Title

The corn protein, Zein, hydrolysate administered into the ileum attenuates hyperglycemia via its dual action on GLP-1 secretion and DPP-IV activity in rats

Precis

Corn Zein hydrolysate both induces GLP-1 secretion and reduces DPP-IV activity in rats

Short Title

GLP-1 releasing peptide on glycemic control

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Key words

Glucagon-like peptide-1; Dipeptidyl peptidase-IV; Dietary peptide

DISCLOSURE STATEMENT: TM has nothing to disclose. TH received grant support (2008) from The Iijima Memorial Foundation For The Promotion Of Food Science And Technology. HH received grant support (2007-2009) from Japan Society for the Promotion of Science (KAKENHI 19380071). TH and HH are inventors on Japan patent filed 2010-053383.

Abstract

We previously showed that a hydrolysate prepared from corn zein (ZeinH) strongly stimulates GLP-1 secretion from the murine GLP-1-producing enteroendocrine cell line and in the rat small intestine, especially in the ileum. Here, we investigated whether ZeinH administered into the ileum affects glucose tolerance via stimulating GLP-1 secretion. To observe the effect of luminal ZeinH itself on GLP-1 secretion and glycemia, intraperitoneal glucose tolerance tests were performed in conscious rats with ileal and jugular catheters, and plasma glucose, insulin, and GLP-1 (total and active) were measured. In addition, plasma dipeptidyl peptidase-IV (DPP-IV) activities in the ileal vein were measured after the administration of ZeinH into the ileal-ligated loop in anesthetized rats. The ileal administration of ZeinH attenuated the glucose-induced hyperglycemia accompanied by the enhancement of insulin secretion, whereas meat hydrolysate (MHY) neither induced insulin secretion nor attenuated hyperglycemia. The anti-hyperglycemic effect was also demonstrated by the oral administration of ZeinH. From these results, it was predicted that the GLP-1-releasing potency of ZeinH was higher than that of MHY. However, both peptides induced similar increase in total GLP-1 concentration after the ileal administration. In contrast, active GLP-1 concentration was increased only in ZeinH-treated rats. In anesthetized rats, ileal administration of ZeinH but not MHY decreased plasma DPP-IV activity in the ileal vein. These results indicate that the ileal administration of a dietary peptide, ZeinH, has the dual functions of inducing GLP-1 secretion and inhibiting GLP-1 degradation, resulting in the enhancement of insulin secretion and the prevention of hyperglycemia in rats.

Introduction

Glucagon-like peptide-1 (GLP-1) is one of the gut hormones that are released in response to nutrient ingestion. GLP-1 stimulates insulin secretion from pancreatic β-cells in the presence of plasma glucose (1). The enhancement of insulin secretion by gut-derived factors, such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), was termed the "incretin effect" (2). GLP-1 also stimulates β-cell proliferation, delays gastric emptying and reduces food intake in rats and humans (2, 3). Recently, stable GLP-1 analogs and dipeptidyl peptidase-IV (DPP-IV) inhibitors that protect active GLP-1 from cleavage by DPP-IV are currently used as additional drugs to control postprandial glycemia in type-2 diabetes (4, 5).

GLP-1 is produced by enteroendocrine L cells, which are located predominantly in the lower part of the intestine (ileum, cecum and colon) (6). After its release into the mesenteric and portal circulation, intact GLP-1 is rapidly degraded (inactivated) by DPP-IV in the plasma (7). It is also inactivated by DPP-IV during passage across the hepatic bed and in the peripheral tissues (8). Despite its short half-life, it is estimated that the incretin effect accounts for ≥ 50% of insulin release after glucose ingestion (9). In addition, GLP-1R$^{-/-}$ mice are characterized by mild fasting hyperglycemia and abnormal glucose tolerance (2). Therefore, GLP-1 secretion triggered by luminal nutrients is potentially important for the postprandial regulation of glucose homeostasis.

Recently, attention has been focused on whether it would be possible to utilize the endogenous GLP-1 stored in L cells to improve glucose tolerance (10). Indeed, several pharmacological compounds, including Berberine (an isoquinoline alkaloid originally isolated from *Coptidis rhizome*) (11), TGR5 (a bile acid-sensing GPCR) agonists (12) and GPR119 agonists (13), are effective at improving glucose tolerance through the enhancement of GLP-1 secretion. Therefore, potent luminal stimulants of GLP-1 secretion are of interest and have a great potential for obtaining better glycemic control in subjects with normal and abnormal glucose tolerance.

Glucose and fatty acids are well known as strong stimulants of GLP-1 secretion (14, 15), but

peptones also induce GLP-1 secretion in rats (16) and enteroendocrine cell lines (17), and whey preload enhances GLP-1 secretion in humans (18). Recently, we found that a hydrolysate prepared from zein, a major corn protein, potently stimulates GLP-1 secretion in the murine GLP-1-producing enteroendocrine cell line GLUTag and in the rat small intestine (19). Zein hydrolysate (ZeinH) in the duodenum indirectly stimulates GLP-1 secretion from ileal L cells probably via the afferent vagus, but in the ileum, ZeinH directly acts on ileal L cells to induce GLP-1 secretion as well as to enhance fat-induced GLP-1 secretion (20).

The purpose of the present study was to investigate whether the potent GLP-1-releasing peptide ZeinH affects glycemia via the stimulation of GLP-1 secretion. We examined the effect of ileal ZeinH on plasma glucose, insulin, and total and active GLP-1 secretion in conscious rats using the intraperitoneal glucose tolerance test (IPGTT). The effect of luminal ZeinH on plasma DPP-IV activity in the ileal vein was also examined by using the ileal-ligated loop in anesthetized rats. Since GLP-1 has the potential to improve pancreatic β-cell function, controlling endogenous GLP-1 secretion by luminal dietary peptides could provide a novel strategy for the prevention and treatment of obesity and diabetes.

Materials and Methods

Materials

Zein hydrolysate (ZeinH) was prepared as described previously (19). Briefly, Zein (Tokyo Chemical Industry, Tokyo, Japan) (50 g) was suspended in deionized water (500 ml) and adjusted to pH 7.0. The suspension was shaken for 60 min at 55°C after the addition of papain (250 mg, Papain F; Asahi Food and Health Care, Tokyo, Japan). It was then treated in boiling water for 20 min to stop the enzyme reaction. After centrifugation and filtration (0.2-μm pore size), the supernatant was lyophilized as ZeinH. Meat hydrolysate (MHY) was purchased from Sigma (St. Louis, MO). ZeinH and MHY had peptide contents of 65.5 and 80.0%, respectively, as determined by the Lowry protein assay, and they had respective molecular weights of 1,600 and <1200 Da, as described previously (19,

21). Total and free amino acid composition of ZeinH and MHY were measured by the method previously described (22) and are shown in Table 1.

Animals

Male Sprague-Dawley rats (7 weeks old) weighing 210–230 g were purchased from Japan SLC (Hamamatsu, Japan). Animals had free access to a semi-purified diet containing 25% casein based on AIN-93G (23) and water in individual cages. All animal experiments were performed after an acclimation period (3–7 d) in a temperature-controlled room maintained at 23 ± 2°C with a 12-h light-dark cycle (0800–2000, light period). The study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Surgical preparation for in vivo experiments (Experiments 1 and 2)

After a 24-h fast, rats were anesthetized with sodium pentobarbital (40 mg/kg body weight, Nembutal Injection, Dainippon Sumitomo Pharma, Osaka, Japan). The right jugular vein was exposed and a silicone catheter (Silascon No. 00, I.D. 0.5 mm, O.D. 1.0 mm; Dow Corning Co., Kanagawa, Japan) was inserted into the vessel and fixed with a thread. The catheter was prefilled with saline containing heparin (50 IU/ml, Ajinomoto, Tokyo, Japan). Another silicone catheter (Silascon No.00, I.D. 0.5 mm, O.D. 1.0 mm; Dow Corning Co.), whose tip housed the small segment (2–3 mm) of a polyethylene tube (Hibiki Fr No. 4; Kunii Co., Tokyo, Japan), was inserted into the proximal ileum (45 cm distal to the ligament of Treitz) and fixed with a thread. The free ends of both catheters were exteriorized dorsally, which allowed the experiment to be carried out under unanesthetized and unrestrained conditions. Rats were used for *in vivo* experiments (Experiments 1 and 2) after a recovery period (3–4 d). The ileal and jugular catheters were flushed daily with saline and heparinized saline, respectively, to maintain their patency. Because the ileal catheter is thinner than ileal tract, the intestinal flow would not be blocked by the ileal catheter. This was confirmed by observing normal feeding and evacuation behaviors before and after the surgical operation, and also by observing the intestinal flow under the anesthesia before killing rats at the end of experiments.

Experiment 1: Effects of ileal peptide administration on plasma glucose and insulin in conscious rats during IPGTT

The glucose solution was administered interperitoneally to examine the effect of luminal peptides themselves on GLP-1-mediated glycemia control. Peptides (MHY and ZeinH) and water (as negative control) were administered into the ileal lumen through the catheter to observe direct effects of peptides on GLP-1 secretion in the ileum and subsequent glucose handling. MHY was chosen as another dietary peptide that has GLP-1-releasing activity *in vitro* (17, 24) and *in situ* (16). After a 24-h fast, a basal (-30 min) blood sample (50 μl) was drawn from the jugular catheter. The catheter was refilled with saline containing heparin (50 IU/ml) between each blood sampling. Just after the basal blood collection, deionized water (2 ml) or test liquids (500 mg MHY, 500 mg ZeinH in 2 ml deionized water) were administered into the ileal lumen through the ileal catheter. A blood sample was drawn (0 min), and then glucose was injected intraperitoneally (1 g/kg) 30 min after the ileal administration. Blood samples were drawn into a syringe containing aprotinin (final concentration 200 kIU/ml, Wako, Osaka, Japan) with heparin (final concentration 50 IU/ml) at 15, 30 and 60 min after i.p. glucose injection. Plasma was separated by centrifugation at 2,500 x *g* for 15 min at 4°C and frozen at –80°C until glucose and hormone measurements. Plasma glucose and insulin were measured by using the Glucose CII-test kit (Wako) and insulin-ELISA kit (AKRIN-010T; Shibayagi, Gunma, Japan), respectively.

Experiment 2: Effects of ileal peptide administration on plasma GLP-1 in conscious rats (IPGTT)

The effect of ileal ZeinH on GLP-1 secretion was examined in conscious rats in a separated experiment because a large volume of plasma was required to measure glucose and total and active GLP-1. Because changes in total GLP-1, which includes both active (7-37) and inactive (9-37) forms, reflect the release of GLP-1 but not its activity as incretin, we also measured active GLP-1 in identical blood samples. IPGTT was performed in conscious rats as in Experiment 1. Blood samples for glucose and total GLP-1 (80 μl) were drawn into a syringe containing aprotinin (final concentration 200 kIU/ml) and heparin (final concentration 50 IU/ml). Blood samples for active GLP-1 (240 μl)

were drawn into a syringe containing EDTA (final concentration 1 mg/ml, Dojindo, Kumamoto, Japan), aprotinin (final concentration 500 kIU/ml) and DPP-IV inhibitor (final concentration 50 μM, catalog no. DPP4-010; Linco Research, St. Charles, MO). Total GLP-1 in the plasma (30 μl) was measured by ELISA kit (Yanaihara Institute, Shizuoka, Japan), and active GLP-1 in the plasma (100 μl) was measured by another ELISA kit (Linco Research).

Experiment 3: Effects of ileal administration of ZeinH and MHY on plasma DPP-IV activity in the ileal vein of anesthetized rats (in situ experiment)

To examine the effect of ileal ZeinH and MHY on plasma DPP-IV activity, blood samples were collected from the ileal vein through the catheter after the administration of water, MHY and ZeinH into the ligated ileal loop in anesthetized rats. After a 24-h fast, rats were anesthetized with ketamine (80 mg/kg body wt i.p., Ketaral, Daiichi Sankyo, Tokyo, Japan) containing xylazine (12 mg/kg i.p., MP Biomedicals, Irvine, CA), and a middle abdominal incision was made.

The small tip (6–7 mm) of a polyethylene catheter (SP 10; ID 0.28 mm, OD 0.61 mm; Natsume Seisakusyo, Tokyo, Japan) connected to a silicone catheter (Silascon no. 00, ID 0.5 mm, OD 1.0 mm; Dow Corning Co.) was inserted into the ileal vein in the ileal mesenteric tissue. The ileal lumen was washed by flushing with saline. The ligated ileal loop (30 cm in length) was prepared between 5 and 35 cm proximal to the cecum, and the proximal and distal ends of the loop were ligated with a silk thread. A basal (–30 min) blood sample (50 μl) was drawn from the ileal vein catheter, and the catheter was refilled with saline containing heparin (50 IU/ml) between each blood sampling. Deionized water (2 ml) or test liquids (500 mg MHY, 500 mg ZeinH in 2 ml) were then directly administered into the loop. Blood was collected (0 min) 30 min after the sample administration, and glucose was administered intraperitoneally (1 g/kg) to reproduce the experimental conditions of Experiments 1 and 2. Blood samples were collected through the ileal vein catheter at 15, 30 and 60 min after the glucose injection.

During the experiment, additional ketamine/xylazine was injected to keep the rats anesthetized,

and body temperature was maintained with heating pads. Blood samples for plasma DPP-IV activity measurement were drawn into a syringe containing heparin (final concentration 50 IU/ml, Ajinomoto).

Plasma DPP-IV activity

DPP-IV activity was determined based on the rate of hydrolysis of a surrogate substrate, Gly-Pro-*p*-nitroaniline (Gly-Pro-*p*NA, Sigma) (25, 26). A 5-µl aliquot of plasma was added to each well of a flat-bottom 96-well plate, followed by the addition of 80 µl of assay buffer (0.25 M Tris-HCl buffer, pH 8.0). The reaction was initiated by the addition of 80 µl of 1.6 mM Gly-Pro-*p*NA in deionized water. After an incubation at 37°C for 60 min, 40 µl of 1 M acetate (pH 5.2) was added to stop the reaction, and the absorbance at 405 nm was measured (A) using a microplate reader (model 680; Bio-Rad Laboratories, Mississauga, ON, Canada). To correct for the influence of hemolysis, a negative control was also prepared for each plasma sample, in which plasma was finally added to the mixture of assay buffer containing substrate and acetate after a 60-min incubation, and the absorbance at 405 nm was measured (B). Plasma DPP-IV activity was determined as the liberation of *p*NA from Gly-Pro-*p*NA by plasma DPP-IV, by subtracting the absorbance (B) from (A). One unit is defined as the liberation of 1 µmol Gly-Pro-*p*NA in one minute.

Experiment 4: Effects of oral ZeinH administration on plasma glucose in conscious rats (IPGTT)

Rats were trained daily by an orogastric administration with distilled water using a feeding tube (Safeed Feeding Tube Fr. 5, 40 cm; Terumo, Tokyo, Japan) during acclimation period. After a 24-h fast, a basal (-15 min) blood was collected (30 µl) from the tail vein, and then deionized water (8 ml/kg) or 250, 500 mg/ml of ZeinH (2 g/kg, 4 g/kg) were orally administered into the stomach by using a feeding tube. Glucose was injected (1 g/kg i.p.) 15 min after the oral administration, and tail vein blood was collected just before (0 min), and 15, 30, 60, 90 and 120 min after the glucose injection. Blood samples were heparinized and plasma glucose concentrations were measured as above.

Statistical analysis

Results are expressed as means ± SE. Statistical significance was assessed by one-way or two-way ANOVA, and significant differences among mean values were determined by the Fisher's post hoc test ($P < 0.05$) or Dunnett's post hoc test ($P < 0.05$).

Results

Experiment 1: Effect of ileal administration of ZeinH and MHY on plasma glucose and insulin concentrations during IPGTT

We first examined the effect of ileal ZeinH administration on plasma glucose and insulin concentrations under IPGTT in conscious rats. Because oral glucose loading can enhance endogenous GLP-1 secretion, and in order to observe the effect of luminal ZeinH itself on GLP-1 secretion and glycemia, we performed IPGTT in the present study. Since plasma glucose returned to nearly baseline level at 60 min after i.p. glucose injection (1 g/kg) in preliminary study, blood samples were collected up to 60 min.

Ileal administration of test liquids slightly increased plasma glucose concentrations (from –30 to 0 min), as shown in Figure 1A. Glucose concentrations at 15 and 30 min in MHY-treated rats were slightly but not significantly lower than those in control rats. In contrast, glucose concentrations at 15 and 30 min in ZeinH-treated rats were significantly lower than the values in control rats. The glucose concentration returned to nearly baseline level at 30 min in ZeinH-treated rats.

The plasma insulin concentration was not affected by ileal administration of water, and it increased from 0.09 nM (0 min) to 0.47 nM at 15 min after i.p. glucose injection in control rats (Fig. 1B). The treatment with MHY slightly but not significantly enhanced insulin concentrations at 0 and 15 min compared with control rats. In contrast, ileal administration of ZeinH significantly increased insulin concentrations at 0 min and 15 min by 6.3- and 2.4-fold, respectively, compared with control rats. Insulin concentrations also tended to be higher in ZeinH-treated rats compared with control and

MHY-treated rats at 30 and 60 min.

Experiment 2: Effects of ileal ZeinH and MHY on plasma total and active GLP-1, and glucose concentrations during IPGTT

We next examined whether ileal ZeinH or MHY stimulated GLP-1 secretion under the same conditions as in Experiment 1. Plasma glucose concentrations at 15 and 30 min in ZeinH-treated rats, but not in MHY-treated rats, were significantly lower than those in control rats. These results are consistent with the results shown in Figure 1A.

Basal values of total GLP-1 were 3.20–3.78 nM, and changes in total GLP-1 levels (ΔGLP-1) in the jugular vein plasma are presented in Figure 2B, because changes after the administration of test liquids were relatively small compared to basal total GLP-1 levels as observed in our previous study (19). The ileal administration of water did not cause any significant changes in the total GLP-1 concentration throughout the blood collection period. In contrast, the ileal administration of ZeinH and MHY induced a significant and sustained increase in total GLP-1 concentration during the period from 0 min to 60 min, except for the time point at 15 min in MHY-treated rats. The increment of total GLP-1 was slightly higher in ZeinH-treated than in MHY-treated rats. Total GLP-1 levels were increased by both MHY- and ZeinH-treatments in a similar manner. These results were not correlated with the insulin or glucose responses shown in Figure 1.

To estimate the activity of GLP-1 as an incretin, plasma active GLP-1 concentrations in the jugular plasma were also measured (Fig. 2C). Basal active GLP-1 concentrations were not significantly different among the three groups (5.78–6.25 pM). Ileal administration of water (at –30 min) and i.p. glucose injection (at 0 min) did not cause any significant changes in the active GLP-1 concentration (Fig. 2C). Ileal administration of MHY showed only a tendency to increase the active GLP-1 concentration at 0 min. ZeinH administered into the ileum sharply increased the active GLP-1 concentration from 5.9 pM at –30 min to 19.3 pM at 0 min, and the values at 0 min and 15 min in ZeinH-treated rats were significantly higher than those in control rats. In contrast to the total GLP-1 responses (Fig. 2B), active GLP-1 responses were correlated with insulin and glucose responses. The

discrepancy between total and active GLP-1 responses induced by ileal MHY and ZeinH might be explained by differences in plasma DPP-IV activity. Therefore, we next examined the effect of ileal administration of ZeinH and MHY on plasma DPP-IV activity in the ileal vein.

Experiment 3: Effects of ileal ZeinH and MHY on plasma DPP-IV activity in the ileal vein of anesthetized rats

By using a catheter inserted into the ileal vein as described previously (19), we collected ileal vein blood before and after ileal administration of test agents. Statistically significant differences were not observed in basal plasma DPP-IV activity (24.0–28.3 mU/ml) among the three groups. The administration of water (control) or MHY into the ligated ileal loop (–30 min) and i.p. injection of glucose (0 min) slightly but not significantly reduced plasma DPP-IV activity (Fig. 3). In contrast, DPP-IV activity at 0 and 15 min in ZeinH-treated rats significantly decreased after ileal administration, and then recovered gradually but did not reached the basal level by 60 min. ZeinH-induced decrements of DPP-IV activity were 26.8% (at 0 min) and 22.1% (at 15 min) compared to the basal level. Values in ZeinH-treated rats were slightly or significantly lower than those in other two groups throughout the experimental period. This finding demonstrates that ZeinH administration into the ileal loop decreased plasma DPP-IV activity in the ileal vein in anesthetized rats.

Experiment 4: Effects of oral ZeinH on glycemia during IPGTT in conscious rats

It was examined the effect of oral administration of ZeinH on the glycemia under IPGTT in conscious rats. ZeinH was administered at the dose of 2, 4 g/kg body weight. The dose of 2 g/kg is comparable to that of 500 mg/rat with the body weight of 250 g in the experiments above. In all three groups, elevated glucose levels at 15 min were gradually lowered from 30 min to 120 min after the glucose injection. Rats treated with oral ZeinH showed significantly lower glucose levels in a dose-dependent manner at 15 and 30 min after the glucose injection. The elevation of glucose concentration at 15 min in 4 g/kg ZeinH-treated rats was around half of that in control rats.

Discussion

Enteroendocrine L cells secrete GLP-1 in response to luminal nutrients, which serves important physiological roles in the maintenance of normal glucose homeostasis, including the potentiation of glucose-stimulated insulin release (1, 2). Exogenously administered GLP-1 exerts powerful anti-diabetic effects, even in type 2 diabetic patients with secondary failure to sulfonylureas (27). Indeed, stable GLP-1 analogs and DPP-IV inhibitors are already licensed for the treatment of type 2 diabetes (28, 29). Therefore, targeting GLP-1 secretion from L cells can provide new opportunities for the improvement of glucose tolerance.

In our previous *in situ* study, the GLP-1-releasing potency of ZeinH was highest in the ileum, where GLP-1-producing L cells are found at a higher density than in the duodenum or the jejunum (19). The interposition of the ileal segment within the jejunum (ileal interposition) has been reported to enhance GLP-1 release and to prevent hyperglycemia via the enhancement of insulin secretion after oral glucose load in non-diabetic rats (30). In that study, orally administered glucose might stimulate GLP-1 secretion from L cells in the interpositioned ileum before glucose absorption in the jejunum. This raises the possibility that pre-stimulation of GLP-1 secretion by ileally administered ZeinH can prevent hyperglycemia *in vivo*.

As expected, ZeinH administered into the ileum effectively attenuated the glycemic response induced by i.p. glucose injection in conscious rats (Fig. 1A). This was confirmed in a repeated experiment (Fig. 2A). Enhanced insulin secretion (Fig. 1B) before and after the glucose injection in ZeinH-treated rats could be responsible for the attenuation of hyperglycemia. Because glucose was injected intraperitoneally in the present study, inhibition of glucose absorption could not be involved in such effect. Therefore, these results demonstrate that a dietary peptide in the ileum can contribute to the prevention of hyperglycemia by enhancing insulin secretion. In contrast, ileal MHY had only a small effect on reducing plasma glucose (Fig. 1A). This reflected insufficient insulin secretion in MHY-treated rats, which was similar to that in control rats (Fig. 1B). MHY is a dietary peptide that has GLP-1-releasing activity *in situ* (16) and *in vitro* (17, 24). In our previous study, MHY was less

effective at inducing GLP-1 secretion than was ZeinH in GLUTag cells (19). Therefore, we expected MHY to induce less GLP-1 secretion than ZeinH in the present study, and this would result in different insulin and glucose responses.

Ileal administration of both ZeinH and MHY induced significant and sustaining increases in total GLP-1 in conscious rats (Fig. 2B). The secretory response of total GLP-1 in MHY-treated rats was unexpectedly similar to that in ZeinH-treated rats. Although MHY induced less GLP-1 secretion than ZeinH *in vitro* at lower concentration (5 mg/ml) (19), the secretion of GLP-1 from ileal L cells might be maximally stimulated by those peptides at the 500 mg/rat in the present study.

In contrast to total GLP-1, the active GLP-1 level was significantly enhanced only by the ileal administration of ZeinH but not by that of MHY (Fig. 2C). This seems to be reflected in the insulin and glucose responses (Fig. 1 and Fig. 2A), but it is not consistent with the total GLP-1 response (Fig. 2B). Since total GLP-1 includes both the active form (7-37) and the partially degraded form (9-37) of GLP-1, the sustained increases of total GLP-1 observed in Fig. 2B might reflect secreted active GLP-1 and accumulated inactive GLP-1. It is possible that large differences between active GLP-1 secretion in ZeinH and MHY treatments result in the small difference observed in total GLP-1.

Changes in active GLP-1 reflect both secreted GLP-1 and biologically active GLP-1 that has not yet been degraded by DPP-IV. The difference between the active GLP-1 (at 0–15 min) levels in MHY- and ZeinH-treated rats could come from the degree of degradation of active GLP-1. That is, the degradation of secreted GLP-1 might be either reduced in ZeinH-treated rats or enhanced in MHY-treated rats. Thus, the present results suggest that the efficacy of enhancing GLP-1 secretion is not necessarily linked to the increase in active GLP-1. Ileal administration of ZeinH significantly increased insulin level at 0 min before the glucose injection without hypoglycemia (Fig. 1B). Such increase in insulin induced by enhanced GLP-1 would be insufficient to cause hypoglycemia in the basal state.

We examined whether ileal ZeinH and MHY affect plasma DPP-IV activity in the ileal vein of anesthetized rats (Experiment 3). It has been reported that approximately half of the secreted active

GLP-1 is already degraded before it enters the systemic circulation (7). DPP-IV exists not only in multiple tissues, such as the liver, kidney and intestine, as a cell-surface membrane-bound protein but also in plasma as a soluble form (31). In the present study, blood collections from the ileal vein enabled us to observe DPP-IV activity in the blood where GLP-1 is released and initial DPP-IV-mediated degradation occurs. Interestingly, ileal administration of ZeinH decreased plasma DPP-IV activity (at 0–15 min), whereas MHY did not cause significant changes in DPP-IV activity (Fig. 3). The slight reductions in the control and MHY groups might be due to the effect of luminal administration. This is the first demonstration that a luminal peptide acutely affects plasma DPP-IV activity. Although the experimental design was not identical to that of Experiment 1, the decrease in plasma DPP-IV activity was coincident with the increase in active GLP-1 (at 0 min) (Fig. 2C) in ZeinH-treated rats. This suggests that the ZeinH-induced increase in active GLP-1 was a consequence of both the stimulation of GLP-1 secretion from L cells and the acute reduction of DPP-IV-mediated degradation of GLP-1. In contrast, MHY only increased total GLP-1 but not active GLP-1, due to the lack of a reduction of DPP-IV activity, which results in a failure of stimulating insulin secretion. A previous paper reported that partial inhibition (20–30%) of plasma DPP-IV activity is sufficient to increase active GLP-1 and to attenuate hyperglycemia by enhancing the incretin effect during the oral glucose tolerance test in lean Zucker rats (32). Therefore, it is likely that the decrement of plasma DPP-IV activity (26.8% at 0 min, 22.1% at 15 min) by luminal ZeinH was sufficient to maintain secreted GLP-1 in the active form.

The mechanism by which ileal ZeinH decreases plasma DPP-IV activity remains to be determined. One possible explanation is the competitive inhibition of DPP-IV by absorbed peptides derived from luminal ZeinH. It has been reported that ingestion of whey protein (WP) inhibits DPP-IV activity in the small intestine in mice (33). In that report, small fragments (di- and tri-peptides) generated by the luminal digestion of whey protein might have become substrates of DPP-IV within the intestinal wall and decreased its activity as competitive inhibitors. In addition, tri-peptides such as diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) have been shown to block DPP-IV-mediated GLP-1

degradation *in vitro* (34, 35) as competitive substrates (36). These reports support our hypothesis that absorbed fragments of ZeinH inhibit DPP-IV activity in the ileal vein. Although ZeinH might be insusceptible to luminal digestion in the ligated ileal loop, brush-border membrane peptidases could hydrolyze it to some degree, resulting in the generation of small peptides that are absorbed and inhibit plasma DPP-IV. The WP-induced decrease of DPP-IV activity was observed only in the small intestinal tissue but not in plasma of the orbital vein (33). In contrast, we could observe a decrease of plasma DPP-IV activity in ZeinH-treated rats by using ileal vein cannulation (Fig. 3).

Zein is a well-known indigestible protein due to its strong hydrophobicity. Although it is slightly less than MHY, ZeinH contained Pro and Ala (Table 1) as well as another Zein hydrolysate prepared with alcalase (37). Because DPP-IV has preferential specificity for X-proline and X-alanine sequences in the N-terminal of small peptides (38), such peptides contained in ZeinH or generated after the digestion of ZeinH might be responsible for DPP-IV inhibition. Free amino acids content was less in ZeinH than MHY, suggesting free amino acids are not responsible for GLP-1 secretion. ZeinH was rich in Leu and Glu (including Gln) compared to MHY. As Leu and Gln are reported to stimulate GLP-1 secretion (39, 40), these amino acids themselves liberated during luminal digestion or peptides containing these amino acids might be involved in ZeinH-induced GLP-1 secretion. Further investigations will be required to clarify active peptides that induce GLP-1 secretion and inhibit DPP-IV, respectively. As a GLP-1-independent mechanism by which luminal ZeinH attenuates hyperglycemina, it is interesting to note that small peptides and free amino acids absorbed from luminal ZeinH might directly stimulate insulin release, since free leucine and arginine are known to have such functions (41).

Oral ZeinH dose-dependently prevented hyperglycemia under IPGTT in conscious rats (Fig. 4). This result further evidences the anti-hyperglycemic effect of ZeinH under physiological condition, and also provides the possibility of application in humans. Although it was predicted that higher dose of ZeinH was required in case of oral administration than that of ileal administration (500 mg/rat), the result in Fig. 4 demonstrated that oral ZeinH at 2 g/kg (~500 mg/rat) is enough to exert its

anti-hyperglycemic activity. It is possible that orally administered ZeinH functioned not only in the ileum to stimulate GLP-1 secretion but also in the upper small intestine to stimulate both GLP-1 and GIP secretion. ZeinH in the duodenum and jejunum induced significant increase in GLP-1 secretion, but smaller than in the ileum in our previous study (19). GIP is another incretin hormone, which is released from enteroendocrine K cells in the duodenum and jejunum by direct contact with luminal nutrients (42, 43). A previous paper has demonstrated peptone-induced GIP secretion in rats (44). If DPP-IV inhibition by ZeinH was occurred in the upper small intestine, degradation of GIP could be prevented as well as that of GLP-1. However, further investigations will be necessary in future to assess these speculations and applications.

Single oral administration of wheat albumin prevents postprandial hyperglycemia in healthy subjects through its α-amylase inhibitory activity (45). In pharmacological therapy, a single oral administration of an α-glucosidase inhibitor, such as acarbose and voglibose, attenuates postprandial glucose and insulin levels (46, 47). The anti-hyperglycemic effects of these compounds depend primarily on the inhibition of carbohydrate digestion and absorption in the small intestine. On the other hand, recent studies have demonstrated that the stimulation of GLP-1 secretion by luminal compounds such as berberin (11), TGR5 agonists (12) and GPR119 agonists (13) is effective for glycemic control. With regard to nutrients, oral glutamine increases GLP-1 and insulin in humans (40). Therefore, such strategies based on an enhancement of endogenous GLP-1 secretion are promising for the prevention of hyperglycemia.

The results of the present study provide the basis for a novel strategy for glycemic control via both stimulating endogenous GLP-1secretion and reducing the degradation of secreted GLP-1. At this time, the anti-hyperglycemic effect is specific for ZeinH. The identification of other peptides with similar functions as ZeinH will be also beneficial for future applications. Although the reduction of DPP-IV activity was observed in the ileal vein in the present study, some reports suggest that GLP-1 acts locally at the site of secretion. The local action of GLP-1 involves the activation of nerve fibers in close proximity to the L cells (7), and postprandial β-cell stimulation by GLP-1 is evoked via a neural

reflex triggered in the hepatoportal system (48, 49).

In summary, we found that ileal administration of ZeinH but not MHY attenuated hyperglycemia by enhancing insulin secretion during IPGTT in conscious rats. Although ZeinH and MHY induced similar increases in total GLP-1, active GLP-1 level was increased only in ZeinH-treated rats. The ileal administration of ZeinH, but not MHY decreased plasma DPP-IV activity in the ileal vein in anesthetized rats. These results indicate that ileal administration of ZeinH both induced GLP-1 secretion and reduced plasma DPP-IV activity, resulting in enhanced insulin secretion. The anti-hyperglycemic activity of ZeinH was also demonstrated in IPGTT with oral administration of ZeinH. Our findings highlight a novel nutritional strategy to improve glycemic control by utilizing the endogenous GLP-1.

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Figure Legends

Figure 1. Plasma glucose and insulin levels during the intraperitoneal glucose tolerance test (IPGTT) in conscious rats after the ileal administration of water, MHY or ZeinH.

Water (2 ml, open square), MHY (500 mg/2 ml, closed triangle), or ZeinH (500 mg/2 ml, closed circle) was administered into the ileum 30 min prior to intraperitoneal glucose injection (1 g/kg). Blood samples were collected from the jugular vein before (–30, 0 min) and after (15, 30, 60 min) the glucose injection. Values are displayed as the means ± SEM of 6 rats in each group. Two-way ANOVA *P* values for glucose (A) and insulin (B) were 0.14 and < 0.01 for the treatments; both < 0.01 for time; and < 0.05 and 0.26 for treatment x time, respectively. Plots at the same time point not sharing the same letter differed significantly between treatments (Fisher's PLSD test, $P < 0.05$).

Figure 2. Plasma glucose, total GLP-1 and active GLP-1 levels during IPGTT in conscious rats after ileal administration of water, MHY or ZeinH.

Water (2 ml, open square), MHY (500 mg/2 ml, closed triangle), or ZeinH (500 mg/2 ml, closed circle) was administered into the ileum 30 min prior to intraperitoneal glucose injection (1 g/kg). Blood samples were collected from the jugular vein before (–30, 0 min) and after (15, 30, 60 min) the glucose injection. Values are displayed as the means ± SEM of 6–8 rats in each group. Two-way ANOVA *P* values for glucose (A), total GLP-1 (B) and active GLP-1 (C) were all <0.01 for the treatment; < 0.01, < 0.01, and < 0.05 for time; and 0.07, 0.23, and 0.11 for treatment x time, respectively. Plus (+) signs indicate significant differences from the basal value (–30 min) in each group (Dunnett's test, $P < 0.05$). Plots at the same time point not sharing the same letter differ significantly between treatments (Fisher's PLSD test, $P < 0.05$).

Figure 3. Changes in plasma DPP-IV activity in the ileal vein in anesthetized rats after ileal administration of water, MHY or ZeinH.

Water (2 ml, open square), MHY (500 mg/2 ml, closed triangle), or ZeinH (500 mg/2 ml, closed circle) was administered into the ligated ileal loop at –30 min, and then glucose (1 g/kg) was injected intraperitoneally at 0 min. Blood samples were collected through the ileal vein catheter before (–30, 0 min) and after (15, 30, 60 min) the glucose injection. Two-way ANOVA *P* values were < 0.05, < 0.05, and 0.76 for treatment, time and treatment x time, respectively. Values are displayed as the means ± SEM of 5–7 rats in each group and are expressed as the percentage of basal (–30 min) activities. *, P < 0.05 compared with basal levels (–30 min) (Dunnett's test, P < 0.05). Plots at the same time point not sharing the same letter differ significantly between treatments (Fisher's PLSD test, P < 0.05).

Figure 4. Plasma glucose level after the oral administration of ZeinH during IPGTT in conscious rats.

Water (8 ml/kg; open square) or 250, 500 mg/ml of ZeinH (2 g/kg; open circle, 4 g/kg; closed circle) was administered orogastrically 15 min prior to intraperitoneal glucose injection (1 g/kg). Blood samples were collected from the tail vein before (–15, 0 min) and after (15, 30, 60, 90, 120 min) the glucose injection. Values are displayed as the means ± SEM of 6-7 rats in each group. Two-way ANOVA *P* values were all < 0.01 for treatment, time and treatment x time, respectively. Plots at the same time point not sharing the same letter differ significantly between treatments (Fisher's PLSD test, P < 0.05).

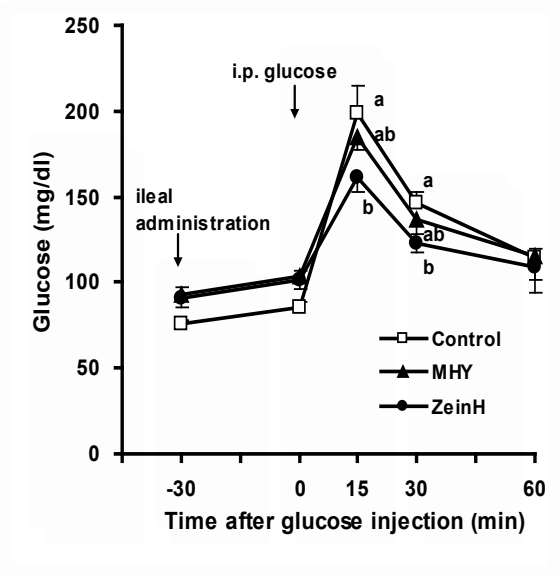
Table 1. ***Free and total amino acid composition of ZeinH and MHY***

	Free (mg/g)		Total (mg/g)	
	ZeinH	MHY	ZeinH	MHY
Ala	2.9	6.8	55.5	92.4
Arg	-	28.9	11.8	103.4
Asp*	-	3.1	62.9	86.7
Glu**	-	9.6	217.1	143.0
Gly	-	5.9	16.2	258.2
His	-	-	5.0	6.9
Ile	10.7	12.1	30.8	26.3
Leu	8.3	14.1	141.1	46.4
Lys	-	7.5	-	47.4
Met	-	-	8.6	-
Phe	-	9.1	47.5	25.9
Pro	3.6	-	70.3	139.6
Ser	-	5.1	42.9	40.8
Thr	-	4.9	20.9	24.1
Tyr	-	-	37.3	6.1
Val	-	6.3	26.8	33.9

Cys and Trp were not detected.
-: not detected, *: includes Asn, **: includes Gln

Figure 1

A


250
200
150
100
50
0
Glucose (mg/dl)
i.p. glucose
ileal
administration
a
ab
b
a
ab
b
Control
MHY
ZeinH
-30
0
15
30
60
Time after glucose injection (min)

B

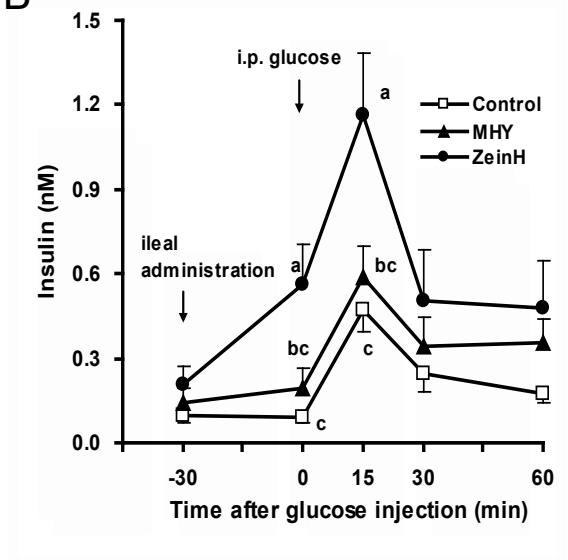

1.5
1.2
0.9
0.6
0.3
0.0
Insulin (nM)
i.p. glucose
a
Control
MHY
ZeinH
ileal
administration
a
bc
bc
c
c
-30
0
15
30
60
Time after glucose injection (min)

Figure 2

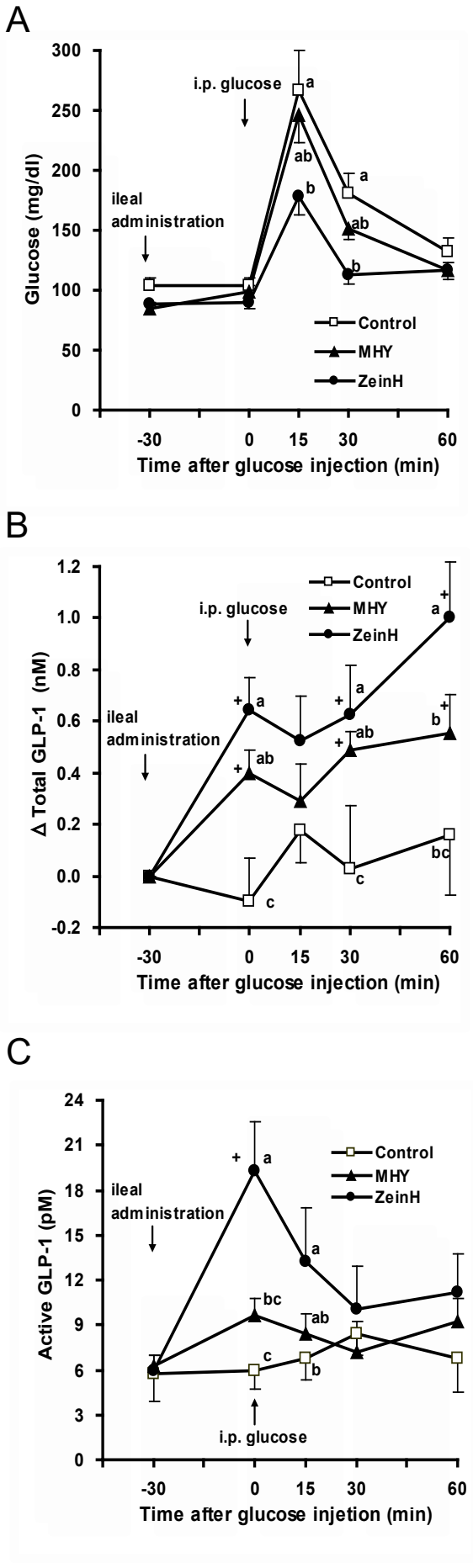

A
300
250
200
150
100
50
0
Glucose (mg/dl)
i.p. glucose
ileal
administration
a
ab
b
a
ab
b
Control
MHY
ZeinH
-30
0
15
30
60
Time after glucose injection (min)
B
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Δ Total GLP-1 (nM)
Control
MHY
ZeinH
i.p. glucose
ileal
administration
+a
+a
+a
+ab
+ab
+b
c
c
bc
-30
0
15
30
60
Time after glucose injection (min)
C
24
21
18
15
12
9
6
3
0
Active GLP-1 (pM)
Control
MHY
ZeinH
ileal
administration
+a
a
bc
ab
c
b
i.p. glucose
-30
0
15
30
60
Time after glucose injetion (min)

Figure 3

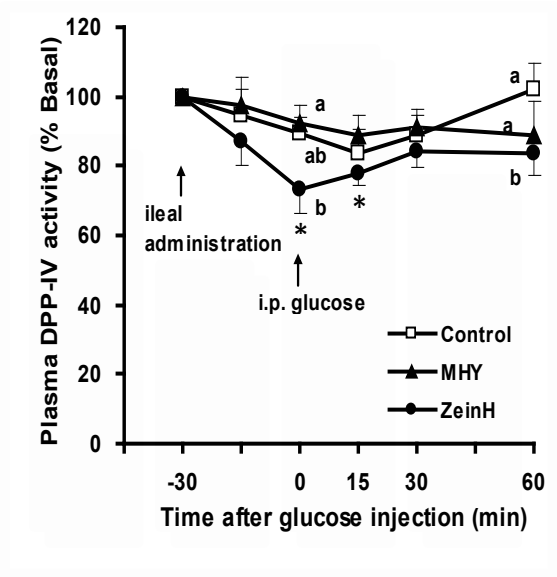
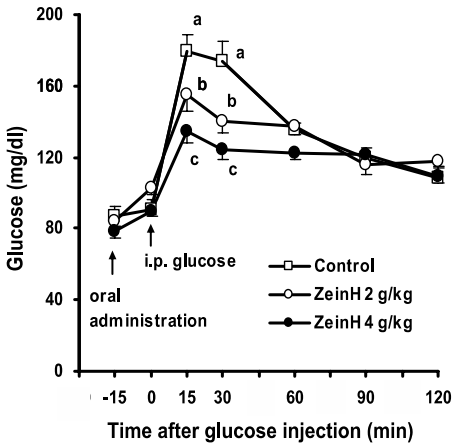

Plasma DPP-IV activity (% Basal)
120
100
80
60
40
20
0
-30
0
15
30
60
Time after glucose injection (min)
ileal
administration
i.p. glucose
a
ab
b
a
a
b
*
*
Control
MHY
ZeinH

Figure 4


200
160
120
80
40
0
Glucose (mg/dl)
a
a
b
b
c
c
i.p. glucose
oral
administration
Control
ZeinH 2 g/kg
ZeinH 4 g/kg
-15
0
15
30
60
90
120
Time after glucose injection (min)