phosphodiesterases and protein kinases. The cell-specific responses evoked by receptor-type GCs are mediated by the cooperative interactions of these proteins [13]. cGMP-dependent protein kinase (cGK) has many structural and functional features that are highly similar to those found in cAMP-dependent protein kinase (cAK), and has similar substrate specificities [14]. Therefore, phosphorylation of cellular proteins by cGK leads to transactivation of multiple target genes, which creates crosstalk between cAMP and cGMP signaling pathway [15,16].

We report here the development of a novel intracellular cGMP detection method utilizing a signaling pathway activating cAMP-response element (CRE)-mediated transcription with phosphorylated CRE binding protein (CREB) and overexpressed cGK in HEK293 cells. Moreover, using this detection method, we have constructed a simple and highly sensitive reporter gene assay system for use in the high throughput screening of receptor-type GC selective agonists.

2. Materials and methods

2.1. Reporter genes construction

MMTV8–29 (VG028), mouse mammary tumor virus (MMTV) genome, was obtained from the Human Healthcare Science Research Resources Bank (Osaka, Japan). To construct the modified promoter ΔMMTV containing a CRE, the glucocorticoid-responsive element (GRE) located in the MMTV promoter was replaced with a CRE derived from the corticotropin-releasing hormone gene, as previously reported [17–19]. In brief, MMTV 5′-LTR regions located at both GRE sites were amplified in separate PCRs. PCR fragments encoding the upstream (position −1200 to −190) and the downstream (position −88 to +1) segments from the GRE were generated using specific sets of primers. The primer sets used for the PCRs are given in Supplementary Table S1. The resulting PCR fragments were subcloned into pGL3-basic-Luc2P, and the luciferase gene in pGL3-basic (Promega, Medison, WI) was substituted with a synthetic destabilized luciferase gene in pGL4.11 (Promega), and was referred to as pGL3b/ΔMMTV-Luc2P. Oligonucleotide cassettes with five tandemly-repeated CRE motifs were synthesized as complementary pairs of primers with HindIII sites at the 5′-end (see Supplementary Table S2 for detailed sequence), and they were subcloned into the pGL3b/ΔMMTV-Luc2P. The plasmid with one unit inserted was designated as pGL3b/ΔMMTV/CRE5-Luc2P and that inserted into three was designated pGL3b/ΔMMTV/CRE15-Luc2P. All constructs were sequenced to be able to confirm the orientation and integrity of the oligonucleotide.

2.2. Cloning of human GC-A, GC-B, GC-C and G-kinase 1β

Total RNA was prepared from HEK293 cells (for isolation of human GC-A), T84 human colonic adenocarcinoma cells (for isolation of human GC-C and G-kinase 1β) and SK-N-MC human neuroblastoma cells (for isolation of human GC-B). The first strand of cDNA was made using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) with an oligo(dT) primer. The full-length coding regions of human GC-A, GC-B, GC-C and G-kinase 1β cDNAs were amplified by PCR using specific primers. The primer sets used for the PCRs are given in Supplementary Table S3. The PCR products were subcloned into mammalian expression vector pcDNA3.1(+) (pcDNA) (Invitrogen). To verify the cDNAs encoding a full-length region, constructed plasmids were subjected by DNA sequencing.

2.3. Cell culture and transfection

HEK293 (RIKEN BRC, Tsukuba, Japan) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were seeded at a density of 1×10^4 cells/cm^2 on type I collagen-coated 12-well or 96-well plates (IWAKI, Japan). Transient transfection was performed using the FuGene6 (Roche, Mannheim, Germany), as described in the manufacturer's instructions.

2.4. cGMP reporter signal detection in living cells

Cells seeded on 12-well plates were transfected with 500 ng of pGL3b/ΔMMTV-Luc2P, pGL3b/ΔMMTV/CRE5-Luc2P or pGL3b/ΔMMTV/CRE15-Luc2P along with either 500 ng of pcDNA or pcDNA/G-kinase 1β. After 36 h transfection, forskolin (Nacalai Tesque, Kyoto, Japan) or 8-bromo-cGMP (Sigma, St. Louis, MO) was added to the culture media containing 0.2 mM D-Luciferin (Nacalai Tesque) in each well at a final concentration of 10 μM and 1 mM, respectively. Four hours later, cells were analyzed with a LAS 4000miniEPUV Luminescent Image Analyzer (Fuji Film, Tokyo, Japan).

2.5. cGMP reporter gene assay in multi well plate

The transfection of cells plated on 96-well plates was done as described above except for using 100 ng of plasmids per well. In addition, as an internal control for transfection efficiency, cells were transfected with 10 ng of the pcDNA/β-galactosidase per well. Thirty-six hours after transfection, the cells were treated with 8-bromo-cAMP (Sigma) or 8-bromo-cGMP, respectively, at a concentration of 1 mM for each. Four hours later, the cells in each well were lysed with 100 μl of Passive Lysis Buffer (Promega). For each well, 50 μl of cell lysate was transferred to a 96-well white plate and 50 μl of Luciferase Assay Substrates (Promega) was added. Luciferase activity (in counts per second, cps) was measured using the Luminous CT-9000D Luminometer (Dia-Iatron, Tokyo, Japan).

2.6. β-Galactosidase activity measurement

Each cell lysate in the cGMP reporter gene assay prepared was subjected to an assay for β-galactosidase activity using the Galacto-Star System (Applied Biosystems, Bedford, MA), as recommended by the supplier. Luciferase reporter gene activities were normalized with the β-galactosidase activities of untreated cells.

2.7. Dose–response analysis of GC receptors employing cGMP reporter gene assay

The human ANP, BNP and CNP were purchased from Peptide Institute Inc. (Osaka, Japan). *E. coli* heat-stable enterotoxin STa was obtained from Sigma. HEK293 cells plated on 96-well plates were transfected with 30 ng of pcDNA/GC-A, pcDNA/GC-B or pcDNA/GC-C along with 30 ng of pcDNA/G-kinase 1β, 30 ng of pGL3b/ΔMMTV/CRE5-Luc2P and 10 ng of pcDNA/β-galactosidase. Thirty-six hours after transfection, the cells were stimulated with appropriate ligands for 4 h at concentrations between 1 pM and 100 nM, except for ANP (1 pM to 1 μM). Cells were subjected to a luciferase assay as described above.

2.8. cGMP enzyme-linked immunosorbent assay (ELISA)

Cells on 96-well plates were transfected with 100 ng of pcDNA encoding human GC-A, GC-B or GC-C. After 36 h transfection, culture media was replaced with assay buffer composed of 1 mM isobutylmethylxanthine (IBMX), 0.1% bovine serum albumin, 137.93 mM NaCl, 5.33 mM KCl, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, and 0.338 mM Na_2HPO_4 with 20 mM Hepes–NaOH (pH 7.5). The human ANP in the assay buffer was added at various concentrations to each well and incubated for 1 h. The intracellular cGMP

was measured with a CatchPoint cGMP fluorescent assay kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. Measurement of fluorescence signals was done with EnVision 2104 Multilabel Reader (Perkin-Elmer, Waltham, MA).

2.9. Data analysis and statistics

Statistical analyses of data from cGK nucleotide specificities, ligand dose-dependencies, β-galactosidase activities and cGMP ELISA were performed with GraphPad Prism 3.0 Software (GraphPad Software, San Diego, CA).

3. Results

3.1. cGMP reporter gene system construction

cGK and cAK have some comparable structural and functional features, and there might be some crosstalk between their signal transduction pathways [15,16]. Some of their signaling pathways are linked with CRE, and induction of these pathways promotes CREB binding of CRE and transactivates certain downstream target genes [16]. Consequently, we have hypothesized that expression of a large amount of cGK would result in functional replacement of cAK signaling, and attempted to develop a novel method enabling the monitoring of intracellular cGMP concentrations utilizing a cAMP/cAK/CREB/CRE signal transduction pathway. We have constructed reporter plasmids containing firefly luciferase gene controlled by a mouse mammary tumor virus (MMTV) with its 5′-LTR substituted glucocorticoid-responsive element with multiple copies of CRE [17–19], referred to as ΔMMTV/CRE reporter genes, and the expression vector encoding human cGK (Fig. 1).

3.2. Detection of intracellular cGMP with ΔMMTV/CRE reporter gene assay in a cGK dependent manner

To examine whether the ΔMMTV/CRE reporter gene in combination with cGK could be detect intracellular cGMP, we evaluated the luciferase activity of HEK293 cells harboring the ΔMMTV/CRE reporter plasmids followed by treatment of forskolin, an activator of adenylate cyclase, or the cGMP analog 8-bromo-cGMP under conditions with and without cGK overexpression. Luciferase chemiluminescence signals were detected in the living cells (Fig. 2A). The results found that the luciferase gene controlled by

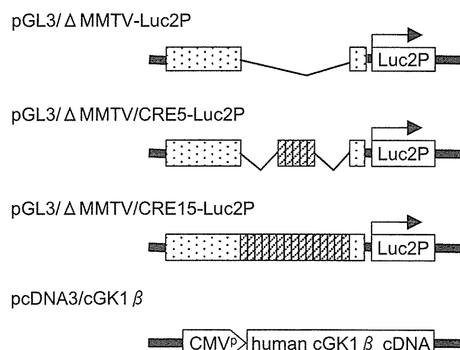

Fig. 1. Scheme of the ΔMMTV/CRE-Luc2P reporter genes and the cGK1β expression vector constructs. The glucocorticoid-responsive element of the MMTV promoter was removed (pGL3b/ΔMMTV) or substituted with multiple copies of CRE (pGL3b/ΔMMTV/CRE5-Luc2P and pGL3b/ΔMMTV/CRE15-Luc2P). MMTV promoter regions and CREs are indicated by dotted boxes and striped boxes, respectively. The human cGK1β cDNA was cloned under the control of an early cytomegalovirus (CMV) promoter (pcDNA/cGK1β).

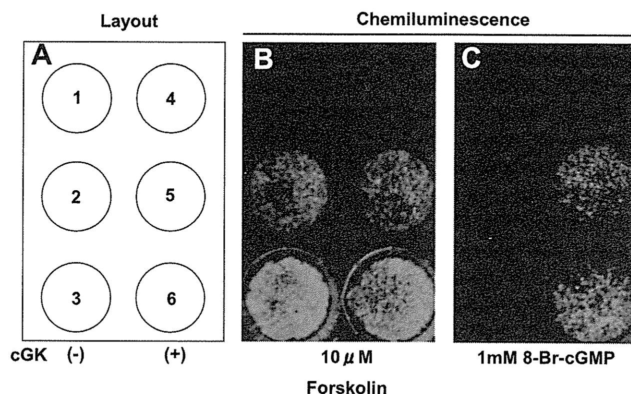

Fig. 2. Detection of 3′,5′-cyclic mononucleotide-induced chemiluminescence signals emitted by intact HEK293 cells harboring ΔMMTV/CRE reporter genes and human cGK1β expression vector. HEK293 cells were transfected with the reporter plasmids (pGL3b/ΔMMTV-Luc2P, 1 and 4; pGL3b/ΔMMTV/CRE5-Luc2P, 2 and 5; pGL3b/ΔMMTV/CRE15-Luc2P, 3 and 6) along with either an empty vector (1–3, (−)) or a cGK1β expression vector (4–6, (+)) (A). Thirty-six hours after transfection, 0.2 mM D-Luciferin plus 10 μM forskolin (B) or 1 mM 8-bromo-cGMP (C) were added. Cells were visualized by a luminescent image analyzer.

the ΔMMTV/CRE promoter was transactivated in response to elevation of intracellular cAMP by addition of forskolin independent of cGK (Fig. 2B). The intensities of luciferase signals were found to be dependent upon the number of CREs. Alternatively, in the case of addition of 8-bromo-cGMP, the luciferase activity was detected only where cGK was expressed at the same time, and was correlated with the number of CREs (Fig. 2C). No signal was detected in the control HEK293 cells (Fig. 2B and C).

3.3. Cyclic mononucleotide specificity of the ΔMMTV/CRE reporter gene assay

To analyze whether the ΔMMTV/CRE reporter gene assay with cGK was capable of detecting intracellular cGMP, HEK293 cells harboring the reporter gene with or without cGK were stimulated with 8-bromo-cAMP or 8-bromo-cGMP and subjected to an analysis of luciferase activity (Fig. 3). Transactivations of the luciferase gene from the ΔMMTV/CRE reporter plasmids were similarly observed under conditions of 8-bromo-cAMP stimulation with or without cGK (Fig. 3). These results indicate that the 8-bromo-cAMP-induced luciferase gene transactivations reflect the endogenous expression of cAK in HEK293 cells. On the other hand, the luciferase activities were detected at very low concentrations by the addition of cGMP without cGK expression, whereas they were markedly increased by cGK overexpression. These results demonstrate that the expression of luciferase activity under the condition of 8-bromo-cGMP is cGK-dependent and that endogenously expressed cAK fails to be activated by cGMP. In addition, we have confirmed that 8-bromo-cGMP-induced cGK activation was specifically inhibited by the addition of cGK antagonist Rp-8-CPT-cGMP (Supplementary Fig. S1), and that cGMP molecules synthesized with guanylate cyclases strictly activated cGK (Supplementary Fig. S2). On the basis of these results, we have designated this intracellular cGMP detection method the cGK × ΔMMTV/CRE reporter gene assay.

3.4. Analysis of dose-dependent activation of GC receptors using the cGK × ΔMMTV/CRE reporter gene assay

To assess whether the cGK × ΔMMTV/CRE reporter gene can be applied in identifying receptor-type GC agonists, we transiently transfected the reporter plasmids into HEK293 cells along with

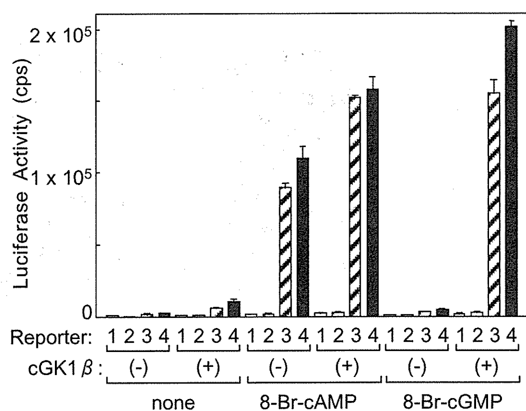

Fig. 3. cGMP reporter gene assay using HEK293 cells harboring cGK × ΔMMTV/CRE reporter genes. HEK293 cells were transiently transfected with either an empty vector (−) or pcDNA/cGK1β (+) along with the reporter gene constructs (1, pGL3b (open bars); 2, pGL3b/ΔMMTV-Luc2P (dotted bars); 3, pGL3b/ΔMMTV/CRE5-Luc2P (striped bars); 4, pGL3b/ΔMMTV/CRE15-Luc2P (closed bars)). Cells were treated with 8-bromo-cAMP or 8-bromo-cGMP at a concentration of 1 mM. The error bars indicate the mean and SEM from at least three experiments performed in triplicate.

receptor-type GC expression vectors. We found that when the cells were stimulated with various doses of each ligand, their luciferase activities increased in a dose-dependent fashion, as shown in Fig. 4A–D. In the case of the combination of ANP and GC-A, the cGMP production of GC-A was measured with an enzyme-linked immunosorbent assay (ELISA) for the purpose of verifying the result of the developed cGK × ΔMMTV/CRE reporter gene assay (Fig. 4A). The concentration values required for one-half of the maximal activation (EC_{50}) obtained from the reporter gene assay and the ELISA were found to be 1.3 and 10 nM, respectively. We were able to detect ANP at less than 100 pM with this reporter gene assay. In addition, using a reporter gene assay we obtained results in BNP, CNP and STa with EC_{50} values of 14.7, 1 and >200 nM against GC-A, GC-B and GC-C, respectively (Fig. 4B–D).

4. Discussion

We report here the construction of a simple and novel intracellular cGMP measurement system using the CRE reporter gene and overexpressed cGK. So far, it has been reported and proposed that the cAMP/cAK signal transduction pathway could interact with the cGMP/cGK signaling pathway in various tissues via activation of the CREB/CRE pathway [14,15,20]. Previous studies analyzing cGK activity using transcription of the reporter gene controlled by the CREB/CRE pathway demonstrated that high doses of cGMP concentration in comparison to the physiological condition were needed to detect a sufficient level of cGK activity. Hence, it was difficult to evaluate whether cGK may have some effects on cAMP/cAK signaling pathways *in vivo* [14]. We therefore postulated this might be due to the following two reasons—firstly, transcriptional abilities of some heterologous promoters containing CRE might be suddenly activated when they exceed a threshold, and secondly, cGK expression is at a very low level in many cell types, and it is difficult to detect cGK-specific activation without non-specific activation of cAK. Therefore, we constructed the method here presented to measure intracellular cGMP utilizing a heterologous promoter, composed of a mouse mammary tumor virus promoter and a CRE, and cGK overexpression, and we designated this system the cGK × ΔMMTV/CRE reporter gene assay (Fig. 1). As expected, reporter gene activity was associated with intracellular cGMP concentration (Figs. 2 and 3 and S1) and with cGK expression (Figs. 2 and 3 and S2). In addition, to our knowledge, this is the first report of measuring cGMP in living cells (Fig. 2). Results from the analysis of dose-dependent activation of GC receptors using the cGK × ΔMMTV/CRE reporter gene assay revealed that this system can detect very small amounts of cGMP agonistic ligands and is linear over a wide range. Comparison of this reporter assay with the

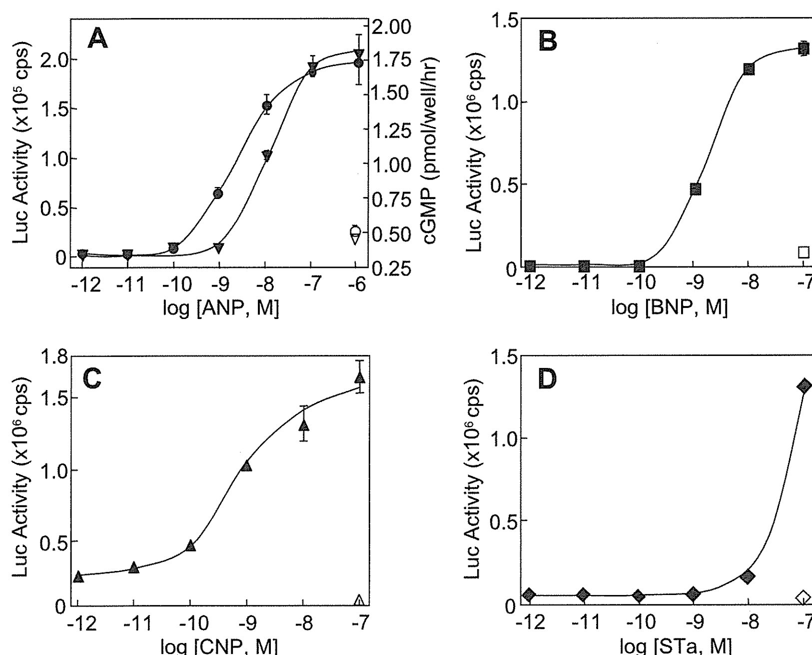

Fig. 4. Responses of HEK293 cells expressing receptor-type GCs specific to each ligand. The dose–response relationships were measured by a cGK × ΔMMTV/CRE reporter gene assay. Cells were transfected with GC-A (A and B), GC-B (C) or GC-C (D) expression vectors along with the reporter gene vectors. Dose–response curves are shown for (A) ANP (filled circles), (B) BNP (filled squares), (C) CNP (filled triangles), and (D) STa (filled diamonds), respectively. The dose–response curve for ANP obtained by ELISA is shown in (A) (filled inverted triangles). The open symbol in each panel shows the response of control HEK293 cells at indicated concentration of each ligand. Each data point shows the mean and SEM from at least three independent measurements. Data are representative of three independent experiments performed in triplicate.

ELISA method indicated that the former method was found to be considerably more sensitive than the latter (Fig. 4). Therefore, this reporter gene assay is capable of evaluating the efficacy of drugs and may be extremely useful in identifying receptor-type GC selective agonists with regard to its high sensitivity, the ability for rapid data acquisition, and simple instrumentation.

Interestingly, the EC_{50} value of STa for GC-C (>200 nM) is much higher when compared with the values of ANP and BNP for the common receptor GC-A (1.3 and 14.4 nM, respectively) and with the value of CNP for GC-B (1 nM) (Fig. 4). One possible reason for this result may be due to a paracrine mode of action of GC-C ligands. Guanylin and uroguanylin, gastrointestinal peptides and endogenous ligands for GC-C, are thought to act on neighboring cells in a paracrine fashion and might require higher doses to activate GC-C receptors than other bioactive peptides [21]. Another possibility is that GC-C may need some additional and specific cellular mechanisms, which are lacking in HEK293, to fully express its receptor activity. These mechanisms would be concerned with the determination of GC-C ligand selectivity between guanylin and uroguanylin *in vivo*. Moreover, this may be one reason why it is that the ligands of GC-D, -E, -F and -G have still not been identified. In any of these cases, this reporter gene system would be useful tool for solving these problems.

The receptor-type GC agonists screened with this reporter gene assay will have some benefit in clinical uses. Many of the physiological effects of ANP and BNP have been reported to exhibit cardioprotective activity, including a diuretic effect and vasodilation [8,9,13]. The infusion of ANP into patients with acute heart failure resulted in hemodynamic responses without tolerance or severe side effects [22]. Although ANP indeed demonstrates cardioprotective effects and its administration may be a clinically useful treatment for heart failure, it only has a half-life of 2 min in the plasma [23]. On the other hand, BNP has a stronger hypotensive effect and a longer half-life in the plasma than does ANP, but its clinical usefulness is limited by adverse effects on renal function, and thus it is mainly used as a better diagnostic marker of heart failure [24]. Hence, to develop GC-A agonists with long half-lives and little or no adverse effects would enable easy maintenance of body fluid volume and blood pressure control, especially in emergency care.

In the case of CNP, its most notable physiological effect via GC-B is to stimulate bone growth, suggesting that CNP could be used in the treatment of osteoporosis and dwarfism. The half-life of CNP in the plasma, however, is about 3 min. GC-B agonists that are stable in the plasma and have few adverse effects would be able to be maintained in long-term administration [13]. Guanylin and uroguanylin peptides have been reported to regulate water and electrolyte absorption in intestinal mucosa epithelial cells, and have an antiproliferative effect on some colon cancer cell types [25]. It may be possible to use GC-C agonists as laxatives and anticancer agents. Therefore, identification of receptor-type GC selective agonists would facilitate the development of a novel class of drugs with improved therapeutic indices and better clinical profiles.

Moreover, the cGK × ΔMMTV/CRE reporter gene assay may also be an applicable method for the following purposes: (1) screening for the specific ligands of GC-D, -E, -F and -G, (2) screening for cGMP-dependent phosphodiesterase inhibitors for the treatment of asthma, alopecia, sexual dysfunction, chronic obstructive pulmonary disease (COPD) and others, and (3) screening for soluble NO-dependent GC agonists to treat patients with hypertension or heart failure. Recently, it has been possible to screen huge amounts of synthetic compounds available from publicly- or commercially-available resources [26]. The reporter gene assay we have reported here would facilitate the high throughput screening of receptor-type GC selective agonists and the development of a novel and more effective class of drugs.

To conclude this report, we have developed a novel reporter gene assay system for measuring intracellular cGMP concentrations using the cGMP/cGK/CRE/CREB signal transduction pathways. We have shown that this system is more sensitive and has a wider range of linearity than do conventional methods. We have demonstrated the possibility of high throughput screening of agonists for receptor-type GCs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.009.

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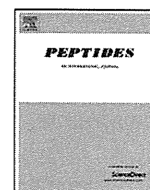

The role of C-terminal part of ghrelin in pharmacokinetic profile and biological activity in rats

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ABSTRACT

Ghrelin is an endogenous ligand for growth hormone secretagogue receptor 1a (GHS-R1a), and consists of 28 amino acid residues with octanoyl modification at Ser^3. The previous studies have revealed that N-terminal part of ghrelin including modified Ser^3 is the active core for the activation of GHS-R1a. On the other hand, the role of C-terminal (8–28) region in ghrelin has not been clarified yet. In the present study, we prepared human ghrelin, C-terminal truncated ghrelin derivatives and anamorelin, a small molecular GHS compound which supposedly mimics the N-terminal active core, and examined GHS-R1a agonist activity *in vitro*, pharmacokinetic (PK) profile and growth hormone (GH) releasing activity in rats. All compounds demonstrated potent GHS-R1a agonist activities *in vitro*. Although the lack of C-terminal two amino acids did not modify PK profile and GH releasing activity, the deletion of C-terminal 8 and 20 amino acids affected them, and ghrelin(1–7)-Lys-NH_2 exhibited very short plasma half-life and low GH releasing activity *in vivo*. In rat plasma, ghrelin(1–7)-Lys-NH_2 was degraded more rapidly than ghrelin, suggesting that C-terminal part of ghrelin protected octanoylation of Ser^3 from plasma esterases. Subdiaphragmatic vagotomy significantly attenuated GH response to ghrelin but not to anamorelin. These results suggest that the C-terminal part of ghrelin has an important role in the biological activity *in vivo*. We also found that ghrelin stimulated GH release mainly via a vagal nerve pathway but anamorelin augmented GH release possibly by directly acting on brain in rats.

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1. Introduction

Ghrelin is a growth hormone (GH) releasing peptide identified as an endogenous ligand for growth hormone secretagogue receptor 1a (GHS-R1a) [11]. The subsequent studies have revealed that ghrelin regulates energy metabolism by stimulating appetite and gastric motility, and suppressing energy expenditure [12]. Ghrelin consists of 28 amino acids, in which Ser^3 residue is n-octanoylated, and this modification is known to be essential for the biological activity of ghrelin [11]. We and others have shown that N-terminal tetrapeptide of ghrelin including the modified Ser^3 is the minimum active core for GHS-R1a agonist activity [2,19,20], and ghrelin(1–7)-Lys-NH_2 displays agonist activity comparable to that of ghrelin [20]. On the other hand, the agonist activity of desacyl-ghrelin, which lacks n-octanoylation at Ser^3, is less than 1/1000 than that of ghrelin [2,20]. These results indicated N-terminal 7 amino acids of ghrelin are important for the activation of GHS-R1a.

Before the identification of ghrelin in 1999, synthetic peptidyl and non-peptidyl molecules called GHS compounds had been extensively investigated, and the chemical structures of these compounds have now been considered to mimic the N-terminal structure of ghrelin [21,24]. One of the non-peptidyl, small molecular GHS compounds, anamorelin, is associated with increased serum GH and insulin-like growth factor-1 (IGF-1) levels, and food intake in healthy subjects [7,14]. These results support the importance of N-terminal part of ghrelin for the activation of GHS-R1a.

In contrast, little is so far known about the biological role of the C-terminal (8–28) region in ghrelin, except that ghrelin binds to human plasma lipoproteins via both its N- and C-terminal parts [4], and that some of amino acids at position 12–24 of ghrelin biochemically interact with GHS-R1a by NMR analysis [17].

Abbreviations: GHS-R1a, growth hormone secretagogue receptor 1a; GHS, growth hormone secretagogue; PK, pharmacokinetic; GH, growth hormone; IGF-1, insulin-like growth factor-1; iv, intravenous; GHRH, growth hormone releasing hormone; $[Ca^{2+}]i$, intracellular calcium concentrations; RIA, radioimmunoassay; $T_{1/2}$, terminal half-life; AUC, area under the curve; SD, standard deviation.

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To explore the function of the C-terminal (8–28) region in ghrelin, we prepared human ghrelin, C-terminal truncated ghrelin derivatives and a small molecular GHS compound, anamorelin [7,14], and evaluated GHS-R1a agonist activity *in vitro*, and pharmacokinetic (PK) profile in rats after a single intravenous (iv) injection in the present study. Biological activity *in vivo* was assessed by GH releasing activity, which is one of the major biological actions of ghrelin [11,12]. Since it has been reported that ghrelin transmits peripheral signals to brain via the afferent vagal nerve [3,18], we also examined the effect of subdiaphragmatic vagotomy on GH responses to ghrelin, its derivatives and anamorelin.

2. Materials and methods

2.1. Peptides and compound

Human ghrelin was synthesized by the chemical condensation of the N-terminal 7 amino acid peptide and a recombinant 21-residue C-terminal fragment as reported previously [16]. C-terminal truncated human ghrelin derivatives, namely, ghrelin(1–26), ghrelin(1–20) and ghrelin(1–7)-Lys-NH_2 were synthesized by solid phase method and purified by HPLC. The third Ser was modified with n-octanoic acid in ghrelin and all ghrelin derivatives. The purity of each peptide was over 98%. Identity of the peptides was assured by amino acid composition analysis, amino acid sequence analysis and electrospray ionization mass spectrometry. Anamorelin hydrochloride was chemically synthesized according to the method of Paul et al. [26]. Human growth hormone releasing hormone (GHRH(1–44)-NH_2) was purchased from Peptide Institute, Inc.

2.2. Calcium-mobilization assay

To evaluate GHS-R1a agonist activities of ghrelin, ghrelin derivatives and anamorelin, we used CHO cells stably expressing rat GHS-R1a, CHO-GHSR62 cells [11]. We have previously confirmed that human ghrelin has almost the same agonist activity as rat ghrelin in rat GHS-R1a expressing cells [19]. The changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) were measured using FlexStation^{TM} (Molecular Devices Corporation) [19–21]. Briefly, CHO-GHSR62 cells were placed on flat-bottom black 96-well plates at 4×10^4 cells/well for 1 day. Prior to the measurement, the cells were incubated with calcium dye solution (Component A in FRIPR Calcium 3 Assay kit, Molecular Devices Corporation) in Hanks' Balanced Salts Solution in 20 mM HEPES, containing 2.5 mM probenecid (Sigma-Aldorich Inc.) and 0.1% bovine serum albumin for 1 h. The test compounds were dissolved in distilled water to prepare 1.0 mM, and diluted with Hanks' buffer. The diluted samples were added to the plate and the changes in $[Ca^{2+}]i$ were measured by FlexStation^{TM} for 2 min. We observed the maximum change in fluorescence ratio over baseline to determine agonist activities using SOFTmax^{TM} PRO (Molecular Devices Corporation), and calculated EC_{50} values using Deltasoft III Ver. 2.247 (Dr. E. Bechtold and BioMetallics, Inc.) from triplicate determinations.

2.3. Animals

We used 7- to 8-week-old female Sprague–Dawley rats (Charles River Japan) in this study. The rats were housed in a humidity- and temperature-controlled environment with an automatic 12-h light/dark cycle. They were fed standard rat chow (CRF-1, Oriental Yeast Co., Ltd.) and tap water *ad libitum*. All the experiments were performed under the approval by the Ethics Committee of Asubio Pharma Co., Ltd.

In all *in vivo* experiments, the rat was weighed and anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg) and, after a catheter was placed in the femoral artery to collect blood samples, subjected to the experiments. The test compounds were weighed and dissolved in 5% mannitol solution at use.

2.4. Pharmacokinetics in rats

The rats received either 10 nmol/kg of ghrelin, ghrelin(1–26), ghrelin(1–20) or 300 nmol/kg of ghrelin(1–7)-Lys-NH_2 by a single iv injection. The blood samples were collected before and 1, 3, 5, 10, 15, and 30 min after the injection and 1/100 volume of 10% EDTA 2Na $2H_2O$ and 1/50 volume of 500 mM AEBSF (Pefabloc SC, Roche Diagnostics K.K.), a protease inhibitor, were added. After centrifugation at 12,000 rpm for 5 min at 4 °C, the plasma samples were collected and 1/10 volume of 1 N HCl was added. Plasma ghrelin immunoreactivity was determined by radioimmunoassay (RIA) using antiserum recognizing the N-terminal region including octanoylated Ser^3 and $[^{125}I]$-labeled $[Tyr^{29}]$-ghrelin as a tracer [10], and ghrelin or each ghrelin derivative was used as the standard. The detection limits of RIA were 0.03 pmol/mL for all peptides. For the PK analysis of anamorelin, anamorelin was administered to the rats at 10 nmol/kg by a single iv injection, and the blood samples were collected at the same time points as for the ghrelin and ghrelin derivatives. The plasma samples were obtained after the addition of 1/100 volume of 10% EDTA 2Na $2H_2O$ to the blood samples and centrifugation. The plasma concentrations of anamorelin were measured by LC–MS (API5000, Applied Biosystems/MDS SCIEX).

PK analysis was performed using WinNonlin Professional Version 4.0.1 (Phasight Corporation) with non-compartment model. We calculated terminal half-life ($T_{1/2}$: min) by the least-squares method.

2.5. Stability of ghrelin and ghrelin(1–7)-NH_2 in rat liver homogenates and plasma

The liver and blood samples were collected from the anesthetized rats. The liver was homogenized with 1000 volume of saline. The plasma samples were obtained after the addition of 1/100 volume of 10% EDTA 2Na $2H_2O$ to the blood samples and centrifugation at 12,000 rpm for 5 min at 4 °C. Ghrelin or ghrelin(1–7)-Lys-NH_2 was added to the liver homogenates and plasma at the concentrations of 0.15 nmol/mL, and incubated at 37 °C. The hydrolysis of octanoylation at Ser^3 in ghrelin and ghrelin(1–7)-Lys-NH_2 was evaluated by the remaining ghrelin-like immunoreactivity in the liver homogenates and plasma using RIA as described in Section 2.4.

2.6. GH response in rats

The test compounds were administered to the anesthetized rats by a single iv injection. The blood samples were collected before and 5, 10, 15 and 30 min after the dosing, and 1/100 volume of 10% EDTA 2Na $2H_2O$ was added. The blood samples were centrifuged at 12,000 rpm for 5 min at 4 °C, and the plasma samples were collected. The plasma GH concentrations were measured using commercially available ELISA kit (EZRMGH-45K, Millipore Co.). We analyzed the time course and the area under the curve (AUC) of plasma GH concentrations.

2.7. GH response in rats with subdiaphragmatic vagotomy

The bilateral subdiaphragmatic vagotomy was performed as previously described [3,28]. The sham rats underwent laparotomy and the exposure of the esophagus alone. About 30 min after the surgery, the test compounds were administered by a single iv

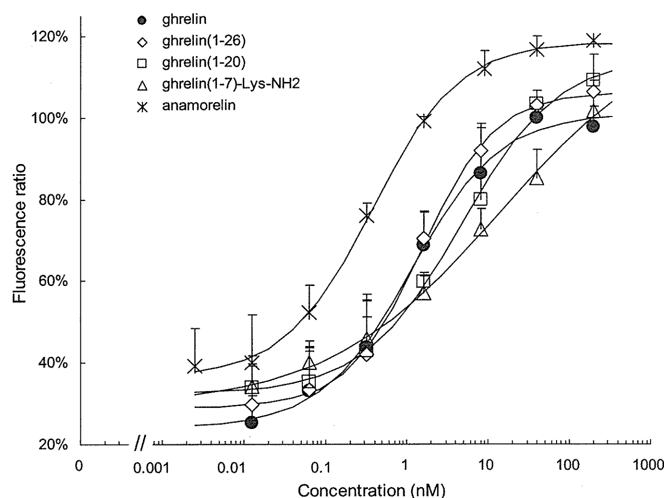

Fig. 1. Dose–response relationships of ghrelin, ghrelin derivatives and anamorelin for the changes in $[Ca^{2+}]i$ in CHO cells expressing rat GHS-R1a. The basal $[Ca^{2+}]i$ without the compound and the maximum increase in $[Ca^{2+}]i$ by ghrelin were regarded as 0 and 100%, respectively. Each value represents the mean ± SD of triplicate determinations.

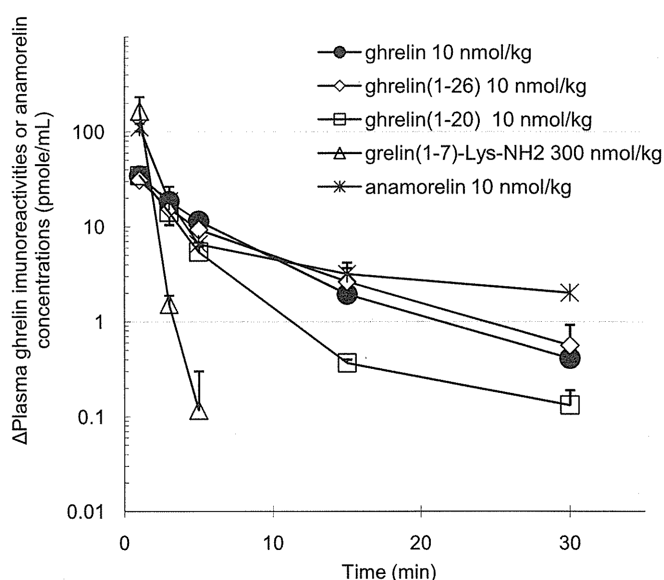

Fig. 2. Changes in plasma ghrelin immunoreactivities or anamorelin concentrations after a single iv administration of ghrelin, ghrelin derivatives or anamorelin in anesthetized rats. Each value represents the mean ± SD of 3 rats.

injection under anesthesia, and plasma GH concentrations were analyzed as described in Section 2.6.

2.8. Data analysis

All data were expressed as the mean or the mean ± standard deviations (SD). The statistical differences between the GH responses in sham rats and those in vagotomized rats were analyzed by Student's *t*-test using EXSUS version 7.6.0.1 (Arm Systex Co., Ltd.).

3. Results

3.1. GHS-R1a agonist activities of ghrelin, ghrelin derivatives and anamorelin

Ghrelin increased $[Ca^{2+}]i$ in CHO cells expressing rat GHS-R1a in a concentration-dependent manner, and the EC_{50} was calculated to be 1.12 nM (Fig. 1, Table 1). Ghrelin(1–26) exhibited agonist activity almost equipotent to that of ghrelin. Ghrelin(1–20) and ghrelin(1–7)-Lys-NH_2 also showed full agonist activities, but the EC_{50} values of these derivatives were less potent than that of ghrelin. Anamorelin potently induced an increase in $[Ca^{2+}]i$, and the EC_{50} was determined to be 0.369 nM, which was 3 times more potent than that of ghrelin.

3.2. PK profile of ghrelin, ghrelin derivatives and anamorelin in rats

Plasma concentrations–time curves after an iv injection of the test compounds are depicted in Fig. 2. The changes in plasma ghrelin immunoreactivity were almost the same among the rats that received either ghrelin or ghrelin(1–26). After the injection of ghrelin(1–20), plasma ghrelin immunoreactivity decreased more rapidly than after ghrelin or ghrelin(1–26). Ghrelin(1–7)-Lys-NH_2 very quickly disappeared from plasma, and plasma ghrelin immunoreactivity declined to concentrations close to endogenous ghrelin levels 5 min after the dosing. The plasma $T_{1/2}$ of ghrelin(1–7)-Lys-NH_2 was 0.4 ± 0.1 min, whereas $T_{1/2}$ of ghrelin and ghrelin(1–26) were 8.5 ± 1.2 or 8.1 ± 2.3 min, respectively. After the iv injection of anamorelin, the plasma concentrations decreased biphasically. During the first 5 min after the injection, plasma anamorelin concentration declined faster than that of ghrelin, thereafter slowly decreased, and $T_{1/2}$ at the terminal phase was calculated to be 21.2 ± 0.2 min, which was around 3 times longer than that of ghrelin.

3.3. Stability of ghrelin and ghrelin(1–7)-Lys-NH_2 in rat liver homogenates and plasma

Ghrelin(1–7)-Lys-NH_2 showed very short plasma half life *in vivo* (Fig. 2), and we next compared the stability of ghrelin and ghrelin(1–7)-Lys-NH_2 in rat liver homogenates and plasma *in vitro*. As shown in Fig. 3A, both ghrelin and ghrelin(1–7)-Lys-NH_2 rapidly decreased in rat liver homogenates in a similar manner. In plasma, around 30% of ghrelin-like immunoreactivities were detected after the incubation of ghrelin for 60 min at 37 °C, but ghrelin(1–7)-Lys-NH_2 was quickly degraded in plasma and decreased to around 3% after 30 min incubation (Fig. 3B).

3.4. GH responses to ghrelin, ghrelin derivatives and anamorelin in rats

Ghrelin and ghrelin(1–26) potently stimulated GH release in rats (Fig. 4). The GH response to ghrelin(1–20) was 3–10 times less potent than those to ghrelin and ghrelin(1–26), and ghrelin(1–7)-Lys-NH_2 exerted a very weak GH releasing activity even at 300 nmol/kg. Anamorelin also increased GH release dose-dependently, but the AUC of plasma GH concentrations were lower than those after ghrelin or ghrelin(1–26) at equimolar doses.

Plasma GH concentrations peaked 15 min after dosing in rats treated with higher doses of ghrelin, ghrelin(1–26) and ghrelin(1–20), but peak GH concentrations were observed 5 min after iv injection in anamorelin treated rats at all doses.

3.5. GH responses to ghrelin, ghrelin(1–20) and anamorelin in rats with subdiaphragmatic vagotomy

It has been reported that ghrelin increased GH secretion via the vagal nerve pathway, and that GH response to ghrelin was greatly attenuated in rats with vagotomy [3]. Since ghrelin(1–26)

Table 1
Amino acids sequences of ghrelin, ghrelin derivatives and chemical structure of anamorelin with EC_{50} values in $[Ca^{2+}]i$ increasing responses in CHO cells expressing rat GHS-R1a.

Test compounds	Amino acid sequence[a] or chemical structure																												EC_{50} (nM)[b]
	1		*							10										20								28	
Ghrelin	G	S	S	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	1.12
Ghrelin(1–26)	G	S	S	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q			1.50
Ghrelin(1–20)	G	S	S	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K									4.61
Ghrelin(1–7)-Lys-NH_2	G	S	S	F	L	S	P	K-NH_2																					13.9
Anamorelin	[1-(2-Methylalanyl)-D-tryptophyl]-3-benzylpiperidine-3(R)-carboxylic acid trimethylhydrazide																												0.369

[a] One-letter amino acid notation is used. Ser at third position (*) was modified with n-octanoic acid in each peptide.
[b] EC_{50} was calculated based on triplicate determinations (Fig. 1).

exhibited almost the same properties as ghrelin both *in vitro* and *in vivo*, and GH releasing activity of ghrelin(1–7)-Lys-NH_2 was very weak even in the normal rats, we eliminated these two derivatives from the study in the rats with vagotomy, and we evaluated GH responses to ghrelin, ghrelin(1–20), and anamorelin. We also tested the effect of GHRH, another hormone stimulating GH release [8], which acts directly on the pituitary. The GH response to GHRH was not affected by vagotomy [3]. We selected 10 nmol/kg for ghrelin and 30 nmol/kg of anamorelin, respectively, as the doses showing sub-maximum GH responses (Fig. 4). The dose of ghrelin(1–20) was set as 10 nmol/kg, which was equal to the dose of ghrelin. The dose of GHRH was selected as 1 nmol/kg based on the previous report [3].

As shown in Fig. 5, GH response to ghrelin was significantly attenuated in the rats with vagotomy compared with that in the sham rats. Although GH response to ghrelin(1–20) at 10 nmol/kg was less potent than that to 10 nmol/kg of ghrelin, it was also significantly diminished in the rats with vagotomy. On the other hand, GHRH and anamorelin stimulated GH release almost equally in both sham and vagotomized rats.

4. Discussion

It has been known that the N-terminal region of ghrelin, including the octanoylated Ser^3, is responsible for the activation of GHS-R1a by ghrelin [2,19,20], and the role of the C-terminal (8–28) region in ghrelin has not been clarified yet. The present study first indicates that the C-terminal part of ghrelin has an important role in the PK profile and biological activity of ghrelin *in vivo*. Our data also suggest that ghrelin stimulates GH release mainly via a vagal nerve pathway but that anamorelin, a small molecular GHS compound, may directly act on the pituitary and/or hypothalamus to promote GH release in rats.

To explore the function of the C-terminal part of ghrelin, we prepared human ghrelin and three C-terminal truncated ghrelin derivatives. Ghrelin(1–26) and ghrelin(1–20) were synthesized to examine the role of C-terminal amino acids and the intramolecular region rich in positively charged amino acids ([11]RVQQRKESKK[20]) (Table 1). We prepared ghrelin(1–7)-Lys-NH_2 because this derivative has been reported to maintain GHS-R1a agonist activity *in vitro* [20], and could be an example of N-terminal peptide. Since it has been considered that GHS compounds mimic the N-terminal structure of ghrelin [21,24], we also prepared anamorelin, one of the small molecular GHS compounds [7,14]. The GHS-R1a agonist activities of ghrelin(1–20) and ghrelin(1–7)-Lys-NH_2 were slightly lower than those of ghrelin and ghrelin(1–26) (Fig. 1, Table 1). When considering that the agonist activity of desacyl-ghrelin, which lacks octanoic acid at Ser^3 [11], is less than 1/1000 than that of ghrelin [2,20], it is suggested that all ghrelin derivatives possess potent GHS-R1a agonist activity *in vitro*. These results indicate that N-terminal part of ghrelin is important for the activation of GHS-R1a as reported previously [2,19,20].

Ghrelin and ghrelin(1–26) exhibited almost the same GHS-R1a agonist activity *in vitro*, and PK profile and GH releasing activity *in vivo*, suggesting that the C-terminal two amino acids do not have a major role for *in vivo* activity at least when administered by a single iv injection to rats. On the other hand, the deletion of C-terminal 8 and 20 amino acids affected PK profile and GH response in rats. The rapid plasma clearance of ghrelin(1–20) compared with ghrelin suggested a certain role of the amino acids at 21–26 in the PK profile of ghrelin. Since we analyzed plasma concentrations of ghrelin and its derivatives by RIA using the antiserum recognized N-terminal part of ghrelin including octanoylated Ser^3, the rapid disappearance of ghrelin(1–20) may represent the more rapid des-octanoylation compared with ghrelin and ghrelin(1–26). Whereas, the deletion of C-terminal 20 amino acids resulted in a marked change in PK

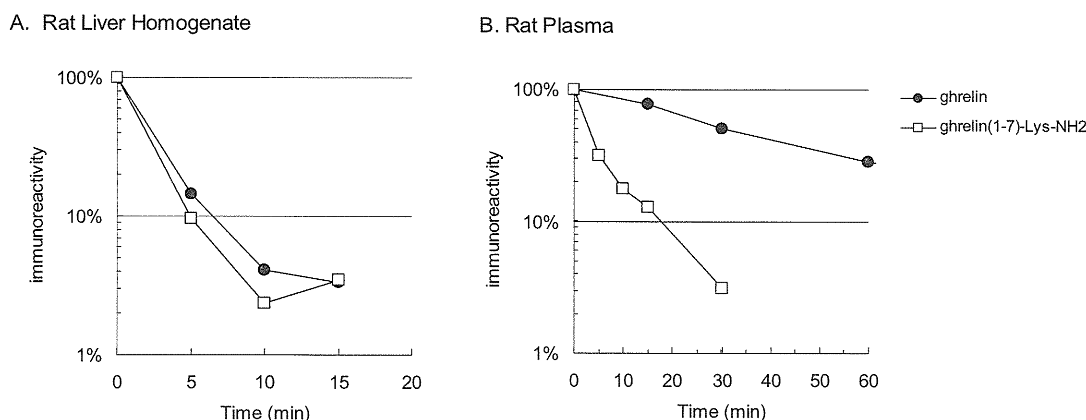

Fig. 3. Stability of ghrelin and ghrelin(1–7)-Lys-NH_2 in rat liver homogenates (A) and plasma (B). The hydrolysis of octanoylated Ser^3 was assessed by RIA using antiserum against N-terminal part of ghrelin. The initial immunoreactivity was regarded as 100%, and % changes after the incubation at 37 °C were analyzed. Each value represents the mean of two experiments.

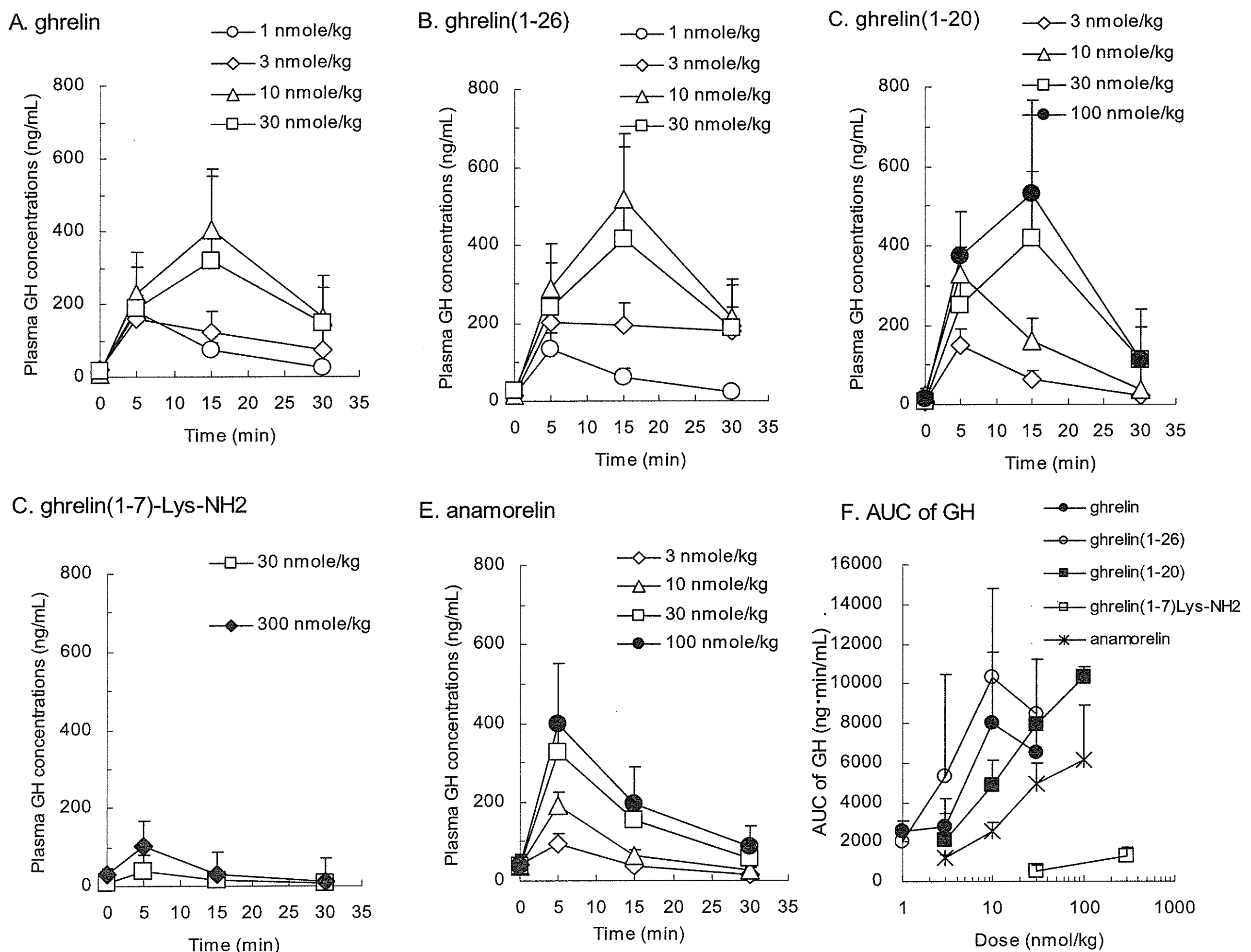

Fig. 4. Effect of ghrelin, ghrelin derivatives or anamorelin on plasma GH concentrations after a single iv administration in anesthetized rats. (A–E) Shows the time course of plasma GH concentrations after the iv injection of ghrelin, ghrelin(1–26), ghrelin(1–20), ghrelin(1–7)-NH_2 and anamorelin, respectively. (F) Shows the increases in AUC of plasma GH concentrations by ghrelin (closed circle), ghrelin(1–26) (open circle), ghrelin(1–20) (closed square), ghrelin(1–7)-Lys-NH_2 (open square), and anamorelin (asterisk). Each value represents the mean ± SD of 3 rats.

profile (Fig. 2). Ghrelin(1–7)-Lys-NH_2 had a very short plasma $T_{1/2}$, which might be associated with its very low GH releasing activity *in vivo*, and indicates the critical role of the amino acids at position 8–20 in ghrelin's PK profile. In rat plasma, ghrelin(1–7)-Lys-NH_2 was degraded more rapidly than ghrelin (Fig. 3B), suggesting that C-terminal part of ghrelin could stabilize octanoylation of Ser^3 in plasma. It has been reported that ghrelin interacts with plasma lipoproteins via both its N- and C-terminal domains [4]. The positively charged amino acids rich in the (11–20) region of ghrelin may interact with negatively charged lipoproteins, and this association may protect the octanoylation of Ser^3 from various esterases in plasma.

A small molecular GHS compound, anamorelin, exerted more potent GHS-R1a agonist activity *in vitro* and longer plasma $T_{1/2}$ in rats compared with ghrelin (Figs. 1 and 2), but the GH releasing activity of anamorelin was lower than that of ghrelin (Fig. 4). Although GHS-R1a has been the sole identified receptor responsible for the GH releasing and orexigenic actions of ghrelin [12,29], the discrepancy between *in vitro* and *in vivo* potency has also been reported for the other GHS compounds [9,22]. In these papers, it was discussed that the discrepancy could not often be explained by differences in PK parameters, which was also the case for our results with anamorelin. Ghrelin elicits GH secretion by a dual action, a direct effect on the pituitary and a modulation of GHRH and somatostatin in the hypothalamus [3,11,30]. Tannenbaum et al. [31] have reported that pretreatment with antiserum against GHRH virtually obliterated the GH response to ghrelin. It has also been reported that the blockade of the vagal afferent abolished ghrelin-induced GH secretion [3]. These results suggest that the vagal mediated hypothalamic GHRH pathway has an important role for GH releasing activity of ghrelin *in vivo* rather than a direct action on the pituitary. In the present study, we found that subdiaphragmatic vagotomy significantly attenuated the GH response to ghrelin, but, as expected, it did not affect the GH response to GHRH. Interestingly, GH response to ghrelin(1–20) was also diminished by vagotomy, but anamorelin stimulated GH release almost equivalently in both sham and vagotomized rats (Fig. 5). These results suggest that ghrelin and ghrelin(1–20) stimulate GH release mainly via a vagal nerve pathway but anamorelin possibly augments GH release by direct action on the pituitary, similarly to GHRH. Since 20–30% of GHRH neurons in hypothalamus express GHS-R mRNA [31,32], anamorelin may directly activate some of GHRH neurons in hypothalamus and stimulate GH release from the pituitary. The difference between vagal mediated action and direct action on pituitary and/or hypothalamus may contribute to the relatively lower GH response and the earlier GH peak time after an iv injection of anamorelin compared with ghrelin (Figs. 4 and 5).

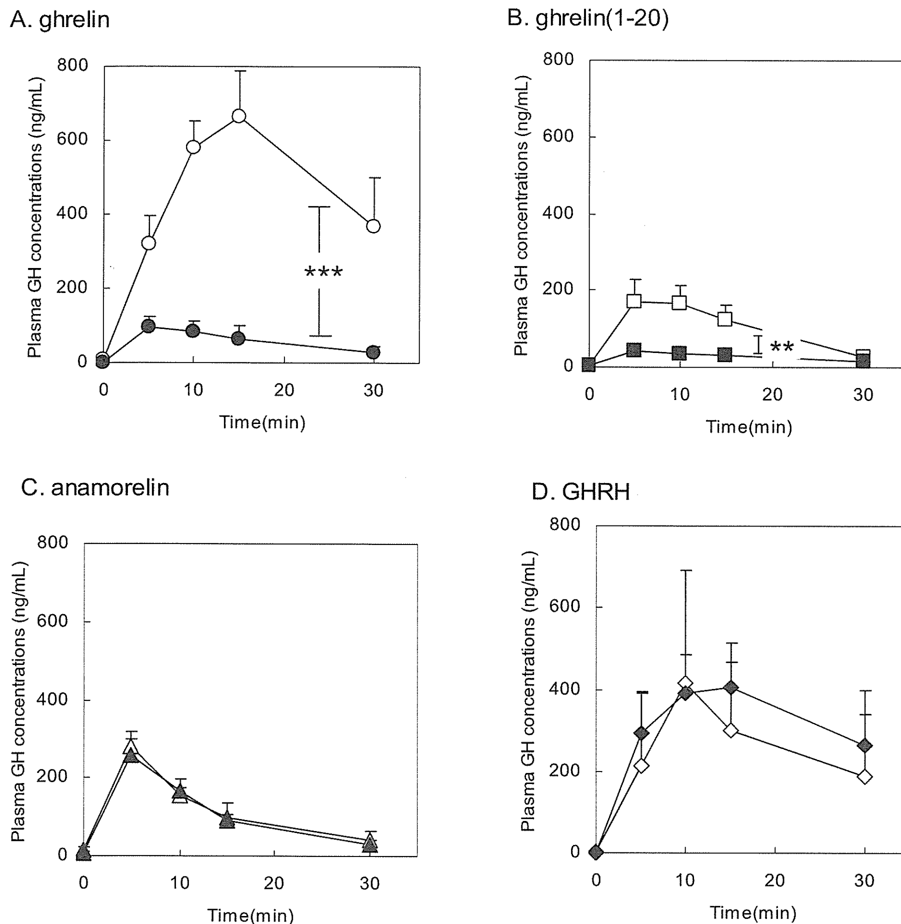

Fig. 5. Effect of vagotomy on GH responses to ghrelin, ghrelin(1–20), anamorelin and GHRH in anesthetized rats. Ghrelin, ghrelin(1–20), anamorelin and GHRH were administered at 10, 10, 30, and 1 nmol/kg by a single iv injection, respectively. Open symbols show GH responses in sham rats, and closed symbols show those in vagotomized rats. Each value represents the mean ± SD of 4 rats for ghrelin, ghrelin(1–20) and anamorelin, 3 rats for GHRH in sham group and 5 rats for GHRH in vagotomized group. Significant differences between AUC of plasma GH concentrations in sham rats and those in vagotomized rats are indicated by asterisks: $^{**}p < 0.01$ and $^{***}p < 0.001$.

Although it has been reported that GHS compounds mimic the N-terminal structure of ghrelin [21,24], anamorelin is a synthetic, small molecular compound and has physicochemical and biochemical properties different from those of ghrelin and ghrelin derivatives. In addition, Kudoh et al. [13] has reported that GHRP-2, a peptidyl GHS compound, increased food intake in normal dogs, but not in dogs with vagotomy. Further studies using ghrelin derivatives and GHS compounds other than ghrelin(1–20) and anamorelin will be required to elucidate the importance of the C-terminal part of ghrelin for the vagal mediated action. At this stage, we speculate that ghrelin, which is mainly produced in the stomach [11], may have a specific structural feature that could efficiently access GHS-R1a on the afferent vagal terminals in the stomach and effectively transmit the various signals including appetite and GH secretion to brain.

It has been reported that vagal nerves are involved in the effects of ghrelin on GH release, food intake, gastric motility and anti-inflammation [3,18,33]. There was a clear difference between GH response to ghrelin and that to anamorelin in the rats with vagotomy (Fig. 5), but among the multiple functions of ghrelin, we only evaluated GH releasing activity in the present study. Ghrelin has a wide variety of additional biological effects such as memory retention, reward, neuroprotection, and inhibition of sympathetic nerve activity via GHS-R1a [1,6,15,23,27,34]. Most of these effects have not been reported for GHS compounds yet, except that GHRP-6, a peptidyl GHS compound, inhibited neuronal death [5,25]. Among the various biological activities of ghrelin, those mediated by the vagal nerve pathway and those unique to ghrelin should also be clarified in the future.

In conclusion, we have indicated that C-terminal part of ghrelin, especially (8–20) region, has an important role in the PK profile and GH releasing activity of ghrelin *in vivo* for the first time. We have also suggested that ghrelin stimulates GH release mainly via a vagal nerve pathway but that anamorelin may directly act on brain to promote GH release in rats.

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Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion

Sachiko Mogami,[1,2] Hidekazu Suzuki,[1] Seiichiro Fukuhara,[1] Juntaro Matsuzaki,[1] Kenji Kangawa,[3] and Toshifumi Hibi[1]

[1]*Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan;* [2]*Tsumura Research Laboratories, Tsumura & Co., Ibaraki, Japan;* [3]*National Cardiovascular Center Institute, Osaka, Japan*

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Mogami S, Suzuki H, Fukuhara S, Matsuzaki J, Kangawa K, Hibi T. Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion. *Am J Physiol Gastrointest Liver Physiol* 302: G359–G364, 2012. First published December 8, 2011; doi:10.1152/ajpgi.00297.2011.—The gastrointestinal (GI) tract is one of the most susceptible organs to ischemia. We previously reported altered gastric motility after gastric ischemia and reperfusion (I/R). However, there have also been few reports of alterations in the eating behavior after gastric I/R. Ghrelin is a GI peptide that stimulates food intake and GI motility. Although ghrelin itself has been demonstrated to attenuate the mucosal injuries induced by gastric I/R, the endogenous ghrelin dynamics after I/R has not yet been elucidated. The present study was designed to investigate the relationship between food intake and the ghrelin dynamics after gastric I/R. Wistar rats were exposed to 80-min gastric ischemia, followed by 12-h or 48-h reperfusion. The food intake, plasma ghrelin levels, gastric preproghrelin mRNA expression levels, and the histological localization of ghrelin-immunoreactive cells were evaluated. The effect of exogenous ghrelin on the food intake after I/R was also examined. Food intake, the plasma ghrelin levels, the count of ghrelin-immunoreactive cells corrected by the percentage areas of the remaining mucosa, and the expression levels of preproghrelin mRNA in the stomach were significantly reduced at 12 h and 48 h after I/R compared with the levels in the sham-operated rats. Intraperitoneal administration of ghrelin significantly reversed the decrease of food intake after I/R. These data show that gastric I/R evoked anorexia with decreased plasma ghrelin levels and ghrelin production, which appears to be attributable to the I/R-induced gastric mucosal injuries. The decrease in the plasma ghrelin levels may have been responsible for the decreased food intake after gastric I/R.

food intake; ghrelin; mucosal injury

GASTROINTESTINAL (GI) TRACT is one of the most susceptible organ systems to ischemia. Various investigations have demonstrated that ischemia and reperfusion (I/R) contribute significantly to the gastric mucosal injuries caused by stress, such as burn stress (17) or hemorrhagic shock (35), nonsteroidal anti-inflammatory drugs (30), and *Helicobacter pylori* (*H. pylori.*) infection (26, 27). We previously demonstrated, not only postischemic mucosal injury, but also transient delay in gastric emptying in a rat model of gastric I/R (28). These changes were found to be associated with disruption of the network of the interstitial cells of Cajal and decrease in neuronal nitric oxide synthase-positive neurons in the smooth muscle layer. On the other hand, there have been no reports on alterations in eating behavior after gastric I/R, at least to our knowledge.

Ghrelin, a 28-residue octanoylated peptide, is an endogenous ligand of the growth hormone secretagogue receptor (18) and is produced and secreted from the A-like cells found mainly in the oxyntic glands of the gastric fundus (8). Gastric ghrelin accounts for the major part of circulating ghrelin, as an ~80% reduction in the circulating levels of ghrelin has been demonstrated after gastrectomy or fundectomy (10). Ghrelin is now known to play a role, not only in growth-hormone release, but also in stimulating gastric motility and food intake (1, 21, 32). Recent studies have also reported the gastroprotective effect of ghrelin; ghrelin has been demonstrated to reduce ethanol-induced gastric ulceration (23), acetic acid-induced chronic gastric and duodenal ulceration (6), and I/R-induced gastric ulceration (11) in rats. Although changes in the plasma ghrelin levels and association with various GI diseases have been reported such as in functional dyspepsia (22), chronic gastritis and gastric ulcer (14), the ghrelin dynamics after gastric I/R has yet to be elucidated.

The present study was designed to investigate the influences of gastric I/R injuries on the food intake and ghrelin dynamics in a rat model of gastric I/R injury.

MATERIALS AND METHODS

I/R. Six-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). All rats were handled according to the guidelines of the Keio University Animal Research Committee (approved protocol No. 078086) and the Experimental Animal Ethics Committee of Tsumura & Co. (approved protocol No. 09–155, 09–157, 10–096, 10–110, 10–156). All rats were used after acclimation for 1 wk and denied access to food for 22–24 h (but allowed free access to water) before the operation. The rats were anesthetized with pentobarbital sodium (50 mg/ kg ip) during the surgery. The abdomen was opened by a midline incision, and the celiac artery was occluded with a small clamp for 80 min. Reperfusion was established for 12 h or 48 h by removal of the clamp. For comparison, some rats were subjected to a sham operation (surgery, but no clamping). Rats were supplied with food after the surgery (returned to normal feeding). Food intake was measured at 12 h after I/R (when gastric emptying was delayed compared with sham-operated rats) and at 48 h after I/R (when gastric emptying was restored) (Fig. 1*A*). In the fasting condition, food deprivation was continued after the surgery when reperfusion was established for 12 h. When reperfusion was established for 48 h, the rats were fed after the operation (normal feeding), but were again deprived of food for 24 h before euthanasia to establish the fasted condition (Fig. 1*B*). To measure plasma ghrelin levels in the fed condition at 48 h after I/R, I/R rats were fed ad libitum after the surgery. Sham-operated rats were given the same amount of food as

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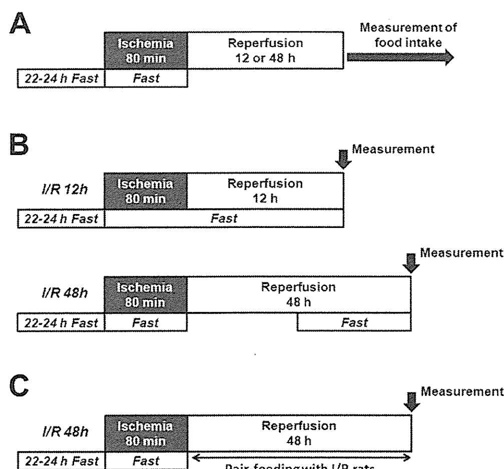

Fig. 1. Experimental protocol. *A*: measurement of cumulative food intake at 12 h or 48 h after gastric ischemia and reperfusion (I/R) in fed condition. *B*: measurement of gastric emptying rates or plasma ghrelin levels at 12 h or 48 h after I/R in fasted condition. *C*: measurement of plasma ghrelin levels at 48 h after I/R in the fed condition.

the I/R rats to eliminate the effect of the difference in the food intake (Fig. 1*C*).

Measurement of food intake. All rats were housed in individual hanging-wire cages. After reperfusion for 12 h or 48 h, the rats were supplied with preweighed food, and the cumulative food intake of each rat was calculated as the difference between the food weights before and after the feeding period. In the experiment to determine the effect of exogenous ghrelin, rat ghrelin (30 nmol/0.5 ml saline per rat; Peptide Institute, Osaka, Japan) or 0.5 ml saline was administered intraperitoneally, immediately before the supply of the preweighed food (Fig. 1*A*).

Evaluation of gastric emptying of solid food. Solid gastric emptying was evaluated using powdered food (13) and glass beads (24, 34). After 24-h food deprivation (Fig. 1*B*), 1 ml of the test meal containing powdered food and glass beads (0.2-mm diameter, BZ-02; AS One, Osaka, Japan) was orally administered to the rats through a Teflon tube (AWG-14) attached to a 1-ml syringe, using a 10Fr Nelaton's catheter. The test meal contained 32 g of ground meal, 40 g of glass beads, and 80 ml of distilled water. Rats were then killed by decapitation 2.5 h after the test meal administration, except for the animals that were killed immediately after the injection to recover the entire dose of the test meal. The gastric contents were then recovered from the stomach, dried, and weighed. The gastric emptying of solid food was calculated as follows: Gastric emptying (%) = [1 − (dried weight of food recovered from stomach / dried weight of food recovered from the stomach immediately after the test meal administration)] × 100.

Measurement of the plasma ghrelin levels. After 24-h food deprivation (Fig. 1*B*) or after 48-h pair feeding (Fig. 1*C*), whole blood samples were obtained from the right ventricle under ether anesthesia in tubes containing EDTA-2Na (1 mg/ ml) and aprotinin (500 kIU/ ml). Samples were promptly centrifuged at 4°C, and the supernatant was acidified with 1 mol/l HCl (1/10 volume) and stored at −80°C until use. The ghrelin level was determined using the Active Ghrelin ELISA Kit, and the desacylghrelin (ghrelin without octanoyl acid modification) level was determined using the Desacyl Ghrelin ELISA Kit (Mitsubishi Chemical Medience, Tokyo, Japan).

Immunohistochemistry. Stomach tissue specimens were fixed in 10% neutralized formalin and embedded in paraffin. After deparaffinization and hydration, the antigens were retrieved by heating for 20 min at 97°C in Dako REAL Target Retrieval Solution (DAKO Japan, Tokyo, Japan). Nonspecific binding was blocked by Protein Block (DAKO Japan). All sections were incubated overnight at 4°C with anti-ghrelin (13–28) antiserum (7) (1:10,000). After being washed with TBS-T, the slides were incubated with peroxidase-labeled dextran polymer conjugated anti-rabbit IgG in Tris-HCl (EnVision/HRP; Dako Japan) for 30 min at room temperature and then visualized after color development using 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution for 3 min. Counterstaining was performed with hematoxylin. The stained sections were observed under a light microscope equipped with a 3CCD digital camera (C7780; Hamamatsu Photonics, Hamamatsu, Japan), and the photomicrographs were obtained in areas without gastric I/R-induced mucosal injuries. DAB-stained ghrelin immunoreactive cells were counted by visual inspection, and hematoxylin-stained nuclei were counted using the ImageJ program (National Institutes of Health, Bethesda, MD). The numbers of ghrelin-IR cells were normalized by dividing by the total number of cells counterstained with hematoxylin. The numbers of ghrelin-IR cells were further corrected by the percentages of the remaining mucosal areas without erosive lesions, which were quantified using the image analysis software. The erosive lesions are indicated by dashed lines in Fig. 4*A*. Corrected IR cells = % of number of ghrelin-IR cells × [(mucosal area without the erosive lesion)/(total area)]. Hematoxylin-eosin (HE) staining was also conducted to evaluate the severity of the injuries induced by I/R.

Preparation of total RNA and quantitative RT-PCR analysis. Total RNA was extracted from the stomach tissue using RNeasy Mini kit (Qiagen, Valencia, CA), and DNase treatment was performed with an RNase-free DNase set (Qiagen). RNA was converted into cDNA using the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). Quantitative RT-PCR analysis was performed using Dice (Takara) with SYBR Premix Ex TaqII (Takara). The primer sequences used were as follows; preproghrelin mRNA: 5′-GGA ATC CAA GAA GCC ACC AGC′ and 5′-GCT CCT GAC AGC TTG ATG CCA-3′; GAPDH mRNA: 5′- GGC ACA GTC AAG GCT GAG AAT G -3′, 5′- ATG GTG GTG AAG ACG CCA GTA -3′. The mRNA expression levels were normalized using the GAPDH mRNA expression levels.

Statistical analysis. All values were expressed as means ± SD. The statistical significance of any differences between two groups was evaluated using unpaired Student's *t*-test. Statistical significance was set at $P < 0.05$, unless otherwise indicated.

RESULTS

Food intake after gastric I/R. Cumulative food intakes were significantly reduced at 12 h after gastric I/R compared with that in the sham-operated rats in the fed condition (Fig. 2*A*). No significant difference was observed in the cumulative food intakes of shorter period, probably because 12 h was not sufficient for recovery from the surgical stress, and the food intake was very small even in the sham-operated rats. Cumulative food intakes (2, 4, 6, and 24 h) were also significantly reduced at 48 h after gastric I/R compared with those in the sham-operated rats in the fed condition (Fig. 2*B*). Decreased food intakes were also observed in the fasting condition after I/R (data not shown).

Gastric emptying of solids after gastric I/R. Gastric emptying rates were investigated using powdered food and glass beads at 48 h after I/R because decreased gastric emptying of liquids at 12 h after I/R was restored at 48 h although food intake was reduced in the I/R rats compared with that in the sham-operated rats. Figure 2*C* shows that the gastric emptying rates of solids in the I/R rats (50.1 ± 15.5%) were comparable with those in the sham-operated rats (57.0 ± 16.9%).

Plasma ghrelin levels. Plasma ghrelin and desacylghrelin levels were measured at 12 and 48 h after gastric I/R in the fasting (Fig. 3, *A* and *B*) and pair-fed (Fig. 3*C*) conditions to eliminate the effect of food intake. As shown in Fig. 3*A*, fasting

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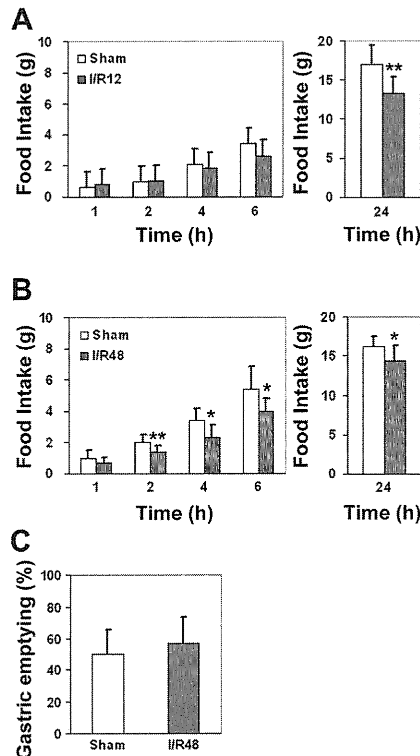

Fig. 2. *A*: effect of gastric I/R on the cumulative food intakes at 12 h after I/R in the fed condition. Sham-operated rats, open bar (n = 8); I/R rats, solid bar (n = 10). *B*: effect of gastric I/R on the cumulative food intakes at 48 h after I/R in the fed condition. Sham-operated rats, open bar (n = 9); I/R rats, solid bar (n = 10). *C*: gastric emptying rates of solids in the sham-operated rats (open bar) and I/R rats (solid bar) at 48 h (Sham, n = 6; I/R, n = 7) after I/R. Data are means ± SD. *$P < 0.05$, **$P < 0.01$ compared with the sham-operated rats by Student's t-test.

plasma ghrelin levels at 12 h and 48 h after I/R were significantly lower than those in the sham-operated rats at the corresponding time-points (sham 12 h, 64.5 ± 13.5 fmol/ml; I/R 12 h, 46.3 ± 9.25 fmol/ml; sham 48 h, 97.2 ± 30.3 fmol/ml; I/R 48 h, 70.9 ± 18.4 fmol/ml). Fasting plasma desacylghrelin levels at 12 h and 48 h after I/R were also significantly lower than those in the sham-operated rats at the corresponding time-points (sham 12 h, 834 ± 137 fmol/ml; I/R 12 h, 663 ± 113 fmol/ml; sham 48 h, 1,092 ± 150 fmol/ml; I/R 48 h, 835 ± 187 fmol/ml), as shown in Fig. 3*B*. Plasma ghrelin (sham, 115 ± 30.0 fmol/ml; I/R, 45.4 ± 23.7 fmol/ml) and desacylghrelin (sham, 1,311 ± 118 fmol/ml; I/R, 577 ± 201 fmol/ml) levels in the fed condition were also significantly decreased compared with those in the pair-fed sham-operated rats at 48 h after I/R (Fig. 3*C*).

Immunohistochemical staining for ghrelin-producing cells. Ghrelin-immunoreactive (IR) cells were counted in the mucosal layer of the fundic gland region (Fig. 4*A*). In case of counting in the mucosal layers of I/R group, the places without the mucosal injuries induced by gastric I/R were selected. The count of ghrelin-IR cells was decreased at 12 h after I/R (Sham, 0.92 ± 0.18%; I/R, 0.58 ± 0.11%, P = 0.0017) but recovered by 48 h (Sham, 0.86 ± 0.17%; I/R, 0.92 ± 0.20%) (Fig. 4*B*). However, because erosive lesion areas were observed at 12 h and 48 h after I/R (Fig. 4*A*, *right*), we corrected the numbers of ghrelin-IR cells by the percentages of the remaining mucosal areas not showing erosive lesions (Fig. 4*C*). The corrected numbers of ghrelin-IR cells were significantly decreased throughout the observation period (44.7 ± 11.1% at 12 h and 78.4 ± 18.6% at 48 h after I/R relative to the value in the sham-operated rats).

Ghrelin production after gastric I/R. The expression levels of preproghrelin mRNA were significantly reduced at 12 h and 48 h (53.4 ± 22.7% and 42.3 ± 16.8% relative to the value in the sham-operated rats) after I/R compared with the levels in the sham-operated rats at the corresponding time points (Fig. 5*A*). Mucosal injuries in the fundic gland regions, where ghrelin-IR cells are mainly distributed, persisted throughout the observation period, as visualized in the HE-stained sections (Fig. 5*B*).

Restoration of decreased food intake by exogenous ghrelin administration. In Fig. 6, ghrelin was administered intraperitoneally (30 nmol/rat) to sham-operated and I/R rats to investigate the effect of exogenous ghrelin on the decreased food intake at 48 h after I/R. In sham-operated rats, food intake was enhanced for 1 h, but not at 2- and 3-h cumulative food intake (Fig. 6*A*). However, administration of ghrelin significantly restored the decreased cumulative food intake (2 and 3 h) in I/R rats (Fig. 6*B*). The effect of decreased food intake restoration by exogenous ghrelin waned 4 h after administration. Administration of 10 nmol ghrelin per rat failed to increase food intake in both sham-operated and I/R rats (data not shown).

DISCUSSION

In the present study, we demonstrated that anorexia was induced after gastric I/R associated with decreased plasma ghrelin levels in rats. Not only the plasma ghrelin level but also ghrelin production was reduced by continuous mucosal injuries. Exogenous ghrelin administration significantly restored the food intake, indicating that it was the decrease in the levels of the orexigenic hormone that induced the anorexia after gastric I/R.

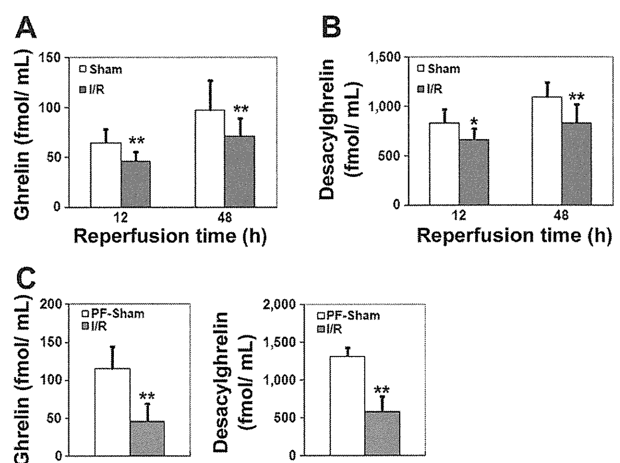

Fig. 3. Fasting plasma ghrelin (*A*) and desacylghrelin (*B*) levels in the sham-operated rats (open bar) and I/R rats (solid bar) at 12 h (Sham, n = 18; I/R, n = 16) and 48 h (Sham, n = 12; I/R, n = 13) after I/R. *C*: plasma ghrelin levels of I/R rats in the fed condition (solid bar, n = 9) and of sham-operated rats in the pair-fed condition (open bar, n = 6) at 48 h after I/R. Data are means ± SD. *$P < 0.05$, **$P < 0.01$ compared with the sham-operated rats by Student's t-test.

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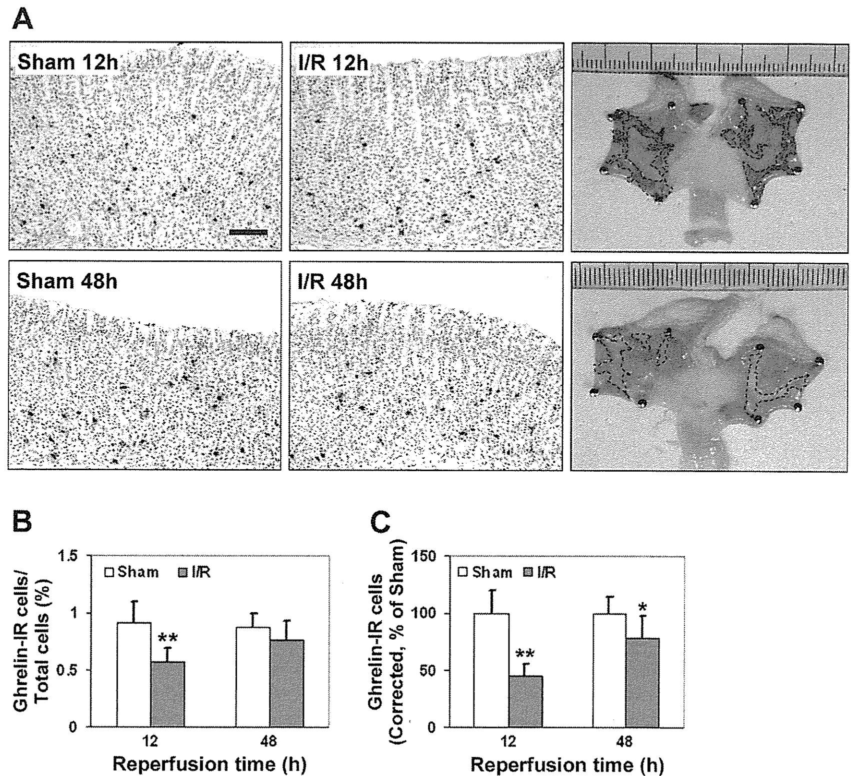

Fig. 4. *A*: representative photomicrograph of ghrelin-immunoreactive (IR) cells in the gastric fundic mucosa of the sham-operated rats and I/R rats at 12 h and 48 h after I/R. The brown-colored cells represent the ghrelin-IR cells. Bar = 100 μm. *Right* photographs of the gastric mucosa obtained from the I/R rats at 12 h and 48 h after I/R. The erosive lesion areas are shown by the dashed lines. *B*: numbers of ghrelin-IR cells in the images from the sham-operated rats (open bar) and I/R rats (solid bar) were counted and normalized by the total number of cells counterstained with hematoxylin, which was quantified using the image analysis software. *C*: numbers of ghrelin-IR cells in the images were corrected by the percentages of the remaining mucosal areas without the erosive lesions, which was quantified using the image analysis software. Sham, $n = 6$; I/R; $n = 7$. Data are means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the sham-operated rats by Student's *t*-test.

Thermal injuries (3) have been reported to induce decreased food intake, and aspirin treatment led to a further and significant decrease of food intake compared with that in the controls (16). A previous study reported that the restoration of gastric ghrelin production was associated with ulcer healing and improvement of the appetite in patients with *H. pylori*-associated active duodenal or gastric ulcer (15). Although these events are reported to induce gastric I/R (17, 26, 27, 30, 35), whether anorexia can be induced by gastric I/R alone remains unclear. The present study is the first report documenting decreased food intake associated with reduced production of ghrelin after gastric I/R.

We previously reported transient delay in gastric emptying of liquids at 12 h after I/R (28), which may be considered as inducing early satiety and contribute to the anorexia. However, the delayed gastric emptying of liquids was normalized at 48 h after I/R. The gastric emptying rates of solids at 48 h were also not significantly different between the sham-operated rats and I/R rats in the present study. The normalized gastric emptying rates do not explain the decrease of food intake at 48 h after I/R; therefore, other factors may also be associated with the anorexia.

Significant decrease in the plasma levels of ghrelin, an orexigenic hormone, in the fasting condition were observed at 12 h and 48 h after I/R in this study. Decreased plasma ghrelin levels are reported to induce anorexia, such as in the lipopolysaccharide-induced food intake and gastric emptying-altered model (31) and cisplatin-induced anorexia model (29). Therefore, we assumed that the decrease in the plasma ghrelin levels may have contributed to the persistent decrease of food intake after I/R in this study. This is also supported by our observation that intraperitoneal administration of exogenous ghrelin restored the food intake at 48 h after I/R.

Ghrelin has been reported to attenuate mucosal injuries induced by gastric I/R (11) and intestinal I/R (33). In the present study, single ghrelin administration, after the formation of mucosal injuries (mucosal injuries were already present after 1-h reperfusion, and exogenous ghrelin was administered at 48

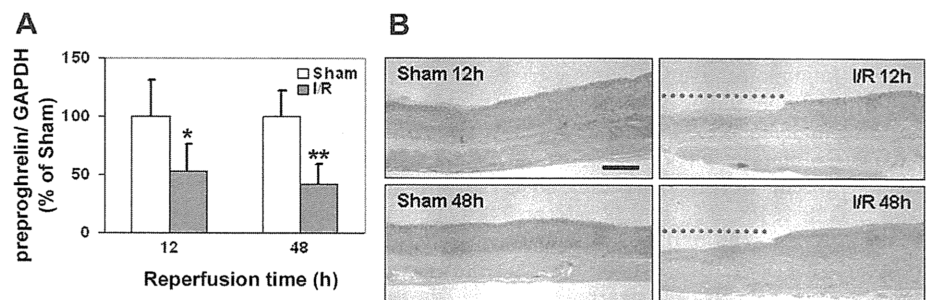

Fig. 5. *A*: expression levels of preproghrelin mRNA in the stomach of the sham-operated rats (open bar) and I/R rats (solid bar) at 12 h (Sham, $n = 6$; I/R, $n = 7$) and 48 h (Sham, $n = 6$; I/R, $n = 7$) after I/R. Data are means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the sham-operated rats by Student's *t*-test. *B*: hematoxylin-eosin staining of gastric tissue (Bar = 500 μm).

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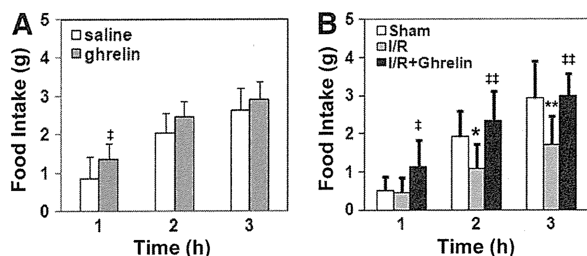

Fig. 6. *A*: effect of exogenous ghrelin (30 nmol/rat ip) in sham-operated rats in the fed condition. Saline-injected sham-operated rats, open bar (n = 10); ghrelin-injected sham-operated rats, shaded bar (n = 9). Data are means ± SD. ‡P < 0.05 compared with the saline group by Student's t-test. *B*: effect of exogenous ghrelin (30 nmol/rat ip) in I/R rats in the fed condition. Sham-operated rats, open bar (n = 9); I/R rats, shaded bar (n = 9); ghrelin-administered I/R rats, solid bar (n = 7). Data are means ± SD. *P < 0.05; **P < 0.01 compared with the sham-operated rats by Student's t-test. ‡‡P < 0.01 compared with the I/R rats by Student's t-test.

h after I/R in this study) restored the gastric I/R-induced decreased food intake; however, the restoration effect lasted only 3 h. Although ghrelin has the potential to attenuate mucosal injuries, it is unlikely that ghrelin can regenerate the gastric mucosa in 3 h. Also, if the restoration effect of exogenous ghrelin in this study is attributable to the attenuation of gastric mucosal injuries, the restoration effect should continue and should not wane after 3 h. Therefore, in this study, we considered that ghrelin administration restored the decreased food intake without attenuating mucosal injuries, indicating that decreased plasma ghrelin level, rather than mucosal injury itself, induces anorexia. However, because ghrelin is expressed in the gastric mucosa, gastric mucosal injury may induce decreased ghrelin production and subsequently induce anorexia. In a previous study, it was reported that lower concentrations of ethanol (but not absolute ethanol) induced increased plasma ghrelin levels despite the increase in the area of hemorrhagic erosions. This may represent the phenomenon of adaptive cytoprotection mediated by mild irritants, and 1 h after ethanol administration was not enough to decrease the ghrelin production in this model (5). Another report showed that the plasma total and active ghrelin levels were significantly higher in cysteamine-treated duodenal ulcer model rats probably attributable to the inhibition of somatostatin secretion, not to the formation of ulcers (12).

We cannot deny the possibility that gastric I/R-induced damages in central ghrelin production and peripherally administered ghrelin might have penetrated the blood-brain barrier and restored the decreased central ghrelin production. However, it is unlikely that a single administration of ghrelin abrogated central injury in 3 h. Ghrelin has an orexigenic effect by activating neuropeptide Y/AgRP (agouti-related protein) neurons through vagal afferent nerves. This signaling pathway is believed to be retained after gastric I/R because ghrelin administration increased food intake in I/R rats in this study although some damages are undeniable.

The ghrelin-IR cells were significantly decreased in number compared with that in the sham-operated rats at 12 h after gastric I/R in this study. According to previous studies, the number of gastric A-like cells is decreased by gastric mucosal injury induced by *H. pylori* infection (25, 29), probably attributable to the large amounts of reactive oxygen species produced during the process of colonization of the host by the bacteria (2, 9). Oxidative stress produced by the xanthine-xanthine oxidase system after gastric I/R may damage the A-like cells. The number of ghrelin-IR cells was restored in the remaining mucosa at 48 h, and we did not detect any cell death by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling in the mucosal layers, except in the areas adjacent to the erosive lesion area (data not shown). Therefore, it is unlikely that the A-like cells were destroyed after I/R and regenerated within 48 h. The expression of ghrelin in the A-like cells might be transiently decreased and the stores of ghrelin in each cell reduced, thereby making the number of ghrelin-immunoreactive cells appear to be decreased, although the precise mechanisms remain to be elucidated. The percentages of ghrelin-immunoreactive cells relative to the total number of cells were restored at 48 h after I/R. However, the numbers of ghrelin-IR cells corrected by the percentage areas not showing the erosive lesions were significantly decreased compared with those in the sham-operated rats at 48 h after I/R. This decrease might have induced the decreased ghrelin production and consequently, decreased plasma ghrelin levels.

The expressions of preproghrelin mRNA in the total stomach were significantly downregulated after I/R, which may explain the decreased plasma ghrelin levels throughout the observation period. We investigated the expression of preproghrelin mRNA in the total stomach, including the mucosal layer and muscle layer, which would reflect the total gastric production. Ghrelin is expressed only in the mucosal layer, thus mucosal injuries alone may decrease the mRNA expression of ghrelin. Erosive legion areas were observed predominantly in the fundic gland region, which is the region in which ghrelin-producing cells are predominantly identified. Thus mucosal injuries might induce decreased ghrelin production and, consequently, decreased plasma ghrelin levels, after gastric I/R.

Expressions of preproghrelin mRNA in the gastric mucosa were previously reported to be increased in response to mucosal injuries, such as those induced by gastric I/R (reperfusion 3 h) (20) and 1 h after ethanol exposure (19), and 3.5-h water-restraint stress (4). Not only preproghrelin mRNA, but also ghrelin protein expression was demonstrated to be increased at 1 h after the ethanol exposure. The expressions were investigated within a short time after the occurrence of the mucosal injuries. In this study, we examined the preproghrelin mRNA expression at 12 h and 48 h after I/R, which could have yielded different results. If the ghrelin protein expression continued to increase after I/R, the number of ghrelin-IR cells in the remaining mucosa would be unlikely to decrease, which was observed in this study. The density of the ghrelin-IR cells did not appear to differ between the sham-operated rats and I/R rats. Therefore, the expression of preproghrelin mRNA might have transiently increased at 3 h but decreased at 12 h after I/R. Also, the expression was determined only in the remaining mucosal layer in previous studies, different from the case in our present study, because we used total stomach to evaluate the total gastric ghrelin production.

In conclusion, gastric I/R caused anorexia associated with a significant decrease of the plasma ghrelin levels, which is attributed to the gastric mucosal injuries induced by I/R. The decrease in the plasma ghrelin levels may have been responsible for the decrease in the food intake after gastric I/R, as it

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was restored by exogenous ghrelin administration. The results of this study show that ghrelin can stimulate food intake in rats with mucosal injuries induced by gastric I/R, suggesting that ghrelin or its analogs may also prove useful for attenuating I/R-induced dysfunctions.

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DISCLOSURES

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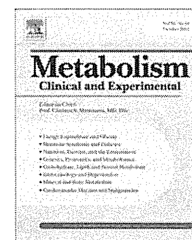

Increased production of active ghrelin is relevant to hyperphagia in nonobese spontaneously diabetic Torii rats

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ABSTRACT

An abnormal eating behavior is often associated with diabetes mellitus in individuals. In the present study, we investigated the mechanisms underlying the relationship among uncontrolled diabetes, food intake, and the production of ghrelin, an orexigenic hormone, in spontaneous diabetic Torii (SDT) rats. Male SDT rats and age-matched control Sprague-Dawley (SD) rats were housed from 8 to 38 weeks of age. Body weight and daily food intake were measured weekly, whereas blood and whole stomach samples were obtained at the age of 8, 25, and 38 weeks in both SDT and SD rats. The SDT rats at both 25 and 38 weeks of age demonstrated significantly lower body weights despite almost doubled food consumption compared with the SD rats of the same age. The SDT rats showed overt hyperglycemia at 25 and 38 weeks of age with concomitant hypoinsulinemia. The plasma active ghrelin levels and the ratio to total ghrelin levels of SDT rats at 38 weeks of age were significantly higher than those of SD rats of the same age. Stomach ghrelin and ghrelin O-acyltransferase messenger RNA expression levels were higher in SDT rats than in SD rats after the induction of diabetes, with a concomitant decrease of stomach ghrelin-immunopositive cell numbers in SDT rats at 38 weeks of age. The SDT rats with uncontrolled hyperglycemia show hyperphagia with a concomitant increase of plasma active ghrelin concentration. This report is the first to clarify the relevance of ghrelin to hyperphagia in diabetic state over an extended period.

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Authors' contributions: H Mifune and Y Nishi contributed to the maintenance of Sprague-Dawley and spontaneous diabetic Torii rats in the animal department. Y Nishi, H Hosoda, and K Kangawa contributed to radioimmunoassay of ghrelin. T Masuyama supervised the maintenance of spontaneous diabetic Torii rats. Y Nishi and Y Tajiri contributed to the reverse transcriptase polymerase chain reaction study of ghrelin and ghrelin O-acyltransferase. Y Tajiri contributed to writing the manuscript and supervised the work together with M Kojima. H Mifune and Y Nishi contributed to this work equally.

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