optimal ventilation/perfusion matching is maintained. Indeed, the quest of identifying or discovering a "cure" for PH should not come at the expense of compromising the homeostatic importance of acute HPV.

In summary, ghrelin has become recognized as an important modulator of numerous physiological homeostatic pathways in health and disease. This study describes ghrelin as an effective prophylactic therapy for attenuating the adverse changes in pulmonary blood flow distribution associated with PH, evident by attenuating endothelial dysfunction and ET-1-mediated vasoconstriction and, consequently, by opposing vascular remodeling. Importantly, these benefits of ghrelin do not come at the expense of compromising the local homeostatic modulation of blood flow, i.e., acute HPV. Further research is now required to establish whether ghrelin can also be an effective therapeutic strategy for restoring normal pulmonary hemodynamics in those patients that already have advanced PH.

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Ethical standards All experiments were approved by the local Animal Ethics Committee of SPring-8 and conducted in accordance with the guidelines of the Physiological Society of Japan.

Conflicts of interest The authors declare that they have no conflicts of interest.

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JB Review

Structure, regulation and function of ghrelin

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Ghrelin is a stomach hormone that acts as an endogenous ligand of orphan G-protein-coupled receptor. Ghrelin is a 28-amino acid peptide existing in two major forms: *n*-octanoyl-modified ghrelin, which possesses an *n*-octanoyl modification on serine-3 and des-acyl ghrelin. Fatty acid modification of ghrelin is essential for ghrelin-induced growth hormone release from the pituitary and appetite stimulation. This acyl-modification of ghrelin is catalysed by ghrelin-*O*-acyl transferase recently identified. Despite the number of innovative advancements in this field of research, there are still many aspects of ghrelin function and biosynthesis process that remain to be clarified. Here, we review the current understanding of the structure, regulation and function of ghrelin; this review is intended for researchers who will be involved in this field in the future.

Keywords: acyl ghrelin/des-acyl ghrelin/GHSs/GHS-R/GOAT.

Abbreviations: ACTH, adrenocorticotropic hormone; AgRP, agouti-related protein; AMPK, 5′ AMP-activated protein kinase; ARC, arcuate nucleus; GH, growth hormone, GHRH, growth hormone releasing hormone; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; GOAT, ghrelin-*O*-acyl transferase; GPCR, G-protein coupled receptor; IP_3, inositol 1,4,5-trisphosphate; MBOATs, the membrane-bound O-acyltransferases; MCFAs, medium-chain fatty acids; MCTs, medium-chain triacylglycerols; MTLRP, motilin-related peptide; NPY, neuropeptide Y; POMC, proopiomelanocortin; PRL, prolactin; 7TM, seven transmembrane domains.

History of Ghrelin Discovery

Kojima *et al.* (*1*) discovered ghrelin in 1999 as a 28-amino acid peptide from the rat stomach extracts. To understand why so many researchers were hunting for this hormone, and why the discovery took such a long time, we must go back to the identification of growth hormone secretagogues (GHSs).

It had been observed that some opioid peptide derivatives had weak growth hormone (GH)-releasing activity. In 1976, Bowers *et al.* (*2*) referred to these compounds as GHSs. Although the activity of early GHSs was very weak, many peptidyl derivatives with more potent GH-releasing activity were synthesized subsequently; including GHRP-6, first reported in 1984 (*3*). The non-peptide GHS L-692,429 was synthesized by Smith and colleagues (*4*) in 1993, suggesting the possibility of clinical use of GHSs because the non-peptide GHS is available for oral administration. Another non-peptide GHS, L-163,191 (MK-0677), was subjected to clinical trials, since it retained sufficient activity even when orally administered (*5*).

During this period, action of GHSs was gradually elucidated. Growth hormone releasing hormone (GHRH), the hormone that promotes GH secretion from GH-secreting cells in the anterior pituitary, acts on the GHRH receptor to increase intracellular cAMP, which serves as a second messenger (Fig. 1) (*6–10*). GHSs also act on a different receptor on GH-secreting cells in the anterior pituitary, increasing the intracellular Ca^{2+} concentration via an inositol 1,4,5-trisphosphate (IP_3) signal transduction pathway (Fig. 1). Growth hormone secretagogue receptor (GHS-R) was identified as a typical G-protein coupled receptor (GPCR) in 1996, and it was subsequently learned that GHSs stimulate phospholipase C, resulting in an increase in IP_3 and intracellular Ca^{2+} (*11*). GHS-R is expressed in the pituitary, hypothalamus and hippocampus; when it was discovered, this receptor was an orphan GPCR for which the natural ligand was not known (*11*, *12*, *13*). Therefore, a search for its endogenous ligand was actively undertaken using the orphan receptor strategy. Identification of the endogenous ligand was not easy, because it was mainly distributed in the stomach whereas GHS-R was mostly distributed in hypothalamus. Eventually, in 1999, ghrelin was identified as the endogenous ligand of GHS-R (*1*).

Structure of Ghrelin and of the Related Substances

Ghrelin

Ghrelin is a peptide consisting of 28 amino acids, and is unusual among peptide hormones of which Ser3 is *n*-octanoylated (Fig. 2a) (*1*). This modification, the first known case in mammals, is essential for ghrelin's activity (*1*).

The human ghrelin gene is localized on chromosome 3p25–26 (*14*). Both human and mouse ghrelin genes

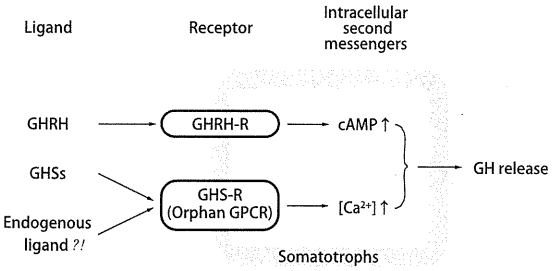

Fig. 1 A second messenger of GHRH and GHSs. GHRH acts on the GHRH receptor to increase intracellular cAMP, which serves as a second messenger. On the other hand, GHSs have also been shown to act on a GHS-R, increasing intracellular Ca^{2+} concentration via an IP_3 signal transduction pathway.

comprise five exons (*15*, *16*). There are two different transcriptional initiation sites in the ghrelin gene; one occurs at −80 and the other at −555 relative to the ATG initiation codon, resulting in two distinct mRNA transcripts (transcript-A and transcript-B) (*15*, *17*). The 28 amino acids of the functional ghrelin peptide are encoded in exons 1 and 2.

In rat and mouse stomach, a second type of ghrelin peptide has been purified and identified as des-Gln14-ghrelin (*18*). In ghrelin genes of these rodents, the codon for Gln14 (CAG) is used as an alternative splice acceptor site to generate two different ghrelin mRNAs. One mRNA encodes the ghrelin precursor, and another encodes a des-Gln14-ghrelin precursor. Except for the deletion of Gln14, des-Gln14-ghrelin is identical to ghrelin. This type of ghrelin also retains the *n*-octanoic acid modification, and has the same activities and potency as ghrelin. However, the level of des-Gln14-ghrelin in the stomach is low. Bovine and ovine ghrelins are 27-amino acid peptides which, like rat des-Gln14 ghrelin, lack the Gln14 residue. In the genes encoding these ghrelins, there is only one AG splice acceptor site between exons 2 and 3, resulting in the production of only one mRNA, which gives rise to the 27-residue ghrelin.

The non-acylated form of ghrelin, des-acyl ghrelin, is also present at significant levels in both stomach and blood (*19*). However, des-acyl ghrelin can neither bind GHS-R nor exhibit GH-releasing activity in rats. Nonetheless, food intake is induced by des-acyl ghrelin, administered by intracerebroventricular injection, to the same extent as ghrelin (*20*). Because the genome database does not contain another GPCR that resembles GHS-R, it is possible that des-acyl ghrelin acts by mechanisms independent of a GPCR. Further study will be required in order to determine the physiological significance of des-acyl ghrelin.

Ghrelin has been identified in many species. The amino acid sequences of human, rat and mouse ghrelin precursors are shown in Fig. 2b. The amino acid sequences of mature ghrelins are well conserved across mammals, including human, rat, mouse, rhesus monkey, mongolian gerbil, cow, pig, sheep and dog (*1*, *16*, *21*, *22*). Specifically, the 10 amino acids in the NH_2-termini are identical, strongly suggesting that this NH_2-terminal region is necessary for the activity of ghrelin. Mammalian ghrelin has a variety of functions—to stimulate GH release, food intake, fat accumulation, etc.

Among birds, chicken ghrelin is composed of 26 amino acids, and possesses 54% sequence identity with human ghrelin. Chicken ghrelin is predominantly expressed in the stomach, where it is present in the proventriculus (*23*). Administration of chicken ghrelin increased plasma GH levels in both rats and chicks, indicating that the stimulatory effect of ghrelin on GH secretion is evolutionarily conserved (*23*). On the other hand, intracerebroventricular injection of chicken ghrelin or of KP-102, a synthetic GHS, strongly suppressed feeding in neonatal chicks during the 2-h post-injection period, whereas ghrelin strongly stimulates feeding in mammals. Furthermore, the suppressive effect of feeding by chicken and rat ghrelin was almost identical in neonatal chicks. Thus, it is possible that the mechanisms for feeding of the neonatal chick are different from mammals.

Among amphibians, bullfrog ghrelin contains either 27 or 28 amino acids due to the differential processing of the COOH-terminal Asn residue (*24*). Bullfrog ghrelin possesses 29% sequence identity to human ghrelin. The unique amino acid sequence feature of bullfrog ghrelin is Thr3, corresponding to Ser3 in the mammalian ghrelins; bullfrog Thr3 is also modified, either by *n*-octanoic or *n*-decanoic acid. Bullfrog ghrelin mRNA is predominantly expressed in the stomach. Bullfrog ghrelin stimulates the secretion of both GH and PRL in dispersed bullfrog pituitary cells with potency 2–3 orders of magnitude greater than that of rat ghrelin. Bullfrog ghrelin, however, was only minimally effective in elevating plasma GH levels following intravenous injection into rats. Thus, although the ability of ghrelin to induce GH secretion is evolutionarily conserved, the structural differences between orthologous ghrelins result in species-specific receptor binding.

Fish ghrelins have been identified in rainbow trout, eel, tilapia and goldfish (*25–29*). Ghrelin has four isoforms in rainbow trout and two molecular forms in eel. In tilapia, ghrelin Ser3 is modified by *n*-decanoic acid, and the COOH-terminal end of the peptide possesses an amide structure. The goldfish ghrelin gene consists of four exons and three short introns. As in other vertebrates, fish ghrelins are also predominantly detected in the stomach. In organ-cultured tilapia pituitary, the release of GH and PRL are stimulated by eel ghrelin at a dose of 0.1 nM, and by tilapia ghrelin at a dose of 10 nM. Intracerebroventricular injection of *n*-octanoylated goldfish ghrelin (residues 1–19) stimulated food intake in goldfish.

GHS-R

The human ghrelin receptor gene has also been identified on chromosome 3, at position q26–27 (*14*). Ghrelin receptor is a typical GPCR, with seven transmembrane domains (7TM); it is expressed as two distinct mRNAs (*11*). The first, GHS-R type 1a, encodes a 7TM GPCR with binding and functional properties consistent with its role as the ghrelin receptor. The other GHS-R mRNA, type 1b, is produced by

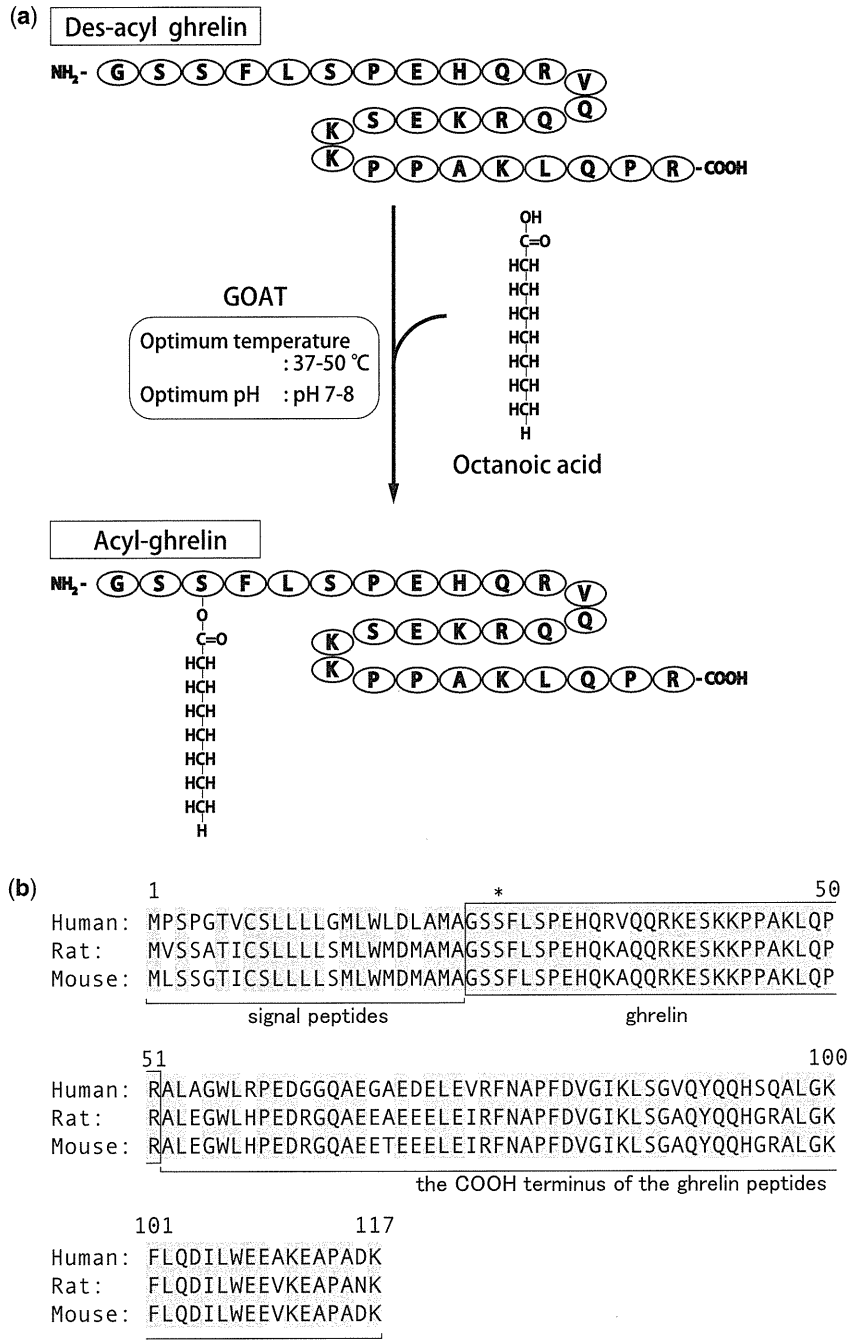

Fig. 2 (a) Structure of human ghrelin and the modification process of octanoic acid by GOAT. In rats, the 11th residue is lysine (K) and the 12th is alanine (a). (b) Amino acid sequences of ghrelin precursors in human, rat and mouse. Identical amino acids are coloured in grey. The asterisk shows the position of acyl-modified Ser3.

alternative splicing. The GHS-R gene consists of two exons; the first exon encodes TM1–TM5, and the second exon encodes TM6–TM7. Type 1b is derived from the first exon alone, and encodes only five of the seven predicted TM domains. The type 1b receptor is thus a COOH-terminal truncated form of the type 1a receptor, and is physiologically inactive.

Among GPCRs, ghrelin receptor is most similar to the motilin receptor (*30–32*). Alignment of the 28-amino acid peptide ghrelin and the 19-amino acid motilin reveals that they share eight amino acids. Although motilin can stimulate the GHS-R at a low level, ghrelin does not activate motilin receptor. After the discovery of ghrelin, the motilin-related peptide

(MTLRP) was identified; the amino acid sequence of MTLRP is identical to that of ghrelin-(1–18) (*33*). The ghrelin receptor is well conserved across all vertebrate species examined, including a number of mammals, bird and fish. This strict conservation suggests that ghrelin and its receptor serve essential physiological functions.

Ghrelin O-acyltransferase

An enzyme that catalyses the acyl-modification of ghrelin was discovered in 2008 by Yang *et al.* (*34*), using an innovative combination of bioinformatics and cell biology (Fig. 2). Using position-specific iterative BLAST and previously reported sequences of membrane-bound *O*-acyltransferases (MBOATs) from diverse species including prokaryotes, plants, humans and mice, they identified 16 MBOATs encoded by the mouse genome. They then isolated clones of all of these and tested them for their ability to catalyse octanoylation of ghrelin expressed in heterologous cell lines. Only one of these enzymes, MBOAT4, was found to be able to octanoylate ghrelin, and this enzyme was renamed ghrelin *O*-acyltransferase (GOAT). Distribution of this enzyme is limited to the gastrointestinal tract and testis, the peripheral tissues that express ghrelin. The optimum temperature of GOAT is 37–50°C, and its optimum pH range is pH 7–8 (*35*).

The origin of the modified medium-chain fatty acids (MCFAs) has not been determined. However, it is known that orally ingested MCFAs are directly utilized for acyl-modification of ghrelin (*36*). Ingestion of either MCFAs or medium-chain triacylglycerols (MCTs) specifically increases production of acyl-modified ghrelin without changing the total (acyl- and des-acyl-) ghrelin level. When mice ingest either MCFAs or MCTs, the acyl group attached to nascent ghrelin molecules corresponds to those of the ingested MCFAs or MCTs. Moreover, *n*-heptanoyl (C7:0) ghrelin, an unnatural form of ghrelin, can be produced in the stomach of mice following ingestion of *n*-heptanoic acid or glyceryl triheptanoate. Thus, it is clear that ingested fatty acids are directly utilized for acyl-modification of ghrelin (*17*).

Recently, Barnett *et al.* (*37*) described the design, synthesis and characterization of GO-CoA-Tat, a peptide-based bisubstrate analog that antagonizes GOAT. GO-CoA-Tat potently inhibits GOAT *in vitro*, in cultured cells and in mice. Intraperitoneal administration of GO-CoA-Tat in the concentration of 8 μmol/kg improves glucose tolerance in 2.5 g/kg of intraperitoneal glucose tolerance test. Moreover, the body weight gain in wild-type mice given MCT diet reduces by treatment with 11 μmol/kg GO-CoA-Tat for 1 month but not ghrelin-deficient mice; thus, its beneficial metabolic effects are due specifically to GOAT inhibition. GOAT is therefore, a useful target for future development of therapeutic compounds.

Obestatin

In 2005, Zhang *et al.* (*38*) used a bioinformatics approach to identify a 23-amino acid peptide derived from the ghrelin peptide precursor; this discovery brought exciting new insights to the gut peptide field. The authors named this peptide 'obestatin' because obestatin has the ability to inhibit food intake in mice by intraperitoneal or intracerebroventricular injection. In addition, the authors reported that peripheral injection of obestatin inhibited jejunal contraction, suppression of gastric emptying and decreased body-weight gain (*39–41*). However, their findings could not be reproduced by several groups, and must therefore be interpreted with caution.

Distribution and Regulation of Ghrelin

The acyl-modification of ghrelin is easily cleaved during sample extraction, and peptide samples are easily digested by a wide range of cellular proteases. Furthermore, in order to correctly measure the plasma concentration of ghrelin, it is necessary to use EDTA and aprotinin when collecting blood samples, and plasma must be collected into 1/10 volume of 1 N HCl (*19*, *42*). If the treated plasma samples are kept at −20 to −80°C, they are stable for at least 6–12 months. When measuring the tissue concentration of ghrelin, it is sufficient to inactivate proteases by boiling the tissues in water for 5–10 min (*43*, *44*). In human, the normal ghrelin concentration of plasma samples is 10–20 fmol/ml for *n*-octanoyl ghrelin and 100–150 fmol/ml for total ghrelin, including both acyl-modified and des-acyl ghrelins (*17*, *45*, *46*). In rats, ghrelin concentration in the stomach is 377.3 ± 55.8 fmol/mg for *n*-octanoyl ghrelin and 1779.8 ± 533.9 fmol/mg for total ghrelin (*17*, *19*). Thus, the concentration of *n*-octanoyl ghrelin is 10–20% that of des-acyl ghrelin.

Ghrelin is present in X/A-like cells, which account for ~20% of the endocrine cell population in adult oxyntic glands (*47*). Ghrelin-immunoreactive cells are also found in the duodenum, jejunum, ileum and colon. In the intestine, ghrelin concentration gradually decreases from the duodenum to the colon. Ghrelin is also secreted from other organs such as hypothalamus and pancreas of rats. In addition, ghrelin mRNA is expressed in various organs (*48*, *49*).

Several cell lines express ghrelin. TT cells, a human thyroid medullary carcinoma cell line, produced ghrelin mRNA; both conditioned medium and cellular extracts of TT cells contain ghrelin peptides (*50*). Cellular extracts of TT cells also contain both *n*-octanoyl ghrelin and des-acyl ghrelin. Other cultured cells that express ghrelin, include the kidney-derived cell line NRK-49F, gastric carcinoid ECC10 cells and the cardiomyocyte cell line HL-1 (*51–53*). Recently, Iwakura *et al.* (*54*) established a ghrelin-producing cell line MGN3-1 from a gastric ghrelin-producing cell tumour derived from transgenic mice in which SV40 Large T antigen was expressed under control of the ghrelin promoter. MGN3-1 cells produce a substantial amount of ghrelin at levels ~5000 times higher than in TT cells. In addition, MGN3-1 cells express two key enzymes: GOAT, for acyl modification and prohormone convertase 1/3 which is required for maturation of ghrelin. Moreover, MGN3-1 cells maintain physiological regulation of ghrelin secretion,

at least in regard to the suppression by somatostatin and insulin, which has been well established in *in vivo* studies. This cell line will be a useful tool for studying both production and secretion of ghrelin, as well as for screening of ghrelin production-modulating drugs.

The most known factor for the regulation of ghrelin secretion is feeding (*45*). Plasma ghrelin concentration increases when fasting, and decreases after food intake. The factors involved in the regulation of ghrelin secretion have not yet been identified. Blood glucose level may be a most probable candidate: oral or intravenous administration of glucose decreases plasma ghrelin concentration (*55*). Because gastric distension by water intake does not change ghrelin concentration, mechanical distension of the stomach alone clearly does not induce ghrelin release (*56*, *57*). Plasma ghrelin concentration exhibits a nocturnal increase. Plasma ghrelin concentration is low in obese people and high in lean people (*55*, *58–62*, *63*). Exogenous GH decreases stomach ghrelin mRNA expression and plasma ghrelin concentration, but does not affect stomach ghrelin stores (*64*).

There may be a relationship between sequence variation in the ghrelin gene and obesity (*65–67*). In humans, two polymorphisms have been reported: Arg51Gln and Leu72Met. For both polymorphisms, allelic frequencies are similar between obese patients and controls. However, obese patients with the Met72 allele became obese earlier than patients homozygous for the wild-type Leu72 allele, suggesting that the polymorphism may affect ghrelin's activity. The Arg51Gln mutation changes the sequence of the COOH-terminal processing site of the ghrelin peptide, within its precursor protein, from Pro-Arg to Pro-Gln; this mutation prevents the normal cleavage necessary to produce mature ghrelin.

Physiological Functions of Ghrelin

GH-releasing activity by ghrelin

Ghrelin is a hormone that has a lot of physiological functions (Table 1). One of its primary functions involves its strong GH-releasing activity (*1*, *68–72*). The maximal stimulation effected by ghrelin is two to three times greater than that of GHRH, in both rats and human. GH release reaches its peak ~5–15 min after intravenous ghrelin injection. A single intracerebroventricular administration of ghrelin also increases rat plasma GH concentration (*70*). There are several models regarding the mechanism of ghrelin's stimulatory effect on GH secretion. Ghrelin stimulates GH release from primary pituitary cells, indicating that ghrelin can act directly on the pituitary (*1*). On the other hand, the involvement of the hypothalamus in ghrelin-mediated stimulation of GH release has also been suggested. Furthermore, the induction of GH release after ghrelin injection is dramatically decreased when the vagus nerve is cut, indicating that the vagus nerve is required for the maximal stimulatory effects of ghrelin (*73*, *74*). A synergistic effect of ghrelin and GHRH is also important. Co-administration of ghrelin and GHRH results in more GH release than does either GHRH or ghrelin alone (*69*, *75*). This finding implies that GHRH is necessary for GH release to be maximally effective in inducing GH release.

Appetite regulation by ghrelin

Ghrelin is only a hunger signal from peripheral tissues. Intravenous and subcutaneous injections of ghrelin increase food intake; likewise, peripherally injected ghrelin stimulates hypothalamic neurons and food intake (*76–81*). Because the rate at which peripheral ghrelin passes the blood–brain barrier has shown to be very low, peripheral ghrelin must activate the appropriate hypothalamic regions via an indirect pathway.

Table I. Physiological functions of ghrelin in human or rats.

Functions	Effects	Organs	Species	References
Pituitary hormone secretions				
GH	↑	pituitary	humans, rats	(*1*, *68–72*)
PRL	↑ (weak)	pituitary	humans	(*72*)
ACTH	↑ (weak)	pituitary	humans	(*72*)
Appetite regulations				
Food intake	↑		humans, rats	(*76*, *78*, *80*, *81*)
AMPK activity	↑	hypothalamus	rats	(*98*)
Lipid metabolisms				
Adiposity	↑		rats	(*99*)
Triglyceride	↑	white adipose tissue, liver	rats	(*100*)
Glucose metabolisms				
Blood glucose	↑		humans	(*96*)
Insulin	↓	Pancreas	humans	(*96*)
Cardiovascular functions				
Blood pressure	↓		humans, rats	(*89*, *90*)
Cardiac output	↑		rats	(*101*)
Gastric functions				
Gastric acid secretion	↑	Stomach	rats	(*102*)
Gastric movement	↑	Stomach	rats	(*88*)
Bone metabolism				
Osteoblast differenciation	↑	Bone	rats	(*103*)
Bone mineral density	↑	Bone	rats	(*103*)

↑, stimulate; ↓, decrease.

The localization of ghrelin receptors on vagal afferent neurons in the rat nodose ganglion suggests that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve (*73*, *82*). As noted above, vagotomy actually inhibits the ability of ghrelin to stimulate food intake (*73*). A similar effect is also observed when capsaicin, a specific afferent neurotoxin, is applied to vagus nerve terminals to induce sensory denervation. Moreover, fasting-induced elevation of plasma ghrelin is completely abolished by sub-diaphragmatic vagotomy or atropine treatment (*74*). In summary, ghrelin is secreted primarily from stomach in response to hunger and starvation, circulates in the blood and serves as a peripheral signal, informing the central nervous system (via vagus nerve) to stimulate feeding.

Ghrelin is also identified in hypothalamus. Ghrelin-containing neurons are found in the arcuate nucleus (ARC) of the hypothalamus, a region involved in appetite regulation (*1*). In fact, intracerebroventricular injection of ghrelin increases cumulative food intake and decreases energy expenditure, resulting in body weight gain (*77*, *79*, *83–85*). This orexigenic effect of hypothalamic ghrelin is regulated through a neuronal network involving food intake. To stimulate the release of the orexigenic peptides, ghrelin-containing neurons send efferent fibers onto neuropeptide Y (NPY)- and agouti-related protein (AgRP)-expressing neurons. On the other hand, to suppress the release of the anorexigenic peptide, ghrelin-containing neurons send efferent fibers onto pro-opiomelanocortin (POMC) neurons (*86*). The ARC is also a target of leptin, an appetite-suppressing hormone produced in adipose tissues (*87*). Leptin directly inhibits appetite-stimulating effects of NPY and AgRP, whereas hypothalamic ghrelin augments NPY gene expression and blocked leptin-induced feeding reduction. Thus, ghrelin and leptin have a competitive interaction in feeding regulation.

Other functions of ghrelin

Intravenous administration of ghrelin increases gastric acid secretion and stimulates gastric motility in a dose-dependent manner (*88*). The maximum response to ghrelin, in terms of gastric acid secretion, is almost as high as that elicited by subcutaneous treatment with histamine (3 mg/kg). These responses to ghrelin were abolished by pre-treatment with either atropine or bilateral cervical vagotomy, but not by a histamine H2-receptor antagonist.

An intravenous bolus of human ghrelin decreased mean arterial pressure without changing the heart rate (*89*, *90*). The decrease in mean arterial pressure induced by ghrelin seems not to occur through direct action on the circulatory system, but by its action on the nucleus of the solitary tract (*91*, *92*). Microinjection of ghrelin into this nucleus significantly decreased the mean arterial pressure and heart rate. This injection also suppressed sympathetic activity.

There are many reports on the regulation of insulin secretion by ghrelin (*48*, *93–95*). Date *et al.* (*48*) reported that ghrelin stimulates insulin release in the presence of high levels of glucose (8.3 mM) that could independently cause insulin release from cultured islet cells. In contrast, ghrelin had no effect on insulin release in the context of a basal level of glucose (2.8 mM). On the other hand, ghrelin reduces insulin secretion and induces hyperglycaemia in humans (*96*). Thus, the regulation of insulin secretion by ghrelin is closely related to the blood glucose level. Ghrelin originating from pancreatic islets may be a major regulator of insulin secretion. Antagonism of the pancreatic ghrelin can enhance insulin release to meet increased demand for insulin in high-fat diet-induced obesity of mice (*97*). Since there is a difference of a result among researchers about the role of the ghrelin on insulin secretion, further research is expected.

After the discovery of ghrelin, it was realized that the stomach is an important organ not only for digestive function, but also for the regulation of energy metabolism and the secretion of GH. In addition, the novel octanoylated structure of ghrelin represented a new finding in biochemistry. The newly identified enzyme that catalyses the acyl-modification of ghrelin, GOAT, strongly provides the secretory machinery of the ghrelin and may herald new progress in our understanding of fatty acid metabolism. The mechanism of ghrelin synthesis still remains unclear, but will hopefully be elucidated by future research.

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Conflict of interest

None declared.

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Neuromedin S regulates cardiovascular function through the sympathetic nervous system in mice

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ABSTRACT

Intracerebroventricular (icv) injection of neuromedin S (NMS) in mice increased the heart rate in a dose-dependent manner. On the other hand, genetically NMS deficient mice (NMS-*KO* mice) exhibited a decreased heart rate and significant extension of the QRS and PR interval in the electrocardiogram complex. Although treatment with a parasympathetic nerve blocker, methylscopolamine, and a sympathetic nerve blocker, timolol, respectively increased and decreased the heart rate in both NMS-*KO* and wild-type mice, the extent of the decrease induced by timolol was smaller in NMS-*KO* than in wild-type mice. In addition, pretreatment with timolol completely inhibited the NMS-induced heart rate increase in wild-type mice. No expression of mRNA for NMS or the NMS receptor was evident in the heart by RT-PCR analysis. These results suggest that endogenous NMS may regulate cardiovascular function by activating the sympathetic nervous system.

1. Introduction

Neuromedin U (NMU), which had been originally discovered as a hypertensive peptide from the porcine spinal cord in 1985, was identified as an endogenous ligand for the orphan receptors FM-3/GPR66 and FM-4/TGR-1 using a reverse-pharmacological technique in 2000 [6,9,10,14,25]. Accordingly, FM-3/GPR66 and FM-4/TGR-1 were subsequently named neuromedin receptor 1 (NMU-R1) and 2 (NMU-R2), respectively. NMU-R1 is distributed mainly in peripheral organs, while central NMU-R1 is distributed only in the suprachiasmatic nucleus (SCN). On the other hand, NMU-R2 is distributed predominantly in the hypothalamus, including the arcuate nucleus (Arc), ventromedial hypothalamus, paraventricular nucleus (PVN) and SCN [7,10,15]. In 2005, we identified neuromedin S (NMS), consisting of 36 amino acid residues, in rat brain as another distinct endogenous ligand for the FM-3/GPR66 and FM-4/TGR-1 receptors [20–22]. Although NMS shares seven amino acid residues of the carboxyl terminal core structure with NMU, and binds to both NMU receptors with an affinity almost equal to that of NMU, the NMS and NMU genes are mapped to separate chromosomes [20–22].

The physiological roles of NMU and NMS include regulation of circadian rhythm [21], an anorexigenic action [11,18], an antidiuretic action, and stimulation of oxytocin and vasopressin release [13,26,27]. The effects of intracerebroventricular (icv) injection of both peptides on feeding and body temperature are abolished in NMU-R2 *KO* mice [24], indicating that the central effects of these peptides are mediated by NMU-R2. As NMU and NMS have the same receptors, icv treatment with NMU and NMS may elicit the same action. However, it has also been demonstrated that the two peptides differ in their potency of action, or exert different actions; for example, NMS has a more potent anorexigenic action than NMU [11], and NMS, but not NMU, is involved in leptin-induced suppression of food intake [18].

It has been demonstrated that icv injection of NMU increases heart rate and blood pressure in conscious or urethane-anesthetized rats, suggesting that central NMU may be involved in cardiovascular function [4,28], and this has led to speculation that central NMS may also have a similar role. On the other hand, it has recently been demonstrated that NMS is directly involved in human cardiovascular function, since mRNA for NMS and NMU-R1, but not

Abbreviations: icv, intracerebroventricular; NMS, neuromedin S; NMS-*KO*, neuromedin S knock out; RT-PCR, reverse transcription polymerase chain reaction; NMU, neuromedin U; NMU-R1, neuromedin receptor 1; NMU-R2, neuromedin receptor 2; SCN, suprachiasmatic nucleus; Arc, arcuate nucleus; PVN, paraventricular nucleus; α-MSH, α-melanocyte-stimulating hormone; CRH, corticotropin-releasing hormone; VIP, vasoactive intestinal peptide.

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for NMU-R2, is expressed in human cardiovascular tissues [19]. In the present study, we examined the possible roles of exogenous and endogenous NMS in cardiovascular function using wild mice and mice genetically deficient in NMS (NMS-*KO* mice).

2. Materials and methods

2.1. Animals

NMS-*KO* mice were originally produced by Dr. M. Kojima (Molecular Genetics, Institute of Life Science, Kurume University, Kurume, Fukuoka, Japan) using the same method as that for NMU *KO* mice described previously [16], and supplied from the National Cardiovascular Center Research Institute (Suita, Osaka, Japan) in 2006. Wild-type C57BL/6J mice of the same strain as the NMS-*KO* mice were purchased from Charles River Japan Inc. (Yokohama, Japan). In this study, male mice aged 12–14 weeks were used. All were housed individually in Plexiglas cages in an animal room maintained under a constant light–dark cycle (lights on 7:00–19:00 h) and temperature (22 ± 1 °C). Food and water were provided *ad libitum*. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care, and the authorization number for this research issued by Miyazaki University Animal Experiment Committee was 2006-053-3.

2.2. Icv injection of NMS

For icv injection, mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, USA; 0.75 mg/10 g body weight) and then mounted in a Narishige mouse brain stereotaxic instrument (Narishige Group, Tokyo, Japan). A stainless steel cannula (guide cannula, length 7 mm, internal diameter 0.4 mm, external diameter 0.7 mm) was then implanted into the lateral left ventricle, the tip being placed at the following stereotaxic coordinates: 0.22 mm posterior to the bregma; 1.0 mm lateral to the midline; 2.0 mm below the dura. The guide cannula was anchored to the skull with machine screws and dental acrylic. After surgery, the animals were housed individually and allowed to recover for 4 days before icv injection, which was performed with a 30-gage injection cannula that extended into the lateral ventricle 0.5 mm beyond the guide cannula. The 30-gage injection cannula was connected to a 10-μl Hamilton syringe via a 10-cm polyethylene tube, and 5 μl of saline solution with or without NMS at various doses (Peptide Inc., Osaka, Japan) was injected into each free-moving mouse at 10:00 h. The mouse was then placed immediately in the holder, and measurements of heart rate and blood pressure were performed at 10, 20 and 30 min after injection.

2.3. Measurement of heart rate and blood pressure in NMS-KO and wild mice

Heart rate and blood pressure were measured non-invasively using a BP-98A instrument (Softron Co., Ltd., Tokyo, Japan) starting from 10:00 h. Each mouse was gently fixed in the instrument holder, and measurements were performed once every 7 days to allow habituation before the experiments. The temperature of the holder was set at 37–39 °C. For data collection, 5 consecutive measurements were performed and then the average value was taken.

2.4. Analysis of the electrocardiogram (ECG) complex in NMS-KO and wild-type mice

To compare the ECG complex between NMS-*KO* and wild mice, ECG recordings were taken using a Power Lab system (Bio Research Center, Nagoya, Japan). The mice were anesthetized with isoflurane using an anesthetic device designed for small animals (Bio Research Center: induction 4.0%, maintenance 2.0%, flow rate 200 ml/min). Measurements were performed after disappearance of the hind limb suspension reflex. All instruments with electrical cords were earthed to prevent data interference by electrical noise. All the animals were placed prone, and measurements were performed using three-lead electrocardiography (right forelimb, left forelimb and left hindlimb electrodes). Data were analyzed using an ECG analysis module.

2.5. Effect of autonomic nerve blocker on heart rate in NMS-KO and wild-type mice

To examine the effect of cardiovascular autonomic nervous activity blockade on the heart rate in NMS-*KO* and wild-type mice, the muscarinic receptor antagonist methyl scopolamine (Sigma–Aldrich Co., St. Louis, USA; 0.1 mg/kg body weight) or the adrenergic receptor antagonist timolol (Sigma–Aldrich Co., 0.5 mg/kg body weight) was dissolved in saline and injected through a polyethylene tube (Becton Dickinson, NJ, USA; internal diameter 0.58 mm, external diameter 0.965 mm) at 10:00 h. Each tube had been fixed subcutaneously to the back of each mouse with 1 cm of the tip externalized, 4 days before the experiments. On the day of the experiment, a 10-cm extension tube was connected to the externalized tube tip, and the mouse was inserted into the holder of the BP-98A with 2 cm of the tip extending from the holder, and maintained in the holder during measurements. To obtain the basal value, measurement was performed at 10 and 20 min, and then the drug was injected using a 10-μl Hamilton syringe. After injection, measurement was performed every 10 min for 60 min.

2.6. Effect of autonomic nerve blocker on NMS-induced change of heart rate in wild-type mice

Wild-type mice were subjected to implantation of a stainless steel cannula into the lateral left ventricle using the method described above. Four days later, timolol (0.5 mg/kg) or methylscopolamine (0.1 mg/kg) was injected subcutaneously at 10:00 h, and then 30 min later, 0.5 nmol NMS or saline was injected icv through the cannula. The heart rate was then monitored immediately for 20 min.

2.7. RT-PCR analysis of NMS, NMU-R1 and NMU-R2 mRNA expression in the heart

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and perfused with heparin (10,000 unit/10 ml) in 0.05 M phosphate-buffered saline for 10–15 min to remove the blood. The heart was immediately dissected out and homogenized in TRIzol reagent (Invitrogen Co., Carlsbad, CA), then total RNA was extracted from each sample using an RNeasy Micro kit (Qiagen, USA) and synthesized into first-strand cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). RT-PCR analysis was performed by the method described previously [17,18]. The primer sets used for mouse NMS, NMU-R1, NMU-R2 and GAPDH were: NMS: sense 5′-ATG AAA CAC CCG CTC CCC CAC TAT TCT-3′, anti-sense 5′-CTA CTG CAA GTT GTT GTC GGT GTA TCT-3′; NMU-R1: sense 5′-AGC TGG GTG CGA GTG CCT GCT ACT TCC-3′, anti-sense 5′-CAG CCG CAG CCC AAT GAG CAG ATA CAG-3′; NMU-R2: sense 5′-TGA TCT TCG TGG TGG GGG TGA TAG GCA-3′, anti-sense 5′-TGG CTC GGA ATG GAT GGA CAA TGG C-3′; GAPDH: sense 5′-CGG CAA GTT CAA CGG CAC A-3′, anti-sense 5′-AGA CGC CAG TAG ACT CCA CGA CA-3′. In this experiment, spinal cord, lung and colon tissue were used as controls.

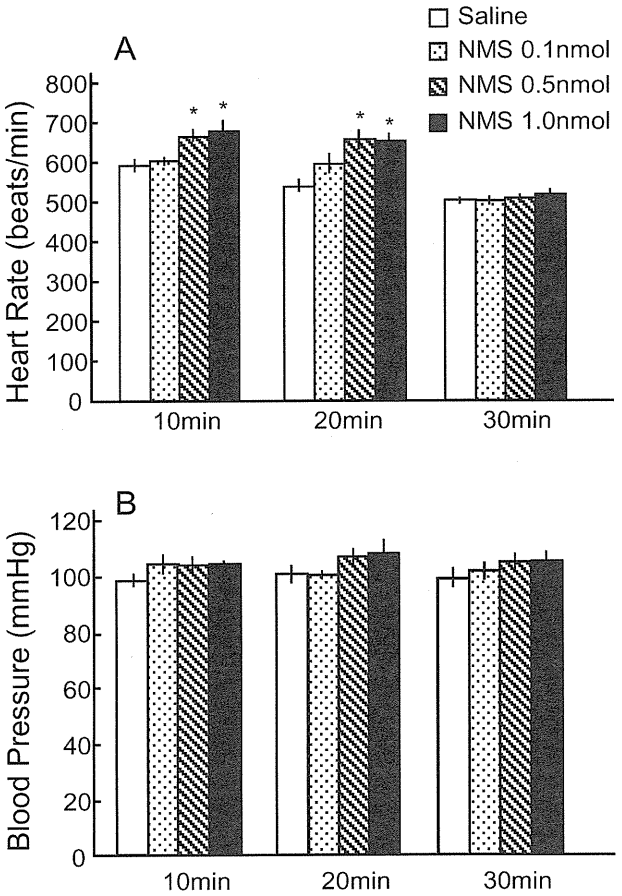

Fig. 1. The effects of icv injection of NMS on heart rate (A) and blood pressure (B) in wild-type mice. All data were measured non-invasively at 10, 20 and 30 min after injection. Each bar and vertical line represent the mean ± SEM ($n = 5$). Asterisks indicate significant differences from the saline group ($^*P < 0.05$).

2.8. Statistical analysis

The data (mean ± SEM) were analyzed statistically by ANOVA with Student's t test. Differences at $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of icv administration of NMS on heart rate and blood pressure

Icv administration of NMS in wild-type mice increased the heart rate in a dose-dependent and time-dependent manner (Fig. 1A). A significant increase of heart rate was observed at NMS doses of 0.5 and 1.0 nmol, but not 0.1 nmol, at 10 and 20 min after treatment, and then the heart rate decreased to the basal level by 30 min. No significant change in blood pressure was observed at any of the doses (Fig. 1B).

3.2. Comparison of heart rate, blood pressure and electrocardiographic complex between NMS-KO and wild-type mice

Heart rate in NMS-*KO* mice was lower than that in wild-type mice (Fig. 2A). No significant inter-group difference in blood pressure was observed (Fig. 2B). Comparison of the electrocardiographic complex between NMS-*KO* and wild-type mice revealed no significant difference in the PR interval, P-amplitude, T-amplitude,

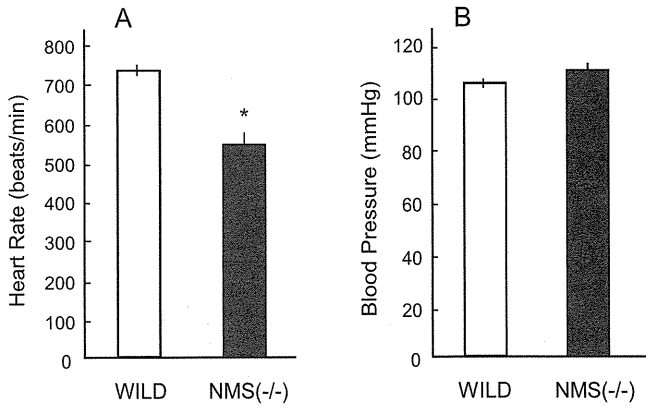

Fig. 2. Evaluation of heart rate (A) and blood pressure (B) in NMS-*KO* mice and wild-type mice. Measurements were conducted non-invasively in the daytime. Each bar and vertical line represent the mean ± SEM ($n = 6$). Asterisks indicate significant differences from wild-type mice ($^*P < 0.05$).

R-amplitude or QT interval (Fig. 3A–E). On the other hand, the QRS interval (Fig. 3F) and RR interval (Fig. 3G) were significantly increased in NMS-*KO* mice.

3.3. Effect of autonomic nerve blockers on heart rate in NMS-KO and wild-type mice

The heart rate of wild-type and NMS-*KO* mice was decreased and increased by treatment with the sympathetic and parasympathetic nerve blocker, respectively (Fig. 4). No effect was observed upon treatment with vehicle only (Fig. 4A). The amplitude of the change in heart rate induced by the parasympathetic nerve blocker in NMS-*KO* mice was larger than that in wild-type mice at 20 and 40 min after injection (Fig. 4D). On the other hand, the amplitude of the change in heart rate induced by the sympathetic nerve blocker was significantly smaller in NMS-*KO* mice than in wild-type mice at 20 and 40 min after injection (Fig. 4E).

3.4. Effect of autonomic nerve blockers on the NMS-induced increase of heart rate in wild-type mice

Pretreatment with the sympathetic nerve blocker timolol and the parasympathetic nerve blocker methylscopolamine resulted in a decrease and increase of the heart rate, respectively, in wild-type mice after icv saline injection at 20 min. Although icv injection of NMS increased the heart rate, this increase was completely blocked in mice pretreated with timolol (Fig. 5). On the other hand, icv injection of NMS augmented the heart rate in mice pretreated with methylscopolamine (Fig. 5).

3.5. Expression of NMS and NMU receptor mRNA in heart, spinal cord, lung in wild-type mice

Both NMS and NMU-R2 mRNA were detected in the spinal cord at relatively high concentrations and in the colon at relatively low concentrations. Neither mRNA was detected in the heart (Fig. 6A and B). NMU-R1 mRNA was detected in the spinal cord, lung and colon at relatively high concentrations, and in the heart at a very low concentration (Fig. 6B).

4. Discussion

The heart rate in NMS-*KO* mice was significantly lower than in wild-type mice under conscious condition, although no difference in blood pressure was evident. On the other hand, icv injection of

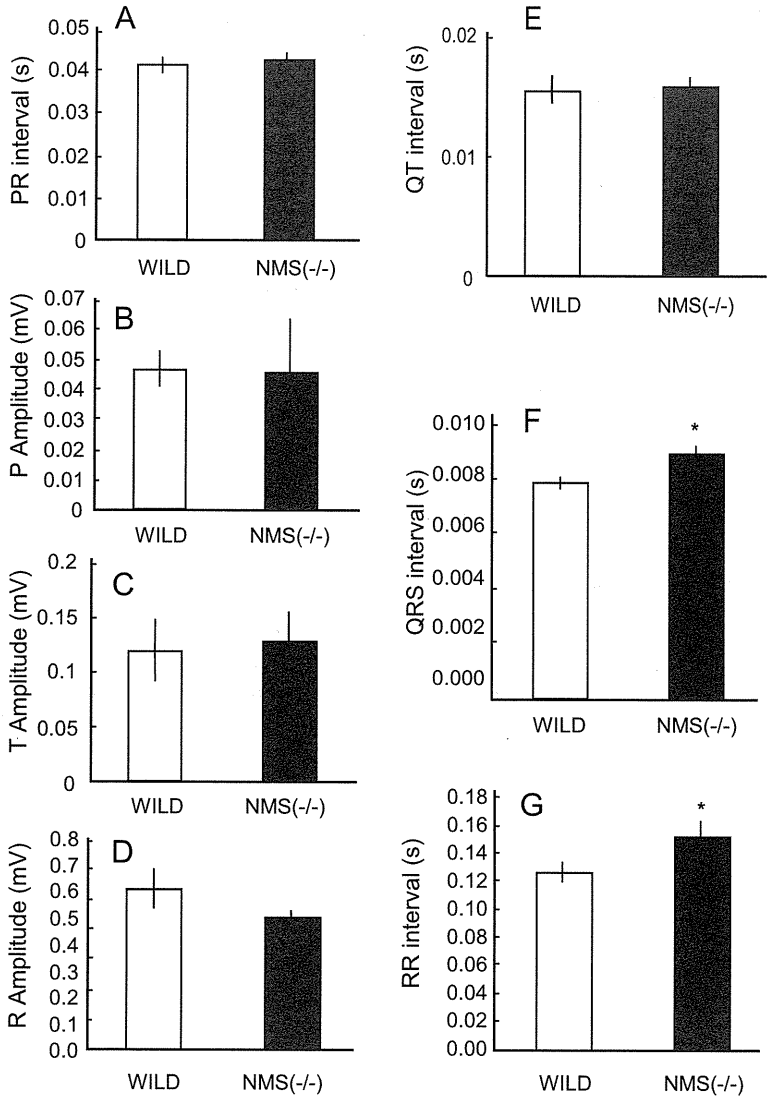

Fig. 3. Comparison of the electrocardiogram complex between NMS-*KO* and wild-type mice. (A) PR interval, (B) P amplitude, (C) T amplitude, (D) R amplitude, (E) QT interval, (F) QRS interval, (G) RR interval. All data were obtained under anesthesia with isoflurane. Each bar and vertical line represent the mean ± SEM ($n = 6$).

NMS significantly increased the heart rate without changing the blood pressure. These results indicate that both endogenous and exogenous NMS affect the heart rate in mice, and that the effect of exogenous NMS is central and not peripheral. No cardiac expression of mRNA for NMS or NMU-R2 was detected in wild-type mice in the present study. In addition, expression of NMU-R1 mRNA was also relatively low in comparison with that in the spinal cord, lung and colon used as control tissues, in agreement with previous reports [7,25]. On the other hand, expression of mRNA for NMS, NMU-R1 and/or NMU-R2 has been recently demonstrated in the heart, but is likely much weaker than in the other organs [2,19,29]. These results indicate that, in mice, cardiovascular regulation by endogenous NMS may be central. Comparison of the electrocardiographic complex between NMS-*KO* and wild mice revealed no significant differences in PR interval, P-amplitude, T-amplitude, R-amplitude or QT interval. However, the QRS interval and RR interval were significantly increased in NMS-*KO* mice. Therefore, one of the reasons for the low heart rate in NMS-*KO* mice may be prolongation of the RR and QRS intervals, rather than any abnormality of heart function. It is unclear why blood pressure was not affected in NMS-*KO* mice, or why icv injection of NMS had no effect on it. NMS might intensify the contractile force of the heart, while relaxing some blood vessels through sympathetic nervous action, such as angiectasis in muscle by sympathetic vasodilation, since icv injection of NMS increased locomotor activity [21].

Administration of a parasympathetic nerve blocker markedly increased the heart rate in NMS-*KO* mice, whereas treatment with a sympathetic nerve blocker decreased it slightly, suggesting that, in this strain, the inhibition of sympathetic nerve activity may be relatively stronger than that of parasympathetic nerve activity, i.e. that there is a decrease of sympathetic, rather than parasympathetic nerve tone. In addition, pretreatment with a sympathetic nerve blocker completely inhibited the increase of heart rate after icv injection of NMS in wild-type mice, whereas heart rate was augmented after treatment with a parasympathetic nerve blocker followed by NMS. These findings strongly suggest that central NMS plays an important role in cardiovascular function via the sympathetic nervous system.

Previously, Chu et al. demonstrated that icv administration of NMU increased blood pressure, heart rate, and the plasma norepinephrine level in conscious rats, suggesting that central NMU regulates sympathetic nerve activity and affects cardiovascular

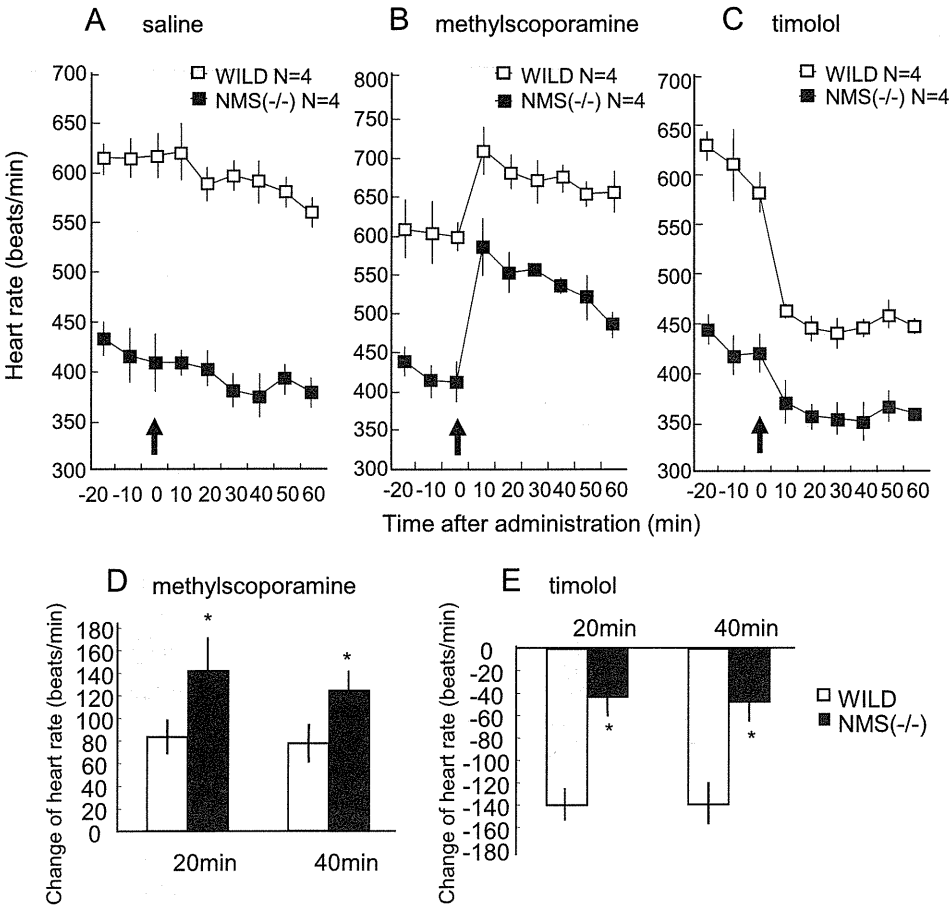

Fig. 4. Effects of saline (A), methylscopolamine (B) and timolol (C) on heart rate in wild-type and NMS-*KO* mice. Heart rate was measured noninvasively when the mice were conscious. Arrow indicates injection time. Each bar and vertical line represent the mean ± SEM ($n = 4$). (D and E) Rate of change in heart rate after treatment with methylscopolamine (D) and timolol (E) in wild-type and NMS-*KO* mice at 20 and 40 min after injection. The data in (D) and (E) were calculated from the value of (B) and (C), respectively. Each bar and vertical line represent the mean ± SEM ($n = 4$). Asterisks indicate significant differences from wild-type mice ($^*P < 0.05$).

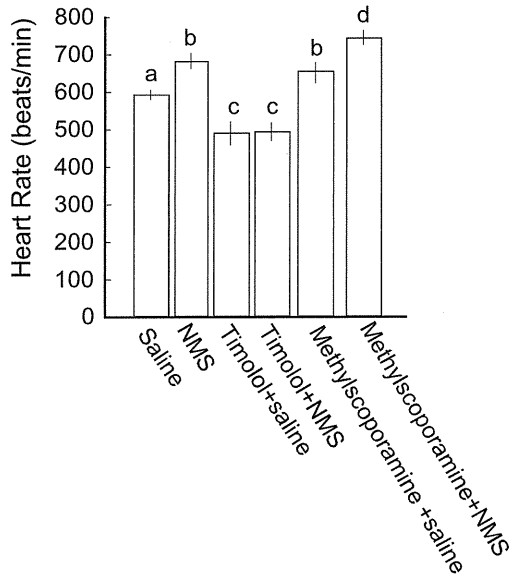

Fig. 5. Effect of autonomic nerve blocker on the NMS-induced increase of heart rate in wild-type mice. Timolol or methylscopolamine was injected subcutaneously at 10:00 h, and then 30 min later 0.5 nmol NMS or saline was injected icv through the cannula. Heart rate was measured immediately for 20 min. Each bar and vertical line represent the mean ± SEM ($n = 5$) ($P < 0.05$: *b* vs. *a*, *c* vs. *a*, *b* vs. *d*).

function [4]. Although it is unknown whether endogenous NMU is involved in cardiovascular function, the present results together with their observations suggest that both NMS and NMU may be involved in cardiovascular function through activation of the sympathetic nervous system.

Although it is unclear how NMS increases the heart rate through sympathetic nervous action, several possibilities can be suggested. NMS may directly stimulate the sympathetic nervous system via output from the circadian oscillator in the SCN. NMS localized in the SCN shows a circadian rhythm with a peak just before the dark period [21]. In addition, NMU-R2 mRNA also shows a circadian rhythm in the SCN with a peak during the dark period [15]. Generally, activity of the sympathetic and parasympathetic nervous systems is increased during the active (dark period) and resting (light period) phases, respectively. Therefore, the rhythm of the autonomic nervous system may be regulated by the SCN through NMS.

On the other hand, NMS may regulate the heart rate through either oxytocin, α-melanocyte-stimulating hormone (α-MSH) or corticotropin-releasing hormone (CRH). It has been demonstrated that NMS stimulates the secretion of oxytocin and CRH from the PVN and α-MSH from the Arc, respectively [8,18,26,27]. The α-MSH is produced within the Arc and released into the PVN by axonal transport, activating oxytocinergic parvocellular neurons in the PVN, and triggering secretion of oxytocin into the intermediolateral cell column of the thoracic spinal cord (T1–T3), which contains preganglionic fibers of the cardiac sympathetic nerves [3]. The released

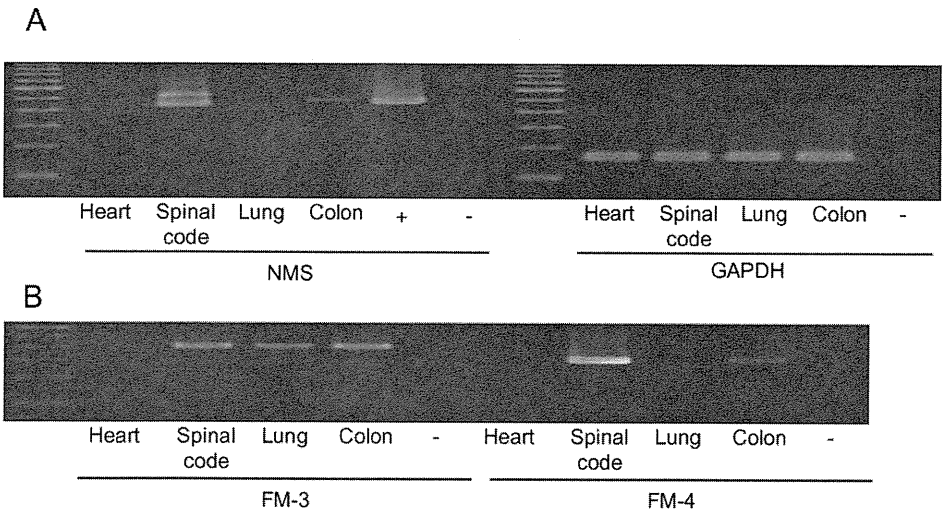

Fig. 6. Expression of mRNA for NMS (A), NMU-R1 and NMU-R2 (B) in the heart, spinal cord, lung and colon of wild-type mice. mRNA extracted from the SCN and distilled water were used as positive (+) and negative (−) controls, respectively.

oxytocin activates the cardiac sympathetic nerves, resulting in an increase of heart rate without any significant effect on blood pressure [30]. This oxytocinergic sympathetic nerve activity is known to be the primary pathway for cardiovascular regulation via the PVN [5]. In addition, CRH is also well known to be an activator of the sympathetic nervous system [1].

It has been reported that the SCN transmits circadian signals through release of vasoactive intestinal peptide to the Arc and regulates the activity of pro-opiomelanocortin neurons [12]. Because icv injection of NMS induces expression of c-fos protein in the vasoactive intestinal peptide (VIP)-positive region of the SCN [21], it is speculated that NMS activates VIP neurons and affects sympathetic nerve activity via α-MSH expression in the Arc. Alternatively, NMS may activate the sympathetic nervous system directly through synaptic transmission from the SCN to the PVN. The PVN and supraoptic nucleus receive direct projections from the SCN, and lesioning of the SCN results in ablation of the circadian rhythm of arginine-vasopressin, oxytocin and CRH secretion [23]. Taken together, the data suggest that one of the reasons for the reduction of sympathetic nerve activity in NMS-*KO* mice may be the disruption of rhythmic signaling from the SCN for regulation of the autonomic nervous system.

Recently, NMS mRNA has been detected in the medulla oblongata, a cardiovascular regulatory center, of pigs by in situ hybridization [29]. Therefore, NMS may play an important role in cardiovascular function through paracrine action in the medulla oblongata. Further studies are required to elucidate the role of NMS in cardiovascular function.

Acknowledgments

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A guanosine 3′,5′-cyclic monophosphate (cGMP) reporter system based on the G-kinase/CREB/CRE signal transduction pathway

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ABSTRACT

Guanylate cyclases constitute a gene family of enzymes that synthesize the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) and play important roles in diverse physiological functions. Here we report a novel, simple and highly sensitive method for measurement intracellular cGMP concentrations using a cAMP-responsive element (CRE) and cGMP-dependent protein kinase (cGK). Transient transfection of the CRE reporter plasmid, encoding a luciferase reporter gene under the control of a modified promoter containing a CRE, and a cGK expression vector into HEK293 cells followed by treatment with 8-bromo-cGMP showed a dose dependent increase in luciferase activity. Moreover, HEK293 cells expressing GC-A or GC-B natriuretic peptide receptors and harboring this reporter system responded to specific ligands in a dose dependent manner. Our results indicate that this reporter gene method enables high throughput screening of receptor-type GC selective agonists in the treatment of cardiovascular diseases and homeostatic dysfunctions.

1. Introduction

The guanylate cyclases (GCs) constitute a family of isoenzymes that play important physiological and pathophysiological roles via the synthesis of guanosine 3′,5′-cyclic monophosphate (cGMP), an intracellular signal transducer, from guanosine triphosphate (GTP), in response to some hormone stimulation. In mammals, seven receptor-type GC (membrane-bound GC) genes and four soluble GC genes have been identified [1,2]. They all share a highly conserved catalytic domain of about 250 amino acids. Three of seven receptor-type GCs are known as bioactive peptide binding proteins and the all soluble GCs are thought to bind nitric oxide and carbon oxide in mammals [1,2].

The known receptor-type GC genes in mammals are referred to as guanylate cyclase-A (GC-A) to -G (GC-G), and their products are all single-chain polypeptides and are composed of four characteristic subdomains: an N-terminal extracellular domain responsible for ligand binding, a short hydrophobic transmembrane domain, an intracellular kinase homology domain and a C-terminal GC catalytic domain [1]. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are both considered to bind to GC-A (also called NPR-A and NPR-1), and C-type natriuretic peptide (CNP) is known to preferentially bind to GC-B (also called NPR-B and NPR-2) rather than to GC-A [3,4]. Guanylin and uroguanylin both are gastrointestinal peptide hormones, and they have been reported to bind to GC-C, an *Escherichia coli* heat-stable enterotoxin receptor [5,6].

Physiological and pathophysiological effects of bioactive peptides that bind to receptor-type GCs have been widely analyzed *in vivo*. ANP is mainly secreted from the cardiac atria, and plays a role in vasodilation, lowering blood pressure and promoting water and sodium excretion [7,8]. BNP has similar biological properties to ANP and appears to be antagonistic to the renin/angiotensin II/aldosterone system [9]. The most obvious physiological effect of CNP, as has been reported, is to stimulate bone growth [10,11]. Guanylin and uroguanylin are thought to regulate water and electrolyte absorption in the gastrointestinal tract [5,6,12]. Therefore, receptor-type GCs are thought to be targets for a range of diseases such as acute and chronic heart failure, hypertension, dwarfism, constipation and arterial sclerosis, and this underscores the necessity of developing a receptor-type GC subtype selective agonist.

Although the physiological importance of bioactive peptides has been widely recognized, there remain many unanswered questions related to the cellular response mediated through the intracellular cGMP signal transduction machinery. This is due to the limited utilization of the cGMP signal transduction system in organs and the instability of cGMP against hydrolysis by cGMP-specific phosphodiesterase, which does not allow the accurate determination of cGMP concentrations. cGMP has been reported to be capable of activating three classes of proteins: ion channels,

Abbreviations: GC, guanylate cyclase; cGK, cGMP-dependent protein kinase; CRE, cAMP-responsive element; CREB, CRE binding protein; cAK, cAMP-dependent protein kinase; MMTV, mouse mammary tumor virus.

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