Peptides 32 (2011) 1876–1886

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Molecular identification of ghrelin receptor (GHS-R1a) and its functional role in the gastrointestinal tract of the guinea-pig

Takio Kitazawa[a,b,*], Tatsuro Nakamura[a,1], Atsuki Saeki[a], Hiroki Teraoka[a], Takeo Hiraga[a], Hiroyuki Kaiya[c]

[a] Department of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[b] Department of Veterinary Science, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[c] Department of Biochemistry, National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan

ARTICLE INFO

Article history:
Received 9 June 2011
Received in revised form 28 July 2011
Accepted 29 July 2011
Available online 6 August 2011

Keywords:
Growth hormone secretagogue receptor 1a
Guinea-pig
Rat
Tissue distribution
Gastrointestinal tract

ABSTRACT

Ghrelin stimulates gastric motility *in vivo* in the guinea-pig through activation of growth hormone secretagogue receptor (GHS-R). In this study, we identified GHS-R1a in the guinea-pig, and examined its distribution and cellular function and compared them with those in the rat. Effects of ghrelin in different regions of gastrointestinal tract were also examined. GHS-R1a was identified in guinea-pig brain cDNA. Amino acid identities of guinea-pig GHS-R1a were 93% to horses and 85% to dogs. Expression levels of GHS-R1a mRNA were high in the pituitary and hypothalamus, moderate in the thalamus, cerebral cortex, pons, medulla oblongata and olfactory bulb, and low in the cerebellum and peripheral tissues including gastrointestinal tract. Comparison of GHS-R1a expression patterns showed that those in the brain were similar but the expression level in the gastrointestinal tract was higher in rats than in guinea-pigs. Guinea-pig GHS-R1a expressed in HEK 293 cells responded to rat ghrelin and GHS-R agonists. Rat ghrelin was ineffective in inducing mechanical changes in the stomach and colon but caused a slight contraction in the small intestine. 1,1-Dimethyl-4-phenylpiperazinium and electrical field stimulation (EFS) caused cholinergic contraction in the intestine, and these contractions were not affected by ghrelin. Ghrelin did not change spontaneous and EFS-evoked [^{3}H]-efflux from [^{3}H]-choline-loaded ileal strips. In summary, guinea-pig GHS-R1a was identified and its functions in isolated gastrointestinal strips were characterized. The distribution of GHS-R1a in peripheral tissues was different from that in rats, suggesting that the functional role of ghrelin in the guinea-pig is different from that in other animal species.

1. Introduction

Ghrelin is an endogenous ligand for growth hormone secretagogue-receptor 1a (GHS-R1a), which was first identified in pigs and humans [19], and is a 28-amino-acid peptide with *n*-octanoyl modification at the third serine residue (Ser^3) [26]. GHS-R is a G-protein-coupled receptor with seven transmembrane regions. Two GHS-R isoforms, a functional receptor, GHS-R1a (ghrelin receptor), and an alternative splice variant with undetermined function, GHS-R1b, have been identified [6]. Ghrelin is mainly produced in G-cells in oxyntic mucosa of the stomach and has potent activity for the release of GH from the pituitary through activation of GHS-R1a. Accumulating evidence has also indicated that ghrelin is an important regulator of glucose metabolism, insulin release and cardiovascular functions, and it has been shown to be a peripheral circulating orexigenic hormone that increases body weight by stimulating food intake and by decreasing fat utilization [27].

Ghrelin and GHS-R1a have some structural similarities with motilin and the motilin receptor, respectively [1,33]. Motilin is a gut hormone that is produced in the duodenum and induces phase III contractions in the stomach through activation of its own receptor (motilin receptor) [12,20]. The similarity between the two gut peptides prompted examination of the physiological roles of ghrelin in regulation of gastrointestinal motility. In rodents, measurement of gastric motility in conscious and non-restrained animals indicated that ghrelin accelerated gastric emptying [23] and augmented spontaneous phase III-like contractions, and vagotomy or capsaicin abolished the ghrelin-induced contractions [13,14,29]. Therefore, vagal afferent and efferent pathways are involved in the gastrointestinal-stimulating action of ghrelin in rodents [13,14,29]. Exogenous ghrelin also accelerates gastric emptying [28] and induces a premature gastric phase III of the migrating motor com-

* Corresponding author at: Department of Veterinary Science, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan. Tel.: +81 11 388 4795; fax: +81 11 387 5890.
E-mail address: tko-kita@rakuno.ac.jp (T. Kitazawa).
[1] Present address: Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan.

0196-9781/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2011.07.026

plex in humans [35]. On the other hand, exogenous ghrelin has no effect on gastrointestinal motility in conscious dogs [32]. Our recent study demonstrated that the guinea-pig is sensitive to ghrelin causing gastric contraction *in vivo* through activation of the capsaicin-sensitive vago-vagal reflex pathway similar to that in rats [29]. Furthermore, ineffectiveness of des-acyl ghrelin and inhibition of ghrelin-induced action by a GHS-R1a antagonist indicated the involvement of GHS-R1a in ghrelin-induced gastric contraction [31]. These differences in ghrelin-induced gastrointestinal action are thought to be species-dependent, but it is possible that the differences reflect a relationship to feeding habit of the animals (rodents and humans, omnivorous; dogs, carnivorous; guinea-pigs, herbivorous). An immunohistochemical study using antibodies for rat ghrelin and rat GHS-R revealed the presence of ghrelin and GHS-R in intestinal enteric nerves of the guinea-pig [38]. In addition, ghrelin has been demonstrated to enhance endothelin-induced contraction in the guinea-pig renal artery [9]. Although these functional and immunohistochemical studies have shown the expression of GHS-R1a in guinea-pig tissues, the structure of guinea-pig GHS-R1a and its distribution and physiological function in the gastrointestinal tract have not been elucidated.

The aim of this study was to identify and characterize GHS-R1a in the guinea-pig. Tissue distribution of the receptor mRNA was determined by using quantitative real-time PCR and was compared with that in the rat. To determine the function of enteric GHS-R1a, the effects of ghrelin on gastrointestinal contractility and stimulation-induced neural responses were examined in isolated smooth muscle preparations.

2. Materials and methods

2.1. Animals and tissue preparations

Hartley guinea-pigs (*Cavia porcellus*) and Wistar rats of both sexes (weighing 200–250 g) were obtained from Sankyo Lab Service (Sapporo, Japan). All experimental procedures were approved by the Medical Ethics Committee of Rakuno Gakuen University. Guinea-pigs and rats were housed in stainless steel cages at a regulated temperature (22 ± 2 °C) and 60–65% relative humidity with a normal 12:12 h light/dark cycle.

2.2. Guinea-pig GHS-R1a cDNA cloning

Total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, NY) from the cerebrum that had been stored in RNAlater (Ambion). Full-length cDNA was determined to amplify an approximately 700-bp fragment using degenerated primers that were designed on the basis of the portions that are highly conserved in other species of GHS-R1a. Then 3′- or 5′-RACE PCR was performed on the basis of the determined nucleotide sequence using a GeneRacer Kit (Invitrogen). Primers used in this study are shown in Table 1.

Cerebrum total RNA (1 μg) was transcribed with GeneRacer 3′-oligo using a QuantiTect RT Kit (QIAGEN, GmbH, Hilden, Germany) (final volume of 20 μl). PCR was performed with 2 μl of a template, a primer set (100 pmol/μl GHS-R-dSES1 and GHS-R-dANT1) and ExTaq DNA polymerase (TaKaRa, Otsu, Japan). The reaction conditions were 94 °C for 2 min followed by 35 cycles of 94 °C for 0.5 min, 54 °C for 0.5 min and 72 °C for 1 min with final extension at 72 °C for 3 min. The amplified product was purified by the Wizard PCR preps DNA purification system (Promega, Madison, WI) and subjected to second-round nested PCR. Nested PCR was performed under the same conditions as those used for primary PCR with another primer set (100 pmol/μl GHS-R-dSES2 and GHS-R-dANT1), 2 μl PCR-prepsed template and ExTaq DNA polymerase.

Table 1
Primers used in this study.

Name	Sequence (5′–3′)
GHS-R-dSES1	AAY YTY TAY CTS TSY AGY ATG GC
GHS-R-dSES2	TTR ATS GCN HCR CTS AGR TAR AA
GHS-R-dANT1	GAY CTS CTS ATY TTY CTS TGY ATG CC
gpGHSR-s1	TTC CAG TTC GTC AGC GAG AGC TGC
gpGHSR-s2	AGC TGC ACC TAC GCC ACG GTG CTC
gpGHSR-AS1	CAC GGT TTG CTT GTG GTT CTG
gpGHSR-AS3	GCT GAC GAA CTG GAA GAG TTT GCA
gpGHSR-full-s	GAT CTG CTC GGT CCT TCG GCG GAG
gpGHSR-code-s	ATG TGG AAC GCG ACG CCC AGC GAG
gpGHSR-code-AS2	TCA TGT ATT GAT GCT AGA CTT TGT
gpGHSR-Q-s	GCT GCG CGC CAA GGT GGT GGT CAC
gpGHSR-Q-AS	TAT CGC CAG CAT TTT CAC GGT TTG
rGHSR-Q-s	CTT TCT ACC GGT CTT CTG CCT
rGHSR-Q-AS	AGC AGA GGA TGA AAG CAA ACA
gpB-act-Q-s	CCA TCA TGA AGT GTG ACG TTG
gpB-act-Q-AS	AGA GTG AGG CCA GGA TAG AGC

The obtained product was subcloned into the pCRII-TOPO vector (Invitrogen), and the nucleotide sequence was determined by automated sequencing (model 3130, Applied Biosystems, Foster City, CA) according to the protocol of the BigDye™ terminator cycle sequencing kit (Applied Biosystems). As a result, a 707-bp GHS-R-like fragment was identified.

For 3′-RACE PCR, primary PCR was performed with a gene-specific primer (GSP), gpGHSR-s1, and a 3′-primer using HotStar Taq Plus Mix (QIAGEN GmbH). The reaction conditions were 95 °C for 5 min followed by 35 cycles of 95 °C for 0.5 min, 57 °C for 0.5 min and 72 °C for 1 min with final extension at 72 °C for 3 min. After PCR preps of the amplified product, nested PCR was performed with another GSP, gpGHSR-s2, and a 3′-nested primer under the same conditions as those used for primary PCR. A 1154-bp GHS-R-like fragment was identified in this process.

To determine the 5′-side cDNA sequence, first-strand cDNAs were synthesized from 2.5 μg cerebrum total RNA with an antisense primer (gpGHSR-Q-AS) using a QuantiTect RT Kit. Primary PCR was conducted using a GSP, gpGHSR-AS1, a 5′-primer and HotStar Taq Plus Mix with amplification conditions of 95 °C for 5 min followed by 35 cycles of 95 °C for 0.5 min, 57 °C for 0.5 min and 72 °C for 1 min with final extension at 72 °C for 3 min. After PCR preps of the product, nested PCR was performed using another GSP, gpGHSR-AS3, a 5′-nested primer and HotStar Taq Plus Mix under the same conditions as those used for primary PCR. A specific 450-bp product was identified.

To determine full-length cDNA, 3′-RACE PCR was performed using cerebrum cDNA for 3′-RACE as a template. HotStar Taq Plus Mix containing 2.5% DMSO was used for amplification with the GSP gpGHSR-full-s and 3′-primer. Then nested PCR was conducted with gpGHSR-full-s and 3′-nest primer. Reaction conditions were 95 °C for 5 min followed by 35 cycles of 95 °C for 0.5 min, 57 °C for 0.5 min and 72 °C for 1 min, and final extension was 72 °C for 3 min.

2.3. Functional analysis of guinea-pig GHS-R1a

To examine functional activity of the identified receptor protein, we cloned an open reading frame (ORF) of the cDNA encoding the protein. RT-PCR was performed under the same conditions as those for full-length cDNA described above except for the use of another primer set, gpGHSR-code-s and gpGHSR-code-AS2. The isolated cDNA was subcloned into pcDNA3.1-V5-His-TOPO mammalian cell expression vector (Invitrogen). A vector having correct orientation of the insert for expression and correct GHS-R1a sequence was sub-cultured, and the plasmid vector was isolated using a HiSpeed Plasmid Midi kit (QIAGEN GmbH) and diluted to 1 μg/μl for a transfection experiment.