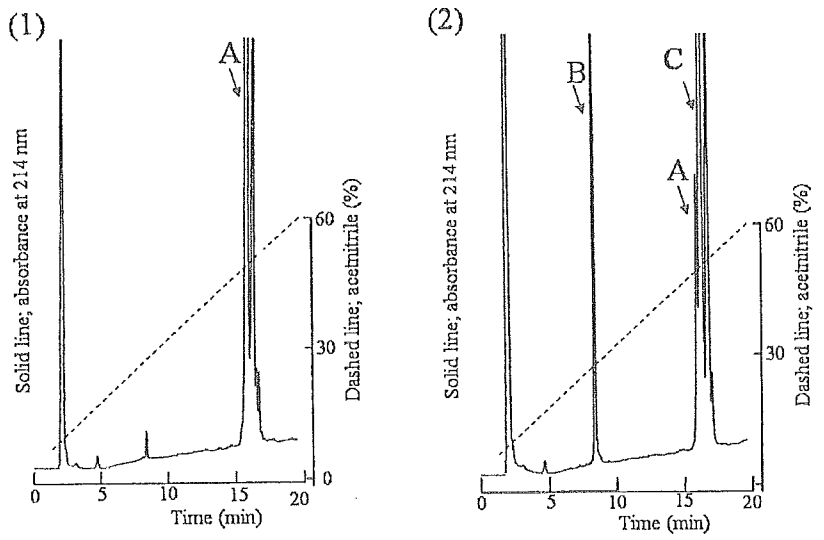

FIGURE 4 HPLC profile of peptides in OmpT reaction. (1) HPLC profile before OmpT reaction. (2) HPLC profile after OmpT reaction. Compared with (1), new peak B appeared and was confirmed as the human ghrelin(8-28) precursor by mass spectrometry. (A) Fusion protein; (B) human ghrelin(8-28) precursor; (C) truncated *E. coli* β-galactosidase. Analytical column was a YMC-PACK PROTEIN-C8.

peak C). We identified this byproduct as [Lys (Boc)^{16,19,20,24}]hGhrelin(16-28). Kex2 may recognize $Q_{14}R_{15}$ and cleave the C terminal of it. As mentioned in Bevan et al.[18], however, cleavage efficiency of Gln-Arg is much lower than Arg-Arg. We could see this byproduct only 5% compared with authentic peak B in our method.

The peptide was further purified by reversed-phase chromatography in order to obtain a highly purified product (Figure 7(I) B). The exact amino acid composition and molecular mass were determined (Table III).

Because this fragment was protected by Boc groups and could possibly be deprotected in the presence of a strong acid, all purification steps were performed in weakly acidic or weakly basic conditions (data not shown). The yield of each process is shown in Table V. The yield in Table V is based on molar yield equivalent to [Lys(Boc)^{16,19,20,24}]hGhrelin(8-28).

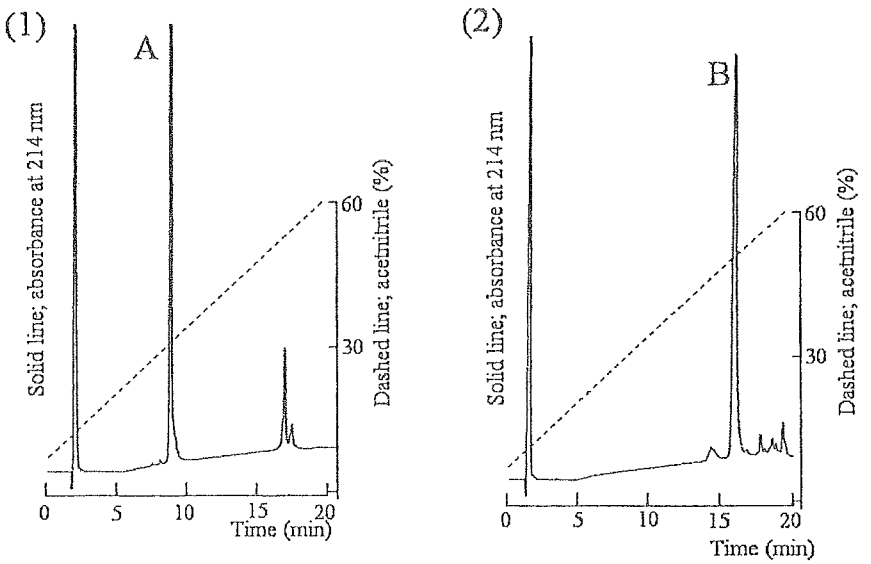

FIGURE 5 HPLC profile of peptides in Boc reaction. (1) HPLC profile before Boc reaction. (2) HPLC profile after Boc reaction. (A) Unprotected human ghrelin(8-28) precursor; (B) [N^{α}-Boc, Lys(Boc)^{16,19,20,24}] hGhrelin(8-28) precursor. Analytical column was a YMC-Pack PROTEIN-C8.

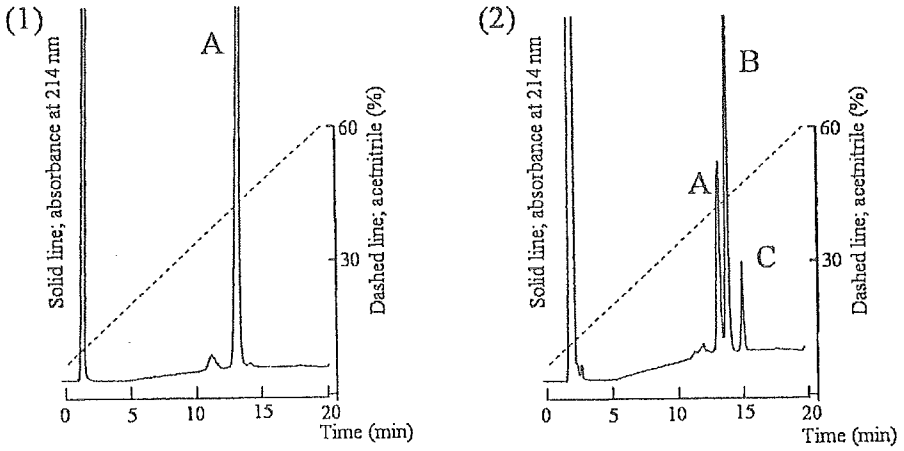

FIGURE 6 HPLC profile of peptides in Kex2 reaction. (1) HPLC profile before Kex2 reaction. (2) HPLC profile after Kex 2 reaction. New peak B was confirmed as [Lys(Boc)^{16,19,20,24}]hGhrelin (8-28) by mass spectrometry. (A) [N^{α}-Boc, Lys(Boc)^{16,19,20,24}] hGhrelin(8-28) precursor; (B) [Lys(Boc)^{16,19,20,24}] hGhrelin(8-28). (C) [Lys(Boc)^{16,19,20,24}] hGhrelin(16-28). Analytical column was a YMC-Pack ODS AP-302.

Condensation and Purification

Figure 7(1) shows the HPLC profiles of the condensation reaction. Peak C (protected human ghrelin) appeared in place of the disappearing peak A ([N^{α}-Boc, Ser(tBu)^{2,6}] hGhrelin(1-7)) and peak B ([Lys(Boc)^{16,19,20,24}]hGhrelin(8-28)) during the reaction. Figure 7(1) D shows the HPLC profile after TFA deprotection.

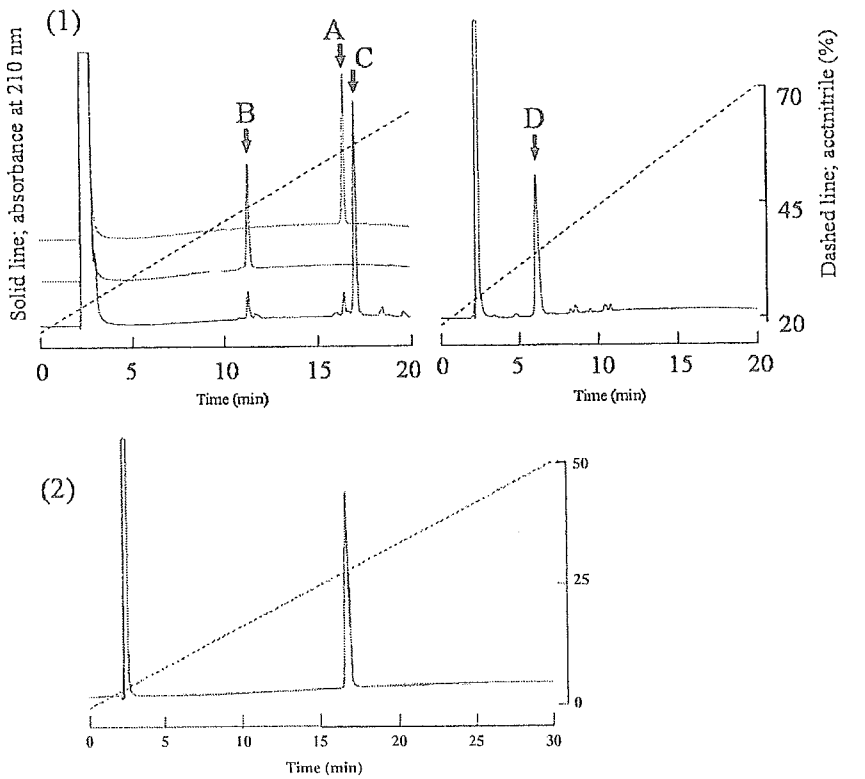

FIGURE 7 HPLC profile of [N^{α}-Boc, Ser(tBu)^{2,6}]hGhrelin(1-7), [Lys(Boc)^{16,19,20,24}]hGhrelin (8-28), and human ghrelin. (1) Purified (A) [N^{α}-Boc, Ser(tBu)^{2,6}]hGhrelin(1-7), (B) [Lys (Boc)^{16,19,20,24}]hGhrelin(8-28), (C) protected human ghrelin after condensation, and (D) human ghrelin after deprotection by TFA. (2) Purified human ghrelin after two-column chromatographic purifications. Analytical column was a YMC-Pack PROTEIN-RP.

Table V Yield of [Lys(Boc)^{16,19,20,24}]hGhrelin(8-28) and hGhrelin(1-28)

Step	Yield (mmol)[a,b]	Yield at each step (%)[a,b]	Yield (%)[a,b]	Purity (%)[a]
Yield and purity of [Lys (Boc)^{16,19,20,24}] hGhrelin(8-28)				
OmpT reaction	3.57	—	—	—
Ion exchange chromatography	3.08	86.5	86.5	94.99
Boc reaction	3.03	98.1	84.8	—
Zn chelate chromatography	2.83	93.7	79.5	95.92
Kex2 reaction	2.62	92.5	73.5	—
RP-chromatography	2.00	76.5	56.2	97.73
Yield and purity of hGhrelin(1-28)				
After condensation and deprotection	1.0	—	—	76.77
Ion exchange chromatography	0.98	98.0	98.0	80.06
RP-chromatography	0.77	78.6	77.0	98.42

[a] Yield and purity are calculated by HPLC analysis compared with a standard sample.
[b] Yield is based on molar yield equivalent to [Lys(Boc)^{16,19,20,24}] hGhrelin(8-28) (top) or hGhrelin(1-28) (bottom).

We obtained crude ghrelin as a white, powdery peptide (purity of 77%). The crude peptide was purified by chromatography to higher than 98% purity (Figure 7(2)). Total yield was about 43% from the OmpT reaction and 77% from the deprotection (Table V).

It was determined that the semisynthetic ghrelin has an amino acid composition, molecular mass, and amino acid sequence identical to that of native human ghrelin (data not shown). Finally, we used GH releasing activity in vitro to check the bioactivity of the semisynthetic ghrelin. EC_{50} about calcium uptake activity of semisynthetic ghrelin (1.2 nM) was almost as same as that of the chemically synthesized one (1.5 nM). We found that human ghrelin prepared by this method was comparable in terms of structure and GH releasing activity with that of chemically synthesized ghrelin.

DISCUSSION

In this report, we present a semisynthesis production method for human ghrelin. There were three hurdles to overcome in establishing this method: 1) determination of a suitable peptide length for chemical synthesis that would be soluble and avoid racemization during condensation reactions; 2) finding appropriate masking groups for each coupling fragment; and 3) in the C-terminal fragment that does not possess the modification, the N-terminal α-amino group should be free, whereas other amino groups in the side chain should be properly protected to ensure effective ligation.

In the course of preparing the N-terminal fragment, we chose ghrelin(1-7) because its C-terminal amino acid is proline. We were able to introduce selectively and quantitatively an octanoyl group to the Ser3 using 2-chlorotrityl resin, known as a weak acid-cleavable resin. The advantage of this type of resin is to suppress the formation of diketopiperazine side reactions.[21] The advantages of our method are 1) all reactions take place on the solid-phase resin and 2) one precipitation with H_2O led to the desired product with high yield (91%) and high purity (90%).

Several strategies could be employed for preparation of C-terminal ghrelin suitable for coupling, such as chemical cleavage,[22,23] the formyl–deformyl method,[24] the thioester method,[25,26] and chemical ligation.[27] In this paper, however, we describe a method for construction of a partially protected peptide segment from expressed peptide using two distinct proteases and the use of this peptide for subsequent condensation with modified synthetic N-terminal segment. We used a truncated form of *E. coli* β-galactosidase as a fusion partner for the purpose of suppressing degradation of the desired peptide in the host cell. A linker sequence containing cleavage sites for the two proteases were inserted between the fusion partner and the C-terminal ghrelin to prepare the partially protected fragment. Several proteases are suitable for this method. In this instance, we used the *E. coli* OmpT protease[16,20] and the Kex2 protease derivative, Kex2-660, from *Saccaharomyces cerevisiae*.[17,28] These two enzymes are well characterized and their cleavage sites can be easily optimized. OmpT protease is an endogenous membrane-bound protease and cleaves a peptide bond at the center of a basic amino acid pair such as Arg-Arg, Lys-Lys, and Arg-Lys.[16,20] It belongs to the Omptin family that includes the OmpP protease of *E. coli* and a pgtE protease of *Salmonella*. It is said that OmpT recognizes

not only cleavage amino acids (P1 and P1′ sites), but also P2, P4, P6, and P2′ positional amino acids.[20,29,30] Especially, acidic amino acid and Pro have been reported as unacceptable amino acids at the P2′ position. In our case, P2′ positions of dibasic sequences were occupied with Glu or Pro except for the target cleavage site in the linker sequence. We suppose that is why OmpT exclusively cleaved the Arg-Arg sequence in the linker region.

Kex2-660 is a derivative of the Kex2 family, with representatives that include Furin and PC1/3. Kex2 cleaves the C-terminal side of a Lys-Arg, Arg-Arg, or Pro-Arg[18,19,28] pair. After several trials shown in Figure 3 and Table IV, we found the most suitable linker sequence for fermentation and cleavage by Kex2 and were able to prepare partially protected hGhrelin(8-28) with high purity and high yield by optimizing purification conditions. Furthermore, in the case of OmpT, it is not necessary to prepare this protease separately, since it is an endogenous protease existing in *E. coli* inclusion bodies.[20] We can possibly apply these enzymes to other peptides by optimizing cleavage sites and/or selecting for substrate specificity.

Preparing these highly purified, properly protected fragments enabled the use of a strong condensation reagent such as HBTU. In addition, the condensation of each peptide fragment has been optimized, and we can now produce ghrelin with a higher quality and a higher yield.

Based on these findings, our method can also possibly be applied to the production of physiologically active peptides with various modified structures such as acylation, glycosylation, and phosphorylation.

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Neuromedin S Is a Novel Anorexigenic Hormone

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A novel 36-amino acid neuropeptide, neuromedin S (NMS), has recently been identified in rat brain and has been shown to be an endogenous ligand for two orphan G protein-coupled receptors, FM-3/GPR66 and FM-4/TGR-1. These receptors have been identified as neuromedin U (NMU) receptor type 1 and type 2, respectively. In this study, the physiological role of the novel peptide, NMS, on feeding regulation was investigated. Intracerebroventricular (icv) injection of NMS decreased 12-h food intake during the dark period in rats. This anorexigenic effect was more potent and persistent than that observed with the same dose of NMU. Neuropeptide Y, ghrelin, and agouti-related protein-induced food intake was counteracted by coadministration of NMS. Icv administration of NMS increased proopiomelanocortin mRNA expression in the arcuate nucleus (Arc) and CRH mRNA in the paraventricular nucleus (PVN). Pretreatment with SHU9119 (antagonist for α-MSH) and α-helical corticotropin-releasing factor-(9–41) (antagonist for CRH) attenuated NMS-induced suppression of 24-h food intake. After icv injection of NMS, Fos-immunoreactive cells were detected in both the PVN and Arc. When neuronal multiple unit activity was recorded in the PVN before and after icv injection of NMS, a significant increase in firing rate was observed 5 min after administration, and this increase continued for 100 min. These results suggest that the novel peptide, NMS, may be a potent anorexigenic hormone in the hypothalamus, and that expression of proopiomelanocortin mRNA in the Arc and CRH mRNA in the PVN may be involved in NMS action on feeding. (*Endocrinology* 146: 4217–4223, 2005)

NEUROMEDIN U (NMU), originally isolated from porcine spinal cord, is a brain-gut peptide that has potent contractile activity on uterine smooth muscle (1). In previous studies, two orphan G protein-coupled receptors, FM-3/GPR66 and FM-4/TGR-1, were identified as NMU receptor type 1 (NMU1R) and type 2 (NMU2R), respectively (2–5). Recently, a novel 36-amino acid neuropeptide was identified in rat brain as another endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 using a reverse-pharmacological technique (6). This neuropeptide was designated neuromedin S (NMS) because it is specifically expressed in the suprachiasmatic nucleus (SCN). Although the NMS shares a C-terminal core structure (seven-amino acid residues) with NMU and activates both recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells, NMS is not a splice variant of NMU because both NMS and NMU genes were mapped to discrete chromosomes. In addition, although NMU mRNA was detected in peripheral and central organs (7), the distribution of NMS was limited to the testis, spleen and SCN (6). NMS was recently suggested to be involved in circadian oscillation systems because intracerebroventricular (icv) administration of NMS induces phase-dependent phase shifts in the circadian rhythm of locomotor activity in rats kept under constant darkness (6).

NMU1R is located in a wide range of peripheral tissues such as intestine, testis, pancreas, uterus, lung, and kidney. On the other hand, expression of NMU2R is limited to areas of the brain such as the paraventricular nucleus (PVN), along the wall of the third ventricle in the hypothalamus and the CA1 region of the hippocampus (2, 5, 8, 9). Immunohistochemical and *in situ* analysis has revealed NMU-immunoreactive neurons or NMU mRNA expression in the ventromedial hypothalamic region including the arcuate nucleus (Arc), pituitary, caudal brainstem region including the nucleus of the solitary tract, area postrema, dorsal motor nucleus of the vagus nerve and inferior olive, and spinal cord (2, 10, 11). NMU-immunoreactive fibers project prominently into the PVN, ventromedial nucleus, dorsomedial nucleus, and Arc. It has been well documented that the PVN and Arc of the hypothalamus play pivotal roles in the regulation of feeding behavior through a complex neuronal network composed of several orexigenic neuropeptides such as neuropeptide Y (NPY), agouti-related protein (AGRP) and ghrelin, and anorexigenic neuropeptides such as α-MSH, cocaine- and amphetamine-regulated transcript, CRH, and leptin (12, 13). Icv administration of NMU suppresses both dark-phase food intake and fasting-induced feeding, suggesting that NMU acts as anorexigenic hormone (2, 3). Conversely, disruption of the *NMU* gene in mice [NMU knockout (KO) mice] resulted in severe obesity (14). Although ob/ob mice (mutant leptin-deficient mice) are known to be obese through a decrease in proopiomelanocortin (POMC) mRNA and an in-

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Abbreviations: AGRP, Agouti-related protein; Arc, arcuate nucleus; α-hCRF, α-helical corticotropin-releasing factor-(9–41); icv, intracerebroventricular; KO, knockout; MUA, multiple-unit activity; NMS, neuromedin S; NMU, neuromedin U; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus.

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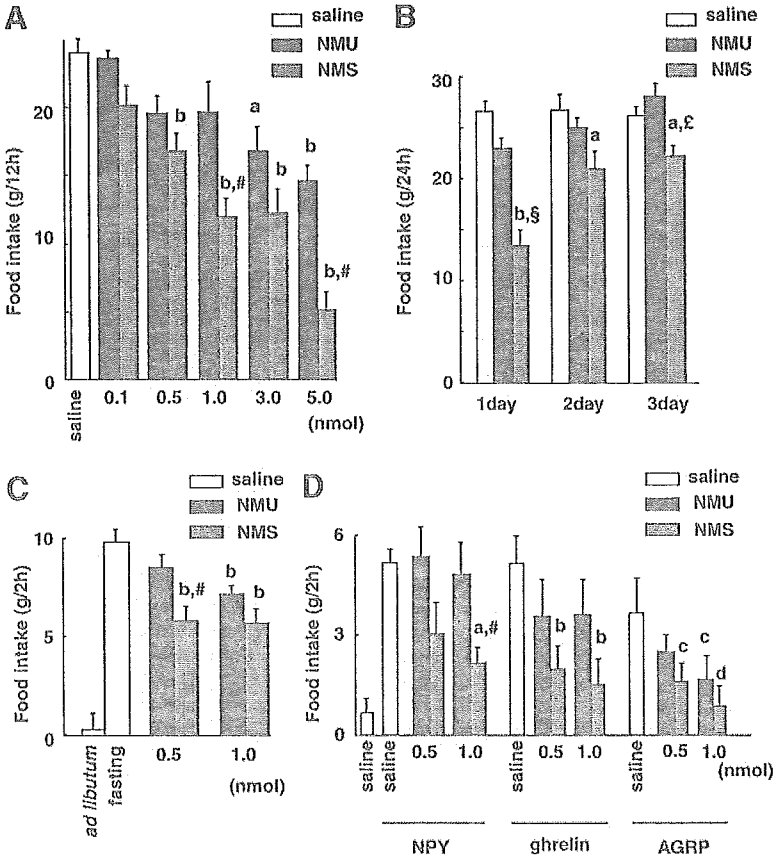

FIG. 1. Comparison of food intake after intracerebroventricular administration of NMS and NMU to rats (n = 8–10 in each group). Each *bar* and *vertical line* represents the mean ± SEM. A, Dark-phase feeding. Food intake of free-feeding rats was examined during a period of 12 h from 1900 h to 0700 h. Each reagent was injected at 1845 h (a, $P < 0.05$; b, $P < 0.01$ *vs.* saline; #, $P < 0.01$ *vs.* NMU at the same dose as NMS). B, The inhibitory effects on food intake after injection. Food intake was examined over a period of 24 h from 1900 h to 1900 h for 3 d after injection. Each reagent (1 nmol) was injected at 1845 h. (a, $P < 0.05$; b, $P < 0.01$ *vs.* saline; £, $P < 0.05$; §, $P < 0.01$ *vs.* NMU at the same dose as NMS). C, Two-hour food intake in rats that had fasted for 8 h and then received each reagent at 0900 h (b, $P < 0.01$ *vs.* saline in fasting rats; #, $P < 0.01$ *vs.* NMU at same dose as NMS in fasting rats). D, Effect of coadministration of NPY (0.5 nmol), ghrelin (0.5 nmol), or AGRP (1 nmol) with NMU or NMS on 2-h food intake in free-feeding rats. Each reagent was injected at 0845 h (a, $P < 0.05$ *vs.* NPY + saline; #, $P < 0.05$ *vs.* NPY + 1.0 nmol NMU; b, $P < 0.01$ *vs.* ghrelin + saline; c, $P < 0.05$; d, $P < 0.01$ *vs.* AGRP + saline).

crease of NPY and AGRP mRNA in the Arc (15–17), obesity in NMU KO mice results specifically from a decrease of CRH mRNA in the PVN. Therefore, NMU and leptin share the mechanism of feeding suppression (14).

The fact that receptors for NMU have a high affinity for NMS suggests that NMS may also act on feeding. The *NMS* gene was mapped to chromosome 2q11.2 in humans, and this locus is consistent with one potential location of the quantitative trait loci implicated in obesity (18). These data also lead to speculation that NMS may play an important role in central regulation of feeding.

To examine whether NMS is involved in feeding regulation, the effects of central administration of NMS and NMU on food intake were investigated in rats, and the cellular mechanisms involved were analyzed.

Materials and Methods

Animals

Male Wistar rats (Charles River Japan, Inc., Yokohama, Japan), weighing 300–350 g, were housed in individual Plexiglas cages in an animal room maintained under a constant light-dark cycle (light on from 0700–1900 h) and temperature (22 ± 1 C) for at least 1 wk. Food and water were provided *ad libitum* except during the fasting experiments. All procedures were done in accordance with the Japanese Physiological Society's guidelines for animal care.

Feeding experiments

Cannulation for icv injection was performed described previously (19). After surgery, all rats were housed individually in Plexiglas cages. During a 6-d postoperative recovery, the rats became accustomed to the handling procedure. In the first experiment, various doses of rat NMS and NMU were dissolved in saline, and 10 μl of solution was injected through a 27-gauge injection cannula connected to a 50-μl Hamilton syringe into each free-moving rat at 1845 h; 12-h food intake was then examined. We also examined the diurnal effect of NMS on food intake by icv injection of NMS at 0900 h. Rat NMS and NMU were synthesized by an Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems, Foster City, CA). In the second experiment, rats were fasted for 8 h from 0100 h at night, and then centrally injected with NMS or NMU (0.5 or 1 nmol) at 0845 h. In the third experiment, single NPY (0.5 nmol), ghrelin (0.5 nmol) or AGRP (1 nmol), and mixed NPY, ghrelin or AGRP + NMS (0.5 or 1 nmol) or NMU (0.5 or 1 nmol) (each peptide was mixed in 10 μl of saline solution) was administered to free-feeding rats at 0845 h and 2-h food intake was measured. NPY, ghrelin and AGRP were purchased from the Peptide Institute, Inc. (Osaka, Japan). In the fourth experiment, 1 nmol NMS was injected 1 h after pretreatment with 1, 5, or 10 μg α-helical corticotropin-releasing factor-(9–41) (α-hCRF) (Sigma, St. Louis, MO) or 0.1, 0.5, or 1 nmol SHU9119 (Bachem, Budendorfm, Switzerland) at 0745 h to 8-h fasted rats or intact rats, and 2-h and 24-h food intake was examined, respectively.

c-Fos immunohistochemistry

Ninety minutes before perfusion, rats were injected with NMS, NMU (1 nmol per rat) or saline (n = 3 per group) in the lateral ventricle to study the immunostaining of c-Fos-expressing neurons. After the rats had been perfused with fixative [4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4)], the brain was removed immediately, fixed in fixative and embedded in O.C.T. compound (Tissue-Tek, Tokyo, Japan) at −20 C. Frozen serial brain sections (40 μm thick) were incubated for 1 d with goat anti-c-Fos antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; final dilution 1:1500) and visualized by the avidin-biotin complex method (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) using 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.005% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6).

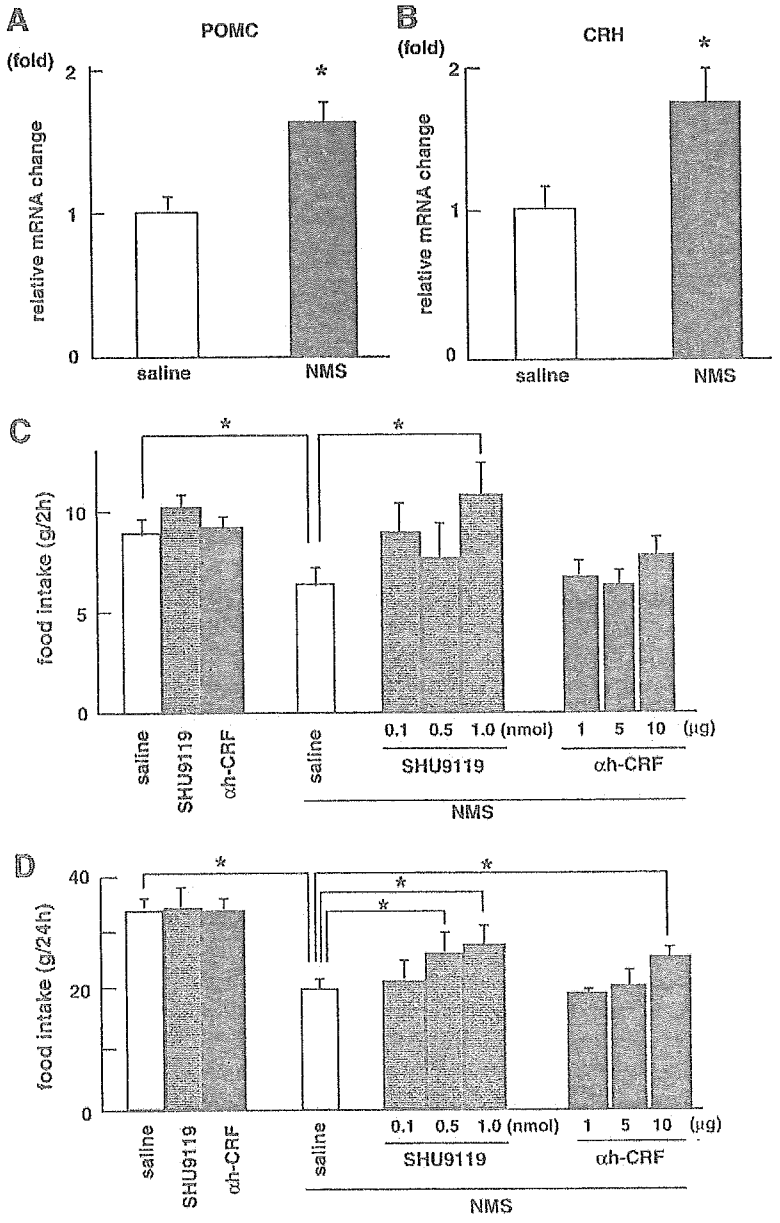

FIG. 2. Involvement of α-MSH and CRH in NMS-elicited feeding behavior. Quantitative RT-PCR on NMS administered rats (n = 8 per group). Level of POMC mRNA (A) and CRH mRNA (B). C, Effect of pretreatments with α-MSH antagonist (SHU9119) or CRH antagonist [α-helical CRF-(9–41)] on food intake reduction by NMS in 8-h fasted rats. Each antagonist was injected at 0745 h, and then NMS was injected at 0845 h. Food intake was examined during a 2-h period from 0900–1100 h. *Asterisks* indicate the significant difference ($P < 0.05$). D, Effect of pretreatments with α-MSH antagonist (SHU9119) or CRH antagonist [α-helical CRF-(9–41)] on food intake reduction by NMS in intact rats. Each antagonist was injected at 0745 h, and then NMS was injected at 0845 h, then, 24-h food intake was examined. *Asterisks* indicate the significant difference ($P < 0.05$).

Quantitative RT-PCR

To quantify POMC and CRH mRNA in the Arc and PVN after icv injection of NMS, 1 nmol NMS was injected into rats at 1845 h, 4 h before collection of Arc and PVN tissue for mRNA extraction. After the brain tissues had been frozen, the Arc and PVN were dissected out. Total RNA was extracted from the Arc and PVN using an RNeasy Mini kit (QIAGEN, Hilden, Germany) and then synthesized into first-strand cDNA. Quantitative RT-PCR was conducted with a LightCycler system (Roche, Basel, Switzerland) using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche). The primer set used for rat POMC was 5′-GACCTCACCACGGAAAGCAACCTG-3′ and 5′-ACTTCCGGGGATTTTCAGTCAAGGG-3′, and for rat CRH was 5′-ATCTCACCTTCCACCTTCTG-3′ and 5′-GTGTGCTAAATGCAGAATCG-3′. Known amounts of rat POMC and CRH cDNA were used to obtain a standard curve. Rat glyceraldehyde-3-phosphate dehydrogenase mRNA was also measured as an internal control. The primer set used for rat glyceraldehyde-3-phosphate dehydrogenase was 5′-CGGCAAGTTCAACGGCACA-3′ and 5′-AGACGCCAGTAGACTCCACGACA-3′.

Multiple unit activity (MUA) recording

Rats were fitted with chronically implanted electrode arrays as described previously (20). Briefly, the electrode assembly consisted of four 75-mm Teflon-insulated platinum (90%)-iridium (10%) wires (A-M Systems, Inc., Sequim, WA) encased in a stainless steel guide tube (650 mm diameter; Inter Medical, Fukuoka, Japan). The stainless steel tube served as a ground. The impedance of each platinum-iridium electrode measured at 1 kHz was 50–100 kΩ. According to the stereotaxic atlas of the rat brain (Paxinos and Watson, Ref. 27) described by Albe-Fessard *et al.*, the electrodes were implanted unilaterally into the left side of the PVN and fixed to the skull with anchor screws and dental cement. At the same time, an icv cannula was implanted slantingly into the right lateral cerebral ventricle. After a recovery period of 5 d, MUA was recorded as follows: signals were passed through a buffer amplifier, amplified by a biophysical amplifier (MEG-2100; Nihon Kohden, Tokyo, Japan) with low and high cutoff frequencies of 500 Hz and 10 kHz, respectively, and displayed on an oscilloscope (DS-8812; Iwatsu, Tokyo, Japan). Neural spikes were discriminated by their amplitude, and the number of spikes was counted with a pulse counter (ET-612J; Nihon Kohden) and inte-

grated for 1 sec. Outputs were recorded as a histogram on a thermal recorder (WR8500; Graphtec, Tokyo, Japan) and with a powerLab (AD Instrument, Castle Hill, Australia), respectively. On the day of the experiment, the MUA electrode was attached to the buffer amplifier under isoflurane inhalation anesthesia (Univentor 400; Univentor, Zejtun, Malta). Rats were maintained under anesthesia with 1.5% isoflurane (Abbott Laboratories, Abbott Park, IL). At 15 min after the beginning of stable MUA volley, rats received icv administration of 1 nmol NMS, NMU, or saline. At 120 min after administration, electrical stimulation was applied for 1 sec through the MUA electrode with pulses (1 mA) from an electric stimulator (RGF-4A; Radionics, Burlington, MA) to check the site of the electrode.

Statistical analysis

The data (mean ± SEM) were analyzed statistically by ANOVA with the *post hoc* Fisher's test. $P < 0.05$ was considered statistically significant.

Results

Intracerebroventricular injection of NMS reduced 12-h food intake during the dark period in a dose-dependent manner (Fig. 1A). This effect of NMS was more potent than that of NMU because a smaller dose of NMS was effective at suppressing feeding (Fig. 1A). We also measured 12-h water intake after NMS or saline injection before the onset of dark period. A quantity of 1 nmol of NMS, but not 0.5 nmol, significantly decreased water intake during dark phase [NMS 1 nmol, 34.75 ± 3.26 ml ($P < 0.05$ *vs.* saline); 0.5 nmol, 44.74 ± 4.89 ml; saline, 47.17 ± 4.54 ml]. Although feeding suppression by 1 nmol NMU recovered completely within 2 d, suppression by the same dose of NMS continued at least for 3 d starting from 1845 h (Fig. 1B). Icv injection of 1 nmol NMS and NMU into 8-h fasted rats also resulted in a decrease in food intake for 2 h. On the other hand, at a dose of 0.5 nmol, only NMS injection suppressed food intake (Fig. 1C).

Although icv injection of NPY, ghrelin, and AGRP significantly increased food intake, this peptide-induced food intake was reduced by coadministration of NMS or NMU (Fig. 1D). In these cases, the suppressive effect with NMS was more potent than that with NMU. We also examined the diurnal effect of NMS on food intake by icv injection of NMS at 0900 h. There was no significant difference in food intake during the 12-h light period on the first, second, and third day between the NMS- and saline-treated groups (first 12-h light period 1.9 ± 0.62 *vs.* 2.4 ± 0.64 g; second 12-h light period 2.4 ± 0.52 *vs.* 2.5 ± 0.44 g; third 12-h light period 2.5 ± 0.72 *vs.* 2.4 ± 0.48 g; NMS *vs.* saline). However, NMS suppressed significantly 12 h dark food intake for 3 d starting from 0900 h.

To understand the cellular mechanisms involved in NMS-induced suppression of feeding, POMC and CRH mRNA expression and the expression of c-Fos protein were investigated. Icv administration of NMS augmented the levels of Arc POMC and PVN CRH mRNA (Fig. 2, A and B). The involvement of POMC and CRH in NMS-induced suppression of feeding was therefore investigated using an antagonist for these peptides. Pretreatment with both SHU9119 (an antagonist for α-MSH) and α-hCRF (an antagonist for CRH) attenuated NMS-induced suppression of food intake in a dose-dependent manner in fasted rats. Whereas only SHU9119 significantly blocked the effect of NMS on 2-h food intake (Fig. 2C), both α-hCRF and SHU9119 blocked the effect of NMS on 24-h food intake (Fig. 2D). The central distributions of c-Fos immunoreactive cell were similar in NMS- and NMU-injected rats. The hypothalamic PVN (Fig. 3, A and D), Arc (Fig. 3, B and E), supraoptic nucleus (Fig. 3, C and F) and SCN (data not shown) expressed the c-Fos protein strongly. In saline-treated rats, no c-Fos immunoreactivity was observed in any of these regions (data not shown).

Neuronal electrical activity in the PVN was then measured before and after icv administration of 1 nmol NMS and NMU using a MUA recording system. This method has practical advantages, in that continuous and real-time analysis of hypothalamic neural activity can performed *in vivo*. In the frequency-time histograms, MUA could be influenced within 5 min by NMS and NMU (Fig. 4, A and B). The most active MUA induced by NMS was observed between 20 min and 100 min and decreased gradually thereafter. Although NMU also increased MUA immediately after injection, the effect was weaker than that of NMS. We analyzed the total spike count at 30-min intervals for 120 min (Fig. 4C). Although a significant increase in the spike count was observed only between 30 and 60 min after icv injection of NMU, the increase continued for at least 120 min in NMS-treated rats. As shown in Fig. 4D, the recording sites of these MUA volleys were located adjacent to the PVN.

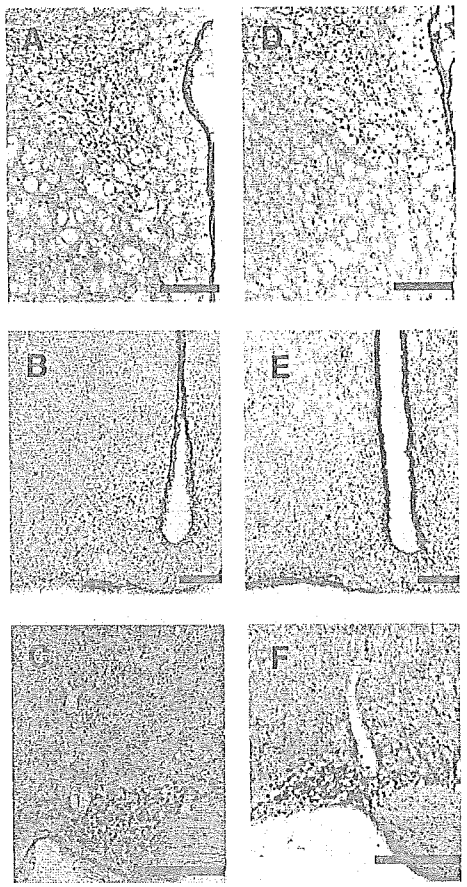

FIG. 3. Representative photomicrographs of c-Fos-immunoreactive cell nuclei in selected brain regions after administration of 1 nmol NMS (A–C) or NMU (D–F). The brain regions shown include the PVN (A and D), Arc (B and E), and supraoptic nucleus (C and F). *Scale bar*, 200 μm (for all panels).

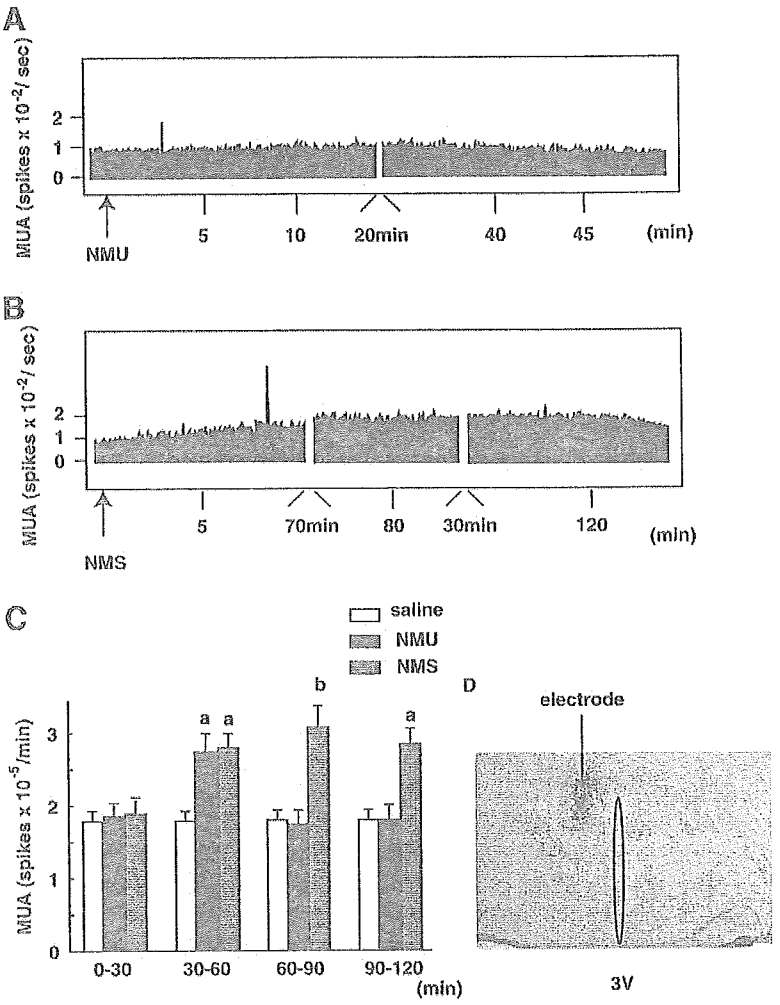

FIG. 4. Effect of icv injection of NMS or NMU on MUA in the PVN. A and B, Frequency-time histograms of firing rate of the PVN after administration of NMS or NMU in representative rats. NMS (A) or NMU (B) was injected 15 min (*arrows*) after stable MUA volley. Abscissa: time (min), ordinate: spike/sec. MUA profiles in one representative rat are shown. C, Summary of the effects of icv injection of NMS or NMU on MUA volley frequency at 30-min intervals for 2-h. Each *column* and *vertical bar* represents the mean ± SEM (n = 3–4) (a, $P < 0.05$ *vs.* saline; b, $P < 0.01$ *vs.* saline). D, Location of the MUA electrode tip. Recording sites of MUA volleys were located adjacent to the PVN.

Discussion

In the present study, the novel peptide, NMS, was demonstrated to be a potent anorexigenic hormone in rats. Central administration of NMS reduced the daily dark period food intake and 8-h fasting-induced food intake. This suppression of feeding is unlikely to be due to any side effects of NMS because NMS-injected rats did not show any abnormal behavior (such as glooming behavior, searching behavior, attaching behavior, and barrel rolling). Although NMU has been well documented to reduce food intake in rats (2, 3), the relative potency of NMS on suppression of food intake was stronger than that of NMU because a smaller dose of NMS significantly suppressed food intake. Considering that NMS contains the core active C terminus of NMU and binds to the same receptors (NMU1R and NMU2R) as NMU (6), NMS-induced suppression of food intake can be assumed. In a previous study, the distribution of NMS mRNA was investigated in various rat tissues by quantitative RT-PCR (6). NMS mRNA was expressed mainly in the hypothalamus, spleen, and testis. In the hypothalamus, however, NMS mRNA was expressed predominantly in the SCN, with only very slight expression in other brain regions including the PVN and Arc. *In situ* hybridization histochemistry also showed that NMS mRNA expression was restricted to the SCN. No hybridization signal was observed in any other brain region. The fact that the relative potency of NMS in suppressing food intake was stronger than that of NMU despite the lower expression of NMS mRNA than NMU mRNA in the PVN and Arc suggests that the feeding regulation effect may differ between NMS and NMU. Especially, in the case of NMS, its action on the PVN and Arc through the NMS projection from the SCN may be important.

When the NMS was injected at 0900 h, there was no significant difference in food intake during the 12-h light period, suggesting the diurnal variation in the anorexigenic effect of NMS. Although the interpretation of these data is difficult because of the very low feeding activity in the beginning of the light period, this diurnal difference may be due to diurnal variation of NMU receptors in SCN (21) or diurnal variation of NMS secretion (6) in autocrine regulation.

It is not known why NMS-induced suppression of food intake is more potent and continues for a longer time than with NMU. There was no difference in the distribution of c-Fos expression between NMS- and NMU-injected rats. However, neural MUA records showed a clear difference between the rats. There was a greater increase in firing rate of PVN neurons in NMS-treated rats than in NMU-treated rats, and this increased effect continued for a long period of

time after NMS injection. This potent and long-term increase of firing rate by NMS may cause the powerful and long-term suppression of food intake. Alternatively, the possibility that NMS may act on another unknown receptor cannot be excluded.

NPY, ghrelin, and AGRP-induced food intake was counteracted by coadministration of NMS, suggesting that the NPY, ghrelin, and AGRP are independently antagonistic with NMS for feeding regulation.

Hanada *et al.* (14) reported that icv injection of NMU in rats did not affect POMC mRNA expression in the Arc but augmented CRH mRNA expression in the PVN. In addition, CRH KO mice did not show any reduction in food intake after NMU injection (22). Therefore, it has been speculated that an increase in CRH, but not α-MSH, is the primary cause of NMU-induced suppression of food intake. In the present study, NMS increased both POMC and CRH mRNA expression. These results indicate that the cellular mechanism of suppression of food intake by NMS may be different from that by NMU, and both CRH and α-MSH may be involved in NMS-induced suppression of food intake. This hypothesis is supported by the following results: pretreatment with antagonists for α-MSH and CRH blocked NMS-induced suppression of food intake.

It is questionable why receptors for NMS and NMU are the same; nevertheless, the downstream mechanism of feeding regulation by NMS and NMU is different. Recent studies demonstrate that NMU, NMS, NMU1R, and NMU2R mRNA each have an intrinsic rhythmic expression in the SCN with a different circadian pattern (6, 21). Because the SCN sends neural projections into the PVN and Arc (23, 24), these different rhythmic expressions may relate to the different effects of NMS and NMU. Of course, as mentioned above, NMS may act on a receptor other than NMU1R and NMU2R. Either way, it is unknown why NMS, but not NMU, stimulates the POMC system in the Arc, but a different downstream mechanism may explain the difference in effectiveness and duration of action between NMU and NMS.

Wren *et al.* (25) reported that leptin was able to stimulate NMU release in hypothalamic explants *in vitro*. In contrast, Hanada *et al.* (14). showed that the anorexigenic effect of NMU is independent of leptin in NMU KO mice because NMU and leptin reduced food intake in ob/ob mice and NMU KO mice, respectively. Wren *et al.* measured NMU content using an antibody raised in a rabbit immunized with synthetic NMU-8. Because NMU-8 is the core active C terminus of NMS and NMU, the antibody must recognize both NMS and NMU. We had also raised antiserum against synthetic NMU-8 and established a RIA for NMU (26). Rat NMS and NMU were equally recognized with the serum on a molar basis (data not shown) and could not separate NMS and NMU in this RIA system. Therefore, NMU release stimulated by leptin in hypothalamic explants presented by Wren *et al.* might be NMS. If this is the case, NMS is the downstream signal pathway for leptin. NMS is a novel anorexigenic hormone, and further investigation of the function of NMS will help in our understanding of weight control mechanisms and should facilitate the study of eating disorders.

Acknowledgments

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Peripheral Interaction of Ghrelin with Cholecystokinin on Feeding Regulation

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Ghrelin and cholecystokinin (CCK) are gastrointestinal hormones regulating feeding. Both transmitted via the vagal afferent, ghrelin elicits starvation signals, whereas CCK induces satiety signals. We investigated the interaction between ghrelin and CCK functioning in short-term regulation of feeding in Otsuka Long-Evans Tokushima fatty (OLETF) rats, which have a disrupted CCK type A receptor (CCK-AR), and their lean littermates, Long-Evans Tokushima Otsuka (LETO) rats. Intravenous administration of ghrelin increased 2-h food intake in both OLETF and LETO rats. Because OLETF rats are CCK insensitive, iv-administered CCK decreased 2-h food intake in LETO, but not in OLETF, rats. Although preadministration of CCK to LETO rats blocked food intake induced by ghrelin, CCK preadministration to OLETF rats did not affect ghrelin-induced food intake. Conversely, preadministration of ghrelin to LETO rats blocked feeding reductions induced by CCK. In electrophysiological studies, once gastric vagal afferent discharges were altered by ghrelin or CCK administration, they could not be additionally affected by serial administrations of either CCK or ghrelin, respectively. The induction of Fos expression in the hypothalamic arcuate nucleus by ghrelin was also attenuated by CCK preadministration. Using immunohistochemistry, we also demonstrated the colocalization of GH secretagogue receptor (GHS-R), the cellular receptor for ghrelin, with CCK-AR in vagal afferent neurons. These results indicate that the vagus nerve plays a crucial role in determining peripheral energy balance. The efficiency of ghrelin and CCK signal transduction may depend on the balance of their respective plasma concentration and/or on interactions between GHS-R and CCK-AR. (*Endocrinology* 146: 3518–3525, 2005)

IN ADULT ANIMALS and humans, body weight usually remains within a relatively narrow range, despite large day-to-day changes in the amount of food consumed. Even when the restriction of food intake or excessive overfeeding induces changes in body adiposity, both body weight and adiposity in humans and animals return to baseline levels after the resumption of regular feeding (1, 2, 3). Multiple peripheral signals (*e.g.* nutrients, nutrient metabolites, or hormones) regulate short-term and long-term food intake and energy balance through diverse but interacting pathways (4). Signals affecting short-term food uptake have significantly different mechanisms than the long-term regulators of energy homeostasis activated in proportion to both body adipose stores and the food consumed over prolonged periods.

Using an intracellular calcium assay of stable cell lines expressing rat GH secretagogue receptor (GHS-R), we recently discovered in rat stomach a novel endogenous ligand for the GHS-R (5) named ghrelin. Ghrelin, produced primarily in endocrine cells of the stomach, is released into circulation (5–7). Whereas multiple gastrointestinal hormones have been implicated in feeding regulation (8–12), ghrelin stimulates appetite, food intake, and GH secretion when administered to humans and rodents (5, 13–17). In humans, the circulating ghrelin levels increase before and decrease after every meal (18–22), suggesting that ghrelin functions as a meal initiator. The effect of ghrelin on feeding is rapid and short-lived, implying that ghrelin functions in short-term regulation of feeding. The inverse correlation between ghrelin levels and body mass index, as well as ghrelin-mediated promotion of adipogenesis, suggests that ghrelin may also participate in long-term regulation of body weight (14, 19, 23–27).

Most gastrointestinal hormones regulating feeding, with the exception of ghrelin, inhibit food intake (28, 29). Cholecystokinin (CCK) decreases meal size in rats and humans when administered peripherally (30–32). This peptide, released from the proximal small intestine, functions as a postprandial satiety signal (33–36). The anorectic effect of CCK is also rapid and short-lived; long-term peripheral administration of CCK does not reduce overall food intake or induce maintained weight loss (37). These results suggest that CCK plays an essential role in the short-term regulation of feeding.

Although ghrelin has an opposite effect on feeding as CCK, this peptide exhibits characteristics similar to CCK on the short-term regulation of feeding. Both ghrelin and CCK, after release from the gastrointestinal tract, transmit starva-

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Abbreviations: CCK, Cholecystokinin; CCK-AR, CCK type A receptor; GHS-R, GH secretagogue receptor; LETO, Long-Evans Tokushima Otsuka; NPY, neuropeptide Y; NTS, the nucleus of the solitary tract; OLETF, Otsuka Long-Evans Tokushima fatty; PBN, parabranchial nucleus.

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tion and satiety signals to the brain through receptors, GHS-R and CCK type A receptor (CCK-AR), respectively, located in the vagal capsaicin-sensitive afferents (38–42). Thus, vagal afferent fibers represent a major target of these peripheral feeding regulators, ghrelin and CCK.

In this study, we examined the functional relationship between ghrelin and CCK in the short-term regulation of food intake using CCK-AR-deficient Otsuka Long-Evans Tokushima fatty (OLETF) rats and their lean littermates, Long-Evans Tokushima Otsuka (LETO) rats. We also investigated the colocalization of GHS-R with CCK-AR in rat vagal afferents. Because iv administration of ghrelin induces Fos expression in the hypothalamic arcuate nucleus of rats through gastric vagal afferents, we examined the induction of Fos expression in the arcuate nucleus by iv administration of ghrelin after CCK treatment. The electrical discharge of gastric vagal afferents is attenuated by ghrelin and stimulated by CCK (38, 42–48). In this study, we evaluated changes in vagal afferent firing induced by iv treatment of ghrelin and CCK after CCK and ghrelin administration, respectively.

Materials and Methods

Animals

Ten-week-old OLETF and lean littermate LETO rats (body weight: OLETF, 403.2 ± 6.0 g; LETO, 392.8 ± 2.6 g; $P > 0.1$; n = 20), obtained from Otsuka Pharmaceutical (Tokushima, Japan), were used in the experiments for feeding. Male Wistar rats (body weight: 361.6 ± 1.3 g; n = 20) (Charles River Japan, Inc., Shiga, Japan) were used for immunohistochemistry, Fos expression, and electrophysiological studies. Rats were housed individually in plastic cages at constant room temperature in a 12-h light (0800–2000)/12-h dark cycle. Animals were given standard laboratory chow and water *ad libitum*. Intravenous cannulas were implanted into the right jugular vein under anesthesia after an ip injection of sodium pentobarbital (80 mg/kg body weight) (Abbott Laboratories, Chicago, IL). Rats were sham-injected before the study and weighed and handled daily. We also injected heparin daily (1 U/100 μl 0.9% saline) into the cannulas of the animals to prevent coagulation. Only animals exhibiting progressive weight gain after surgery were used in subsequent experiments. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Preparation of anti-GHS-R serum

The [Cys0]-rat GHS-R [342–364] peptide was synthesized using the Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems, Foster City, CA), then purified by reverse phase-HPLC. The synthesized peptide (10 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (6 mg) (mcKLH; Pierce, Rockford, IL) in conjugation buffer (Pierce). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and was used to immunize New Zealand white rabbits by intracutaneous and sc injection. Animals were boosted every 2 wk and bled 7 d after each injection. The specificity of the antisera was confirmed by immunoreactivity of GHS-R-expressing (CHO-GHSR62 cells), but not of control cells.

Feeding experiments

Experiments were performed 1 wk after iv cannulation. First, CCK (Peptide Institute, Inc., Osaka, Japan) was dissolved in 0.9% saline, and this solution (10 pmol-5 nmol/100 μl) was administered iv at 1000 h to LETO rats after fasting for an 8-h period to determine the lowest effective dose of CCK on feeding (n = 10 per group). Second, a solution of rat ghrelin (Peptide Institute) dissolved in 0.9% saline (1.5 nmol/100 μl or 3 nmol/100 μl) was administered iv at 1000 h to OLETF and LETO rats fed *ad libitum* (n = 10 per group). After injection, rats were immediately returned to their cages, then 2-h food intake was measured. Third, a solution of CCK dissolved in 0.9% saline (1 nmol/100 μl) was administered iv at 1000 h to OLETF and LETO rats after fasting for an 8-h period (n = 10 per group). After returning rats to their cages immediately after injection, 2-h total food intake was measured. Fourth, after fasting for an 8-h period, OLETF and LETO rats were treated with CCK (1 nmol/100 μl). They were not given any food until the next injections. Animals were subsequently given ghrelin (3 nmol/100 μl) or saline (100 μl) 30 min after CCK injection (n = 10 per group). After ghrelin or saline injection, 2-h food intake was measured. Fifth, after an 8-h fasting period, LETO rats were first treated with ghrelin (3 nmol/100 μl), then subsequently given CCK (1 nmol/100 μl) or saline (100 μl) 30 min after ghrelin injection (n = 10 per group). The rats were fasted between ghrelin and CCK or saline injections. After this second injection, 2-h food intake was measured. These feeding studies were performed in a crossover design. Rats were allowed at least 4 d without injections between experimental days.

Immunohistochemical double-staining

Three Wistar rats, weighing 300–350 g, were perfused transcardially with 0.1 M phosphate buffer (pH 7.4), then with 4% paraformaldehyde in a 0.1 M phosphate buffer. The nodose ganglia were sectioned into 12 μm-thick slices at −20 C using a cryostat. Sections were stored at −80 C. Primary neurons were also obtained from the nodose ganglia of five Wistar rats, ranging from 5–6 wk of age. These neurons were submitted to collagenase dispersion as described (49, 50), then seeded and cultured for 4 d in polyethylenimine-coated Lab-Tek chamber slides (Electron Microscopy Sciences, Hatfield, PA) in complete DMEM (25 mM glucose) containing 5% newborn calf serum, 5% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 30 ng/ml nerve growth factor 2.5S (Sigma Chemical Co., St. Louis, MO), and 2 mM L-glutamine at 37 C in 5% CO_2. The medium was replaced every 2 d. Slides were washed in 0.01 M PBS (pH 7.4), then fixed in 10% formaldehyde. The slides of both the primary culture and sectioned nodose ganglia were incubated overnight at 4 C in rabbit anti-GHS-R antiserum (dilution 1/1000). Antibody staining was detected using Alexa Fluor 594-conjugated chicken antirabbit IgG (Molecular Probes, Inc., Eugene, OR). Samples were subsequently incubated with anti-CCK-AR antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; dilution 1/100), then with Alexa Fluor 488-conjugated donkey antigoat IgG (Molecular Probes, Inc.). Slides were observed by fluorescence microscopy (BH2-RFC; Olympus, Tokyo, Japan). The number of neurons expressing GHS-R or CCK-AR immunoreactivity in the nodose ganglion was quantified by counting two randomly selected visual fields in two sections from each of the three rats.

Fos expression and image analysis

The lowest effective dose of ghrelin or CCK on feeding was used for Fos expression studies. CCK (1 nmol/100 μl) or saline was injected iv into three male Wistar rats weighing 341.6 ± 1.3 g. Ghrelin (1.5 nmol/100 μl) was then injected into these rats 30 min after CCK or saline injection. Ninety minutes after ghrelin or saline injection, rats were perfused transcardially with fixative containing 4% paraformaldehyde. The brain was sectioned into 40-μm-thick samples. Immunohistochemistry of Fos was performed as described (51). Quantitation of Fos-immunoreactive cells in the nucleus of the solitary tract (NTS), parabranchial nucleus (PBN), and hypothalamic arcuate nucleus (bregma: −11.30 to −14.60 for NTS, −9.16 to −10.04 for PBN, −2.30 to −3.30 for the arcuate nucleus from Paxinos and Watson's rats brain atlas) was performed bilaterally. Fos-expressing cells of the arcuate nucleus for a 0.7-mm right triangle (0.245 mm^2) were counted in every fifth section (200 μm frequency) (five tissue sections per rats) using a cell counting program written for NIH Image (version 1.62; National Institutes of Health, Bethesda, MD).

Electrophysiological study

Multiunit neural discharge in gastric vagal afferent fibers was recorded extracellularly. Male Wistar rats, fasted for an 8-h period, were anesthetized by an ip injection of urethan (1 g/kg) (Sigma). The electrophysiological study was performed under anesthetization throughout. The rat trachea was intubated, and the electrocardiogram was recorded. Body temperature was maintained at 37 C. Standard methods of extracellular recording from vagal nerve filaments were used, as described in detail elsewhere (52). After laparotomy, a small catheter (Intramedic PE-10; Clay Adams, Parsippany, NJ) was inserted into the

inferior vena cava. After gastric branches of the vagus nerve were visualized, we placed filaments isolated from the peripheral cut end of the ventral branch for recording of afferent nerve activity on a pair of silver wire electrodes. Silver wire electrodes, connected through an alternating current-coupled differential amplifier (DAP-10E; Dia Medical Systems, Co., Tokyo, Japan) to an oscilloscope and magnetic tape recorder, were used for display and storage of the neural activity. A window discriminator (DSE-325A) converted spikes to constant amplitude pulses for analysis of spike frequency with a rate meter that reset at 5-sec intervals. Output from the rate meter was recorded on a chart paper (8K20 recorder; NEC-SAN E1 Co., Tokyo, Japan). Ghrelin (0.03 pmol–1.5 nmol/100 μl) or CCK (0.01 pmol–1 nmol/100 μl) was administered iv to rats through a catheter inserted into the inferior vena cava (n = 10 per group). After administration, nerve discharges from the multiunit afferents were recorded for 60 min and analyzed. In addition, CCK (1 nmol/100 μl) or ghrelin (1.5 nmol/100 μl), considered as a standard dose for feeding (38), was administered iv to rats through a catheter inserted into the inferior vena cava (n = 5 per group) before recording the multiunit afferent nerve discharges for 30 min. After the subsequent iv administration of either ghrelin (1.5 nmol/100 μl) or CCK (1 nmol/100 μl) to these rats, multiunit afferent nerve discharges were recorded for 30 min and analyzed.

Statistical analysis

Groups of data (mean ± SEM) were compared using ANOVA and *post hoc* Fisher's test. $P < 0.05$ was considered to be significant.

Results

Effects of ghrelin and CCK on feeding in OLETF or LETO rats

We first tested various doses of CCK ranging from 10 pmol to 5 nmol in a food intake experiment using LETO rats fasted for an 8-h period (Fig. 1A). The lowest effective dose of CCK administered iv was 1 nmol, which also applied to feeding examination using Wistar rats (data not shown). Therefore, we used 1 nmol as a standard dose of CCK in a subsequent series of experiments. The lowest effective dose of ghrelin on feeding in Wistar rats was 1.5 nmol (38); however, a single iv administration of 1.5 nmol ghrelin to OLETF and LETO rats did not induce feeding (LETO: saline, 0.35 ± 0.19 g; 1.5 nmol ghrelin, 0.28 ± 0.14 g; $P > 0.7$, n = 10; OLETF: saline, 0.36 ± 0.19 g; 1.5 nmol ghrelin, 0.33 ± 0.21 g; $P > 0.9$, n = 10). Because a single iv administration of 3.0 nmol ghrelin significantly increased food intake in both OLETF and LETO rats (Fig. 1B), we used this dose as a standard dose of ghrelin in feeding experiments. Although a single iv administration of CCK significantly decreased food intake in LETO rats, it did not affect food intake in CCK-AR-deficient OLETF rats (Fig. 1C). When CCK was administered iv to LETO rats 30 min before ghrelin administration, we could not observe a ghrelin-induced increase in food intake (Fig. 2A). Administration of CCK iv to OLETF rats 30 min before ghrelin treatment, however, induced similar increases in food intake as those seen in rats without pretreatment (Fig. 2A). Conversely, when ghrelin was administered iv to LETO rats 30 min before CCK treatment, the CCK-induced feeding reduction could not be observed (Fig. 2B).

Immunohistochemistry

GHS-R- and CCK-AR-immunoreactive neurons were found throughout the nodose ganglion (Fig. 3A). Approximately 70% of GHS-R-immunoreactive neurons in the nodose ganglion also expressed CCK-AR (Fig. 3, B–D). Double-staining studies also demonstrated the colocalization of GHS-R with CCK-AR in cultured nodose ganglion neurons (Fig. 3, E–G). GHS-R immunoreactivity was observed in GHS-R-expressing CHO cells (CHO-GHSR62 cells), but not in control CHO cells (data not shown). No GHS-R-specific immunoreactivity could be detected in the nodose ganglion using either normal rabbit serum or antiserum absorbed with excess synthetic GHS-R [342–364] (Fig. 3H).

Fos expression

A single iv administration of ghrelin induced Fos protein in the arcuate nucleus of the hypothalamus of rats (Fig. 4A). Fos-positive neurons were mainly distributed from the anterior to the middle region of the arcuate nucleus. Ghrelin-induced Fos expression in the NTS and PBN was not found (data not shown). When CCK was administered to rats iv 30 min before ghrelin treatment, the number of Fos-expressing neurons was significantly decreased compared with that induced by ghrelin alone (Fig. 4, B and E) and was not different from that of control rats (Fig. 4, C and E).

Electrophysiological study

Intravenous administration of ghrelin to rats significantly suppressed gastric vagal afferent activity (Fig. 5A), whereas iv administration of CCK significantly enhanced the afferent activity (Fig. 5B). The lowest effective doses of ghrelin and CCK were 0.3 pmol and 0.1 pmol, respectively (Fig. 5, A and

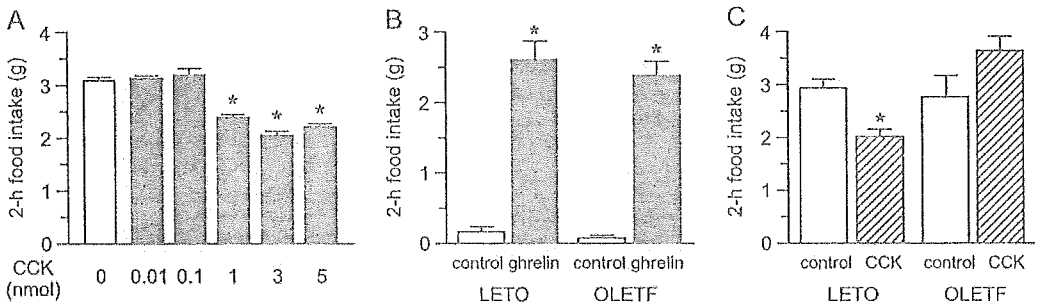

FIG. 1. The effect of iv administration of ghrelin or CCK on food intake in LETO and OLETF rats. A, Two-hour food intake (mean ± SEM) of 8-h fasting rats after a single iv administration of CCK (0.01–5 nmol). *, $P < 0.0005$ *vs.* control. B, After a single iv administration of ghrelin (3 nmol) or saline to LETO and OLETF rats, 2-h food intake from 1000–1200 h was measured. *, $P < 0.0001$ *vs.* control. C, After a single iv administration of CCK (1 nmol) or saline to LETO and OLETF rats after an 8-h fasting period, 2-h food intake from 1000–1200 h was measured. Control rats were administered saline iv. *, $P < 0.001$ *vs.* control.

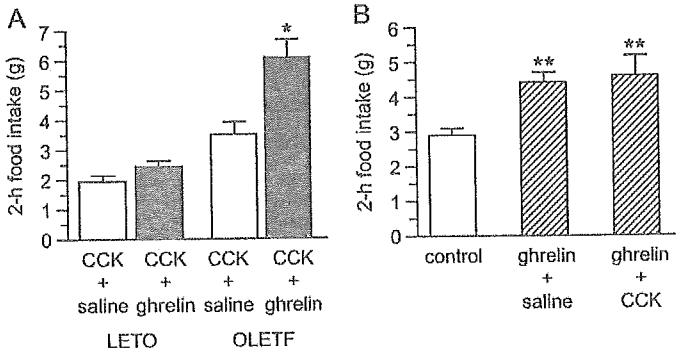

FIG. 2. The interaction between ghrelin and CCK on food intake in LETO and OLETF rats. A, After a single iv administration of ghrelin or saline after CCK treatment of LETO and OLETF rats after an 8-h fasting period, 2-h food intake from 1000–1200 h was measured. *, $P < 0.01$ *vs.* OLETF rats administered saline after CCK treatment. B, After a single iv administration of CCK or saline after ghrelin or saline treatment of LETO rats after an 8-h fasting period, 2-h food intake was measured from 1000–1200 h. Control rats were administered saline iv. **, $P < 0.001$ *vs.* control.

B). To investigate the interaction between ghrelin and CCK in electrophysiological studies, we used 1.5 nmol ghrelin and 1 nmol CCK, considered as their respective standard doses for feeding. When ghrelin was administered to rats after CCK treatment, gastric vagal afferent activity was not suppressed (Fig. 5C). Conversely, when CCK was administered to rats after ghrelin treatment, the CCK-mediated enhancement of afferent activity could not be observed (Fig. 5D).

Discussion

Signals produced within gastrointestinal tract affect feeding patterns (53). Distension of the stomach inhibits feeding, while nutrients in the small intestine induce the release of several gastrointestinal hormones, including CCK, an intestinal anorectic peptide (8, 9). The vagus nerve plays an important role in regulating feeding behavior by transmitting chemosensory and mechanosensory information from the viscera (54). Both neural and humoral signals for satiety and starvation generated in the gastrointestinal tract can be conveyed to the brain via the vagal afferent nerve and the blood circulation.

In the present study, we investigated peripheral interaction of ghrelin, an orexigenic gut peptide, with CCK on feeding regulation. Ghrelin, discovered in the stomach, stimulates food intake and GH secretion after iv administration (5, 16, 38, 55). Because the existence of an orexigenic peptide-based system in the periphery has yet to be discovered, ghrelin is thought to be the first peptide acting in the periphery as a starvation signal. Plasma ghrelin levels are up-regulated under conditions of negative energy balance including starvation, whereas they are down-regulated under conditions of positive energy balance (19, 56–59). Glucose load and food intake lead to a rapid fall in plasma ghrelin concentration, indicating that endogenous ghrelin serves as an indicator of short-term energy balance (19). Very recently, Cummings *et al.* (22) showed the preprandial increase of ghrelin levels among humans without time- or food-related cues and the overlap between these levels and hunger scores. These findings indicate ghrelin would be a candidate for peripheral meal initiator. In contrast, CCK, the most well-studied gastrointestinal peptide functioning in feeding, transmits a satiety signal to the NTS via vagal afferents (41, 43, 60). CCK decreases food intake when peripherally administered to rats (30). In humans, postprandial CCK levels are increased about five times higher than fasting levels (35, 61, 62). Thus, CCK has been thought to be a meal terminator.

Ghrelin is produced not only in the stomach but also in the hypothalamus (5, 63). Centrally administered ghrelin also stimulates both food intake and GH secretion (5, 13, 15, 64–66), and the ghrelin receptor is expressed in neuropeptide Y (NPY)- and GHRH-producing neurons in the hypothalamic arcuate nucleus, where it is incompletely isolated from the general circulation by the blood-brain barrier (67).

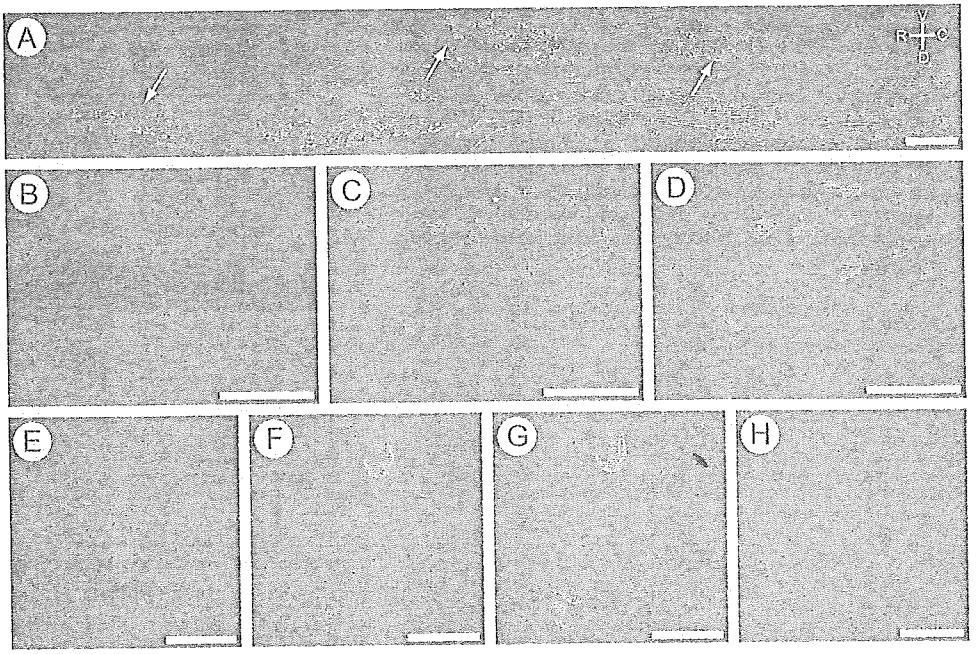

FIG. 3. Colocalization of GHS-R with CCK-AR in neurons of the nodose ganglion. A, GHS-R-immunoreactive neurons (*arrows*) are distributed throughout the nodose ganglion. Antisera for GHS-R [342–364] (A, B, D, E, and G) and CCK-AR (C, D, F, and G) were used to assess the (D) immunofluorescence double staining of GHS-R and CCK-AR in the nodose ganglion or (G) immunofluorescence double staining of GHS-R and CCK-AR in primary cultured neurons of the nodose ganglion. H, No GHS-R-immunoreactivity was observed with antiserum absorbed with excessive synthetic GHS-R [342–364]. R, Rostral; C, caudal; D, dorsal; V, ventral. *Scale bar*, 200 μm (A), 100 μm (B–H)

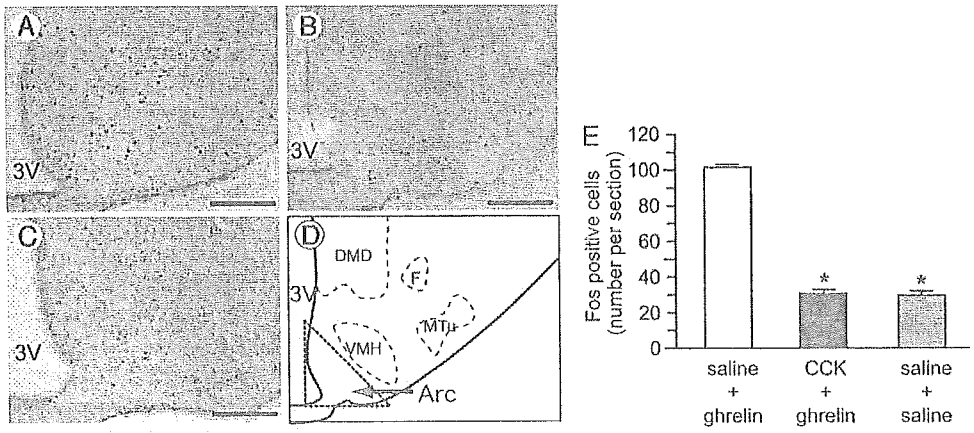

FIG. 4. Fos expression in the arcuate nucleus induced by ghrelin administration. A, Fos expression in response to iv ghrelin administration to rats after saline treatment. B, Fos expression in response to iv ghrelin administration after CCK treatment. C, Fos expression in response to iv saline to rats. D, A schematic drawing of an area in which Fos-positive neurons were counted. Fos-expressing neurons in a 0.7-mm right triangle (0.245 mm^2) were evaluated. Arc, arcuate nucleus; DMD, dorsomedial nucleus, dorsal; F, fornix; MTu, medial tuberal nucleus; VMH, ventromedial hypothalamic nucleus; 3V, third ventricle. *Scale bar*, 50 μm. C, The number of cells per section (bilateral). Data are expressed as mean ± SEM (n = 3). *, $P < 0.0001$ *vs.* rats administered ghrelin after saline treatment.

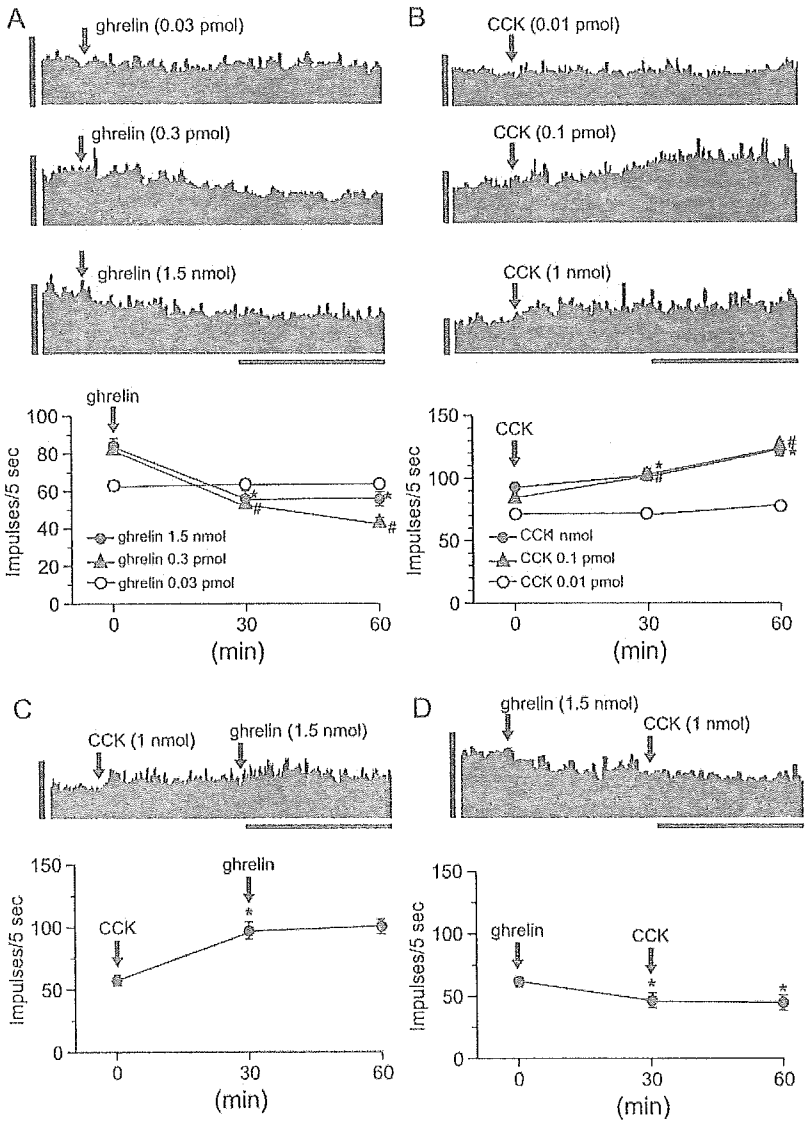

FIG. 5. The electrophysiological effect of ghrelin and CCK on gastric vagal afferent activity. A, Alterations of gastric vagal afferent discharge after a single iv administration of ghrelin (0.03 pmol–1.5 nmol). *, $P < 0.05$ *vs.* value at 0 min of 1.5 nmol ghrelin. #, $P < 0.05$ *vs.* value at 0 min of 0.3 pmol ghrelin. B, Alterations of gastric vagal afferent discharge after a single iv administration of CCK (0.01 pmol–1 nmol). *, $P < 0.05$ *vs.* value at 0 min of 1 nmol CCK. #, $P < 0.05$ *vs.* value at 0 min of 0.1 pmol CCK. C, Ghrelin (1.5 nmol) administration after CCK treatment (1 nmol) does not attenuate gastric vagal afferent activity. *, $P < 0.05$ *vs.* value at 0 min. D, CCK (1 nmol) administration after ghrelin (1.5 nmol) treatment does not activate gastric vagal afferent activity. *, $P < 0.05$ *vs.* value at 0 min. Representative data of gastric vagal afferent discharge rates are shown in the *upper panels*. *Vertical bar*, 100 impulses/5 sec; *horizontal bar*, 30 min.

These findings indicate that central ghrelin, peripheral ghrelin, or both may increase food intake and GH secretion via NPY and GHRH directly. However, we recently demonstrated that blockade of the gastric vagal afferent abolished ghrelin-induced feeding, GH secretion, and activation of NPY and GHRH neurons (38). These data suggest a possibility that ghrelin's signals for starvation and GH secretion are conveyed to the brain via the gastric vagal afferent system. Therefore, ghrelin and CCK, both produced within the gastrointestinal tract, exert opposite effects on feeding behavior through the vagal afferent, thereby regulating food intake on a short-term basis as a meal initiator and terminator, respectively.

In this study, we examined the interaction of ghrelin with CCK in the regulation of feeding behavior using CCK-AR-deficient OLETF rats. Ghrelin increased food intake in both OLETF and their lean littermates, LETO rats. In contrast, CCK decreased food intake in LETO rats fasted for 8-h period, but did not affect food intake in OLETF rats. These findings indicate that CCK-AR is required for CCK, but not ghrelin, regulation of feeding and that exogenous CCK reduces food intake of rats whose endogenous ghrelin levels are increased. Preadministration of CCK to LETO, but not to OLETF, rats blocked the food intake induced by peripheral administration of ghrelin. Conversely, the preadministration of ghrelin to LETO rats blocked the feeding reduction induced by peripheral CCK administration. These findings suggest that the effect of CCK or ghrelin administered after ghrelin or CCK, respectively, on feeding, might not be displayed, while some information to determine feeding behavior induced by exogenously preadministered ghrelin or CCK is transmitting via the vagal afferent system to the brain. When ghrelin or CCK was administered to rats, each plasma level transiently increases over their physiological ranges, which may also have cause complete blockade of the effect of serially administered ghrelin or CCK on feeding.

We also investigated the colocalization of GHS-R with CCK-AR in the rat nodose ganglion. Receptors of the vagal afferent are generated by nodose ganglion neurons and are transported to the nerve terminal through axonal transport (68, 69). Although we failed to demonstrate the colocalization of GHS-R and CCK-AR in the nerve terminal, immunohistochemical double staining of the nodose ganglion demonstrated that the majority of the GHS-R-immunoreactive neurons express CCK-AR. These findings suggest that the vagus nerve plays a major role in determining the peripheral parameters of energy balance.

Signals mediated by ghrelin secretion by the stomach are thought to be transmitted to the hypothalamus of the brain via the NTS, as iv administration of ghrelin induces Fos expression in the arcuate nucleus of the hypothalamus (38). Ghrelin suppresses gastric vagal afferent discharges when administered iv (38), whereas CCK enhances these discharges (43–48). Preadministration of CCK reduced the number of Fos-immunoreactive neurons induced by ghrelin. Very recently, Kobelt *et al.* (70) showed that peripherally administered CCK simultaneously with ghrelin inhibited ghrelin-induced feeding and ghrelin-induced Fos expression in the hypothalamic arcuate nucleus. These results are consistent with our data presented here. In addition, treatment with ghrelin after CCK administration did not affect the vagal afferent discharges induced by CCK. The effect of some peptides on vagal afferent discharge is known to be rapid (71, 72). However, in our experimental system, the changes in firing rate of the vagal afferent fibers induced by several substances continued over 60 min (38, 42, 73–79). These findings suggest that alteration of the firing rate counted by the interval and/or number of firing fibers may be caused by several messengers after peptides bound to their receptors. For example, a single somatostatin administration to rats actually increased the vagal afferent discharge for over 60 min. The afferent discharge stimulated by somatostatin was canceled by an injection of a monoclonal antibody for somatostatin before, but is ineffective after, the somatostatin injection (79). These results suggest the involvement of a unique postreceptor mechanism in the chemoreception as responsible for this long-acting effect of somatostatin on the afferent discharge. Such a postreceptor mechanism may apply to the time course of the ghrelin-induced decrease or CCK-induced increase of the vagal afferent discharge, although the precise mechanism remains to be elucidated. Recently, Królczyk *et al.* (80) performed electrophysiological recordings in both fasted and fed rats and demonstrated that the firing rate of the vagal afferent discharge in fasted rats was lower than that in fed rats. In that study, the increase in the firing rate after food administration to the fasted rats lasted for 15 min. Considering that ghrelin concentration increases in the fasting state and CCK concentration increases after feeding, exogenous administrations of ghrelin and CCK may induce in part starvation and satiety conditions on the basis of circulating hormones, respectively. The actual linkage of these peripheral signals with the vagal afferent pathway is likely to be more complicated given the remarkable number of neurotransmitters, neuropeptides, and neuromodulators. Feeding is a complicated interaction of many factors such as orexigenic or anorectic signals, emotion, learning, memory, *etc.* We believe that the alternation of the firing rate of the vagal afferent induced by ghrelin, CCK, or the combination of ghrelin with CCK is only a part of feeding regulation. Although it is difficult to clearly explain the reasons why the afferent activity lasts for such a long period after the single administration of ghrelin or CCK, we suggest that the long-acting effect on the afferent discharge may provide sufficient time for the brain to receive feeding-related conditions originating throughout the body. In addition, there may be a limitation on connecting the electrophysiological findings of rats under anesthetization with the feeding behavior of free-moving rats.

In summary, this study demonstrates that ghrelin administration after CCK treatment does not induce feeding; CCK administration after ghrelin treatment does not reduce it. We assume some mechanism whereby the intracellular signaling pathway induced by preadministered ghrelin or CCK interferes with signal transmission of serially administered CCK or ghrelin. In addition, the efficiency of ghrelin and CCK signal transport may depend on the balance in the plasma concentrations of these factors. In normal subjects, plasma ghrelin levels rise before the onset of meals and decline 30 min after feeding. In obese subjects, however, these declinations in plasma ghrelin levels are absent (81). The lack of

suppression of ghrelin secretion after a meal may be a critical factor in the pathophysiology of obesity and eating disorders. CCK is released postprandially, eliciting satiety signals (82–85). Plasma CCK concentrations in lean subjects fed a solid meal peak around 60 min after eating (86, 87). CCK also interacts synergistically in rats with other hormones released postprandially, including insulin, leptin, and glucagon (88–90). Abnormalities in the release of or sensitivity to ghrelin and/or CCK may be involved in alterations of food intake. Further investigation of the mechanisms controlling ghrelin and CCK release will help our understanding of the multifactorial regulation of feeding behavior, potentially leading to new treatments for obesity and eating disorders.

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Adrenomedullin Enhances Therapeutic Potency of Mesenchymal Stem Cells After Experimental Stroke in Rats

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