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Ghrelin and obestatin promote the allergic action in rat peritoneal mast cells as basic secretagogues

Tatsuya Hirayama^a, Tsutomu Kawabe^{a,b,*}, Miyoko Matsushima^{a,b}, Yuko Nishimura^a, Yuko Kobe^a, Yui Ota^a, Kenji Baba^c, Kenzo Takagi^a

^a Department of Medical Technology, Nagoya University School of Health Sciences, 1-1-20 Daikou-minami, Higashi-ku, Nagoya 461-8673, Japan

^b MEXT Innovative Research Center for Preventive Medical Engineering, Nagoya University, Furou-cho, Chikusa-ku, Nagoya 464-8603, Japan

^c Department of Respiratory Medicine, Aichi Medical University School of Medicine, 21 Karimata, Yazako, Nagakute-cho, Aichi 480-1195, Japan

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ABSTRACT

Ghrelin is an endogenous ligand of the type 1a growth hormone secretagogue receptor (GHSR1a) that regulates energy balance. Ghrelin and obestatin, derived from the post-translational processing of preproghrelin, are involved in a diverse range of biological activities, yet their effect on the immune system is not fully understood. In the present study, we investigated the roles of ghrelin and obestatin on mast cell degranulation and found that both ghrelin and obestatin induce the release of histamine from rat peritoneal mast cells. This induced histamine release was inhibited by the pertussis toxin, an inhibitor of $G\alpha_i$ protein, and extracellular Ca^{2+} . Rat peritoneal mast cells and rat basophilic leukemia (RBL-2H3) cells did not express the ghrelin receptor GHSR1a, suggesting that histamine release induced by ghrelin occurs via a receptor-independent mechanism. We report here that ghrelin and obestatin, belonging to the family of basic secretagogues, stimulate mast cells independent of a receptor, and this may play a crucial role at the site of allergy or inflammation.

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1. Introduction

Ghrelin and obestatin are bioactive peptides that originate from the post-translational processing of the 117-amino acid precursor, preproghrelin [20,42]. Ghrelin, a 28-amino acid acylated peptide, is secreted predominantly from X/A-like enteroendocrine cells of the stomach and has been identified as the endogenous ligand of the type 1a growth hormone secretagogue receptor (GHSR1a) [20]. GHSR1a is widely expressed in the brain and peripheral tissues [12,16] and in various forms of cancers [7]. Ghrelin exerts diverse biological actions through GHSR1a, including effects on growth hormone secretion, cell proliferation, increase of appetite, and inflammation [6,30,36,39]. Ghrelin plays an important role in immune cell function as a negative regulator of inflammatory cytokine expression [6,39].

Obestatin has been reported to have opposite actions to ghrelin in energy balance, food intake and intestinal motility, which can occur via GPR39 [22,41,42], a receptor specific for obestatin. GPR39 belongs to the ghrelin family that includes the ghrelin receptor,

neurotensin receptor, and motilin receptor [35]. However, in the situation of inflammation, recent reports show that ghrelin and obestatin have similar biological actions despite having opposite actions in energy balance [17,24].

Mast cells play an important role in inflammatory and allergic reactions through the release of inflammatory mediators stored in granules such as histamine, serotonin, and proteases, or *de novo* synthesized molecules such as prostaglandins, leukotrienes, and cytokines [9,15,33,37]. Mast cell activation has two main signaling pathways: immunoglobulin E (IgE)-dependent and -independent [9,33]. The IgE-dependent pathway occurs in response to antigens and is mediated at the cell surface by cross-linking IgE to the high affinity IgE receptor FcεRI [15,33]. One of the IgE-independent pathways is mast cell activation evoked by basic secretagogues [9]. Basic secretagogues activate the pertussis toxin (PTX)-sensitive G proteins, $G\alpha_{i2}$ and $G\alpha_{i3}$, and stimulate phospholipase Cβ through their βγ subunits. This leads to mast cell exocytosis and the synthesis and release of arachidonic acid metabolites [9,10]. Most basic secretagogues are either endogenous or exogenous peptides, and are characterized by a net positive charge. Ghrelin and obestatin have an overall positive charge. This suggests that they might be involved in mast cell activation in inflammatory conditions where they exhibit similar actions, which is contrary to their opposite biological activities in the regulation of energy balance, food intake and intestinal motility.

* Corresponding author at: Department of Medical Technology, Nagoya University School of Health Sciences, 1-1-20 Daikou-minami, Higashi-ku, Nagoya 461-8673, Japan. Tel.: +81 52 719 1547.

E-mail address: kawabe@met.nagoya-u.ac.jp (T. Kawabe).

In this study, we investigated the effects of ghrelin and obestatin on histamine release from rat peritoneal mast cells. We also determined the effect of PTX and extracellular Ca^{2+} on ghrelin- and obestatin-induced mast cell degranulation.

2. Materials and methods

2.1. Animals and reagents

Male Wistar rats (300–400 g) were purchased from Japan SLC (Shizuoka, Japan). Animals were maintained in a room regulated for temperature ($22\text{--}24^\circ\text{C}$), humidity ($55 \pm 5\%$) and light (12-h light–dark cycle; lights on at 7:00), with food and water available ad libitum for at least 3 days before the experiments. All procedures were performed with the approval of the Animal Experimentation Committee, Graduate School of Medicine, Nagoya University, in accordance with the Guidelines for Animal Experimentation of Nagoya University.

Rat ghrelin and obestatin were purchased from the Peptide Institute (Osaka, Japan). All other chemicals were obtained commercially and were used at analytical grade without the need for further purification.

2.2. Cell culture

Rat basophilic leukemia (RBL-2H3) cells were purchased from American Type Culture Collection. RBL-2H3 cells were maintained in MEM supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

2.3. Crude peritoneal mast cell preparation

Under anesthesia, the rat peritoneal cavity was washed with 20 ml HEPES Tyrode's solution containing 140 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4 , 1.2 mM NaHCO_3 , and 0.56 mM glucose. After gentle massage of the abdomen for a few minutes, peritoneal exudate cells (PEC) containing mast cells were obtained. The collected PEC were washed twice with HEPES Tyrode's solution by centrifugation ($800 \times g$, 4°C , 10 min). The pellets were then resuspended in ice-cold HEPES Tyrode's solution at a concentration of 5×10^5 cells/ml. Viability of PEC (greater than 95%) was confirmed by trypan blue exclusion and the proportion of mast cells in PEC (approximately 10%) by toluidine blue staining.

2.4. Purification of mast cells from PEC

Mast cells were purified from PEC as described previously [26]. Briefly, mast cells were purified to >95% by Percoll (GE Healthcare Bioscience, Buckinghamshire, UK) density gradient centrifugation at $800 \times g$ for 10 min at 4°C . Purified mast cells (3×10^6 cells) were washed three times in HEPES Tyrode's solution following Percoll treatment.

2.5. Cell activation

To determine the dose-dependent effect of ghrelin and obestatin on histamine release, PECs were incubated for 15 min with different concentrations of each peptide solution (10^{-8} to 10^{-5} M). After incubation, all test tubes were immediately placed in an ice-cold water bath and then centrifuged at $4000 \times g$ for 3 min at 4°C to collect the supernatant. The effects of extracellular Ca^{2+} on histamine release induced by ghrelin or obestatin were examined at different concentrations of Ca^{2+} (0.5–2 mM). To assess the effect of PTX, an inhibitor of Gi protein, on histamine release, 100 ng/ml of PTX was added 2 h before incubation with ghrelin or obestatin.

2.6. RT-PCR

RT-PCR was performed as described previously with a few modifications [32]. Briefly, total RNA was isolated using TRIZOL reagent and reverse-transcribed to cDNA using random hexamers and SuperScript II, according to the manufacturer's instructions. cDNA was amplified with Taq polymerase using 28 cycles for β -actin and 35 cycles for GHSR1a under the following conditions: denaturation at 94°C for 30 s, annealing at 55°C (β -actin) or 62°C (GHSR1a) for 30 s, and extension at 72°C for 60 s.

The sequence of each primer was as follows: β -actin (465 bp): sense 5'-TTC TAC AAT GAG CTG CGT GTG GC-3'; antisense 5'-CTC ATA GCT CTT CTC CAG GGA GGA-3'; GHSR1a (897 bp): sense 5'-CAC CAC CAA CTT CTA CCT GTC CA-3'; antisense 5'-TGG CGG AGA GAT GGG ATG TGC TGT CA-3'.

The PCR products were electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide staining under an ultraviolet transilluminator.

2.7. Western blotting

RBL-2H3 cells and PEC were collected and resuspended in lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA-2Na, and 1% Triton X-100. Total protein was determined by the bicinchoninic acid (BCA) technique. Then, 50 μg of protein was subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was incubated with 4% skim milk for 1 h at room temperature followed by primary antibodies overnight at 4°C . The membrane was subsequently incubated with the secondary antibody, either horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, for 1 h at room temperature. The specific protein bands on the membrane were visualized using the Enhanced Chemiluminescence kit (GE Healthcare Bioscience, Buckinghamshire, UK) according to the manufacturer's instructions.

2.8. Histamine release assay

Histamine content in the supernatants was determined using high-performance liquid chromatography (HPLC), as previously described [27]. HPLC was performed on a Shimadzu LC-10A system equipped with a fluorescence detector (RF10A XL, Shimadzu; Em: 450 nm, Ex: 330 nm) and an LC-10A liquid pump under the following conditions: column, Cosmosil Packed Column (150 mm $\times$ 4.6 mm); mobile phase, 50% methanol in 0.02 M acetate buffer (pH 4.0); flow rate, 0.8 ml/min; oven temperature, 40°C . To quantify the total histamine content, cells were treated with 1% Triton X-100, and the extract was analyzed for histamine release. Histamine release was expressed as a percentage of the total histamine content in cells, and the spontaneous release was subtracted from the stimulated cell release.

2.9. Statistical analysis

Data are expressed as mean $\pm$ SD of the net percent of total histamine release. Statistical analysis was performed using StatView (Abacus Concept, Berkeley, CA, USA).

3. Results

3.1. Ghrelin and obestatin induce histamine release from PEC

Examination of the effects of ghrelin and obestatin on histamine release revealed that histamine is released from PEC in a dose-dependent manner (Fig. 1). At the highest concentration of ghrelin and obestatin tested (10^{-5} M), histamine increased by $36.0 \pm 2.0\%$

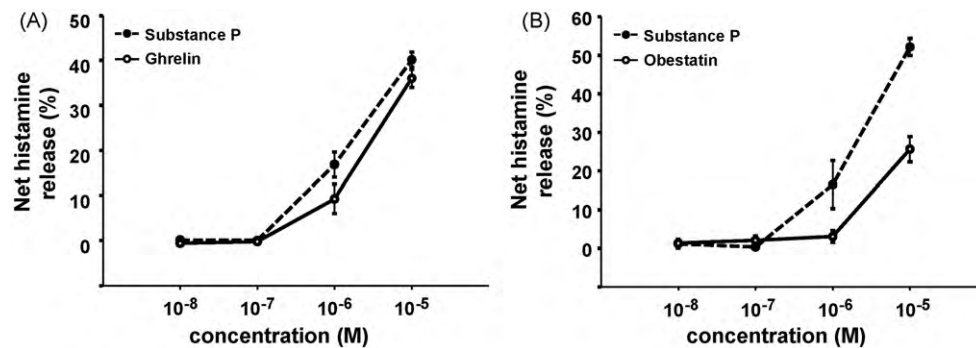


Fig. 1. Dose-dependent effects of ghrelin and obestatin on histamine release from rat peritoneal mast cells. After 10-min preincubation, PECs were stimulated with various concentrations of ghrelin (A) or obestatin (B) (10^{-8} to 10^{-5} M) for 15 min, and release of histamine was determined. Substance P was used as a positive control. Values are expressed as the mean $\pm$ SD of percentages of total histamine. Results shown are representative of three independent experiments.

and $25.6 \pm 3.3\%$, respectively. In all experiments, cell viability was similar to that of the control, indicating that peptide-induced histamine release was not due to cytotoxicity (data not shown).

3.2. Histamine release induced by ghrelin and obestatin is mediated by G protein

To determine the pathways involved in ghrelin- and obestatin-induced histamine release from mast cells, PEC were pretreated with PTX, an inhibitor of $G\alpha_i$ protein, prior to activation with ghrelin or obestatin. As shown in Fig. 2A and B, PTX pretreatment reduced both ghrelin- and obestatin-induced histamine release from $37.9 \pm 4.1\%$ and $32.6 \pm 1.2\%$ to $3.1 \pm 0.5\%$ and $1.2 \pm 0.2\%$, respectively. These results show that histamine release induced by ghrelin or obestatin was mediated by $G\alpha_i$ protein in peritoneal mast cells.

3.3. Effects of extracellular Ca^{2+} on histamine release induced by ghrelin and obestatin in PEC

G_i protein-dependent mast cell activation induced by cationic peptides, such as substance P and mastoparan, has been shown to be inhibited by extracellular Ca^{2+} [9]. As ghrelin- and obestatin-induced histamine release from PEC also occurred via a G_i -dependent pathway, we determined the effect of extracellular Ca^{2+} on inhibition. The presence of extracellular Ca^{2+} inhibited ghrelin- and obestatin-induced histamine release from PEC in a dose-dependent manner (Fig. 3A and B). These results suggest that ghrelin and obestatin might share the Ca^{2+} -sensitive pathway in the regulation of histamine release from mast cells that occurs with substance P and mastoparan [9].

3.4. Expression of GHSR1a in mast cells

To characterize receptor expression in mast cells, we tested for the presence of the ghrelin receptor GHSR1a on the mRNA and protein levels by RT-PCR and western blotting, respectively. We examined two types of mast cells: connective tissue for rat peritoneal mast cells and mucosal cells for RBL-2H3 cells. Neither types of mast cells expressed GHSR1a mRNA or protein (Fig. 4). These results suggest that ghrelin-induced histamine release in rat peritoneal mast cells is not likely to be mediated via receptor-dependent mechanisms.

3.5. Effect of ghrelin on obestatin-induced histamine release from rat peritoneal mast cells

Ghrelin and obestatin have been reported to exert opposite biological actions in food intake and growth hormone secretion [43]. We investigated the effect of ghrelin on histamine release induced by obestatin from PEC. As shown in Fig. 5, we found that ghrelin had an additive effect on mast cell degranulation when induced by obestatin.

4. Discussion

Although ghrelin and obestatin are derived from the same gene, they have different effects on the regulation of energy balance [42]. In order to determine whether ghrelin and obestatin also have different effects on the regulation of immune function, we investigated the role of these two peptides on mast cell degranulation.

In the present study, we show for the first time that both ghrelin and obestatin dose-dependently increase histamine release

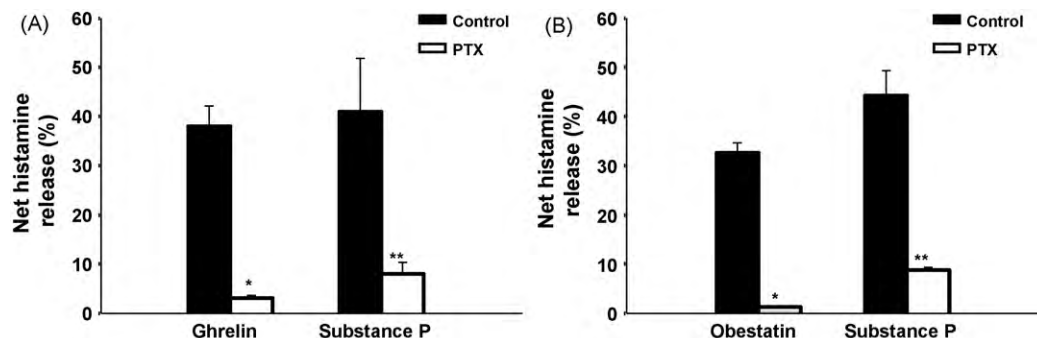


Fig. 2. Effects of PTX on histamine release induced by ghrelin and obestatin from rat peritoneal mast cells. PECs were exposed to PTX (100 ng/ml) for 2 h. After incubation, PECs were stimulated with ghrelin (10^{-5} M) or obestatin (10^{-5} M) for 15 min, and release of histamine was determined. Substance P (10^{-5} M) was used as a positive control. Values are expressed as the mean $\pm$ SD of percentages of total histamine. Results are representative of three independent experiments. * $p < 0.05$, significantly different from the data for the control.

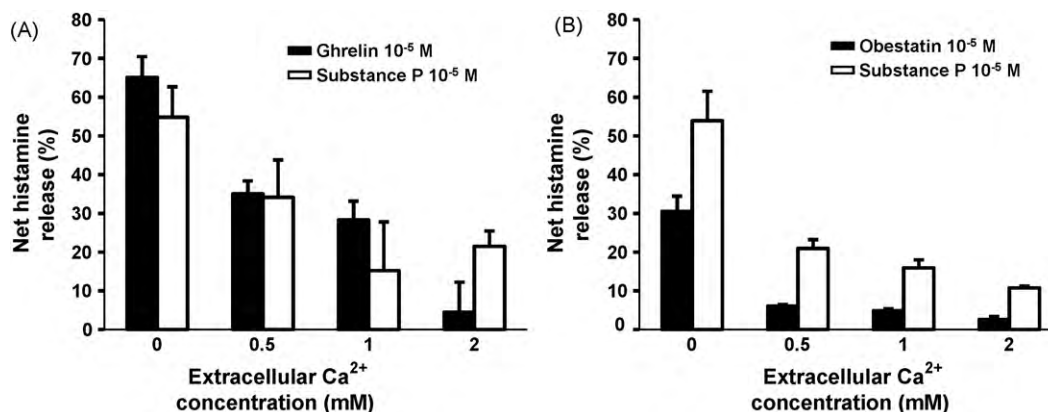


Fig. 3. Effects of extracellular Ca^{2+} on histamine release induced by ghrelin and obestatin from rat peritoneal mast cells. PECs were exposed to various concentrations of extracellular Ca^{2+} (0, 0.5, 1.0, or 2.0 mM) for 10 min. After incubation, PECs were stimulated with ghrelin (10^{-5} M) or obestatin (10^{-5} M) for 15 min, and release of histamine was determined. Substance P (10^{-5} M) was used as a positive control. Values are expressed as the mean $\pm$ SD of percentages of total histamine. Results are representative of three independent experiments. ** $p < 0.05$, significantly different from data for 0 mM of extracellular Ca^{2+} .

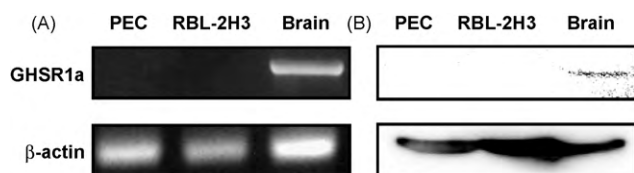


Fig. 4. Expression of GHSR1a in peritoneal mast cells and RBL-2H3 cells. The expression of GHSR1a mRNA (A) and protein (B) was determined by RT-PCR and western blotting, respectively. Rat brain was used as a positive control.

from rat peritoneal mast cells, which can be inhibited by extracellular Ca^{2+} and PTX, an inhibitor of $\text{G}\alpha_i$ protein. To establish whether ghrelin activated mast cells through a receptor-dependent or -independent pathway, we examined the expression of the ghrelin receptor, GHSR1a. We found almost no expression of GHSR1a in the rat mast cells. Furthermore, the potency of ghrelin and obestatin on mast cell activation was similar to that of the basic secretagogues, including mastoparan [29], nociceptin [18], histatin 5 [40], and platelet factor 4 fragment [34]. Thus, it is conceivable that ghrelin and obestatin also belong to the family of basic secretagogues.

Ghrelin and obestatin have opposite actions in energy balance, food intake, and body weight [22,41,42]. However, there are some

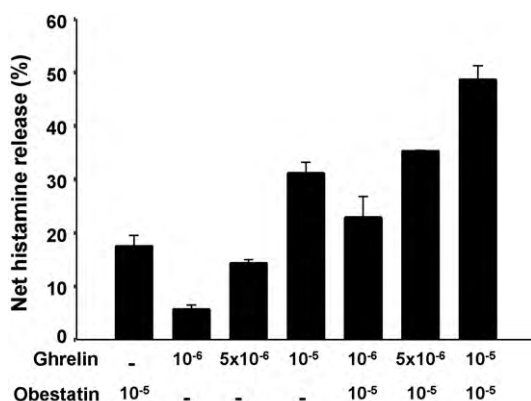


Fig. 5. Effect of ghrelin on obestatin-induced histamine release from rat peritoneal mast cells. After 10 min preincubation, PECs were stimulated with obestatin (10^{-5} M) and various concentrations of ghrelin (0, 10^{-6} , 5×10^{-6} , and 10^{-5} M) for 15 min, and release of histamine was determined. Values are expressed as the mean $\pm$ SD of percentages of total histamine. Results are representative of three independent experiments.

controversies regarding these actions, suggesting that the intricate balance of ghrelin and obestatin is important for the regulation of energy homeostasis [5,31]. In this study, we showed that both ghrelin and obestatin induced the release of histamine in mast cells. Ghrelin is known to have anti-inflammatory actions, including the inhibition of TNF- α -induced IL-8 production and inhibition of NF- κ B signaling in human endothelial cells [25]. The potential activity of ghrelin as an anti-inflammatory mediator has also been demonstrated *in vivo* with several animal models including inflammatory bowel disease [13], arthritis [4,14], sepsis, and endotoxemia [4,38]. Obestatin is known to antagonize the physiological actions of ghrelin. For example, obestatin reverses ghrelin-induced serotonin release in rat hypothalamus [2]. Furthermore, it is also reported that serum levels of ghrelin are decreased and obestatin increased in rheumatoid arthritis [19]. However, a recent study showed that ghrelin and obestatin exhibit similar actions. Ghrelin and obestatin increased ICAM-1 expression as well as the binding of oxidized LDL to thioglycollate-elicited mouse peritoneal macrophages [17]. These peptides also exerted protective effects on cardiac dysfunctions and on intestinal ischemia-reperfusion injury [1,3,11,24]. These observations support our results that both ghrelin and obestatin promote the degranulation of mast cells in allergic inflammation.

Since both ghrelin and obestatin are thought to activate mast cells as basic secretagogues, it might be difficult for them to stimulate the release of histamine from mast cells under physiological conditions: ghrelin at 130 pM and obestatin at 7 pM [28]. However, it has been reported that low concentrations of the basic secretagogues (i.e., substance P or compound 48/80) can promote mast cell degranulation in the presence of IgE-dependent stimulation [21,23]. Therefore, low concentrations of basic secretagogues might have some effects on mast cells and exaggerate allergic symptoms in allergic inflammation. Since ghrelin is known to impair activation and differentiation of Th1 cells [6] as well as IL-17 secretion in T cells [8], it might also be involved in regulating the balance of Th1/Th2 in favor of Th2 immunity, and its local elevation might play a role at the sites of allergic inflammation and/or immune responses.

Some of the basic secretagogues like neurotensin have been reported to promote mast cell secretion via the endogenous specific receptor pathway [9]. In order to investigate the effect of GHSR1a on mast cell activation by ghrelin, we examined the expression of GHSR1a in rat mast cells. This result that no GHSR1a expression was found in rat mast cells further suggests that ghrelin might induce mast cell degranulation via the specific receptor-independent pathway.

In conclusion, ghrelin and obestatin-induced histamine release from rat peritoneal mast cells via a receptor-independent, $G\alpha_i$ - and extracellular Ca^{2+} -dependent pathway. These results suggest that ghrelin and obestatin activate mast cells as basic secretagogues and play an important role in allergic reactions.

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