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Influence of the intragastric administration of [D-Lys3]-GHRP-6 on the pro-proliferative effects of endogenous ghrelin in the small intestine of the rat

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Summary

Little is known about the in vivo influence of the blockade of the growth hormone secretagogue receptor Ia (GHS-R1a) on the gut structure. Data obtained in in vitro studies can be misinterpreted and can generate a confusing picture of the effects of ghrelin on the gastrointestinal structure. In a living organism the remodeling processes in the gastrointestinal tract is affected by complex regulatory mechanisms governed by locally produced hormones and peptides, as well as by the enteric and central nervous system. To our knowledge, there are as yet no published reports on the influence of ghrelin receptor blockades on the morphology of the alimentary system. The aim of the study was therefore to determine the effect of the GHS-R1a antagonist [D-Lys3]-GHRP-6 on the structure of the gastrointestinal (GI) system in the rat. Studies were performed on 12 male Wistar rats aged approx. 2 months with an initial body mass of approx. 180-200 g. The rats were kept on a 12/12 hour light/dark cycle at a temperature of 22 ± 2°C, and had free access to a standard rat diet and water. The animals were divided into two groups: control and experimental. The control group received physiological saline, and the experimental group were administered 100 nmol/kg b.wt. of [D-Lys3]-GHRP-6, a GHS-R1a antagonist (Peptides International, USA&Canada), intragastrically one dose/day during 4 weeks. The animals were fasted during the night before killing. After euthanasia the GI tract was rapidly removed, and the weight and length of the stomach, pancreas, liver, and small intestine were measured. Samples of the pancreatic tissue, duodenum, jejunum (25%, 50%, 75% of length), and ileum were taken for histological analyses. The paraffin sections were stained with hematoxylin and eosin, and a morphometric analysis was performed with the use of light microscopy. Significant differences in the surface area of pancreatic acinar cells and significantly increased mucosa thickness, villi length and crypt depth in the proximal jejunum were found in the rats intragastrically treated with [D-Lys3]-GHRP-6. However, changes in body weight, weight of the organs, and intestine length were not significant. In conclusion, the blockade of the GHS-R1a by [D-Lys3]-GHRP-6 did not abolish the pro-proliferative effect of endogenous ghrelin on the intestinal mucosa in the proximal jejunum, and increased the surface area of pancreatic acinar cells. The mechanisms behind these changes are not fully understood, and further research is needed for a better understanding of this phenomenon.

Keywords: GHS-R1a antagonist, intestinal mucosa, pancreas, acinar cells, ghrelin

Acyl ghrelin was identified as the endogenous cognate ligand for the growth hormone secretagogue receptor GHS-R1a in 1999 by Kojima et al. (18). Ghrelin is a 28-amino acid peptide that is produced mainly by mucosal X/A-cells in the oxyntic glands of the stomach, which was initially identified in rodents (18, 19). Ghrelin is produced in much smaller amounts also in the duodenum, small intestine, cecum, and pancreas, as well as in the heart and aorta (8, 15, 18). Moreover, some regions of the brain are involved in ghrelin synthesis, as ghrelin-containing neurons were identified in the pituitary gland, as well as in the arcuate nucleus of the hypothalamus (16, 21, 23).

Ghrelin has been known as a multifunctional hormone. The major actions of this peptide include stimulation of the growth hormone (GH), insulin secretion

(18, 28, 31), regulation of appetite and nutrient ingestion (13, 35), improvement of digestive motility (2, 6). When injected into mice (2, 14), rats (6, 10), humans (6), or dogs (15), ghrelin accelerates gastric emptying of a solid meal.

One of the most important actions of ghrelin is its regulatory role for long-term energy homeostasis and short-term food intake (7). Ghrelin is an appetite-stimulating gastrointestinal hormone (37). It acts as a circulating orexigenic signal, and has also been implicated in preprandial hunger and meal initiation (29). It has been shown that all nutrient types (i.e., carbohydrates, proteins, fats) can inhibit ghrelin secretion similarly, and that ingested nutrients may exert their inhibitory effects on ghrelin secretion luminally or systemically (12). Endogenous ghrelin has an important role in insulin secretion. Glucose-stimulated insulin secretion is reduced with exogenous ghrelin in healthy humans (32).

The GSHR-1a receptor is abundantly distributed in organs of the gastrointestinal (GI) system, namely the pancreas and intestine (1, 9). Protective properties of acyl ghrelin against gastric and intestinal mucosa lesions in rats exposed to noxious agents have been shown after central and peripheral administration of this peptide. This ghrelin-induced protective effect is due to its ability to induce vasodilatation and an increase in oxygen and nutrient flow to the mucosa (4, 5, 33). However, the generation of mucosal prostaglandin E_2 (20), and sensory nerve transmission may be involved in this action (4, 5, 20, 36).

Significant research has been done on ghrelin mitogenic properties *in vitro* in a variety of non-transformed and transformed cell systems. Several studies have shown that both acyl and deacyl-ghrelin inhibit apoptosis and promote cell survival (3, 11, 14, 35).

On the one hand, Waseem and coworkers (35) demonstrated that ghrelin promotes intestinal epithelial cell proliferation *in vitro*. According to their findings, ghrelin stimulates intestinal cell proliferation in a dose-dependent manner, and this effect is exerted via specific receptor binding. Interestingly, cell culture pretreatment with ghrelin's receptor antagonists D[Lys-3]-GHRP-6 completely abrogated the mitogenic effect of ghrelin. However, D[Lys-3]-GHRP-6 has not been demonstrated to be receptor subtype-specific (24).

On the other hand, *in vivo* studies performed on neonatal pigs, which were administered ghrelin enterally confirmed some pro-proliferative effects of ghrelin on the intestinal mucosa (30, 38). These authors showed that ghrelin is present in colostrum and milk, and that it is necessary for GIT development. Neonatal pigs fed a milk replacer formula lacking ghrelin showed a slowdown in the intestinal mucosa development, which was prevented by intragastric ghrelin treatment. Moreover, in rats that developed a hypotrophic gut after elemental diet feeding, a pro-proliferative effect of exogenous ghrelin has been shown (27).

Recent data from RNA interference technique studies performed to suppress the GHS-R1a showed that the pro-proliferative effect of both ghrelin and des-acyl ghrelin in intestinal cells is not affected by the silencing RNA directed at GHS-R1a (35). These authors hypothesize that in this case ghrelin may exert its non-endocrine biological actions (cell proliferation and apoptosis) through a yet unknown GHS-receptor subtype independently of the GHS-R1a. Although Waseem et al. (35) do not deny that pro-proliferative effects can be achieved by ghrelin binding to GSH-R1a, they point out that the presence of this receptor is not necessary for ghrelin and des-acyl ghrelin to promote the pro-proliferative effect in intestinal cells.

Little is known about the *in vivo* influence of the GHS-R1a blockade on gut remodeling. *In vitro* studies create a confusing picture of the effects of ghrelin on the gastrointestinal structure, which can be misinterpreted. In a living organism the remodeling processes in the gastrointestinal tract are affected by complex regulatory mechanisms governed by the locally produced hormones and peptides, as well as the enteric and central nervous system. To our knowledge, there are as yet no published reports on the influence of ghrelin receptor blockades on the alimentary system morphology. The aim of the study was therefore to determine the effect of the ghrelin antagonist [D-Lys3]-GHRP-6 on the structure of the GI system in the rat.

Material and methods

The experimental procedures used in this study were approved by the 2nd Local Animal Welfare Committee at the University of Life Sciences in Lublin, Poland.

Studies were performed on 12 male Wistar rats aged approx. 2 months with an initial body mass of approx. 180-200 g. The rats were housed under conditions of controlled illumination (12:12 hour light/dark cycle, lights on/off 7:00 a.m./7:00 p.m.) and temperature (22 ± 2°C). The animals were maintained in colony cages until the start of the experiment and given *ad libitum* access to a standard rat diet and water, and were deprived of food during the night before killing.

The animals were divided into two groups: control and experimental. The control group received physiological saline, and the experimental group was administered intragastrically 100 nmol/kg b.wt. of [D-Lys3]-GHRP-6, a ghrelin antagonist (Peptides International, Louisville, USA & Canada), one dose every day during 4 weeks. Body weight was measured before and at the end of the experiment. The animals were killed by an overdose of carbon dioxide, and the GI tract was rapidly removed. The stomach, pancreas, liver, and small intestine were weighed and measured. Samples of the pancreatic tissue, duodenum, jejunum (25%, 50%, 75% of the length), and ileum were collected and gently flushed with physiological saline. The tissue samples were fixed in Bouin solution for 3 days and subsequently dehydrated in a series of increasing ethanol concentration (POCH, Gliwice, Poland). The specimens were treated twice with xylen (POCH, Gliwice, Poland)

and embedded in Paraplast regular (Sigma-Aldrich). For histological analyses, paraffin sections of 4 μm were made in a Microtome (Microm, Germany) and stained with hematoxylin/eosin. A morphometric analysis of the specimens was performed by Microimage v4.0. software with the use of a light microscope (Zeiss, Germany) fitted with a camera (RC5 Zeiss, Germany) connected to a computer.

The results are presented as mean ± SD. Data were statistically analyzed by one-way analysis of variance (ANOVA) with Statistica 5.0 software. Differences among each treatment group were tested by Tukey's multiple comparison test, and were considered significant at $P \leq 0.05$.

Results and discussion

Here, we report the effects of the intragastric administration of the GHS-R1a antagonist [D-Lys3]--GHRP-6 on the gastrointestinal system in Wistar rats fed a standard diet.

At the beginning of the study, the body weight of the rats was in the 180-200 g range. After 4 weeks of experiments, the body weight increased by about 100 g in both control and [D-Lys3]-GHRP-6 treated rats, and did not differ significantly. The absence of significant differences in body weight between the rats of both groups is in agreement with studies by Pfluger et al. (25), which were performed on wild-type and mutant mice lacking both ghrelin and ghrelin receptors. It was shown that only double knockout animals differed significantly in body weight and length from wild-type mice after 10 weeks of a standard chow diet. Single mutation of either ghrelin or ghrelin receptors did not cause changes in body weight or length under these conditions (25). Since our rats were fed a standard rat diet and were not knockout animals, body weight changes did not take place. Although a tendency toward a lower relative weight of the pancreas ($P = 0.071$) was observed in the [D-Lys3]-GHRP-6 treated rats, no significant differences were observed in the stomach and liver weight or in intestine relative length (Tab. 1).

The histological measurements of the small intestine are shown in table 2. Although there is a tendency toward higher values all along the small intestine in the [D-Lys3]-GHRP-6 treated rats, no significant differences in the mucosa thickness, villi length, crypt depth or the muscle thickness of the duodenum, middle jejunum, and ileum in the rats could be shown. Interestingly, in the [D-Lys3]-GHRP-6 treated rats a significant increase in mucosa thickness (862.7 ± 76.0 *vs.* 730.2 ± 42.3 μm, $P < 0.01$), villi length (623.0 ± 40.4 *vs.* 535.52 ± 49.0 μm, $P < 0.01$), and crypt depth (229.0 ± 14.1 *vs.* 204.4 ± 8.1 μm, $P < 0.05$) was found in the proximal part of the jejunum. Moreover, the depth of the crypts in the distal part of the jejunum was significantly affected by the treatment (Tab. 2). Our data clearly show that the intragastric administration of the ghrelin antagonist [D-Lys3]-GHRP-6 resulted in an increase in the gut mucosa size, that is, villi and crypt enlargement in the proximal part of the jejunum. Additionally, in the distal part of the jejunum an increased crypt depth was also found, although villi length and mucosa thickness were not affected. Although, tendencies toward higher values of the mucosa measurements in the duodenum and the middle part of the jejunum were seen, no statistically significant changes were found. Moreover, the mucosa

Tab. 1. Relative organ weight (g/100 g b.wt.) and relative small intestine length (cm/100 g b.wt.) in the control rats and in the experimental rats treated with the GHS-R1a antagonist [D-Lys3]-GHRP-6 (n = 6, mean ± SD)

	Stomach	Liver	Pancreas	Small intestine	Duodenum	Jejunum	Ileum
Control	0.5 ± 0.1	3.0 ± 0.2	0.3 ± 0.1	27.5 ± 3.0	1.5 ± 0.2	25.7 ± 3.0	0.3 ± 0.02
[D-Lys3]-GHRP-6	0.5 ± 0.1	2.6 ± 0.3	0.2 ± 0.1	25.7 ± 3.8	1.6 ± 0.1	23.7 ± 3.8	0.4 ± 0.1

Tab. 2. Histological measurements of the small intestine, mucosa thickness, villi length, crypt depth, and muscle thickness (μm) in the control rats and in the experimental rats treated with the GHS-R1a antagonist [D-Lys3]-GHRP-6 (n = 6, mean ± SD)

		Mucosa	Villi	Crypts	Muscle layer
Duodenum	Control	791.7 ± 133	578.4 ± 147	220.9 ± 19.7	100.5 ± 41.4
	[D-Lys3]-GHRP-6	899.4 ± 90.8	630.9 ± 62.7	237.0 ± 38.3	88.0 ± 7.2
Proximal jejunum	Control	730.2 ± 42.3	535.5 ± 9.0	204.4 ± 8.1	63.0 ± 10.6
	[D-Lys3]-GHRP-6	862.7 ± 76.0 *	623.0 ± 40.4*	229.0 ± 14.1*	65.8 ± 5.1
Middle jejunum	Control	688.2 ± 46.3	472.9 ± 37.1	229.1 ± 20.5	73.7 ± 15.2
	[D-Lys3]-GHRP-6	682.6 ± 71.6	471.0 ± 58.0	209.5 ± 18.5	66.7 ± 10.6
Distal jejunum	Control	586.5 ± 48.3	391.1 ± 43.5	209.8 ± 19.0	63.0 ± 11.6
	[D-Lys3]-GHRP-6	655.5 ± 68.0	411.5 ± 26.6	248.6 ± 23.7*	73.0 ± 18.4
Ileum	Control	596.7 ± 67.4	372.2 ± 62.1	235.8 ± 35.8	85.5 ± 11.2
	[D-Lys3]-GHRP-6	551.9 ± 67.9	340.2 ± 15.4	230.1 ± 19.5	90.2 ± 11.4

Explanation: * – significant differences in the parameters of mucosa in the corresponding segments of the small intestine between the control and GHS-R1a treated rats at the level of $P < 0.05$

Tab. 3. Pancreatic acini and acinar cell surface area, and protein content, proteolytic activity in pancreatic and gastric mucosa homogenates in control and experimental rats treated with the GHS-R1a antagonist [D-Lys3]-GHRP-6 (n = 6, mean ± SD)

Group	Pancreatic acini surface area (μm^2)	Pancreatic acinar cell surface area (μm^2)	Protein in pancreatic homogenates (mg/g)	Trypsin activity in pancreatic homogenates (U/mg)	Protein in gastric mucosa (mg/g)	Proteolytic activity in gastric mucosa (U/mg)
Control	1004 ± 17.8	128.4 ± 0.8	15.8 ± 1.96	0.89 ± 0.20	11.8 ± 1.25	1.40 ± 0.23
[D-Lys3]-GHRP-6	1145 ± 138	143.9 ± 1.8*	15.1 ± 1.60	0.70 ± 0.25	12.5 ± 1.24	1.50 ± 0.32

Explanation: * – significant differences between the control and GHS-R1a treated rats at the level of $P < 0.05$

thickness, villi length, and crypt depth in the ileum of the control and ghrelin antagonist treated rats showed similar values. The local changes in the jejunum are difficult to explain and need further investigation to elucidate the mechanism by which [D-Lys3]-GHRP-6 affects the intestinal mucosa selectively.

The ability of ghrelin to promote intestinal cell proliferation has been shown in both *in vitro* and *in vivo* studies, and this effect was abolished by the ghrelin antagonist [D-Lys3]-GHRP-6 *in vitro* (35). As far as we know, this is the first *in vivo* study in which a chronic blockade of the GHS-R has been performed to evaluate its influence on the structure of intestinal mucosa. Our data clearly show that the ghrelin receptor antagonist administered intragastrically did not abrogate the pro-proliferative effects of endogenous ghrelin, which suggests that these effects are exerted not only trough the GHS-R1a. This is in accordance with the results of the studies by Waseem et al. (35), who used RNA interference techniques to suppress the GHS-R1a in intestinal cells and showed that the pro-proliferative effect of ghrelin was not affected. These authors hypothesize that ghrelin may exert its non-endocrine biological effects through a yet unknown receptor independently of the GHS-R1a. Additionally, *in vitro* studies have shown that ghrelin neither stimulates the release of the growth hormone from intestinal epithelial cells nor increases IGF-1 production, which suggests that the pro-proliferative effect is direct and independent of GH/IGF-1 (35).

The protein content and proteolytic activity of the gastric mucosa of the GHS-R1a treated rats did not differ from those of the control rats (Tab. 3). This shows that the ghrelin antagonist [D-Lys3]-GHRP-6 did not influence the protein content or the proteolytic activity of the gastric mucosa.

The measurements of the pancreatic acini showed a tendency toward higher values of the surface area in the [D-Lys3]-GHRP-6 treated rats, suggesting a trophic effect on the exocrine pancreas. This is supported by the measurements of the acinar cells, which showed significantly higher values of the surface area in the [D-Lys3]-GHRP-6 treated rats (Tab. 3). These changes affected neither the protein content nor the trypsin activity in pancreatic homogenates (Tab. 3).

To our knowledge, this is the first study to show such an effect of [D-Lys3]-GHRP-6 on pancreatic tissue, but it is not clear if this effect is mediated by ghrelin receptors in acinar cells. It has been demonstrated by the RT- PCR technique that the pancreas expresses the GHS-R (13). Moreover, a ghrelin system consisting of both a ligand and a receptor has been found to exist in the AR42J cells (22). These authors postulated that this system may regulate the exocrine function by paracrine and/or autocrine mechanisms.

Kapica et al. (17) and Zhang et al. (39) demonstrated that ghrelin and pentaghrelin are potent inhibitors of stimulated pancreatic exocrine secretion in anaesthetized rats, but this effect is achieved through a mechanism dependent on intrapancreatic neurotransmission. In our studies, the protein content and trypsin activity in pancreatic homogenates was not altered, but the surface area of acinar cell was increased. This suggests a stimulating effect of the ghrelin antagonist [D-Lys3]--GHRP-6, whose mechanisms have yet to be elucidated. The effect of ghrelin on the exocrine pancreas function is believed to be regulated at multiple levels and is mediated by intrapancreatic neural transmission, as well as by receptors on acinar cells (39).

It is noteworthy that in the [D-Lys3]-GHRP-6 treated rats the level of ghrelin in blood was almost double that in the control rats (26). One possible explanation for the proliferative effects observed in our studies is that the high amount of endogenous ghrelin circulating in the body of the treated rats exerted a pronounced influence through a hypothetic receptor, other than GSH--R1a, as proposed by Waseem and co-workers (35).

We conclude that the blockade of the GHS-R1a by the ghrelin antagonist [D-Lys3]-GHRP-6 did not abolish the pro-proliferative effect of endogenous ghrelin on the intestinal mucosa, and led to an increase in the pancreatic acinar cell surface area. This suggests that the pro-proliferative effects of ghrelin are mediated not only by the GHS-R1a, but also by another unknown receptor. Further research is needed for a better understanding of this phenomenon.

The blockade of the GHS-R1a by the ghrelin antagonist [D-Lys3]-GHRP-6 did not abolish the pro-proliferative effect of endogenous ghrelin on the intestinal mucosa, and led to an increase in the pancreatic acinar cell surface area.

The pro-proliferative effects of ghrelin are mediated not only by the GHS-R1a, but also by another unknown receptor.

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