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Effect of ghrelin receptor antagonist on meal patterns in cholecystokinin type 1 receptor null mice

Jennifer Lee, Elizabeth Martin, Gabriel Paulino, Guillaume de Lartigue, Helen E. Raybould *

Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, UC Davis, Davis, CA 95616, USA

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ABSTRACT

Vagal afferent neurons (VAN) express the cholecystokinin (CCK) type 1 receptor (CCK_1R) and, as predicted by the role of CCK in inducing satiation, $CCK_1R-/-$ mice ingest larger and longer meals. However, after a short fast, $CCK_1R-/-$ mice ingesting high fat (HF) diets initiate feeding earlier than wild-type mice. We hypothesized that the increased drive to eat in $CCK_1R-/-$ mice eating HF diet is mediated by ghrelin, a gut peptide that stimulates food intake. The decrease in time to first meal, and the increase in meal size and duration in $CCK_1R-/-$ compared to wild-type mice ingesting high fat (HF) diet were reversed by administration of GHSR1a antagonist D-(Lys3)-GHRP-6 ($p<0.05$). Administration of the GHSR1a antagonist significantly increased expression of the neuropeptide cocaine and amphetamine-regulated transcript (CART) in VAN of HF-fed $CCK_1R-/-$ but not wild-type mice. Administration of the GHSR1a antagonist decreased neuronal activity measured by immunoreactivity for fos protein in the nucleus of the solitary tract (NTS) and the arcuate nucleus of both HF-fed wild-type and CCK1R−/− mice. The data show that hyperphagia in $CCK_1R-/-$ mice ingesting HF diet is reversed by blockade of the ghrelin receptor, suggesting that in the absence of the CCK_1R, there is an increased ghrelin-dependent drive to feed. The site of action of ghrelin receptors is unclear, but may involve an increase in expression of CART peptide in VAN in HF-fed $CCK_1R-/-$ mice.

1. Introduction

Ghrelin is produced primarily by enteroendocrine cells in the gastric epithelium and is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a). Plasma levels of ghrelin are high during fasting; in particular, there is a preprandial peak consistent with a role for ghrelin in meal initiation [1] and acute peripheral administration of exogenous ghrelin increases food intake in humans and experimental animals [2]. However, the site of action of ghrelin endogenously released from gastric endocrine cells or peripherally administered ghrelin is not clear. GHSR1a are located on several populations of neurons shown to be involved in the regulation of food intake, including the arcuate nucleus of the hypothalamus, the brainstem and vagal afferent neurons (VAN) [3–7]. Functional ablation of VAN via perineural capsaicin treatment, total subdiaphragmatic or selective gastric vagotomy inhibits the ability of ghrelin to increase food intake in mice and rats [5,8,9] and truncal vagotomy associated with gastric surgery inhibited the stimulatory effect of ghrelin in humans [10], although this has not been verified in one study in rats [11]. In addition, ghrelin has been shown to influence VAN function; ghrelin inhibits the neuronal discharge of gastric mechanoreceptor fibers and increases the discharge of subdiaphragmatic vagal afferents innervating the intestine to distention stimulus [12,13].

There are data to suggest that cholecystokinin (CCK), a gut peptide that inhibits food intake, and ghrelin interact in the control of food intake. Prior administration of ghrelin inhibits the effects of CCK to reduce feeding, and administration of CCK prior to ghrelin inhibits the ability of ghrelin to induce feeding [8,14] The site of this interaction between these two peptides is unknown. A similar interaction between CCK and ghrelin was reported on vagal afferent fiber discharge [5]. Moreover, ghrelin inhibits CCK- or feeding-induced alteration of peptide expression by vagal afferent neurons [4]; CCK induces expression of cocaine- and amphetamine-regulated transcript (CART) peptide in VAN, an effect inhibited by administration of ghrelin. Thus CCK and ghrelin interact at the level of the vagus nerve, yet whether there are functional consequences of this interaction on feeding behavior remain unknown.

We and others have previously shown that $CCK_1R-/-$ mice lack short term satiety, resulting in the ingestion of longer and larger meals [15,16]. Moreover, we extended these observations to show that the hyperphagia was more pronounced in animals ingesting HF diet. An unexpected finding was a marked decrease in the time to the first meal after a short (6 h) fast in $CCK_1R-/-$ mice, particularly when the mice were ingesting a diet high in fat and calories [16]. These data suggests that lack of the CCK_1R can disrupt orexigenic signaling, but the possible mechanism and pathway by which this occurs are unknown. Given the possible role of ghrelin in meal initiation, and

* Corresponding author at: Vet Med: APC, 1321 Haring Hall, UC Davis, Davis, CA 956161, USA. Tel.: +1 530 754 6555; fax: +1 530 752 7690.
E-mail address: heraybould@ucdavis.edu (H.E. Raybould).

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previous studies showing interaction of CCK and ghrelin in the regulation of feeding and VAN function, we hypothesized that the increase in time to first meal in $CCK_1R-/-$ mice may be mediated by ghrelin and that this interaction may occur at the level of VAN.

To test this hypothesis, we measured meal patterns in wildtype and $CCK_1R-/-$ mice and determined the role of GHSR1a using the specific receptor antagonist, D-(Lys3)-GHRP-6 [17]. To determine whether there were any changes in the vagal afferent pathway, we measured expression of CART peptide in VAN using immunocytochemistry. To determine the possible site of action of blockade of the ghrelin receptor on meal patterns, we also measured neuronal activation using immunohistochemistry for c-fos protein in $CCK_1R-/-$ and wild-type mice in the nucleus of the solitary tract (NTS), the region where vagal afferent terminates in the brainstem, and in the arcuate nucleus of the hypothalamus, a site of expression of GHSR1a and a possible site of action of ghrelin to increase food intake.

2. Materials and methods

2.1. Animals

Age matched (25–30 g body weight, 8 weeks) adult male $CCK_1R-/-$ mice (Mouse Biology Program, UC Davis) and their wild-type controls 129Sv (Taconic, Oxnard, CA) mice were used in all experiments [18]. All mice were individually housed on wire bottom cages and maintained on a 12-h light, 12-h dark cycle (lights on at 3:00 am for feeding behavior; otherwise lights on at 6:00 am) in a temperature-controlled room (23 °C). For experiments where neural tissues were collected, animals had ad libitum access to water and either standard laboratory chow (Lab Diet 5001), or 38% high fat (HF) diet (Cat. No. D12451Research Diets). Animals used for meal pattern studies were fed either a 10% low fat (LF) or 45% high fat (HF) diet (Bioserv Custom Dustless Precision Pellets, Frenchtown, NJ) for 2 weeks. Prior to treatment or experiment day, all mice were placed in wire-bottom cages during short term or overnight fasts. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of California, Davis.

2.2. Drugs/Peptides

D-(Lys3)-GHRP-6 (Cat. # H-3108) was purchased from Bachem (King of Prussia, PA) and reconstituted in 0.9% saline and administered intraperitoneally (ip) following either a short term (6 h) or overnight fast on wire-bottom cages.

2.3. Food intake analysis

Meal pattern analysis was performed as previously described [16]. Briefly, mice were fed either LF or HF isocaloric (3.4 kcal/g), isonitrogenous (21% of energy) 20 mg pelleted diets. Mice (n = 3–6 per treatment group) were fasted daily in wire bottom cages for 6 h during the light cycle (9:00 am to 3:00 pm). Each experiment lasted 15 days. Mice were acclimatized to the diets and the feeding paradigm for 5 days. Following the acclimation period, meal pattern data were recorded for 10 consecutive days with treatment injections. Body weight was measured at 3:00 pm prior to placement in the meal pattern analysis cages. Feeding patterns (meal duration and meal size) were measured continuously from 3:00 pm to 9:00 am using food intake monitoring cages (The Habitest® System, Coulbourn Instruments, Allentown, PA). In this system, infrared pellet-sensing photo beams control the pellet dispensers and pellets are delivered in response to removal of the previous pellet. Data were recorded from EZ count software and analyzed using Spike2 (version 5.07, Cambridge Electronic Design 1988–2004), SigmaStat (version3.11, Systat Software Inc. 2004) and Graph Pad Prism® (version 3.02, GraphPad Software Inc. 1994–2000). The parameters for defining a meal (acquisition of at least 4 pellets within 10 min, preceded or followed by 10 min of no feeding) were based on two previously published studies using mice and our previous study [15,16,19].

2.4. Immunohistochemistry of CART in nodose ganglia neurons

Mice were fed HF diets for two weeks (n = 4–6 per group) with free access to water. Nodose ganglia collected for CART immunostaining were taken from LF saline-treated mice, and from HF fed mice of both genotypes treated with saline or D-(Lys3)-GHRP6 (2.8 μg/kg). Following an overnight fast, mice were given an intraperitoneal injection of saline or drug treatment and after 90 min, were deeply anesthetized (sodium pentobarbital, Beuthanasia 0.05 mL/rat) and transcardially perfused with 0.1% heparinized 0.9% NaCl at 4 °C, followed by 1 mL/g body weight of 4% paraformaldehyde dissolved in PBS (PFA-PBS) at 4 °C. Nodose ganglia were collected and post-fixed for 2 h in 4% PFA, then transferred and stored at 4 °C in 25% sucrose-PBS until further processing. Frozen nodose ganglia were sectioned at 10 μm on a cryostat and placed onto Fisher Superfrost/Plus slides. Slides were blocked with 20% goat serum-PBS for 30 min, followed by primary antibody incubation of rabbit-anti-rat-CART (1:200, Cat. No. H00360, Phoenix Pharmaceuticals, Inc., Burlingame, CA) at 37 °C. Following three serial washes in PBS at room temperature, donkey or goat anti-rat AlexaFluor 488 secondary antibody was applied and incubated at 37 °C for 30 min. Slides were washed overnight and coverslips mounted using Fluoro-Gel mounting media (Cat. No. 17985–10, Electron Microscopy Sciences, Hatfield, PA), dried overnight at room temperature, and stored at −20 °C until ready for imaging. Confocal images were made using an Olympus FV1000 Laser Scanning Confocal Microscope (Olympus America Inc., Melville, NY) at 20× oil objective and analyzed for neuronal labeling using Scion Image (Beta 4.0.2, Scion Corporation, 2000). 5–8 photomicrographs were analyzed per mouse; the level of CART expression was quantified by determination of the number of positively labeled pixels normalized to total number of pixels and expressed as percent positive labeled pixels.

2.5. c-fos immunohistochemistry in the hindbrain and forebrain

Mice were fed a 45% high fat diet, (129sv and $CCK_1R-/-$, n = 4–6 for all groups) for two weeks ad libitum. Animals were fasted overnight on wire-bottom cages with ad libitum water. Animals were then given an intraperitoneal injection of drug treatment of 0.1 μL total volume of either saline or D-(Lys3)-GHRP-6 (2.8 μg/kg). After 90 min, animals were deeply anesthetized with sodium pentobarbital (Beuthanasia 0.05 mL/animal) and transcardially perfused with 4% paraformaldehyde. Whole brains were removed and stored in 4% paraformaldehyde for post-fixation, then transferred to PBS and stored at 4 °C until vibratome sectioning.

Regions of the hindbrain and forebrain were cut rostrocaudally and categorized into areas of the fourth ventricle, area postrema, and post-area postrema for the hindbrain [18,20] and the third ventricle was used as a landmark for the arcuate nucleus in the forebrain. 100 μm sections were cut using a Series 1000 Vibratome in cold PBS and blocked with goat serum-PBS (2% goat serum, 0.2% Triton X-100, 0.1% bovine serum albumin in PBS) for 2 h at 37 °C. Next, sections were incubated with c-fos primary antibody (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h at 37 °C, serially washed three times in PBS and treated with biotinylated goat anti-rabbit IgG secondary antibody (Vector Labs, Burlingame, CA) for 1 h at 37 °C. Following two serial washes of PBS, sections were incubated with ABC solution from the Standard *Elite* Vectastain ABC Kit (Vector Labs) for 1.5 h at 37 °C. Ni-3′3′-diaminobenzidine (Cat. No. D-8000-5 G, Sigma Aldrich Chemicals) was dissolved in PBS (30 mg/100 mL) and added to sections for 5 min followed by the addition of 30% H_2O_2 to each section with the reaction stopped after 5 min with three serial washes using cold PBS. All reagents contained penicillin streptomycin (Cat. No. 15140–122, Gibco, Carlsbad, CA) antibiotic treatment to prevent