A novel role of G protein coupled receptor, family C, group 5, member B (GPRC5B) in the regulation of ghrelin expression and secretion

(グレリン発現と分泌における GPRC5B の役割に関する研究)

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DECLARATION

I hereby declare that this PhD thesis entitled “A novel role of G protein coupled receptor, family C, group 5, member B (GPRC5B) in the regulation of ghrelin expression and secretion” was written by me for the degree of Doctor of Philosophy under the guidance and supervision of Dr. Ichiro Sakata, Ph.D., Associate Professor, Graduate School of Science and Engineering, Saitama University, Japan.

Parts of this thesis have been published as research articles in Cell Biology International (entitled “The effect of glutamate on ghrelin release in mice”).

For the present thesis, which I am submitting to Saitama University, no degree, diploma, or distinction has been conferred on me before, either at this or any other University. No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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ABSTRACT

Ghrelin is a 28-amino acid peptide that is mainly produced in the stomach and is purified as an endogenous ligand of the growth hormone secretagogue receptor (GHSR). Ghrelin plays many important roles in the regulation of feeding behavior and energy homeostasis. Ghrelin plasma levels change depending on the nutritional and metabolic status of the body; ghrelin levels increase dramatically before each meal and decline rapidly after feeding. However, ghrelin secretion mechanisms are not fully understood. To determine the novel factors that are involved in the regulation of ghrelin secretion, we focused on the G protein-coupled receptor, family C, group 5, member B (GPRC5B). We studied the role of GPRC5B on ghrelin expression and secretion in stomach ghrelinoma (SG-1), pancreatic ghrelinoma (PG-1), and primary gastric mucosal cells. Results of quantitative RT-PCR analysis revealed that SG-1, PG-1, and stomach tissue exhibit transcriptional expression of GPRC5B strongly. Glutamate, a putative GPRC5B agonist candidate, inhibited ghrelin secretion in SG-1, PG-1, and primary cells in a dose-dependent manner. However, glutamate treatment significantly inhibited mRNA expression of ghrelin, but not ghrelin O-acyltransferase (GOAT) and prohormone convertase 1 (PC1). L-glutamate treatment significantly decreased ghrelin expression, but D-glutamate did not affect ghrelin expression and secretion. Small interfering RNA (siRNA) targeting GPRC5B blocked the inhibitory effect of glutamate on ghrelin secretion. Furthermore, pretreatment with glutamate blocked the effect of the norepinephrine (NE)-induced ghrelin upregulation in primary cultures of gastric mucosal cells. Additionally, the results showed that glutamate treatment significantly increased the levels of phosphorylated extracellular signal-regulated kinase (ERK) compared to the control group. Co-administration

of glutamate with an ERK pathway inhibitor reversed the inhibitory effect of glutamate on acyl-ghrelin secretion in PG-1 and SG-1 cells. Glutamate treatment increased mRNA expression levels of cAMP response element-binding protein2 (CREB2) in both SG-1 and PG-1 cell lines. Furthermore, glutamate decreased both food intake and plasma acyl-ghrelin concentrations in mice. These results suggest that glutamate is an important regulator of ghrelin signaling and that GPRC5B signaling is involved in the inhibition of ghrelin expression and secretion in gastric ghrelin cells.

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Chapter 1

1.1. Introduction

1.1.1 Ghrelin

Ghrelin, a 28-amino acid peptide, was first identified as a peptide hormone produced in rat and human stomachs in 1999 by Kojima et al. [1, 2]. The stomach is the major source of plasma ghrelin, and approximately 70% of circulating ghrelin is produced in the mucosal layer of the stomach [3], although ghrelin is also expressed in the duodenum, jejunum, ileum, colon, pancreas, kidney, pituitary gland, lungs, and hypothalamus. Ghrelin stimulates the secretion of growth hormones [1, 4-6] and plays well-defined roles as a stimulator of food intake [7-12] and body weight gain [13] when administered peripherally or centrally [13, 14]. In addition, ghrelin is known to stimulate gastric acid secretion [15-17], gastric motility [18-20], cardiovascular function [21], and cell proliferation [22, 23]. Ghrelin can also influence pancreatic exocrine and endocrine function and glucose metabolism [24]. Plasma concentrations of ghrelin change according to the nutritional and metabolic status of the body [25]. More specifically, plasma ghrelin concentrations increase during fasting and dramatically decrease after feeding, whereas feeding and nutritional abundance suppress ghrelin secretion [25, 26].

Ghrelin exists in two molecular forms, namely, acyl-ghrelin and des-acyl ghrelin [13, 27]. Des-acyl ghrelin is octanoylated at the third serine position by ghrelin O-acyltransferase (GOAT) to produce an acylated form that can bind to its receptor growth hormone, secretagogue receptor 1a (GHSR, ghrelin receptor), to exert its biological function [24, 25,

28]. Ghrelin-producing cells, which are classified into open- and closed-types, are present in the mucosa but not in the myenteric plexus of the stomach, duodenum, ileum, cecum, and colon [9]. Small, round, closed-type ghrelin cells known as X/A-like cells, which are located in the stomach, constitute the majority of ghrelin-producing cells, whereas open-type cells gradually increase in number from the stomach to the lower gastrointestinal tract [9, 29]. These findings suggest that the mucosal layer of the stomach is the primary source of plasma ghrelin. Previous studies have shown that human ghrelin gene contains six exons encoding a 511-bp mRNA transcript and four introns [30-32]. Preproghrelin (117 AA) contains 23-AA signal peptide and 94-AA proghrelin [33]. Proghrelin is composed of a 28-AA ghrelin peptide and a 66-AA C-ghrelin peptide [34, 35]. C-ghrelin can be further processed to produce a 23-AA peptide called obestatin [34, 35]. Signal peptidase, prohormone convertase 1/3 (PC1/3), and carboxypeptidase-B-like enzyme cleave preproghrelin at Arg 23, Arg 51 (generating ghrelin 1–28) [36], and Pro50 (generating ghrelin 1–27), respectively to produce the final form of ghrelin [36, 37].

1.1.2. Regulatory mechanisms of ghrelin secretion

Although the complete regulatory mechanisms of ghrelin secretion remain to be elucidated, food intake has been recognized to inhibit mRNA expression of ghrelin and reduce its circulating levels [38, 39]. In addition, food deprivation has been demonstrated to increase ghrelin concentration in rats [40]. Furthermore, several hormones have been reported to be involved in the regulation of ghrelin expression and secretion [41-48]. Previous studies have confirmed that epinephrine, norepinephrine, endothelin, and secretin stimulate ghrelin secretion, whereas cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), peptide YY

(PYY), and somatostatin inhibit ghrelin secretion [49]. Insulin is considered as one of the major factors that regulate the circulating ghrelin levels. Recently, Gagnon et al. reported that insulin activates the PI3K-AKT pathway and decreases intracellular cAMP levels, resulting in suppressed ghrelin secretion in primary cultures of stomach cells from rats [50]. Leptin administration suppressed ghrelin secretion in rats [48, 51, 52]. Furthermore, ghrelin is reported to directly act on α-cells and stimulate glucagon secretion by α-cells [53]. Ghrelin secretion was also found to be regulated by the autonomic nervous system, which is predominantly controlled by sympathetic nerves [43]. Adrenaline and noradrenaline directly stimulate ghrelin secretion by binding to β1 receptors on ghrelin cells [43, 49, 50, 54, 55]. A previous study showed that ghrelin levels change in response to food intake, suggesting that ghrelin concentrations depend on nutrient uptake. Sakata et al. reported that glucose administered at high levels inhibited ghrelin secretion, whereas low glucose administration stimulated ghrelin release in primary cultures of gastric mucosal cells from mice [41]. Duodenal and jejunal infusions of lipids or amino acids also reduced ghrelin levels in rats [56]. Moreover, administration of free fatty acids or proteins decreased plasma ghrelin levels in humans [57]. Previous studies showed that long-chain fatty-acid receptor FFAR4 (GPR120), which is expressed in ghrelin-producing cells [54, 58-60], suppresses ghrelin secretion *in vitro* [54, 60]. Ghrelin-producing cells also express short-chain fatty acid receptors, namely, FFAR2 (GPR43), and FFAR3 (GPR41) [54]. Short-chain fatty acids (such as acetate or propionate) suppress ghrelin secretion by activating FFAR2 [54]. However, the detailed regulatory mechanisms by which nutrients regulate ghrelin secretion remain unclear. In this study, we hypothesized that glutamate, which has been reported as candidate agonist for GPRC5B, regulates ghrelin secretion in gastric ghrelin cells.

1.1.3. Stomach ghrelinoma (SG-1) and pancreatic ghrelinoma cells (PG-1)

Ghrelin-producing cell lines were generated using the transgenic mice overexpressing the SV40 T-antigen (TAg) in ghrelin cells [43]. The SV40 T-antigen-coding region was inserted downstream of the start codon of preproghrelin, thereby allowing both the transcription and translation of SV40 T-antigen under the control of preproghrelin regulatory elements. Transgenic mice showed high levels of plasma acyl ghrelin and des-acyl ghrelin and developed pancreatic and gastric tumors [43]. SG-1 cell lines were produced from gastric tumors, and PG-1 cell lines were produced from pancreatic tumors. SG-1 and PG-1 cells are morphologically similar but have different growth requirements. SG-1 cells require low oxygen, 5% O_2, and 5% CO_2 conditions, whereas PG-1 cells can grow under normal culture conditions with 5% CO_2 and standard O_2 concentrations. Ghrelin and GOAT mRNAs were found to be highly expressed in SG-1 and PG-1 cells. These two cell lines also showed strong transcriptional expression of PC1, PC2, and chromogranin A [43].

1.1.4. G protein-coupled receptor, family C, group 5, member B (GPRC5B)

GPRC5B is a member of the retinoic acid-inducible gene 2 (Raig2) subfamily of type 3 (family C) GPCRs. GPRC5B encodes an orphan G protein-coupled receptor and exhibits significant sequence similarity with metabotropic glutamate receptors in their seven transmembrane domain regions [61]. Soni et al. reported that GPRC5B is highly expressed in both human and mice pancreatic islets [62]. The group also examined GPRC5B expression in mouse tissues [62], and the results showed that GPRC5B mRNA was moderately expressed in the brain, liver, and kidney and less expressed in the lungs and heart [62]. A recent genome-wide association study showed a strong association between the human *GPRC5B* gene and

body mass index, suggesting that *GPRC5B* is a critical regulator of human metabolism [63]. Another study showed that highly fed *GPRC5B*-knockout mice showed faster glucose removal, lower circulating levels of insulin and glucose, and 18% lower body weight than wild-type mice [64]. Recently, GPRC5B has been demonstrated as a glutamate receptor candidate that influences both glucose-induced insulin secretion and β-cell survival in mouse islets [62].

Gene chip screening using primary gastric ghrelin-producing cells collected from ghrelin-GFP transgenic mice showed that *GPRC5B* is strongly expressed in ghrelin cells [65]. Therefore, in this study, we examined the involvement of GPRC5B signaling on ghrelin secretion both *in vivo* and *in vitro*.

1.2. Materials and Methods

1.2.1. Cell lines

Two types of ghrelin cells were used in this experiment, namely, stomach ghrelinoma cells (SG-1), which originate from the stomach, and pancreatic ghrelinoma cells (PG-1), which are derived from the pancreas. SG-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 50:50 (Cellgro, Manassas, VA, USA) containing 10% fetal bovine serum (FBS) and supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C with 5% CO_2 and 5% O_2. On the other hand, PG-1 cells were cultured in DMEM/F12 50:50 containing 10% fetal bovine serum supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) in an incubator at 37 °C with 5% CO_2.

1.2.2. Chemicals

L-glutamate, D-glutamate, and norepinephrine (NE) were purchased from Sigma (St. Louis, MO, USA). Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Sodium octanoate was purchased from Wako (Osaka, Japan).

1.2.3. RT-PCR

Total RNA was extracted from SG-1 cells, PG-1 cells, and brain and stomach tissues using ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. DNA contamination was removed via DNase digestion (Promega, Madison, WI, USA). cDNA was synthesized from 1 μg of DNase-treated total RNA using Superscript® III Reverse Transcriptase (Invitrogen) and random primers (#48190-011, Invitrogen). The following primers were designed to amplify the mouse *GPRC5B* gene (fragment size: 418 bp): sense

primer, 5′-AGTTCAAACGGTGGAAGGTG-3′ and antisense primer, 5′-CCCAGGCTGCTAGATCTTTG-3′; and mouse β-actin (internal control, fragment size: 210 bp): sense primer, 5′-CTGGGTATGGAATCCTGTGG-3′ and antisense primer, 5′-GTACTTGCGCTCAGGAGGAG-3′. Reactions were performed on an iCycler (Bio Rad, Hercules, CA, USA) instrument. Amplicon size and specificity were confirmed via 2% agarose gel electrophoresis.

1.2.4. Primary culture of gastric mucosal cells

Adult male C57BL/6J mice were sacrificed via cervical dislocation, and stomachs were exposed and tied off proximally from the esophagus and distally from the duodenum by surgical suturing. A 5-mm incision was made in the non-glandular forestomach. The stomach was then emptied by gently pushing out the food through the incision using blunt forceps. Next, the stomach was turned inside out by pushing the distal part of the stomach through the incision in the forestomach. A blunt 20-gauge metal needle was then attached to a syringe and inserted into the small hole, and surgical suturing was performed to tighten the incisional opening. The stomach was inflated by injecting cold DMEM/F12 50:50 containing 10% FBS. Afterwards, the needle was removed, and the incisional opening was completely tied off using the previously placed suture. The inflated, inside-out stomachs were temporarily placed in DMEM/F12 50:50 containing 10% FBS until all stomachs were processed. Next, isolated stomachs were added to 30 mL of digestion solution containing 100 mg of Dispase II (Roche Diagnostics, Indianapolis, IN) and subsequently incubated in a water bath at 37 °C for 90 min. The mucosal layer of each stomach was scraped using a polyethylene transfer pipette (1-4634-01; AS ONE, Osaka, Japan) and added to the culture media. Cell suspensions were transferred

into a 15-mL conical tube and centrifuged at 1200 rpm for 5 min. The supernatant was removed, and 3 mL of 0.25% trypsin with 300 μL of ethylenediaminetetraacetic acid (EDTA) (Gibco, Tokyo, Japan) was added, followed by incubation at 37 °C for 5 min. To inactivate trypsin, 10 mL of DMEM/F12 supplemented with 10% FBS was added to the solution. Cells were then resuspended and filtered through a 40-μm nylon mesh (352340, Cell Strainer; BD Falcon, Bedford, MA). Filtered cells were recentrifuged at 1200 rpm for 5 min. Supernatants were discarded, and cell pellets were resuspended in 5 mL of DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Counts of the isolated cells were determined using a hemocytometer. Cells at 1×10^5 cells/well were seeded in 24-well plates. Ghrelin secretion experiments were performed the following day. Media were aspirated before the addition of 200 μL of serum-free 2.5 mM D-glucose/DMEM (Gibco) containing 50 μM sodium octanoate with glutamate. After incubation at 37 °C for 3 h, culture media were collected and centrifuged at 3000 rpm for 5 min at 4 °C. Afterwards, 100 μL of supernatant was transferred to a separate tube, to which 10 μL of 1 N HCl was immediately added. Samples were stored at -80 °C until analysis.

1.2.5. Ghrelin secretion experiment in SG-1 cells and PG-1 cells

On day 0, PG-1 and SG-1 cells were plated at 1×10^6 cells/well in 12-well plates with DMEM/F12 50:50 containing 10% fetal bovine serum and supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) containing 50 μM sodium octanoate. On day 1, the medium was removed, and wells were added with 400 μL of serum-free DMEM (GIBCO, Tokyo, Japan) containing 2.5 mM D-glucose and 50 μM sodium octanoate with chemicals. After incubation at 37 °C for 3 h, the medium was collected and centrifuged at 3000 rpm for 5

min at 4 °C. Next, 100 μL of the supernatant was transferred to a separate tube, to which 10 μL of 1 N HCl was added immediately. Samples were stored at -80 °C until analysis.

1.2.6. Quantitative RT-PCR

On day 0, SG-1 cells were plated in 12-well plates at 1×10^6 cells/well in DMEM/F12 50:50 containing 10% fetal bovine serum supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) containing 50 μM sodium octanoate. Cells were then treated with 1 mM glutamate for 3 h. The medium was aspirated, and RNA was extracted using ISOGEN (Nippon gene, Tokyo, Japan). A total of 1 μg of RNA was used for cDNA synthesis using Superscript® III Reverse Transcriptase (Invitrogen). Real-time PCR was performed on a LightCycler (Roche Diagnostics) instrument using SYBR premix Ex Taq (Takara Bio, Shiga, Japan) using the following primers: mouse preproghrelin (fragment size: 67 bp): forward, 5′-CCCAGGCATTCCAGGTCAT-3′ and reverse, 5′-AACTGCAGATGGTGCCTGAAG-3′; mouse GOAT (fragment size: 71 bp): forward, 5′-TCCACAGCCTGGCTCTTTAAAC-3′ and reverse, 5′-GCCGCGTGGAGGAGAGA-3′; mouse prohormone convertase 1 (PC1) (fragment size: 113 bp): forward, 5′-GGCACCTGGACATTGAAAATTAC-3′ and reverse, 5′-TTCATGTGCTCTGGTTGAGAAGA-3′; and mouse GAPDH: forward, 5′-TGTGTCCGTCGTGGATCTGA-3′ and reverse, 5′-CCTGCTTCACCACCTTCTTGA-3′. PCR amplification was performed under the following cycling conditions: initial template denaturation for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. mRNA levels were expressed relative to GAPDH (internal control). The same RT-PCR protocol was performed on PG-1 cells.

1.2.7. Measurement of ghrelin concentrations

Acyl-ghrelin concentrations were determined using a rat acylated ghrelin enzyme immunoassay kit (Bergin Pharma, SPI bio, France) according to the manufacturer's guidelines. Absorbance data were collected on a BIO-RAD iMAR^{KTM} Microplate Reader spectrophotometer using Microplate Manager® 6 Software (BIO-RAD).

1.2.8. Statistical analysis

All data were expressed as mean ± SEM, and statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Data were standardized as percentage values relative to the control group and analyzed using Student's *t*-test, one-way ANOVA with Tukey's, or Dunnett's post-hoc tests. Differences were considered statistically significant at $P < 0.05$.

1.3. Results

1.3.1. GPRC5B mRNA expression in SG-1 cells, PG-1 cells, and stomach tissue.

RT-PCR was performed to measure GPRC5B mRNA expression in SG-1 cells, PG-1 cells, and gastric tissues. GPRC5B mRNA was found to be strongly expressed in SG-1 cells, PG-1 cells, and gastric tissue (Fig. 2). We previously performed gene chip screening using enriched ghrelin cells to determine relative levels of mRNAs within fluorescence-activated cell sorting (FACS)-separated pools of gastric mucosal cells. Results showed that GPRC5B was strongly expressed in ghrelin cells [65] (data not shown). Brain samples were used as positive control, whereas distilled water (DW) was used as negative control. β-actin was used as an internal control.

1.3.2. Effect of glutamate on ghrelin secretion in SG1 cells, PG-1 cells, and primary gastric mucosal cells

To determine the role of GPRC5B in ghrelin secretion, glutamate, a candidate agonist of GPRC5B, was added to the cells. After collecting the culture media, acyl ghrelin levels were measured using an ELISA kit. Results showed that glutamate significantly inhibited acyl-ghrelin secretion in SG-1 cells (Fig. 3). Similarly, glutamate inhibited acyl ghrelin secretion in PG-1 cells (Fig. 4). Afterwards, glutamate was added to the media containing ghrelin cells collected from stomach tissues of mice. After collecting the culture media, acyl ghrelin levels were measured following the procedure for SG-1 and PG-1 cells. Interestingly,

acyl ghrelin levels were significantly inhibited by glutamate treatment in primary cultured gastric mucosal cells (Fig. 5).

1.3.3. Role of glutamate on preproghrelin, GOAT, and PC1 mRNA expression in SG-1 cells

Quantitative PCR was performed to determine the effect of glutamate on transcriptional expression of preproghrelin, GOAT, and PC1. Results showed no significant changes in GOAT and PC1 mRNA levels, but preproghrelin mRNA levels were significantly decreased after 3 h of incubation with glutamate in SG-1 cells (Fig. 6A-C).

1.3.4. Effect of glutamate on preproghrelin, GOAT, and PC1 mRNA expression in PG-1 cells

The effect of glutamate on mRNA expression of preproghrelin, GOAT, and PC1, in PG-1 cells was verified. Results from quantitative PCR showed that glutamate significantly inhibited mRNA expression of preproghrelin in PG-1 cells but did not affect GOAT and PC1 mRNA expression (Fig. 7A-C).

1.3.5. Effect of D-glutamate on mRNA expression of preproghrelin and acyl-ghrelin secretion in PG-1 cells

Next, we determined whether D-glutamate and/or L-glutamate can inhibit acyl ghrelin levels and preproghrelin expression in PG-1 cells. For this experiment, we used similar doses of D-glutamate and L-glutamate and measured acyl ghrelin levels and ghrelin mRNA expression. Results showed that L- glutamate but not D-glutamate affects acyl ghrelin

concentrations and RNA levels of preproghrelin (Fig. 8A-B). However, D-glutamate showed similar mRNA expression levels to that of the control group (Fig. 8A-B).

1.4. Discussion

1.4.1. GPRC5B regulates ghrelin secretion in gastric ghrelin cells

Ghrelin is a 28-amino acid peptide that regulates metabolism by activating orexigenic neural circuits [66]. Plasma ghrelin levels rise 1–2 h before each meal and immediately decline after feeding; this transient change can be modified by altering the feeding regimen in sheep [67]. Zhao et al. reported that adrenaline and noradrenaline stimulate ghrelin secretion in mice, and ghrelin-secreting cells strongly express mRNA encoding beta-1 adrenergic receptors [43]. Fasting-induced increase in plasma ghrelin was also found to be blocked by reserpine treatment, which depletes sympathetic neurons of adrenergic neurotransmitters [43]. The rise in plasma ghrelin levels during fasting was blocked by administration of atenolol, a selective beta-1-adrenergic antagonist, thereby suggesting that sympathetic neurons directly act on beta-1 adrenergic receptors to induce ghrelin secretion [43]. In addition, results of quantitative PCR analysis showed that G protein-coupled receptors (GPCRs) are highly expressed in ghrelin cells and play an important role in regulating ghrelin secretion [68]. Several GPCRs have been reported to exert stimulatory effects on ghrelin secretion, whereas other GPCRs exert inhibitory effects [68]. Moreover, previous studies showed that glucose-dependent insulinotropic polypeptide (GIP), secretin, melanocortin 4, and calcitonin gene-related peptide (CGRP) receptors are expressed in ghrelin cells and stimulate ghrelin secretion [68]. On the other hand, somatostatin receptors (SSTRs), the lactate receptor (GPR81), and receptors for short chain fatty acids (FFAR2) and long chain fatty acids (FFAR4) were found to inhibit ghrelin secretion. The decrease in ghrelin secretion after feeding is partially induced by long chain fatty acids that act directly on gastric GPR120-expressing ghrelin cells [60]. The

above studies demonstrate GPCRs as important regulators of ghrelin secretion. Therefore, we focused on GPCR5B, which was found to be expressed in ghrelin-producing cells.

Nutrients such as carbohydrates, protein, and fatty acids are important factors that inhibit ghrelin secretion after feeding. In humans and rats, oral administration of glucose decreases plasma ghrelin concentrations [42], indicating that ghrelin-producing cells directly respond to plasma glucose levels [42]. In rats, proteins were shown to have the highest appetite-suppressing effect among the three macronutrients [69]. Another study also showed that macronutrients regulate ghrelin signaling; among the three macronutrients (carbohydrate, protein, and lipids), carbohydrates, and proteins were shown to strongly inhibit concentrations of acyl ghrelin and total ghrelin in humans [70]. All the above findings suggest that amino acids are important in the regulation of food intake and acyl ghrelin secretion.

We hypothesized that glutamate, which has been reported as a candidate agonist for GPRC5B, regulates ghrelin secretion. To validate this hypothesis, we used ghrelin-producing cell lines, namely, SG-1 and PG-1 [43] and tested ghrelin secretion. Results showed that SG-1 and PG-1 cells strongly express GPRC5B transcripts. After incubation with glutamate, the secretion of acyl-ghrelin was significantly inhibited in ghrelin SG-1, PG-1, and gastric mucosal cells. The effect of glutamate administration on acyl ghrelin secretion was almost similar among ghrelin cell lines and primary gastric mucosal cells, suggesting that glutamate is involved in the regulation of acyl ghrelin secretion. In addition, glutamate inhibited mRNA expression of preproghrelin in ghrelin cell lines. On the other hand, glutamate did not significantly affect GOAT and PC1 expression. Ghrelin is derived from its precursor protein, preproghrelin, and is processed by alternative mRNA splicing and/or proteolytic processing [71]. PC1 (commonly referred to as PC1/3) is the primary enzyme involved in the processing

of protein precursors [72]. PC1/3 is the only enzyme that is reported to be involved in the processing of ghrelin *in vivo*, consistent with *in vitro* data showing that PC1/3 can cleave recombinant proghrelin produced from bacteria [76]. Ghrelin requires n-octanoylation modification on the third serine residue, which is necessary for binding to its receptor growth hormone secretagogue receptor 1a (GHSR, ghrelin receptor) [72, 76]. This acyl modification is mediated by a membrane-bound O-acyltransferase, ghrelin O-acyltransferase (GOAT) [72, 76]. Taken together with our results, GPRC5B regulates ghrelin mRNA transcription and ghrelin secretion, but does not affect mRNA expression of GOAT and PC1. Moreover, we observed that D-glutamate did not affect ghrelin secretion and mRNA expression of preproghrelin in ghrelin cell line. Glutamate exists in two forms, namely, L-glutamate and D-glutamate [73]. L-glutamate is the naturally occurring form of glutamate, whereas D-glutamate is the artificially or chemically processed glutamate [73]. Our current results suggest that L-glutamate specifically binds to GPRC5B, indicating that GPRC5B is a critical regulator of ghrelin signaling. Glutamate administration inhibited both acyl ghrelin secretion and ghrelin expression, suggesting that GPRC5B regulates acyl ghrelin secretion by inhibiting preproghrelin expression.

1.5. Summary

GPRC5B mRNA was found to be strongly expressed in SG-1 cells, PG-1 cells, and gastric tissue. Glutamate, a candidate agonist of GPRC5B, markedly inhibited ghrelin secretion *in vitro*. Moreover, GPRC5B inhibited ghrelin secretion in primary cultured gastric mucosal cells. Glutamate did not influence the mRNA expression of GOAT and PC1. However, glutamate treatment suppressed preproghrelin expression. On the other hand, D-glutamate did not affect ghrelin secretion and transcriptional expression of preproghrelin mRNA.

Chapter 2

2.1. Introduction

G protein-coupled receptors (GPCRs) constitute the largest protein family of transmembrane receptors that act in response to molecules outside the cell and activate internal signal transduction pathways and, ultimately, cellular responses [74]. The common role of GPCRs is the transmission of signals to the cell interior through interactions with molecules that change the structure of the transmembrane domains and/or extracellular and intracellular regions [75]. GPCRs contain seven transmembrane segments (7TM) and function as sensors for neurotransmitters, hormones, and paracrine lipid messengers, nutrients, and metabolites [76]. Some GPCRs were previously determined to be expressed in ghrelin cells and play important roles in the regulation of ghrelin signaling [68]. Quantitative PCR analysis of FACS-purified gastric ghrelin cells identified a series of GPCRs that regulate ghrelin secretion [54]. GPCRs that stimulate ghrelin secretion were mainly G-coupled and include the β1-adrenergic receptor, the GIP receptor, secretin receptor (SCTR), sensory neuropeptide receptor CGRP, and melanocortin 4 receptor (MC4R) [54]. On the other hand, GPCRs that inhibit ghrelin release were determined to be Gq- and/or Gi-coupled and include somatostatin receptors (SSTRs), lactate receptor (GPR81), and receptors for short chain fatty acids (FFAR2) and long chain fatty acids (FFAR4) [54]. A recent study showed that GPR120 mRNA is expressed in ghrelin-producing SG-1 cells and that its ligands significantly inhibit ghrelin secretion. The ERK/MAPK pathway is also involved in GPR120-mediated inhibition of ghrelin secretion. [60]. Furthermore, a previous study showed that the FFAR2 and FFAR4 receptors inhibit ghrelin secretion through Gαi/o-mediated mechanisms [54].

GPRC5B belongs to an evolutionarily conserved subgroup of the C family of GPCRs that include GPRC5A, GPRC5B, GPRC5C, and GPRC5D, whose expression is induced by all-trans retinoic acid [62]. GPRC5B displays exhibits significant sequence similarity with metabotropic glutamate receptors [77]. However, the exact physiological roles and natural ligands of GPRC5B remain unknown. Moreover, GPRC5B is a glutamate receptor candidate that inhibits insulin secretion in MIN6 cells [78].

To confirm the involvement of GPRC5B in the regulation of ghrelin expression and secretion, we conducted GPRC5B small-interfering RNA (siRNA) knockdown and *in vivo* experiments in mice.

2.2. Materials and Methods

2.2.1. Cell lines

Pancreatic ghrelinoma cells (PG-1) were used in this experiment. PG-1 cells were cultured in DMEM/F12 50:50 containing 10% fetal bovine serum and supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in an incubator with 5% CO_2.

2.2.2. Chemicals

L-glutamate, L-cysteine, glycine, and norepinephrine (NE) were purchased from Sigma (St. Louis, MO, USA). Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Sodium octanoate was purchased from Wako (Osaka, Japan). GPRC5B small interfering RNA (siRNA) (sc-62410), control siRNA (sc-37007), and GPRC5B primers for RT-PCR (sc-62410-PR) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2.3. small-interfering RNA (siRNA) knockdown of GPRC5B

A pool of three small interfering RNAs (siRNAs; 19–25 nucleotides) targeting *Gprc5b* mRNA (Santa Cruz Biotechnology Inc.) was used. On day 0, PG-1 cells were plated at a concentration of 2×10^5 cells/well in 12-well plates with DMEM/F12 50:50 containing 10% fetal bovine serum and supplemented with 50 μM sodium octanoate. On day 1, cells were transfected with 50 nM siRNA and control siRNA using Lipofectamine™ 2000. After 24 h, the medium was discarded, and cells treated with vehicle or 5 mM L-glutamate for 3 h. Culture media were then processed as described above. Total RNA was extracted from the

cells and used for RT-PCR analysis. Image J software (NIH, Bethesda, MD, USA) was used to analyze RT-PCR images to compare gene expression between control and GPRC5B siRNA-transfected cells.

2.2.4. *In vivo* experiments

Male C57BL/6J wild-type (WT) mice (Nihonikagaku, Tokyo, Japan) (9-12 weeks old) were used for the experiments. Animals were housed individually in plastic cages and provided with *ad libitum* access to food and drinking water. The animal room was maintained at 21–24 °C on a 12-h light:dark cycle (lights were turned on from 0800 h to 2000 h). All procedures were approved and performed in accordance with the guidelines of the Saitama University Committee on Animal Research and the UTSW Medical Center Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiment.

Twelve mice were divided into four groups and starved overnight. Following overnight (16 h) starvation, mice were intraperitoneally (i.p) injected with saline (150 µL/mouse), L-glutamate, L-cysteine, or glycine diluted in saline at a dose of 2 mmol/kg body weight. Food intake was measured at 1 h post administration, and blood was collected from the tail vein to measure plasma acyl-ghrelin concentrations. Blood was collected in polypropylene tubes containing 0.5 M EDTA, pH 8.0 (Gibco), and 1 mM PHMB (Sigma) to prevent the degradation of acyl-ghrelin. Blood was centrifuged at 3500 rpm for 10 min at 4 °C, and the supernatant was transferred to a separate tube and added with 1 N HCl (10% v/v of total plasma). Samples were stored at -80 °C until analysis.

2.2.5. Measurement of ghrelin concentrations

Acyl-ghrelin concentrations were determined using a rat acylated ghrelin enzyme immunoassay kit (Bergin Pharma, SPI bio, France) according to the manufacturer's guidelines. Absorbance data were collected on a BIO-RAD iMAR^{KTM} Microplate Reader spectrophotometer using Microplate Manager® 6 Software (BIO-RAD).

2.2.6. Statistical analysis

All data were expressed as mean ± SEM, and statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Data were standardized as percentage values relative to the control group and analyzed using Student's *t*-test, one-way ANOVA with Tukey's, or Dunnett's post-hoc tests. Differences were considered statistically significant at $P < 0.05$.

2.3. Results

2.3.1. GPRC5B siRNA knockdown of ghrelin secretion in PG-1 cells

We examined the effect of GPRC5B siRNA knockdown on ghrelin secretion in PG-1 cells. Incubation with GPRC5B siRNA for 24 h decreased GPRC5B mRNA expression levels to approximately 50% of that in the control group (Fig. 9A, B). We also measured acyl-ghrelin secretion after siRNA knockdown of GPRC5B. The inhibitory effect of L-glutamate on ghrelin secretion was completely reversed in PG-1 cells treated with GPRC5B siRNA (Fig. 10).

2.3.2. Effect of glutamate on food intake and plasma acyl-ghrelin levels in mice

We next examined the regulatory role of GPRC5B in food intake and plasma acyl-ghrelin levels in mice. Intraperitoneal administration (i.p.) of L-glutamate significantly reduced food intake by about 30% (Fig. 11) compared to the control group. Moreover, administration of L-cysteine, which is known as an anorexigenic amino acid, significantly decreased food intake by about 42.3% (Fig. 11) compared to the control group. In addition, L-glutamate and L-cysteine administration significantly decreased plasma acyl-ghrelin levels by about 50% and 56.8%, respectively, compared to the control group (Fig. 12). However, glycine, which was administered to mice in the negative control group, did not affect food intake and plasma acyl-ghrelin levels in mice (Fig. 11 and 12).

2.3.3. Dose-dependent effect of glutamate on food intake and plasma acyl ghrelin levels in mice

We tested the dose-dependent regulatory effects of glutamate on food intake and plasma acyl ghrelin levels in mice. Three different doses of glutamate (0.5, 1, and 2 mmol/kg) were administered intraperitoneally in mice. At 1 h after glutamate administration, food intake was measured, and plasma samples were collected for measurement of plasma acyl ghrelin levels. Results showed that intraperitoneal administration of L-glutamate significantly reduced food intake (Fig. 13) in mice. Plasma acyl ghrelin concentrations were measured using an ELISA kit. Results showed that mice administered with 0.5, 1, and 2 mmol/kg L-glutamate significantly had significantly lower plasma acyl ghrelin concentrations than saline-treated mice (Fig. 14). Treatment with 1 mmol/kg L-glutamate resulted in stronger inhibition of acyl ghrelin secretion than treatment with 0.5 mmol/kg L-glutamate. In addition, mice treated with 2 mmol/kg L-glutamate showed lower acyl ghrelin levels than mice treated with 0.5 and 1 mmol/kg L-glutamate. Thus, these results suggest that glutamate inhibits plasma acyl ghrelin levels in a dose-dependent manner (Fig. 14).

2.4. Discussion

GPRC5B expression was significantly inhibited by GPRC5B siRNA treatment, and the inhibitory effect of glutamate on acyl ghrelin secretion was blocked by siRNA knockdown of GPRC5B. These results indicate that L-glutamate inhibition of ghrelin in gastric ghrelin cells is mediated via *GPRC5B*. A previous study showed that amino acids and glutamate are involved in the regulation of gastric motility, pancreatic secretion, and food intake [79]. Proteins and amino acids are also known to be more potent than carbohydrates and fats in inducing short-term satiety in humans [79]. In addition, another study reported that amino acids supplied by dietary proteins are important for regulation of food intake [67]. Moreover, protein- and amino acid-dependent mechanisms were shown to be involved in the control of food and protein intake [67]. Neuropeptide Y (NPY) and Agouti-related peptide (AgRP) play crucial roles in ghrelin-induced increase in food [80, 81]. Ghrelin directly stimulates the activity of NPY/AgRP neurons and stimulate the release of neuropeptides and neurotransmitters, thereby increasing food intake and decreasing energy expenditure [66]. Amino acids have also been reported to be involved controlling food intake in rats [82, 83]. Moreover, Cummings et al. reported that plasma ghrelin concentrations increase under conditions of food deprivation and decrease after food intake [38]. Taken together, these results indicate that amino acids are involved in the regulation of food intake, which is important in controlling ghrelin secretion.

GPRC5B is known to inhibit ghrelin secretion in ghrelin cell lines and primary gastric mucosal cells. Thus, we hypothesized that GPRC5B exerts physiological effects on appetite. As expected, our data demonstrated that L-glutamate decreased fasting-induced food intake in

mice. In addition, glutamate also significantly decreased fasting-induced increase in plasma acyl-ghrelin concentrations in mice. Recently, G-protein coupled receptors that are sensitive to amino acids were shown to be expressed in the gastrointestinal tract, indicating their possible involvement in regulating appetite [84]. Jordi et al. reported that glutamate is a potent anorexigenic amino acid and that glutamate administration inhibits food intake in rat [83]. Our present findings are almost consistent with the above mentioned results from previous studies.

A previous study demonstrated that ghrelin cells express FFAR2 and FFAR3 [54]. FFAR2 was shown to efficiently decrease basal ghrelin secretion in a dose-dependent manner in the primary gastric mucosal cells [54]. Propionate and acetate were also demonstrated to efficiently inhibit ghrelin secretion mainly through the FFAR2 receptor [54]. In addition, ghrelin secretion is inhibited by lactate and is mediated by the highly enriched GPR81 receptor [54]. Gong Zhi et al. [60] showed that mice treated with a GPR120 agonist showed decreased fasting-induced plasma acyl-ghrelin elevation *in vivo* compared to control mice, indicating that GPR120 is physiologically important for maintaining homeostasis of circulating ghrelin levels. Our data also showed that similar to GPR120, GPRC5B inhibited both food intake and plasma acyl ghrelin secretion in mice. In addition, we observed that glutamate suppressed food intake and plasma acyl ghrelin secretion in mice in a dose-dependent manner. Thus, our results indicate that glutamate directly regulates gastric ghrelin secretion and *GPRC5B* may be physiologically important for ghrelin regulation. However, glutamate concentration varies between 50–100 μM and plasma glutamate concentration was not affected after feeding [86]. Moreover, glutamatergic neurons have been found in cholinergic enteric neurons in the stomach of rats [87] and glutamatergic neurons were shown to be heterogeneously distributed in both myenteric ganglia and circular muscles in the

stomach [87]. Most of the glutamate-containing axons in the intestinal and stomach walls originate from cell bodies within the myenteric and submucous plexus [87]. Taken together with previous results, our study suggests that glutamate is produced by glutamatergic neurons in gastric tissue and acts as a natural ligand for *GPRC5B*. Thus, glutamate can bind to *GPRC5B* to suppress ghrelin secretion and food intake.

2.5. Summary

Transcriptional expression of *GPRC5B* was inhibited by *GPRC5B* small interfering RNA (siRNA) knockdown. The inhibitory effects of *GPRC5B* on ghrelin secretion were blocked by treatment with siRNA targeting *GPRC5B*. Glutamate significantly inhibited food intake and plasma acyl-ghrelin secretion in mice. Moreover, glutamate inhibited food intake and plasma acyl ghrelin secretion in a dose-dependent manner.

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Chapter 3

3.1. Introduction

Several signaling mechanisms of ghrelin has been reported [43, 50, 54, 85]. Zhao et al. showed that forskolin stimulated ghrelin secretion by activating adenylyl cyclase (AC) in PG-1 cells [43]. The release of intracellular calcium is known to mediate NE-stimulated ghrelin secretion in SG-1 cells [85]. Our results showed that glutamate inhibited ghrelin secretion in ghrelin cell lines and primary gastric mucosal cells. Moreover, glutamate treatment blocked NE-induced ghrelin secretion in primary cells. Gong zhi et al. [60] reported that GPR120 inhibits ghrelin secretion via the ERK pathway and participates in a cross-talk with NE-induced ghrelin secretion. Thus, we first investigated the association between GPRC5B-induced ghrelin suppression and the ERK pathway.

The mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathways transmit signals from the cell surface to the transcriptional factors in the nucleus for regulation of gene expression. This pathway is composed of MAPK kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK, also referred to as ERKs [86]. The RAF/MEK/ERK, JNK, and p38 MAPK pathways are known as the three major MAPK/ERK pathways [86]. Moreover, the MAPK/ERK pathways are not directly coupled with the receptors on the cell surface. In fact, these pathways require effector molecules to transmit the signals from the receptors. These effector molecules are called small guanine nucleotide-binding proteins or G proteins. The activated form of a G protein is coupled with GTP, while

the inactive form is coupled with GDP [86]. The MAPK pathways have been demonstrated to participate in crosstalk with several other signaling pathways such as the PKA pathways [86].

The ERK/MAPK pathways are involved in numerous cellular events. A previous study showed that the ERK pathway is involved in regulating the expression of transcription factors and also regulating gene expression patterns that promote cell growth, differentiation, or mitosis [87]. Moreover, the ERK pathway is known to regulate adipogenesis in human adipose tissue-derived mesenchymal stem cells [88] and cell proliferation in mammalian cells [87]. Pierce KL et al. revealed that GPCRs activate numerous complex cascades to activate MAPKs [89]. The group also reported that stimulation of GPCRs leads to tyrosine phosphorylation of RTKs, such as the EGFR, which ultimately results in ERK activation [89].

cAMP response element-binding protein (CREB) is a cellular transcription factor that binds to certain DNA sequences to increase or decrease the transcription of downstream genes [90]. cAMP response element binding protein 1 (CREB1) is activated via the activation of the protein kinase A (PKA) intracellular signaling pathways, and cAMP response element binding protein 2 (CREB2) acts as a transcriptional repressor of mitogen-activated protein kinase (MAPK) [90]. Bartsch D et al. found that CREB2, also known as activating transcription factor 4 (ATF4), functions as a repressor of long-term memory storage [91]. Microarray data revealed that two subtypes of CREB (CREB1 and CREB2) are expressed in ghrelin cells and that CREB acts as a downstream regulator of the ERK pathway. Thus, we next focused on the role of CREB in glutamate inhibition of ghrelin secretion in ghrelin cells.

In this chapter, we studied the signal transduction mechanisms that mediate glutamate-induced inhibition of ghrelin secretion using ghrelin cells.

3.2. Materials and Methods

3.2.1. Cell lines

Two types of ghrelin cells were used in this experiment, namely, stomach ghrelinoma cells (SG-1), which originate from the stomach, and pancreatic ghrelinoma cells (PG-1), which are derived from the pancreas. SG-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 50:50 (Cellgro, Manassas, VA, USA) containing 10% fetal bovine serum (FBS) and supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C with 5% CO_2 and 5% O_2. On the other hand, PG-1 cells were cultured in DMEM/F12 50:50 containing 10% fetal bovine serum supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) in an incubator at 37 °C with 5% CO_2.

3.2.2. Chemicals

L-glutamate, Norepinephrine (NE), H-89, PD-98059, and wortmannin were purchased from Sigma (St. Louis, MO, USA). Sodium octanoate was purchased from Wako (Osaka, Japan). Primary anti-mouse phosphor-p44/42 MAPK (Erk1/2) (Thr202/Try204) was purchased from cell signaling (#9101, Danvers, MA, USA) and HRP-labeled secondary antibody was purchased from promega (Madison, WI, USA).

3.2.3. Ghrelin secretion experiment

On day 0, PG-1 cells were plated at 1×10^6 cells/well in 12-well plates with DMEM/F12 50:50 containing 10% fetal bovine serum and supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) containing 50 μM sodium octanoate. On day 1, the

medium was removed, and wells were added with 400 μL of serum-free DMEM (GIBCO, Tokyo, Japan) containing 2.5 mM D-glucose and 50 μM sodium octanoate with chemicals. PD98059 (ERK inhibitor), H89 (PKA inhibitor) and Wortmannin (PI3K inhibitor) were added and the plate was incubated at 37°C for 1 h. L-glutamate was then added and the cells were incubated at 37°C for 3 h. After incubation at 37 °C for 3 h, the medium was collected and centrifuged at 3000 rpm for 5 min at 4 °C. Next, 100 μL of the supernatant was transferred to a separate tube, to which 10 μL of 1 N HCl was added immediately. Samples were stored at -80 °C until analysis.

Primary cultured gastric mucosal cells were seeded at a concentration of 1×10^5 cells/well in 24-well plates. Ghrelin secretion experiments were performed the following day. Media were aspirated before the addition of 200 μL of serum-free 2.5 mM D-glucose/DMEM (Gibco) containing 50 μM sodium octanoate with glutamate. After incubation at 37 °C for 3 h, culture media were collected and centrifuged at 3000 rpm for 5 min at 4 °C. Afterwards, 100 μL of supernatant was transferred to a separate tube, to which 10 μL of 1 N HCl was immediately added. Samples were stored at -80 °C until analysis.

3.2.4. Western blotting

PG-1 cells were washed twice with cold PBS and added with 0.2 mL of RIPA buffer containing 1× phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Samples were placed on ice for 30 min, and the resulting mixture was centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was collected, and protein concentration was measured using BCATM Protein Assay kit (Thermo Fisher, Waltham, MA). For each sample, equal amounts of protein

(10 μg) were run on 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), and the resolved proteins were transferred onto PVDF membranes. Immunoblotting was performed by incubating the membranes with primary anti-mouse phosphorylated p44/42 MAPK (ERK1/2) (Thr^{202}/Tyr^{204}) (no. 9101; Cell Signaling Technology, Danvers, MA) diluted to 1:200 and primary anti-mouse p44/42 MAPK (Erk1/2) (no. 9102; Cell Signaling Technology) diluted to 1:200, followed by probing with horseradish peroxidase (HRP)-labeled secondary antibody (Promega, Madison, WI) diluted to 1:50,000. Luminata Forta Western HRP substrate (Millipore, Billerica, MA) was used to detect specific antigen-antibody binding. Images were analyzed using Image J software (National Institutes of Health, Bethesda, MD). The same experimental protocol was performed using SG-1 cells.

3.2.5. Quantitative RT-PCR

On day 0, PG-1 cells were plated in 12-well plates at 1×10^6 cells/well in DMEM/F12 50:50 containing 10% fetal bovine serum supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) containing 50 μM sodium octanoate. Cells were then treated with 1 mM glutamate for 3 h. The medium was aspirated, and RNA was extracted using ISOGEN (Nippon gene, Tokyo, Japan). A total of 1 μg of RNA was used for cDNA synthesis using Superscript® III Reverse Transcriptase (Invitrogen). Real-time PCR was performed on a LightCycler (Roche Diagnostics) instrument using SYBR premix Ex Taq (Takara Bio, Shiga, Japan) using the following primers: mouse cAMP responsive element-binding protein1 (CREB1) (fragment size: 250 bp): forward, 5′-CTGATTCCCAAAAACGAAGG-3′ and reverse, 5′-TGTACCCCATCCGTACCATT-3′; mouse cAMP responsive element-binding protein2 (CREB2) (fragment size: 292 bp): forward, 5′- GAAACCTCATGGGTTCTCCA -3′

and reverse, 5′- GCCAATTGGGTTCACTGTCT -3′; and mouse GAPDH: forward, 5′-TGTGTCCGTCGTGGATCTGA-3′ and reverse, 5′-CCTGCTTCACCACCTTCTTGA-3′. PCR amplification was performed under the following cycling conditions: initial template denaturation for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. mRNA levels were expressed relative to GAPDH (internal control). The same RT-PCR protocol was performed on SG-1 cells.

3.2.6. Measurement of ghrelin concentration

Acyl-ghrelin concentrations were determined using a rat acylated ghrelin enzyme immunoassay kit (Bergin Pharma, SPI bio, France) according to the manufacturer's guidelines. Absorbance data were collected on a BIO-RAD iMAR^{KTM} Microplate Reader spectrophotometer using Microplate Manager® 6 Software (BIO-RAD).

3.2.7. Statistical analysis

All data were expressed as mean ± SEM, and statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Data were standardized as percentage values relative to the control group and analyzed using Student's *t*-test, one-way ANOVA with Tukey's, or Dunnett's post-hoc tests. Differences were considered statistically significant at $P < 0.05$.

3.3. Results

3.3.1. Intracellular signaling in ghrelin secretion

To elucidate the GPRC5B signaling pathway mechanisms underlying ghrelin secretion, PG-1 cell cultures were treated with three intracellular signaling inhibitors, namely, H89, PD98059 (ERK inhibitor), and wortmannin (AKT inhibitor). As expected, results showed that glutamate treatment inhibited ghrelin secretion. Mice co-administered with glutamate and H89 showed ghrelin levels similar to those in the glutamate treated-group (Fig. 15). Similarly, co-administration of glutamate with wortmannin did not reverse glutamate-mediated inhibition of acyl-ghrelin secretion (Fig. 15). However, co-administration of glutamate with PD98059 significantly reversed glutamate-induced acyl-ghrelin inhibition (Fig. 15). Norepinephrine (NE) was used to treat the positive control group.

3.3.2. Role of glutamate in regulating phosphorylated ERK levels in PG-1 cells and SG-1 cells

To verify the effect of glutamate on phosphorylation levels of ERK, PG-1 cells were treated with glutamate, and levels of phosphorylated ERK were determined by western blotting following the same protocol used in SG-1 cells. We observed that PG-1 cells treated with glutamate had higher levels of phosphorylated ERK compared to the control (Fig. 16A) and significantly higher p-REK/ERK ratios (Fig. 16B). Glutamate-treated SG-1 cells also showed significantly higher levels of phosphorylated ERK and higher p-REK/ERK ratio (Fig. 17A, B).

3.3.3. Effect of glutamate on mRNA expression of CREB1 and CREB2 in PG-1 cells and SG-1 cells

To determine the roles of CREB1 and CREB2, PG-1 cells were treated with glutamate and subjected to mRNA expression analysis using quantitative PCR. Glutamate-treated PG-1 cells showed no significant changes in CREB1 mRNA levels, but showed significant CREB2 upregulation after 3 h of incubation (Fig. 18A, B). SG-1 cells also showed similar expression patterns to those of PG-1 cells (Fig. 19A, B).

3.3.4. Effect of GPRC5B signaling on NE-induced ghrelin elevation

To test the effect of GPRC5B signaling on NE-induced ghrelin elevation, primary cultured gastric mucosal cells were pretreated with glutamate for 1 h before NE stimulation. NE (10 μM) significantly increased acyl-ghrelin secretion, and co-administration of glutamate and NE significantly reversed NE-induced acyl-ghrelin secretion (Fig. 20).

3.4. Discussion

3.4.1. GPRC5B signaling mechanisms involved in ghrelin secretion

We focused on the relationship between the ERK pathway and GPRC5B-mediated suppression of ghrelin secretion and found that ERK pathway inhibitors significantly reversed GPRC5B-inhibited ghrelin secretion. In addition, results showed that glutamate significantly increased the levels of phosphorylated ERK. Treatment with either a PKA-pathway inhibitor or an AKT pathway inhibitor did not reverse GPRC5B-mediated inhibition of ghrelin secretion. These results suggest that GPRC5B signaling for ghrelin secretion is mediated by the MAPK/ERK pathway, which is known to stimulate cell biological activities, such as cell proliferation, differentiation, migration, and hormone release [92-95]. By contrast, the MAPK/ERK pathway is involved in GPR120-mediated inhibition of acyl ghrelin secretion [60], consistent with the results obtained from the present study. We also found that GPRC5B inhibits ghrelin secretion is mediated by the ERK pathway in gastric ghrelin cells, indicating that the MAPK/ERK pathway also exerts inhibitory roles to regulate cell function. These results suggest that like GPR120, GPRC5B inhibits acyl ghrelin secretion through the ERK pathway in both SG-1 and PG-1 cells.

3.4.2. Crosstalk between GPRC5B and adrenergic receptor signaling pathways in regulating ghrelin secretion

In this study, NE-induced ghrelin secretion was blocked by glutamate, suggesting a crosstalk between the GPRC5B and NE signaling pathways. The PKA and MAPK pathways

do not act independently of each other; however, there are multiple cross-talk events between two pathways [94]. cAMP can influence ERK signaling transduction through the cAMP-dependent protein kinase (PKA) [86]. Several GPCRs that are expressed in ghrelin cells can interact with each other to regulate ghrelin secretion. β1 adrenergic receptor has been reported to stimulate ghrelin secretion [43]. Our results showed that glutamate significantly blocks the NE-induced ghrelin secretion in primary cultured gastric mucosal cells, suggesting crosstalk between the NE and GPRC5B signaling pathways in regulating ghrelin secretion. However, the exact molecular mechanisms remain unclear. Thus, we investigated whether NE inhibits ERK activity by acting on PKA and involved in the effect of GPRC5B on ghrelin secretion.

3.4.3. The role of CREB2 in GPRC5B-mediated inhibition of ghrelin secretion

CREB is a member of the basic region leucine zipper (bZIP) family of proteins and is the major cAMP response element (CRE)-binding protein that mediates diverse transcriptional regulatory effects [96]. CREB is a crucial regulator of DNA-binding proteins (transcription factors) that interact with specific DNA binding elements in gene promoter regions, thereby activating or repressing their transcription [97]. CREB has two subtypes, namely, cAMP response element-binding protein 1 (CREB1) and cAMP response element-binding protein 2 (CREB2). CREB acts as a downstream regulator of the ERK pathway and plays important role in transcription factor regulation [98]. We measured the mRNA levels of CREB1 and CREB2 after glutamate treatment in both SG-1 and PG-1 cells and found that glutamate increases CREB2 expression but does not affect CREB1 expression in ghrelin cells. Hao et al. reported

that CREB1 is activated via the activation of the protein kinase A (PKA) intracellular signaling pathways and that CREB2 acts as a transcriptional repressor by inducing the phosphorylation of CREB2 by mitogen-activated protein kinase (MAPK) [90]. Taken together with our results, GPRC5B is potentially involved in stimulating CREB2 expression and CREB2 upregulation can inhibit the transcription of ghrelin, which results in the inhibition of ghrelin expression and secretion in ghrelin cells.

3.5. Summary

Glutamate inhibits ghrelin secretion in ghrelin cells by regulating extracellular signal-regulated kinase (ERK) activity. Glutamate significantly increased mRNA expression of CREB2. Moreover, pretreatment with glutamate blocked the effect of NE-induced ghrelin elevation in primary cultured gastric mucosal cells.

4.0. Conclusion

In conclusion, our findings indicate that GPRC5B is highly expressed in ghrelin cells and gastric tissue. GPRC5B was shown to be involved in the regulation of ghrelin expression and secretion in ghrelin cells. L-glutamate, but not D-glutamate, is involved in the regulation of ghrelin expression in ghrelin cells. In addition, the inhibitory effect of glutamate on ghrelin secretion was blocked by small interfering RNA (siRNA) knockdown of GPRC5B. Moreover, glutamate treatment decreased both food intake and plasma ghrelin levels *in vivo*. We also found that glutamate inhibited ghrelin secretion via the ERK pathway. Furthermore, CREB2, a downstream regulator of the ERK pathway, is involved in GPRC5B-mediated inhibition of ghrelin secretion. The above results suggest that GPRC5B signaling is a critical regulator of plasma ghrelin expression and secretion and that glutamate may directly regulate ghrelin secretion in ghrelin cells via GPRC5B.

However, the natural ligand of GPRC5B remains to be identified, although Soni et al. recently showed that GPRC5B exhibits the characteristics of a novel putative glutamate receptor [66]. Indeed, we observed that glutamate inhibits gastric ghrelin secretion and expression *in vitro*. Glutamate administration decreased plasma ghrelin concentrations in mice, suggesting that glutamate directly regulates gastric ghrelin. Based on the current findings, it is tempting to propose that L-glutamate is a natural ligand of GPRC5B and that GPRC5B is a novel L-glutamate receptor that inhibits ghrelin secretion. Therefore, further studies are needed to identify the natural GPRC5B ligand, and detailed morphological examination of glutamatergic neurons within the gastric mucosal layer is warranted to determine the physiological role of GPRC5B in ghrelin signaling.

ABBREVIATIONS

AC, Adenylate cyclase;

AgRP, Agouti-related peptide

AKT, phosphorylated serine-threonine kinase;

ANOVA, Analysis of variance;

ARC, Arcuate Nucleus

cAMP, Adenosine 3′,5′ cyclic monophosphate;

CCK, cholecystokinin;

CGRP receptor, neuropeptide receptor;

CO_2, Carbon dioxide;

CREB, CAMP Responsive Element Binding Protein;

CREB1, CAMP Responsive Element Binding Protein 1;

CREB2, CAMP Responsive Element Binding Protein 2;

DMEM, Dulbecco's Modification of Eagle's Medium;

EDTA, Ethylenediaminetetra-acetic acid;

EGFR, epidermal growth factor receptor;

ELISA, Enzyme-linked immunosorbent assay;

ERK, extracellular signal-regulated kinase;

FACS, Fluorescence-activated cell sorting;

FBS, fetal bovine serum;

FFAR1, Free Fatty Acid Receptor 1;

FFAR2, Free Fatty Acid Receptor 2;

FFAR3, Free Fatty Acid Receptor 3;

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase;

G-proteins, small guanine nucleotide-binding proteins;

GDP, guanosine diphosphate;

GHS-R, growth hormone secretagogue receptor;

GIP receptor, gastric inhibitory polypeptide receptor;

GLP-1, Glucagon-like peptide-1;

GOAT, ghrelin-O-acyl transferase;

GPCR, G-protein coupled receptor;

GPR81, G protein-coupled receptor 81;

GPR120, G-protein coupled receptor 120;

GPRC5A, G Protein-Coupled Receptor Class C Group 5 Member A;

GPRC5B, G protein coupled receptor, family C, group 5, member B;

GPRC5C, G Protein-Coupled Receptor Class C Group 5 Member C;

GPRC5D, G Protein-Coupled Receptor Class C Group 5 Member D;

GTP, Guanosine-5'-triphosphate;

HCL, Hydrochloric acid;

MAPK, mitogen-activatedprotein kinase;

MAPKK, MAPK kinase;

MAPKKK,MAPK kinase kinase;

MC4R, melanocortin 4 receptor;

mRNA, Messenger RNA ;

NE, norepinephrine;

NPY, Neuropeptide Y

O_2, Oxygen;

PBS, phosphate buffered saline;

PC1, prohormone convertase 1;

PC2, prohormone convertase 2;

PCR, Polymerase chain reaction;

p-ERK, phosphorylated extracellular signal-regulated kinase;

PG-1 cells, pancreatic ghrelinoma cells;

PHMB, p-hydroxymercuribenzoic acid;

PKA, protein kinase A;

PVDF, polyvinylidene difluoride

PYY, peptide YY;

RIPA buffer, radio immunoprecipitation assay buffer;

RT-PCR, Reverse transcription polymerase chain reaction;

RTK, Receptor tyrosine kinases;

SCTR, secretin receptor;

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gels;

SEM, Standard error of the mean;

SG-1 cells, stomach ghrelinoma cells;

siRNA, small interfering Ribonucleic Acid;

SSTR, Somatostatin receptor;

7TM, 7-Transmembrane receptor;

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REFERENCES

1. Kojima, M., et al., *Ghrelin is a growth-hormone-releasing acylated peptide from stomach.* Nature, 1999. **402**(6762): p. 656-60.
2. Kojima, M. and K. Kangawa, *Ghrelin discovery: a decade after.* Endocr Dev, 2013. **25**: p. 1-4.
3. Jeon, T.Y., et al., *Changes in plasma ghrelin concentration immediately after gastrectomy in patients with early gastric cancer.* J Clin Endocrinol Metab, 2004. **89**(11): p. 5392-6.
4. Seoane, L.M., et al., *Ghrelin elicits a marked stimulatory effect on GH secretion in freely-moving rats.* Eur J Endocrinol, 2000. **143**(5): p. R7-9.
5. Tolle, V., et al., *In vivo and in vitro effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat.* Neuroendocrinology, 2001. **73**(1): p. 54-61.
6. Sakata, I. and T. Sakai, *Ghrelin cells in the gastrointestinal tract.* Int J Pept, 2010. **2010**.
7. Nakazato, M., et al., *A role for ghrelin in the central regulation of feeding.* Nature, 2001. **409**(6817): p. 194-8.
8. Wren, A.M., et al., *Ghrelin enhances appetite and increases food intake in humans.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5992.
9. Wren, A.M., et al., *Ghrelin causes hyperphagia and obesity in rats.* Diabetes, 2001. **50**(11): p. 2540-7.
10. Wang, Q., et al., *Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin.* Mol Metab, 2014. **3**(1): p. 64-72.
11. Gil-Campos, M., et al., *Ghrelin: a hormone regulating food intake and energy homeostasis.* Br J Nutr, 2006. **96**(2): p. 201-26.
12. Murakami, N., et al., *Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats.* J Endocrinol, 2002. **174**(2): p. 283-8.

13. Tschop, M., D.L. Smiley, and M.L. Heiman, *Ghrelin induces adiposity in rodents.* Nature, 2000. **407**(6806): p. 908-13.

14. Iwakura, H., K. Kangawa, and K. Nakao, *The regulation of circulating ghrelin - with recent updates from cell-based assays.* Endocr J, 2015. **62**(2): p. 107-22.

15. Masuda, Y., et al., *Ghrelin stimulates gastric acid secretion and motility in rats.* Biochem Biophys Res Commun, 2000. **276**(3): p. 905-8.

16. Fukumoto, K., et al., *Synergistic action of gastrin and ghrelin on gastric acid secretion in rats.* Biochem Biophys Res Commun, 2008. **374**(1): p. 60-3.

17. Yakabi, K., J. Kawashima, and S. Kato, *Ghrelin and gastric acid secretion.* World J Gastroenterol, 2008. **14**(41): p. 6334-8.

18. Zheng, J., et al., *Ghrelin regulates gastric phase III-like contractions in freely moving conscious mice.* Neurogastroenterol Motil, 2009. **21**(1): p. 78-84.

19. Nakamura, T., T. Onaga, and T. Kitazawa, *Ghrelin stimulates gastric motility of the guinea pig through activation of a capsaicin-sensitive neural pathway: in vivo and in vitro functional studies.* Neurogastroenterol Motil, 2010. **22**(4): p. 446-52, e107.

20. Perboni, S. and A. Inui, *Appetite and gastrointestinal motility: role of ghrelin-family peptides.* Clin Nutr, 2010. **29**(2): p. 227-34.

21. Okumura, H., et al., *Vasodilatory effect of ghrelin, an endogenous peptide from the stomach.* J Cardiovasc Pharmacol, 2002. **39**(6): p. 779-83.

22. Duxbury, M.S., et al., *Ghrelin promotes pancreatic adenocarcinoma cellular proliferation and invasiveness.* Biochem Biophys Res Commun, 2003. **309**(2): p. 464-8.

23. Maccarinelli, G., et al., *Ghrelin regulates proliferation and differentiation of osteoblastic cells.* J Endocrinol, 2005. **184**(1): p. 249-56.

24. van der Lely, A.J., et al., *Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin.* Endocr Rev, 2004. **25**(3): p. 426-57.

25. Klok, M.D., S. Jakobsdottir, and M.L. Drent, *The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review.* Obes Rev, 2007. **8**(1): p. 21-34.

26. Camina, J.P., et al., *Regulation of ghrelin secretion and action.* Endocrine, 2003. **22**(1): p. 5-12.

27. Sakata, I., et al., *Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract.* Peptides, 2002. **23**(3): p. 531-6.

28. Kojima, M., A. Hamamoto, and T. Sato, *Ghrelin O-acyltransferase (GOAT), a specific enzyme that modifies ghrelin with a medium-chain fatty acid.* J Biochem, 2016. **160**(4): p. 189-194.

29. Zhao, Z. and T. Sakai, *Characteristic features of ghrelin cells in the gastrointestinal tract and the regulation of stomach ghrelin expression and production.* World J Gastroenterol, 2008. **14**(41): p. 6306-11.

30. Kanamoto, N., et al., *Genomic structure and characterization of the 5'-flanking region of the human ghrelin gene.* Endocrinology, 2004. **145**(9): p. 4144-53.

31. Seim, I., et al., *Revised genomic structure of the human ghrelin gene and identification of novel exons, alternative splice variants and natural antisense transcripts.* BMC Genomics, 2007. **8**: p. 298.

32. Nakai, N., et al., *Identification of promoter region of ghrelin gene in human medullary thyroid carcinoma cell line.* Life Sci, 2004. **75**(18): p. 2193-201.

33. Korbonits, M., et al., *Ghrelin--a hormone with multiple functions.* Front Neuroendocrinol, 2004. **25**(1): p. 27-68.

34. Pemberton, C., et al., *C-terminal pro-ghrelin peptides are present in the human circulation.* Biochem Biophys Res Commun, 2003. **310**(2): p. 567-73.

35. Bang, A.S., et al., *Characterisation of proghrelin peptides in mammalian tissue and plasma.* J Endocrinol, 2007. **192**(2): p. 313-23.

36. Delporte, C., *Structure and physiological actions of ghrelin.* Scientifica (Cairo), 2013. **2013**: p. 518909.

37. Wellman, M.K., et al., *Novel Regulator of Acylated Ghrelin, CF801, Reduces Weight Gain, Rebound Feeding after a Fast, and Adiposity in Mice.* Front Endocrinol (Lausanne), 2015. **6**: p. 144.

38. Cummings, D.E., et al., *A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans.* Diabetes, 2001. **50**(8): p. 1714-9.

39. Tschop, M., et al., *Post-prandial decrease of circulating human ghrelin levels.* J Endocrinol Invest, 2001. **24**(6): p. RC19-21.

40. Gualillo, O., et al., *Effect of food restriction on ghrelin in normal-cycling female rats and in pregnancy.* Obes Res, 2002. **10**(7): p. 682-7.

41. Sakata, I., et al., *Glucose-mediated control of ghrelin release from primary cultures of gastric mucosal cells.* Am J Physiol Endocrinol Metab, 2012. **302**(10): p. E1300-10.

42. Shiiya, T., et al., *Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion.* J Clin Endocrinol Metab, 2002. **87**(1): p. 240-4.

43. Zhao, T.J., et al., *Ghrelin secretion stimulated by {beta}1-adrenergic receptors in cultured ghrelinoma cells and in fasted mice.* Proc Natl Acad Sci U S A, 2010. **107**(36): p. 15868-73.

44. Sakata, I., et al., *Gastric estrogen directly induces ghrelin expression and production in the rat stomach.* J Endocrinol, 2006. **190**(3): p. 749-57.

45. Barkan, A.L., et al., *Ghrelin secretion in humans is sexually dimorphic, suppressed by somatostatin, and not affected by the ambient growth hormone levels.* J Clin Endocrinol Metab, 2003. **88**(5): p. 2180-4.

46. Shimada, M., et al., *Somatostatin suppresses ghrelin secretion from the rat stomach.* Biochem Biophys Res Commun, 2003. **302**(3): p. 520-5.

47. Silva, A.P., et al., *Regulation of ghrelin secretion by somatostatin analogs in rats.* Eur J Endocrinol, 2005. **152**(6): p. 887-94.

48. Zhao, Z., et al., *Gastric leptin, but not estrogen and somatostatin, contributes to the elevation of ghrelin mRNA expression level in fasted rats.* J Endocrinol, 2008. **196**(3): p. 529-38.

49. de la Cour, C.D., P. Norlen, and R. Hakanson, *Secretion of ghrelin from rat stomach ghrelin cells in response to local microinfusion of candidate messenger compounds: a microdialysis study.* Regul Pept, 2007. **143**(1-3): p. 118-26.

50. Gagnon, J. and Y. Anini, *Insulin and norepinephrine regulate ghrelin secretion from a rat primary stomach cell culture.* Endocrinology, 2012. **153**(8): p. 3646-56.

51. Kamegai, J., et al., *Effects of insulin, leptin, and glucagon on ghrelin secretion from isolated perfused rat stomach.* Regul Pept, 2004. **119**(1-2): p. 77-81.

52. Lippl, F., et al., *Direct effect of leptin on gastric ghrelin secretion.* Horm Metab Res, 2005. **37**(2): p. 123-5.

53. Chuang, J.C., et al., *Ghrelin directly stimulates glucagon secretion from pancreatic alpha-cells.* Mol Endocrinol, 2011. **25**(9): p. 1600-11.

54. Engelstoft, M.S., et al., *Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells.* Mol Metab, 2013. **2**(4): p. 376-92.

55. Gagnon, J. and Y. Anini, *Glucagon stimulates ghrelin secretion through the activation of MAPK and EPAC and potentiates the effect of norepinephrine.* Endocrinology, 2013. **154**(2): p. 666-74.

56. Overduin, J., et al., *Role of the duodenum and macronutrient type in ghrelin regulation.* Endocrinology, 2005. **146**(2): p. 845-50.

57. Gormsen, L.C., et al., *Free fatty acids decrease circulating ghrelin concentrations in humans.* Eur J Endocrinol, 2006. **154**(5): p. 667-73.

58. Lu, X., et al., *Postprandial inhibition of gastric ghrelin secretion by long-chain fatty acid through GPR120 in isolated gastric ghrelin cells and mice.* Am J Physiol Gastrointest Liver Physiol, 2012. **303**(3): p. G367-76.

59. Janssen, S., et al., *Sensing of fatty acids for octanoylation of ghrelin involves a gustatory G-protein.* PLoS One, 2012. **7**(6): p. e40168.

60. Gong, Z., et al., *G protein-coupled receptor 120 signaling regulates ghrelin secretion in vivo and in vitro.* Am J Physiol Endocrinol Metab, 2014. **306**(1): p. E28-35.

61. Chung, H.J., et al., *G protein-coupled receptor, family C, group 5 (GPRC5B) downregulation in spinal cord neurons is involved in neuropathic pain.* Korean J Anesthesiol, 2014. **66**(3): p. 230-6.

62. Soni, A., et al., *GPRC5B a putative glutamate-receptor candidate is negative modulator of insulin secretion.* Biochem Biophys Res Commun, 2013.

63. Speliotes, E.K., et al., *Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index.* Nat Genet, 2010. **42**(11): p. 937-48.

64. Kim, Y.J., et al., *GPRC5B activates obesity-associated inflammatory signaling in adipocytes.* Sci Signal, 2012. **5**(251): p. ra85.

65. Walker, A.K., et al., *Expression of Serum Retinol Binding Protein and Transthyretin within Mouse Gastric Ghrelin Cells.* PLoS One, 2013. **8**(6): p. e64882.

66. Cowley, M.A., et al., *The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis.* Neuron, 2003. **37**(4): p. 649-61.

67. Sugino, T., et al., *A transient surge of ghrelin secretion before feeding is modified by different feeding regimens in sheep.* Biochem Biophys Res Commun, 2002. **298**(5): p. 785-8.

68. Muller, T.D., et al., *Ghrelin.* Mol Metab, 2015. **4**(6): p. 437-60.

69. Fromentin, G., et al., *Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins.* Nutr Res Rev, 2012. **25**(1): p. 29-39.

70. Foster-Schubert, K.E., et al., *Acyl and total ghrelin are suppressed strongly by ingested proteins, weakly by lipids, and biphasically by carbohydrates.* J Clin Endocrinol Metab, 2008. **93**(5): p. 1971-9.

71. Seim, I., et al., *Ghrelin gene-related peptides: multifunctional endocrine / autocrine modulators in health and disease.* Clin Exp Pharmacol Physiol, 2010. **37**(1): p. 125-31.

72. Zhu, X., et al., *On the processing of proghrelin to ghrelin.* J Biol Chem, 2006. **281**(50): p. 38867-70.

73. Man, E.H. and J.L. Bada, *Dietary D-amino acids.* Annu Rev Nutr, 1987. **7**: p. 209-25.

74. Amisten, S., et al., *An atlas and functional analysis of G-protein coupled receptors in human islets of Langerhans.* Pharmacol Ther, 2013. **139**(3): p. 359-91.

75. Trzaskowski, B., et al., *Action of molecular switches in GPCRs--theoretical and experimental studies.* Curr Med Chem, 2012. **19**(8): p. 1090-109.

76. Engelstoft, M.S., et al., *A gut feeling for obesity: 7TM sensors on enteroendocrine cells.* Cell Metab, 2008. **8**(6): p. 447-9.

77. Joost, P. and A. Methner, *Phylogenetic analysis of 277 human G-protein-coupled receptors as a tool for the prediction of orphan receptor ligands.* Genome Biol, 2002. **3**(11): p. RESEARCH0063.

78. Soni, A., et al., *GPRC5B a putative glutamate-receptor candidate is negative modulator of insulin secretion.* Biochem Biophys Res Commun, 2013. **441**(3): p. 643-8.

79. Tome, D., et al., *Protein, amino acids, vagus nerve signaling, and the brain.* Am J Clin Nutr, 2009. **90**(3): p. 838S-843S.

80. Date, Y., et al., *The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats.* Gastroenterology, 2002. **123**(4): p. 1120-8.

81. Chen, H.Y., et al., *Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein.* Endocrinology, 2004. **145**(6): p. 2607-12.

82. Tome, D., *Protein, amino acids and the control of food intake.* Br J Nutr, 2004. **92 Suppl 1**: p. S27-30.

83. Jordi, J., et al., *Specific amino acids inhibit food intake via the area postrema or vagal afferents.* J Physiol, 2013. **591**(22): p. 5611-21.

84. Wellendorph, P., L.D. Johansen, and H. Brauner-Osborne, *Molecular pharmacology of promiscuous seven transmembrane receptors sensing organic nutrients.* Mol Pharmacol, 2009. **76**(3): p. 453-65.

85. Mani, B.K., et al., *Role of calcium and EPAC in norepinephrine-induced ghrelin secretion.* Endocrinology, 2014. **155**(1): p. 98-107.

86. Stork, P.J. and J.M. Schmitt, *Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation.* Trends Cell Biol, 2002. **12**(6): p. 258-66.

87. Zhang, W. and H.T. Liu, *MAPK signal pathways in the regulation of cell proliferation in mammalian cells.* Cell Res, 2002. **12**(1): p. 9-18.

88. Lim, S., et al., *Wedelolactone inhibits adipogenesis through the ERK pathway in human adipose tissue-derived mesenchymal stem cells.* J Cell Biochem, 2012. **113**(11): p. 3436-45.

89. Pierce, K.L., L.M. Luttrell, and R.J. Lefkowitz, *New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades.* Oncogene, 2001. **20**(13): p. 1532-9.

90. Hao, L., Z. Yang, and Y. Bi, *Stochasticity and bifurcations in a reduced model with interlinked positive and negative feedback loops of CREB1 and CREB2 stimulated by 5-HT.* Math Biosci, 2016. **274**: p. 73-82.

91. Bartsch, D., et al., *Aplysia CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change.* Cell, 1995. **83**(6): p. 979-92.

92. Chang, L. and M. Karin, *Mammalian MAP kinase signalling cascades.* Nature, 2001. **410**(6824): p. 37-40.

93. Geest, C.R. and P.J. Coffer, *MAPK signaling pathways in the regulation of hematopoiesis.* J Leukoc Biol, 2009. **86**(2): p. 237-50.

94. Gerits, N., et al., *Relations between the mitogen-activated protein kinase and the cAMP-dependent protein kinase pathways: comradeship and hostility.* Cell Signal, 2008. **20**(9): p. 1592-607.

95. Hirasawa, A., et al., *Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120.* Nat Med, 2005. **11**(1): p. 90-4.

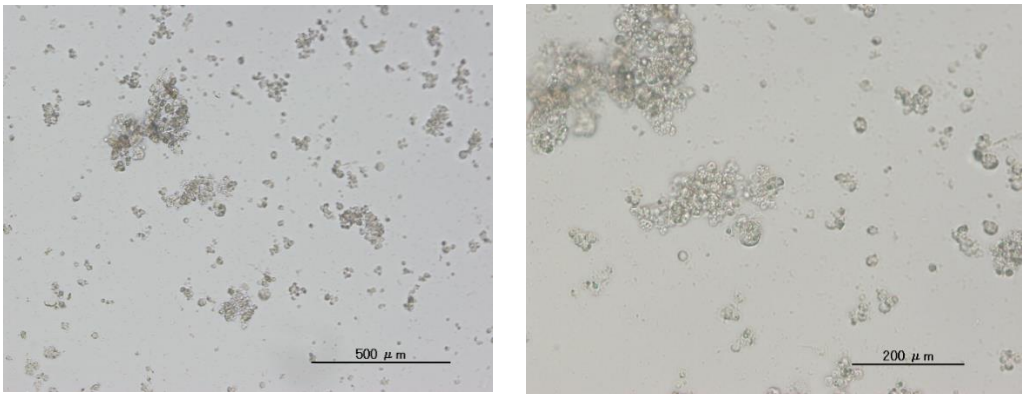
96. Thiel, G., et al., *Role of basic region leucine zipper transcription factors cyclic AMP response element binding protein (CREB), CREB2, activating transcription factor 2 and CAAT/enhancer binding protein alpha in cyclic AMP response element-mediated transcription.* J Neurochem, 2005. **92**(2): p. 321-36.

97. Alberini, C.M., *Transcription factors in long-term memory and synaptic plasticity.* Physiol Rev, 2009. **89**(1): p. 121-45.

98. Chang, F., et al., *Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention.* Leukemia, 2003. **17**(7): p. 1263-93.

Figures

A



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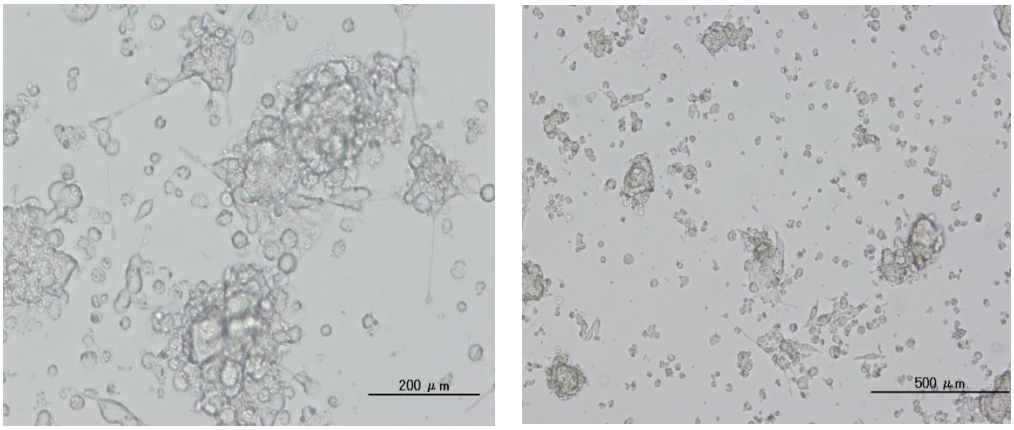

Fig. 1. Microscopy images of stomach and pancreatic ghrelinoma cells.

Stomach ghrelinoma cells (SG-1) (A) and pancreatic ghrelinoma cells (PG-1) (B) were small and round and floated in the culture medium. Cells were observed as single cells or clumps of cells.

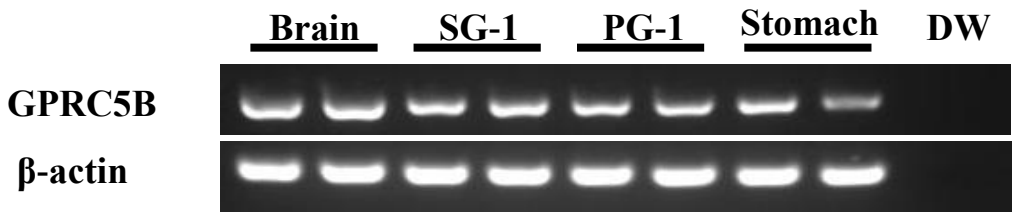

Fig. 2. GPRC5B mRNA expression in SG-1 cells, PG-1 cells, and stomach tissue.

PCR products and specificity of amplification were confirmed via 2% agarose gel electrophoresis. *GPRC5B* was expressed in SG-1 cells, PG-1 cells, gastric tissue, and brain tissue (positive control). Distilled water (DW) was used as negative control, and β-actin was used as an internal control.

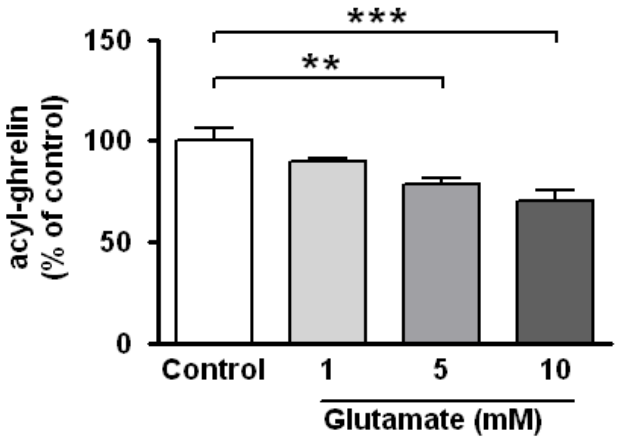

Fig. 3. Effect of glutamate treatment on acylated ghrelin secretion in SG-1 cells.

Acyl-ghrelin secretion was examined after 3 h of treatment with varying doses of glutamate. Glutamate significantly suppressed ghrelin secretion. Data are presented as means ± SEM. n = 6, *p<0.05; **p<0.01; ***p<0.001.

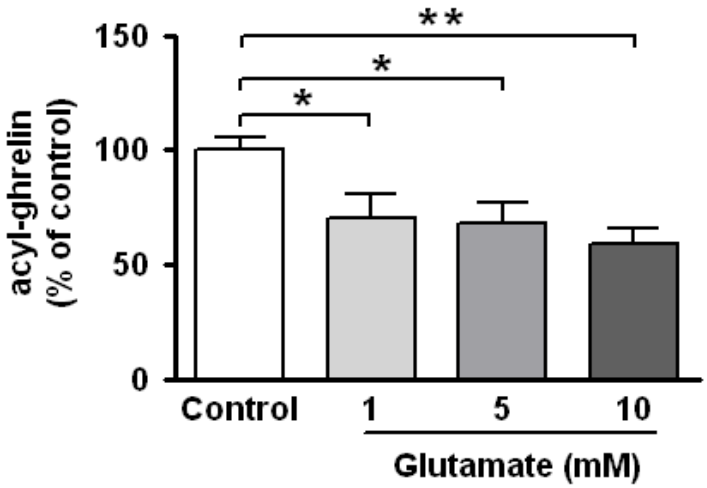

Fig. 4. Effect of glutamate treatment on acylated ghrelin secretion in PG-1 cells.

Acyl-ghrelin levels were examined after 3 h of treatment with different doses of glutamate. Glutamate significantly suppressed ghrelin secretion in PG-1 cells. Data are presented as means ± SEM. n = 6, *p<0.05; **p<0.01; ***p<0.001.

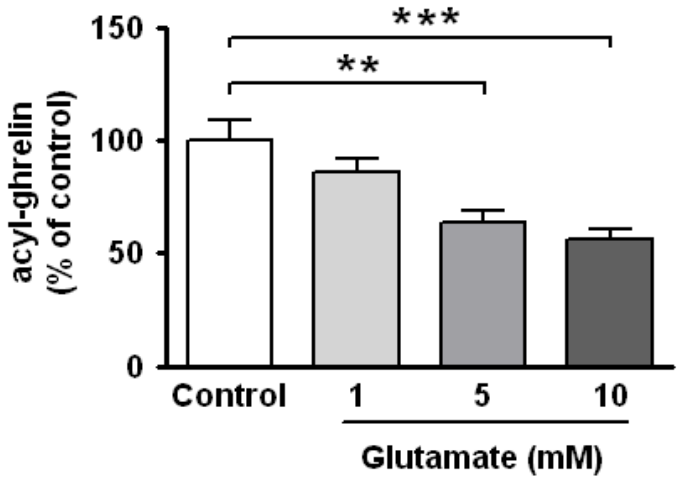

Fig. 5. Effect of glutamate treatment on acylated ghrelin secretion in primary cultured gastric mucosal cells.

Acyl-ghrelin secretion was examined after 3 h of glutamate treatment. Glutamate significantly suppressed acyl-ghrelin secretion in primary cultured gastric mucosal cells. Data are presented as means ± SEM. n = 6, **p<0.01; ***p<0.001.

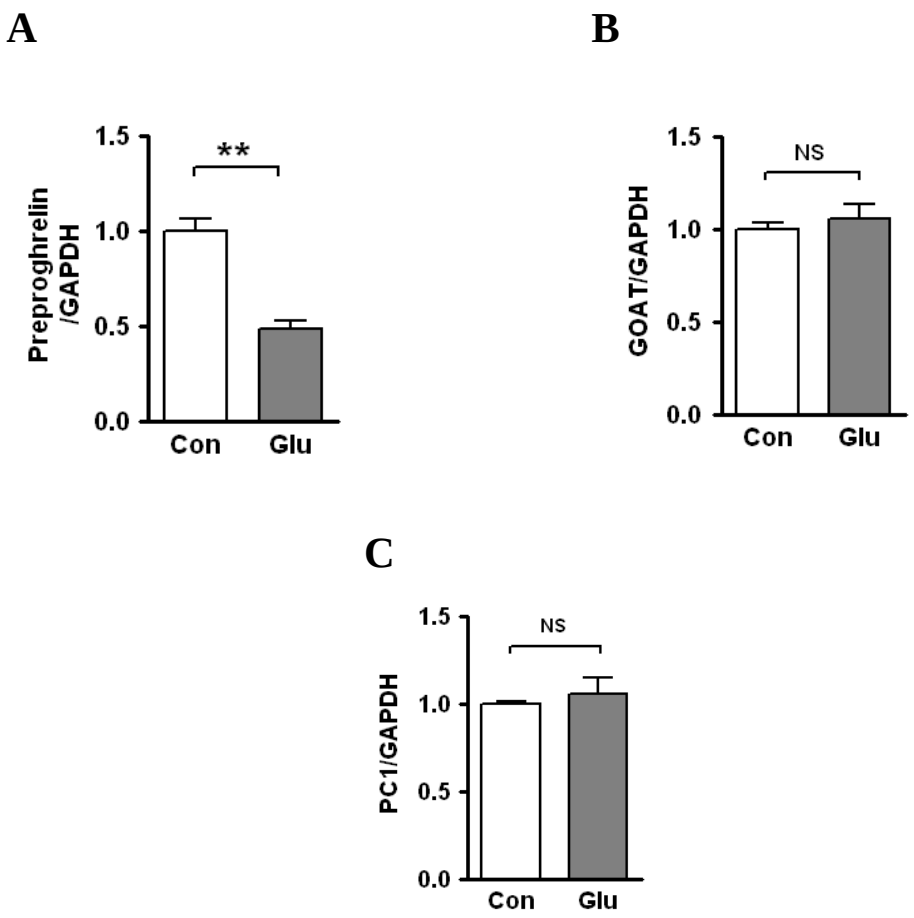

Fig. 6. Effect of glutamate treatment on mRNA expression of preproghrelin, GOAT, and PC1 in SG-1 cells.

Effect of glutamate on mRNA levels of preproghrelin (A), GOAT (B), and PC1 (C) were determined by quantitative RT-PCR. Glutamate treatment did not significantly change mRNA expression levels of GOAT and PC1 but significantly decreased preproghrelin mRNA expression. Data are presented as means ± SEM. n = 6. **p<0.01; Con: Control, Glu: Glutamate, NS= non-significant.

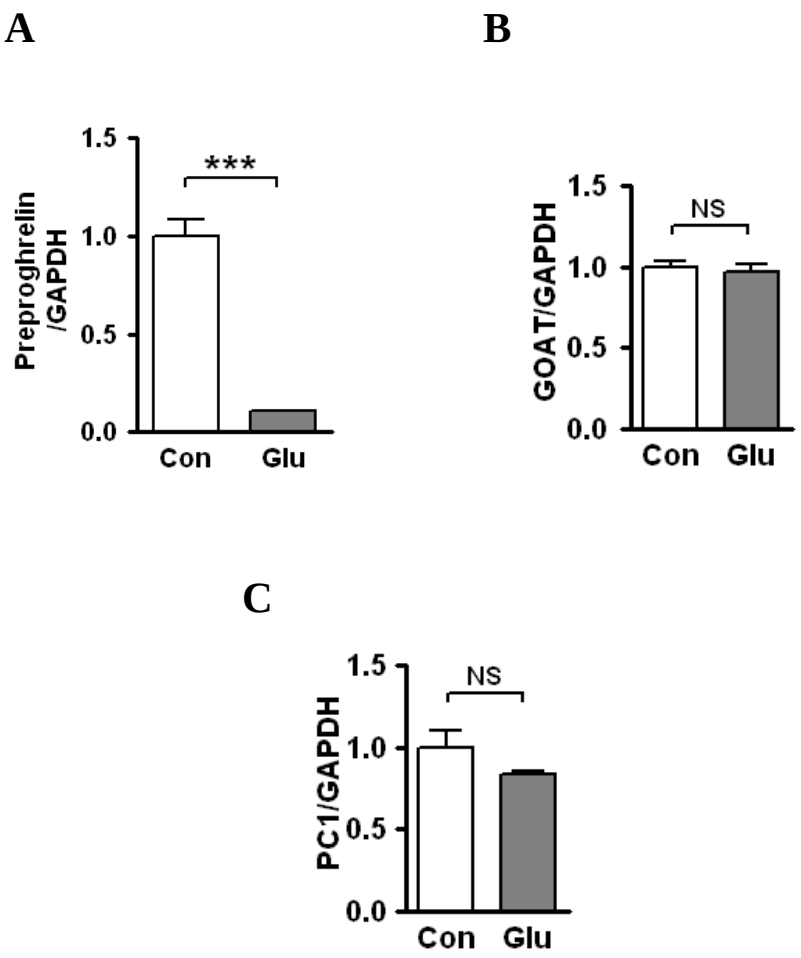

Fig. 7. Effect of glutamate treatment on mRNA expression of preproghrelin, GOAT, and PC1 in PG-1 cells.

Effect of glutamate on mRNA expression of preproghrelin (A), GOAT (B), and PC1 (C) mRNA based on quantitative RT-PCR. Glutamate treatment did not significantly change mRNA levels of GOAT and PC1 but significantly decreased preproghrelin mRNA expression levels. Data are presented as means ± SEM. n = 6. ***$p<0.001$; Con: Control, Glu: Glutamate, NS= non-significant.

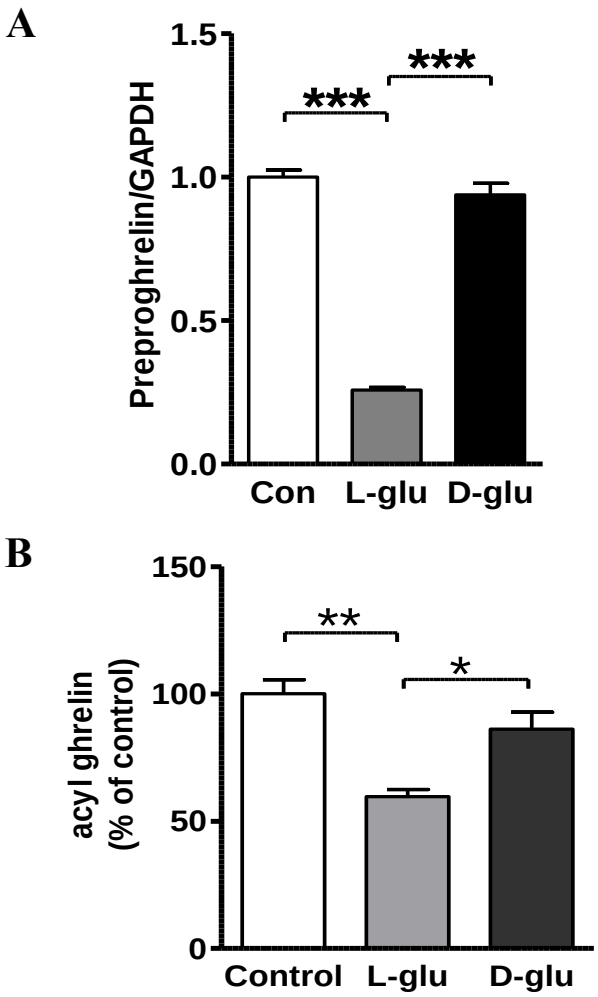

Fig. 8. Effect of D-glutamate treatment on mRNA expression of preproghrelin and acyl-ghrelin levels in PG-1 cells.

The effect of D-glutamate on preproghrelin mRNA expression and acyl-ghrelin secretion was measured. Preproghrelin expression and acyl ghrelin levels was downregulated by L-glutamate but was not affected by D-glutamate treatment. Data are presented as means ± SEM. n = 3. *p<0.05; **p<0.01; ***p<0.001; L-glu: L-glutamate, D-glu: D-glutamate.

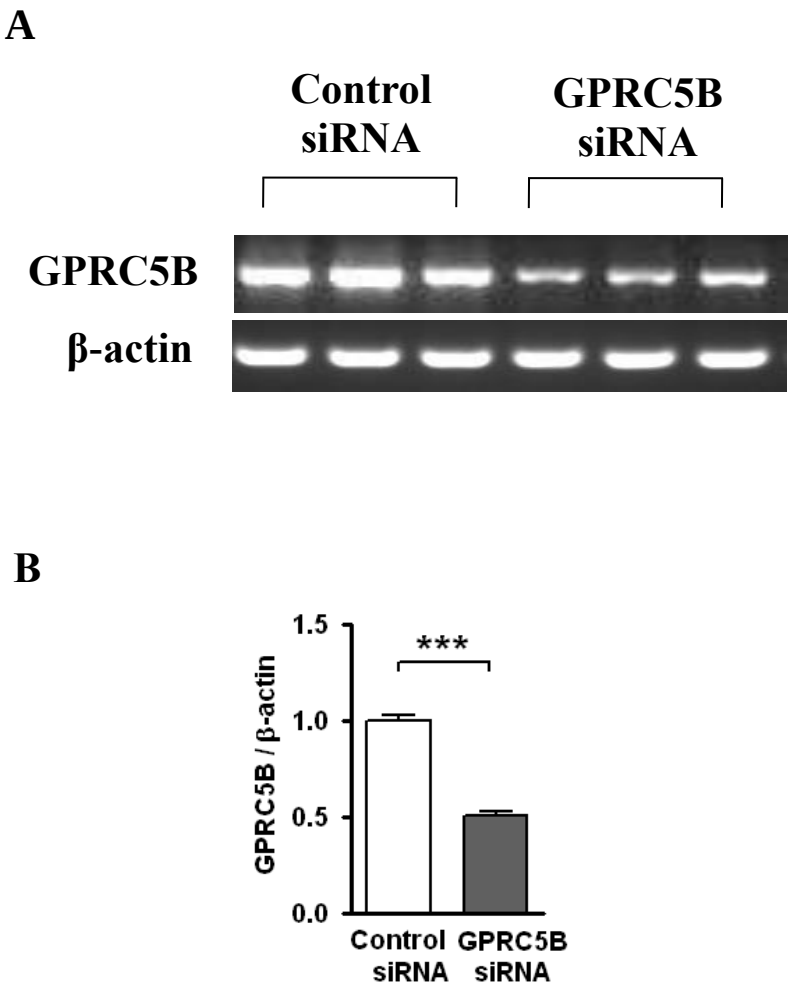

Fig. 9. GPRC5B mRNA knockdown in PG-1 cells.

Representative gel image showing downregulated GPRC5B mRNA levels following siRNA knockdown (A). GPRC5B siRNA knockdown significantly decreased GPRC5B mRNA expression to approximately 50% (B). Data are presented as means ± SEM. n = 3, ***$p<0.001$.

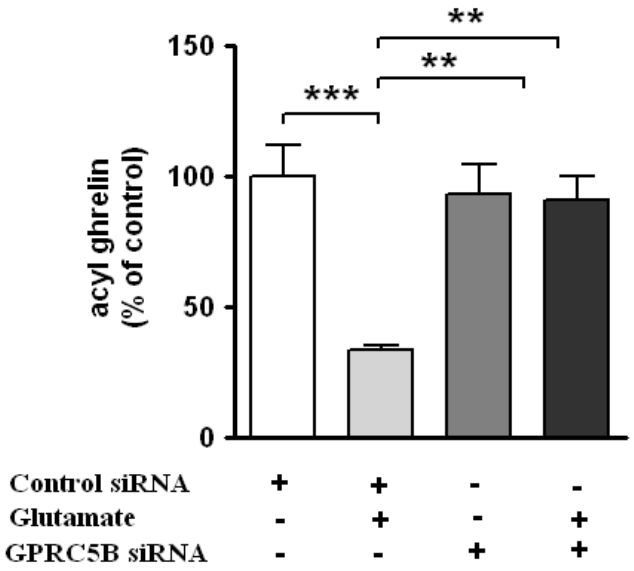

Fig. 10. Effect of glutamate on ghrelin secretion in GPRC5B-knockdown PG-1 cells.

GPRC5B siRNA knockdown markedly reversed glutamate-induced inhibition of acyl-ghrelin secretion. Data are presented as means ± SEM. n = 3, **p<0.01; ***p<0.001.

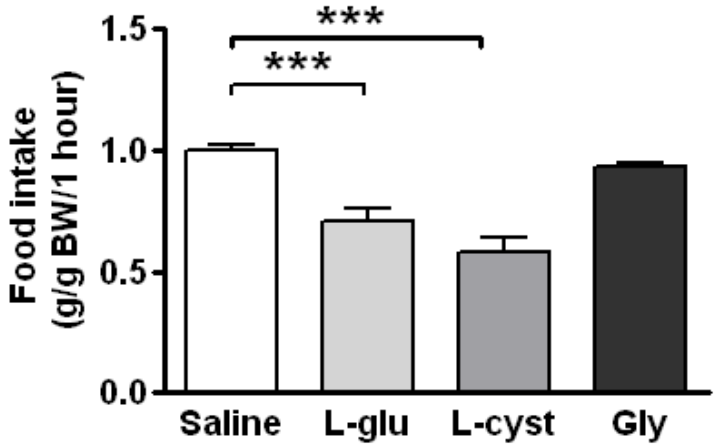

Fig. 11. Glutamate suppresses food intake in mice.

Mice were injected with glutamate, vehicle, L-cysteine, or glycine after 16 h of fasting and were given a fixed amount of food. Food intake was measured at 1 h following administration. Glutamate significantly inhibited food intake in mice. L-cysteine-treated mice were used as positive control, while glycine-treated mice were used as negative controls. Data are presented as means ± SEM. n = 3, ***$p<0.001$. L-glu: L-glutamate, L-cyst: L-cysteine, Gly: Glycine.

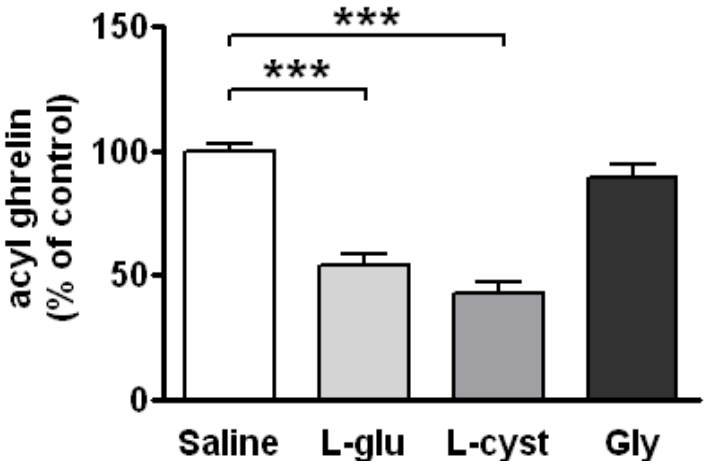

Fig. 12. Glutamate suppresses plasma acyl ghrelin secretion in mice.

Mice were injected with glutamate, vehicle, L-cysteine, or glycine after 16 h of fasting and were given a fixed amount of food. At 1 h following administration, blood was collected from caudal vein for measurement of plasma ghrelin concentrations. Glutamate treatment significantly reduced plasma acyl-ghrelin levels. Mice treated with L-cysteine served as positive control group, while mice treated with glycine served as the negative control group. Data are presented as means ± SEM. n = 3, ***$p<0.001$. L-glu: L-glutamate, L-cyst: L-cysteine, Gly: Glycine.

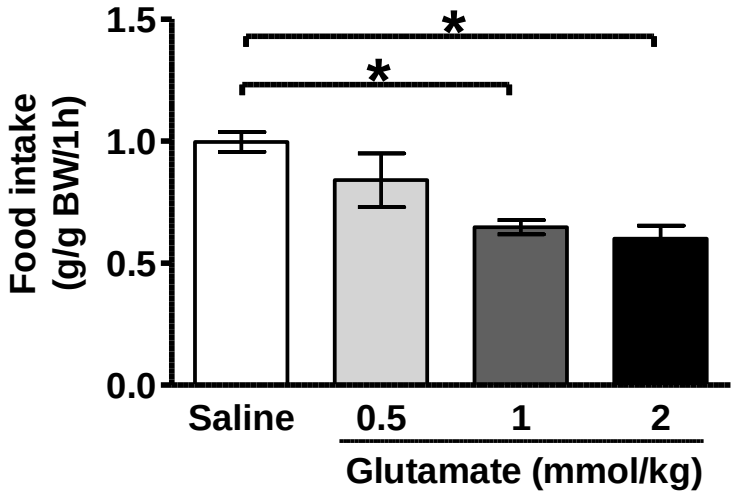

Fig. 13. Glutamate suppresses food intake in mice in a dose-dependent manner.

L-glutamate administration suppressed food intake in mice. Mice were injected with L-glutamate or saline after 16 h of fasting and were given a fixed amount of food. Food intake was measured at 1 h following glutamate administration. L-glutamate significantly inhibited food intake in mice in a dose-dependent manner. Data are presented as means ± SEM. *$p<0.05$.

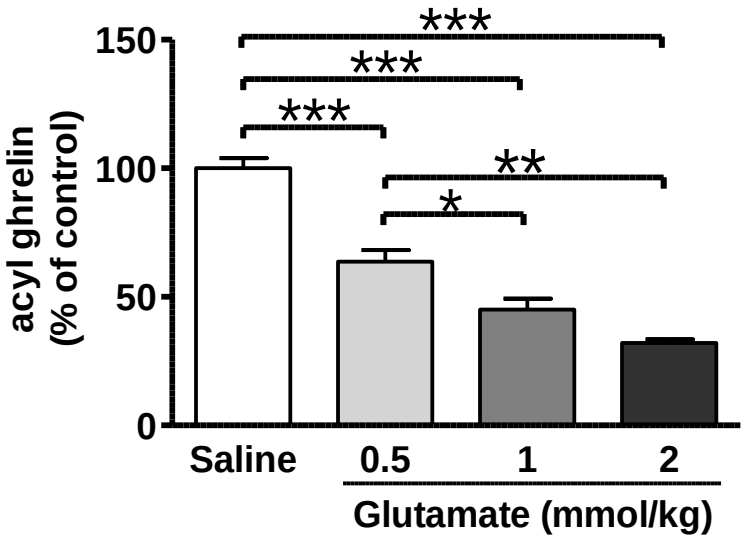

Fig. 14. Glutamate suppresses plasma acyl ghrelin production in mice in a dose dependent manner.

L-glutamate administration suppressed plasma acyl ghrelin secretion in mice. Mice were injected with L-glutamate or saline after 16 h of fasting and given a fixed amount of food. At 1 h following administration, blood was collected from the caudal vein for measurement of plasma acyl ghrelin concentrations. L-glutamate significantly reduced plasma acyl-ghrelin levels in a dose-dependent manner. Data are presented as means ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

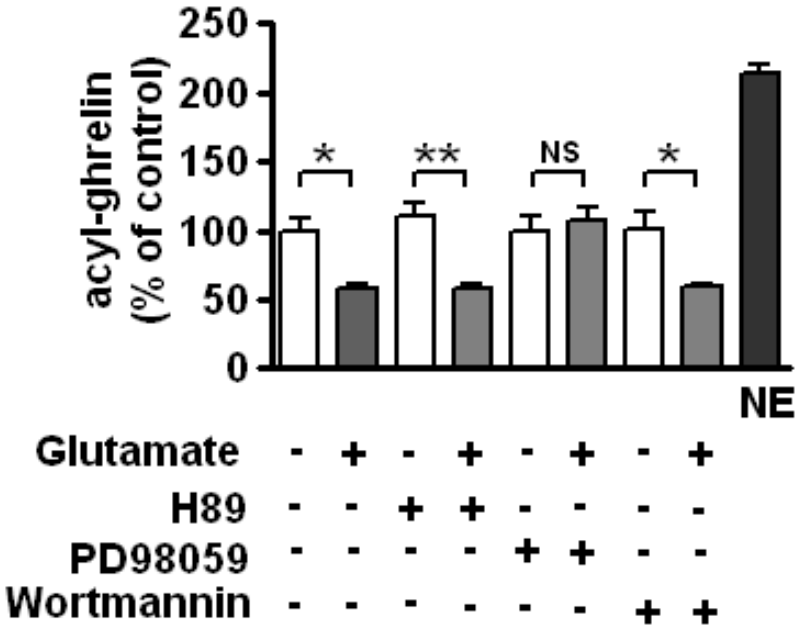

Fig. 15. GPRC5B signaling mechanisms in ghrelin secretion in PG-1 cells.

Co-administration of glutamate with different pathway inhibitors was performed. Co-administration of glutamate with PD98059 completely reversed glutamate-inhibited ghrelin secretion. On the other hand, mice co-treated with glutamate and H89 or wortmannin showed ghrelin secretion levels similar to mice in the glutamate-treated group. NE was used as positive control group. Data are presented as means ± SEM. n = 3, *$p < 0.05$; **$p < 0.001$; NS=non-significant.

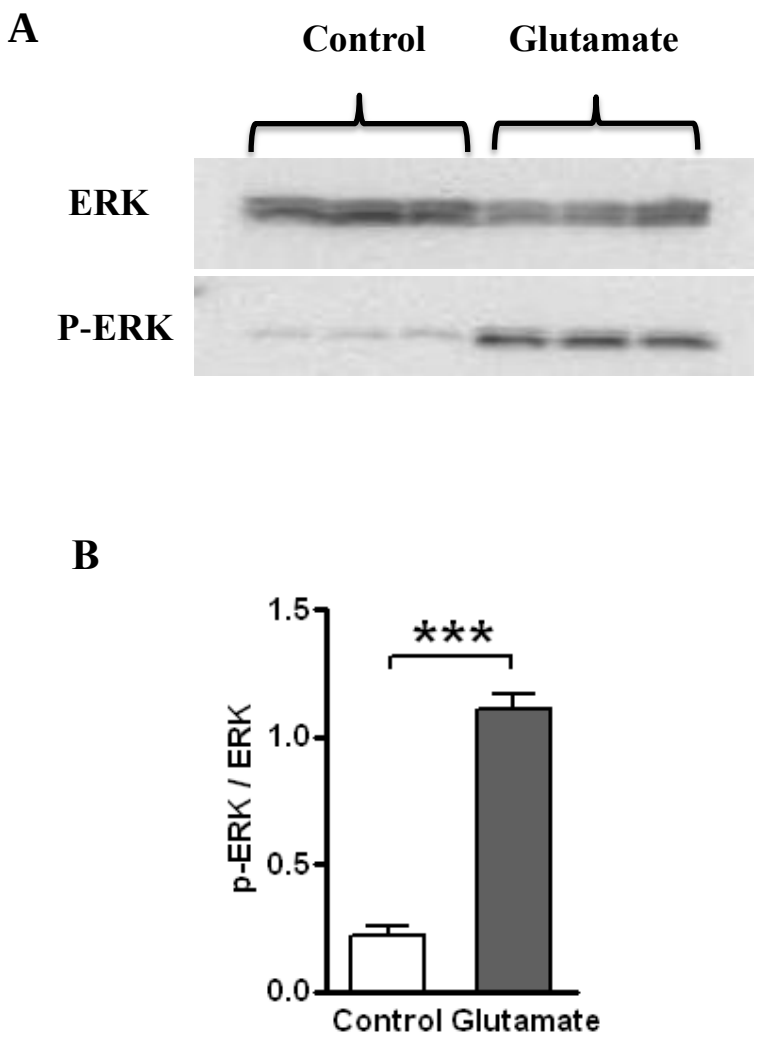

Fig. 16. The ERK pathway is involved in the regulation of GPRC5B in ghrelin secretion in PG-1 cells.

Levels of phosphorylated ERK following glutamate treatment were measured by western blotting. Glutamate-treated mice showed increased levels of phosphorylated ERK compared to the control (A). Glutamate significantly increased p-ERK/ERK ratios (B). Data are presented as means ± SEM. n = 3, ***$p < 0.0001$.

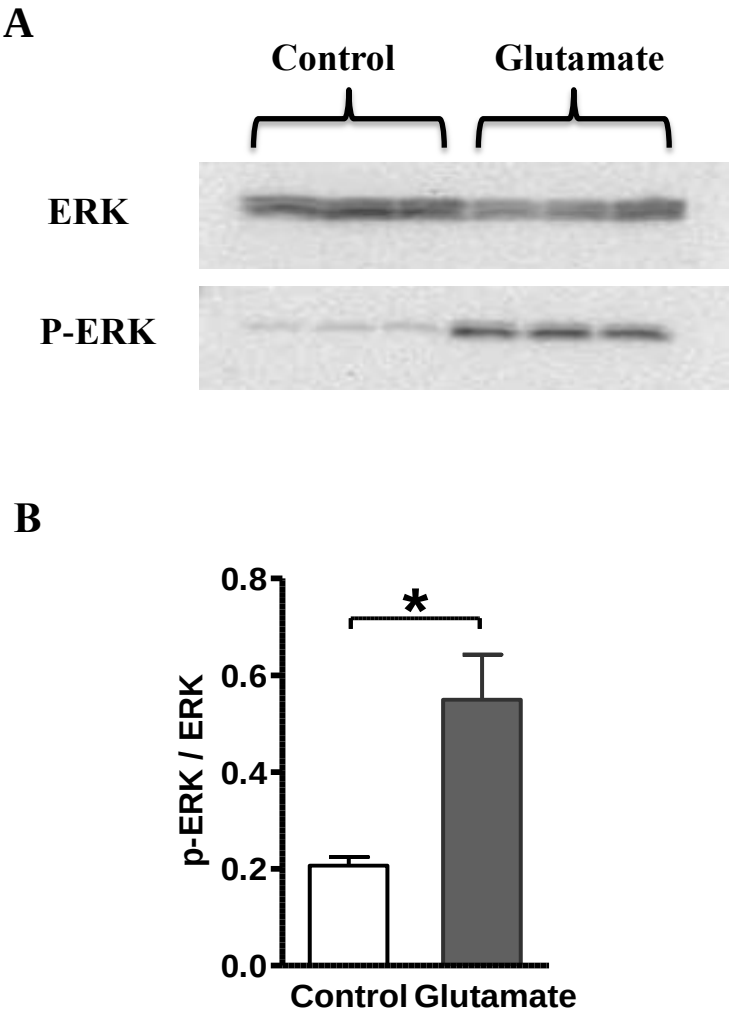

Fig. 17. The ERK pathway is involved in the regulation of GPRC5B in ghrelin secretion in SG-1 cells.

Levels of phosphorylated ERK following glutamate treatment were measured by western blotting. Glutamate administration increased levels of phosphorylated ERK compared to the control (A). Glutamate significantly increased the ratio of p-ERK in ratio of ERK (B). Data are presented as means ± SEM. n = 3, ***$p < 0.0001$.

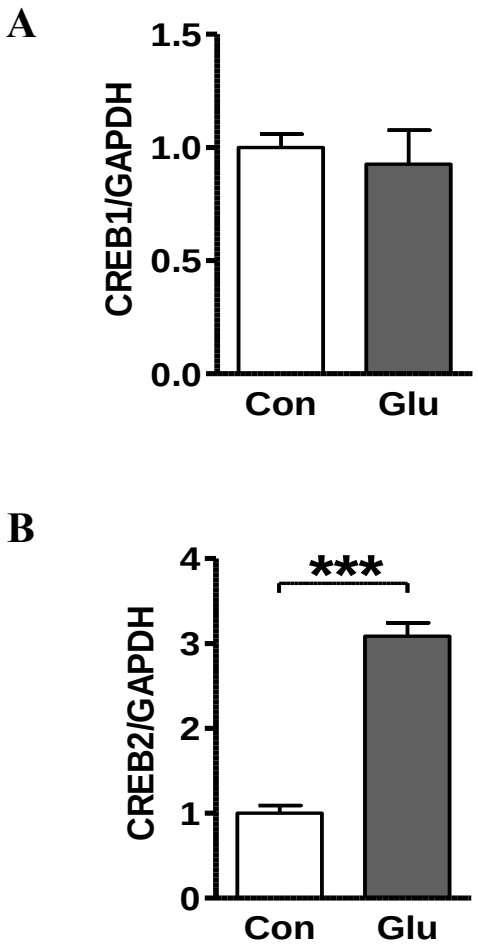

Fig. 18. Effect of glutamate treatment on CREB1 and CREB2 mRNA expression levels in PG-1 cells.

The effect of glutamate on transcriptional expression of CREB1 and CREB2 was determined via quantitative RT-PCR. Glutamate did not alter CREB1 mRNA expression levels but increased CREB2 mRNA levels. Data are presented as means ± SEM. n = 3. *** $p<0.001$. Con: Control, Glu: Glutamate.

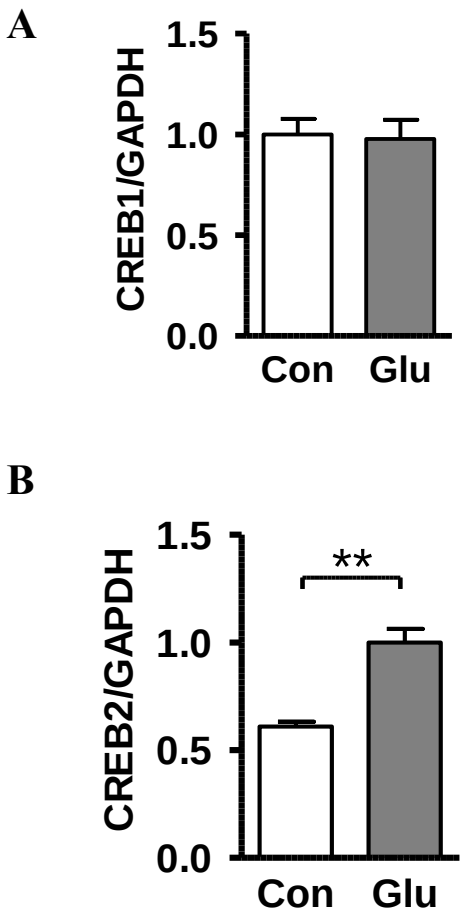

Fig. 19. Effect of glutamate treatment on mRNA expression levels of CREB1 and CREB2 in SG-1 cells.

The effects of glutamate on transcriptional expression of CREB1 and CREB2 were determined via quantitative RT-PCR. Glutamate administration did not affect CREB1 mRNA expression levels but increased CREB2 expression. Data are presented as means ± SEM. n = 3. ***p<0.001. Con: Control, Glu: Glutamate.

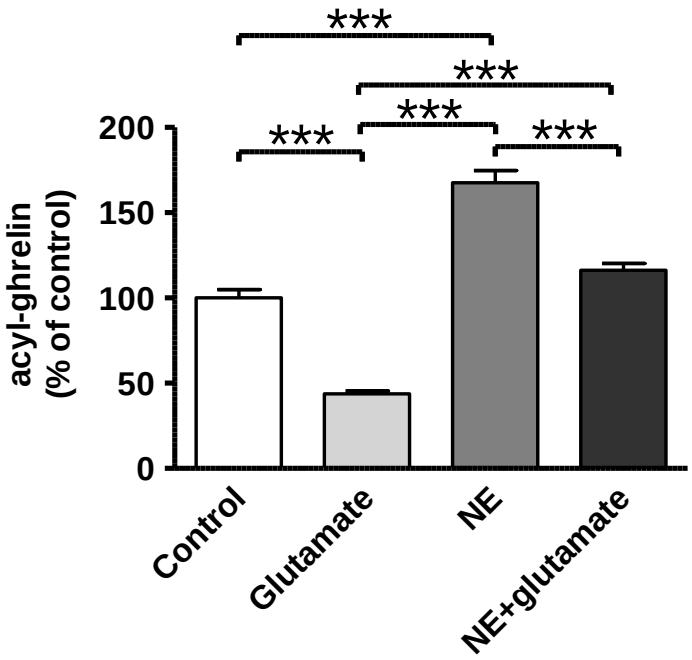

Fig. 20. Effect of glutamate on NE-induced ghrelin secretion in primary cultured gastric mucosal cells.

Cells were pretreated with glutamate for 1 h before NE (10 μM) stimulation. Glutamate treatment significantly reversed NE-induced ghrelin elevation. Data are presented as means ± SEM. n = 3, ***$p < 0.001$.

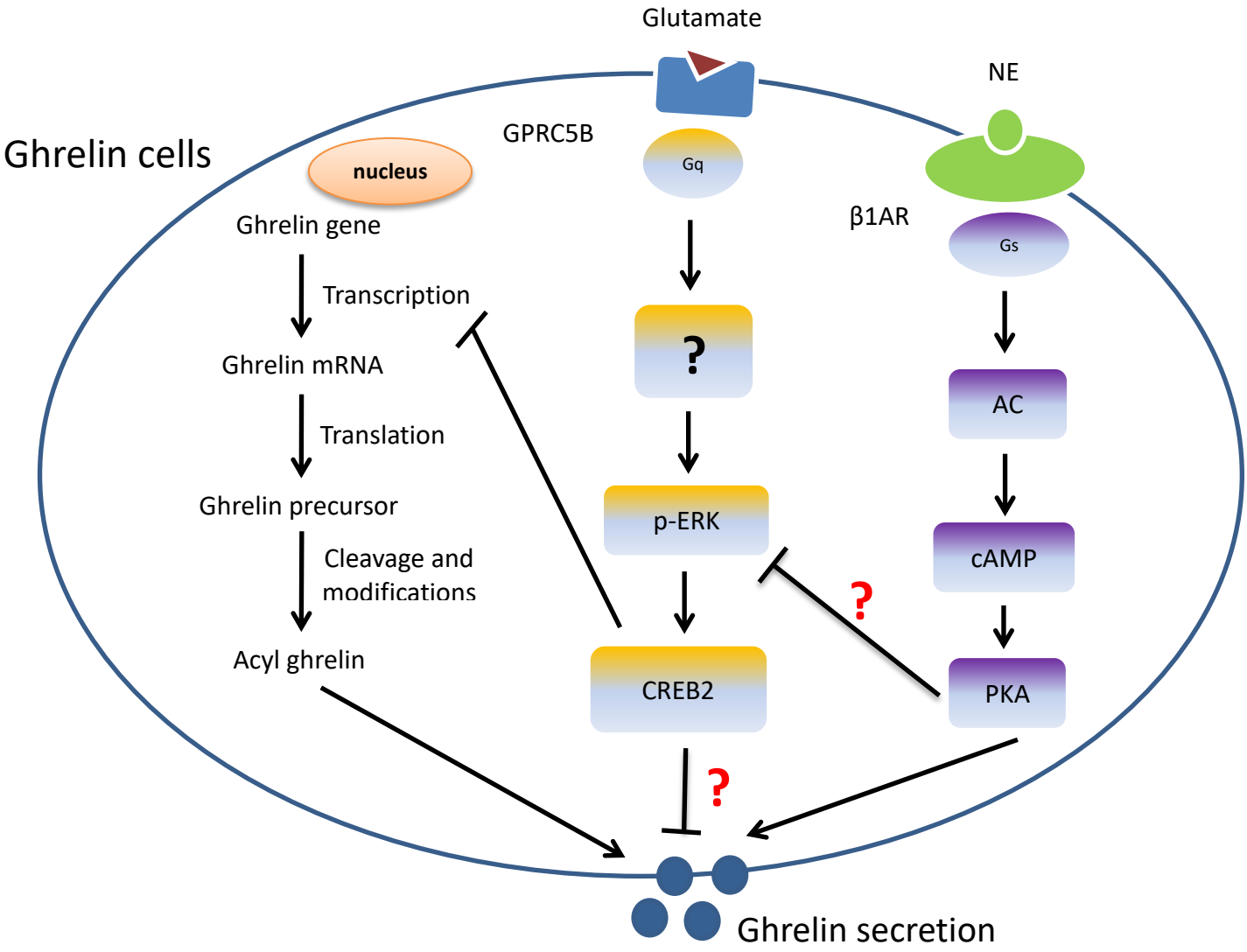

Fig. 21. In ghrelin cells, proghrelin is processed into the 28-AA acyl-ghrelin by GOAT and prohormone convertase 1 (PC1). Our results showed that glutamate treatment significantly inhibited preproghrelin expression and inhibited ghrelin secretion via the ERK pathway. In addition, glutamate induced CREB2 mRNA upregulation. The inhibitory effect of GPRC5B may be mediated via CREB2, which inhibits transcription and thus the production of acyl-ghrelin or directly inhibits ghrelin secretion. NE stimulates ghrelin secretion by increasing cAMP production and activating PKA. Furthermore, pretreatment with glutamate blocked NE-induced increase in ghrelin levels. These results suggest crosstalk between the two pathways, and it is hypothesized that cAMP blocks ERK activity via PKA. Further studies are warranted to elucidate the detailed regulatory mechanisms underlying ghrelin secretion.