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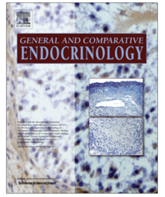

Involvements of the lateral hypothalamic area in gastric motility and its regulation by the lateral septum

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ABSTRACT

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) pre-dominantly produced in the stomach. Recent studies have shown that it may promote food intake and gastric motility. We aim to explore effects of ghrelin on the gastric distension (GD) sensitive neurons and gastric motility in the lateral hypothalamic area (LHA), and the possible regulation by the lateral septum. Extracellular single unit discharges were recorded and the gastric motility was monitored by administration of ghrelin into LHA and electrical stimulation of lateral septum. Expression of GHS-R was determined by polymerase chain reaction (PCR), western blot and immunohistochemistry staining. Projection of nerve fiber and expression of ghrelin were observed by retrograde tracer and fluo-immunohistochemistry staining. Results revealed that there were GD neurons in the LHA, and administration of ghrelin could excite both GD-excitatory (GD-E) and GD-inhibited (GD-I) neurons in the LHA. The gastric motility was significantly promoted by administration of ghrelin into LHA with a dose dependent manner, which could be completely abolished by treatment with ghrelin receptor antagonist [D-Lys-3]-GHRP-6 or BIM-28163. c-Fos expression was significantly increased after ghrelin administration to the LHA. Electrical stimulation of the lateral septum could significantly excite GD neurons responsive to ghrelin in the LHA as well as promote gastric motility. However, those effects could be absorbed by pre-treatment of [D-Lys-3]-GHRP-6. GHSR-1a expression in the LHA had no change after ghrelin administration to the LHA or electrical stimulating lateral septum. Electrical lesion of the LHA resulted in the decrease of gastric motility. GHS-R and Ghrelin/FG-double labeled neurons were observed in the LHA and lateral septum, respectively. It is suggested that the LHA may involve in promoting gastric motility via ghrelin. The Lateral septum projects to the LHA and exerts some regulating function on the LHA.

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1. Introduction

Ghrelin was discovered as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R); it is pre-dominantly produced in the stomach and potently stimulates growth hormone (GH) secretion (Kojima et al., 1999). Eventually, ghrelin has also been detected in other organs, including the small intestine, pituitary, kidney, pancreas and immune system (Gnanapavan et al., 2002), as well as in neurons in the hypothalamus (Cowley et al., 2003). Apart from its GH-releasing activity, ghrelin has many other metabolic and endocrine effects, such as inducing appetite, promoting gastric motility and gastric acid secretion, influencing sleep and behavior, regulating the endocrine and exocrine functions of pancreas, controlling cell proliferation etc. (Van der Lely et al., 2004; Morpurgo et al., 2005; Korbonits et al., 2004; Grove and Cowley, 2005). Because of a structural resemblance to motilin, ghrelin is known as the motilin-related peptide (Asakawa et al., 2001). Research reveals that endogenous ghrelin regulate spontaneous phase III-like contractions of the rat stomach (Ariga et al., 2007). Intravenous administration of ghrelin stimulates gastric acid secretion and gastric motility in rats (Masuda et al., 2000). Central and peripheral administration of ghrelin increases the gastric emptying rate and the frequency of phase III of the interdigestive migrating myoelectric complex (MMC) (Trudel et al., 2002; Edholm, 2004). The food intake and gastric motility stimulating effects of ghrelin are mediated by feeding regulatory hypothalamic centers (Diéguez, 2010).

The hypothalamus is the main part of the central nervous system in regulating food intake and energy metabolism with dense neuronal connections to higher-order brain regions (Shioda et al., 2008). The Lateral hypothalamic area (LHA) is a large and heterogeneous area with several distinct nuclear groups and is one of the most extensively interconnected area of the hypothalamus,

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allowing it to receive a vast array of interoceptive and exteroceptive information and to modulate cognitive, skeletal motor, autonomic, and endocrine functions (Berthoud and Münzberg, 2011). Local administration of ghrelin into the LHA increases food intake and wakefulness (Szentirmai et al., 2007), as well as induces Fos immunoreactivity in feeding-related brain areas, including the hypothalamic paraventricular, arcuate, and dorsomedial nuclei, amygdala, and nucleus of the solitary tract (Olszewski et al., 2003). Central pre-treatment with anti-orexin antibody attenuates peripheral ghrelin-induced increase in food intake (Toshinai et al., 2003). The connectivity and functional specificity of the LHA are still under investigation.

Septal nuclei, the vital structures of the limbic system, are the integral part of Papez loop and basolateral limbic circuit closely linked with hypothalamus and midbrain. Recent studies have shown that septal nuclei participate in the regulation of food intake and digestive function (Scopinho et al., 2008; de Arruda Camargo et al., 2010). Up to now, an involvement of septal nuclei especially the lateral septum in the regulation of gastric motility has not been demonstrated yet. In this study, GHSR-1a expression was detected in LHA of rats by PCR, western blotting and immunohistochemistry. The effects of both ghrelin administration to LHA and electrical stimulation of lateral septum on GD neuron electrical activity and gastric motility were examined by extracellular electrophysiologic recording and gastric motility measurement in conscious rats. Ghrelin neurons projecting from lateral septum into the LHA was traced by retrograde tracing and immunohistochemistry.

2. Materials and methods

2.1. Animals

Three hundred and forty-one male Wistar rats (250–300 g, provided by Qingdao Marine Drug Institution) were used for the experiments. The rats were housed in a regulated environment (22–28 °C, exposed to lights on from 8:00 a.m. to 8:00 p.m.). Standard laboratory chow pellets and tap water were available ad libitum. All animal experiments were approved and carried out according to the guidelines for animal experimentation established by the Institutional Animal Care and Use Committee at Qingdao University.

2.2. Retrograde tracing and immunohistochemistry

Rats were anesthetized with 10% chloral hydrate (3 ml/kg, i.p.) and mounted on a stereotaxic apparatus (Narashige SN-3, Tokyo, Japan) with its core temperature maintained at −37 °C by a feedback-controlled heating pad. A single pressure injection of 0.1 μl 4% (w/v) FG (Fluorochrome, Sigma, St. Louis, MO, USA; dissolved in distilled water) was made stereotaxically into the unilateral LHA (bregma: P: 1.3–2.3 mm, L (R): 1.5–2.5 mm, H: 8.0–9.0 mm) at coordinates derived from the atlas of Paxions and Watson (Paxinos and Watson, 1998). 7 days after injection, the rats were perfused transaortically with 100 ml of 0.9% saline, followed by 300 ml of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed immediately and post-fixed for 2 h in 4% paraformaldehyde in PBS, then cryoprotected in 30% sucrose for 2 days at 4 °C. The brain was cut serially into 15 μm thick frontal sections on a freezing microtome (Kryostat 1720; Leica, Germany).

After the sections were incubated with primary anti-ghrelin antibody (polyclonal, 1:300; Chemicon International, Temecula, CA, USA), anti-GHSR-1a antibody (polyclonal, dilution: 1:400; Phoenis Pharmaceuticals, CA, USA), anti-orexin antibody (monoclonal, dilution: 1:500; Phoenis Pharmaceuticals, CA, USA), anti-melanin-concentrating hormone (MCH) antibody (monoclonal, dilution: 1:500; Phoenis Pharmaceuticals, CA, USA) or anti-c-Fos antibody (polyclonal, dilution: 1:1000; Invitrogen, Carlsbad, CA, USA) at 4 °C for 40 h, they were incubated with fluorochrome-labeled secondary antibody (Cy^3-conjugated goat anti-rabbit IgG, dilution: 1:500; FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG, dilution: 1:50, Jackson ImmunoreseLHh, West Grove, PA, USA) for 2 h. The sections were mounted with Citifluor (Citifluor, London, UK). All fluorophores were visualized and photographs were taken under a Leica DMRB/Bio-Rad MRC 1024 krypton–argon laser scanning confocal microscope (Olympus, Tokyo, Japan).

2.3. Measurement of GHSR-1a by real time PCR

Rats were fasted for 24 h and then killed for tissue isolation. Total RNA was extracted from the LHA and hippocampus respectively using a TRIzol Plus RNA Purification kit (Invitrogen, CA, USA) according to the manufacturer's instructions. 1 μg of total RNA was reverse transcribed using first-strand cDNA synthesis kit (Pharmacia Biotech, Piscataway, NJ, USA). Real-time quantitative PCR was performed on a GeneAmp 5700 Sequence detection system (Applied Biosystems, Warrington, UK) using SYBR Green I as double-stranded DNA-specific binding dye for continuous fluorescence monitoring. Amplification was carried out in a total volume of 25 μl containing 2 × PCR Master Mix (Applied Biosystems, Warrington, UK), 2 μl of 1:4 diluted cDNA and 5 $\mu mol\,L^{-1}$ of each specific primer. PCR primers for rat GHSR-1a (312 bp) were 5′-GAGATCGCTCAGATCAGCCAGTAC-3′ (sense), 5′-TAATCCCCAAACT-GAGGTTCTGC-3′(antisense) and for housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 134 bp) were 5′-CGGCAAGTTCAACGGCACAG-3′(sense), 5′-ACTCCACGACA-TACTCAGCAC-3′ (antisense). The PCRs were cycled 40 times through denaturation (95 °C, 15 s) and annealing (64 °C for GHSR-1a or 60 °C for GAPDH, 60 s). Data was analyzed by Gene Amp 5700 SDS software (Applied Biosystems, Warrington, UK). Relative quantitate was performed by calculating the difference of the threshold cycle ($\Delta C_t = C_{tGHSR\text{-}1a} - C_{tGAPDH}$) of GHSR-1a and GAPDH.

2.4. Measurement of GHSR-1a by Western blot

0.1 g of the LHA or the hippocampus tissue samples was lysed and homogenized in 1 ml of lysis buffer for 30 min on ice and cleared by centrifugation at 14,000 rpm for 15 min at 4 °C. 50 μg of proteins were fractionated on Tris–tricine gradient gels (10–20%; Bio-Rad), transferred to nitrocellulose membrane (Roth), blocked with 5% milk in TBST (10 mM Tris · HCl, pH 7.5, 150 $m\,mol\,L^{-1}$ NaCl, and 0.1% Tween 20) for 1 h. Blots were incubated with specific rabbit anti-rat GHSR-1a IgG (1:5,000; Alpha Diagnostic International, San Antonio, TX, USA) overnight at 4 °C. After washing five times in TBST for 10 min, blots were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:2000; New England Biolabs) for 1 h at room temperature. A chemiluminescent peroxidase substrate (ECL, Amersham Biosciences) was applied according to the manufacturer's instructions, and the membranes were exposed briefly to X-ray film.

2.5. Extracellular discharge recordings

The process of balloon implantation in stomach and cranial surgery were as described previously (Xu et al., 2008). A latex balloon attached to polyethylene tubing (PE-240) surgically was placed in the stomach to produce gastric distension by increasing the volume of the balloon. Briefly after midline laparotomy, gastric contents were washed out with warm isotonic saline through a

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