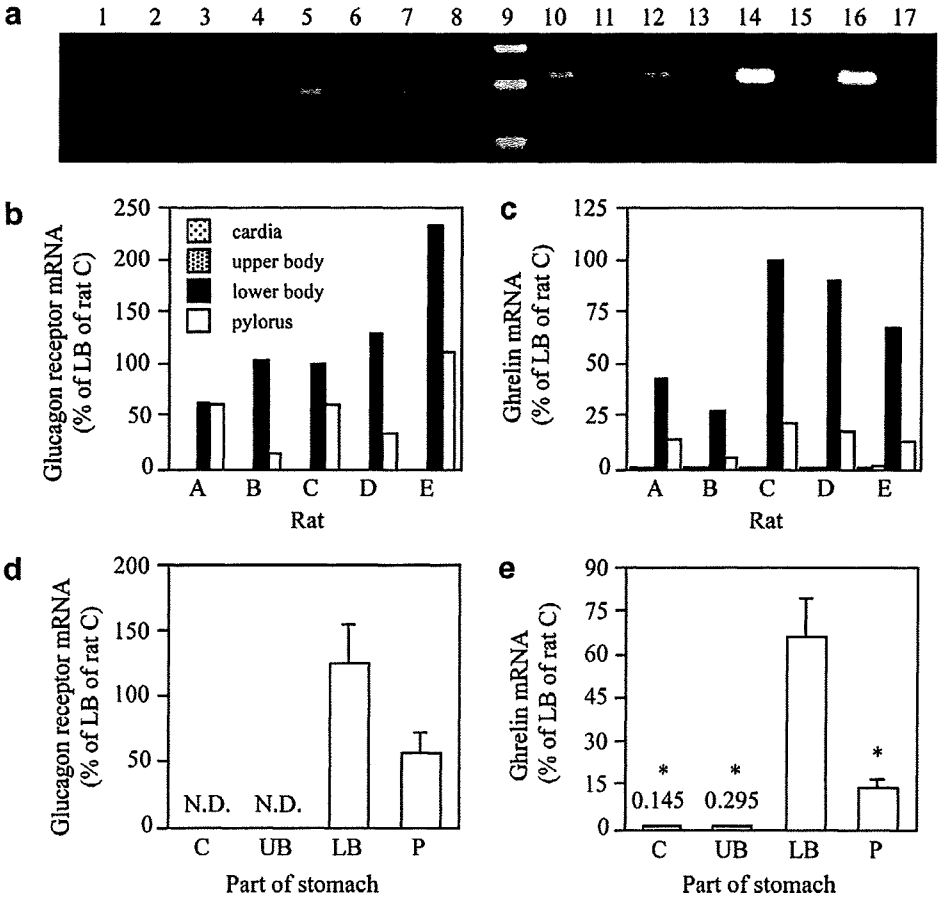


Fig. 1. Comparison of glucagon receptor and ghrelin mRNA levels in cardia (C), upper body (UB), lower body (LB), and pylorus (P) of stomach. Tissues were resected from rats A to E at 10:00, 11:30, 13:00, 15:00, and 15:00 h, respectively. (a) Representative results of RT-PCR. Lanes 1, 3, 5, and 7: glucagon receptor mRNA expression in C, UB, LB, and P, respectively. Lanes 10, 12, 14, and 16: ghrelin mRNA expression in C, UB, LB, and P, respectively. Lanes 2, 4, 6, 8, 11, 13, 15, and 17: RT-minus controls. Lane 9: size markers (150, 100, and 50 bp). (b–e) Relative values of LB of rat C were standardized to 100%. (b,c) Values of individual rats. Ghrelin mRNA is expressed in cardia and upper body in all rats examined although it is hardly visible in the (c). (d,e) Same data are plotted as means ± SEM for five rats. N.D., not detectable; $^{*}P < 0.01$ between C and LB, UB and LB, and LB and P.

tor were detected as spots (Fig. 2e). This is probably because the antibody for glucagon receptor recognizes the extracellular domain of the receptor molecules that are expressed in scattered fashions in cell membrane. While most cells revealed immunoreactions for either acyl-ghrelin or glucagon receptor alone, a few cells were reactive for both antibodies (Fig. 2c and f).

*Effects of glucagon on plasma levels of acyl- and desacyl-ghrelin*

Plasma concentrations of acyl- and desacyl-ghrelin in the gastric vein at the start of experimental period, at −20 min in Fig. 3, were 78.7 ± 17 and 3593 ± 405 fmol/ml, respectively (means ± SEM, $n = 8$). Saline administration did not cause significant changes in plasma acyl-ghrelin levels (Fig. 3a). Desacyl-ghrelin levels revealed significant changes over time by saline administration and was significantly higher at 50 min than at 0 min (Fig. 3b). Glucagon, on the other hand, induced significant increases in both acyl- and desacyl-ghrelin levels within 10 min following administration (Fig. 3c and d). The increases in both hormone levels were transient and acyl-ghrelin levels thereafter remained similar to the basal level at 0 min until the end of experimental period (Fig. 3c). In contrast, desacyl-ghrelin levels gradually rose again from 30 min and were significantly higher than the basal level at 40, 50, and 60 min (Fig. 3d).

*Effects of glucagon on ghrelin mRNA expression in the lower body of stomach*

Glucagon induced a significant 1.9-fold elevation of the ghrelin mRNA level 20 min after administration, compared to saline-injected controls. Ghrelin mRNA levels were not significantly different between saline- and glucagon-administer rats at 5 or 120 min (Fig. 4a). The baseline of ghrelin mRNA levels, that is, levels in saline-administer rats, significantly ascended from 20 min to 120 min by 1.7-fold (Fig. 4b).

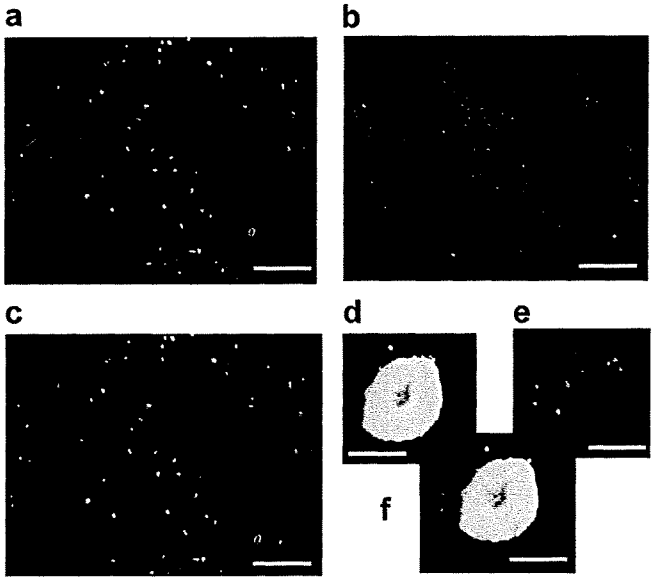


Fig. 2. Double staining for ghrelin and glucagon receptor in rat stomach. Localization of acyl-ghrelin (a; stained green) and glucagon receptor (b; stained red) in lamina propria mucosae of rat stomach. Co-localization of acyl-ghrelin and glucagon receptor is observed in some cells (c; stained yellow). The length of scale bars represent 100 μm. (d–f) Higher magnifications of cells stained for both acyl-ghrelin and glucagon receptor. Acyl-ghrelin (d), glucagon receptor (e) and both (f). The length of scale bars represent 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

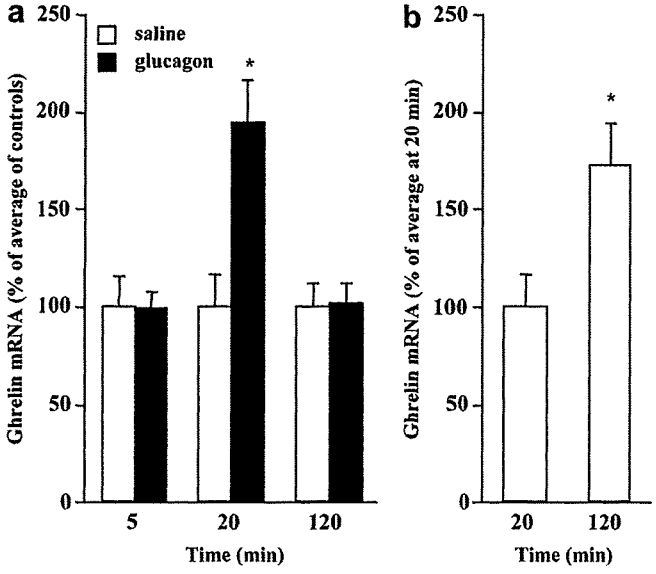


Fig. 4. Effects of glucagon on ghrelin mRNA expression in the lower body of stomach. (a) Ghrelin mRNA levels 5, 20, and 120 min after administration of saline (control) or glucagon. Relative values of control rats were standardized to 100%, and data are expressed as means ± SEM for five rats. $^{*}P < 0.01$ compared with control. (b) Comparison of ghrelin mRNA levels between 20 and 120 min after administration of saline. Relative values at 20 min were standardized to 100%, and data are expressed as means ± SEM for five rats. $^{*}P < 0.05$ compared with 20 min.

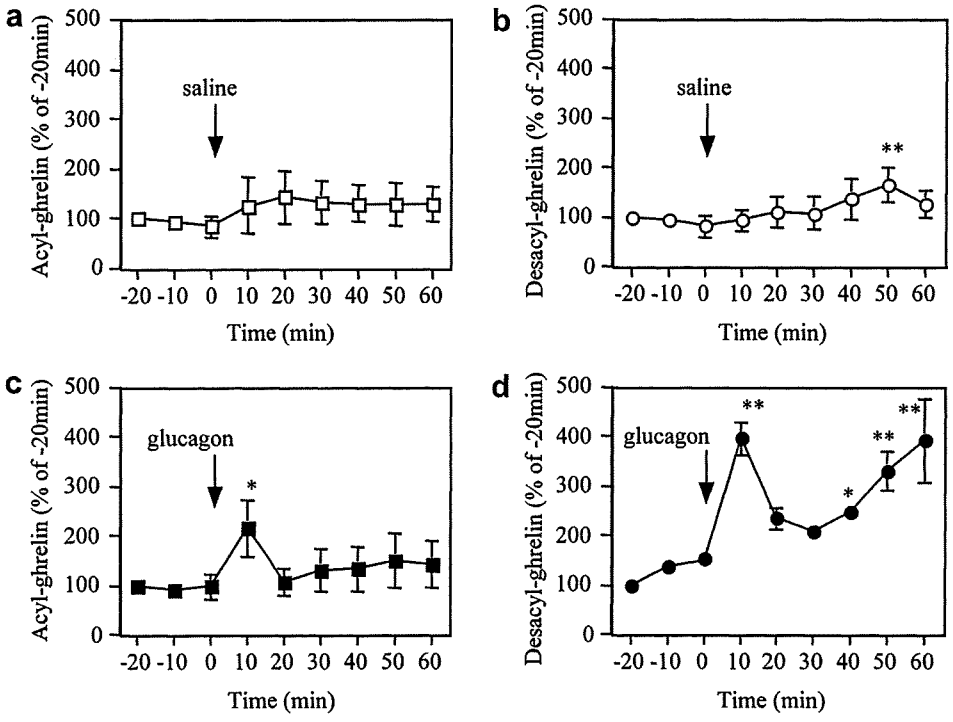


Fig. 3. Effects of glucagon on plasma levels of acyl- and desacyl-ghrelin. Saline or glucagon was administer immediately after blood collection at 0 min as indicated by arrows. The relative value at −20 min in each rat was standardized to 100%. Data are expressed as means ± SEM for four rats. Repeated-measures ANOVA indicates significant changes in hormone levels over time in the (b) ($P < 0.05$), (c) ($P < 0.05$), and (d) ($P < 0.0001$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 0 min.

## Discussion

Ghrelin-producing cells in the stomach have been identified mainly from the neck to the base of the oxyntic glands, especially in the X/A-like cells [12]. The present study showed that ghrelin mRNA was expressed at relatively high levels in lower body and pylorus, and at lower levels in cardia and upper body of the rat stomach. The upper body of the rat stomach mostly consists of stratified flattened epithelium, while lower body and pylorus consist of lamina propria mucosae enriched with gastric glands. The reginal dependency of the ghrelin mRNA expression in the present study is in agreement with the previous finding. Immunohistochemical analyses also confirmed the expression of acyl-ghrelin predominantly in lamina propria mucosae of rat stomach.

Glucagon receptor mRNA is expressed widely throughout rat tissues [16] and its expression is increased by glucose and somatostatin, whereas it is decreased by glucocorticoids and agents stimulating intracellular cAMP in rats [17]. However, little information was available concerning the regional distribution of glucagon receptor within the stomach. The present study demonstrated that expression of glucagon receptor mRNA revealed similar regional distribution to that of ghrelin mRNA except for little or no expression in cardia and upper body. The glucagon receptor mRNA level in lower body of stomach was lower than that in liver by approximately 6-fold (data not shown). Immunohistochemical analyses indicated the predominant expression of the receptor proteins in lamina propria mucosae of rat stomach. In addition, some stomach cells appeared to express both acyl-ghrelin and glucagon receptors. The similarity in regional expression profiles of ghrelin and glucagon receptor mRNAs and the existence of cells expressing both proteins in the stomach would support a possibility of interactions between ghrelin production and glucagon.

Our previous study using free-feeding rats demonstrated that both acyl- and total ghrelin levels exhibited a diurnal pattern of release, with bimodal peaks occurring at 15:00 h in the light period and at 06:00 h in the dark period [6]. The results in the present study are, at least in part, consistent with these findings in that ghrelin mRNA levels in stomach tissues from rats C to E (tissue resection at 13:00, 15:00, and 15:00 h, respectively) were totally higher than those from rats A and B (tissue resection at 10:00 and 11:30 h, respectively). Based on these observations, effects of glucagon on ghrelin secretion and mRNA expression were investigated by glucagon administration between 12:00 and 13:00 h, the period where the plasma ghrelin level is in an increasing phase. The results suggest that glucagon induces acute and transient increases in the secretion of both acyl- and desacyl-ghrelin from the stomach. Since ghrelin mRNA levels were not changed by glucagon prior to these increases in hormone secretion, it is likely that glucagon stimulates release, but not synthesis, of both forms of ghrelin in this period. Furthermore, plasma desacyl-ghrelin levels, but not acyl-ghrelin levels, revealed significant increases from 40 min after glucagon administration. This increase in desacyl-ghrelin secretion was accompanied by a preceding increase in the ghrelin mRNA level observed at 20 min. It is thus suggested that glucagon also has an ability to increase synthesis and release of desacyl-ghrelin selectively in a prolonged fashion. However, the increase in ghrelin mRNA level by glucagon treatment was no longer observed 120 min after administration. This time point corresponds to 14:00–15:00 h, the period exhibiting a peak in the diurnal pattern of plasma ghrelin levels [6], and the basal level of ghrelin mRNA was indeed higher than that at 20 min after administration. Therefore, the ineffectiveness of glucagon on ghrelin mRNA expression at this time point might be due to the end of the increasing phase of ghrelin production.

Recent findings indicated that promotor activity of ghrelin gene was stimulated by glucagon and its second messenger cAMP [18,19] and that glucagon exerted acute stimulation of total ghrelin release from the isolated perfused rat stomach [9]. Prolonged 3-day treatment of rats with glucagon was also shown to increase ghrelin mRNA levels in stomach [19]. These results, together with those in the present study, suggest that glucagon is involved in ghrelin production and secretion in the stomach.

The concentration of desacyl-ghrelin in the gastric vein was higher than that of acyl-ghrelin by 45-fold, which is consistent with previous observations [2,6]. The interpretation of the physiological significance of the stimulatory effect of glucagon on desacyl-ghrelin secretion is difficult. Regulatory mechanisms of octanoylation and des-octanoylation of ghrelin are unclear. While *n*-octanoylation at Ser3 residue of the molecule is essential for the expression of biological activities of ghrelin though GHS-R [1], recent studies have presented biological effects of non-octanoylated desacyl-ghrelin through pathways independent of GHS-R. Administration of desacyl-ghrelin was demonstrated to inhibit food intake via signaling through corticotropin-releasing factor 2 receptor in rats [20]. On the contrary, central but not peripheral administration of desacyl-ghrelin was shown to induce food intake in rats [21]. The effect of desacyl-ghrelin on food intake is therefore obscure so far.

From the results in the present study, a question may remain why saline administration caused slight but significant changes over time in plasma levels of desacyl-ghrelin. Since electrical vagal stimulation in isolated perfused rat stomach has been reported to lead to consistent elevation of total ghrelin secretion [22], it is possible that vagal stimulation occurred during surgical treatments and/or blood collection and such an artificial effect led to the elevation of desacyl-ghrelin secretion. If this is the case, it might be assumed that modes of vagal regulation on acyl- and desacyl-ghrelin are dintinct since little elevation was observed in acyl-ghrelin levels in the same rats.

In summary, the present study has shown that glucagon receptor mRNA is expressed with similar regional distribution as that of ghrelin in the rat stomach. Furthermore,

biphasic effects of glucagon on ghrelin secretion have been suggested, that is, stimulating acute release of both forms of ghrelin and thereafter upregulating systhesis and release of desacyl-ghrelin selectively in the rat stomach. Since physiological effects of desacyl-ghrelin especially on food intake remain to be controversial, further studies are required to elucidate the physiological role of glucagon as a regulator of ghrelin secretion.

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# Peptidomic Identification and Biological Validation of Neuroendocrine Regulatory Peptide-1 and -2*

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**Recent advances in peptidomics have enabled the identification of previously uncharacterized peptides. However, sequence information alone does not allow us to identify candidates for bioactive peptides. To increase an opportunity to discover bioactive peptides, we have focused on C-terminal amidation, a post-translational modification shared by many bioactive peptides. We analyzed peptides secreted from human medullary thyroid carcinoma TT cells that produce amidated peptides, and we identified two novel amidated peptides, designated neuroendocrine regulatory peptide (NERP)-1 and NERP-2. NERPs are derived from distinct regions of the neurosecretory protein that was originally identified as a product of a nerve growth factor-responsive gene in PC12 cells. Mass spectrometric analysis of the immunoprecipitate using specific antibodies as well as reversed phase-high performance liquid chromatography coupled with radioimmunoassay analysis of brain extract demonstrated the endogenous presence of NERP-1 and NERP-2 in the rat. NERPs are abundant in the paraventricular and supraoptic nuclei of the rat hypothalamus and colocalized frequently with vasopressin but rarely with oxytocin. NERPs dose-dependently suppressed vasopressin release induced by intracerebroventricular injection of hypertonic NaCl or angiotensin II *in vivo*. NERPs also suppressed basal and angiotensin II-induced vasopressin secretion from hypothalamic explants *in vitro*. Bioactivity of NERPs required C-terminal amidation. Anti-NERP IgGs canceled plasma vasopressin reduction in response to water loading, indicating that NERPs could be potent endogenous suppressors of vasopressin release. These findings suggest that NERPs are novel modulators in body fluid homeostasis.**

Peptide hormones or neuropeptides function as cell-to-cell signaling molecules to mediate a variety of physiological phenomena. These bioactive peptides are cleaved from precursor proteins via limited cleavage and often undergo post-translational modifications to perform their functions (1). Technological advancement in mass spectrometry, along with an ever increasing number of genomes being sequenced, has made it possible to study the peptidome or a whole set of endogenously processed peptides. In fact, peptidomic approaches have been applied to the analysis of peptides found in mammalian tissues or body fluids, leading to the description of a number of previously uncharacterized peptides (2–4).

In mammalian peptidomic studies, however, most peptides identified are fragments of intracellular proteins. Even in the studies designed to identify pituitary and hypothalamic peptides using specific sample preparation methods (5, 6), the peptides identified have turned out to be N-terminally or C-terminally deleted or extended fragments of relatively abundant precursors of known peptide hormones or secretory proteins. This is because mass spectrometry schemes detect only abundant molecules or easily ionized molecules, although tandem mass spectrometry has the potential to efficiently identify peptides present in complex mixtures. Thus, candidates for novel bioactive peptides present in trace amounts remain elusive in peptidomic identification studies. Another critical issue in peptidomics is that we cannot infer biological activity just from the sequence of a target peptide; it is practically impossible to synthesize and test all the peptidomic-identified peptides for assessing bioactivity.

We thought that one solution to increase the probability of identifying potentially bioactive peptides is to focus on secretory peptides with a post-translational modification characteristic of bioactive peptides. In this study, we targeted C-terminal amidation, which is shared by many known bioactive peptides or peptide hormones (7). By analyzing peptides released by a human cell line of endocrine origin, we discovered two C-terminally amidated peptides derived from the neurosecretory protein VGF (8). Although VGF has long been considered a precursor of bioactive peptides, functional studies are limited to C-terminal peptides as yet (9, 10). Biological functions of other VGF-related peptides, identified by recent peptidomic

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studies, have not been investigated (11, 12). To get a clue for identifying their biological functions, we prepared antisera against these peptides and performed immunohistochemical studies to identify peptide-producing tissues and cells in the rat. We took advantage of the well documented findings on the localization and production sites of peptide hormones and deduced the possible biological functions of candidate peptides in relation to these known peptide hormones. This study would provide a new approach to the peptidomics-aided discovery of mammalian bioactive peptides.

## EXPERIMENTAL PROCEDURES

*Mass Spectrometric Analysis*—The supernatant of human medullary thyroid carcinoma TT cells (13) cultured in serum-free media for 6 h was harvested and immediately processed using a Sep-Pak C18 cartridge (Waters) as described (14). The resultant eluate was applied to a gel filtration column (Superdex Peptide PE7.5/300, GE Healthcare) to obtain peptide-rich fractions. These were subjected to reductive alkylation, desalted, and fractionated by RP-HPLC[4] into 50 fractions. Each fraction was analyzed by off-line nano-electrospray ionization MS/MS with a Q-Tof II mass spectrometer (Micromass, Milford, MA) and by matrix-assisted laser desorption ionization-time of flight MS/MS with a Proteomics 4700 mass spectrometer (Applied Biosystems, Foster City, CA). Each MS/MS spectrum was used to probe the NCBI and Swiss-Prot databases with Mascot MS/MS ion search software (Matrix Science, Boston, MA) and was also interpreted by SeqMS (15).

*Peptide Synthesis*—All peptides were synthesized on an Abacus peptide synthesizer (Sigma Genosys) using Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) strategy, purified by RP-HPLC, and verified for correct synthesis by mass spectrometry and amino acid analysis. Purity of the peptides was confirmed on separate HPLC systems. Synthetic rat NERPs were used in all the *in vivo* and *in vitro* administration experiments.

*Antibody Preparation and Radioimmunoassay (RIA)*—A C-terminal octapeptide common to human and rat NERP-1 (QGLAQVEA-$NH_2$) was conjugated with keyhole limpet hemocyanin (Pierce) by the glutaraldehyde method. Cysteinyl C-terminal decapeptides of rat NERP-2 (CQGGARQRDLG-$NH_2$) and human NERP-2 (CQGGARQRGLG-$NH_2$) were each coupled with maleimide-activated keyhole limpet hemocyanin (Pierce) through its thiol groups. Rabbits were immunized with each conjugate emulsified with an equal volume of Freund's complete adjuvant. Radioimmunoassay was carried out as reported (16) using $^{125}$I-radiolabeled YLLQQGLAQVEA-$NH_2$ (human and rat NERP-1), YLLQGGARQRDLG-$NH_2$ (rat NERP-2), or YQGGARQRGLG-$NH_2$ (human NERP-2). A half-maximum inhibition concentration of ligand binding in each RIA was 20 fmol/tube (human and rat NERP-1), 10 fmol/tube (rat NERP-2), or 20 fmol/tube (human NERP-2). Specificity of the three RIAs for human/rat NERP-1 and rat NERP-2 was examined with immunized peptides with C-terminal Gly extension, C-terminal rat VGF-(588–617) and human VGF-(586–615), rat VGF-(556–585), and human VGF-(554–583) corresponding to a C-terminally extended form of TLQP-21 (10) and 13 known bioactive peptides listed below, including 10 C-terminally amidated peptides. Vasopressin, neuromedin U, neurokinin A, calcitonin, calcitonin gene-related peptide, calcitonin receptor-stimulating peptide, adrenomedullin, proadrenomedullin N-terminal 20-amino acid peptide, peptide histidine isoleucine, corticotropin-releasing factor, angiotensin II, leucine-enkephalin, and methionine-enkephalin-Arg-Gly-Leu.

*Immunological Detection of NERPs*—Rat hypothalamus was extracted and condensed with a Sep-Pak C18 cartridge as described previously (17). An aliquot of cartridge eluate was examined by RIA to quantify each NERP. The remaining portion was separated by RP-HPLC and assessed by RIA to identify individual immunoreactive (ir-) NERPs. To determine major endogenous forms of rat NERPs, Sephadex G-50 gel-filtrated fractions of rat brain extracts (1.1- and 5.9-g eq for NERP-1 and NERP-2, respectively) were immunoprecipitated with anti-NERP antibodies and then analyzed on a surface-enhanced laser desorption ionization mass spectrometer (Ciphergen, Fremont, CA). Immunoprecipitate from TT cell extract was prepared and mass analyzed as described (14).

*Intracerebroventricular Administration*—Male Wistar rats (aged 9–10 weeks, from Charles River Laboratories, Shiga, Japan) were maintained in individual cages under controlled temperature (21–23 °C) and light (light on 08:00–20:00) conditions with *ad libitum* access to food and water. Cannulation and intracerebroventricular (icv) administration were performed as described (18). Test materials for icv administration were dissolved in 10 $\mu$l of artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 1.24 mM $KH_2PO_4$, 2 mM $CaCl_2$, 25.9 mM $NaHCO_3$, and 10 mM glucose, pH 7.3. All animal experiments were repeated three to five times and performed in accordance with the guidelines for animal care from the Japanese Physiological Society.

*Immunohistochemistry*—Brains were removed from colchicine (200 $\mu$g)-treated rats following perfusion with either 2% paraformaldehyde (PFA) or 4% PFA containing 0.1% glutaraldehyde, respectively, for immunofluorescence microscopy or electron microscopy. Immunofluorescence staining and immunogold electron microscopy were performed as described previously (19, 20). For light microscopy, peripheral tissues from rats perfused with 2% PFA were stained with antibodies against NERP-1 (1:2,500), rat NERP-2 (1:5,000), oxytocin (1:15,000; Chemicon, Temecula, CA), and vasopressin (1:80,000; Peninsula Laboratories, Torrance, CA). Samples were visualized as described (21). Control studies were done with normal rabbit serum or NERP antisera that had been pretreated with 10 $\mu$g of synthetic NERPs.

*In Situ Hybridization*—VGF and vasopressin mRNA levels in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from rats deprived of water for 48 h were examined by *in situ* hybridization with $^{33}$P 3′ end-labeled deoxyoligonucleotide probes specific for VGF (complementary to bases 1741–1785

[4] The abbreviations used are: RP-HPLC, reversed phase-high performance liquid chromatography; AII, angiotensin II; ir, immunoreactive; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NERP, neuroendocrine regulatory peptide; PFA, paraformaldehyde; PVN, paraventricular nucleus; RIA, radioimmunoassay; SON, supraoptic nucleus; TOF, time-of-flight; RP-HPLC, reversed phase-high performance liquid chromatography; aCSF, artificial cerebrospinal fluid.

and 1825–1870 of rat VGF nucleotides; GenBank™ accession number M74223) and vasopressin (complementary to bases 1843–1868 of rat vasopressin nucleotides) as described (18). Autoradiographic images were analyzed on an MCID imaging analyzer (18). VGF mRNA intensity is expressed relative to that of control rats drinking water *ad libitum* ($n = 5$ per group).

*Vasopressin Secretion and Measurements*—Rats ($n = 8–14$ per group) received an icv injection of test peptide 5 min before icv injection of either hypertonic NaCl (8.5 μmol of NaCl/10 μl of aCSF) or AII (0.1 nmol/10 μl of aCSF). Plasma vasopressin was measured using an RIA kit (Mitsubishi Chemical, Tokyo, Japan) in blood samples taken 10 min after the hypertonic saline or AII injection. Static incubation of PVN and SON explants punched out from the hypothalamus was performed as reported previously with minor modifications (22). They were sequentially stimulated (each at a final concentration of $10^{-6}$ M for 5 min) as indicated in Fig. 5. Stimulation periods were separated by 5-min recovery periods. At the end of each experiment, KCl was added at a final concentration of $6 \times 10^{-2}$ M to confirm depolarization-induced secretion. Perifusion assays were replicated 5–8 times.

*Effects of NERPs on Plasma Vasopressin in Rats*—Rats ($n = 8$ per group) deprived of water for 48 h were decapitated 10 min after icv administration of NERP-1 or NERP-1-Gly (1nmol/rat) to measure plasma vasopressin. Prior to immunoneutralization studies of NERPs, water (5 ml/100 g body weight) was loaded to rats ($n = 8$ per group) by oral injection through a stomach tube. Fifteen minutes after water loading, rats received an icv injection of anti-NERP-1-IgG (0.1 μg), anti-NERP-2-IgG (0.1 μg), or control IgG (0.1 μg). Rats were decapitated 45 min after immunoneutralization, and plasma vasopressin was measured.

*Statistical Analysis*—All data are expressed as means ± S.E. Groups of data were compared with analysis of variance and the post-hoc Fisher's test. $p < 0.05$ were considered to be statistically significant.

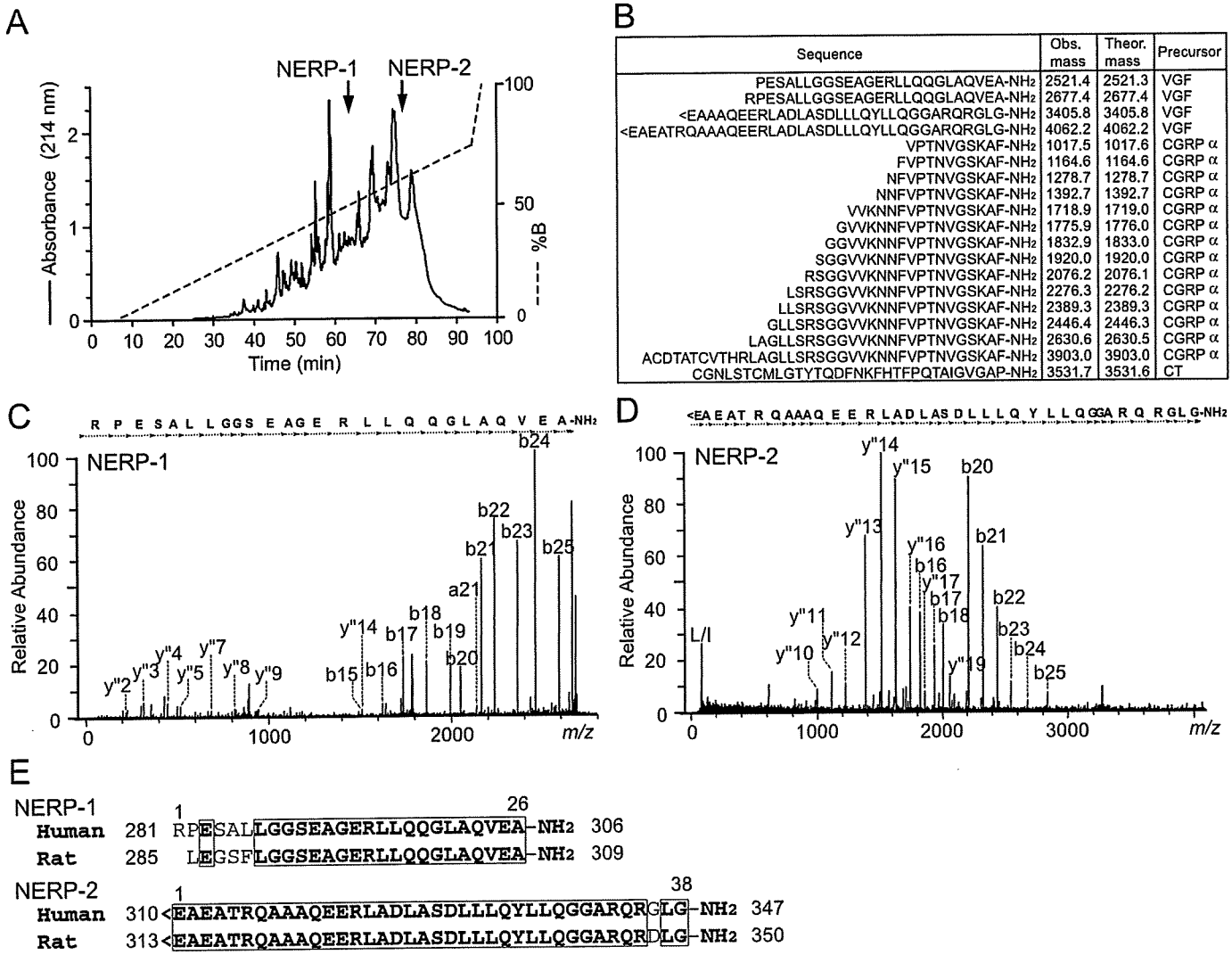
## RESULTS

*Peptidomic Identification of NERPs from Culture Supernatant of TT Cells*—We analyzed peptides secreted from human medullary thyroid carcinoma TT cells, because this cell line is known to actively secrete the C-terminally amidated peptide hormones calcitonin gene-related peptide α and calcitonin (13). The supernatant of the cell line cultured in a serum-free medium for 6 h was concentrated and subjected to gel filtration chromatography to obtain a peptide-rich fraction, whose cysteine residues were then converted to carboxyamidomethyl cysteine using dithiothreitol and iodoacetamide. This peptide fraction was separated by conventional RP-HPLC to 50 fractions (Fig. 1*A*), each of which was analyzed with tandem mass spectrometric techniques for identification. We identified 19 C-terminally amidated peptides (Fig. 1*B*), of which 15 peptides were the entire or partial sequences corresponding to calcitonin gene-related peptide α and calcitonin. The identification of a series of 13 calcitonin gene-related peptide α-derived (CRGPα) peptides with sequential N-terminal deletions is consistent with the fact that this cell line produces this peptide α at higher levels than calcitonin (13). Of note, we discovered two novel amidated peptides with monoisotopic masses of 2677.4 and 4062.2, both of which were derived from distinct regions of the neurosecretory protein VGF; one is from human VGF-(281–306), and the is other from VGF-(310–347) (NCBI accession number gi|17136078) (Fig. 1, *C* and *D*). Shorter fragments of both peptides were also identified (Fig. 1*B*). Based on their localization and physiological role described below, we designated these peptides as neuroendocrine regulatory peptide (NERP)-1 and NERP-2.

*Antisera against NERP-1 and NERP-2*—The rat VGF sequences registered in the NCBI data base, as represented by gi|13591864 and gi|1352860, suggest that the rat precursor comprised of 617 amino acids generates amidated peptides as well. To characterize rat endogenous peptides, we prepared antibodies specific to the C-terminal region of each peptide; an octapeptide common to human and rat NERP-1 (QGLAQVEA-$NH_2$) and decapeptides of rat NERP-2 (QGGARQRDLG-$NH_2$) and human NERP-2 (QGGARQRGLG-$NH_2$) were used for immunization. We confirmed that each antiserum strictly recognizes the C-terminal amide structure but does not show more than 0.1% cross-reactivity with its C-terminally Gly-extended peptide or another NERP. Furthermore, the antisera did not recognize rat C-terminal VGF-(588–617) peptide or human C-terminal VGF-(586–615) peptide even at 10 μM, indicating that they do not detect the intact VGF precursor.

*Characterization and Identification of NERP-1 and NERP-2*—In the Sephadex G-50 gel filtration of rat brain extracts, ir-NERP-1 and ir-NERP-2 were observed as distinctive peaks in the region of relative molecular mass <6 kDa (data not shown). These NERP-1- and NERP-2-immunoreactive fractions were further characterized by mass analysis of immunoprecipitates using these antibodies (Fig. 2, *A* and *B*). Based on the observed mass of the immunoprecipitates, we concluded that rat NERP peptides are derived from the VGF precursor (gi|13591864, Gly at residue 342), with the major endogenous forms of rat NERP-1 and NERP-2 being 25 and 38 amino acids long, respectively (Fig. 1*E*). The rat hypothalamus ir-NERPs behaved identically to synthetic rat NERP-1 or NERP-2 on RP-HPLC (Fig. 2*C*). Immunoprecipitation experiments with TT cell extract also showed the dominant peaks that correspond to human NERP-1 (2677 Da) and NERP-2 (4062 Da). These findings suggest that the processing and amidation of NERPs occur intracellularly before secretion, as is known with amidated bioactive peptides secreted by endocrine cells (7).

*NERPs Suppress Vasopressin Release*—ir-NERPs were highly abundant in the rat hypothalamus (NERP-1, 14.40 ± 1.05 pmol/g wet weight; NERP-2, 11.33 ± 0.80 pmol/g wet weight, $n = 5$). Cell bodies with strong immunostaining of NERPs were observed in the SON (Fig. 3, *A–D*) and PVN (Fig. 3, *E–J*), the nuclei that produce vasopressin and oxytocin (23, 24). Immunofluorescence microscopy showed that NERPs frequently colocalized with vasopressin, which controls body fluid homeostasis, but rarely with oxytocin (Fig. 3, *A–D*). Within the PVN, immunostaining of NERPs was detected in both magnocellular and parvocellular divisions, whereas that of arginine vasopressin was mainly observed in the magnocellular division. Thus, arginine vasopressin-pos-




| Sequence | Obs. mass | Theor. mass | Precursor |
|---|---|---|---|
| PESALLGGSEAGERLLQQGLAQVEA-NH2 | 2521.4 | 2521.3 | VGF |
| RPESALLGGSEAGERLLQQGLAQVEA-NH2 | 2677.4 | 2677.4 | VGF |
| <EAAAQEERLADLASDLLLQYLLQGGARQRGLG-NH2 | 3405.8 | 3405.8 | VGF |
| <EAEATRQAAAQEERLADLASDLLLQYLLQGGARQRGLG-NH2 | 4062.2 | 4062.2 | VGF |
| VPTNVGSKAF-NH2 | 1017.5 | 1017.6 | CGRP α |
| FVPTNVGSKAF-NH2 | 1164.6 | 1164.6 | CGRP α |
| NFVPTNVGSKAF-NH2 | 1278.7 | 1278.7 | CGRP α |
| NNFVPTNVGSKAF-NH2 | 1392.7 | 1392.7 | CGRP α |
| VVKNNFVPTNVGSKAF-NH2 | 1718.9 | 1719.0 | CGRP α |
| GVVKNNFVPTNVGSKAF-NH2 | 1775.9 | 1776.0 | CGRP α |
| GGVVKNNFVPTNVGSKAF-NH2 | 1832.9 | 1833.0 | CGRP α |
| SGGVVKNNFVPTNVGSKAF-NH2 | 1920.0 | 1920.0 | CGRP α |
| RSGGVVKNNFVPTNVGSKAF-NH2 | 2076.2 | 2076.1 | CGRP α |
| LSRSGGVVKNNFVPTNVGSKAF-NH2 | 2276.3 | 2276.2 | CGRP α |
| LLSRSGGVVKNNFVPTNVGSKAF-NH2 | 2389.3 | 2389.3 | CGRP α |
| GLLSRSGGVVKNNFVPTNVGSKAF-NH2 | 2446.4 | 2446.3 | CGRP α |
| LAGLLSRSGGVVKNNFVPTNVGSKAF-NH2 | 2630.6 | 2630.5 | CGRP α |
| ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH2 | 3903.0 | 3903.0 | CGRP α |
| CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH2 | 3531.7 | 3531.6 | CT |

FIGURE 1. **Peptidomic identification of NERPs.** *A*, RP-HPLC of the secretory peptides obtained from culture supernatant of TT cells. NERP-1 (2677.4 Da) and NERP-2 (4062.2 Da) were eluted at the positions indicated by *arrows*. *B*, summary of C-terminally amidated peptides. <*E*, pyroglutamic acid; *Obs*, observed; *Theor*, theoretical. *C* and *D*, identification of NERP-1 (*C*) and NERP-2 (*D*) by nano-electrospray ionization MS/MS. MS/MS spectra were obtained from the precursor ions at *m/z* 893.5 ($[M + 3H]^{3+}$ ion) (*C*) and *m/z* 1016.5 ($[M + 4H]^{4+}$ ion) (*D*). The resultant spectra, deconvoluted with MaxEnt3TM (Micromass), were interpreted by SeqMS (15). The y″*n* and bm ions, where *n* and *m* denote arbitrary positions counted from the C and N termini, indicate C- and N-terminal ions, respectively, which were produced by cleavage of peptide bonds during MS/MS. *L/I* in the spectrum (*D*) denotes the immonium ion of Leu or Ile, which have the same mass. *E*, sequence alignment of human and rat NERPs. Residue numbering is based on the human (gi|17136078) and rat (gi|13591864) VGF precursor.

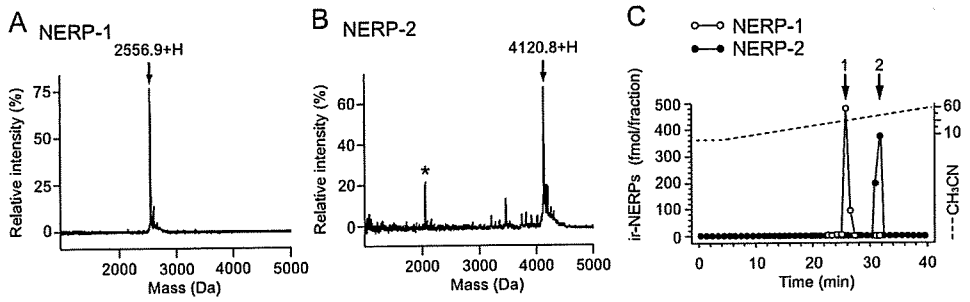



FIGURE 2. **Major endogenous forms of rat NERPs determined by surface-enhanced laser desorption ionization mass spectrometry and RP-HPLC.** Peptides immunoprecipitated with an antibody against NERP-1 (*A*) or NERP-2 (*B*) from rat brain extracts were analyzed. The values correspond to the molecular masses of 25-amino acid-long NERP-1 (*A*, VGF-(285–309)) and 38-amino acid-long NERP-2 (*B*, VGF-(313–350)) of rat VGF (gi|13591864). An *asterisk* denotes a doubly charged ion. *C*, characterization of ir-NERPs from rat hypothalamus by RP-HPLC coupled with RIAs. *Arrows 1* and *2* indicate the elution positions of synthetic rat NERP-1 and NERP-2.

itive cells are concluded to contain NERPs at high frequency. Immunogold electron microscopy revealed the colocalization of NERPs with vasopressin in storage granules (Fig. 3, *A* and *C*, *insets*). Based on these results, we supposed that we could elucidate a biological function of NERPs in the context of vasopressin physiology.

VGF mRNA levels in both the PVN and SON were up-regulated upon water deprivation in rats (Fig. 4, *A* and *B*), accompanied by the up-regulation of vasopressin mRNA levels (PVN, 153.0 ± 13.6%; SON, 161.9 ± 12.4%; % of controls, $p < 0.01$). These *in vivo* and immunocytochemical observations suggest that NERPs are involved in the central control of body fluid balance. Consistent with previous reports (25, 26), icv injection of hypertonic NaCl or AII increased plasma vasopressin levels in rats (Fig. 4*C*, *2nd* and

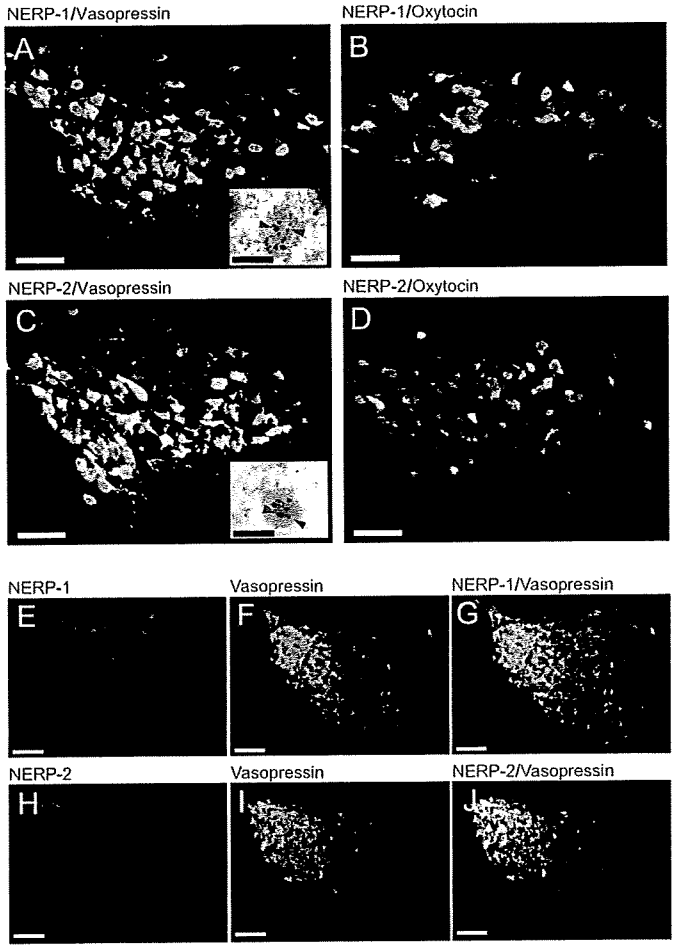



FIGURE 3. **NERPs colocalize with vasopressin in the PVN and SON of rats.** *A–D*, NERPs-specific immunohistochemistry of the SON. NERP-1 and NERP-2 (both in *red*) frequently colocalize with vasopressin (*green*) but rarely with oxytocin (*green*). *Scale bars*, 50 μm. *Inset*, immunoelectron micrographs indicating the colocalization of NERPs (10-nm gold particles, *red arrowhead*) with vasopressin (5-nm gold particles, *green arrowhead*) in an SON neuron. *Scale bars*, 1 μm. *E–J*, immunofluorescence staining of NERPs in the PVN. NERP-1 and NERP-2 (both in *red*) colocalize with vasopressin (*green*), especially in the magnocellular division. *Scale bars*, 100 μm.

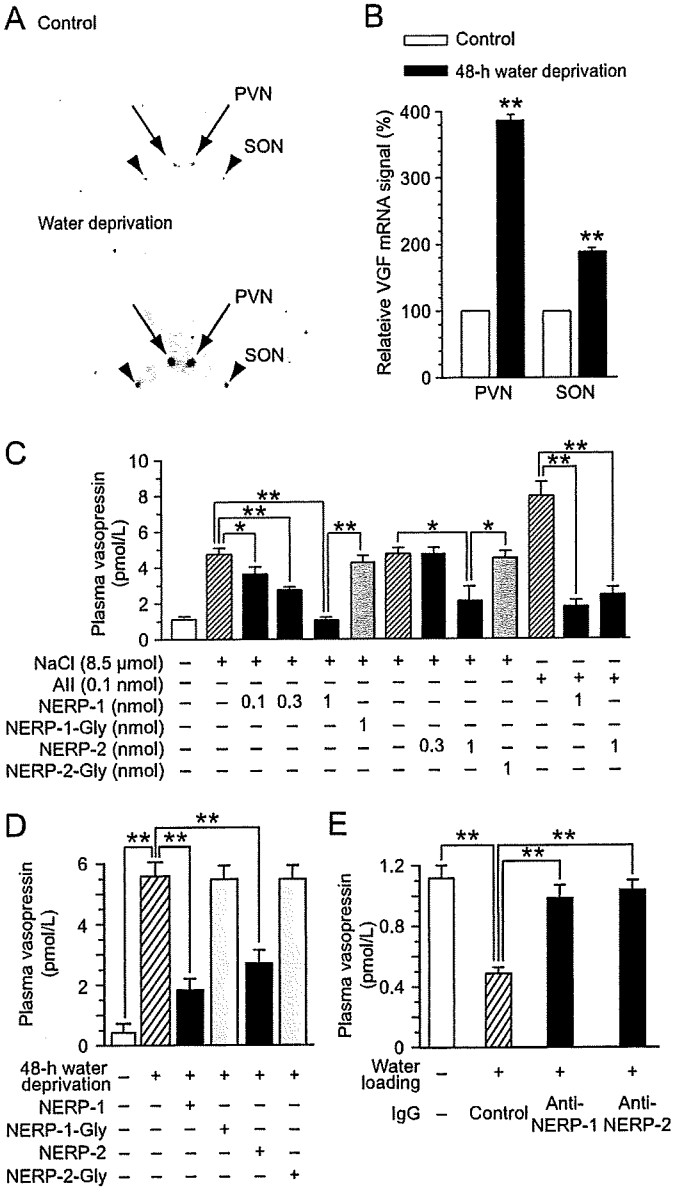



FIGURE 4. **NERPs suppress vasopressin secretion *in vivo* in rats.** *A*, increased VGF gene expression in the PVN and SON following 48 h of water deprivation. *B*, quantitative densitometric analysis of *A*. *C*, effect of icv-administered NERPs on rat plasma vasopressin levels in response to icv stimulation with hypertonic NaCl or AII. *D*, intracerebroventricular administration of NERPs, but neither NERP-1-Gly nor NERP-2-Gly, to rats suppressed plasma vasopressin elevation induced by water deprivation. *E*, intracerebroventricular administration of anti-NERP-1 IgG or anti-NERP-2 IgG reversed plasma vasopressin suppression induced by water loading. *, $p < 0.05$; **, $p < 0.01$.

*11th lanes*). This stimulation was dose-dependently suppressed by icv injection of NERP-1 before injection of the vasopressin secretagogues (Fig. 4*C*). Similar effects were observed with NERP-2, but its potency was weaker than that of NERP-1 because 0.3 nmol of NERP-2 was not effective. Neither nonamidated NERP-1 (NERP-1-Gly) nor nonamidated NERP-2 (NERP-2-Gly) suppressed vasopressin secretion (Fig. 4*C*). The increase in plasma vasopressin levels caused by water deprivation was also suppressed by icv-administered NERP-1 or NERP-2 (Fig. 4*D*). Furthermore, icv administration of anti-NERP-1 IgG or anti-NERP-2 IgG significantly reversed plasma vasopressin suppression induced by acute water loading (Fig. 4*E*), suggesting that NERPs function as endogenous peptides to regulate vasopressin secretion. Next, we examined the *in vitro* effect of NERPs on vasopressin secretion using hypothalamic explants. NERP-1 suppressed basal and AII-induced vasopressin secretion from the PVN and SON (Fig. 5). NERP-2 was likewise effective, but NERP-1-Gly or NERP-2-Gly was not (Fig. 5).

## DISCUSSION

In this study, to expedite the identification of potentially bioactive peptides, we analyzed peptides present in the supernatant of cultured cells, rather than analyzing peptides extracted from tissues. In addition, the benefit of our approach is that we examined an endocrine cell line that secretes C-terminally amidated peptide hormones such as calcitonin or calcitonin gene-related peptide at a high rate. Because C-terminal amidation is a post-translational modification most often shared by bioactive peptides (7), we thought that potentially bioactive peptides could be identified easier using this chemical feature. This tag has been used to discover a series of bioactive peptides such as

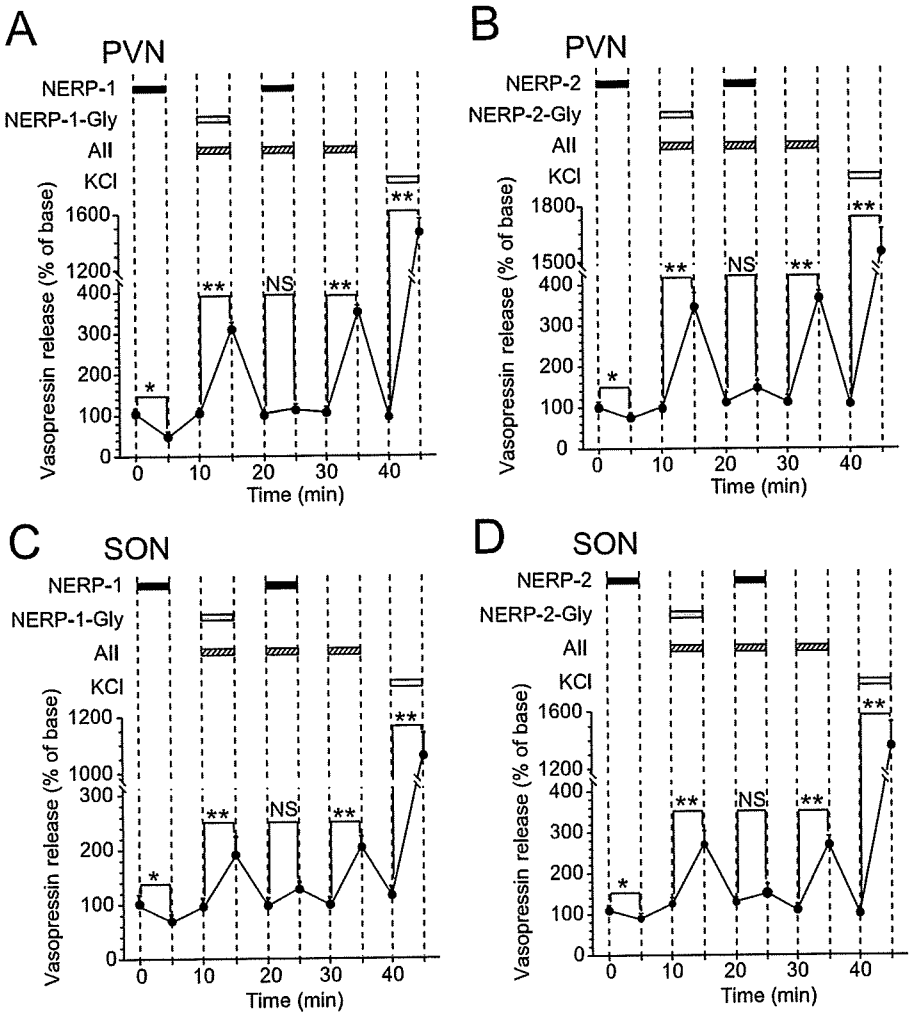



FIGURE 5. **NERPs suppress vasopressin secretion from PVN (*A* and *B*) and SON (*C* and *D*) explants.** *Black, gray, shaded,* and *white bars* indicate the NERPs, NERPs-Gly, AII, and KCl administration periods, respectively. *, $p < 0.05$; **, $p < 0.01$; NS, not significant.

galanin or neuropeptide Y (27). Using this "peptide first" approach, it remains difficult to infer biological activity just from the sequence of a peptide even if it is C-terminally amidated. Fortuitously, localization of NERPs in specific hypothalamic nuclei allowed us to speculate on their possible biological roles in relation to known peptide hormone, vasopressin, and to analyze them with *in vitro* and *in vivo* experiments described here.

VGF, originally identified in rat pheochromocytoma PC12 cells as a nerve growth factor-responsive gene, encodes a 617-amino acid protein in rodents (8). Immunohistochemical studies in PC12 cells revealed that the protein is stored in dense core granules and secreted through the regulated pathway (28, 29). Because VGF harbors several paired basic amino acid residues targeted by prohormone convertases, it has long been considered a precursor for several bioactive peptides (9). Using antibodies raised against the C terminus of the intact VGF protein, some VGF-derived peptides have been reported and shown to possess biological activity; C-terminal peptides TLQP-62 and AQEE-30 enhance synaptic activity in a whole-cell patch clamp recording on rat hippocampal cells (30), and AQEE-30 and LQEQ-19 enhance penile erection (31). More recently, the study has extended to the identification of TLQP-21 that increases energy expenditure by stimulating autonomic activation of adrenal medulla and adipose tissues (10).

In contrast to these conventional approaches, several peptidomics studies have reported the identification of VGF-derived peptides in brain tissues or cerebrospinal fluid (11, 12). Some VGF peptides are reported to be a marker for Alzheimer disease (32, 33). Except for the aforementioned VGF peptides, however, no VGF-related peptides have been demonstrated to be bioactive. Current peptidomic studies use tandem mass spectrometric techniques for peptide identification, in which C-terminal amidation is considered as a possible modification that target peptides could undergo. Because mass spectrometry schemes tend to detect abundant peptides or easily ionized peptides, it would make sense that no C-terminally amidated peptides from VGF have been reported to date.

It should also be mentioned that NERP-2 might have escaped *in silico* prediction of bioactive peptides that principally takes into account only paired basic amino acid residues for processing sites; human and rat NERP-2 are cleaved at the amino acid residues $^{345}$GLG↓GRG$^{350}$ and $^{348}$DLG↓GRG$^{353}$, respectively. The identification of these processing sites also demonstrates a methodological advantage of a peptidomic approach, and *in silico* prediction in turn may also be reinforced by accumulating data about endogenous peptides that peptidomics is going to provide.

Vasopressin synthesized in the PVN and SON magnocellular neurosecretory cells is packed in the secretory granules. Axons from these cells terminate in the posterior pituitary from which vasopressin is secreted into the systemic circulation to control renal excretion of water. NERPs were frequently colocalized with vasopressin in the secretory granules of the PVN and SON. VGF mRNA levels increased along with vasopressin mRNA in response to water deprivation, suggesting that NERPs participate in the hypothalamic control of plasma osmolarity balance. Both NERP-1 and NERP-2 suppressed vasopressin release stimulated by icv administration of hypertonic saline or AII *in vivo*. In *in vitro* experiments, NERPs also abolished AII-induced vasopressin release from the PVN and SON. All these actions were observed with C-terminally amidated forms only. Both anti-NERPs IgGs canceled plasma vasopressin reduction in

response to water loading, indicating that NERPs could be potent endogenous suppressors of vasopressin release.

Vasopressin release is regulated by the electrical activity of vasopressin neurons, which are modulated by various neurotransmitters and neuromodulators (25, 26). The major neural signals to vasopressin neurons are excitatory and inhibitory postsynaptic currents generated by presynaptic release of glutamate and γ-aminobutyric acid, respectively. AII and NaCl potentiate excitatory postsynaptic currents in vasopressin neurons, thereby stimulating vasopressin secretion (25). Although cell-surface receptors or target proteins of NERPs have not been identified yet, the actions of NERP to suppress AII- and NaCl-induced vasopressin release from the hypothalamus may suggest that they presynaptically inhibit the glutamatergic inputs or enhance GABAergic inputs to vasopressin neurons. Further investigation using whole-cell patch clamp recordings of PVN or SON slice preparations to examine the effect of NERPs on synaptic inputs to vasopressin neurons should elucidate the mechanisms by which NERPs modulate vasopressin release.

In conclusion, NERPs are novel bioactive peptides involved in body fluid homeostasis; they appear to modulate the actions and secretions of other neuropeptides. This study exemplifies the ability of focused peptidomics to facilitate the discovery of mammalian bioactive peptides. Further studies of NERPs and their receptors will pave the way for elucidating unknown extracellular signaling mechanisms as well as understanding the physiological roles of NERPs in body fluid homeostasis.

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# Ghrelin stimulates growth hormone secretion and food intake in aged rats

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**Abstract**

Age-related decreases in energy expenditure have been associated with the loss of skeletal muscle and decline of food intake, possibly through a mechanism involving changes of growth hormone (GH) secretion and feeding behavior. Age-related declines of growth hormone secretion and food intake have been termed the somatopause and anorexia of ageing, respectively. Ghrelin, a 28-amino-acid peptide, was isolated from human and rat stomachs as an endogenous ligand of growth hormone secretagogue receptor. Ghrelin stimulates growth hormone release and food intake when peripherally administered to rodents and humans. Here, we investigate the relationship between age-related decline of growth hormone secretion and/or food intake and ghrelin function. Ghrelin (10 nmol/kg body weight) was administered intravenously to male 3-, 12-, 24-and 27-month-old Long-Evans rats, after which growth hormone concentrations and 2 h food intake were measured. An intravenous administration of ghrelin to rats increased food intake in all generations. In addition, to orexigenic effect by ghrelin, intravenous administration of ghrelin elicited a marked increase in plasma GH levels, with the peak occurring 15 min after administration. These findings suggest that the aged rats maintain the reactivity to administered exogenous ghrelin.
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*Keywords:* Ghrelin; Feeding behavior; Growth hormone secretion; Ageing

## 1. Introduction

The decline in blood levels of growth hormone (GH) with ageing are commonly referred to as the somatopause (Anawalt and Merriam, 2001; Lamberts et al., 1997). Because GH changes are associated with declines in physical abilities, attempts are often made to save the decline of physical abilities with ageing by GH replacement. However, the relative ratio of risk to benefit in GH replacement requires further discussion. Underlying mechanism of age-related somatopause, therefore, has to be investigated to find an ideal method of intervention.

Ghrelin, a 28-amino-acid peptide, was isolated from human and rat stomachs as an endogenous ligand of growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). Ghrelin stimulates growth hormone release when peripherally or centrally administered to rats and when applied directly to rat primary pituitary cells (Date et al., 2000; Kojima et al., 1999; Toshinai et al., 2006; Wren et al., 2000). Plasma ghrelin levels decline with ageing due to impaired function of the gastric mucosa reducing the thickness of the membrane, the length of the glands, and the number of the endocrine cells in mice (Sandstrom et al., 1999). Previous human studies indicated that stomach ghrelin secretion decreases with ageing (Rigamonti et al., 2002) and that ghrelin-induced GH secretion is reduced in aged subjects compared to younger subjects (Broglio et al., 2003). In contrast to human data, plasma ghrelin concentrations and stomach ghrelin contents in aged rats are significantly higher than in young rats (Englander et al., 2004). In addition, ghrelin-induced GH secretion is higher compared to young rats. However, since these findings were provided from a cross-sectional study, the relationship between age-related dynamics of ghrelin and somatopause remains undefined.

*Abbreviations:* CCK, cholecystokinin; GH, growth hormone; GHS-R, growth hormone secretagogue receptor; GHRP-6, GH-releasing hexapeptide; IGF-1, insulin-like growth factor-1; ip, intraperitoneal; iv, intravenous; LETO, Long-Evans Tokushima Otsuka

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Anorexia is commonly associated with ageing (MacIntosh et al., 2000; Morley, 2001a) and may be related to age-related decline of plasma ghrelin (Rigamonti et al., 2002). Normal ageing is associated with a decrease in appetite and energy intake, which has been termed the anorexia of ageing (Morley and Thomas, 1999; Morley, 2001b). Generally, after age 70–75 years, the reduction in energy intake exceeds energy expenditure in humans, resulting in weight loss where loss of muscle (sarcopenia) predominates and predisposes older subjects to protein energy malnutrition (Baumgartner et al., 1998; Morley, 2001b). The observed malnutrition and sarcopenia correlates with increased morbidity, and the number of hospitalizations with extended stays (Sullivan, 1998). The causes of the physiological anorexia typified during ageing are unknown; they are probably multifactorial and include a reduction in feeding drive with increased activity of satiety signals. Ghrelin stimulates food intake as well as GH secretion (Asakawa et al., 2001; Lawrence et al., 2002; Nakazato et al., 2001; Shintani et al., 2001; Tschöp et al., 2000; Wren et al., 2000, 2001). Treatment with exogenous ghrelin or ghrelin mimetics may prove beneficial in the anorexia of ageing. To investigate the relationship between age-related decline of GH secretion and food intake and ghrelin function, ghrelin (10 nmol/kg body weight) was administered intravenously to 3-, 12-, 24- and 27-month-old rats, after which GH concentrations and 2 h food intake were measured.

## 2. Materials and methods

### 2.1. Animals

Male Long-Evans Tokushima Otsuka (LETO) rats (4-week-old) were obtained from Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). All animals were housed individually in plastic cages at constant room temperature (23 °C) in a 12 h light (8 AM–8 PM)/12 h dark cycle and were given standard laboratory chow and water *ad libitum*. Experiments were conducted on rats at 3, 12, 24, and 27 months of age ($n = 10$). In this study, anesthesia was carried out by an intraperitoneal (ip) injection of sodium pentobarbital (75 mg/kg body weight) (Abbot Lab., Chicago, IL). Rats were used as follows. Sterilized intravenous (iv) cannulae were implanted into the right jugular vein 1 week before the experiments of feeding and GH response on rats at 3, 12, 24, and 27 months of age. All rats recovered from surgery within 1 week, showing food intake amounts similar to pre-surgery levels and progressive weight gain. These rats were then used in the experiments. Rat ghrelin (Peptide Institute Inc., Osaka, Japan) or saline was administered icv to rats fed *ad libitum*. The 2 h food intake amounts were then measured. This feeding test was performed using a crossover design experiment in which animals were randomized to receive either test substance with a washout period of 2 days between each administration. Two days after the feeding test, ghrelin (10 nmol/kg body weight) was administered iv to these rats which were anesthetized by sodium pentobarbital for the GH response test. After these tests, the iv cannulae were removed from the rats using sterilized devices. To prevent suppuration by infection, we frequently disinfected the rat, and exchanged cages after the operation. Rats were bred in previously described conditions until reaching the age of the following test. All procedures were approved by University of Miyazaki Animal Care and Use Committee and were in accordance with the Japanese Physiological Society's guidelines for animal care.

### 2.2. Food intake

During 3 days before administration, 24 h food intake amount was measured each day. Ghrelin (10 nmol/kg body weight) or saline was administered iv to rats at 10:00 AM through an iv cannula. The 2 h food intake amount was then measured. Also, relative amount of ghrelin-induced food intake was evaluated by the ratio of ghrelin-induced food intake to average of 24 h food intake amount during the 3 days. All of the rats used in these experiments were satisfactorily acclimated to handling before iv injections.

### 2.3. GH response

After anesthesia by an ip injection of sodium pentobarbital, ghrelin (10 nmol/kg body weight) was administered iv to rats at 11:30 AM through an iv cannula. Blood samples (60 μl) were obtained from the tail vein, which was cut 15 mm from the tail end at a depth of about 2 mm by knife, at 0, 15, 30 and 60 min after administration. The plasma concentration of GH was determined with a Biotrak Rat GH RIA kit (Amersham, Buckinghamshire, UK).

### 2.4. Statistic analysis

Data (mean ± S.E.M.) were analyzed by ANOVA (analysis of variance) and the *post hoc* Scheffe-F test. Differences were considered to be significant when the $P$ values were less than 0.05.

## 3. Results

### 3.1. Changes of age-related body weight and food intake

Body weight increased gradually in LETO rats from 3- to 24-month of age. The body weight in 27-month-old LETO rats was significantly decreased compared to 24-month-old LETO rats (Fig. 1A). Food intake for 24 h did not change from 3- to 24-month-old LETO rats, while 24 h food intake in 27-month-old LETO rats was significantly decreased compared to 24-month-old LETO rats (Fig. 1B).

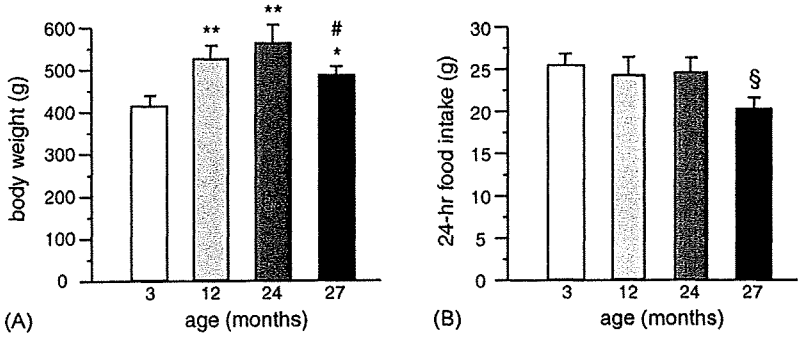


Fig. 1. Changes of body weight (A) and 24 h food intake (B) with ageing. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. 3-month-old rats, $^{\#}P < 0.05$ vs. 24-month-old, $^{\S}P < 0.01$ vs. 3-, 12-, or 24-month-old rats.

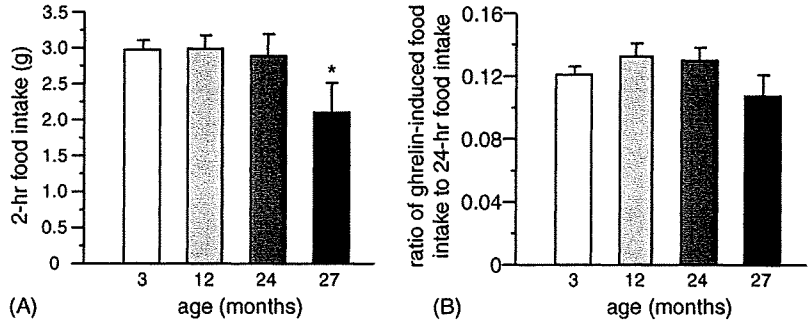


Fig. 2. (A) Effect of iv administration of ghrelin (10 nmol/kg body weight) on 2 h food intake in 3-, 12-, 24- and 27-month-old rats. $^{*}P < 0.01$ vs. 3-, 12-, or 24-month-old rats. (B) No effect of ageing on the ration of ghrelin-induced 2 h food intake to 24 h food intake.

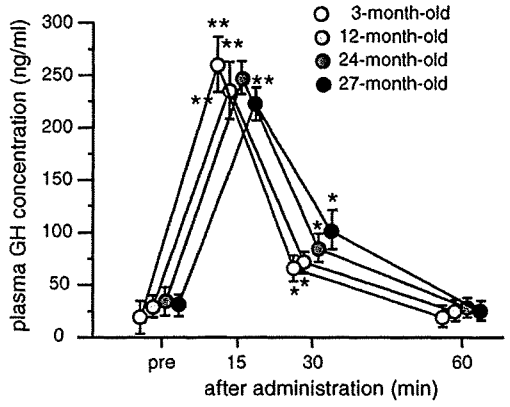


Fig. 3. Effect of iv administration of ghrelin (10 nmol/kg body weight) on the plasma GH concentration in 3-, 12-, 24- and 27-month-old rats. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. pre-administration.

### 3.2. Changes of age-related ghrelin-induced food intake

We examined the effects of ageing on ghrelin-induced food intake. While an iv administration of saline to LETO rats did not induced food intake in all generations, an iv administration of ghrelin to LETO rats increased food intake in all generations. The amounts of ghrelin-induced food intake in 27-month-old LETO rats were significantly decreased compared to the other generations (Fig. 2A). However, the ratio of ghrelin-induced food intake to 24 h food intake was the same among the generations (Fig. 2B).

### 3.3. Changes of age-related ghrelin-induced GH secretion

We studied the release of GH in response to peripheral ghrelin administration at all generations of LETO rats. Iv administration of ghrelin elicited a marked increase in plasma GH levels, with the peak occurring 15 min after administration (Fig. 3). The level of ghrelin-induced GH secretion was not different among the generations.

## 4. Discussion

The circulation level of insulin-like growth factor-1 (IGF-1) is increased by the increase in plasma GH concentration. GH and IGF-1 promote cell survival and proliferation through independent and different pathways (Baixeras et al., 2001). The amplitude of pulsatile GH release from the anterior pituitary gland secretion is attenuated with ageing, and the attenuation of GH release induces decrease in IGF-1 (Ho et al., 1987; Minisola et al., 1993). These age-related reductions are commonly referred to as the somatopause (Anawalt and Merriam, 2001; Lamberts et al., 1997). The somatopause during ageing has been partially explained by the reduction in GH response to peptidyl or nonpeptidyl synthetic ghrelin mimetics, GH-releasing hexapeptide (GHRP-6) or MK-0677, and GH releasing hormone (Abribat et al., 1991; Aloi et al., 1994; Ceda et al., 1986; Chapman et al., 1996; Muccioli et al., 2002; Sonntag et al., 1983; Spik and Sonntag, 1989). The GH responses to acute iv administration of ghrelin in elderly subjects were lower than those in young adult subjects (Broglio et al., 2003). In addition, expression of GHS-R messenger ribonucleic acid is reduced in the aged human hypothalamus, which is consistent with their reduced GH response to ghrelin (Muccioli et al., 2002). Plasma ghrelin concentrations reduce in humans as they age (Rigamonti et al., 2002); therefore, lower ghrelin production in addition to reduced GHS-R levels suggest that somatopause may reflect impairment in the ghrelin signaling pathway. In contrast to humans, stomach ghrelin production and secretion are increased, and GH release in response to exogenous ghrelin is enhanced in aged rats (Englander et al., 2004). Therefore, age-related decline in GH secretion may not be due to a reduction in stomach ghrelin secretion or a stimulatory action on GH release. The present study demonstrated that iv administration of ghrelin increased GH secretion in all LETO rats investigated for 27 months at 15 min after administration. In addition, the levels of GH response to ghrelin were not affected with the months of age in rats. These findings suggest that the aged rats maintain a high reactivity to ghrelin stimulation, and that aged rats secure storage of GH in the anterior pituitary gland.

Longitudinal studies have demonstrated a decline in energy intake with ageing (Hallfrisch et al., 1990; Koehler, 1994). For example, a study involving a three-decade follow-up of 105 male humans aged 27–65 years demonstrated a decrease in daily energy intake of up to 25% (Hallfrisch et al., 1990). A 7-year longitudinal study in subjects aged 64–91 years also demonstrated a decrease in energy intake of 19.3 kcal/d per year in women and 25.1 kcal/d per year in men (Koehler, 1994).

The reduction in energy intake with ageing exceeds energy expenditure, resulting in weight loss involved sarcopenia (Baumgartner et al., 1998; Morley, 2001b). Indeed, the satiating effects of cholecystokinin (CCK), a gastrointestinal-derived anorectic peptide, increased with ageing and fasting and postprandial CCK concentrations are higher in healthy elderly subjects compared to young adults (MacIntosh et al., 1999, 2001). In contrast to age-related increase of CCK function, previous cross-sectional studies indicated that stomach ghrelin secretion and ghrelin-induced GH secretion decreased in aged subjects compared to younger subjects (Broglio et al., 2003; Rigamonti et al., 2002). The efficiency of ghrelin and CCK signal transduction depend on the balance of their respective plasma concentration and/or on interactions between GHS-R and CCK type A receptor (Date et al., 2005). Thus, enhanced effects of CCK and/or reduced effects of ghrelin may contribute to the development of anorexia and in some cases protein malnutrition during ageing. Therefore, ghrelin coupled with its anabolic effects via the GH/IGF-1 axis indicate that rescue of reduced GHS-R activity by treatment with exogenous ghrelin or ghrelin mimetics may contribute to retard the progress of anorexia of ageing. We indicate that iv administration of ghrelin increases food intake in all generations and that the ratio of ghrelin-induced food intake to 24 h food intake was the same among the generations. These results suggest that peripheral administration of ghrelin may prevent age-dependent decline in energy intake in animals.

Recent studies demonstrated that circulating ghrelin bound to the membranes of cardiomyocytes, adipocytes, and osteocytes dependently or independently of the GHS-R (Baldanzi et al., 2004; Bedendi et al., 2003; Delhanty et al., 2006). Ghrelin functions as an anti-catabolic agent in peripheral tissues, involving adipogenesis, osteogenesis, and cell proliferation (Baldanzi et al., 2004; Bedendi et al., 2003; Delhanty et al., 2006). Therefore, ageing process represented by catabolic-anabolic imbalance in peripheral tissues may increase ghrelin utilization to maintain cell functions. The present study indicated the possibility of suppressing the age-related decline of GH secretion and food intake by ghrelin. Further studies will be necessary to clarify whether a chronic administration of ghrelin prevents age-related regression involved somatopause, sarcopenia, and anorexia.

In conclusion, our results indicate that peripheral administration of ghrelin increases GH secretion and food intake in all generations. Somatopause and anorexia of ageing are associated with declines in physical abilities. Therefore, ghrelin replacement may improve physical abilities to stimulate GH secretion and feeding in aged animals. The present study will provide novel insights into the physiological function of ghrelin in ageing process.

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# Plasma Ghrelin Concentrations in Different Clinical Stages of Diabetic Complications and Glycemic Control in Japanese Diabetics

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**Abstract.** Ghrelin is an acylated 28-amino-acid peptide that stimulates food intake, GH secretion, and gastric motility. Experimental studies have suggested that ghrelin plays roles in glucose homeostasis, atherosclerosis, and microangiopathy. We investigated possible involvement of ghrelin in micro- and macro-vascular diabetic complications and glycemic control in diabetic patients. Fasting and postprandial plasma ghrelin concentrations after a test meal were measured in 108 and 61 Japanese diabetic patients, respectively. Plasma ghrelin concentrations were negatively correlated with body mass index (BMI) ($r = -0.309$, $P = 0.002$) or $HbA_{1c}$ ($r = -0.264$, $P = 0.0065$). Plasma ghrelin levels in patients with diabetic nephropathy who showed high serum creatinine levels (s-Cre) were significantly higher than those in patients who showed normal s-Cre ($P<0.02$). In patients with diabetic triopathy, plasma ghrelin concentrations were significantly lower than those in patients without diabetic complications ($P<0.05$). Stepwise multiple regression analyses revealed that s-Cre, BMI, and $HbA_{1c}$ were independently associated with plasma ghrelin levels. A postprandial decrease of ghrelin was observed in patients with normal $CV_{R\text{-}R}$ values or those with normal body weight, whereas it was not seen in obese patients or in patients with low $CV_{R\text{-}R}$ values. Suppression rates of ghrelin 30–60 min after a test meal in obese patients were significantly lower than those in normal-weight patients. These findings suggest that ghrelin secretion is suppressed by long-term hyperglycemia and that obesity influences the regulation of ghrelin secretion.

*Key words*: Diabetes, Diabetic complication, Ghrelin, $HbA_{1c}$, Hyperglycemia

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**GHRELIN**, a GH-releasing peptide isolated from human and rat stomachs, is an acylated 28-amino-acid peptide that stimulates food intake, GH secretion, and gastric motility in many species, including humans [1–4]. Ghrelin is considered to be involved in the regulation of eating behavior and energy homeostasis. For example, chronic ghrelin administration increased the body fat content of rodents [5]. Ghrelin is mainly secreted from endocrine cells in the stomach mucosa, but is also widely expressed in various tissues and cells, including pancreatic β cells [6].

Plasma ghrelin concentrations are negatively correlated with body mass index (BMI) in healthy subjects [7–9] and type 2 diabetic patients without diabetic complications [10]. Low plasma ghrelin concentrations are associated with insulin resistance in patients with polycystic ovary syndrome [11] and in obese children and adolescents [12]. On the other hand, high plasma ghrelin concentrations have been reported in patients who are emaciated due to cancer [13], chronic heart failure [14], or anorexia nervosa [15].

Ghrelin secretion is upregulated under negative energy balance conditions, whereas it is downregulated in a state of positive energy balance. Plasma ghrelin levels were shown to rise before meals and rapidly

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decrease after food consumption, glucose intake, or intravenous glucose infusion in humans and rodents [6, 10, 15]. These results indicate that ghrelin secretion is suppressed, at least, under short-term hyperglycemic and/or hyperinsulinemic conditions. The relationship between ghrelin and long-term hyperglycemia, however, has yet to be clarified. In addition, there is data suggesting that ghrelin may have a favorable effect on endothelial function. The ghrelin receptor was upregulated in the coronary arteries of humans with atherosclerosis [16] and ghrelin was shown to improve endothelial dysfunction through a GH-independent mechanism in rats [17]. Ghrelin also inhibited the angiogenic activity of rat brain microvascular endothelial cells [18]. These findings suggest that ghrelin has an important role in endothelial function and may prevent diabetic complications such as atherosclerosis and microangiopathy.

In the present study, we investigated the relationship between plasma ghrelin concentrations in diabetic patients and micro- and macro-vascular diabetic complications as well as the relationship between plasma ghrelin concentrations and glycemic control.

## Materials and Methods

### *Subjects*

One hundred and eight Japanese patients (56 men and 52 women) with diabetes mellitus (11 type 1 and 97 type 2 patients) based on the criteria of the American Diabetes Association [19] were enrolled in our study. The subjects were randomly selected from out- and in-patients who were treated at the Miyazaki Medical College Hospital between 2000 and 2004. Table 1 shows their clinical characteristics. All patients were classified into lean (BMI<18.5), normal-weight (18.5≤BMI<25), or obese (BMI≥25) according to the criteria of the Japan Society for the Study of Obesity. Fifty-one patients were treated with insulin, 30 patients with oral hypoglycemic agents, and 27 patients with diet alone. All patients were clinically stable at the time of evaluation and had no evidence of gastrointestinal disease or cachectic state such as cancer, thyroid disease, liver disease, or infection. Diabetic retinopathy was diagnosed by ophthalmologists using fundus examinations and photography. All patients were classified into one of three grades: (1) no signs of diabetic retinopathy (NDR); (2) simple diabetic retinopathy (SDR); or (3) proliferative diabetic retinopathy (PDR) including pre-proliferative retinopathy [20]. Diabetic nephropathy was classified based on the criteria of the Japan Diabetes Society. Seventy-one patients with normoalbuminuria were classified into stage 1; 16 patients with microalbuminuria stage 2; 14 patients with overt albuminuria but no elevation of serum creatinine levels (s-Cre) stage 3; and seven patients with elevated s-Cre (>1.4 mg/dl) who were not receiving hemodialysis or continuous ambulatory peritoneal dialysis stage 4. Normoalbuminuria, microalbuminuria, and overt albuminuria were defined as <30 mg/day, 30–300 mg/day, and >300 mg/day, respectively. Diabetic neuropathy was diagnosed in the presence of clinical evidence of peripheral sensorimotor neuropathy plus abnormal nerve conduction in at least two or more peripheral

**Table 1.** Clinical characteristics of diabetic patients

| | Lean | Normal | Obese | Total |
|---|---|---|---|---|
| n (M/F) | 8 (4/4) | 61 (31/30) | 39 (21/18) | 108 (56/52) |
| Age (y) | 57.1 ± 2.3 | 56.6 ± 1.8 | 56.7 ± 2.1 | 56.7 ± 1.3 |
| BMI | 17.4 ± 0.2 | 22.3 ± 0.2 | 28.1 ± 0.8 | 24.0 ± 0.4 |
| Duration of diabetes (y) | 14.4 ± 2.9 | 10.1 ± 1.1 | 10.1 ± 1.3 | 10.4 ± 0.8 |
| Type of diabetes (1/2) | 0/8 | 10/51 | 1/38 | 11/97 |
| $HbA_{1c}$ (%) | 7.6 ± 0.7 | 8.2 ± 0.3 | 8.0 ± 0.3 | 8.1 ± 0.2 |
| Treatment (diet/OHA/insulin) | 0/2/6 | 10/18/33 | 17/10/12 | 27/30/51 |
| Retinopathy (none/SDR/PDR) | 0/2/6 | 35/15/11 | 22/9/8 | 57/26/25 |
| Neuropathy (+/–) | 6/2 | 31/30 | 25/14 | 62/46 |
| Nephropathy (1/2/3/4) | 4/2/1/1 | 38/11/7/5 | 29/4/5/1 | 71/17/13/7 |
| Macroangiopathy (+/–) | 1/7 | 10/51 | 6/33 | 17/91 |

Data are expressed as means ± SEM. BMI, body mass index; OHA, oral hypoglycemic agents; SDR, simple diabetic retinopathy; PDR, proliferative diabetic retinopathy.

nerves. Diabetic macroangiopathy was diagnosed based on a positive history of ischemic heart disease, cerebral infarction, or arteriosclerosis obliterans.

*Protocol*

The Institutional Committee of Miyazaki Medical College approved the protocol, and all subjects gave informed consent before participation. Blood samples were collected from all the patients at 0800 h after they fasted overnight. Sixty-one diabetic patients were given a test meal (30% of their daily energy intake containing 50% carbohydrate, 30% fat, and 20% protein) at 0800 h. Their blood samples were collected at 0, 30, 60, and 120 min after eating a test meal.

*Laboratory methods*

Plasma ghrelin concentrations were measured using a radioimmunoassay developed by our laboratory, which targeted the C-terminal region of human ghrelin as described [10]. Inter- and intra-assay variations were less than 8% and 6%, respectively. The levels of plasma glucose, serum insulin, serum C-peptide, serum creatinine, urinary albumin, urinary creatinine, and $HbA_{1c}$ were measured using standard laboratory methods.

*Coefficient of variation of R-R intervals ($CV_{R-R}$)*

$CV_{R-R}$ was calculated using the following formula using an electrocardiogram FDX-4521 (Fukuda Denshi, Tokyo, Japan): $CV_{R-R}$ = (standard deviation/mean value of R-R intervals) × 100 (%). $CV_{R-R}$ was calculated during a 60-sec period when it appeared stable on an electrocardiogram.

*Statistical analyses*

Statistical differences among more than two groups were evaluated using one-way analysis of variance (ANOVA) and a post hoc Fisher's test. Values are expressed as means ± SEM. Unpaired Student's t-test and Wilcoxon's correction for unequal variances were used for comparisons between two groups. Correction coefficients were calculated by linear regression analysis. Stepwise multiple regression analyses were used to determine the contributing factors to plasma ghrelin level. We used the following independent variables: age, sex, BMI, $HbA_{1c}$, s-Cre, and fasting glucose. A $P$ value of less than 0.05 was considered statistically significant. Statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

## Results

Plasma ghrelin concentrations in lean diabetic patients were significantly higher than those in the other two groups, and the plasma ghrelin concentrations in normal-weight patients were significantly higher than those in obese patients (Fig. 1A). As plasma ghrelin concentration was negatively correlated with BMI (Fig. 1B), we examined the relationships between the levels of plasma ghrelin and nephropathy in the lean,

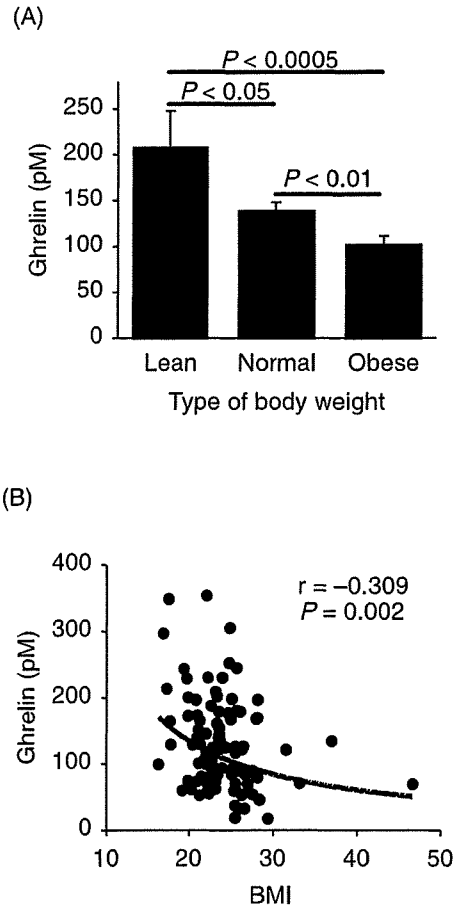


**Fig. 1.** Relationship between the plasma ghrelin concentration and BMI in type 1 and 2 diabetic patients. (A) Plasma ghrelin concentrations in lean (n = 8), normal-weight (n = 61), and obese patients (n = 39). (B) Correlation between plasma ghrelin concentration and BMI.

normal-weight, and obese patients separately. Plasma ghrelin concentrations in normal-weight patients with stage 4 nephropathy were significantly higher than those in normal-weight patients with other stages of nephropathy (Fig. 2A). A similar result was seen in obese patients (data not shown). Plasma ghrelin concentration was positively correlated with s-Cre levels in normal-weight patients (Fig. 2B) as well as those in obese patients (data not shown). Plasma ghrelin concentration was negatively correlated with creatinine clearance (Ccr) in both normal-weight patients (Fig. 2C) and obese patients (data not shown). We could not analyze these parameters in the lean group due to the small number of patients.

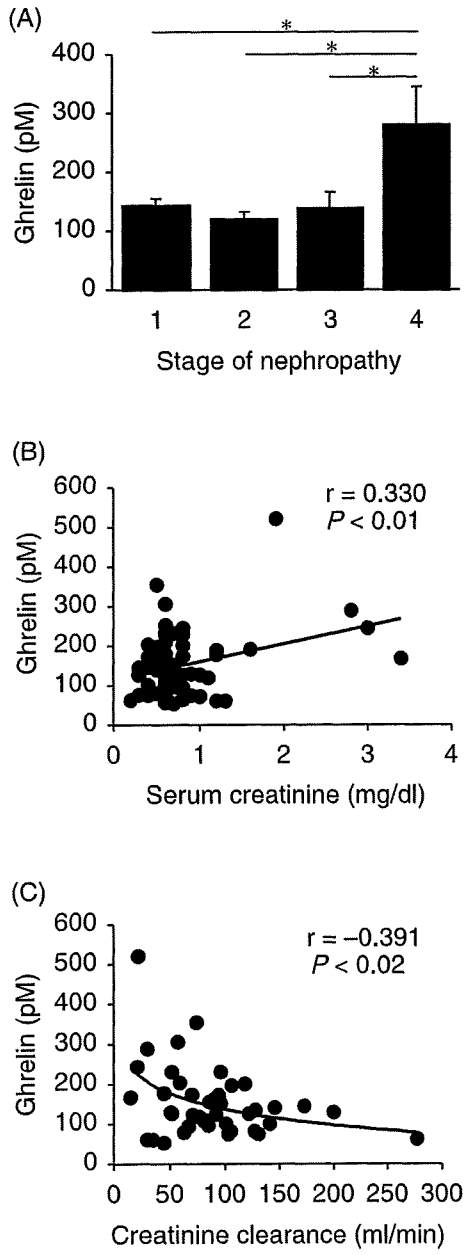


**Fig. 2.** Plasma ghrelin concentrations in normal-weight diabetic patients with or without diabetic nephropathy. (A) Plasma ghrelin concentrations for each stage of nephropathy. *$P$<0.02 vs. stage 4. (B) Correlation between plasma ghrelin concentration and serum creatinine. (C) Correlation between plasma ghrelin concentration and creatinine clearance.

Next, we examined the relationship between plasma ghrelin concentrations and diabetic complications. We compared all groups except for stage 4 nephropathy patients, because plasma ghrelin concentrations in patients with high s-Cre were significantly higher than those in patients with normal s-Cre. Plasma ghrelin concentrations in normal-weight patients were not affected by the presence of retinopathy, neuropathy, or macroangiopathy (Fig. 3A–C). Similar findings were observed in obese patients (data not shown). Plasma ghrelin concentrations in normal-weight patients with diabetic triopathy (*i.e.* retinopathy, nephropathy, and neuropathy) were significantly lower than those in the normal-weight patients without complications (Fig. 3D). Plasma ghrelin concentration was negatively correlated with $HbA_{1c}$ in all subjects (Fig. 4). To further define the relationship between plasma ghrelin level and various parameters in diabetic patients, stepwise multiple regression analysis was employed. In this analysis, plasma ghrelin level was used as a dependent variable and age, sex, BMI, $HbA_{1c}$, fasting plasma glucose, and s-Cre were used as independent variables in all subjects. When plasma ghrelin level was examined, s-Cre entered the regression first ($r = 0.326$), followed by BMI ($r = 0.420$), and $HbA_{1c}$ ($r = 0.467$) (Table 2). Plasma ghrelin concentrations in normal-weight patients ($n = 36$) significantly decreased at 30, 60, and 120 min after eating a test meal (Fig. 5A). In obese patients ($n = 21$), however, the level of plasma ghrelin was suppressed only at 120 min after eating a test meal. Suppression rates from the basal value of plasma ghrelin concentration at 30 and 60 min after a test meal in obese patients were significantly lower than those in normal-weight patients (Fig. 5A). Plasma ghrelin concentrations in normal-weight patients with normal $CV_{R\text{-}R}$ values (≥2%, $n = 16$) significantly decreased at 30, 60, and 120 min after eating a test meal. In patients with low $CV_{R\text{-}R}$ values (<2%, $n = 19$), however, a significant decrease in the ghrelin concentration was not seen at 30 and 60 min after eating a test meal (Fig. 5B).