ORIGINAL

Ghrelin in small intestine, its contribution to regulation of food intake and body weight in cross-intestinal parabiotic rats

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Abstract. Ghrelin has been shown to be associated with feeding behavior in humans and rodents. It has been suggested that ghrelin may play a role behind the effect of bariatric surgery. Inbred rats were made into parabiotic pairs so that they shared a single abdominal cavity. A further operation is performed later in which the small intestines are transected and re-connected so that one rat continually lost nutrition to its partner. Changes in food intake and body weight were recorded. Seven weeks later, content of ghrelin in the plasma, stomach and upper intestines were measured in the paired rats. Rats which lost nutrients to its counterpart (Loss rats) ingested significantly more food than sham control rats ($p<0.001$). Rats which gained nutrient (Gain rats) ingested less than controls ($p<0.001$). There was no significant difference in body weight, blood glucose, insulin, free fatty acids and triglycerides between the paired rats. There was significantly higher levels of ghrelin in the plasma ($p<0.008$) and the intestine of the Loss rats ($p<0.02$). There were no difference in ghrelin in the stomach between parabiotic rats and sham operated controls. The ghrelin content of the plasma and intestines were significantly higher in the Loss rats, which ate more, and normal in the Gain rats, which ate less than controls. Because no remarkable changes in the ghrelin content were observed in the stomach, difference in the quality of the chime may affect the local synthesis and release of ghrelin.

Key words: Ghrelin, Parabiosis, Feeding, Behavior

GHRELIN, an endogenous ligand for growth hormone secretagogue receptors (GHS-R), is a gastrointestinal hormone primarily isolated from the stomach [1]. Ghrelin which has been also identified in other tissues including the intestines, [2] has received much attention about its possible role in the pathogenesis of obesity [7, 8, 9, 10].

The various studies involving ghrelin to this day have provided evidence about its functional role in feeding regulation. Central as well as peripheral administration of ghrelin have been shown to increase food intake and body weight in rodents [11, 12, 13, 14]. It has also been demonstrated that serum level of ghrelin is increased by fasting [15, 16] and decreased by refeeding [13]. These previous studies indicate that ghrelin plays an important role in the regulation of energy metabolism.

However, in human studies, contradictory results have been reported. Obese human subjects showed lower level of serum ghrelin than lean human subjects [8]. Some have suggested that smaller fluctuation in the serum ghrelin concentration of obese subjects contribute to the excessive feeding [9]. In contrast, a serum level of ghrelin is known to decrease in human subjects who have undergone bariatric surgery. Patients with bariatric surgery display significant weight loss accompanied by lower serum ghrelin levels and its reduced fluctuation [15].

The secretion of ghrelin may be triggered by direct stimulation to the gastro-intestinal wall by nutrients in the lumen. Another possible mechanism is that it is triggered by neural or hormonal signals from elsewhere in the body after nutrition is absorbed by the intestines. In human and scheduled-fed animal, pre-prandial increase of serum ghrelin was observed without immediate mechanical stimuli in gastrointestinal tract [17, 18] indicating a mediation of the central nervous system in production and/or secretion of ghrelin.

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Whereas, the instant postprandial decrease [19] and the anatomical location of ghrelin producing cells [1, 5] suggest the involvement of mechanical stimuli or change in post-absorptive humoral factors in inhibitory regulation of ghrelin through the sensors along the gastrointestinal lumen.

In order to further elucidate the nature of this hormone, we employed Koopmans' cross-intestinal parabiotic rat model [22]. In this model, one rat continually gained or lost nutrient in the intestine from or to the other rat, respectively, through the transplanted intestine. Regardless of their increased or decreased ingestion of nutrient, the animals show little difference in body weight and major blood parameters such as glucose and insulin at four weeks post operation. However, animals eat dramatically more or less than their corresponding pairs. This model is used to study various aspects of feeding behavior, but the mechanisms by which its food intake is modulated is as yet unknown. We hypothesized that change in ghrelin of gastrointestinal tract might have a role in modulating their feeding behavior. To address this issue, we analyzed the relationship between food intake and changes in local and systemic ghrelin levels in parabiotic rats.

Animals

Adult, male [12-14 weeks old, 250-300g) Lewis rats were kept in a clean, air conditioned environment at $22\pm1^{\circ}\text{C}$ in 12 hour light-dark cycles throughout the experiment. The rats were normally kept in standard cages with bedding. Liquid food was given everyday at 4pm and removed at 11am (a span of 19 hours) to correspond with the natural feeding cycle of the rats. Water was provided in a separate bottle *ad libitum*. All work involving rats was done in accordance with the principles set forth in the NIH Guide for the Care and Use of Laboratory Animals and all animal protocols were approved by the Animal Care and Use Committee of the University of Oita School of Medicine.

Surgery

Inbred Lewis rats matched for body weight to within 5g of each other were paired and surgically made into parabiotic rats which shared a common abdominal cavity. The rats were allowed to recuperate for two weeks during which they were adapted to liquid diet (Ensure Liquid, Abbot) and feeding measurements were taken to ensure that there was no initial lateral difference. They were then given secondary operations in which a

segment of the upper gut from one rat was transplanted into the other rat in a pedicle transposition so that the food ingested through the mouth of the recipient rat will first pass through the segment transplanted from the donor rat before it is reintroduced into the corresponding gut of the recipient rat. The mesentarium is left intact on the transplanted segment along with the local nerves and blood vessels (Fig. 1). Thus the food absorbed from this segment will nourish the individual of its origin. The recipient rat, which lost luminal nutrition to the paired rat, was denoted the Loss rat, while the donor rat, which gained luminal nutrition beyond what it ingested orally, was named the Gain rat. The details of the surgical procedure are described elsewhere [22]. Sham operated controls were likewise paired and their guts were similarly sectioned but re-sutured into the original position. In cases where samples from sham operated controls were damaged or lost, we used non-operated rats given the same liquid diet for a corresponding number of weeks.

Feeding Measurements

Food intake was measured in restraining cages which allowed each animal to drink liquid diet only from the bottle allotted to it. They were placed in restraining cages for the same duration of time as their normal feeding hours. A minimum of three days' interval was inserted between measurements so as to avoid the effect of restraint on the feeding. At least three measurements were taken on each pair and an average was used as the representative value for each pair.

Sacrifice and Assays

Six pairs were sacrificed for ghrelin assay. The animals were sacrificed, separated, weighed for individual body weight, and 6-8mL of blood was sampled from each animal. The blood was centrifuged with EDTA to collect plasma for ghrelin assay. The plasma was frozen at -80°C until RIA assay. The stomach and anatomically equivalent sections of the upper gut were also sampled. The tissues were placed in test tubes containing distilled water and boiled at 100°C for 3 minutes and immediately put on ice. Distilled water, 100% ascorbic acid and 0.5N hydrochloric acid were added so that ascorbic acid will account for 6.25% of final volume and HCl will account for 2% of final volume. The tissues were then homogenized and kept at 4°C until RIA assay. Protein content of the supernatant was quantified using BioRad Protein assay kit. RIA values were

Fig. 1a 1. Two Lewis rats matched for body weight were sutured together so that they share a common abdominal cavity. 2. A section of the upper jejunum was transected but with the mesenterium left intact. The jejunum of the recipient was transected in the equivalent position. 3. The upper and lower intestines of the donor were connected to create a shorter intestine while the transected gut segment was introduced into the digestive tract of the recipient to create a longer intestine.

Fig. 1b A section of the intestine of the “gain” rat was surgically introduced into the intestinal tract of the “loss” rat in a pedicle transposition. The “loss” rat continuously lost nutrition into the bloodstream of the “gain” rat. The “gain” rat consequently ate less and the “loss” rat ate more. Anatomically equivalent areas of the stomach and the intestines were sampled upon sacrifice.

standardized to per milligram protein bases.

The RIA assay for ghrelin was performed at Department of Internal Medicine 3, Miyazaki Medical University using in-house ghrelin antibodies as described elsewhere [21].

Serum glucose, triglycerides, free fatty acids and insulin were assayed by SRL Inc.

Statistics

Mann-Whitney-U test was employed in the statistics. StatView 5.0 was used for the calculations.

Results

Changes in body weight and food intake of parabiotic rats

Fig. 2 shows the body weight of the rats upon sacrifice. The body weights of Gain and Loss rats were 413.2g (± 11.0 SE) and 410.1g (± 19.9 SE) respectively. There were no significant differences in body weight between gain and loss rats at 4 weeks after secondary surgery and the deviation was comparable to that of the sham operated controls. However, there was a small but significant increase in the body weight of the Gain rats over the Loss

rats after 6 weeks (data not shown). All samples were taken from rats 4 weeks after secondary surgery to avoid interference of body weight differences.

Changes in food intake

Fig. 3 shows the changes in food intake in parabiotic rats. There was no difference in the food intake within the parabiotic pairs before the intestinal surgery. There was a significant difference ($p < 0.005$) in food intake after the intestinal transplants. Food intake in Loss and Gain rats significantly increased or decreased, respectively, compared with that in sham-operated control rats ($p < 0.001$). It should be noted that the animals were given two weeks to recuperate from the first surgery before pre-operative feeding measurements were taken and another four weeks after the second surgery before post-operative feeding measurements were taken. The post-operative rats were therefore 4 weeks older than the pre-operative rats on average. Thus the post-operative parabiotic rats and sham-operated controls ate slightly more than the pre-operative animals. For this reason, the post-operative food intake of the Gain rats is not significantly lower than the pre-operative baseline, but is significantly lower than that of the post-operative controls which were of the same age (Koopmans used older rats in excess of 350g, which were not in steady supply at our locale, and was free from the changes in food intake due to growth of the animals).

Changes in ghrelin content in gastrointestinal tract of parabiotic rats

The RIA measurements of ghrelin in the upper gut showed significantly higher level in the Loss rats (586.4fmol/mg protein ± 14.6 SE) than the Gain rats (469.7fmol/mg protein ± 14.6 SE) ($p < 0.008$) as well as sham control rats (411.2fmol/mg protein ± 42.0 SE) ($p < 0.02$) (Fig. 5). There were no significant difference in ghrelin level between Gain rats and sham controls. We also measured the ghrelin in the lower gut but there was no significant difference (data not shown). There were no significant differences in ghrelin concentration in the stomach between gain and loss rats and also between each parabiotic rats and controls (Fig. 6)

Changes in conventional parameters

As with previous reports using the same model, there were no differences in serum glucose, insulin, triglycerides and free fatty acids between the paired rats.

Fig. 2 In grams of body weight at sacrifice. There was no significant difference in the body weight of the matched pairs at sacrifice when separated along the suture lines.

Fig. 3 In grams of liquid diet eaten. Approx. 1Kcal/gram. There was no significant difference before the intestines were re-routed. The Gain side ate significantly less and the Loss side significantly more after the surgery. The post-operative rats were 4 weeks older than the pre-operative rats on average and were consequently larger and ate more. For this reason, the post-operative food intake of the Gain rats is not significantly lower than the pre-operative baseline, but is significantly lower than that of the post-operative controls which were of the same age (Koopmans used older rats in excess of 350g, which were not in steady supply at our locale, and was free of this discrepancy).

Fig. 4 Plasma ghrelin levels were significantly increased in the loss side in the parabiotic group. An average of 12 similarly fed, but non-operated, rats is presented as control.

Fig. 5 The ghrelin content of the intestinal tissue (in fmol/mg protein) was significantly higher in the Loss group. An average of 12 similarly fed, but non-operated, rats is presented as control.

Fig. 6 There was no significant difference in the ghrelin content in the stomach tissue (in pmol/mg protein). An average of 12 similarly fed, but non-operated, rats is presented as control.

Discussion

These experiments were conducted circa 1999–2003. The original objective was to show that ghrelin had a role in modulating the feeding behavior of cross-intestine parabiotic rats and thereby suggest that bariatric surgery in humans may also function by a similar mechanism. Cummings *et al* reported, in 2002, ghrelin data from human gastric bypass patients that was partly what this animal experiment was meant to predict [15].

In cross intestinal parabiotic rats, the Loss rats lose nutrition into the bloodstream of the Gain rats through a segment of intestine transplanted in a pedicle transposition (Fig. 1). Since Gain rats absorb nutrition from the Loss rats on top of the nutrition that it ingests by itself, a great difference in energy intake between Gain and Loss rats is expected. However, a dramatic change in food intake adjusts for the difference in absorption with little or no resultant difference in body weight

or serum borne metabolites at four weeks after the cross-intestinal surgery. It has not been explained how parabiotic rats recognize the changes in their nutrient absorption and reflect it on their feeding behavior. It is unlikely that changes in humoral factors such as glucose and insulin affect food intake since they show no remarkable difference.

Ghrelin has been shown to be involved in the regulation of feeding behavior. So we focused on ghrelin as an intestine-derived signal to regulate food intake in parabiotic rats. Results showed that the plasma ghrelin was significantly higher in the Loss rats than in the Gain rats as well as sham control rats (Fig. 4). However, ghrelin level in Gain rats showed no remarkable change from controls. The Loss rats that ate more showed higher levels of plasma ghrelin, while the gain rats that ate less showed normal levels. Ghrelin being an orexigenic substance [20, 28, 29, 30], the correlation between ghrelin level and food intake suggests that ghrelin may be involved in the regulation of feeding behavior in response to intestinal transplantation.

Thus, it is probable that parabiotic treatment has induced change in plasma ghrelin levels through affecting its production and/or release from gastrointestinal tract. We found no difference in the ghrelin content in stomachs between gain and loss rats, even though the stomachs were exposed to different amounts of food. This result suggests that food volume per se may not affect ghrelin levels in the stomach. It is known that serum ghrelin is increased preprandially and decreased postprandially [19, 28, 29] indicating suppressive effect of food on the production of ghrelin. In the post-prandial phase, direct contact of nutrition against the wall of the digestive tract or changes in post-absorptive humoral factors could trigger the suppression of ghrelin. If humoral factors regulate ghrelin, then we should see the same changes in ghrelin content in the stomach as well. But we found no difference in the stomach ghrelin, while there was significant difference in the intestinal ghrelin. Thus, it seems more likely that the change in ghrelin is controlled locally by the intestinal tract itself.

Small intestines sampled in the present study for measurement of ghrelin are anatomically equivalent areas while each segment of the intestine changed their relative positions upstream or downstream of intestinal tract. The segment downstream is exposed to chyme that has been further digested and more depleted nutrition than the segment upstream. Anatomically equiv-

alent segments of the intestine exposed to lower concentrations of nutrition showed higher content of ghrelin than segments exposed to higher concentration of nutrition.

Small intestine in Gain rats maintains its upstream position in the intestinal tract even after transplantation. It is therefore reasonable that no remarkable change in ghrelin levels was observed in the transplanted segment of the intestines of Gain rats. But food intake of Gain rats were decreased compared to controls. It can be speculated that ghrelin is not the only factor controlling food intake and other factors such as cholecystokinin (CCK) and GLP-1 may inhibit food intake of Gain rats in response to local food stimulation.

How the ghrelin signal reaches the brain is still open to debate. Present study demonstrated that blood ghrelin level in Loss rats increased from 480.5pg/mL to 558.4pg/mL, an increase of mere 16%, a relatively small change compared to other studies. It is unlikely that this increased blood ghrelin may affect food intake because this alternation is too small compared with physiological periplandial change in blood ghrelin level of rats [12, 13]. Rather, it is possible that the afferent neuronal pathway is involved in its signal transmission to the brain. It has been shown that stomach ghrelin relays its signal to the brain *via* gastric vagal afferent nerves [23]. Signal transmission from small intestine mediated by vagal afferent nerve has been also identified in intestinal peptides such as CCK [24] and GLP-1[25]. So it can be hypothesized that small change in local intestinal ghrelin level may affect food intake by signal transmission *via* vagal afferent nerve. Other studies dispute the contribution of the vagal afferent nerve for ghrelin's effect on food intake. It has been shown that completely vagotomized rats can respond to intraperitoneal injections of very large doses of ghrelin (up to 40 microgram/Kg) [26]. This study found substantial changes in food intake with much smaller changes in systemic ghrelin for which the vagal afferent nerve may be a vital component. The surgical manipulation could not have had any more influence to the vagal nerves of the Loss and Gain rats than to the sham-operated controls since the surgery itself was identical except for the way the intestines were re-connected. (Although this report shows ghrelin data from non-operated controls, the feeding and body weight data are

from sham operated controls). Surgical manipulation can affect ghrelin in other ways. Another study which employed Bilroth II and Roux-en-Y reconstruction on gastrectomized rats have shown that in the absence of stomach-derived ghrelin, intestine-derived ghrelin can increase and fill in for the loss [27]. In this study, however, stomach-derived ghrelin was not different from that of sham operated controls. The Gain rats in a parabiotic pair do gain weight when compared to Loss rats after six weeks, but since these rats were sacrificed at four weeks before significant body weight differences appear, weight related factors such as leptin probably did not influence the outcome.

The above study may provide several insights about the role of gastrointestinal tract in regulation of feeding behavior as follows. Parabiotic rats may compensate their altered energy intake induced by intestinal transplantation through the modulation of their food intake. Change in ghrelin levels of the small intestine may be involved in the regulation of feeding behavior in response to intestinal transplantation. Intestinal tract may sense the nutritional content of the chime to regulate synthesis and/or release of ghrelin. Further study is necessary to clarify the local mechanism to affect intestinal ghrelin and its signaling pathway to the brain.

Disclosure

The authors have no conflicts of interests.

Ethics

All work involving rats was done in accordance with the principles set forth in the NIH Guide for the Care and Use of Laboratory Animals and all animal protocols were approved by the Animal Care and Use Committee of the University of Oita School of Medicine.

Dedication

This work is dedicated to the memory of Henry S. Koopmans, PhD. (1944-2010), former professor at the University of Calgary who developed the cross-intestinal parabiotic rat model.

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