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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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27 **Abstract**

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

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

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

49

50 **INTRODUCTION**

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

53 decreased, from 350 g/day in the 1950s to 150 g/day in the 2000s¹. This decrease is likely
54 due to the increased consumption of alternative staple foods, such as breads and noodles.
55 In addition, as excessive consumption of digestible starch can cause glucose intolerance
56 (which leads to diabetes mellitus), there is a recent trend in Japan towards reduced
57 consumption of boiled white rice.

58 The incidence of metabolic syndrome is increasing throughout the world, including in the
59 Asian countries. In Japan, increasing consumption of a Western diet is thought to be the
60 main cause of the increasing rates of obesity, glucose intolerance, diabetes, dyslipidemia,
61 and cardiovascular diseases. A positive association between the consumption of white rice
62 and the risk of type-2 diabetes has been reported^{2,3}, although this concept remains
63 controversial⁴. However, as described above, there were less diabetic patients in 1950-60's
64 when people consumed much more rice than today. Therefore, it is possible that the
65 consumption of rice has a beneficial effect on glucose and/or lipid metabolism.

66 Regarding the functional factors present in rice, resistant starch⁵ and gamma-oryzanol⁶
67 reportedly improve glucose homeostasis. Several studies have also demonstrated that
68 dietary rice protein effectively reduces serum cholesterol levels⁷⁻¹¹, through the increased
69 excretion of bile acids or neutral sterols. The protein content in rice is 5–7% by weight, and
70 this protein consists of glutelin (80%), globulin (4–15%), albumin (1–5%), and prolamin (2–
71 8%). A recent study showed that dietary rice protein can ameliorate the progression of early
72 stage diabetic nephropathy in a rat model of diabetes¹². Although these studies have
73 investigated the effects of continuous ingestion of rice proteins on lipid metabolism and
74 kidney function, the acute effects of rice proteins on glucose metabolism are not well
75 understood.

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

79 effect), which helps to attenuate postprandial hyperglycemia. GLP-1 also has proliferative
80 and protective effects on pancreatic beta cells, as well as multiple effects outside the
81 pancreas including in the liver, adipose tissue, cardiovascular, and central nervous
82 systems¹³. Recently, GLP-1 receptor agonists and dipeptidyl peptidase-IV (DPP-IV)
83 inhibitors have been used to treat type 2 diabetes^{14,15}. As GLP-1 and glucose-dependent
84 insulintropic polypeptide (another incretin) are immediately inactivated by plasma DPP-IV,
85 inhibitors of DPP-IV can help preserve the physiological activity of both these peptides¹⁶.

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

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

99

100 **Materials and Methods**

101 **Materials**

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

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

106

107 **Preparation of rice protein hydrolysates**

108 Purified rice endosperm protein (REP) was prepared using a previously reported alkali
109 extraction method²⁰. Briefly, milled rice flour was mixed with a 0.2% NaOH solution at room
110 temperature for 1 h to extract the protein fraction, and the mixture was then centrifuged.
111 The supernatant was neutralized with 1 M HCl to precipitate the protein fraction; the
112 collected protein fraction was repeatedly washed with distilled water, and then lyophilized
113 as REP. The purity of the REP was >90%, as determined using the Kjeldahl method.

114 Purified rice bran protein (RBP) was prepared from commercially available RBP powder
115 (TSUNO-RBP, TSUNO CO., LTD). The RBP powder was suspended in water, the pH of the
116 suspension was adjusted to 5.7 using H₂SO₄, and the suspension was then stirred for 1 h at
117 95°C. The precipitated protein was collected by centrifugation, washed with deionized
118 water and further centrifugation. Lyophilized protein fraction was used as RBP, and its
119 protein purity was 80%, determined using the Kjeldahl method.

120 Papain digests of REP and RBP were prepared as previously described¹⁹. In short, 50 g
121 of rice protein was suspended in deionized water (500 mL), and the pH of the suspension
122 was adjusted to 7.2 using NaOH. After 250 mg of papain (papain F, Asahi Food and Health
123 Care, Tokyo, Japan) was added, the suspension was shaken for 60 min at 55°C. The
124 suspension was then treated in boiling water for 20 min to stop the enzymatic reaction.
125 After filtration with a 0.45-μm filter, the pH was adjusted to 7.0, and the solution was
126 lyophilized.

127 Pepsin digests of REP and RBP were prepared as follows²¹⁻²⁴: 50 g of rice protein was
128 suspended in 0.02N phosphoric acid (1,000 mL), and the pH of the solution was adjusted to
129 1.85 with 20N phosphoric acid. After the addition of 250 mg pepsin (from porcine gastric
130 mucosa, Sigma, St. Louis, MO), the suspension was shaken for 30 min or 60 min at 37°C,

131 and then treated in boiling water for 20 min to stop the enzymatic reaction. The pH of the
132 suspension was adjusted to 7.0 with $\text{Ca}(\text{OH})_2$. After centrifugation at $3,300 \times g$, the
133 supernatant was collected and frozen overnight at -30°C . When the supernatant was
134 thawed the following day, it was centrifuged again, subjected to filtration (using a $0.45 \mu\text{m}$
135 filter) to remove the calcium phosphate, and finally lyophilized.

136 Papain was chosen because papain-treated Zein (ZeinH) had potent GLP-1 secretory
137 activity *in vitro* and *in vivo*¹⁹, and pepsin was chosen to partially mimic gastric digestion.
138 The degree of hydrolysis (DH) of rice protein hydrolysates was determined by using the
139 trinitrobenzenesulfonic method^{25,26}. The values of DH were as follows: 6.3% for REP
140 papain digest (60 min), 7.3% for REP pepsin digest (30 min), 8.2% for REP pepsin digest
141 (60 min), 5.3% for RBP papain digest (60 min), 9.8% for RBP pepsin digest (30 min), and
142 10.3% for RBP pepsin digest (60 min).

143 Rice protein hydrolysates were analyzed using HPLC system (Waters 2695 system,
144 Waters, Milford, MA) and an octadecyl silica column (CAPCELL PAK C18 MGII, $5 \mu\text{m}$, 4.6
145 $\text{mm} \times 250 \text{ mm}$, Shiseido Co., Ltd., Tokyo, Japan) with a linear gradient of acetonitrile/water
146 from 5 to 40% v/v for 45 min, from 40 to 100% v/v for following 20 min (from 45 to 65 min),
147 and then 100% v/v for 5 min.

148

149 **Cell culture**

150 The GLP-1 producing murine cell line GLUTag (a gift from Dr. D. J. Drucker, University of
151 Toronto, Canada) was cultured in Dulbecco's modified Eagle's medium (Invitrogen, cat. no.
152 12100-038), which was supplemented with 10% fetal bovine serum, 100 U/mL penicillin,
153 and 100 $\mu\text{g/mL}$ streptomycin. Cultures were grown in a humidified 5% CO_2 atmosphere at
154 37°C , and cells were routinely subcultured using trypsinization upon reaching 80–90%
155 confluency.

156

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 diet²⁷ 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 $23 \pm 2^{\circ}\text{C}$ 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 control¹⁹. Supernatants were
176 collected from the wells after the exposure, and centrifuged at $800 \times g$ 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

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

184

185 **Animal Experiments (Experiments 2-4)**

186 To measure plasma glucose, GLP-1, and insulin, blood samples were collected into a 1.5
187 mL tube containing aprotinin (final concentration, 500 kIU/mL), heparin (final concentration,
188 50 IU/mL), and a DPP-IV inhibitor (final concentration, 50 μ M; DPP4-010; Millipore Co,
189 Billerica, MA). Plasma was separated by centrifugation at $2,300 \times g$ for 10 min at 4°C, and
190 then frozen at –80°C until testing. Plasma glucose concentrations were measured using the
191 Glucose CII test kit (Wako), insulin was measured using an ELISA kit (AKRIN-010T;
192 Shibayagi Co, Ltd, Gunma, Japan), and active GLP-1 and total GLP-1 were measured
193 using the respective ELISA kits (EGLP-35K and EZGLP1T-36K; Millipore Co, Billerica, MA).

194

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

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

202

203 Experiment 3: The effects of oral REPH or RBPH on plasma glucose, GLP-1, insulin, and 204 gastric emptying under the oral glucose tolerance test

205 To examine the effects of oral REPH or RBPH on the glycemic response to oral glucose
206 load, the oral glucose tolerance test (OGTT) was performed in conscious rats. After basal
207 blood collection (0 min), glucose solution (2 g/kg body weight) or the solution containing
208 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)

235 After an overnight fast, rats were anesthetized with sodium pentobarbital (50 mg/kg body
236 weight, Somnopentyl Injection; Kyoritsu Seiyaku Co, Tokyo, Japan), and a midline
237 abdominal incision was made. Body temperature was maintained with heating pads during
238 the experiment. The small tip (6–7 mm) of a polyethylene catheter (SP 10; ID 0.28 mm, OD
239 0.61 mm; Natsume Seisakusyo, Tokyo, Japan) connected to a silicone catheter (Silascon
240 no. 00, ID 0.5 mm, OD 1.0 mm; Dow Corning Co.) was inserted into the ileal vein of the
241 mesenteric tissue, and the ileal lumen was washed with saline. The ligated ileal loop (length,
242 30 cm) was prepared between 5 and 35 cm proximal to the cecum, and the proximal and
243 distal ends of the loop were ligated with a silk thread. A basal blood sample (0 min, 50 μ L)
244 was drawn from the ileal vein catheter, and the catheter was refilled with sterilized saline
245 containing heparin (50 IU/mL; Ajinomoto, Tokyo, Japan) between each sampling. Two
246 milliliters of saline, REPH (250 mg/mL), or RBPH (250 mg/mL) was then administered
247 directly into the loop. Blood samples (50 μ L) were collected through the ileal vein catheter
248 at 15, 30, 45, and 60 min after the ileal administration to measure the plasma activity of
249 DPP-IV. For active GLP-1 and total GLP-1 measurement, ileal vein blood samples were
250 collected 60 min after the ileal administration. Blood samples for DPP-IV activity
251 measurement were drawn into a syringe containing heparin sodium salt (final concentration,
252 50 IU/mL; Nacalai Tesque, Kyoto, Japan), and samples for active or total GLP-1
253 measurement were drawn into a syringe containing aprotinin (final concentration, 500
254 kIU/mL), heparin (final concentration, 50 IU/mL), and DPP-IV inhibitor (final concentration,
255 50 μ M). The plasma was separated by centrifugation at $2,300 \times g$ (10 min at 4°C), and then
256 frozen until testing.

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

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

271

272 **Statistical analysis**

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

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285 **Results**

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

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

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

305 We next examined whether REPH- or RBPH-stimulated secretion of GLP-1 affected
306 glycemia via the incretin effect (the main function of GLP-1) under the oral glucose
307 tolerance test (OGTT). Oral gavage with REPH resulted in attenuation of the glycemic
308 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).
309 However, no significant difference in the area under the curve (AUC) was observed for
310 each group (data not shown). The plasma GLP-1 concentration only increased in the 2.0

g/kg REPH group, and that was significantly higher from 30 min to 90 min compared to that in the control group (Fig. 4B). However, the plasma insulin concentration at 15 min was suppressed by REPH in a dose-dependent manner (Fig. 4C). The plasma insulin concentration in the 2.0 g/kg group was lowest at 15 min, although this concentration was higher than the concentrations in the control group at 60 and 90 min. The acetaminophen test is used to assess the gastric emptying rate, as acetaminophen is absorbed rapidly in the intestine after emptied from the stomach^{28,29}. The change in plasma acetaminophen concentration was lower in the 1.0 g/kg REPH group (at 15 min and 30 min) and in the 2.0 g/kg REPH group (from 15 min to 90 min) (Fig. 4D), which indicated that gastric emptying was delayed by oral REPH.

The glycemic response during oral glucose load showed a dose-dependent effect, and it was significantly suppressed by oral RBPH gavage at 15 min (1.0–2.0 g/kg) and 30 min (0.5–2.0 g/kg) (Fig. 5A), compared with the control treatment. However, the AUC did not differ for each group (data not shown). The plasma GLP-1 concentration was significantly increased in the 2.0 g/kg RBPH group (from 30 min to 60 min) compared to the control treatment. The insulin response at 15 min tended to be reduced by oral RBPH, although this result was not statistically significant. The changes in plasma acetaminophen levels were reduced in a dose-dependent manner by RBPH as well as REPH.

Although increased GLP-1 and reduced glycemic responses were observed treatment with oral REPH and RBPH, the involvement of GLP-1 (the incretin effect) was unclear in OGTT, due to the delayed gastric emptying response (Fig. 4 and 5). Therefore, we conducted IPGTT to eliminate the possible effect of delayed gastric emptying and luminal glucose-induced GLP-1 secretion on the glycemic response, as well as to evaluate the effect of oral REPH or RBPH on GLP-1 secretion (Fig. 6 and 7).

Under IPGTT, 2.0 g/kg oral REPH resulted in the attenuation of the glycemia at 15 min and 30 min (Fig. 6A), although the AUC did not differ for each group (data not shown). The

337 plasma GLP-1 concentrations were increased in a dose-dependent manner by REPH. In
338 addition, 2.0 g/kg REPH induced continuous elevation of plasma GLP-1 from 15 to 60 min,
339 and these levels were significantly higher than the levels in the control group (Fig. 6B). Oral
340 REPH (1.0 g/kg) also resulted in the elevation of plasma GLP-1 at 0 min and 30 min (Fig.
341 6B). Plasma insulin concentrations tended to be increased by 2.0 g/kg REPH (Fig. 6C).

342 We also conducted IPGTT to examine the effect of oral RBPH on GLP-1 secretion and
343 glycemic control. In this experiment, RBPH or REPH was orally administered immediately
344 before the intraperitoneal injection of glucose (0 min), as RBPH failed to reduce the
345 glycemic response in the preliminary study when it was provided at other time points (e.g.,
346 15 min or 30 min before the intraperitoneal injection of glucose).

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

352 GLP-1 is rapidly inactivated by DPP-IV in the plasma³², and a recent study has
353 demonstrated that peptides derived from rice bran can inhibit DPP-IV activity³³. Therefore,
354 we investigated whether luminal REPH or RBPH affected the plasma activity of DPP-IV in
355 the ileal vein, and thereby prolonged the activity of intact plasma GLP-1. The plasma
356 activity of DPP-IV in the ileal vein decreased gradually after ileal administration of REPH or
357 RBPH (Fig. 8A). In the REPH group, the plasma activity of DPP-IV at 30 min was
358 significantly lower than that in the control group. In the RBPH group, the DPP-IV activity at
359 45 min was significantly lower than the basal (0 min) activity. We also measured the active
360 and total GLP-1 concentrations in the ileal vein plasma 60 min after the ileal administration
361 of REPH or RBPH. Although the total GLP-1 concentration was only significantly elevated
362 in the RBPH group (Fig. 8B), the concentrations of active GLP-1 were significantly elevated

363 in the REPH and RBPH groups, compared with the control group (Fig. 8C). Because the
364 incremental degrees in GLP-1 levels for the REPH and RBPH groups (compared to the
365 control group) were larger for active GLP-1 (~3×) than for total GLP-1 (2–2.5×), we
366 calculated the ratio of active GLP-1 to total GLP-1 (Fig. 8D). The ratios in the REPH group
367 (51.4%) and in the RBPH group (54.3%) were approximately 2× higher than that in control
368 group (28.4%), and this difference was statistically significant.

369

370

371 Discussion

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

385 GLUTag cells are widely used as an enteroendocrine cell model to investigate the effect
386 of various molecules on the secretion of GLP-1, including glucose³⁴, fatty acids³⁵,
387 peptides¹⁹ and amino acids³⁶. We observed that hydrolysates prepared from REP and RBP,
388 particularly the 30 min pepsin digests (REPH and RBPH), potently stimulated GLP-1
389 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

416 increased GLP-1 secretion (Fig. 6B and 7B). Because REPH or RBPH was administered in
417 the absence of oral (luminal) glucose, the increased GLP-1 secretion should be induced by
418 luminal rice peptides. In our previous¹⁸ and preliminary study, oral administration of
419 hydrolysates prepared from meat, soy protein or wheat gluten was not effective to reduce
420 glycemic response under IPGTT. Thus, the present results clarified that rice peptides can
421 potently induce GLP-1 secretion, thereby lowering the glycemic response through the
422 incretin effect.

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

432 Although we observed that REPH and RBPH reduced the glycemic response in the early
433 period, the plasma glucose concentrations in the REPH and RBPH groups were slightly
434 increased in the late period compared to the control group (90 and 120 min). A previous
435 study has reported that exendin-4 (a GLP-1 receptor agonist) increased plasma glucose
436 concentrations via the sympathetic nervous system⁴². Similar pathways affected by REPH
437 or RBPH might be related to the late increase in plasma glucose.

438 DPP-IV is one of serine proteases, and is known to inactivate GLP-1, GLP-2, and
439 glucose-dependent insulintropic polypeptide⁴³. Recently, oral inhibitors of plasma DPP-IV
440 have been used to treat type 2 diabetes mellitus. In addition, several studies have reported
441 that the activity of DPP-IV is reduced by various dietary peptides, including corn zein

442 hydrolysate¹⁷, whey hydrolysate⁴⁴, casein-derived peptides⁴⁵, and rice bran peptides³³,
443 possibly acting as competitive inhibitors. We observed that luminal administration of rice
444 protein hydrolysates attenuated the plasma DPP-IV activity in anesthetized rats (Fig. 8A).
445 Because peptides produced from defatted rice bran have potent inhibitory effects on
446 DPP-IV *in vitro*³³, it was possible that such peptides were absorbed into the ileal vein, and
447 inhibited the plasma DPP-IV activity.

448 Moreover, ileal administration of REPH and RBPH both stimulated GLP-1 secretion and
449 increased the ratio of active GLP-1 to total GLP-1 (Fig. 8B-D), which might be attributed to
450 the reduced plasma activity of DPP-IV. Dietary peptides, including REPH, RBPH, and corn
451 zein hydrolysate¹⁷, may contribute to the reduced postprandial glycemia through their
452 synergistic effects on GLP-1 secretion and degradation. Glucose and fatty acids are also
453 known to induce GLP-1 secretion. However, these molecules would not affect the plasma
454 activity of DPP-IV. The synergistic effects that we observed might be unique to dietary
455 peptides.

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

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

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

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

479

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484

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Figures

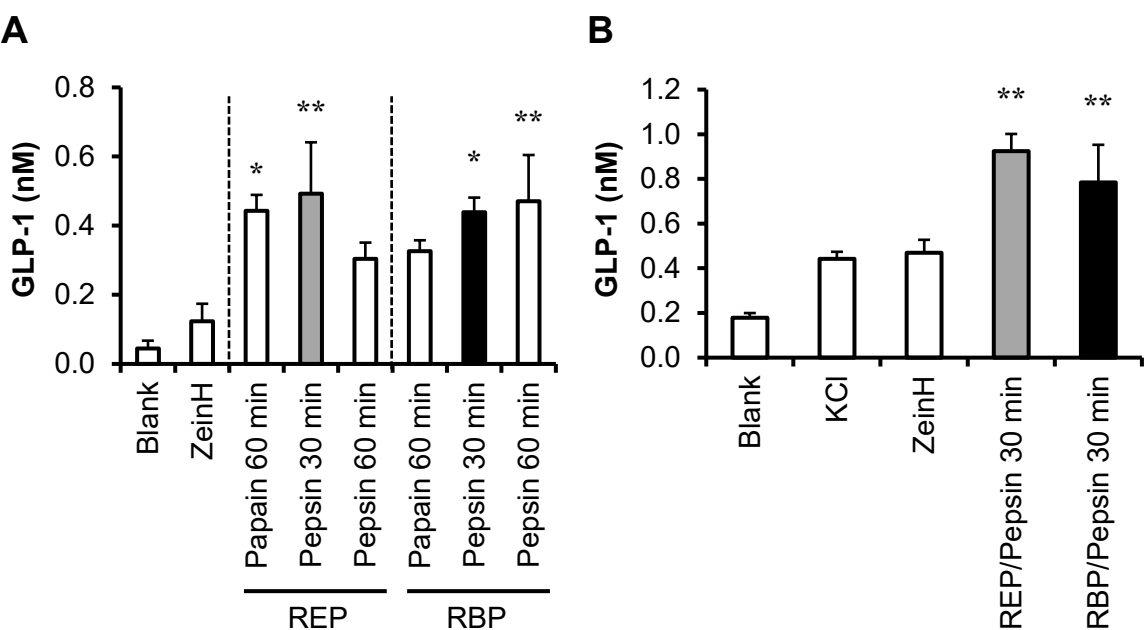


Fig. 1. GLP-1 secretion from GLUTag cells by various protein hydrolysates

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 $\pm$ SEM (n = 4). Asterisks (*) indicate significant differences compared to the blank treatment (*P < 0.05, **P < 0.01; Dunnett's test).

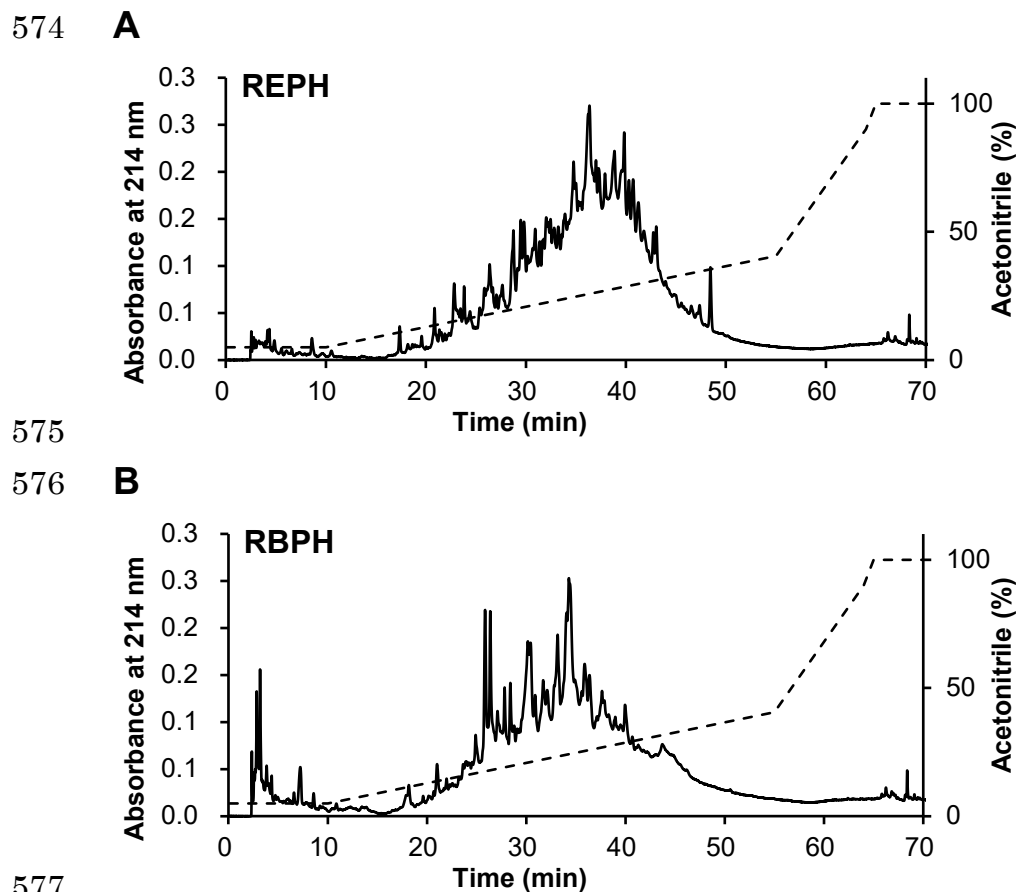
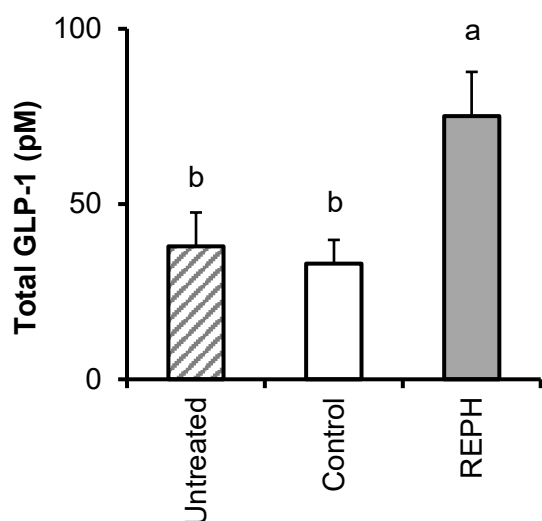
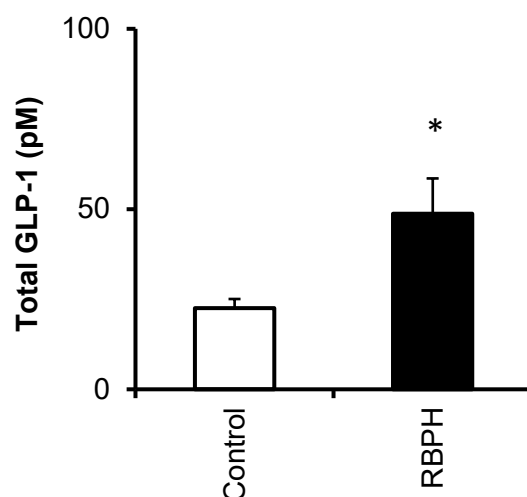


Fig. 2. HPLC chromatograms of REPH and PBPH.

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.

585

A**B**

586

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

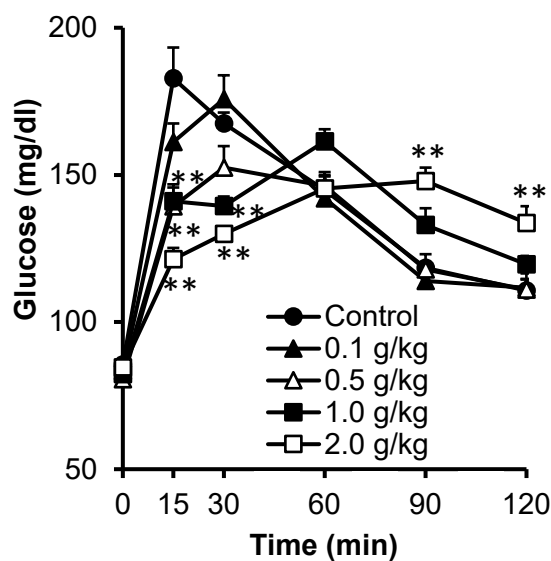
589 Blood samples were collected from portal vein after oral administration of REPH (2 g/kg),
 590 RBPH (2 g/kg), or water (control), and the plasma concentration of total GLP-1 was
 591 measured. As the untreated control, blood samples were collected from rats that did not
 592 receive any oral treatment.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

596

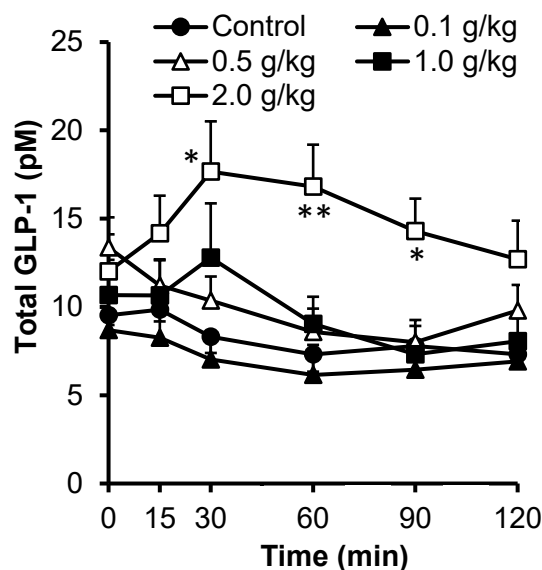
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A



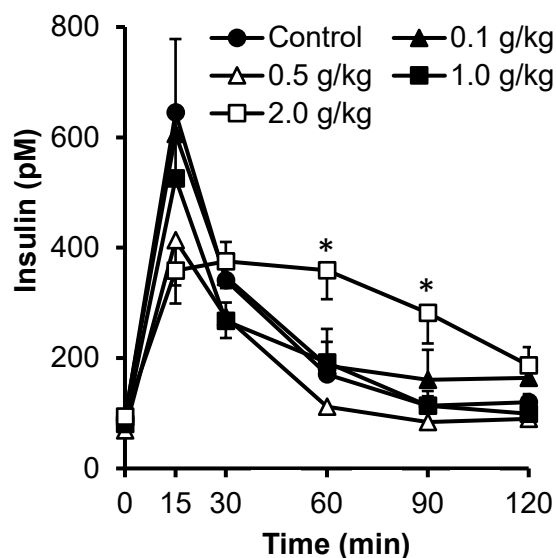
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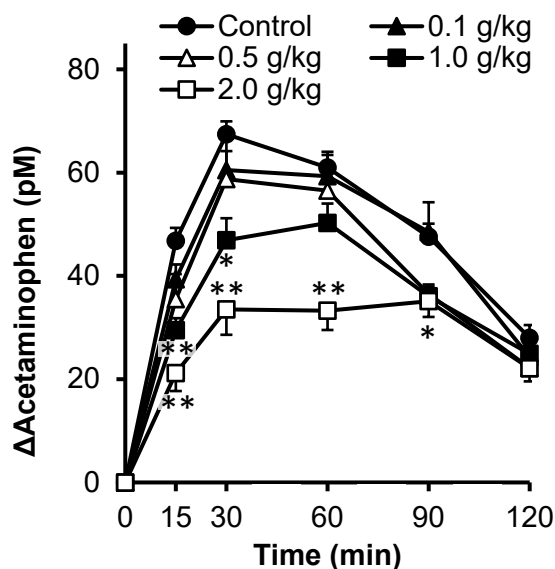
599

600

C



D



601

602

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

605 Glucose solution as control (●, 2 g/kg, 10 mL/kg), or the solution containing REPH at 0.1
 606 g/kg (▲), 0.5 g/kg (△), 1.0 g/kg (■), or 2.0 g/kg (□) was administered orally to rats after an
 607 overnight fast. Acetaminophen (100 mg/kg) was added to each solution. Blood samples
 608 were collected from the tail vein before (0 min) and after the oral administration, as
 609 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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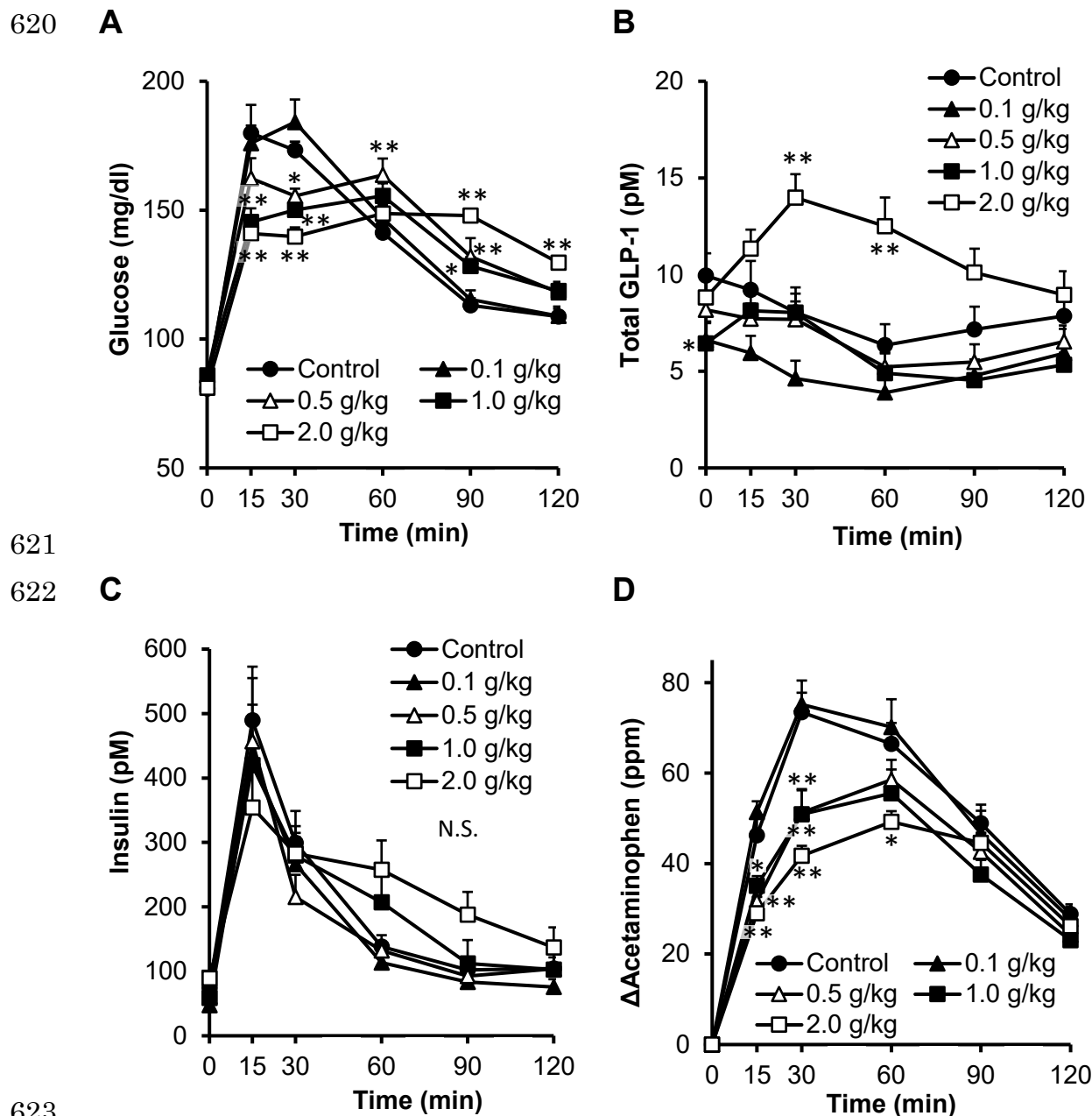


Fig. 5. Changes in plasma glucose, total GLP-1, insulin, and acetaminophen after oral administration of RBPH under the oral glucose tolerance test.

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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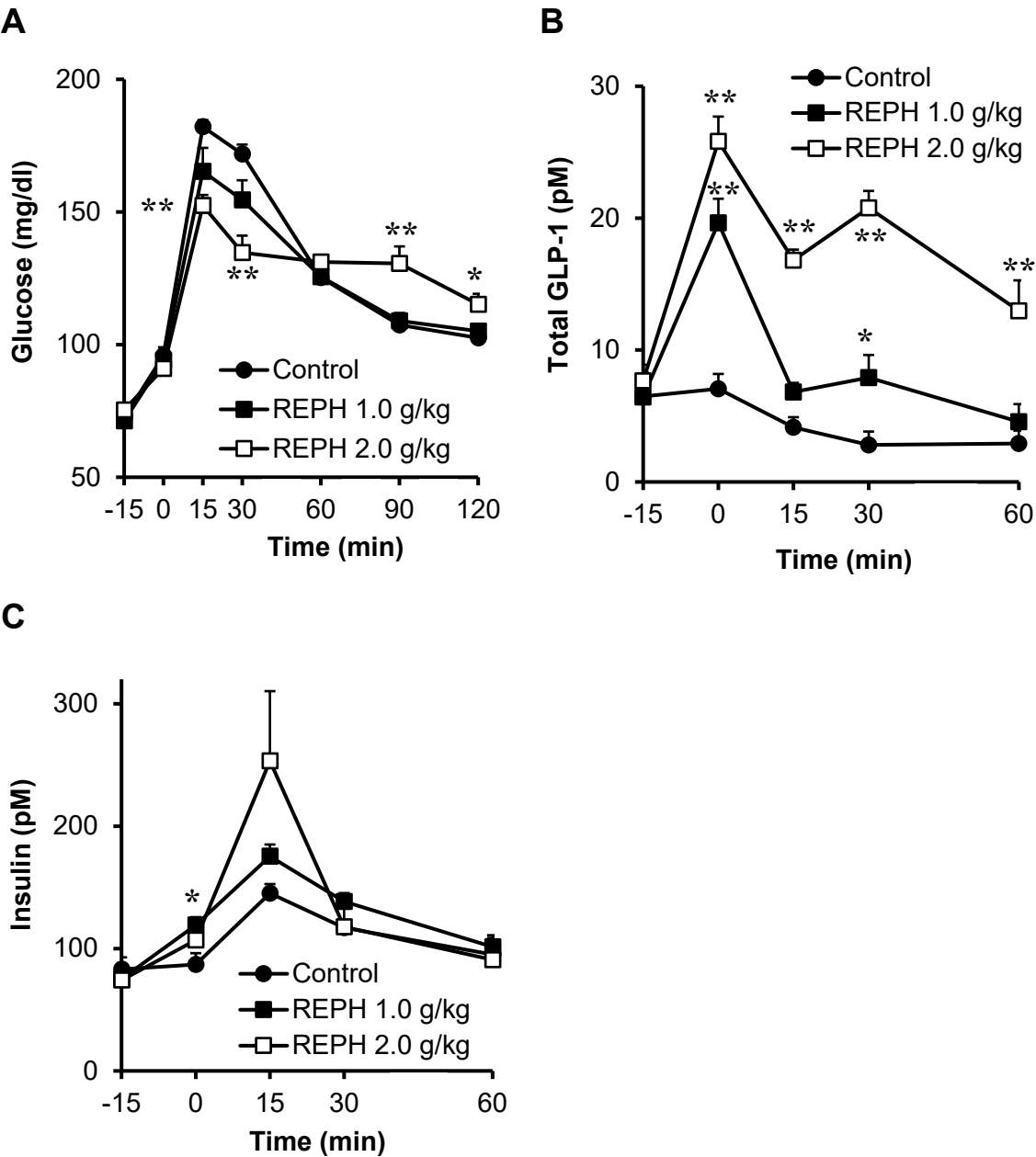
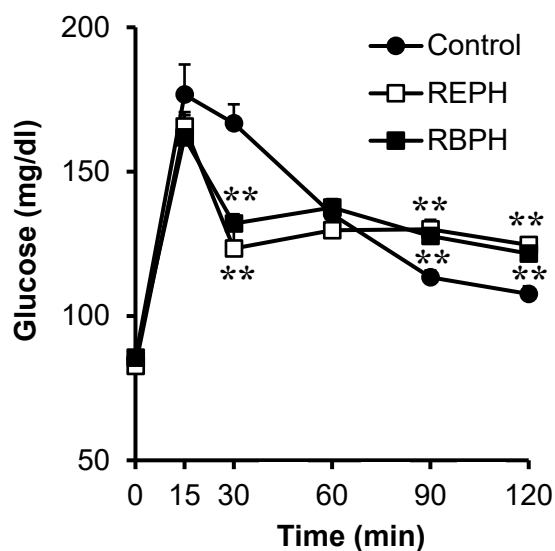
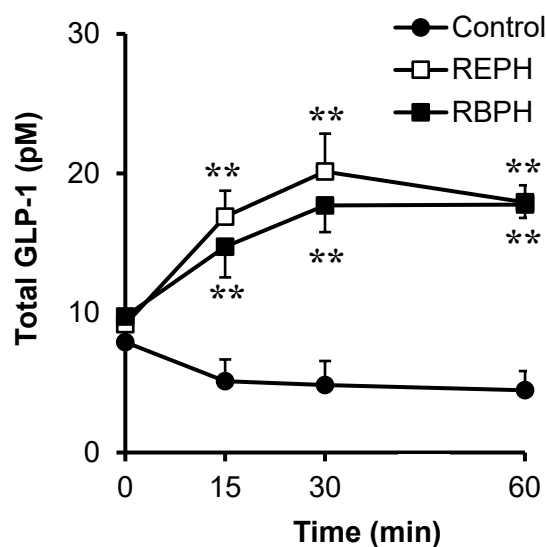


Fig. 6. Changes in plasma glucose, total GLP-1, and insulin after oral administration of REPH under the intraperitoneal glucose tolerance test.

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

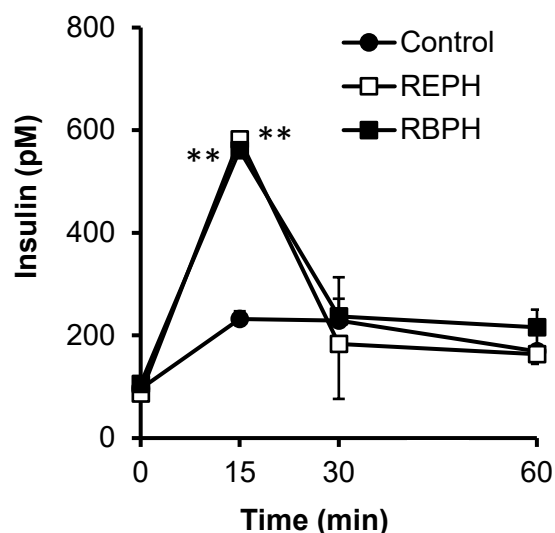
653 0.273 (treatment), <0.01 (time) and <0.01 (treatment × time) for insulin (**C**). Values are
654 mean ± SEM (n = 4–6). Asterisks (*) indicate significant differences compared to the control
655 treatment at the same time point (*P < 0.05, **P < 0.01; Dunnett's test).
656
657

658

A**B**

659

660

C

661

662 **Fig. 7. Changes in plasma glucose, total GLP-1, and insulin levels after the oral**663 **administration of rice peptides under IPGTT.**

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

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

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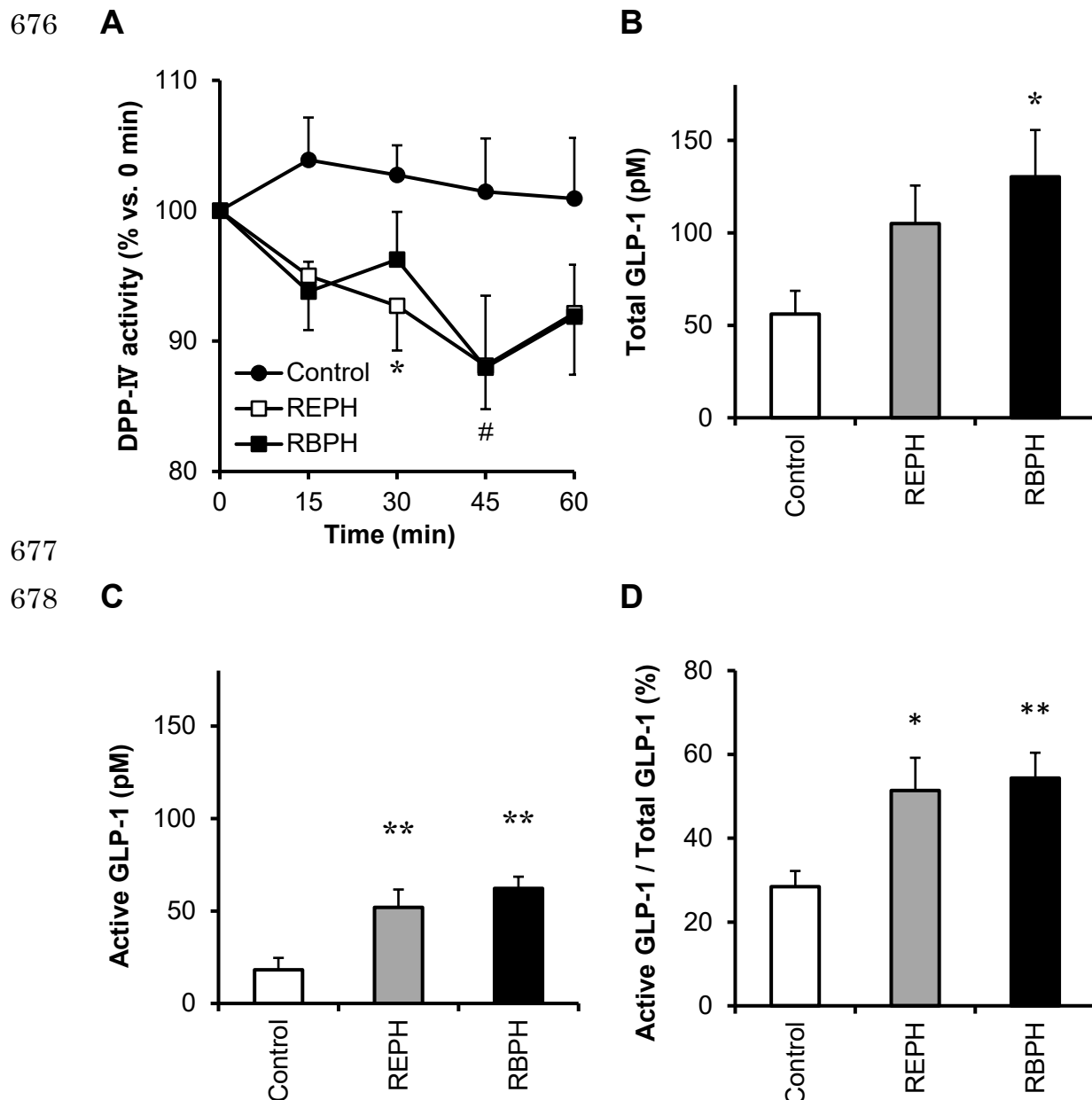
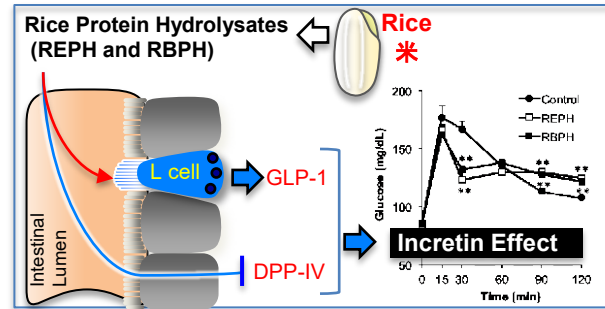


Fig. 8. Changes in the plasma DPP-IV activity, GLP-1 levels, and the ratio of active GLP-1 to total GLP-1 in the ileal vein of anesthetized rats after ileal administration of REPH or RBPH.

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

688 (D). The two-way ANOVA P-values for the plasma activity of DPP-IV (A) were <0.05
689 (treatment), <0.05 (time), and 0.287 (treatment × time). Values are mean ± SEM (n = 6–9).
690 Asterisks (*) indicate significant differences compared to the control treatment (P < 0.05;
691 Dunnett's test), and hash marks (#) indicate significant differences compared to the 0 min
692 value (P < 0.05; Dunnett's test).
693



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