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Title

Rice protein hydrolysates stimulate GLP-1 secretion, reduce GLP-1 degradation, and lower the glycemic response in rats.

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Abstract

Rice has historically been consumed in Asia as a major source of carbohydrates, however, little is known regarding the functional roles of rice protein as a dietary factor. In the present study, we investigated whether peptides derived from rice proteins could stimulate GLP-1 secretion, and that results in reducing glycemia via the incretin effect in normal rats. Hydrolysates were prepared from the protein fraction of rice endosperm or rice bran, and the effects of these hydrolysates on GLP-1 secretion were examined in a murine enteroendocrine cell line GLUTag. Plasma was collected after oral administration of the rice protein hydrolysates, under anesthesia, or during glucose tolerance tests in rats. In anesthetized rats, plasma dipeptidyl peptidase-IV (DPP-IV) activity was measured after ileal administration of the rice protein hydrolysates.

GLP-1 secretion from GLUTag cells was potently stimulated by the rice protein hydrolysates, especially by the peptic digest of rice endosperm protein (REPH) and that of rice bran protein (RBPH). Oral administration of REPH or RBPH elevated plasma GLP-1 concentrations, which resulted in the reduction of glycemia under the intraperitoneal glucose tolerance test. In addition, the plasma DPP-IV activity was attenuated after ileal administration of REPH or RBPH, which resulted in a higher ratio of intact (active) GLP-1 to total GLP-1 in the plasma.

These results demonstrate that rice proteins exert potent stimulatory effects on GLP-1 secretion, which could contribute to the reduction of postprandial glycemia. The inhibitory effect of these peptides on the plasma DPP-IV activity may potentiate the incretin effect of GLP-1.

INTRODUCTION

Rice is a staple food throughout Asia, including Japan, and is recognized as a major source of carbohydrates (energy). However, its consumption in Japan has recently

decreased, from 350 g/day in the 1950s to 150 g/day in the 2000s[1]. This decrease is likely due to the increased consumption of alternative staple foods, such as breads and noodles. In addition, as excessive consumption of digestible starch can cause glucose intolerance (which leads to diabetes mellitus), there is a recent trend in Japan towards reduced consumption of boiled white rice.

The incidence of metabolic syndrome is increasing throughout the world, including in the Asian countries. In Japan, increasing consumption of a Western diet is thought to be the main cause of the increasing rates of obesity, glucose intolerance, diabetes, dyslipidemia, and cardiovascular diseases. A positive association between the consumption of white rice and the risk of type-2 diabetes has been reported[2,3], although this concept remains controversial[4]. However, as described above, there were less diabetic patients in 1950-60's when people consumed much more rice than today. Therefore, it is possible that the consumption of rice has a beneficial effect on glucose and/or lipid metabolism.

Regarding the functional factors present in rice, resistant starch[5] and gamma-oryzanol[6] reportedly improve glucose homeostasis. Several studies have also demonstrated that dietary rice protein effectively reduces serum cholesterol levels[7-11], through the increased excretion of bile acids or neutral sterols. The protein content in rice is 5–7% by weight, and this protein consists of glutelin (80%), globulin (4–15%), albumin (1–5%), and prolamin (2–8%). A recent study showed that dietary rice protein can ameliorate the progression of early stage diabetic nephropathy in a rat model of diabetes[12]. Although these studies have investigated the effects of continuous ingestion of rice proteins on lipid metabolism and kidney function, the acute effects of rice proteins on glucose metabolism are not well understood.

Glucagon-like peptide-1 (GLP-1) is a gut hormone that is produced and released from enteroendocrine L-cells, which are scattered throughout the intestinal epithelium. The primary function of GLP-1 is to enhance glucose-induced insulin secretion (the incretin

effect), which helps to attenuate postprandial hyperglycemia. GLP-1 also has proliferative and protective effects on pancreatic beta cells, as well as multiple effects outside the pancreas including in the liver, adipose tissue, cardiovascular, and central nervous systems[13]. Recently, GLP-1 receptor agonists and dipeptidyl peptidase-IV (DPP-IV) inhibitors have been used to treat type 2 diabetes[14,15]. As GLP-1 and glucose-dependent insulinotropic polypeptide (another incretin) are immediately inactivated by plasma DPP-IV, inhibitors of DPP-IV can help preserve the physiological activity of both these peptides[16].

Recent studies have revealed that, along with glucose and fatty acids, some of dietary proteins and peptides have potent stimulatory effects on the secretion of GLP-1. We have previously reported that a single oral administration of a corn protein zein hydrolysate (ZeinH) effectively lowered rats' glycemic response by stimulating GLP-1 secretion, although this results was not observed with meat hydrolysate[17,18]. Whey protein and glutamine can also induce GLP-1 secretion in animals and humans, although not all dietary proteins/peptides induce a similar "incretinotropic" effect[18,19].

We conducted the present study to investigate the ability of rice protein hydrolysates to stimulate GLP-1 secretion *in vitro* and *in vivo*. The hydrolysates were prepared using rice proteins extracted from the bran and endosperm, and were orally administered respectively to rats. We also evaluated the effect of the hydrolysates on the glycemic response, under oral and intraperitoneal glucose loads. Finally, we examined the effect of luminal administration of the rice protein hydrolysates on plasma DPP-IV activity.

Materials and Methods

Materials

Cell culture consumables (Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin-streptomycin, and trypsin-EDTA solution) were purchased from Invitrogen (Carlsbad, CA, US). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis,

MO, US). Other reagents were purchased from Wako (Osaka, Japan), unless specified.

Preparation of rice protein hydrolysates

Purified rice endosperm protein (REP) was prepared using a previously reported alkali extraction method[20]. Briefly, milled rice flour was mixed with a 0.2% NaOH solution at room temperature for 1 h to extract the protein fraction, and the mixture was then centrifuged. The supernatant was neutralized with 1 M HCl to precipitate the protein fraction; the collected protein fraction was repeatedly washed with distilled water, and then lyophilized as REP. The purity of the REP was >90%, as determined using the Kjeldahl method.

Purified rice bran protein (RBP) was prepared from commercially available RBP powder (TSUNO-RBP, TSUNO CO., LTD). The RBP powder was suspended in water, the pH of the suspension was adjusted to 5.7 using H_2SO_4, and the suspension was then stirred for 1 h at 95°C. The precipitated protein was collected by centrifugation, washed with deionized water and further centrifugation. Lyophilized protein fraction was used as RBP, and its protein purity was 80%, determined using the Kjeldahl method.

Papain digests of REP and RBP were prepared as previously described[19]. In short, 50 g of rice protein was suspended in deionized water (500 mL), and the pH of the suspension was adjusted to 7.2 using NaOH. After 250 mg of papain (papain F, Asahi Food and Health Care, Tokyo, Japan) was added, the suspension was shaken for 60 min at 55°C. The suspension was then treated in boiling water for 20 min to stop the enzymatic reaction. After filtration with a 0.45-μm filter, the pH was adjusted to 7.0, and the solution was lyophilized.

Pepsin digests of REP and RBP were prepared as follows[21-24]: 50 g of rice protein was suspended in 0.02N phosphoric acid (1,000 mL), and the pH of the solution was adjusted to 1.85 with 20N phosphoric acid. After the addition of 250 mg pepsin (from porcine gastric mucosa, Sigma, St. Louis, MO), the suspension was shaken for 30 min or 60 min at 37°C,

and then treated in boiling water for 20 min to stop the enzymatic reaction. The pH of the suspension was adjusted to 7.0 with $Ca(OH)_2$. After centrifugation at 3,300 × *g*, the supernatant was collected and frozen overnight at –30°C. When the supernatant was thawed the following day, it was centrifuged again, subjected to filtration (using a 0.45 μm filter) to remove the calcium phosphate, and finally lyophilized.

Papain was chosen because papain-treated Zein (ZeinH) had potent GLP-1 secretory activity *in vitro* and *in vivo*[19], and pepsin was chosen to partially mimic gastric digestion. The degree of hydrolysis (DH) of rice protein hydrolysates was determined by using the trinitrobenzenesulfonic method[25,26]. The values of DH were as follows: 6.3% for REP papain digest (60 min), 7.3% for REP pepsin digest (30 min), 8.2% for REP pepsin digest (60 min), 5.3% for RBP papain digest (60 min), 9.8% for RBP pepsin digest (30 min), and 10.3% for RBP pepsin digest (60 min).

Rice protein hydrolysates were analyzed using HPLC system (Waters 2695 system, Waters, Milford, MA) and an octadecyl silica column (CAPCELL PAK C18 MGII, 5 μm, 4.6 mm x 250 mm, Shiseido Co., Ltd., Tokyo, Japan) with a linear gradient of acetonitrile/water from 5 to 40% v/v for 45 min, from 40 to 100% v/v for following 20 min (from 45 to 65 min), and then 100% v/v for 5 min.

Cell culture

The GLP-1 producing murine cell line GLUTag (a gift from Dr. D. J. Drucker, University of Toronto, Canada) was cultured in Dulbecco's modified Eagle's medium (Invitrogen, cat. no. 12100-038), which was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were grown in a humidified 5% CO_2 atmosphere at 37°C, and cells were routinely subcultured using trypsinization upon reaching 80–90% confluency.

157 **Animals**

158 Male Sprague Dawley (SD) rats (6 weeks old, 150–170 g) were purchased from Japan
159 SLC (Hamamatsu, Japan). All animals were housed in individual cages, had free access to
160 water, and received a semipurified AIN-93G diet[27] containing 25% casein. All animal
161 experiments were performed after an acclimation period (4–7 days) in a
162 temperature-controlled room that was maintained at 23 ± 2°C with a 12-hour light, 12-hour
163 dark cycle (light period, 8:00 AM to 8:00 PM).

164 This study was approved by the Hokkaido University Animal Committee, and the animals
165 were maintained in accordance with the Guide for the Care and Use of Laboratory Animals
166 of Hokkaido University.

167

168 **Cell Culture Experiment (Experiment 1)**

169 Experiment : GLP-1 Secretion by GLUTag Cells

170 GLUTag cells were grown for 2 days in 48-well culture plates at a density of 1.25×10^5
171 cells/well, until they reached 80–90% confluency. Cells were washed twice with HEPES
172 buffer (140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM
173 D-glucose, 1 mg/mL BSA, pH 7.4) to remove the culture media, and then exposed to the
174 test agents (60 min at 37°C), which were dissolved in the same buffer. In this experiment,
175 70 mM potassium chloride (KCl) was used as a positive control[19]. Supernatants were
176 collected from the wells after the exposure, and centrifuged at 800 × *g* for 5 min at 4°C to
177 remove the remaining cells, then stored at –50°C until the GLP-1 concentration was
178 measured with a commercial enzyme immunoassay kit (Yanaihara Institute, Shizuoka,
179 Japan). The GLP-1 assay detects intact GLP-1 (7-36) and metabolite GLP-1 (9-36) with
180 100% and 95.6% cross-reactivity, respectively. Cross-reactivities for GLP-1 (1-37), GLP-1
181 (1-36) amide, and GLP-1 (7-37) are <0.1%, 0.3%, and <0.1%, respectively. The minimum
182 detection limit for the assay is 60 pM for GLP-1 (7-36) amide. The intra- and interassay

coefficients for this kit in rat plasma are 5.36–6.60% and 5.51–18.87%, respectively.

Animal Experiments (Experiments 2-4)

To measure plasma glucose, GLP-1, and insulin, blood samples were collected into a 1.5 mL tube containing aprotinin (final concentration, 500 kIU/mL), heparin (final concentration, 50 IU/mL), and a DPP-IV inhibitor (final concentration, 50 μM; DPP4-010; Millipore Co, Billerica, MA). Plasma was separated by centrifugation at 2,300 × *g* for 10 min at 4°C, and then frozen at –80°C until testing. Plasma glucose concentrations were measured using the Glucose CII test kit (Wako), insulin was measured using an ELISA kit (AKRIN-010T; Shibayagi Co, Ltd, Gunma, Japan), and active GLP-1 and total GLP-1 were measured using the respective ELISA kits (EGLP-35K and EZGLP1T-36K; Millipore Co, Billerica, MA).

Experiment 2: The effects of oral REPH or RBPH on plasma GLP-1 in rats

REPH, RBPH (both, 2 g/kg body weight), or water (12 mL/kg body weight) was orally administrated to rats by using a feeding tube (5 Fr; Atom Medical Co, Tokyo, Japan). A blood sample (1 mL) was collected from the portal vein (under isoflurane anesthesia) 15 min after the oral administration. As untreated control, portal blood was collected from rats without oral administration. The concentration of total GLP-1 in the plasma was measured as described above.

Experiment 3: The effects of oral REPH or RBPH on plasma glucose, GLP-1, insulin, and gastric emptying under the oral glucose tolerance test

To examine the effects of oral REPH or RBPH on the glycemic response to oral glucose load, the oral glucose tolerance test (OGTT) was performed in conscious rats. After basal blood collection (0 min), glucose solution (2 g/kg body weight) or the solution containing REPH (2 g/kg body weight) or RBPH (2 g/kg body weight) was orally administered (10

209 mL/kg body weight) by using a feeding tube. These test solutions contained acetaminophen
210 (100 mg/kg body weight) to assess the gastric emptying rate[28,29]. Blood samples (120 μL)
211 were collected from the tail vein 15, 30, 60, 90, and 120 min after the oral administration,
212 and the plasma glucose, insulin, and total GLP-1 levels were measured as described above.
213 The plasma acetaminophen concentration was measured using an acetaminophen
214 detection kit (01601-96; Kanto Kagaku, Tokyo, Japan).
215
216 Experiment 4: The effects of oral REPH or RBPH on plasma glucose, GLP-1, and insulin
217 under the intraperitoneal glucose tolerance test
218 The intraperitoneal glucose tolerance test (IPGTT) was used to evaluate the GLP-1–
219 mediated glycemic control induced by oral REPH or RBPH. After an overnight fast, blood
220 samples were collected from the tail vein before and after intraperitoneal glucose injection
221 (–15, 0, 15, 30, 60, 90, and 120 min), and the plasma levels of glucose, insulin, and total
222 GLP-1 were measured as described above.
223 To examine the dose-response effect for REPH, it was dissolved in deionized water (1 or
224 2 g/kg body weight), and was administered orally (12 mL/kg body weight) using a feeding
225 tube. Deionized water was used as a negative control. Fifteen minutes after oral
226 administration, a blood sample was collected, and then the glucose solution was injected
227 intraperitoneally (1 g/kg body weight, 0 min).
228 In a separated experiment, the glucose solution was injected intraperitoneally (1 g/kg
229 body weight, 0 min) immediately after oral administration of REPH (2 g/kg body weight),
230 RBPH (2 g/kg body weight), or deionized water (12 mL/kg body weight). Blood samples
231 were then collected as described above.
232
233 Experiment 5: The effects of ileal administration of REPH or RBPH on plasma DPP-IV
234 activity in the ileal vein of anesthetized rats (in situ experiment)

After an overnight fast, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, Somnopentyl Injection; Kyoritsu Seiyaku Co, Tokyo, Japan), and a midline abdominal incision was made. Body temperature was maintained with heating pads during the experiment. 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 of the mesenteric tissue, and the ileal lumen was washed with saline. The ligated ileal loop (length, 30 cm) 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 blood sample (0 min, 50 μL) was drawn from the ileal vein catheter, and the catheter was refilled with sterilized saline containing heparin (50 IU/mL; Ajinomoto, Tokyo, Japan) between each sampling. Two milliliters of saline, REPH (250 mg/mL), or RBPH (250 mg/mL) was then administered directly into the loop. Blood samples (50 μL) were collected through the ileal vein catheter at 15, 30, 45, and 60 min after the ileal administration to measure the plasma activity of DPP-IV. For active GLP-1 and total GLP-1 measurement, ileal vein blood samples were collected 60 min after the ileal administration. Blood samples for DPP-IV activity measurement were drawn into a syringe containing heparin sodium salt (final concentration, 50 IU/mL; Nacalai Tesque, Kyoto, Japan), and samples for active or total GLP-1 measurement were drawn into a syringe containing aprotinin (final concentration, 500 kIU/mL), heparin (final concentration, 50 IU/mL), and DPP-IV inhibitor (final concentration, 50 μM). The plasma was separated by centrifugation at 2,300 × *g* (10 min at 4°C), and then frozen until testing.

DPP-IV activity was determined based on the rate of hydrolysis of a surrogate substrate (Gly-Pro-*p*-nitroaniline, Gly-Pro-pNA, Sigma), as previously described[30,31]. Five microliters of plasma were added to each well of a flat-bottomed 96-well plate, and then 80 μL of assay buffer (0.25 M Tris-HCl buffer, pH 8.0) was added. The reaction was started by adding 80

μL of 1.6 mM Gly-Pro-pNA in deionized water. After an incubation at 37°C for 60 min, 40 μL of 1 M acetate (pH 4.2) was added to stop the reaction, and the absorbance at 405 nm was measured ("absorbance A") using a microplate reader (infinite 200; Tecan Group Ltd., Männedorf, Switzerland). To correct for the influence of hemolysis, a negative control was also prepared for each plasma sample, and the absorbance at 405 nm was measured ("absorbance B") when the plasma added to the assay buffer containing substrate and acetate after the 60-min incubation. The plasma activity of DPP-IV was defined as the liberation of *p*NA from Gly-Pro-*p*NA, and was calculated by subtracting "absorbance B" from "absorbance A". One unit of DPP-IV activity was defined as the liberation of 1 μmol of *p*NA in 1 min.

Statistical analysis

All results were expressed as mean ± SEM. Statistical significance was assessed using one-way or two-way ANOVA to assess the main effects (treatment and time), as well as the interaction effect (treatment × time). The significance of differences between the mean values was evaluated using Tukey-Kramer's test, Dunnett's test, or Student's t-test, as appropriate and described in figure legends.

Results

GLUTag cells were exposed to various protein hydrolysates dissolved in HEPES buffer, and each rice protein hydrolysate was observed to stimulate GLP-1 secretion with varying potency (Fig. 1A). Among the REP hydrolysates, the papain digest (Papain 60 min) and pepsin 30 min digest (Pepsin 30 min) had similar potency, although the 60 min pepsin digest (Pepsin 60 min) had a lesser effect. Among the RBP hydrolysates, the pepsin digests had higher potency compared to the papain digest. In addition, the 30 min pepsin digests of REP and RBP induced a relatively higher GLP-1 secretion compared to the positive control peptide (zein hydrolysate) (Fig. 1B)[19].

As the 30 min pepsin digests of REP and RBP potently stimulated GLP-1 secretion from GLUTag cells, and their yield was higher than that of papain digestion, we selected the 30 min pepsin digestion for REP and RBP in the subsequent experiments. Each hydrolysate was designated as REPH and RBPH, respectively.

To examine the effect of the rice protein hydrolysates on plasma GLP-1 levels *in vivo*, we collected portal blood 15 min after oral administration of REPH or RBPH (2 g/kg body weight). The plasma GLP-1 concentration in REPH-treated rats was significantly higher than that in control (water-treated) rats (Fig. 3A), and it was also significantly higher than that in untreated (no oral administration) rats. In a separated experiment, the portal GLP-1 concentration in RBPH-treated rats was significantly higher compared to that in control (water-treated) rats (Fig. 3B).

We next examined whether REPH- or RBPH-stimulated secretion of GLP-1 affected glycemia via the incretin effect (the main function of GLP-1) under the oral glucose tolerance test (OGTT). Oral gavage with REPH resulted in attenuation of the glycemic response to oral glucose load at 15 min (0.5–2.0 g/kg) and 30 min (1.0–2.0 g/kg) (Fig. 4A). However, no significant difference in the area under the curve (AUC) was observed for each group (data not shown). The plasma GLP-1 concentration only increased in the 2.0

311 g/kg REPH group, and that was significantly higher from 30 min to 90 min compared to that
312 in the control group (Fig. 4B). However, the plasma insulin concentration at 15 min was
313 suppressed by REPH in a dose-dependent manner (Fig. 4C). The plasma insulin
314 concentration in the 2.0 g/kg group was lowest at 15 min, although this concentration was
315 higher than the concentrations in the control group at 60 and 90 min. The acetaminophen
316 test is used to assess the gastric emptying rate, as acetaminophen is absorbed rapidly in
317 the intestine after emptied from the stomach[28,29]. The change in plasma acetaminophen
318 concentration was lower in the 1.0 g/kg REPH group (at 15 min and 30 min) and in the 2.0
319 g/kg REPH group (from 15 min to 90 min) (Fig. 4D), which indicated that gastric emptying
320 was delayed by oral REPH.
321 The glycemic response during oral glucose load showed a dose-dependent effect, and it
322 was significantly suppressed by oral RBPH gavage at 15 min (1.0–2.0 g/kg) and 30 min
323 (0.5–2.0 g/kg) (Fig. 5A), compared with the control treatment. However, the AUC did not
324 differ for each group (data not shown). The plasma GLP-1 concentration was significantly
325 increased in the 2.0 g/kg RBPH group (from 30 min to 60 min) compared to the control
326 treatment. The insulin response at 15 min tended to be reduced by oral RBPH, although
327 this result was not statistically significant. The changes in plasma acetaminophen levels
328 were reduced in a dose-dependent manner by RBPH as well as REPH.
329 Although increased GLP-1 and reduced glycemic responses were observed treatment
330 with oral REPH and RBPH, the involvement of GLP-1 (the incretin effect) was unclear in
331 OGTT, due to the delayed gastric emptying response (Fig. 4 and 5). Therefore, we
332 conducted IPGTT to eliminate the possible effect of delayed gastric emptying and luminal
333 glucose-induced GLP-1 secretion on the glycemic response, as well as to evaluate the
334 effect of oral REPH or RBPH on GLP-1 secretion (Fig. 6 and 7).
335 Under IPGTT, 2.0 g/kg oral REPH resulted in the attenuation of the glycemia at 15 min
336 and 30 min (Fig. 6A), although the AUC did not differ for each group (data not shown). The

plasma GLP-1 concentrations were increased in a dose-dependent manner by REPH. In addition, 2.0 g/kg REPH induced continuous elevation of plasma GLP-1 from 15 to 60 min, and these levels were significantly higher than the levels in the control group (Fig. 6B). Oral REPH (1.0 g/kg) also resulted in the elevation of plasma GLP-1 at 0 min and 30 min (Fig. 6B). Plasma insulin concentrations tended to be increased by 2.0 g/kg REPH (Fig. 6C).

We also conducted IPGTT to examine the effect of oral RBPH on GLP-1 secretion and glycemic control. In this experiment, RBPH or REPH was orally administered immediately before the intraperitoneal injection of glucose (0 min), as RBPH failed to reduce the glycemic response in the preliminary study when it was provided at other time points (e.g., 15 min or 30 min before the intraperitoneal injection of glucose).

Oral administration of RBPH or REPH resulted in the attenuation of the glycemic response at 30 min, and the potency was similar for both hydrolysates (Fig. 7A). Plasma GLP-1 concentrations were increased by both hydrolysates in a similar manner, which lasted until 60 min and significantly higher than control treatment (Fig. 7B). Plasma insulin concentrations were also significantly increased by RBPH and REPH at 15 min (Fig. 7C).

GLP-1 is rapidly inactivated by DPP-IV in the plasma[32], and a recent study has demonstrated that peptides derived from rice bran can inhibit DPP-IV activity[33]. Therefore, we investigated whether luminal REPH or RBPH affected the plasma activity of DPP-IV in the ileal vein, and thereby prolonged the activity of intact plasma GLP-1. The plasma activity of DPP-IV in the ileal vein decreased gradually after ileal administration of REPH or RBPH (Fig. 8A). In the REPH group, the plasma activity of DPP-IV at 30 min was significantly lower than that in the control group. In the RBPH group, the DPP-IV activity at 45 min was significantly lower than the basal (0 min) activity. We also measured the active and total GLP-1 concentrations in the ileal vein plasma 60 min after the ileal administration of REPH or RBPH. Although the total GLP-1 concentration was only significantly elevated in the RBPH group (Fig. 8B), the concentrations of active GLP-1 were significantly elevated

in the REPH and RBPH groups, compared with the control group (Fig. 8C). Because the incremental degrees in GLP-1 levels for the REPH and RBPH groups (compared to the control group) were larger for active GLP-1 (~3×) than for total GLP-1 (2–2.5×), we calculated the ratio of active GLP-1 to total GLP-1 (Fig. 8D). The ratios in the REPH group (51.4%) and in the RBPH group (54.3%) were approximately 2× higher than that in control group (28.4%), and this difference was statistically significant.

Discussion

In this study, we investigated whether rice protein-derived dietary peptides could stimulate GLP-1 secretion both in an enteroendocrine cell line and in rats, and whether this effect might contribute to the improvement of glycemia. Rice protein hydrolysates (including REPH and RBPH) potently stimulated GLP-1 secretion from GLUTag cells, and oral administration of REPH or RBPH resulted in significant elevation of GLP-1 concentrations in the portal and tail vein plasma. The incretin effect by oral REPH or RBPH was clearly observed under IPGTT, as well as a continuous elevation of plasma GLP-1. In addition, the plasma DPP-IV activity was decreased by the ileal administration of REPH or RBPH in anesthetized rats. This observation was also supported by the higher ratio of intact (active) GLP-1 to total GLP-1. These results revealed that peptides derived from rice proteins can stimulate secretion of GLP-1 *in vivo*, which effectively lowers the glycemic response. In addition, the inhibitory effect of these peptides on the plasma DPP-IV activity may potentiate the incretin effect of secreted GLP-1.

GLUTag cells are widely used as an enteroendocrine cell model to investigate the effect of various molecules on the secretion of GLP-1, including glucose[34], fatty acids[35], peptides[19] and amino acids[36]. We observed that hydrolysates prepared from REP and RBP, particularly the 30 min pepsin digests (REPH and RBPH), potently stimulated GLP-1 secretion in GLUTag cells (Fig. 1). Intriguingly, the potency of GLP-1 secretion varied by

390 enzyme (pepsin or papain) and the digestion length (30 min or 60 min) despite the same
391 origin of rice proteins (REP or RBP). For RBP, the peptide sequence, which is cleaved
392 preferably by papain, might contribute to the triggering of GLP-1 secretion. The weaker
393 GLP-1 secretion produced by the 60 min digests in REP hydrolysates (compared to the 30
394 min digest) suggests that oligo- or larger peptides, rather than small peptides or free amino
395 acids, might be responsible for this effect. HPLC analysis (Fig. 2) demonstrated both REPH
396 and RBPH contain so many peptide fragments, which may be due to partial digestion by
397 relatively short time (30 min) treatment with pepsin. REPH mainly contains hydrophobic
398 peptides but RBPH contains both hydrophilic and hydrophobic peptides. The specific
399 structures of the active peptides should be clarified in future studies.
400 Oral administration of REPH or RBPH induced a significant increase in GLP-1
401 concentration in the portal (Fig. 3) and tail veins (Fig. 4-7) with or without oral glucose, as
402 demonstrated in experiments 2-4. Under OGTT, both REPH (Fig. 4) and RBPH (Fig. 5)
403 lowered glycemic response accompanied with decreasing tendency of insulin response.
404 The glucose-lowering effect under OGTT is likely due to the decrease of glucose absorption
405 in the small intestine, rather than insulin-dependent glucose uptake into the peripheral
406 tissues. This interpretation is supported by the ability of REPH and RBPH to suppress
407 gastric emptying assessed by the acetaminophen test (Fig. 4D and 5D). As GLP-1 is known
408 to suppress gastric emptying as well as other gut hormones (e.g., cholecystokinin,
409 serotonin, peptide-YY)[37], increased GLP-1 secretion might also be involved in the
410 glucose-lowering effect of REPH and RBPH.
411 IPGTT (Fig. 6-7) is an appropriate method to evaluate the GLP-1 secretion and incretin
412 effects of orally administered factors, as it avoids various confounding factors, such as
413 glucose outflow from the stomach, glucose absorption in the small intestine, and luminal
414 glucose-induced GLP-1 secretion. In contrast to OGTT, oral administration of REPH or
415 RBPH enhanced insulin response under IPGTT (Fig. 6C and 7C), which is likely due to the

increased GLP-1 secretion (Fig. 6B and 7B). Because REPH or RBPH was administered in the absence of oral (luminal) glucose, the increased GLP-1 secretion should be induced by luminal rice peptides. In our previous[18] and preliminary study, oral administration of hydrolysates prepared from meat, soy protein or wheat gluten was not effective to reduce glycemic response under IPGTT. Thus, the present results clarified that rice peptides can potently induce GLP-1 secretion, thereby lowering the glycemic response through the incretin effect.

In contrast to REPH (Fig. 6), oral RBPH 15 min before the intraperitoneal glucose injection failed to attenuate the glycemic response (data not shown), which suggests that there exists an optimal time for respective dietary peptides to exert their GLP-1-mediated glucose-lowering effect. This optimal time is likely dependent on the digestibility and stability of the peptides in the stomach and small intestine. Rice endosperm protein contains mainly glutelin (an alkaline soluble protein) and prolamin (an alcohol soluble protein), while rice bran protein contains mainly globulin (a salt soluble protein)[38-41]. These differences in the protein component of REP and RBP might affect their properties *in vivo*, although further studies are needed to test this hypothesis.

Although we observed that REPH and RBPH reduced the glycemic response in the early period, the plasma glucose concentrations in the REPH and RBPH groups were slightly increased in the late period compared to the control group (90 and 120 min). A previous study has reported that exendin-4 (a GLP-1 receptor agonist) increased plasma glucose concentrations via the sympathetic nervous system[42]. Similar pathways affected by REPH or RBPH might be related to the late increase in plasma glucose.

DPP-IV is one of serine proteases, and is known to inactivate GLP-1, GLP-2, and glucose-dependent insulinotropic polypeptide[43]. Recently, oral inhibitors of plasma DPP-IV have been used to treat type 2 diabetes mellitus. In addition, several studies have reported that the activity of DPP-IV is reduced by various dietary peptides, including corn zein

hydrolysate[17], whey hydrolysate[44], casein-derived peptides[45], and rice bran peptides[33], possibly acting as competitive inhibitors. We observed that luminal administration of rice protein hydrolysates attenuated the plasma DPP-IV activity in anesthetized rats (Fig. 8A). Because peptides produced from defatted rice bran have potent inhibitory effects on DPP-IV *in vitro*[33], it was possible that such peptides were absorbed into the ileal vein, and inhibited the plasma DPP-IV activity.

Moreover, ileal administration of REPH and RBPH both stimulated GLP-1 secretion and increased the ratio of active GLP-1 to total GLP-1 (Fig. 8B-D), which might be attributed to the reduced plasma activity of DPP-IV. Dietary peptides, including REPH, RBPH, and corn zein hydrolysate[17], may contribute to the reduced postprandial glycemia through their synergistic effects on GLP-1 secretion and degradation. Glucose and fatty acids are also known to induce GLP-1 secretion. However, these molecules would not affect the plasma activity of DPP-IV. The synergistic effects that we observed might be unique to dietary peptides.

Several dietary components, including peptides (whey, casein, zein) and amino acids (glutamine, ornithine, leucine), have been reported to induce GLP-1 secretion *in vitro* and *in vivo*[19,46-49]. However, limited studies have examined the effects of these peptides and amino acids on the insulin and glycemic responses together with GLP-1 response. Furthermore, the effects of ingested dietary factors on DPP-IV have also not been studied. The synergistic effects that we have suggested require further investigation, particularly regarding the identification of specific peptide sequences that are responsible for triggering GLP-1 secretion and reducing the plasma DPP-IV activity.

Rice is typically consumed in Asia as a source of carbohydrates rather than a source of protein, given its low protein content (5-7%). However, our results raise a novel hypothesis that rice proteins contribute to preventing postprandial hyperglycemia (which is caused by the easily digested rice starch). To test this hypothesis, future studies must evaluate

whether long-term ingestion of rice proteins or peptides improves glucose homeostasis. In addition, these studies should evaluate rice peptides as functional dietary factors that can help prevent obesity and glucose intolerance through their synergistic effects.

In conclusion, protein hydrolysates prepared from rice endosperm or rice bran potently stimulated GLP-1 secretion in an enteroendocrine cell model and in normal rats. Oral administration of the protein hydrolysates effectively reduced the glycemic response by increasing the levels of GLP-1 and insulin. In addition, luminal administration of these peptides attenuated the plasma DPP-IV activity, which was reflected by increased ratio of intact GLP-1 to total GLP-1. These results reveal a novel function of rice peptides as a dietary factor that can reduce postprandial glycemia through increase in the secretion of GLP-1 and decrease in plasma DPP-IV activity.

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Figures

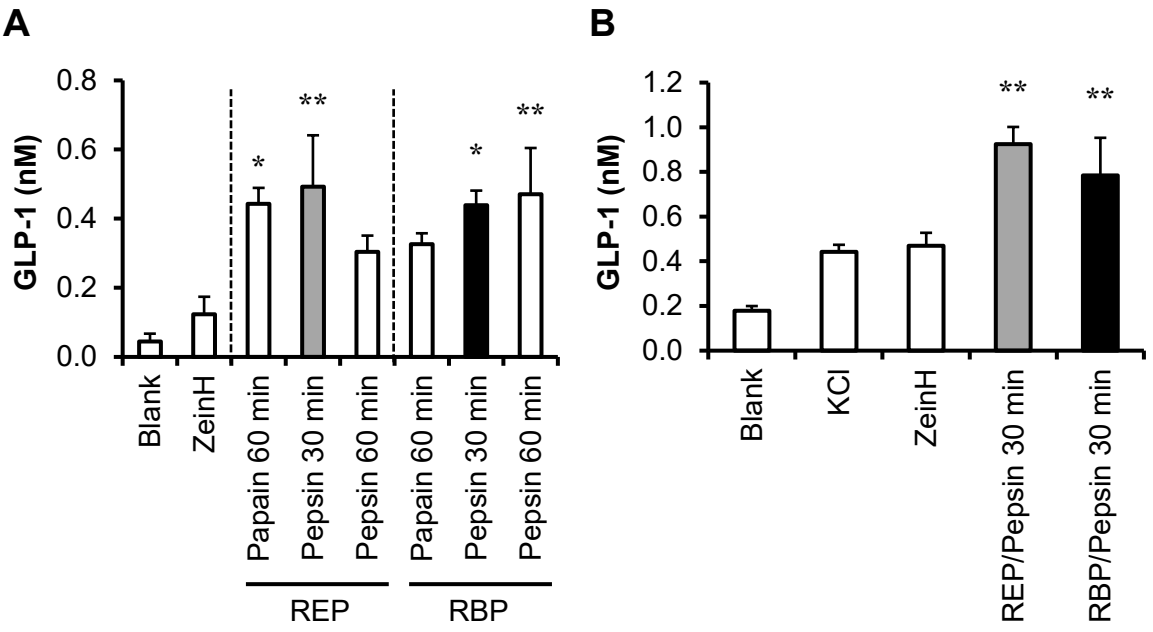

Fig. 1. GLP-1 secretion from GLUTag cells by various protein hydrolysates

GLUTag cells were exposed to various protein hydrolysates (10 mg/mL), or 70 mM KCl for 60 min. The supernatants were collected and assayed using a GLP-1 enzyme immunoassay. REP, rice endosperm protein; RBP, rice bran protein; ZeinH, corn zein hydrolysate. Values are mean ± SEM (n = 4). Asterisks (*) indicate significant differences compared to the blank treatment (*P < 0.05, **P < 0.01; Dunnett's test).

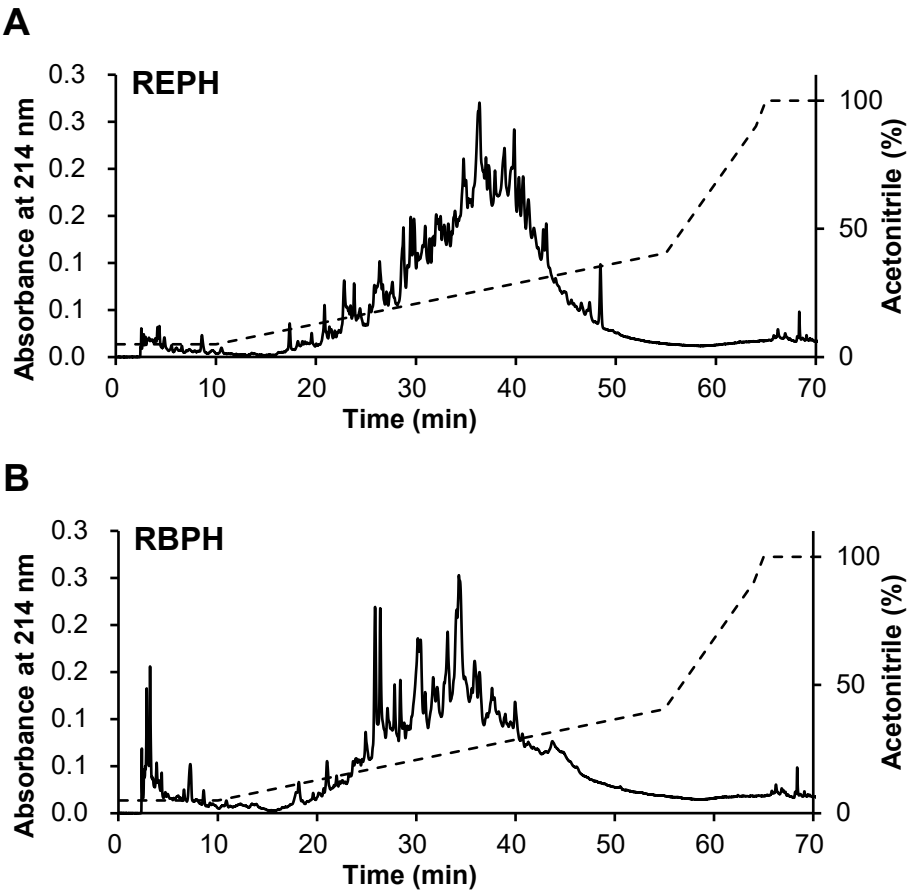

Fig. 2. HPLC chromatograms of REPH and PBPH.

REEPH (A) and RBPH (B) were respectively subjected to reverse phase HPLC analysis using octadecyl silica column with a linear gradient of acetonitrile/water (broken line), and the optical absorbance at 214 nm (solid line) was monitored.

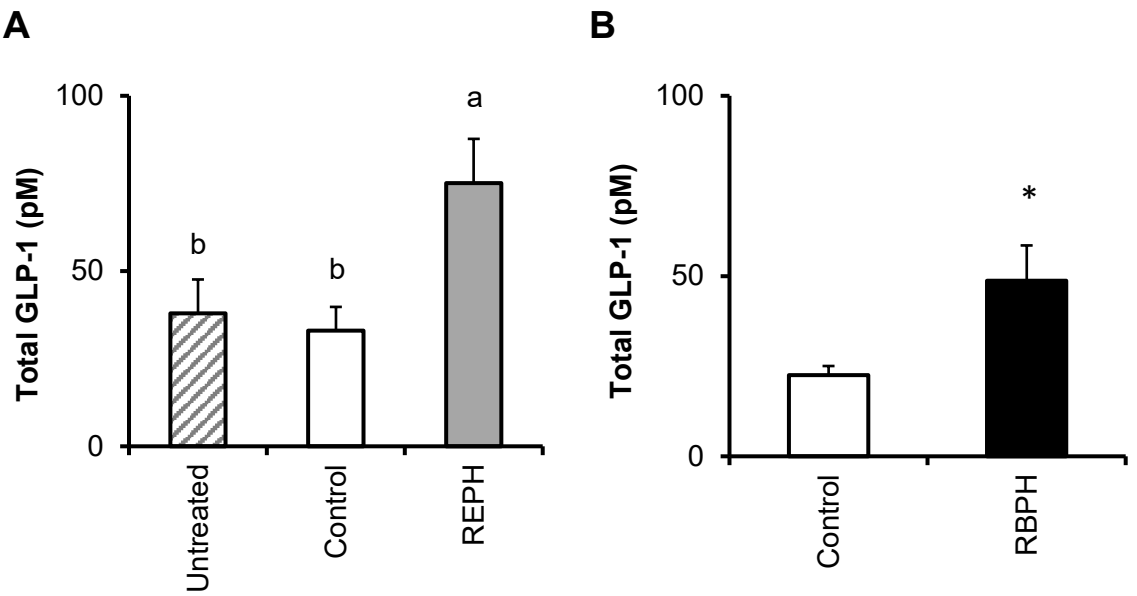

Fig. 3. Total GLP-1 concentration in the portal vein 15 min after oral administration of REPH or RBPH

Blood samples were collected from portal vein after oral administration of REPH (2 g/kg), RBPH (2 g/kg), or water (control), and the plasma concentration of total GLP-1 was measured. As the untreated control, blood samples were collected from rats that did not receive any oral treatment. Values are mean ± SEM (n = 4–6). (**A**) Bars that do not share the same letter are significantly different ($P < 0.05$; Tukey–Kramer's test). (**B**) The asterisk (*) indicates a significant difference compared to the control treatment (*$P < 0.05$; Student's t-test).

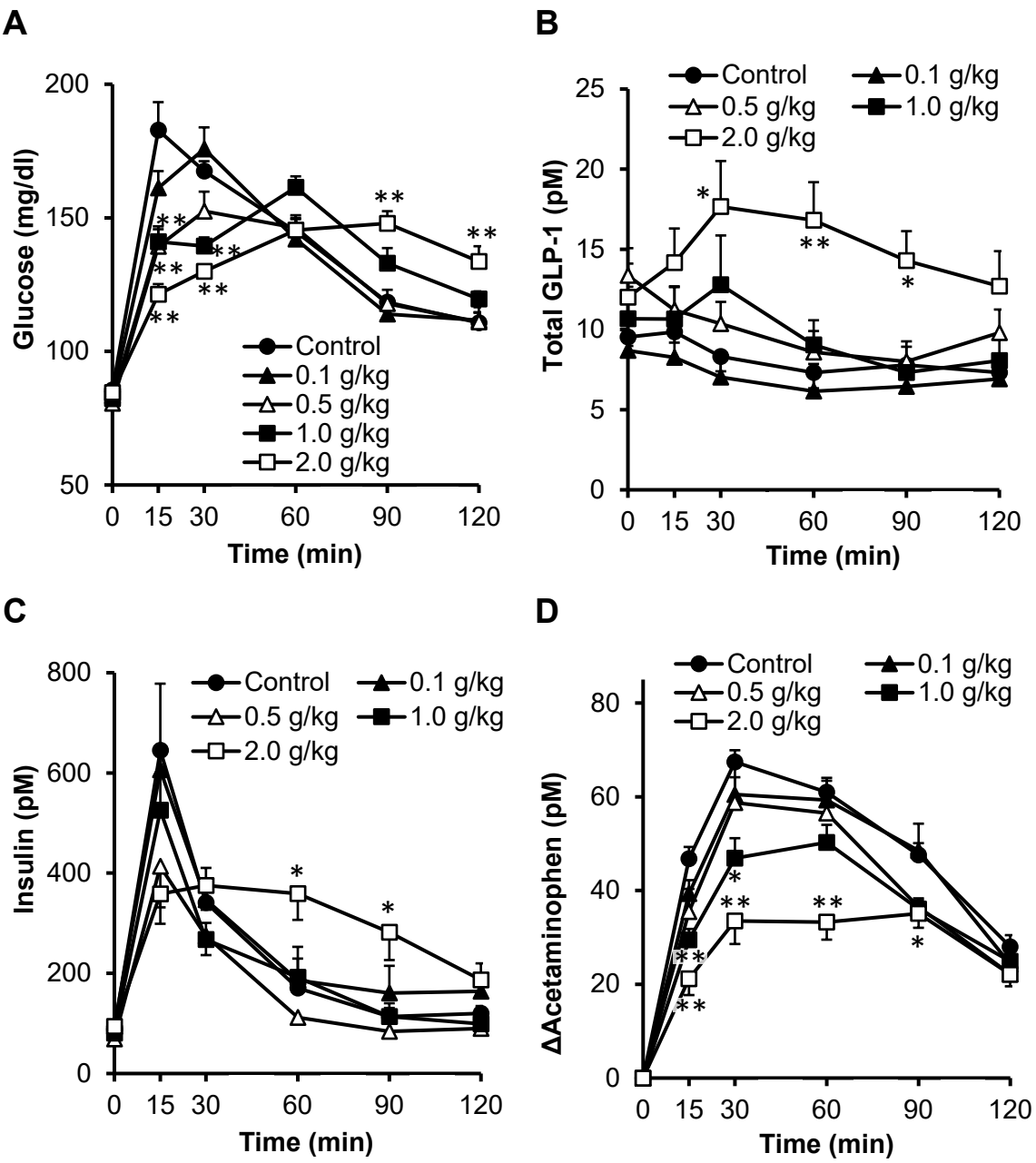

Fig. 4. Changes in plasma glucose, total GLP-1, insulin, and acetaminophen after the oral administration of REPH under the oral glucose tolerance test.

Glucose solution as control (●, 2 g/kg, 10 mL/kg), or the solution containing REPH at 0.1 g/kg (▲), 0.5 g/kg (△), 1.0 g/kg (■), or 2.0 g/kg (□) was administered orally to rats after an overnight fast. Acetaminophen (100 mg/kg) was added to each solution. Blood samples were collected from the tail vein before (0 min) and after the oral administration, as indicated. Plasma concentrations of glucose, total GLP-1, insulin, and acetaminophen were

610 measured. The two-way ANOVA P-values were 0.137 (treatment), <0.01 (time), and <0.01
611 (treatment × time) for glucose (**A**); <0.05 (treatment), <0.01 (time), and <0.01 (treatment ×
612 time) for total GLP-1 (**B**); 0.133 (treatment), <0.01 (time), and <0.01 (treatment × time) for
613 insulin (**C**); and <0.01 (treatment), <0.01 (time), and <0.01 (treatment × time) for
614 acetaminophen (**D**). Values are mean ± SEM (n = 5–6). Asterisks (*) indicate significant
615 differences compared to the control treatment at the same time point (*P < 0.05, **P <
616 0.01; Dunnett's test).
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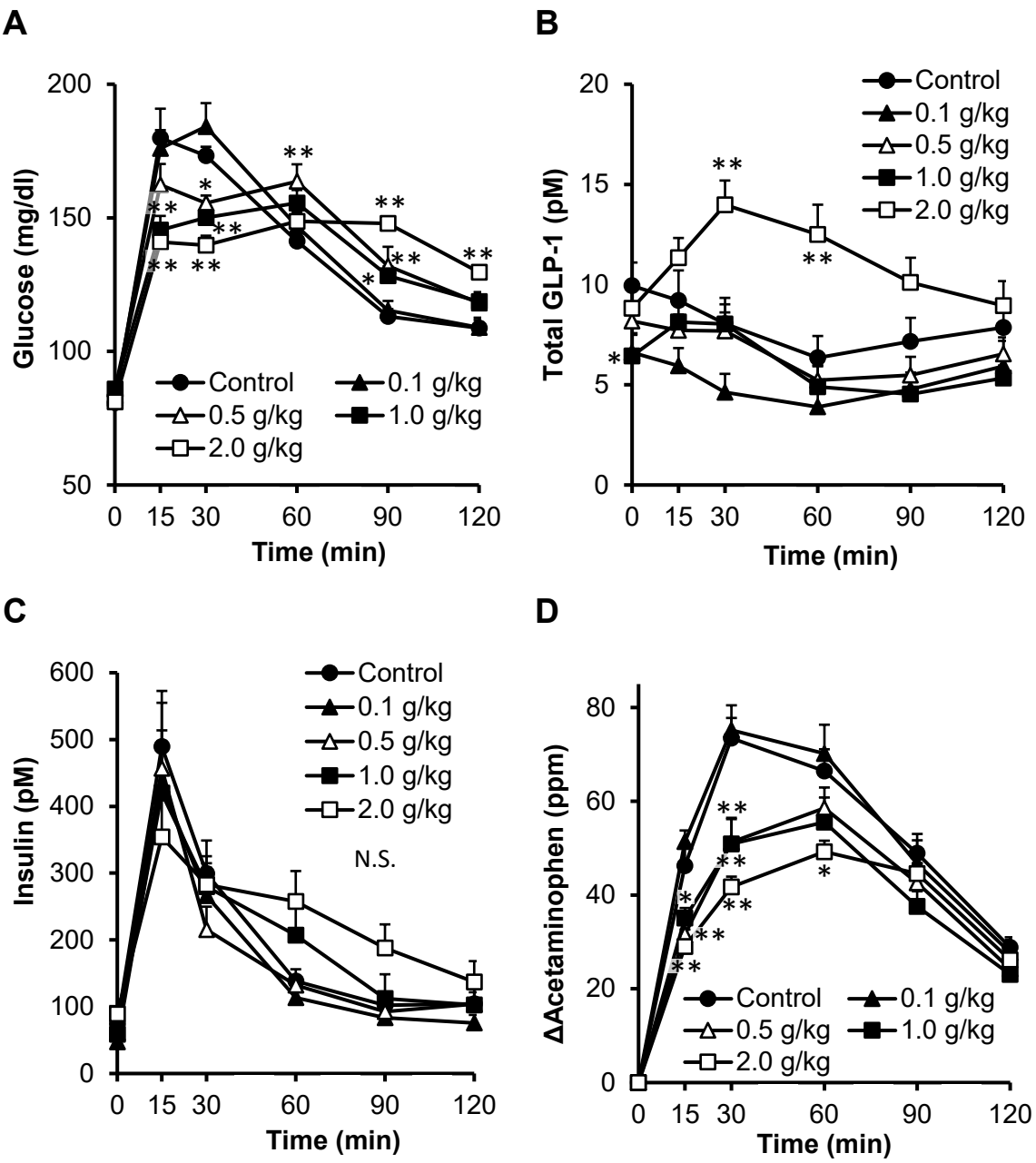

Fig. 5. Changes in plasma glucose, total GLP-1, insulin, and acetaminophen after oral administration of RBPH under the oral glucose tolerance test.

Glucose solution as control (●, 2 g/kg, 10 mL/kg), or the solution containing RBPH at 0.1 g/kg (▲), 0.5 g/kg (△), 1.0 g/kg (■), or 2.0 g/kg (□) was administered orally to rats after an overnight fast. Acetaminophen (100 mg/kg) was added to each test solution. Blood samples were collected from the tail vein before (0 min) and after the oral administration, as indicated. Plasma concentrations of glucose, total GLP-1, insulin, and acetaminophen were

632 measured. The two-way ANOVA P-values for glucose (**A**) were 0.488 (treatment), <0.01
633 (time), and <0.01 (treatment × time); <0.01 (treatment), <0.01 (time), and <0.01 (treatment
634 × time) for total GLP-1 (**B**); 0.791 (treatment), <0.01 (time), and 0.269 (treatment × time) for
635 insulin (**C**); and <0.01 (treatment), <0.01 (time), and <0.01 (treatment × time) for
636 acetaminophen (**D**). Values are mean ± SEM (n = 6–9). Asterisks (*) indicate significant
637 differences compared to the control treatment at the same time point (*P < 0.05, **P <
638 0.01; Dunnett's test).
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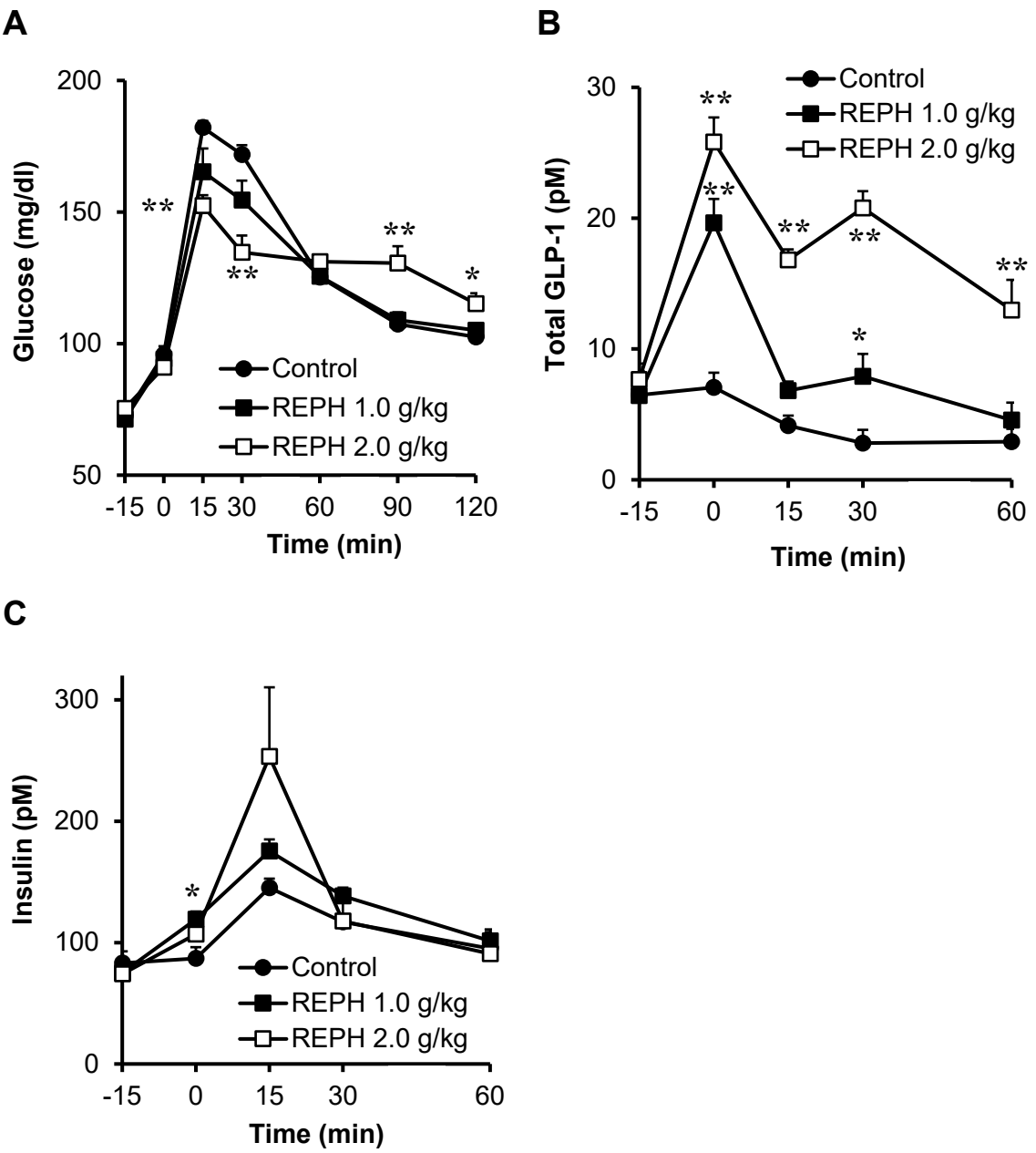

Fig. 6. Changes in plasma glucose, total GLP-1, and insulin after oral administration of REPH under the intraperitoneal glucose tolerance test.

Water as control (●, 12 mL/kg) or REPH at 1.0 g/kg (■) or 2.0 g/kg (□) was administered orally 15 min before an intraperitoneal glucose injection (1 g/kg). Blood samples were collected from the tail vein before and after the oral administration, and the plasma concentrations of glucose, total GLP-1, and insulin were measured. The two-way ANOVA P-values for glucose (**A**) were 0.481 (treatment), <0.01 (time), and <0.01 (treatment × time); <0.01 (treatment), <0.01 (time), and <0.01 (treatment × time) for total GLP-1 (**B**); and

653 0.273 (treatment), <0.01 (time) and <0.01 (treatment × time) for insulin (**C**). Values are
654 mean ± SEM (n = 4–6). Asterisks (*) indicate significant differences compared to the control
655 treatment at the same time point (*P < 0.05, **P < 0.01; Dunnett's test).
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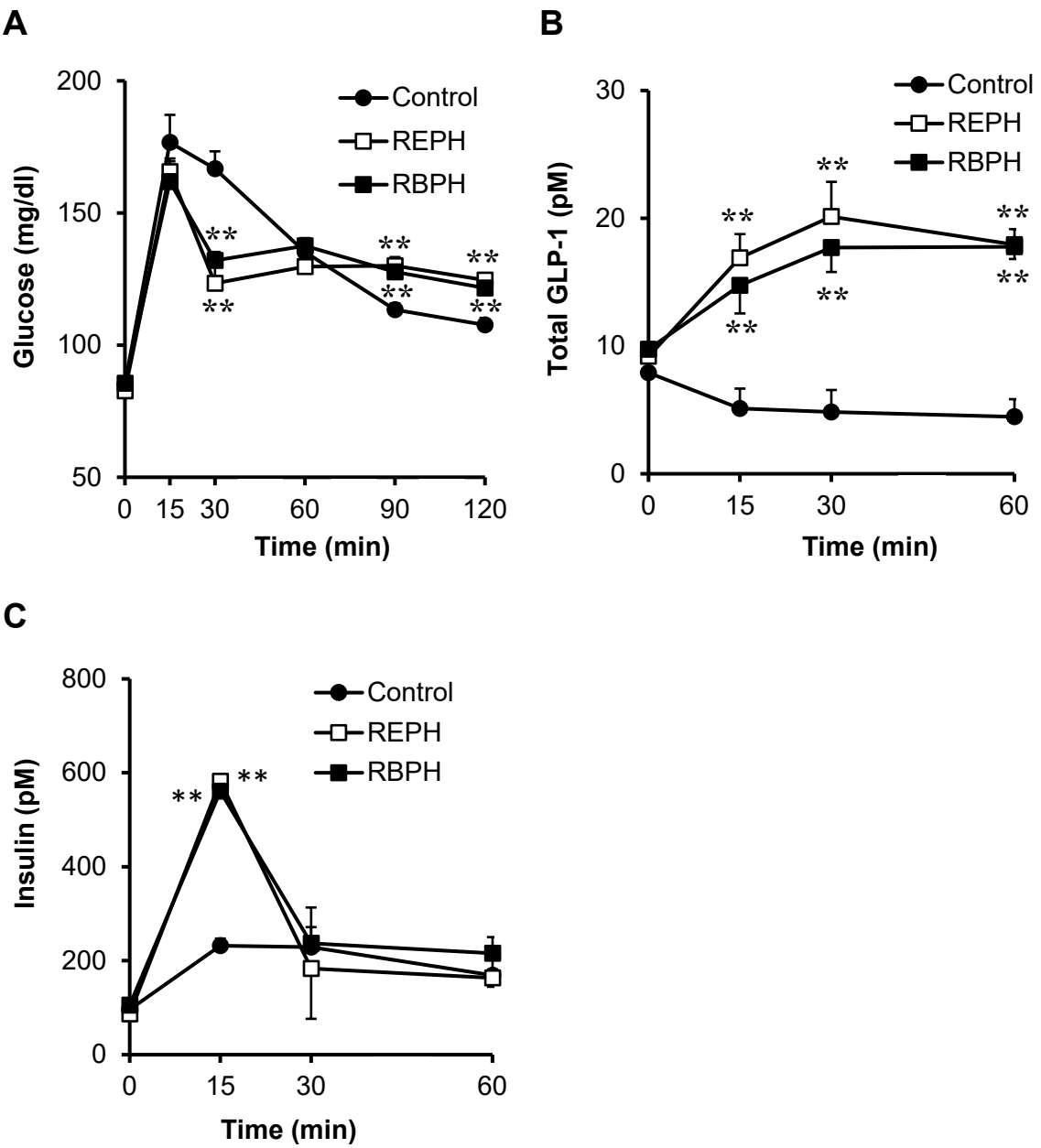


Fig. 7. Changes in plasma glucose, total GLP-1, and insulin levels after the oral administration of rice peptides under IPGTT.

Water as control (●, 12 mL/kg), REPH at 2.0 g/kg (□), or RBPH at 2.0 g/kg (■) was administered orally immediately before an intraperitoneal glucose injection (1 g/kg). Blood samples were collected from the tail vein before and after the oral administration, and the plasma concentrations of glucose, total GLP-1, and insulin were measured. The two-way ANOVA P-values for glucose (**A**) were 0.534 (treatment), <0.01 (time), and <0.01 (treatment × time); <0.01 (treatment), <0.01 (time), and <0.01 (treatment × time) for total

GLP-1 (**B**); and 0.118 (treatment), <0.01 (time) and <0.01 (treatment × time) for insulin (**C**). Values are mean ± SEM (n = 5–6). Asterisks (*) indicate significant differences compared to the control treatment at the same time point (*P < 0.05, **P < 0.01; Dunnett's test).

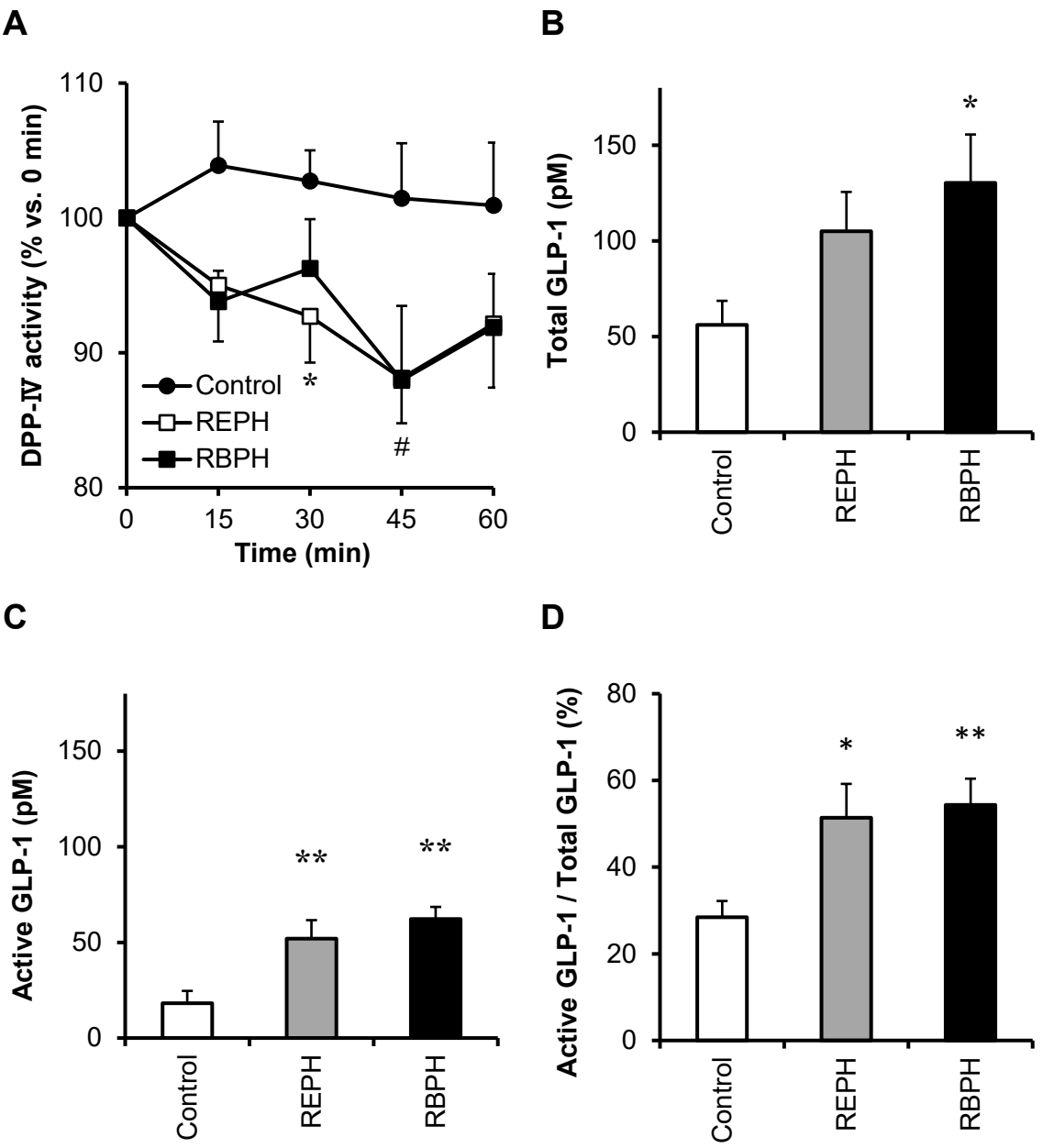
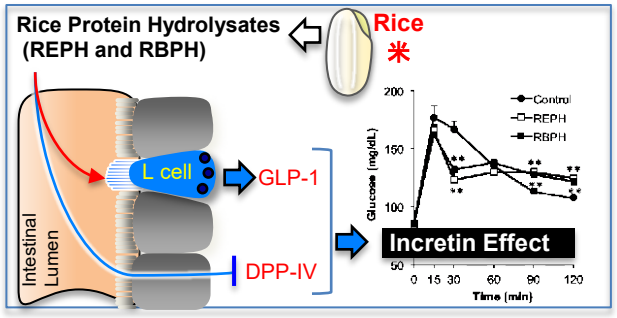


Fig. 8. Changes in the plasma DPP-IV activity, GLP-1 levels, and the ratio of active GLP-1 to total GLP-1 in the ileal vein of anesthetized rats after ileal administration of REPH or RBPH.

A: Saline as control (●, 2 mL/head), REPH (□, 500 mg/2 mL), or RBPH (■, 500 mg/2 mL) was injected into the ligated ileal loop at 0 min. Blood samples were collected through the ileal vein catheter before (0 min) and after (15, 30, 45, and 60 min) the ileal administration of test solutions. **B-D**: Total (**B**) and active (**C**) GLP-1 levels were evaluated 60 min after the ileal administration of REPH or RBPH, as well as the ratio of active GLP-1 to total GLP-1

(**D**). The two-way ANOVA P-values for the plasma activity of DPP-IV (**A**) were <0.05 (treatment), <0.05 (time), and 0.287 (treatment × time). Values are mean ± SEM (n = 6–9). Asterisks (*) indicate significant differences compared to the control treatment (P < 0.05; Dunnett's test), and hash marks (#) indicate significant differences compared to the 0 min value (P < 0.05; Dunnett's test).



Single oral administration of rice protein hydolysates stimulated GLP-1 secretion and reduced glycemic response in awake rats. Luminal REPH and RBPH reduced DPP-IV activity in the mesenteric vein.