Regulation of GH secretagogue receptor gene expression in the rat nodose ganglion

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Abstract

It has been shown that the ghrelin receptor, GH secretagogue receptor (GHS-R), is synthesized in neurons of the nodose ganglion and then transmitted to axon terminals, where it binds to ghrelin. The orexigenic signal of ghrelin secreted from the stomach is transmitted to the brain via the vagal afferent nerve. To explore the regulation of GHS-R synthesis in the nodose ganglion, we examined whether or not GHS-R type a mRNA expression shows circadian rhythm, and is affected by starvation, vagotomy, or i.v. administration of gastrointestinal peptides. Nodose ganglion GHS-R mRNA levels showed a diurnal rhythm, being high during periods of light and low during darkness. Although starvation tended to

increase the level of GHS-R mRNA, a more significant increase was observed upon re-feeding. Vagotomy decreased the level of GHS-R mRNA significantly in comparison with animals that underwent a sham procedure. Cholecystokinin and gastrin increased the level of GHS-R mRNA after 2 h, but after 4 h, the level decreased. These results suggest that GHS-R synthesis in the nodose ganglion is regulated centrally and peripherally by neuronal and humoral information, and that these dynamic changes of GHS-R mRNA expression may be involved in the regulation of feeding by ghrelin.

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Introduction

Ghrelin is an orexigenic peptide secreted from endocrine cells of the stomach (Date *et al.* 2000). It has a characteristic structure, comprising 28 amino acid residues with a serine residue modified with octanoic acid at position 3, which is essential for ligand–receptor interaction (Kojima *et al.* 1999). The growth hormone secretagogue receptor type a (GHS-R1a) is the only receptor specific for acylated ghrelin that has been identified so far. The homologous receptor, referred to as GHS-R type b (GHS-R1b), also exists but does not possess receptor activity for acylated ghrelin due to a lack of transmembrane regions 6 and 7 and thus a lack of intracellular signaling (Howard *et al.* 1996).

The orexigenic and anorexigenic signals of gastrointestinal peptides are transmitted via either a neuronal or a humoral pathway, or both. The former pathway consists of afferent vagal fibers that pass through the nodose ganglia to terminate on the nucleus of the solitary tract (NTS), ultimately transmitting to the hypothalamus, whereas the latter reaches the hypothalamus directly via the blood circulation (Woods 2004). The GHS-R1a receptor is expressed in neurons of the nodose ganglia, and the receptor protein is conveyed to afferent terminals by axonal transport (Date *et al.* 2002). In order to exert its effect, ghrelin is also understood to be transmitted via the neuronal pathway, whereby, after being

secreted from the stomach, it interacts with GHS-R1a expressed at afferent terminals and the stimulus is relayed via the NTS to the hypothalamus. In fact, blockade of the vagal afferent by vagotomy or administration of capsaicin abolishes facilitation of feeding and GH secretion, and also activation of neuropeptide Y (NPY)- and growth hormone-releasing hormone (GHRH)-producing neurons by i.v. administration of ghrelin, suggesting that the predominant action of ghrelin occurs via the neuronal pathway (Date *et al.* 2002).

The route through the vagal afferent nerve transmits various signals, including those resulting from mechanical (distention and contraction) stimuli, chemicals such as nutrients in the gut lumen and neurohormonal stimuli (Konturek *et al.* 2004). Neurohormonal information is mediated by various receptors expressed in the nodose ganglia. Besides ghrelin receptors, many studies have demonstrated the presence of cholecystokinin (CCK) type A (CCK1-R) and type B receptors (CCK2-R; Corp *et al.* 1993, Moriarty *et al.* 1997), neuropeptide YY2 receptors (Koda *et al.* 2005), the long and short forms of the leptin receptor (Buyse *et al.* 2001), orexin-A receptors (Burdyga *et al.* 2003), and cannabinoid receptors (Burdyga *et al.* 2004). However, few studies have investigated how the expression of these receptors is controlled in the nodose ganglia.

In the present study, therefore, we investigated agents that might be responsible for the regulation of GHS-R mRNA

expression in the nodose ganglion by focusing on 1) the natural diurnal pattern of GHS-R mRNA levels, and how these levels are affected by 2) starvation and re-feeding, 3) vagotomy, and 4) i.v. administration of gastrointestinal peptides.

Materials and Methods

Animals

Male Wistar rats aged 9 weeks (Charles River Japan Inc., Shiga, Japan) were purchased and acclimated under our laboratory conditions mentioned below for 2 weeks before experiments. All animals were kept at a constant room temperature of $23 \pm 1^\circ\text{C}$ under a light cycle of 12 h light:12 h darkness (lights on at 0700 h) and provided with standard laboratory chow and water available *ad libitum*, except where otherwise noted. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care and the Fund for the Replacement of Animals in Medical Experiments guidelines for studies involving the use of laboratory animals.

Experimental designs and procedures

In the first experiment, the diurnal pattern of GHS-R mRNA levels in the nodose ganglia was investigated in five rats. Sampling was performed every 4 h for 24 h, starting from 0900 h. The animals were then decapitated and the right and left nodose ganglia were excised and immediately frozen in liquid nitrogen, both ganglia being pooled and considered as one sample. Frozen samples were stored at -80°C before the extraction of total RNA.

In the second experiment, the effects of starvation and subsequent re-feeding on GHS-R mRNA levels were examined. The animals were deprived of food for 24 h from 1100 h, and then sampling was immediately performed on six rats. For the re-feeding part of the experiment, six rats were fed for 2 h after 22 h of food deprivation, and gastric contents were confirmed in all of the animals at sampling. As a control, six rats were fed *ad libitum* for 24 h and killed at 1100 h. Sampling procedures were the same as those described for the first experiment.

In the third experiment, the effect of vagotomy was examined. Twelve rats were anesthetized with ether, and only the left cervical vagal nerve was transected, while the right nerve was kept intact. Sham operations were also performed on 12 animals, in which the right cervical vagus nerve was exposed but not transected. The incision was then closed, and sampling was performed 12 or 24 h after surgery. In both vagotomized and sham rats, two nodose ganglia from the same side of two animals were pooled and regarded as one sample. Dissected nodose ganglia were immediately frozen in liquid nitrogen and stored at -80°C before the extraction of total RNA.

In the fourth experiment, the effects of i.v. administration of CCK, gastrin, somatostatin, ghrelin, and saline were

examined. Following light anesthesia with ether, each of six rats received a single injection of each hormone (3 nmol in 200 μl saline) via the femoral vein at 1700 h, and sampling was performed 2 (1900 h) or 4 h (2100 h) later. Sampling procedures were the same as those used in the first experiment.

Quantitative real-time PCR

Total RNA was isolated from rat nodose ganglia using an RNeasy Micro Kit (Qiagen Inc.) according to the manufacturer's instructions. After treatment with DNase and elution with RNase-free water, RNA was quantified by measurement of absorbance (A_{260}/A_{280}). cDNA was synthesized by reverse transcription from 1 μg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time PCR was performed using Assays-on-Demand Gene Expression products (Perkin-Elmer Life Sciences, MA, USA) and iQ Supermix (Bio-Rad) in an iCycler (Bio-Rad). Assays-on-Demand Gene Expression products consisted of a TaqMan MGB probe (FAM dye labeled) and PCR primers. The probe sequences were: GHS-R1a, 5'-TGAAGATGCTTGCTGTGGTGGTGT-3'; GAPDH, 5'-GAAACCCATCACCATCTTCCAGGAG-3'; and 18S ribosomal RNA, 5'-TGGAGGG-CAAGTCTGGTGCCAGCAG-3'; while information about the sequences of the PCR primers was not declared by the supplier (GenBank accession numbers, GHS-R: NM_032075, AB001982, GAPDH: NM_017008, AF106860). Five microliters of the cDNA diluted 20-fold after reverse transcription were used as a template for each PCR with 10 μl iQ Supermix containing 2 $\times$ PCR buffer and 1 μl Assays-on-Demand Gene Expression products in a total volume of 20 μl . The PCR cycling was performed at 95°C for 3 min (denaturation) and then at 95°C for 15 s, and 60°C for 1 min for a total of 50 cycles.

Statistical analysis

Examinations of GHS-R mRNA levels for a diurnal pattern and the effects of vagotomy were evaluated by one-way ANOVA, and *post hoc* comparisons were made using the Tukey-Kramer test. The effects of starvation, and those of administration of CCK, gastrin, somatostatin, and ghrelin, were evaluated by Student's *t*-test. Differences at $P < 0.05$ were considered significant.

Results

A clear diurnal pattern was observed in the expression of GHS-R mRNA in the nodose ganglia, an increase being evident in the light phase (from 0500 to 1300 h) and a decrease in the darkness phase (from 1700 to 0100 h). The initiation of the increase or decrease occurred exactly 2 h before the room lights were switched on or off. A significant

peak of GHS-R mRNA expression occurred at 1300 h in the light phase ($P < 0.05$; Fig. 1).

When the rats were starved for 24 h, the GHS-R mRNA level increased about twofold, but this was not significantly different from the level in rats fed *ad libitum*. However, contrary to our expectation, the GHS-R mRNA level showed a further increase in rats that were re-fed for 2 h after 22 h of starvation, and the value was significantly different from that in rats fed *ad libitum* ($P < 0.05$) and in rats that were starved ($P < 0.05$; Fig. 2A). We checked the GHS-R mRNA levels using 18S as an internal control in this experiment. As shown in Fig. 2B, the results were quite similar to those for GHS-R mRNA levels using GAPDH as an internal control.

When the rats were vagotomized unilaterally, the level of GHS-R mRNA in the nodose ganglia was decreased significantly on the vagotomized side at 24 h after surgery ($P < 0.05$; Fig. 3).

Although GHS-R mRNA levels were influenced by i.v. administration of CCK and gastrin, the effects between 2 and 4 h after treatment were opposite. At 2 h after i.v. administration of CCK and gastrin, GHS-R mRNA levels were significantly higher than those in rats treated with saline ($P < 0.05$). On the other hand, at 4 h after administration of CCK and gastrin, GHS-R mRNA expression was lower than in rats treated with saline ($P < 0.05$), and no significant differences were evident between rats that had been treated with somatostatin and ghrelin, and rats treated with saline (Fig. 4A and B).

Discussion

The ghrelin receptor, GHS-R, is widely localized in both the central nervous system and the periphery (Guan *et al.* 1997). A detailed study on the localization of GHS-R mRNA

Figure 1 Diurnal pattern of GHS-R mRNA levels in the rat nodose ganglion revealed by real-time RT-PCR. Rats were maintained at a constant temperature under a 12 h light:12 h darkness regime (lights on 0700–1900 h) as indicated by black and white bars. Results are means $\pm$ S.E.M. ($n = 5$). * $P < 0.05$ compared with the value at 1700 h.

Figure 2 Effect of starvation and re-feeding after starvation on GHS-R mRNA levels in the rat nodose ganglion. The animals were deprived of food for 24 h from 1100 h, and then sampling was immediately performed on six rats. For the re-feeding part of the experiment, six rats were fed for 2 h after 22 h of food deprivation. As a control, six rats were fed *ad libitum* for 24 h and killed at 1100 h. Results are means $\pm$ S.E.M. ($n = 6$). * $P < 0.05$ compared with controls. The relative value of GHS-R mRNA levels was obtained for internal control of (A) GAPDH and (B) 18S ribosomal RNA.

expression in the central nervous system of rats and mice revealed expression in the hypothalamic nuclei, which are involved in the regulation of body weight and food intake (Zigman *et al.* 2006). Several other studies have examined the regulation of GHS-R expression in the hypothalamus and pituitary. Glucocorticoids and thyroid hormones were shown to stimulate the level of GHS-R mRNA *in vivo* and *in vitro* in primary cultured pituitary cells (Tamura *et al.* 2000, Kamegai *et al.* 2001), while insulin-like growth factor-I (IGF-I) decreased the level of GHS-R mRNA (Kamegai *et al.* 2005). In rat mammosomatotroph pituitary GH₄ cells, treatment with thyroid hormone and/or estradiol significantly enhanced the activity of the GHS-R 5'-flanking region (promoter region). In contrast, treatment with glucocorticoid significantly inhibited the GHS-R promoter (Peterseenn *et al.* 2001). The reason for the discrepancy in the effect of glucocorticoid on GHS-R mRNA expression in primary cultured pituitary cells and in GH₄ cells is unknown.

Figure 3 Effect of unilateral vagotomy on GHS-R mRNA levels in the rat nodose ganglion. Only the cervical vagal nerve on the left side was transected in all vagotomized (VX) rats. At 12 and 24 h after the operation, the right and left nodose ganglia were collected and analyzed separately. Results are means $\pm$ S.E.M. ($n=6$). Unilateral vagotomy induced a significant decrease in the level of GHS-R mRNA in the nodose ganglia on the vagotomized side at 24 h after surgery (a versus b). Although unilateral vagotomy decreased GHS-R mRNA expression in nodose ganglia on the intact opposite side, the difference was not significant in comparison with rats that underwent a sham operation. Different letters indicate $P<0.05$.

On the other hand, another study has shown that leptin and ghrelin induced a respective decrease and increase of GHS-R mRNA expression specifically in the arcuate nuclei of fasted rats (Nogueiras *et al.* 2004). Although a role of GHS-R expression in the nodose ganglion is also evident in the regulation of food intake, no information has yet been published about the alteration pattern of GHS-R expression or its regulation at the pre- and post-transcriptional levels.

The present study revealed a clear diurnal pattern in the expression of GHS-R mRNA in the nodose ganglia, an increase being evident in the light phase and a decrease in the dark phase. These findings suggest that GHS-R mRNA expression could be regulated physiologically in association with food intake upon entraining to a dark/light cycle, and that some factors might facilitate or inhibit GHS-R expression. We have previously reported that the plasma level of ghrelin exhibits two diurnal peaks, one occurring at 1500 h in the light phase and the other at 0600 h in the dark phase, and that these peaks correspond to the times when the gastric contents are minimal and maximal respectively (Murakami *et al.* 2002). This result suggests that ghrelin secretion is increased under conditions of both gastric emptying and filling, leading to one explanation that the peak observed during gastric emptying is to stimulate food intake whereas the other is to stimulate gastric acid secretion. In the present study, the significant peak of GHS-R mRNA expression occurred at 1300 h in the light phase, which was 2 h before the ghrelin level peaked at 1500 h. This peak level of GHS-R mRNA before an increase of the plasma ghrelin level seems to be reasonable for induction of food intake via the nodose ganglia, where GHS-R mRNA expression would

Figure 4 Effect of i.v. administration of CCK, gastrin, somatostatin, and ghrelin on GHS-R mRNA levels in the rat nodose ganglia. Sampling was carried out at (A) 2 h and (B) 4 h after the treatments. Results are means $\pm$ S.E.M. ($n=6$). * $P<0.05$ compared with controls.

increase, followed by facilitation of receptor protein synthesis and transport of the receptor to the afferent terminals to react with ghrelin secreted from the stomach.

In both rats and humans, ghrelin is considered to be a starvation signal, whose level increases upon fasting, and decreases immediately after food ingestion. This led us to the hypothesis that GHS-R expression might also change in the same way as the ligand during starvation. In fact, starvation for 24 h induced a twofold increase in the level of GHS-R mRNA, but this was not significantly different from the level in rats fed *ad libitum*. However, contrary to our expectation, the GHS-R mRNA level showed a further increase in rats that were re-fed after 22 h of starvation, and the value was significantly different from that in rats fed *ad libitum* and in rats

starved. This phenomenon might be related to our observation that GHS-R mRNA expression was significantly elevated within 2 h after i.v. administration of CCK. The function of CCK is opposite to that of ghrelin, as the former is an anorexigenic hormone secreted from the duodenum and whose level increases after meals (Gibbs *et al.* 1973, Moran & Kinzig 2004, Rehfeld 2004). Therefore, an increase in the level of GHS-R mRNA might be due to an increase in the CCK level upon re-feeding. However, as mentioned below, further investigations will be required to confirm this.

GHS-R mRNA levels increased 2 h after administration of CCK and gastrin, and decreased 4 h after administration, whereas no significant change was observed between rats that had been treated with somatostatin and ghrelin. In our previous study, we noted a similar phenomenon whereby CCK and gastrin rapidly stimulated the secretion of ghrelin from the stomach within 40 min of administration (Murakami *et al.* 2002). Generally, as ghrelin acts in an opposite way to CCK and gastrin during fasting or after a meal, it can be speculated that CCK and gastrin would also inhibit the secretion of ghrelin and expression of its receptor at 2 h after administration, in a similar way to that observed in the present study at 4 h after administration. However, our results indicate that these hormones may possess stimulatory and inhibitory effects on ghrelin and GHS-R mRNA levels. Additional investigations will be required to determine the conditions that would allow rapid transient upregulation of ghrelin secretion and expression of its receptor upon treatment with CCK and gastrin. Gastrin, which is secreted from the stomach and stimulates gastric acid secretion (Dockray 2004), also showed trends similar to those of CCK. Two types of receptors, CCK1-R and CCK2-R, mediate the effects of CCK (Innis & Snyder 1980), but only the CCK1-R is related to feeding regulation by CCK via the afferent vagus (Moran *et al.* 1992). Gastrin has high affinity for the CCK2-R but not for the CCK1-R (Dockray 2004) and, interestingly, both receptors are expressed in neurons of the afferent vagus (Corp *et al.* 1993, Konturek *et al.* 2004). Recently, Date *et al.* (2005) reported that the CCK1-R is localized in afferent neurons expressing GHS-R, implying an interaction between these receptors, although there are no data concerning the relationship between the CCK2-R and GHS-R localization in afferent neurons. On the basis of this evidence, it is speculated that either the CCK1-R or the CCK2-R can mediate the effect of CCK or gastrin on GHS-R expression. On the other hand, in the present study, somatostatin did not cause any change in GHS-R expression. Somatostatin elicits a decrease of ghrelin secretion, and consequently meal size, by acting directly on ghrelin-secreting gastric cells in a paracrine manner (Shimada *et al.* 2003). Since somatostatin receptors are expressed at terminals of the hepatic, but not gastric, afferent vagus (Nakabayashi *et al.* 1995), neurons that express GHS-R are unlikely to be identical to those expressing somatostatin receptors. Considering these results and previous observations, transcription of the *GHS-R* gene is thought to be regulated by the factors that act on receptors in the neurons expressing GHS-R. There was also no

effect of ghrelin on its GHS-R mRNA expression in this study, although ghrelin exerted an increase of GHS-R mRNA expression in the arcuate nuclei of fasted rats (Nogueiras *et al.* 2004) and a decrease of that in pituitary cells (Luque *et al.* 2004), indicating that modulation of GHS-R mRNA expression by ghrelin seemed to be tissue specific.

In Fig. 5, we have summarized the regulation of GHS-R mRNA expression. The levels of GHS-R mRNA in the nodose ganglia were increased in the light phase and decreased in the dark phase, and upregulated by starvation and re-feeding, suggesting that expression of GHS-R mRNA is under physiological control. Unilateral vagotomy significantly decreased the level of GHS-R mRNA expression, as did administration of CCK and gastrin. In the present study, the regulatory mechanism involving these factors remained unclear. However, as the GHS-R-producing cells in the nodose ganglia are sensory neurons, as shown in Fig. 5, several mechanisms can be suggested, as follows. CCK and gastrin receptors are expressed in vagal afferent neurons, implying that regulation of *GHS-R* gene expression may be responsible for transmission of hormonal signals via vagal afferents. As the nodose ganglia include many satellite cells, they may play a role in GHS-R mRNA expression by receiving information

Figure 5 Model for the regulation of GHS-R mRNA expression in nodose ganglia. The GHS-R1a receptor is produced in sensory neurons of the nodose ganglia. The receptor proteins are conveyed to afferent terminals by axonal transport. Ghrelin interacts with GHS-R1a expressed at afferent terminals and the stimulus is relayed via the NTS to the hypothalamus. GHS-R mRNA expression is regulated by circadian rhythm, starvation, CCK, gastrin, and other factors, possibly via extracellular fluid or satellite cells. CCK and gastrin receptors are expressed in vagal afferent neurons, implying that the regulation of *GHS-R* gene expression may be responsible for the transmission of hormonal signals via vagal afferents.

from the central nervous system. In the nodose ganglia, some nutrients (such as glucose, fatty acids, and amino acids) and hormones that diffuse into tissue fluid from the blood may regulate the expression of GHS-R mRNA. Further studies are required to elucidate the regulatory mechanism of expression of GHS-R mRNA in the nodose ganglia.

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