented data on stimulating effect of GHREL on CRH, ACTH and steroid secretion were observed *in vivo* in GHREL administered subjects. However, until yet the role of native GHREL in regulation of HPA axis function remained unclear. A tool allowing to study the involvement of native GHREL in physiologic regulation of HPA axis function was recently described by Barnett et al. [4]. As known, production of n-octanoyl-modified GHREL, the active form of the peptide, in cultured cells requires prohormone processing protease and GHREL O-acyltransferase (GOAT), as well as n-octanoic acid. As emphasize by Romero et al. [43], GOAT "is the unique enzyme that acylates ghrelin in a highly conserved manner" and "its inhibition or stimulation would not affect physiological processes other than ghrelin acylation". Barnett et al. [4] invented a peptide-based bisubstrate analog that antagonizes GOAT. This analog, GO-CoA-Tat (GS-Dap3(Oct. CoA)-Phe-Leu-Ser-Pro-Glu-His-Gln-Ahx-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) potently

Abbreviations: ACTH, adrenocorticotropin; CRH, corticotrophin releasing hormone; DMSO, dimethyl sulfoxide; EIA, enzyme-immuno assay; FBS, fetal bovine serum; FCS, fetal calf serum; GH, growth hormone; GHREL, ghrelin; GOAT, ghrelin O-acyl transferase; GOATi, ghrelin O-acyl transferase inhibitor [GO-CoA-Tat (GS-Dap3(Oct. CoA)-Phe-Leu-Ser-Pro-Glu-His-Gln-Ahx-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg)]; HPA, hypothalamo-pituitary-adrenal; OA, octanoic acid; PCR, polymerase chain reaction; POMC, proopiomelanocortin; PVN, paraventricular nucleus; QPCR, quantitative PCR; RIA, radio-immuno assay; RT-PCR, reverse transcription polymerase chain reaction.

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inhibits GOAT *in vitro*, in cultured cells, and in mice. This analog appeared to be a selective GOAT antagonist. Having this tool, we performed studies on the possible role of endogenous GHREL in regulating HPA axis in the rat. It appeared that administration of the above mentioned GOAT inhibitor (GOATi) resulted in a notable decrease in plasma ACTH, aldosterone and corticosterone concentrations in the rat. Moreover, GOATi inhibited CRH release from hypothalamic explants. Thus, obtained results seem to suggest that in the rat endogenous GHREL exerts tonic stimulating effect on hypothalamic CRH release.

2. Materials and methods

2.1. Animals and reagents

Wistar rats from the Laboratory Animals Breeding Center, Department of Toxicology, Poznań University of Medical Sciences were used. Animals were maintained under standardized conditions of light (14:10 h light–dark cycle, illumination onset at 06.00 a.m.) at 23 °C with free access to standard pellets and tap water. The Local Ethics Committee for Animal Studies approved the study protocol. Ghrelin O-acyltransferase inhibitor (GOATi) was purchased from Phoenix Pharmaceuticals, Inc. (cat no. 032-37) and octanoic acid (OA) from Sigma–Aldrich (cat no. C2875). If not otherwise stated, all other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA) or from POCh (Gliwice, Poland).

2.2. Experiments in vivo

Adult female rats (100–115 g BW) were administered daily for 13 days with sc injection of 0.2 ml 0.9% saline. At day 14 they were given an ip injection of GOATi [8 nmol/100 g BW], OA [8 nmol/100 g BW] or vehicle [0.66% DMSO in 0.9% NaCl, 0.2 ml]. GOATi and OA were dissolved in DMSO. Rats were decapitated 30 and 60 min after injection. The trunk blood was collected in the presence of EDTA [1 mg/ml], and plasma was separated and stored at −36 °C until biochemical assays. The hypothalami, pituitaries, adrenals and stomach fundus samples were dissected for total RNA isolation.

2.3. Isolated adrenocortical cells

Method of obtaining isolated rat adrenocortical cells was that described earlier [22,33–35,44,45,52,64,65]. Briefly, adrenal glands from adult intact female rats were decapsulated to separate zona glomerulosa (ZG) from the zona fasciculata and reticularis (ZF/R). Dispersed cells were obtained by collagenase (type I) digestion and mechanical disaggregation [21]. Dispersed cells obtained from 6 to 8 rats were pooled to obtain a single cell suspension, and 6 cell suspensions for each incubation experiment were employed. Cells were counted with the CASY – Cell Counter and Analyser Systems, Model TT, Schaerfe System GmbH, Reutlingen, Germany. Aliquots of each cell suspension (10^4 cells/ml in Krebs–Ringer bicarbonate buffer with 0.3% glucose and 0.2% bovine serum albumin) were incubated with ACTH [1×10^{-7} mol/l] (Cortrosyn, Organon) and various GOATi concentrations. Incubations were carried out in a shaking bath at 37 °C for 60 min, in an atmosphere of 95% O_2 and 5% CO_2. At the end of the experiment, the incubated tubes were centrifuged at 4 °C and the supernatant was stored at −36 °C.

2.4. Adrenocortical cell culture

Details of primary culture of rat adrenocortical cells were presented earlier [22,44,45,52,65,67]. For the preparation of one primary culture of adrenocortical cells twelve 21-day-old male Wistar rats were used. After decapitation adrenals were immediately removed, placed in Dulbecco's MEM/Nutrient mix from Gibco (15.57 g/l) and the fat and connective tissue were cleaned away. Each adrenal was cut into small pieces. Tissue fragments were dissociated to cell suspensions using enzymatic digestion in Dulbecco's MEM/Nutrient mix supplemented with 1 g/l collagenase (type I, Sigma), 0.1 g/l trypsin inhibitor (Sigma), 0.3 g/l BSA (Sigma) and 4.75 g/l HEPES (Sigma) for 30 min at 37 °C in a shaking water bath. The cells were harvested by centrifugation and suspended in Dulbecco's medium with 1.125 g/l sodium bicarbonate (POCH), 10% fetal bovine serum (FBS) (Gibco) and designated concentration of antibiotics (penicillin–streptomycin–fungizone mixture; Sigma). Cells were counted as above and so prepared suspensions were placed in 96-well cluster dishes (NUNC Brand Products), 10,000 cells per well, and cultured for 96 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every 24 h. At day 4 cells were incubated with 6 μM GOATi or 0.01% OA. Concentrations of applied compounds were chosen from an earlier publication [4]. As a positive controls cells were incubated in the presence of ACTH [1×10^{-7} M]. These experiments were performed with or without FBS. Incubation was carried out at 37 °C in 5% CO_2 and 95% air condition. The collected culture medium was stored at −36 °C until corticosterone assay.

2.5. Adrenocortical cell proliferation assay

Proliferation rate of cultured rat adrenocortical cells was estimated by means of the xCelligence Real-Time Cell Analyzer (RTCA) DP (Roche) (www.xcelligence.roche.com). The system measures real-time changes in electrical impedance across microelectrodes integrated into the bottom of tissue culture E-Plates. The impedance measurement provides quantitative information about the biological status of the cells, including cell number, viability, and morphology. Studies were performed on cells prepared for the classic culture, as described above. They were cultured in the above described medium in wells of E-Plates for 3 days. GOATi, OA and ACTH were added at h 48 of culture, in the same concentrations as in classic culture. Number of cells was recorded every 15 min for subsequent 24 h.

2.6. CRH release by hypothalamic explants

With minor modifications assays were performed as described earlier [24,42,59]. The hypothalami and pituitaries were dissected from the brains of male Wistar rats (250–300 g) and placed in cold Krebs–Ringer bicarbonate buffer (pH 7.4; 4 °C). Slices (approximately 1 mm^3) were prepared from the hypothalami by chopping by hand with a razor blade and the slices from each hypothalamus were incubated separately. After preincubation for 30 min in 0.4 ml of Krebs–Ringer bicarbonate buffer, the hypothalamic fragments were incubated for two successive 30 min periods in Krebs–Ringer bicarbonate buffer with 0.3% glucose and 0.2% bovine serum albumin. Tested substances – GOATi and OA were added each at the concentration of 6 μM. During the second incubation period, hypothalami were incubated in Krebs–Ringer bicarbonate buffer containing 60 mM KCl and tested substances (6 μM GOATi or OA). Incubation media were carefully aspirated and frozen at −36 °C until biochemical assays.

2.7. ACTH release by anterior pituitary gland

Under dissecting microscope neural lobe was removed, adenohypophyses placed in cold Krebs–Ringer bicarbonate buffer (pH 7.4; 4 °C) and sliced as described above. Slices from each adenohypophysis were incubated separately. After preincubation for 30 min in 0.4 ml of Krebs–Ringer bicarbonate buffer, they were incubated for two successive 30 min periods in Krebs–Ringer bicarbonate buffer with 0.3% glucose and 0.2% bovine serum albumin. GOATi