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Fusogenic Liposome can be Used as an Effective Vaccine Carrier for Peptide Vaccination to Induce Cytotoxic T Lymphocyte (CTL) Response

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We reported previously that fusogenic liposome (FL) introduced antigen protein encapsulated in the liposome directly into the cytoplasm of the antigen presenting cells, and that it induced immune responses. In the present study, we encapsulated TAX38-46, an HTLV-I derived protein and an antigen peptide model, into FL. The ability to induce effective cytotoxic T lymphocytes (CTL) responses in immunized mice was evaluated. Results showed FL could induce CTL response effectively and suggested that FL is a potential peptide vaccine carrier.

Key words fusogenic liposome; cytotoxic T lymphocyte (CTL); peptide vaccine

Induction of cytotoxic T lymphocytes (CTL) that kill tumor cells is a critical role of immunotherapeutic agents for cancer. Most cancer vaccine strategies have focused on induction of CTL and various approaches, including DNA, virus vector or peptide vaccine, have been tested.[1] In general, the advantages of a peptide vaccine are the induction of CTL by the epitope and the safety, stability and simplicity of peptide production. However, peptides by themselves are rather weak immunogens. Peptides usually require the addition of an adjuvant for inducing immunogenicity, and recently, incomplete Freund's adjuvant (IFA) has been widely used as a vaccine adjuvant in clinical research.[2] However, IFA is only useful for inducing humoral immunity and thus it does not induce effective cell-mediated immune responses. In contrast, complete Freund's adjuvant (CFA) is used for CTL induction, although it cannot be applied clinically due to serious side effects, such as inflammation.[3]

To induce cell-mediated immune responses, a peptide must be delivered through the cytoplasm to the MHC class I processing pathway. However, peptides are unable to pass through the cytoplasm alone.[4] Therefore, we hypothesized that a vaccine carrier is required, which can deliver antigens into the cytoplasm and which exhibits adjuvant activity. We reported previously that fusogenic liposome (FL) introduces antigen protein encapsulated in the liposome directly into the cytoplasm, and that it can induce effective immune responses.[5—7] FL is a fusion liposome in which proliferating ability is inactivated, but which retains its cell membrane fusing ability. Therefore, FL can deliver encapsulated molecules into cells. Furthermore, FL possesses immune stimulating activity.[5] In this context, we considered FL as an ideal peptide vaccine carrier.

In the present study, we chose the TAX protein epitope (TAX 38-46; H-2D^{k-} restricted epitope in TAX, amino acid residues 38-46, sequence ARLHRHALL.[10]), which is an immunodominant target antigen derived from the human T-cell leukemia virus type I (HTLV-I), as a model peptide. HTLV-I, a retro virus, is known to cause Adult T-cell leukemia (ATL).[8] ATL is characterized by poor prognosis after chemotherapy and no effective therapy exists. Immunotherapy, which can induce strong anti HTLV-I CTL, has been proposed as an optimal approach to ATL treatment.[9] However, an effective immunotherapeutic approach has not been developed to date.

MATERIALS AND METHODS

Cells and Animals L929 cells were cultured with RPMI-1640 containing with 10% fetal calf serum (FCS). Female C3H mice were purchased from Nippon SLC (Kyoto, Japan) and used at 6 weeks-old stage.

Fusogenic Liposome Encapsulated TAX 38-46 TAX 38-46 and FITC conjugated TAX 38-46 were purchased from SIGMA (Japan). FL was prepared as described previously.[5—7] Briefly, lipid mixture (L-α-dimyristoyl phosphatidic acid/phosphatidylcholine/cholesterol in molar ratio of 1 : 4 : 5) was hydrated with phosphate buffered saline (PBS) or PBS containing TAX 38-46 or FITC conjugated TAX 38-46. Peptides containing liposome was prepared from these hydrated mixture by using a hand-held extruder with two layers of cellulose acetate membranes (pore size, 800 nm in diameter) (ADVANTEC, Osaka, Japan), and washed with PBS by centrifugation (20000 rpm, 40 min, 4 °C) in order to remove free peptides. These liposomes were mixed with UV-inactivated Sendai virus and incubated at 37 °C for 2 h with shaking. FL was purified by sucrose gradient centrifugation (24000 rpm, 2 h, 4 °C). The diameter of FL was detected by using ZETA-SIZER 3000HS (Malvern, U.K.). The concentration of peptide in FL was determined by measuring the fluorescence intensity of FITC.

^{51}Cr Release Assay For the CTL assay, TAX38-46 (50 μg), empty FL, TAX38-46 emulsified with CFA (contains 50 μg

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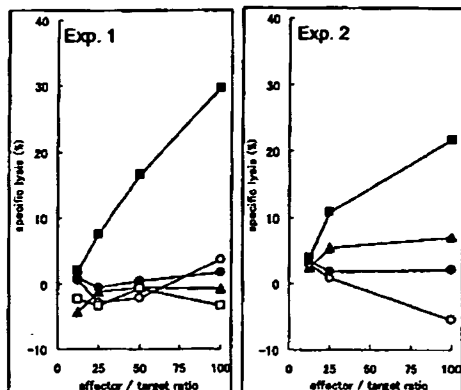

Fig. 1. TAX Peptide Encapsulated in FL Induced TAX Specific CTL

Nine days after the final immunization, mononuclear cells from the spleen of mice immunized with PBS (closed circle), TAX 38-46 alone (open circle), empty FL (open square), TAX 38-46 emulsified with CFA (closed triangle), or TAX 38-46 encapsulated in FL (closed square) were isolated and restimulated with MMC-treated TAX38-46 pulsed L929 for 5 d to enhance the frequency of antigen specific CTLs. CTL activity against TAX38-46 pulsed L929 (positive targets) or L929 (negative targets) was measured by ^{51}Cr release assay. Results were expressed as a percentage of specific lysis. Percentage of specific lysis=(percentage of positive target lysis)−(percentage of negative target lysis).

TAX38-46), or TAX38-46 encapsulated in FL (contains 30 μg TAX38-46) diluted to in total were injected into back of C3H mice (i.d.), respectively. Mice were immunized once a week for three weeks, and the spleen was harvested 9 d after the last immunization. Splenocytes were mixed with mitomycin C (MMC) treated TAX38-46 pulsed L929 for 5 d, and CTL assay was determined as follows. L929 cells (5×10^6) were pulsed with TAX 38-46 (positive targets) for 1 h at 37 °C or not (negative targets), and labeled with 200 μCi of ^{51}Cr for 2 h at 37 °C. Splenocytes were incubated with target cells (positive or negative targets) for 2 h at 37 °C. CTL activity was determined by measuring ^{51}Cr levels in the supernatants using a gamma counter. The specific lysis was determined as follows: percentage of specific lysis= (percentage of positive target lysis)−(percentage of negative target lysis).

RESULTS AND DISCUSSION

We prepared the FL encapusulating TAX 38-46. The diameter of FL was 880.1±9.5 nm. We calculated the concentrarion of peptide in FL using FITC conjugated TAX38-46. One milliliter of FL suspension at OD_{540} of 1.0 contained 29.8 μg of TAX 38-46. FL encapsulating TAX 38-46 was immunized with 100 μl at OD_{540} of 10.0.

Figure 1 demonstrates that the induction of TAX-specific CTL occurred only in response to TAX38-46-FL. CTL induction could not be detected in the TAX38-46-CFA administered group, in the TAX38-46 group, or in the empty FL group. Likewise, there was no CTL response in the group immunized with the mixture of TAX 38-46 and empty FL (data not shown). Previous reports have demonstrated that FL can deliver peptide directly into cytoplasm and that it possesses immune stimulating ability.

In a future study, we will investigate the control of peptide distribution in the cytoplasm and attempt to induce a stronger CTL response. Peptides that target the endoplasmic reticulum (ER) are able to induce stronger CTL responses because MHC class I molecules are expressed on the ER membrane.[11—13] However, because FL cannot control peptide distribution in the cytoplasm, we were unable to target the ER specifically. Therefore, the use of both FL and ER targeting sequences will more effectively induce CTL.

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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 co-administration 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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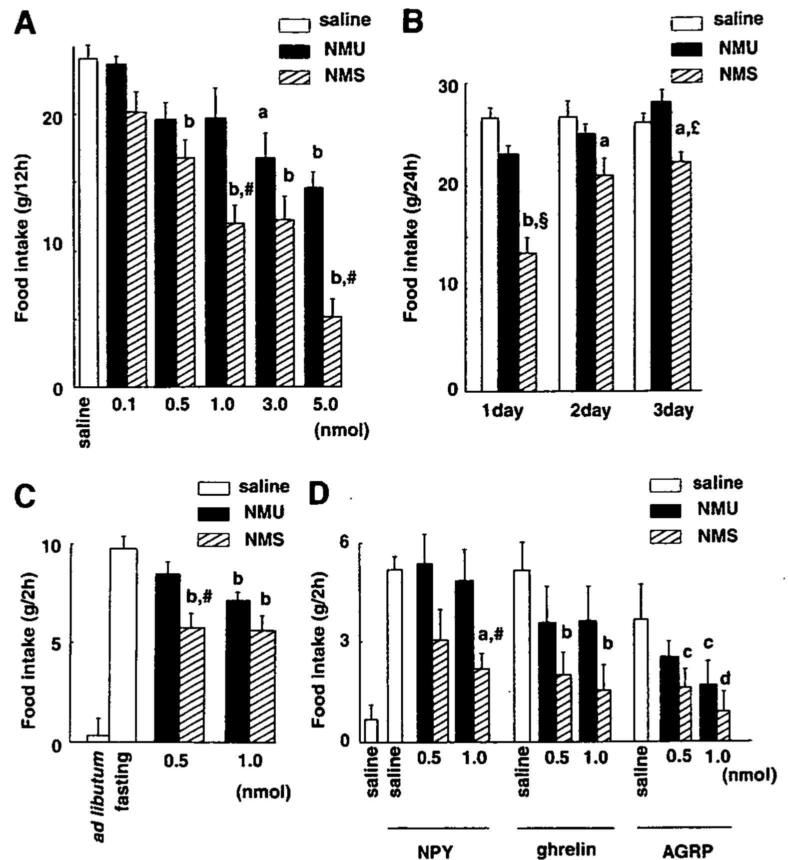

FIG. 1. Comparison of food intake after intracerebroventricular administration of NMS and NMU to rats (n = 8–10 in each group). Each *bar* and *vertical line* represents the mean ± SEM. A, Dark-phase feeding. Food intake of free-feeding rats was examined during a period of 12 h from 1900 h to 0700 h. 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).

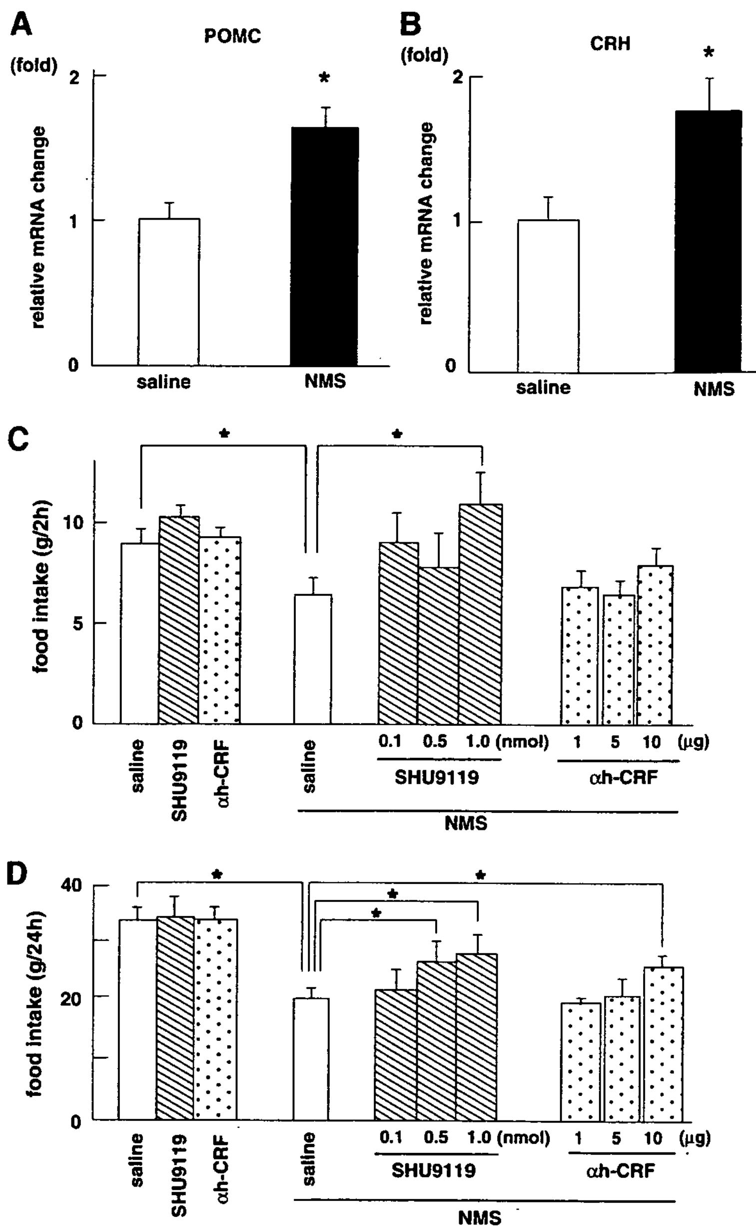

FIG. 2. Involvement of α-MSH and CRH in NMS-elicited feeding behavior. Quantitative RT-PCR on NMS administered rats (n = 8 per group). Level of POMC mRNA (A) and CRH mRNA (B). C, Effect of pretreatments with α-MSH antagonist (SHU9119) or CRH antagonist [α-helical CRF-(9–41)] on food intake reduction 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′-GTGTGCTAAATGCA-GAATCG-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′-AGACGCCAGTA-GACTCCACGACA-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.

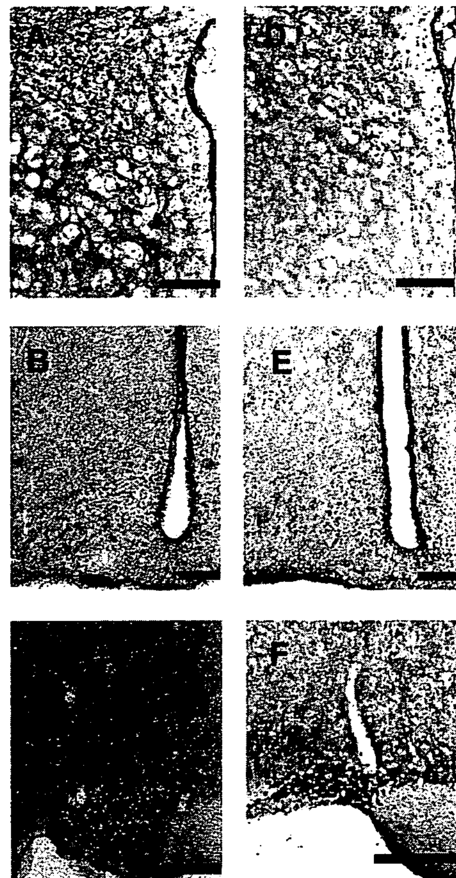

FIG. 3. Representative photomicrographs of c-Fos-immunoreactive cell nuclei in selected brain regions after administration of 1 nmol NMS (A–C) or NMU (D–F). The brain regions shown include the PVN (A and D), Arc (B and E), and supraoptic nucleus (C and F). *Scale bar*, 200 μm (for all panels).

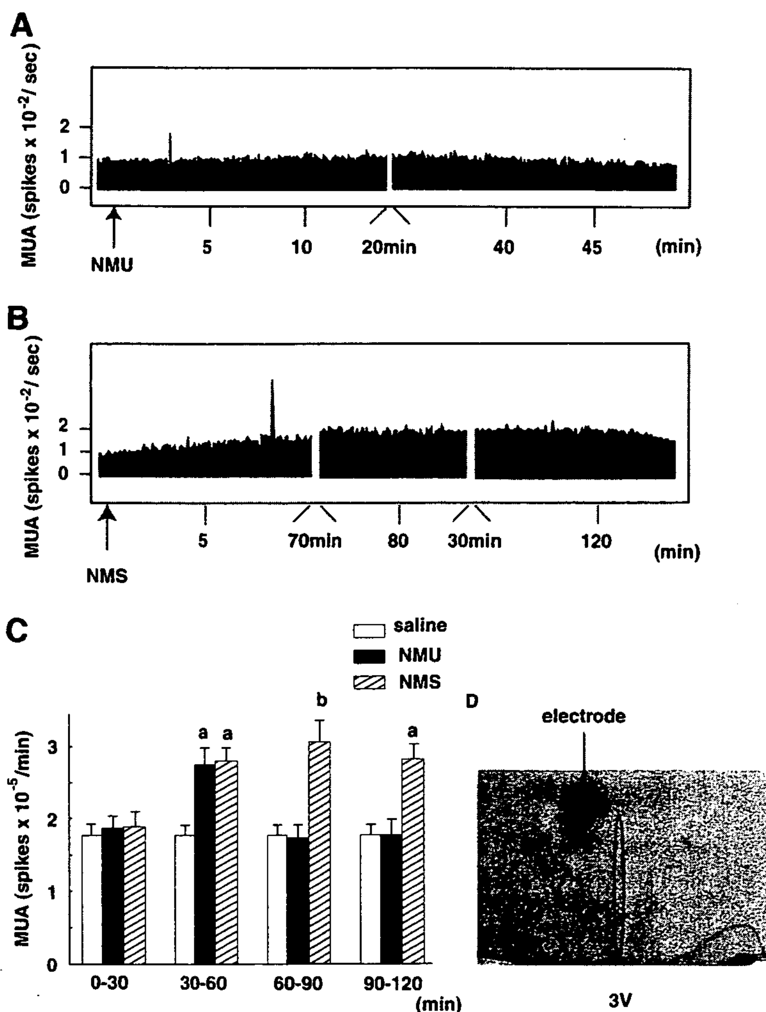

FIG. 4. Effect of icv injection of NMS or NMU on MUA in the PVN. A and B, Frequency-time histograms of firing rate of the PVN after administration of NMS or NMU in representative rats. NMS (A) or NMU (B) was injected 15 min (*arrows*) after stable MUA volley. Abscissa: time (min), ordinate: spike/sec. 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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Effect of neuromedin S on feeding regulation in the Japanese quail

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Abstract

Neuromedin S (NMS) was recently isolated from the brains of humans, mice and rats as an endogenous ligand for the orphan G protein-coupled receptors FM-3 and FM-4, which have been identified as neuromedin U (NMU) receptors 1 and 2, respectively. To investigate the role of NMS in avian species, we elucidated the effect of intracerebroventricular (i.c.v.) administration of rat NMS on food intake, body weight, body temperature and gross locomotor activity in adult Japanese quails. NMS significantly decreased food intake (and consequently body weight) in a time-dependent manner during 12-h light period, but increased both body temperature and gross locomotor activity. On the other hand, i.c.v. injection of rat NMU showed the reverse effects of NMS in Japanese quail. These results suggest that NMS may play an important role in regulating food intake and sympathetic nerve activity in the Japanese quail.

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Keywords: Food intake; Body temperature; Neuromedin S; Japanese quails

Recently, a novel 36-amino acid residue neuropeptide was isolated from rat brain and was identified as an endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 using a reverse-pharmacological technique [9]. FM-3/GPR66 and FM-4/TGR-1 have been already identified as neuromedin U (NMU) receptor type-1 (NMUR1) and NMU receptor type-2 (NMUR2), respectively [3]. The novel peptide was designated neuromedin S (NMS), because it is specifically expressed in the suprachiasmatic nucleus (SCN) [9]. Although NMS shares a C-terminal core structure (seven amino acid residues) with NMU, and activates recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells, it is not a splice variant of NMU because the genes for NMS and NMU have been mapped to discrete chromosomes [9].

The physiological functions of NMU have recently been clarified. Its most marked effect is on feeding regulation [3,7,10]. Intracerebroventricular (i.c.v.) administration of NMU decreases both the daily food intake during dark period and fasting-induced food intake in rats [5,12]. Conversely, injection of anti-NMU IgG increases dark-phase feeding compared with preimmune serum IgG [7]. Recently, our group has also demonstrated that NMU-knockout mice become obese [2]. These results indicate that NMU is a potent endogenous anorexigenic peptide in rats. In addition to feeding regulation, NMU increases gross locomotor activity, body temperature and heat production in rats, suggesting that it is a catabolic signaling molecule [10]. We previously reported that synthetic Japanese quail NMU decreased food intake and increased both body temperature and gross locomotor activity in Japanese quails [11], thus implying that avian NMU also plays important physiological roles.

As mentioned above, NMS shows homology of the C-terminal core structure with NMU and activates recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells. It may therefore also play important roles in feeding regulation, locomotor activity and body temperature. With this possibility in mind, we compared the effects of NMS with those of NMU in avian species. In this study, we chose to use adult Japanese quails instead of chickens because the growth curve in chickens is steep and so their body weight and food intake vary widely on a daily basis, whereas in the adult Japanese quail, the growth curve is relatively constant and therefore body weight and food intake are not subject to such great variability. In addition, it is possible to chronically implant an i.c.v. cannula into adult Japanese quails.

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Adult male Japanese quails (*Coturnix coturnix japonica*), weighing 110–120 g, were reared in individual net cages (*W*: 14 × *L*: 26 × *H*: 17 cm) in a room with a 12-h light (300 lx)/12-h dark (dim light, 25 lx) period (lights on at 07:00 h), at a temperature of 28 ± 1 °C. The birds were given free access to food and water. Rat NMS or rat NMU (Peptide Institute, Osaka, Japan) was dissolved in 0.9% saline and several doses were administered i.c.v. to each of six free-feeding male birds in each experimental group. Each experiment was set for measurement only one parameter to avoid the effect of one parameter on the other. All the experiments were performed twice in order to confirm the results obtained in each experiment. We performed 1-week interval between the first and second time experiment to avoid the residual effects of repeated injection. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

For implantation of the i.c.v. cannula, each bird was anesthetized with 5% sodium pentobarbital (1.4 μl/g body weight) and placed in a stereotaxic frame. A stainless steel guide cannula (outer diameter: 550 μm; length: 14 mm) was stereotaxically implanted into the third cerebral ventricle using a modification of a previously reported method [1]. The coordinates were 5 mm anterior to the interaural axis and 6.5 mm below the dura at the midline. One stainless steel anchoring screw was fixed to the skull, and the guide cannula was secured in place with acrylic dental cement. The birds were returned to their individual cages and allowed to recover for at least 4 days. They were acclimatized to handling every day before the start of the experiments. The i.c.v. injections were administered through the implanted guide cannulae without anesthesia or restraining of the birds. At the end of the experiments, proper placement of the cannulae was verified by administering Evans Blue dye (10 μl), followed by sacrifice and brain sectioning (20 μm intervals). Data for birds lacking dye in the third ventricle were excluded from the analysis.

Before the feeding experiment, the birds were weighed and assigned to an experimental group based on their body weight. The average body weight (110–120 g) in each group was kept as uniform as possible. To examine the orexigenic or anorexic effect of NMS, rat NMS (0.1, 0.5 or 1.0 nmol/10 μl saline) or saline (control) was administered i.c.v. at 07:00 h. Food consumption was determined in the free-fed birds at 2, 4 and 12 h after administration by measuring the disappearance of food from a pre-weighed feeder placed in each individual cage. Care was taken to collect and weigh any spillage, thus making the determination of food intake as accurate as possible.

The quails' body temperature was measured at 0 min (before injection), then at 5, 10, 20, 40, 60 and 120 min after i.c.v. injection of rat NMS, rat NMU (each at doses of 0.1, 0.5 or 1.0 nmol/10 μl saline) or saline vehicle ($n = 6$ in each group) at 10:00 h using a previously reported method [1]. Briefly, temperature was measured electronically with a small sensor (measurable range: 25–50 °C; measurement error: 0.05 °C) connected to a line (outer diameter: 0.7 mm; length: 45 cm). The sensor tip was inserted into the cloaca, and part of the line was fixed to the body of the bird.

Locomotor activity was measured in each bird under light/dark conditions for 1 week, and thereafter under constant dim light at an intensity of about 30 lx. Locomotion was measured using a rat locomotor activity recording system (Muromachi Co. Ltd., Tokyo, Japan) comprising infrared sensors, an interface and a computer [8]. The infrared sensors were placed above the cages and measured all locomotor activity (e.g. eating, perch-hopping and flying). Each cage with its infrared sensor was placed in an isolated chamber with a controlled light/dark cycle. Data were collected at 15-min intervals and analyzed using CompactACT AMS software (Muromachi Co.). Rat NMS, rat NMU (each at doses of 0.1, 0.5 or 1.0 nmol/10 μl saline) or saline vehicle was administered i.c.v. at 10:00 h ($n = 8$ per group). After the injections, the birds were immediately returned to their individual cages. Locomotor activity counts were made every 15 min and summed for the 2-h period following administration.

All results are expressed as mean ± S.E.M. The data were analyzed using analysis of variance and the post hoc Fisher's test.

I.c.v. administration of NMS 0.5 and 1 nmol significantly ($P < 0.05$) decreased food intake in a time-dependent manner compared with saline alone (Fig. 1A). This anorexigenic action of NMS was apparent by 2 h and continued for 12 h after i.c.v. administration. The effect was no longer observable on the following day (data not shown). Concomitantly, a significant ($P < 0.05$) decrease in body weight was observed at 2, 4 and 12 h after i.c.v. injection of NMS (Fig. 1B). The decrease in body weight was more pronounced than the decrease in food intake, and became quite considerable by 12 h after the injection. The effect of a smaller dose of NMS (0.01 nmol i.c.v.) was examined, but this dose effected no significant change in food intake ($n = 6$; data not shown).

I.c.v. injection of NMS also significantly ($P < 0.05$) increased body temperature and locomotor activity (Fig. 1C and D). An increment of about 2 °C was observed in body temperature 40–60 min after i.c.v. injection of 1.0 nmol NMS. Although 0.1 nmol NMS also caused an increase in body temperature, the change was not significantly different from that seen with saline alone. Locomotor activity was increased 1.5-fold during the 2-h period following i.c.v. injection of 1 nmol NMS.

When the effects of i.c.v. injection of the same doses of rat NMU and rat NMS on food intake, body temperature and locomotor activity were compared in Japanese quails, opposite effects were observed. Fig. 2 shows that rat NMU produced an increase in food intake but decreases in body temperature and locomotor activity.

The present study demonstrates that rat NMS suppresses food intake but promotes locomotor activity and increases body temperature in avian species. The suppression of feeding is unlikely to be due to any side effect of NMS, since the quails in the treated group did not show any abnormal behavior. The noticeable decrease in body weight after i.c.v. injection of NMS may be due to both a decrease in food intake and an increase in energy expenditure. These results therefore suggest that central NMS may play important roles in the regulation of feeding and the sympathetic nervous system in avian species.

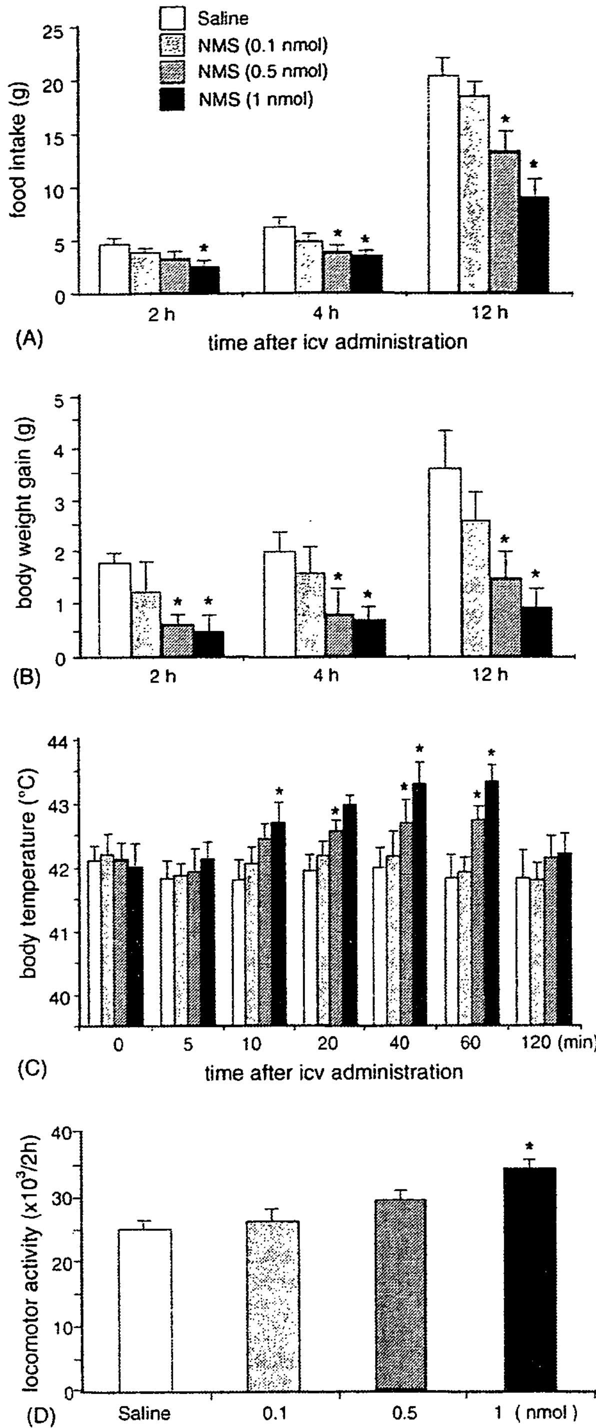

Fig. 1. Effect of intracerebroventricular (i.c.v.) administration of rat NMS on food intake (A), body weight change (B), body temperature (C), and gross locomotor activity (D) in the Japanese quail. Saline (vehicle control) or NMS (0.1, 0.5 or 1.0 nmol) was injected i.c.v. at 07:00 h for food intake assessments or 10:00 h for body temperature and gross locomotor activity assessments. Each bar and vertical line represents the mean ± S.E.M. ($n = 12$ for food intake assessments; $n = 6$ for body temperature assessments and $n = 8$ for gross locomotor activity assessments). *Significantly different from the saline-treated group; $P < 0.05$.

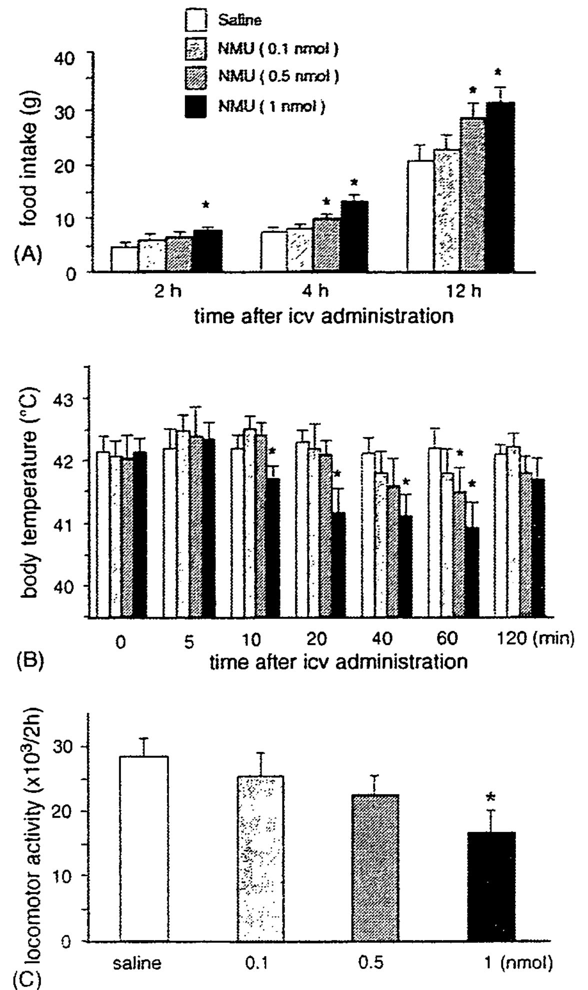

Fig. 2. Effect of i.c.v. administration of rat NMU on food intake (A), body temperature (B) and gross locomotor activity (C) in the Japanese quail. Saline (vehicle control) or NMU (0.1, 0.5 or 1.0 nmol) was injected i.c.v. at 07:00 h for food intake assessments or 10:00 h for body temperature and gross locomotor activity assessments. Each bar and vertical line represents the mean ± S.E.M. ($n = 12$ for food intake assessments; $n = 6$ for body temperature assessments and $n = 8$ for gross locomotor activity assessments). *Significantly different from the saline treated group; $P < 0.05$.

In a previous study, the distribution of NMS mRNA in various rat tissues was investigated using a quantitative reverse-transcriptase polymerase chain reaction technique [9]. NMS mRNA was expressed mainly in the hypothalamus, spleen and testis. Within the hypothalamus, NMS mRNA was expressed predominantly in the SCN; there was only very slight expression in other brain regions, such as the paraventricular nucleus (PVN) and arcuate nucleus (Arc) [9]. 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 [9]. In the case of NMS, therefore, its action on the PVN and Arc through the NMS projection from the SCN may be important.

Recently, we observed that i.c.v. NMS also suppressed food intake in rats [4]. In that case, cFos expression was detected in preopiomelanocortin (POMC)-neuron in the arcuate nucleus and corticotropin-releasing hormone (CRH)-secreting cells in

the paraventricular nucleus. This suggests that neuron containing POMC (a precursor of α-melanocyte-stimulating hormone; α-MSH) and CRH may be the targets for suppression of food intake by NMS, because CRH and α-MSH are known to be anorexigenic hormones in chicken [6,13]. However, further study is required to elucidate the mechanism of action of NMS in avian species.

Because NMS contains the active core C-terminus of NMU and binds to the same receptors (NMU1R and NMU2R), rat NMS and rat NMU would be expected to have very similar actions on food intake, locomotor activity and body temperature in Japanese quails. However, opposite effects were observed. Previously, we reported that Japanese quail NMU, but not rat NMU, suppressed food intake in Japanese quails, and that pretreatment with rat NMU inhibited the Japanese quail NMU-induced suppression of food intake [11]. Rat NMU therefore appears to have an antagonistic action on Japanese quail NMU, possibly through competition for NMU receptors. If this is so, why did rat NMS not show similar antagonism? The reason for the discrepancy is unclear from the present study; however, the following considerations may provide possible explanations. First, the structure of avian NMS may be close to that of rat NMS. If this is so, rat NMS may not act antagonistically at NMU receptors, and may be able to have same physiological function as avian NMS. Although we tried cloning Japanese quail NMS using essentially the same method as that used for cloning Japanese quail NMU [11], we were unsuccessful and could not therefore perform direct experiments with Japanese quail NMS. Second, there may be a specific receptor for NMS other than the NMU1R and NMU2R, and NMS may act on feeding and locomotion through it.

In conclusion, NMS, a novel peptide, appears to play important roles in the regulation of feeding, locomotor activity and body temperature in avian species. As this is the first paper to describe the actions of NMS in avian species, further research will be required to elucidate the exact mechanisms of action of NMS and any further physiological functions that it may have.

Acknowledgments

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Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus

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Abstract

Ghrelin, an acylated peptide originally identified in rat stomach as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R), stimulates both food intake and growth hormone (GH) secretion. Ghrelin is predominantly synthesized by a subset of endocrine cells in the oxyntic gland of human and rat stomach. Previous studies using immunohistochemistry have shown that ghrelin is also present in the hypothalamic arcuate nucleus, a region critical for the control of feeding and GH secretion, but its expression pattern in this region and the details of its molecular form has yet to be clarified. In this report, we examined the presence of ghrelin in the arcuate nucleus using reverse-phase liquid chromatography combined with radioimmunoassay (RIA) and immunohistochemistry. Neurons in the arcuate nucleus were observed to react positively to ghrelin antibodies. In addition, we confirmed the existence of ghrelin mRNA expression using the reverse-transcription polymerase chain reaction (RT–PCR). We also observed the colocalization of GHS-R with neuropeptide Y (NPY) and growth-hormone-releasing hormone (GHRH) in the arcuate nucleus. The present study clearly indicates that ghrelin is synthesized in the arcuate nucleus, which will further our understanding of ghrelin's actions in the central nervous system, including feeding behavior and GH secretion.
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Keywords: Ghrelin; GHS-R; Arcuate nucleus; HPLC; RIA; Immunohistochemistry

1. Introduction

Ghrelin, a 28-amino acid peptide with an *n*-octanoyl modification that is indispensable for its activity, was originally discovered in human and rat stomach as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) [1]. Currently, ghrelin homologues have been identified in fish, amphibians, birds, and many mammals. Ghrelin is predominantly produced in the endocrine cells of the stomach and is then released into circulation. Gastrectomy in rats decreases plasma ghrelin concentrations by approximately 80%, indicating that the stomach is the main source of circulating ghrelin [2]. When administered either centrally or peripherally, ghrelin stimulates GH secretion, food intake, and body weight gain [1,3–8]. Several groups have demonstrated that intracerebroventricular administration of ghrelin induces food intake by way of neuropeptide Y (NPY) and agouti-related protein (AGRP) produced in the hypothalamic arcuate nucleus [8–10]. Central effects of ghrelin on feeding are also mediated in part by orexin-A and -B produced in the lateral hypothalamus [11]. These findings suggest that ghrelin is also synthesized in some regions of the brain involved in both feeding and GH secretion. We have already shown using immunohistochemistry that ghrelin-producing neurons are present in the arcuate nucleus, a region critical for feeding and GH secretion [1]. Further verification of the presence of ghrelin and its

Abbreviations: AGRP, agouti-related protein; CH_3CN, acetonitrile; GH, growth hormone; GHS-R, growth hormone secretagogue receptor; NPY, neuropeptide Y; RIA, radioimmunoassay; RP-HPLC, reverse-phase high-performance liquid chromatography; RT–PCR, reverse-transcription polymerase chain reaction.

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receptor in the arcuate nucleus would solidify current models of ghrelin's central activity.

In the present study, we analyzed and characterized ghrelin-immunoreactive molecules in the arcuate nucleus by reverse-phase high-performance liquid chromatography (RP-HPLC) combined with radioimmunoassay (RIA). Using immunohistochemistry, we investigated the ghrelin-immunoreactive neurons in the arcuate nucleus. We also examined the expression of ghrelin mRNA in the arcuate nucleus by reverse-transcription polymerase chain reaction (RT–PCR). We also studied the colocalization in neurons of the ghrelin receptor, GHS-R, with NPY and growth-hormone-releasing hormone (GHRH). Using these methods, we demonstrate that ghrelin is synthesized in the arcuate nucleus.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 300–350 g (Charles River Japan, Shiga, Japan) were used in all experiments. Rats, housed individually in plastic cages at constant room temperature in a 12-h light (07:00–19:00)/12-h dark cycle, were given standard laboratory chow and water ad libitum. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. The protocol was approved by the Miyazaki Medical College Animal Care Research Committee.

2.2. Ghrelin radioimmunoassay (RIA)

Acylated ghrelin content was measured by radioimmunoassay (RIA) recognizing *n*-octanoylated ghrelin [12]. To generate anti-ghrelin antisera, synthetic [Cys^{12}]-ghrelin [1–11] peptide was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH; Pierce, Rockford, IL). This antigenic conjugate solution was administered to three New Zealand white rabbits. The anti-rat ghrelin [1–11] antiserum (#G606) specifically recognized *n*-octanoylated ghrelin and did not recognize des-acyl ghrelin. Synthetic rat [Tyr^{29}]–ghrelin [1–28] was radioiodinated using the lactoperoxidase method. The ^{125}I-labeled peptide was purified on a TSK ODS SIL 120A column by RP-HPLC. Diluted samples or standard peptide solutions (100 μl) were incubated for 24 h with 100-μl diluted antiserum (final dilution of anti-ghrelin [1–11] antiserum, 1:620,000). Following addition of the tracer solution (16,000 cpm in 100 μl), mixtures were incubated for 24 h. Samples were assayed in duplicate; all procedures were done at 4 °C. The limit of detection of rat ghrelin [1–28] on the standard RIA curve was 0.5 fmol per tube. The respective intra- and interassay coefficients of variation at 50% binding for ghrelin RIA were 3.5% and 3.2%. The recoveries of rat ghrelin [1–28] (1 ng) and ^{125}I-rat ghrelin [1–28] (5000 cpm) added to the plasma samples extracted using Sep-Pak C-18 cartridges were 92.2±0.4% (S.E.M.) and 88.9±0.6% (S.E.M.), respectively.

2.3. Quantification of ghrelin in arcuate nucleus

Arcuate nuclei were punched out from the brains of 50 male Wistar rats following anesthesia with pentobarbital (Nembutal, Abbot Laboratories, Chicago, IL) after an overnight 12-h fast. These samples were then boiled at 100 °C for 3 min and applied to a Sep-Pak cartridge. The eluates were subjected to ghrelin RIA, as described above. Portions of the Sep-Pak eluates were applied to RP-HPLC on a TSK ODS SIL 120A column (4.6×150 mm, Tosoh, Tokyo, Japan). RP-HPLC was performed for 40 min at 1.0 ml/min with a linear gradient of acetonitrile (CH_3CN; 10–60%) in 0.1% TFA. All HPLC fractions were quantified by ghrelin RIA.

2.4. Preparation of anti-GHS-R serum

A [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) and then purified by RP-HPLC. The synthesized peptide (10 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH, Pierce; 6 mg) in conjugation buffer (Pierce). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and used to immunize New Zealand white rabbits by intra- and subcutaneous injection. Animals were boosted every 2 weeks and bled 7 days after each injection. The specificity of the antiserum was confirmed by its immunoreactivity against GHS-R-expressing (CHO-GHSR62 cells) but not control cells.

2.5. Immunohistochemistry

Three male Wistar rats weighing 250–300 g were used for immunohistochemical study. To enhance the immunostaining of GHS-R-expressing neurons, colchicine (100 μg/rat) was injected into the lateral ventricle 30 h before perfusion. Rats were perfused transcardially with 0.1 M phosphate buffer (pH 7.4) and then with 4% paraformaldehyde in 0.1 M phosphate buffer. The hypothalamus was sectioned into 40-μm thick slices at −20 °C using a cryostat and then treated with 0.3% hydrogen peroxide for 1 h to inactivate endogenous peroxidases. The hypothalamic sections were incubated for 2 days at 4 °C, with anti-ghrelin antiserum diluted 1:1000 or with anti-GHS-R antiserum diluted 1:1000. The pituitary sections were incubated for 2 days at 4 °C with anti-GHS-R antiserum diluted 1:1000. All of the sections were stained using the avidin–biotin complex method, as described previously [13]. We subsequently performed double staining for GHS-

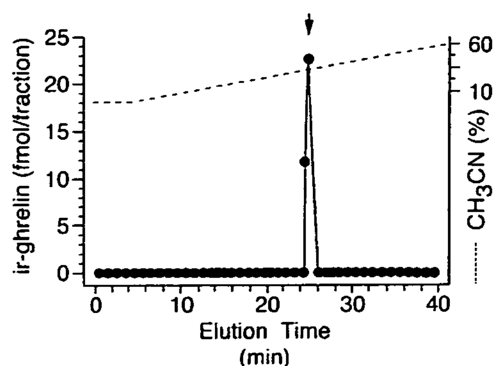

Fig. 1. Representative RP-HPLC profile of ghrelin extracted from rat arcuate nucleus. Wet weight (250 mg) of rat arcuate nucleus was analyzed. Arrow indicates the elution position of *n*-octanoylated ghrelin.

R vs. either NPY or GHRH in some sections of the hypothalamic arcuate nucleus. We also performed double staining for GHS-R vs. GH in some sections of the anterior pituitary. After the sections of arcuate nucleus and anterior pituitary were stained with anti-NPY antiserum (Diasorin, Stillwater, MN; final dilution 1:500) or anti-GHRH antiserum (Chemicon International, Temecula, CA; final dilution 1:1000) and with anti-GH antiserum (NIDDK, National Hormone and Peptide Program, Torrance, CA; final dilution 1:5000), respectively, they were washed with 100 mM glycine–HCl buffer (pH 2.2). Next, the sections of arcuate nucleus and anterior pituitary were stained with anti-GHS-R antiserum using an SG (blue/gray) substrate kit (Vector Laboratories, Burlingame, CA). To test for antiseral specificity, preabsorption tests were done using anti-ghrelin that had been absorbed with 10 μg of ghrelin and GHS-R antiserum that had been absorbed with 10 μg of GHS-R.

2.6. RT–PCR for ghrelin

Total RNA was extracted from the arcuate nuclei of three Wistar rats by the acid guanidinium thiocyanate–phenol–chloroform (AGPC) method [14]. First-strand cDNA was synthesized from 2.5 μg RNA and 7 μM oligo-$(dT)_{18}$ primer with ReverTra Ace-α-™ (Toyobo, Osaka, Japan). The resulting cDNA was subjected to PCR amplification with 2 μM each of the sense and antisense primers and 2.5 units of Pyrobest DNA polymerase (Takara Shuzo, Shiga, Japan). PCR primers for ghrelin were 5′-TTGAGCCCAGAGCACCAGAAA-3′ (sense) and 5′-AGTTGCAGAGGAGGCAGAAGCT-3′ (antisense), corresponding to nucleotide numbers 112–132 and 437–458 (Reference 4, GenBank). PCR was conducted in a reaction volume of 25 μl for 35 cycles comprising denaturation for 5 s at 94°C, annealing for 10 s at 65 °C, and extension for 1 min at 72 °C. The PCR products were electrophoresed on a 2% agarose gel (FMC BioProducts, Rockland, ME).

3. Results

3.1. HPLC characterization of ghrelin-immunoreactive molecules and its content in the arcuate nucleus

RP-HPLC coupled with ghrelin RIA was used to analyze the presence of immunoreactive ghrelin molecules in the arcuate nucleus. A large peak corresponding to immunoreactive ghrelin was eluted at the position of *n*-octanoylated ghrelin in arcuate nucleus tissue extract (Fig. 1). The ghrelin content measured by RIA in the arcuate nucleus was 0.56 pg/mg tissue extract.

3.2. Immunohistochemistry

Neuronal cell bodies immunoreactive for ghrelin were found in the arcuate nucleus (Fig. 2A and B). No immunoreactivity for ghrelin was detected in the arcuate nucleus when normal rabbit serum or antisera preabsorbed with an excess of ghrelin was applied (data not shown). Neurons immunostained with GHS-R antisera were also present in the arcuate nucleus (Fig. 3A). GHS-R immunoreactivity colocalized with that of NPY (Fig. 3B) and GHRH (Fig. 3C) in some neurons in the arcuate nucleus. GHS-R was also abundantly expressed in the pituitary,

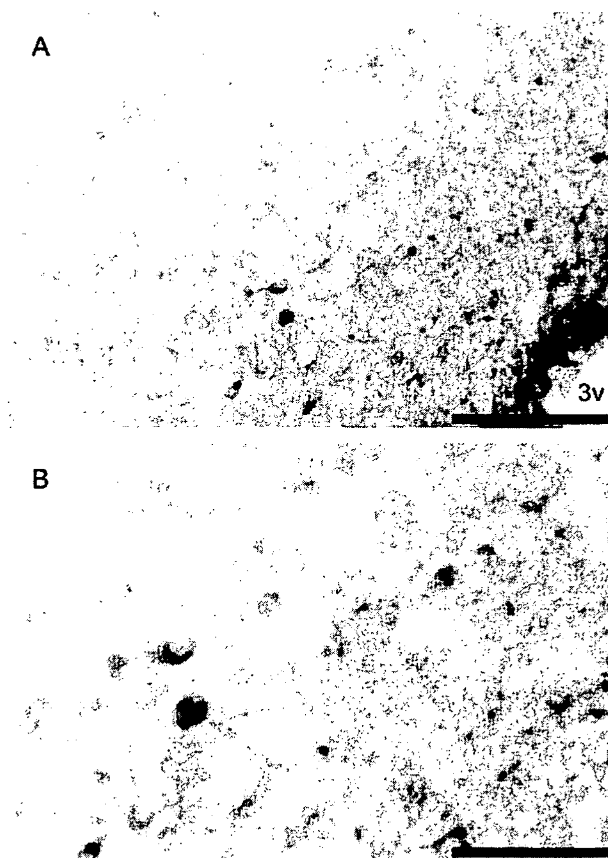

Fig. 2. Immunohistochemical localization of ghrelin in the arcuate nucleus. (A) Ghrelin-immunoreactive neurons in the arcuate nucleus. Bar, 50 μm. (B) High magnification of panel (A). Bar, 25 μm.

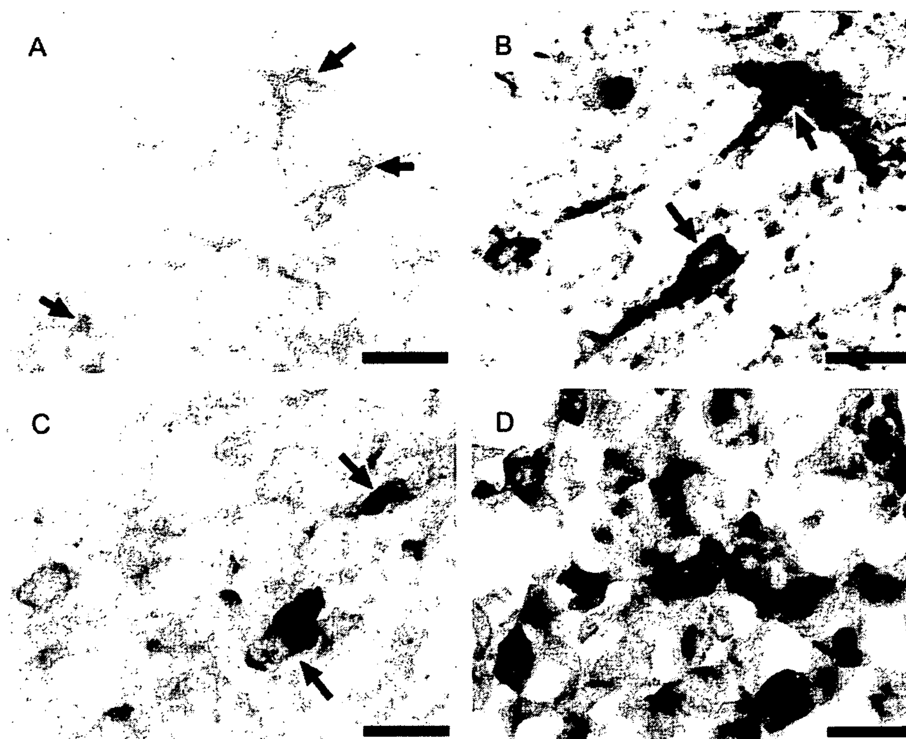

Fig. 3. Immunohistochemical localization of GHS-R in arcuate nucleus and the anterior pituitary. (A) GHS-R-immunoreactive neurons in the arcuate nucleus (arrows). (B) GHS-R-immunoreactive neurons (blue-black) colocalized with NPY neurons (brown; arrows) and (C) GHRH neurons (brown; arrows) in the arcuate nucleus. (D) GHS-R-immunoreactive cells (blue-black) colocalized with GH-producing cells (brown) in the pituitary. Bar, 20 μm in panel (A) to panel (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

where it colocalized with GH-producing cells (Fig. 3D). No GHS-R immunoreactivity was detected in the arcuate nucleus or pituitary gland when normal rabbit serum or antisera preabsorbed with an excess of GHS-R was applied (data not shown).

3.3. RT–PCR amplification of ghrelin transcript

Using ghrelin-specific primers, an RT–PCR product corresponding to the predicted 347-bp size of the ghrelin transcript was present in a rat arcuate nucleus RNA sample (Fig. 4, left panel) but not present when no template was used in the reaction (Fig. 4, right panel).

4. Discussion

Ghrelin is a recently discovered gastrointestinal hormone that appears to play a major role in regulating energy balance [1,3–8]. Ghrelin is predominantly produced in the X/A-like cells of the stomach and may link the gastrointestinal system with hypothalamic control of energy balance, growth, and digestive functions [15]. Central administration of ghrelin has been shown to stimulate GH secretion as well as food intake, fat deposition, and body growth [1,3–8]. Central administration of ghrelin also activates various nuclei in rat hypothalamus, including critical regions for GH and energy homeostasis [8]. These findings strongly suggest the existence of neurons that produce ghrelin and/or its receptor in the brain. In situ hybridization histochemistry has demonstrated expression of the mRNA encoding the ghrelin receptor GHS-R in the pituitary, hypothalamus, pancreas, stomach, and other tissues [16], but immunohistochemical localization of GHS-R has yet to be confirmed.

The present study shows that ghrelin-immunoreactive neurons exist in the ventral portion of the arcuate nucleus, which is consistent with a previous published report [1]. Furthermore, we demonstrated ghrelin immunoreactivity in the arcuate nucleus using RP-HPLC combined with RIA. Ghrelin mRNA expression was also found in the arcuate nucleus. These results imply that the arcuate nucleus may be a major source of ghrelin in the central nervous system of rats. In addition, GHS-R was expressed in NPY- and

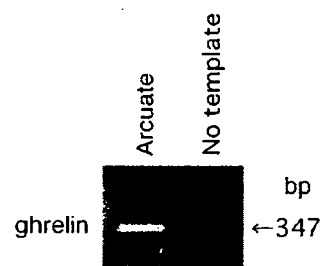

Fig. 4. Representative agarose gel showing the RT–PCR product corresponding to ghrelin mRNA amplified from rat arcuate nucleus.

GHRH-immunoreactive neurons of the rat arcuate nucleus, suggesting that central ghrelin directly affects these neurons which enhance feeding and GH secretion. We also demonstrated that GH-producing cells in the anterior pituitary express GHS-R. This finding indicates that ghrelin also induces GH secretion via an endocrine as well as a neural pathway.

In summary, the present study demonstrated the expression of an active *n*-octanoylated form of ghrelin in the arcuate nucleus. Given the expression of GHS-R in NPY- and GHRH-producing neurons, central ghrelin is expected to play an important role in appetite stimulation, energy homeostasis, and GH secretion. Such identification of ghrelin in the brain will facilitate the investigation of the link between peripheral factors relaying starvation or satiety signals and central ghrelin pathways. Further examination of the distribution of GHS-R-immunoreactive cells throughout the brain and peripheral tissues could lead to discovery of novel functions of ghrelin.

Acknowledgments

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Peptidome Database and Identification of New Endogenous/Bioactive Peptides

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Peptidome database is aimed to comprehensively accumulate the fact data for the peptides present in the specific cells, tissues and organisms. In this database, the peptide data are stored based on their hydrophobicity, charges and molecular masses. By minimizing the degradation, the data are deduced to reflect the endogenous forms of the peptides. Thus, this database is expected to provide a new basement for peptide research, especially for identifying new endogenous and bioactive peptides.

Keywords: peptidome, fact database, endogenous peptides, 2-dimensional HPLC, mass spectrometry.

Introduction

Peptides play crucial roles in many physiological events as hormones, neurotransmitter and local mediators, but no database for endogenous peptides is available. This is mainly due to the following facts; i) peptide contents are extremely low, ii) most peptides in the cells are degradation products of proteins, and iii) peptides are easily susceptible to proteolysis during extraction and purification. Moreover, peptides are generated from precursor proteins by specific cleavages, and the cleavage sites are often different in different tissues. Modification of the peptides, such as amidation, is also essential for eliciting biological activity. As the processing, including cleavage and modification, is the most important feature of bioactive peptides and is difficult to deduce from the DNA sequences, fact data for endogenous peptides must be accumulated in order to advance peptide research.

Despite these problems and difficulties, we demonstrated that endogenous peptides can be detected at substantial levels by minimizing the degradation of proteins and peptides. By separating peptides by 2-dimensional (2D) chromatography composed of ion exchange and reverse phase high performance liquid chromatography (HPLC), a huge number of peptides were detected by the recently advanced mass spectrometers. In 1999, we undertook the Peptidome project, which is aimed to comprehensively analyze all peptides in the cells and tissues, to store the data based on