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Title	Continuous feeding of a combined high-fat and high-sucrose diet, rather than an individual high-fat or high-sucrose diet, rapidly enhances the glucagon-like peptide-1 secretory response to meal ingestion in diet-induced obese rats
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Continuous feeding with a combined high fat/high sucrose diet, rather than an individual high fat or high sucrose diet, rapidly enhances the GLP-1 secretory response to meal ingestion in diet-induced obese rats

Abstract

Glucagon-like peptide-1 (GLP-1) is secreted by enteroendocrine L-cells in response to nutrient ingestion. To date, GLP-1 secretion in diet-induced obesity is not well characterized. We aimed to examine GLP-1 secretion in response to meal ingestion during the progression of diet-induced obesity and to determine whether a combined high fat/high sucrose diet, an individual high fat or high sucrose diet affects adaptive changes in the postprandial GLP-1 response. Rats were fed a control diet, high fat diet (30% weight, HiFat), a high sucrose diet (40% weight, HiSuc), or a high fat/high sucrose diet (HFS, 30% fat and 40% sucrose) for 5 weeks. Meal tolerance tests (MTTs) were conducted to determine postprandial glucose, insulin, and GLP-1 responses to standard (control) diet ingestion every 2 weeks. After 5 weeks, body weight gain of the HiFat (232.3 ± 7.8 g, $P = 0.021$) and HFS groups (228.0 ± 7.8, $P = 0.039$), but not of the HiSuc group (220.3 ± 7.9, $P = 0.244$), were significantly higher than the control group (200.7 ± 5.4 g). In MTT after 2 weeks, GLP-1 concentration was significantly elevated only in the HFS group (17.2 ± 2.6 pM, $P < 0.001$) in response to meal ingestion, but the HiFat group (16.6 ± 3.7 pM, $P = 0.156$) had a similar response to the HFS group. After 4 weeks, GLP-1 concentrations were similarly elevated at 15 min in the HFS (14.1 ± 4.4, $P = 0.010$), HiFat (13.2 ± 2.0, $P < 0.001$), and HiSuc groups (13.0 ± 3.3, $P = 0.016$), but the HFS (9.8 ± 1.0, $P = 0.019$) and HiFat groups (8.3 ± 1.5, $P = 0.010$) had significant elevation also at 30 min. These results demonstrate that continuous ingestion of excessive fat and sucrose rapidly enhances the GLP-1 secretory response to luminal nutrients, and the high fat diet may have potent effect compared to the high sucrose diet on

GLP-1 secretory responses. The increment of postprandial GLP-1 and insulin secretion may have a role in normalizing postprandial glycaemia and slowing the establishment of glucose intolerance.

Keywords: Glucagon-like peptide-1; Luminal nutrients; L-cell; Meal tolerance test; Obesity

Introduction

Obesity is rapidly increasing in the global population and has become a major public health problem [1]. Dietary habits are now considered key factors related to obesity development, especially excessive energy consumption from a high fat and/or a high sugar diet [2,3]. Obesity leads to metabolic syndromes, such as hyperinsulinemia, hypertension, hyperlipidaemia, and type II diabetes mellitus [4,5]. Glucagon-like peptide-1 (GLP-1), a product of the proglucagon (*gcg*) gene, is a gut-derived hormone, which is produced and secreted from enteroendocrine L cells in response to nutrient ingestion, including protein, glucose, fatty acids, and dietary fibre [5-9]. GLP-1 has been recognized as an incretin hormone that reduces the postprandial glycaemic response by stimulating insulin secretion [10]. Existing data on GLP-1 responses in humans to nutrient ingestion (enhanced, unchanged, and decreased) in obesity, prediabetes, and diabetes patients remains unclear [11,12]. GLP-1 secretion in response to oral glucose was enhanced in type II diabetic patients [13]. In contrast, GLP-1 secretion was impaired in obese subjects and type II diabetic patients [14,15], whereas GLP-1 secretion in response to oral glucose was unchanged in type II diabetic patients [16].

In order to understand how GLP-1 responses are modified in the process of diet-induced obesity, it is relevant to adopt animal models continuously fed an obesogenic diet, rather than using genetically obese/diabetic models. Continuous feeding (8 weeks) of a high fat and high sucrose diet increased postprandial GLP-1 secretion in response to normal meal administration, as previously reported [17].

Although the impact of feeding animal models with a high fat and/or high sucrose diet has been previously examined, the alteration of the postprandial GLP-1 response during obesity development has not been clearly characterized [18-20].

The aims of this study were to clarify the GLP-1 response to meal ingestion during the progression of diet-induced obesity and to determine whether a combined high fat/high sucrose diet or an individual high fat or high sucrose diet contributed to adaptive changes in postprandial GLP-1 secretion. In this study, the rats were given a standard (control) diet as a meal tolerance test (MTT), instead of the typical oral glucose loading (OGTT).

Experimental methods

Animals and diet

Five-week-old male Sprague-Dawley rats (weighing 160-200 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were fed with the American Institute of Nutrition (AIN)-93G (control) diet for 1 week as an acclimation period [21]. Each rat was housed in an individual cage in a temperature- and humidity-controlled environment with a 12 h light-dark cycle (8.00 a.m.-8.00 p.m. light period) and was allowed free access to diet and water (*ad libitum*). After one-week acclimation period, fasting glucose and GLP-1 concentrations were measured. Then, the rats were divided into four groups to have matched body weight, plasma glucose, and GLP-1 concentrations. Rats in each group were given the control, high fat/high sucrose (HFS, 30% fat weight and 40% sucrose weight) [17], high fat (HiFat, 30% fat), or high sucrose (HiSuc, 40% sucrose) diet (Table 1) for 5 weeks. The initial body weight, fasting glucose, and GLP-1 concentrations were 168.7 ± 3.1 g, 73.2 ± 2.4 mg/dL, 18.0 ± 3.0 pM in the control group (n=10), 169.3 ± 2.5 g, 72.0 ± 3.3 mg/dL, 17.7 ± 3.1 pM in HFS group (n=8), 169.1 ± 4.3 g, 71.5 ± 4.3 mg/dL, 17.8 ± 1.8 pM in HiFat group (n=10), and 167.7 ± 2.8 g, 71.5 ± 2.0

70 mg/dL, 20.0 ± 3.2 pM in HiSuc group (n=8), respectively. The final compositions of 30% fat in the
71 HiFat diet and 40% sucrose in the HiSuc diet were adjusted to be equal to those in the HFS diet. Body
72 weight and food intake were measured every 2 days. All experimental animal procedures used in this
73 study were approved by the institutional animal care committee, and the animals were maintained in
74 accordance with the institutional animal guidelines for the care and use of laboratory animals.

75 *Meal tolerance test (MTT)*

76 Meal tolerance test (MTT) was performed on rats after they were fed the test diets for 2 and 4
77 weeks, in order to determine the postprandial glucose, insulin, and GLP-1 responses to standard meal
78 ingestion. The rats were fasted for 16 h, and then basal (0 min) blood was collected from the tail vein.
79 Then, the rats were given the control diet (10 g/kg body weight) for 30 min, and blood samples from
80 the tail vein were collected at 15, 30, 60, 90 and 120 min after providing the diet. In this study, only the
81 rats that ingested more than 90% of the given diet were used to assess the postprandial responses.
82 Blood samples were collected in chilled tubes containing heparin (final concentration 50 IU/mL;
83 Ajinomoto Company, Inc., Tokyo, Japan) and aprotinin (final concentration 500 Kallikrein inhibitor
84 units (KIU)/mL; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma was separated by
85 centrifugation at 2300 x *g* for 10 min at 4°C and then stored at -80°C until analysis. The plasma
86 glucose level was determined using the Glucose CII Test Kit (Wako Pure Chemical Industries, Osaka,
87 Japan). Plasma insulin and GLP-1 concentrations were analysed using the Rat Insulin ELISA (AKRIN-
88 010T; Shibayagi Company Limited, Gunma, Japan) and Multi Species GLP-1 Total ELISA
89 (EZGLP1T-36K; Merck Millipore, Darmstadt, Germany), respectively. The GLP-1 Total ELISA
90 detects both GLP-1 (7-36) and GLP-1 (9-36) and has no significant cross-reactivity with GLP-2, GIP,
91 glucagon, oxyntomodulin. The minimum detection limit of the assay is 1.5 pM. The intra-assay
92 precision is < 5%, and the inter-assay precision is < 12%, respectively. The homeostatic model

93 assessment of insulin resistance (HOMA-IR) was used to assess insulin resistance. It was calculated by
94 the following equation [22], using glucose and insulin values at the fasting state and those values of the
95 area under the curve during MTT:

96 $$\text{HOMA-IR} = \text{insulin } (\mu U/mL) \times \text{glucose } (mg/dL)/2430$$

97 Where: 1 mg insulin = 26 IU

98 *Blood and tissue collection*

99 After receiving the test diet for 5 weeks, the rats were fasted overnight (16 h). Blood samples
100 from the portal vein were taken under sodium pentobarbital anaesthesia (50 mg/kg of body weight,
101 somnopentyl injection; Kyoritsu Seiyaku Corporation, Tokyo, Japan) and collected in a chilled syringe
102 containing heparin (final concentration 50 IU/mL), aprotinin (final concentration 500 KIU/mL), and
103 DPP-IV inhibitors (final concentration 50 μmol/L; DPP4-010; Merck Millipore, Darmstadt, Germany).
104 The rats were then sacrificed by exsanguination. Intestinal segments were carefully dissected and
105 washed with a cold saline solution (0.9% NaCl); then, 2 cm segments of the jejunum, ileum, and colon
106 were taken from the middle region to measure GLP-1 content. The caecal tissues were washed with a
107 cold saline solution and divided equally into 2 parts; then, 2 cm from the middle region was collected
108 for GLP-1 measurement. All intestinal segments were rapidly frozen in liquid N_2 and stored at -80°C
109 until measurement of the GLP-1 content. The mesenteric, retroperitoneal, and epididymal adipose
110 tissues were individually weighed and expressed as the visceral adipose tissue weight. Plasma was
111 stored as described above in order to measure the glucose, insulin, total GLP-1, triacylglycerol, and
112 total cholesterol levels. Plasma triacylglycerol and cholesterol concentrations were measured using the
113 Triglyceride E Test and Cholesterol E Test kits (Wako Pure Chemical Industries, Osaka, Japan),
114 respectively.

115 *Measurement of the glucagon-like peptide-1 (GLP-1) content in intestinal tissue*

116 Intestinal segments (2 cm length) were immersed in an ethanol-acid solution (absolute
117 ethanol:water:12 M HCl = 74:25:1, 5 mL/g of intestinal tissue segments) [23] and then cut into small
118 pieces. The tissue samples were homogenized at 25,000 rpm (Ultra Turrax homogenizer T18, IKA,
119 Staufen, Germany) for 2 min and placed at 4°C for 24 h. The homogenates were then centrifuged at
120 2000 x *g* for 20 min. The supernatant was collected for measuring the total GLP-1 and protein contents
121 using the Multi Species GLP-1 Total ELISA (EZGLP1T-36K; Merck Millipore, Darmstadt, Germany)
122 and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA), respectively. The
123 supernatant was diluted 10-fold for protein measurement, 500-fold (jejunum, ileum, and colon
124 segments), and 625-fold (colon segment) for GLP-1 measurement with normal saline solution.

125 *Statistical analysis*

126 The primary endpoint of this study was nutrient-induced GLP-1 responses in rats treated with
127 various (obesogenic) diets. The secondary endpoint was to assess glycaemic and insulin responses,
128 body weight and adipose tissue weight changes. The results were expressed as mean ± standard error of
129 the mean (SEM). The sample size was calculated based on the experimental design (two-way repeated
130 measure ANOVA) in MTTs for examining postprandial GLP-1 responses as the primary outcome
131 measure, by using G*Power software (version 3.1.9.2) as effect size f = 0.4 and Power = 0.8.
132 Significant effects of time (TI), treatment (TR) and the interactions of time and treatment (TI x TR)
133 were assessed by two-way repeated measure ANOVA in the results of MTTs. One-way ANOVA and
134 Tukey-Kramer's test were used to assess the significant difference among the treatments for parametric
135 data (assessed by Goodness of Fit test). A Kruskall–Wallis test and Dunn's multiple range test were
136 used for MTT data containing non-parametric data. Dunn's with control test was used to determine the
137 significant differences from baseline (0 min) value within the same group. *P* values less than 0.05 was

138 considered to be statistically significant. Statistical analysis was performed using JMP Pro version 13
139 software (SAS Institute, Inc., Cary, NC, USA).
140
141 **Results**
142 *Basal glucose, insulin, GLP-1 levels, and homeostasis model assessment of insulin resistance (HOMA-*
143 *IR) after feeding with the test diet for 2 and 4 weeks.*
144 After receiving the test diet for 2 and 4 weeks, the rats were fasted overnight (16 h) before
145 conducting the MTT. After 2 weeks of feeding, basal glucose (84.1 ± 4.5 - 90.0 ± 3.6 mg/dL, P =
146 0.396), insulin (0.11 ± 0.01 - 0.20 ± 0.06 nM, P = 0.329), GLP-1 (12.1 ± 1.5 - 20.4 ± 2.6 pM, P =
147 0.070), and HOMA-IR (0.54 ± 0.07 - 1.11 ± 0.34, P = 0.287) were not significantly different among all
148 of the treatment groups (Fig 1A, B, C, and D). After 4 weeks, the basal insulin level and HOMA-IR
149 tended to be higher in the HFS group (0.26 ± 0.03, P = 0.218 and 1.45 ± 0.20, P = 0.200) compared to
150 control group (0.17 ± 0.02 nM, and 0.90 ± 0.10) (Fig 1F and H).
151 *Postprandial glycaemic, insulin, and GLP-1 responses during the MTT*
152 In this study, MTT was used instead of the oral glucose tolerance test (OGTT) in order to
153 determine postprandial responses to ingestion of a standard diet (control diet). MTT can be used to
154 mimic dietary exposure in normal life and voluntary ingestion is more relevant than enforced gavage
155 feeding. Although HiSuc group had relatively lower concentrations, postprandial glycemic responses
156 were overall similar in all groups (Fig. 2A and E) after receiving the test diet for 2 weeks. The AUC of
157 insulin increased largely in the HFS group (1.15 ± 0.19 nM, P = 0.015), compared to that of the control
158 group (0.46 ± 0.04 nM). The HiFat group (0.93 ± 0.35, P = 1.000) also showed a similar trend, but less
159 so than the HFS group (Fig. 2F). Insulin levels at 60 min were significantly higher in the HFS (0.62 ±
160 0.08 nM) than those in the HiSuc group (0.23 ± 0.03 nM, P = 0.022) and control group (0.24 ± 0.03 nM,

161 $P = 0.016$) (Fig. 2B). The basal levels of GLP-1 of control, HFS, HiFat, and HiSuc groups were 20.4 ±
162 2.6, 12.1 ± 1.5, 12.6 ± 2.3, and 18.2 ± 3.8 pM respectively. Due to varied basal GLP-1 levels in each
163 treatment group (Fig. 1), changes in GLP-1 concentrations from the basal value (ΔGLP-1) were
164 presented to illustrate postprandial GLP-1 response in this study. The GLP-1 level significantly
165 increased after 15 min in HFS (17.2 ± 2.6 pM, $P < 0.001$), compared to basal level. HiFat groups also
166 showed similar level (16.6 ± 3.7 pM, $P = 0.156$) without significant difference, while the HiSuc group
167 (6.6 ± 4.8 pM, $P = 1.000$) was more similar to the control group (7.3 ± 2.3 pM, $P = 0.169$) (Fig. 2C).
168 The AUC of HOMA-IR was calculated using the glycaemic and insulin response (0-120 min) during
169 the MTT to estimate the degree of insulin resistance in the postprandial state. The results revealed that
170 the HFS group (22.4 ± 1.0, $P = 0.008$) had the highest AUC of HOMA-IR, followed by the HiFat group
171 (18.3 ± 7.3, $P = 1.000$), whereas the HiSuc group (8.2 ± 0.9, $P = 1.000$) showed a similar level to the
172 control group (8.9 ± 1.1) (Fig. 2H).

173 After receiving the test diet for 4 weeks, AUC of postprandial glycaemia in the HFS (301.6 ±
174 6.1 mg/dL), HiFat (293.0 ± 5.9 mg/dL), and HiSuc (291.6 ± 7.4 mg/dL) groups were almost similar to
175 that in the control group (291.3 ± 6.6 mg/dL, $P = 0.579$). The HFS group (1.53 ± 0.18 nM, $P = 0.010$)
176 had a higher postprandial insulin AUC than the control group (0.77 ± 0.06 nM), while the HiFat group
177 (1.17 ± 0.14 nM, $P = 0.676$) showed an intermediate level between the HFS and control group (Fig.
178 3B). The GLP-1 response and HOMA-IR AUC in the HiSuc group showed similar values to those of
179 the HiFat group, in contrast to the result of the MTT after 2 weeks (Fig. 2). The basal levels of GLP-1
180 of control, HFS, HiFat, and HiSuc groups were 15.0 ± 1.9, 13.7 ± 3.7, 11.8 ± 2.9 and 11.9 ± 2.7
181 respectively. Postprandial GLP-1 levels at 15 min were significantly higher in all of the treatment
182 groups (HFS, HiFat, and HiSuc, 13.0 ± 3.3 - 14.1 ± 4.4 pM, $P < 0.05$), compared to the basal level (Fig.
183 3C). Significant effects of treatment (TR) on postprandial GLP-1 secretion were also detected by two-

184 way repeated measure ANOVA in both MTTs (P = 0.031 and P = 0.003, respectively) (Fig. 2C and
185 3C). Although significant differences were not detected, ΔAUC of GLP-1 in the HFS, HiFat, and
186 HiSuc groups tended to increase when compared to the control group (P = 0.161), as shown in Fig. 3G.

187 *Effect of feeding a chronic high fat and/or high sucrose diet on body weight gain, food intake, and fat*
188 *accumulation*

189 After receiving the test diet for 5 weeks, body weight gain and total energy intake of the HiFat
190 (232.3 ± 7.8 g, P = 0.021 and 2966.3 ± 56.8 kcal, P = 0.039) and HFS groups (228.0 ± 7.8 g, P = 0.039
191 and 2958.6 ± 60.3 kcal, P = 0.032) were significantly higher than those of the control group (200.7
192 ±5.4 g and 2736.6 ± 61.7 kcal), as shown in Table 2. Epididymal adipose tissue weight in the HFS
193 group (9.7 ± 0.5 g, P = 0.040) was significantly higher than the control (7.5 ± 0.6 g). The HiSuc
194 feeding group showed similar values for these parameters as those seen in the control group.

195 *Peptide hormones, cholesterol, and triacylglycerol levels in the portal vein after feeding with the test*
196 *diet for 5 weeks.*

197 On the final day of the experiment, blood samples were collected from the portal vein after
198 overnight fasting. The results showed that the HFS group had the highest values for the glucose (107.3
199 ± 3.9 mg/dL, P = 0.030), insulin (1.38 ± 0.20 nM, P = 0.069), total cholesterol (35.9 ± 3.5 mg/dL, P =
200 0.023), and TG levels (76.1 ± 3.1 mg/dL, P = 0.028) (Fig. 4). The HiFat group overall showed similar
201 results, but slightly lower values for these parameters, compared to the HFS group. The HiSuc group
202 had results similar to the control group. The GLP-1 levels in the HFS (69.3 ± 9.0 pM, P = 0.325) and
203 HiFat groups (61.3 ± 8.6 pM, P = 0.812) were slightly higher than the control group (51.8 ± 6.2 pM),
204 but without significant differences (Fig. 4C).

205 *GLP-1 content in the intestinal tissues of rats after feeding with the test diet for 5 weeks.*

206 In the jejunum segment, the HFS group (4.8 ± 0.4 pmol/mg protein) had a significantly higher
207 GLP-1 content than that of the control group (3.2 ± 0.2 pmol/mg protein, $P = 0.004$), while in the other
208 segments (ileum, cecum, and colon) significant differences were not observed (Fig. 5A, B, C, and D).
209

210 **Discussion**

211 Excess energy consumption, especially from a high fat and/or high sugar diet, is a major risk
212 factor for the development of metabolic disorders, including obesity, insulin resistance, and type II
213 diabetes in rodent [20,24-26]. Therefore, diets containing high fat and/or high sucrose are commonly
214 used to establish obesity development in animal models [24,27-29]. In this study, a HFS diet (30% fat
215 and 40% sucrose), HiFat diet (30% fat diet), or HiSuc diet (40% sucrose) were provided to rats for a
216 total of 5 weeks in order to determine whether a combined high fat/high sucrose diet, an individual high
217 fat or high sucrose diet contributed to adaptive changes in postprandial GLP-1 secretion and obesity
218 development.

219 As expected, body weight gain and total energy intake of the HFS and HiFat groups were
220 higher than the control group, while the HiSuc group was similar to the control group. The results were
221 also consistent with a previous report, in which chronic feeding of HFS and HiFat caused additional
222 body weight gain and obesity development [27-33]. Consumption of a high caloric diet directly relates
223 to fat accumulation in various tissues and also obesity development [27,29,34]. Indeed, the epididymal
224 adipose tissue weight of the HFS and HiFat groups increased largely when compared with the HiSuc
225 group. These results suggest that the high fat diet, rather than high sucrose diet has a potent impact on
226 obesity (adiposity) development in rodent. Nevertheless, a combination of a high fat and high sucrose
227 diet had an intense impact on obesity development, compared to a high fat diet alone.

228 Nowadays, several methodologies, such as the OGTT and MTT, are available to assess
229 postprandial glucose and insulin responses [35,36]. However, a previous study supported the fact that
230 the MTT is more reflective of postprandial metabolic responses than the OGTT [37]. In addition, we
231 considered that the voluntary ingestion was more appropriate than enforced gavage feeding to mimic
232 the dietary exposure in normal life. Therefore, in the present study, MTT, using ingestion of the
233 standard (control) diet at a dose of 10 g/kg body weight, was performed in order to evaluate
234 postprandial glycaemia, insulin, and GLP-1. Unfortunately, some rats did not consume more than 90%
235 weight of the provided diet (20.3 to 89.7% of provided diet) which could be a limitation of the current
236 study. The data from these rats were not included since glycaemic and gut hormone responses primarily
237 depend on amount of food ingested. Because it was difficult to interpret the data from all of rats with
238 largely varied food consumption, we decided to omit the data from those rats. It is assumed that lower
239 diet consumption resulted in lower GLP-1 secretion compared to rats consumed more than 90% of the
240 diet since GLP-1 secretion depends on the caloric load administered [38-39.] Nevertheless, significant
241 effects of treatment (diet) were detected by two-way repeated ANOVA in MTTs. Accordingly, we
242 consider that oral administration of meal solution would be suitable to assess postprandial responses
243 instead of voluntary feeding in MTT using animal models. Although it was difficult to control, the
244 results obtained from the MTT with voluntary feeding should have significant meaning to understand
245 postprandial glycaemic and gut hormone responses.

246 Continuous feeding of the HFS and HiFat diet for 2 weeks highly affected the postprandial
247 insulin response and slightly enhanced postprandial GLP-1 response. Likewise, we found that the AUC
248 of HOMA-IR was largely increased by feeding with the HFS diet for 2 weeks. This indicates that
249 feeding with the HFS diet immediately triggered the development of insulin resistance. Interestingly,
250 continuous consumption of the HiSuc diet for 4 weeks illustrated that the postprandial GLP-1, insulin,

251 and HOMA-IR parameters eventually matched those observed for the HiFat feeding group. It can be
252 concluded that chronic feeding with the HiSuc diet gradually increased the postprandial insulin
253 response and HOMA-IR index. These findings are supported by a previous study, which demonstrated
254 that the HiFat diet caused severe metabolic dysfunction faster than the HiSuc diet [24].

255 After feeding with the test diet for 2 weeks, the HFS feeding groups showed significant
256 elevation of the postprandial GLP-1 level, while the other groups did not. As all rats were given the
257 identical control diet for the MTT, differences in the GLP-1 response should be attributed to the
258 differences in the rat phenotype. In addition, basal GLP-1 levels did not differ significantly throughout
259 the experimental period. One of limitations of the present study is that significant differences were not
260 detected in absolute GLP-1 values between treatments (Fig. 2D and 3D). However, by observing
261 incremental GLP-1 (ΔGLP-1), it was found that postprandial GLP-1 responses differed between groups
262 (assessed by two-way repeated measure ANOVA). These results suggest that the sensitivity of GLP-1-
263 producing L-cells to luminal nutrients (protein, carbohydrates, or fatty acids) was enhanced by chronic
264 feeding with the HFS diets. The HiFat and HiSuc feeding group also had a similar response after
265 receiving the test diet for 4 weeks, suggesting that ingestion of excessive fat and sucrose rapidly
266 enhances the sensitivity of GLP-1-producing cells to luminal nutrients rather than individual fat or
267 sucrose alone. GLP-1 is secreted in response to macro-nutrient ingestion (proteins, carbohydrates, and
268 triglycerides) [8,40,41], and we used a control diet containing all of the nutrients in the present study.
269 Therefore, specific diet components that contributed to enhanced postprandial GLP-1 secretion in
270 obese animals were not identified. It would be interesting to investigate which nutrient enhances the
271 sensitivity of L cells under diet-induced obesity.

272 To date, it is still controversial whether GLP-1 is increased or diminished during obesity
273 development. In this study, we found that the postprandial GLP-1 level was slightly increased in the

274 HFS and HiFat feeding groups, consistent with previous studies [17,31,32]. In contrast, other studies
275 have demonstrated that chronic feeding with a high fat diet diminished the GLP-1 secretion response to
276 oral glucose and impaired the function of GLP-1-producing L cells [19,42]. The different results
277 observed by each research group might be due to differences in the experimental design, such as the
278 treatment period, diet composition, rodent species, etc.

279 From the MTT experiments, postprandial glycaemic responses were apparently unchanged by
280 chronic feeding with the high fat and/or high sucrose diet throughout the experimental period, whereas
281 postprandial insulin and GLP-1 secretion increased progressively. Because significant differences were
282 not detected in absolute GLP-1 levels among treatment groups, we then speculated that the incremental
283 GLP-1 (ΔGLP-1) levels played an important role in blood glucose regulation through enhanced insulin
284 secretion. This may be partially explained by increased GLP-1 response as observed. In addition, a
285 recent study suggest that treatment with GLP-1 receptor antagonist exendin (9-39) (Ex9) reduced body
286 weight gain in rats fed a high fat diet without affecting food intake, compared to untreated rats
287 suggesting that Ex9 increased energy expenditures [43]. Moreover, increasing of GLP-1 secretion in
288 high fat fed rats lead to hyperinsulinemia, thus promoting energy storage (decreasing energy
289 expenditure) and contributing to body weight gain [43]. We therefore speculate that HFS and HiFat
290 groups had higher body weight gain, compared to control group might involve hyperinsulinemia and
291 lowering energy expenditure. However, previous studies demonstrated that high fat diet-fed rats
292 exhibited impaired- GLP-1 signaling [44] and reduced GLP-1 production [45], which possibly
293 promoted hyperphagia and increased body weight gain. Those reports focused on intestinal GLP-1
294 content and GLP-1 signaling instead of GLP-1 secretion. It is interesting to examine whether
295 postprandial GLP-1 responses increased or decreased in other DIO models (obese-prone and -resistant
296 rats). The GLP-1 content of the jejunum segment in the HFS group was higher than that of the control

297 group; whereas in other intestinal segments, significant differences were not detected, indicating that
298 the jejunum was more prone to adapting to an obesogenic diet feeding than other intestinal segments. It
299 can be assumed that the L cells in the jejunum might be the major source of enhanced postprandial
300 GLP-1 secretion, based on the present result. Supporting our results, postprandial GLP-1 secretion is
301 reported to be a direct action of the luminal contents on enteroendocrine L cells in the distal jejunum
302 [46,47]. Although the majority of enteroendocrine L-cells are located in the distal intestine, our result
303 suggests that L cells in the proximal intestine play a role in the rapid secretion of GLP-1 in response to
304 meal ingestion. Moreover, L-cell numbers in jejunoileum (alimentary channel) are reportedly higher
305 than the distal gut regions (colon) after Roux-en-Y Gastric Bypass (RYGB) [48], suggesting that L-cell
306 numbers in upper jejunum part were adaptively increased after the surgery. In addition, the RYGB
307 contributed to intestinal hypertrophy in rats, which might be due to nutrients-induced stimuli in the
308 jejunum part [48]. It seems consistent with our findings that jejunum part is prone to adapt to stimulant
309 rather than other intestinal segments.
310

311 **Conclusions**

312 Postprandial GLP-1 secretion was elevated in rats continuously fed the HFS diet within 2 weeks.
313 Continuous feeding of the HiFat or HiSuc diet for 4 weeks, exhibited a similar effect on the
314 postprandial GLP-1 response, suggesting that excessive ingestion of high fat/high sucrose diet rapidly
315 caused adaptive changes in nutrient sensitivity in GLP-1-producing cells, rather than individual high fat
316 or high sucrose diet alone. However, HiFat diet likely has relatively potent effect on the GLP-1
317 response compared to HiSuc diet. This may have a role in the normalization of postprandial glycaemia
318 and the slowing of the establishment of glucose intolerance.
319

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Conflict of Interest: The authors declare that they have no conflicts of interest.

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462 **Table 1.** Test diet composition

Ingredient	Control	HFS	HiFat	HiSuc
	g/kg of diet			
Cornstarch	397.486	–	167.486	97.486
Casein[1]	200	200	200	200
Dextrinized cornstarch[2]	132	–	132	132
Sucrose	100	399.486	100	400
Soybean oil	70	70	70	70
Lard oil	–	230	230	–
Fibre (cellulose)[3]	50	50	50	50
Mineral mixture[4]	35	35	35	35
Vitamin mixture[4]	10	10	10	10
L-Cystine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
tert-Butylhydroquinone	0.014	0.014	0.014	0.014
Protein (energy %)	20.5	15.9	15.9	20.5
Carbohydrate	63.6	31.3	31.3	63.6
Fat (energy %)	15.9	52.8	52.8	15.9
Energy density (kcal/g)	3.96	5.11	5.11	3.96

463 [1] Acid Casein (Fonterra, Ltd., Auckland, New Zealand);

464 [2] TK-16 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan);

465 [3] Avicel PH102 (Asahi Kasei Chemicals Corporation, Tokyo, Japan);

466 [4] Mineral and vitamin mixtures were prepared according to the AIN-93G formulation.

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472 **Table 2.** Initial body weight, body weight gain, visceral adipose tissue (mesenteric, epididymal, and
473 retroperitoneal) weight, and energy intake after feeding with the test diet for 5 weeks

	Control	HFS	HiFat	HiSuc
Initial weight (g)	168.7 ± 3.1 ^{NS}	169.3 ± 2.5	169.1 ± 4.3	167.7 ± 2.8
Body weight gain (g)	200.7 ±5.4^{b}	228.0 ± 7.8^{a}	232.3 ± 7.8^{a}	220.3 ± 7.9^{ab}
Visceral fat (g)	25.0 ± 1.6 ^{NS}	29.7 ± 1.5	29.2 ± 2.0	23.6 ± 1.4
Mesenteric fat (g)	7.2 ± 0.5 ^{NS}	7.8 ± 0.4	7.8 ± 0.5	6.7 ± 0.4
Epididymal fat (g)	7.5 ± 0.6^{bc}	9.7 ± 0.5^{a}	9.3 ± 0.8^{ab}	6.9 ± 0.5^{c}
Retroperitoneal fat (g)	10.4 ± 0.6^{NS}	12.1 ± 0.7	12.1 ± 0.8	10.0 ± 0.6
Total energy intake (kcal)	2736.6 ± 61.7^{b}	2958.6 ± 60.3^{a}	2966.3 ± 56.8^{a}	2809.9 ± 40.0^{ab}

474 Visceral fat weight is the sum of the mesenteric, epididymal, and retroperitoneal fat weight. The values
475 were expressed as the mean ± SEM for n = 8-10 rats (control = 10, HFS = 10, HiFat = 8, HiSuc = 8).
476 The superscripts without the same letters differed significantly between treatments ($P < 0.05$, Tukey-
477 Kramer's test). NS indicates that there was no significant difference among the treatments.

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484 **Figure legends**

485 **Fig. 1. Basal glucose, insulin, GLP-1 levels, and HOMA-IR after feeding with the test diet for 2**
486 **and 4 weeks.**

487 Rats were given the control, HFS, HiFat, or HiSuc diet (*ad libitum*) for 2 and 4 weeks. On the
488 day of the experiment, blood samples were collected from the tail vein after overnight fasting (16 h),
489 before conducting the MTT. The values were expressed as the mean ± SEM for n = 4-9 (A-D; control =
490 9, HFS = 8, HiFat = 4, HiSuc = 7, E-H; control = 9, HFS = 6, HiFat = 5, HiSuc = 6) rats. NS indicates
491 that there was no significant difference among the treatments (Tukey-Kramer's test, $P < 0.05$).

492

493 **Fig. 2. Postprandial glucose, insulin, GLP-1 level, and HOMA-IR during the meal tolerance test**
494 **(MTT), after feeding with the test diet for 2 weeks.**

495 Rats were given the control, HFS, HiFat, or HiSuc diet (*ad libitum*) for 2 weeks; then, the MTT
496 was performed. After overnight fasting, basal blood (0 min) was taken, followed by feeding of the
497 control diet (10 g/kg body weight) for 30 min, and blood samples were collected until 120 min. The
498 values were expressed as the mean ± SEM for n = 4-9 rats (control = 9, HFS = 8, HiFat = 4, HiSuc = 7).
499 Two-way repeated measure ANOVA *P* values for time (TI), for treatment (TR), and for the interactions
500 of time and treatment (TI x TR) are shown in each panel (A, B, C). The superscripts without the same
501 letters differed significantly between treatments ($P < 0.05$, Dunn's multiple range test). Asterisks (*)
502 indicate significant differences from the basal value (0 min) in each group ($P < 0.05$, Dunn's with
503 control). NS indicates that there was no significant difference among the treatments.

504

505 **Fig. 3. Postprandial glucose, insulin, GLP-1 level, and HOMA-IR during the meal tolerance test**
506 **(MTT), after feeding with the test diet for 4 weeks.**

507 Rats were given the control, HFS, HiFat, or HiSuc diet (*ad libitum*) for 4 weeks; then, MTT was
508 performed. After overnight fasting, basal blood (0 min) was taken, followed by feeding of the control
509 diet (10 g/kg body weight) for 30 min, and blood samples were collected until 120 min. The values
510 were expressed as the mean ± SEM for n = 5-9 (control = 9, HFS = 6, HiFat = 5, HiSuc = 6) rats. Two-
511 way repeated measure ANOVA *P* values for time (TI), for treatment (TR), and for the interactions of
512 time and treatment (TI x TR) are shown in each panel (A, B, C). The superscripts without the same
513 letters differed significantly between treatments ($P < 0.05$, Dunn's multiple range test). Asterisks (*)
514 indicate significant differences from the basal value (0 min) in each group ($P < 0.05$, Dunn's with
515 control). NS indicates that there was no significant difference among the treatments.

516

517 **Fig. 4. Portal glucose, insulin, GLP-1, cholesterol, and triglyceride (TG) levels after feeding with**
518 **the test diet for 5 weeks.**

519 Rats were given the control, HFS, HiFat, or HiSuc diet (*ad libitum*) for 5 weeks. After overnight
520 fasting, blood samples were collected from the portal vein under sodium pentobarbital anaesthesia (50
521 mg/kg of body weight). The values were expressed as the mean ± SEM for n = 8-10 (control = 10, HFS
522 = 10, HiFat = 8, HiSuc = 8) rats. The superscripts without the same letters differed significantly
523 between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant
524 difference among the treatments.

525

526 **Fig. 5. GLP-1 content in intestinal tissues after feeding with the test diet for 5 weeks.**

527 After feeding with the test diets for 5 weeks, each intestinal segment: (A) jejunum, (B) ileum,
528 (C) cecum, and (D) colon was collected. The values were expressed as the mean ± SEM for n = 8-10
529 (control = 10, HFS = 10, HiFat = 8, HiSuc = 8) rats. The superscripts without the same letters differed
530 significantly between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no
531 significant difference among the treatments.

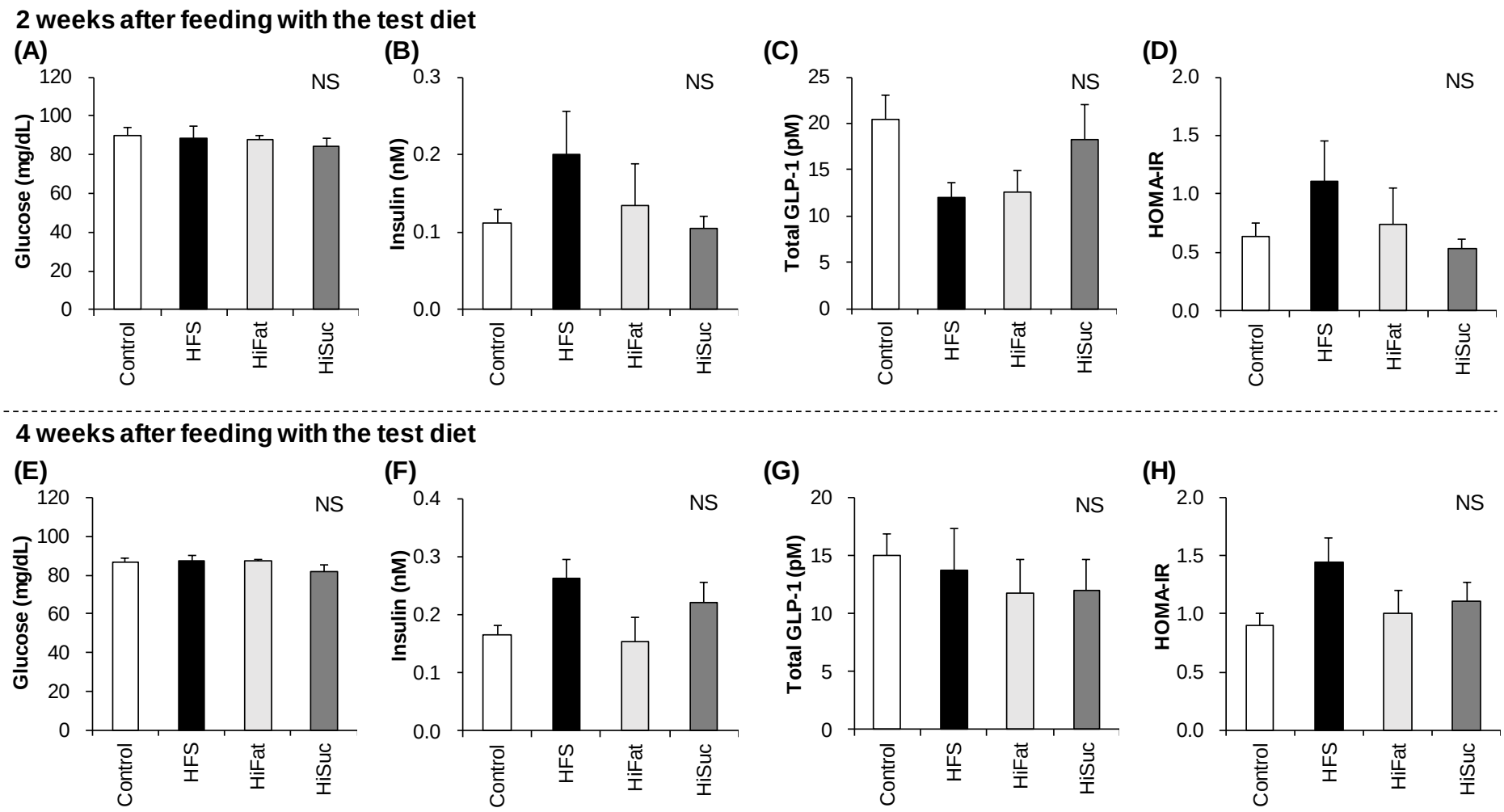

2 weeks after feeding with the test diet
(A)
Glucose (mg/dL)
120
100
80
60
40
20
0
NS
Control
HFS
HiFat
HiSuc
(B)
Insulin (nM)
0.3
0.2
0.1
0.0
NS
Control
HFS
HiFat
HiSuc
(C)
Total GLP-1 (pM)
25
20
15
10
5
0
NS
Control
HFS
HiFat
HiSuc
(D)
HOMA-IR
2.0
1.5
1.0
0.5
0.0
NS
Control
HFS
HiFat
HiSuc
4 weeks after feeding with the test diet
(E)
Glucose (mg/dL)
120
100
80
60
40
20
0
NS
Control
HFS
HiFat
HiSuc
(F)
Insulin (nM)
0.4
0.3
0.2
0.1
0.0
NS
Control
HFS
HiFat
HiSuc
(G)
Total GLP-1 (pM)
20
15
10
5
0
NS
Control
HFS
HiFat
HiSuc
(H)
HOMA-IR
2.0
1.5
1.0
0.5
0.0
NS
Control
HFS
HiFat
HiSuc

Fig. 1.

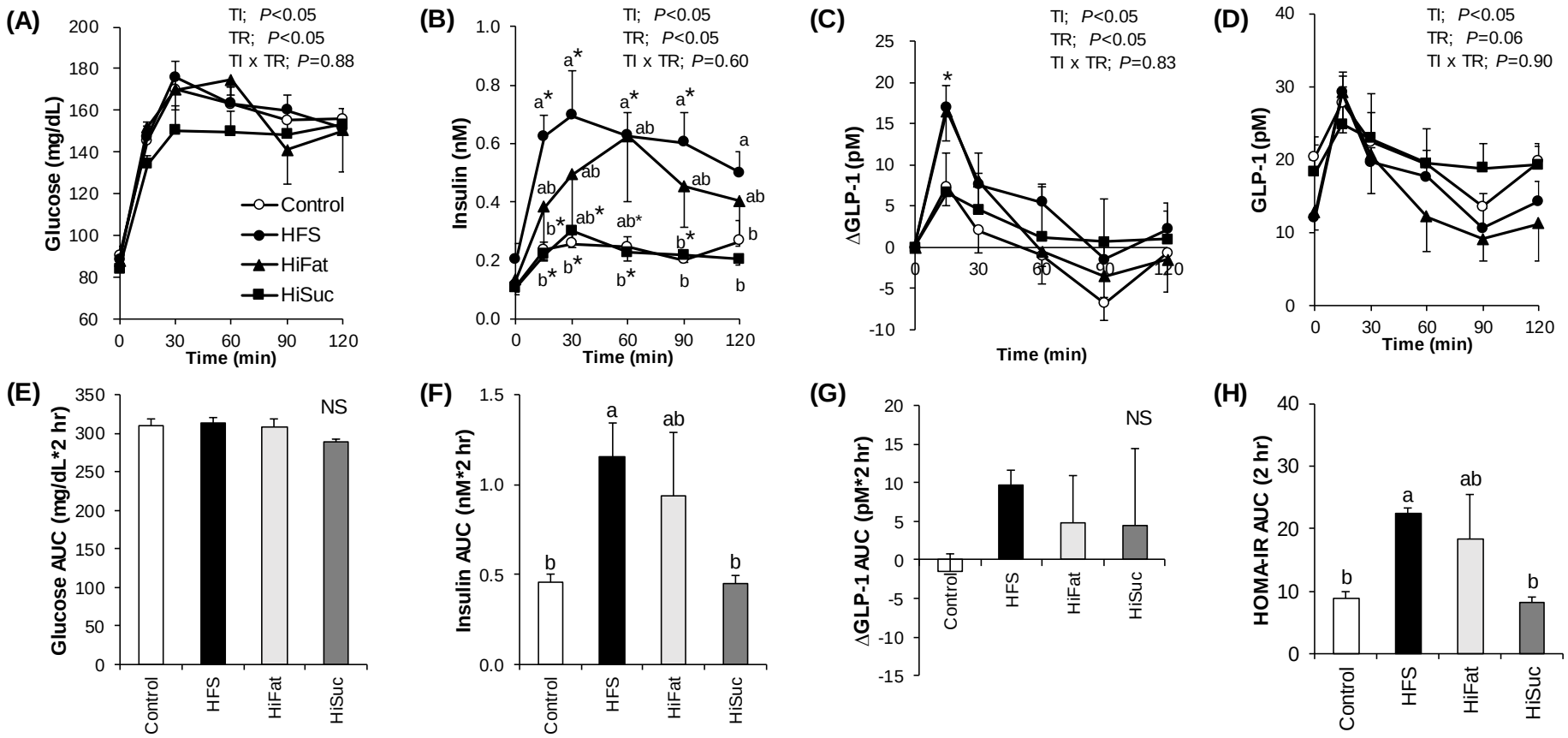

(A)
TI; P<0.05
TR; P<0.05
TI x TR; P=0.88
Glucose (mg/dL)
Time (min)
Control
HFS
HiFat
HiSuc
(B)
TI; P<0.05
TR; P<0.05
TI x TR; P=0.60
Insulin (nM)
Time (min)
(C)
TI; P<0.05
TR; P<0.05
TI x TR; P=0.83
ΔGLP-1 (pM)
Time (min)
(D)
TI; P<0.05
TR; P=0.06
TI x TR; P=0.90
GLP-1 (pM)
Time (min)
(E)
Glucose AUC (mg/dL*2 hr)
NS
Control
HFS
HiFat
HiSuc
(F)
Insulin AUC (nM*2 hr)
Control
HFS
HiFat
HiSuc
(G)
ΔGLP-1 AUC (pM*2 hr)
NS
Control
HFS
HiFat
HiSuc
(H)
HOMA-IR AUC (2 hr)
Control
HFS
HiFat
HiSuc

Fig. 2.

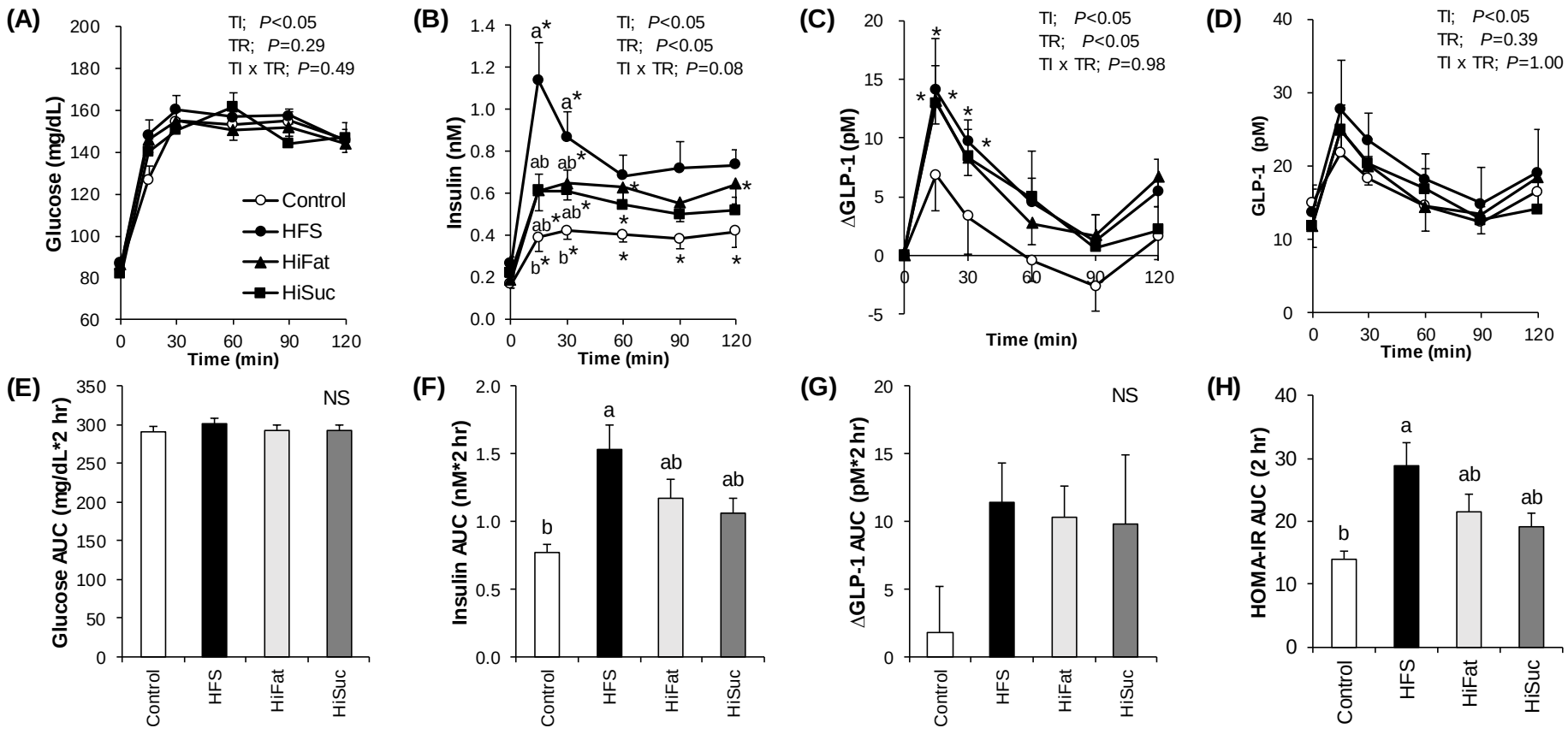

(A)
TI; P<0.05
TR; P=0.29
TI x TR; P=0.49
Glucose (mg/dL)
Time (min)
Control
HFS
HiFat
HiSuc
(B)
TI; P<0.05
TR; P<0.05
TI x TR; P=0.08
Insulin (nM)
Time (min)
(C)
TI; P<0.05
TR; P<0.05
TI x TR; P=0.98
ΔGLP-1 (pM)
Time (min)
(D)
TI; P<0.05
TR; P=0.39
TI x TR; P=1.00
GLP-1 (pM)
Time (min)
(E)
Glucose AUC (mg/dL*2 hr)
NS
(F)
Insulin AUC (nM*2 hr)
(G)
ΔGLP-1 AUC (pM*2 hr)
NS
(H)
HOMA-IR AUC (2 hr)
Control
HFS
HiFat
HiSuc

Fig. 3.

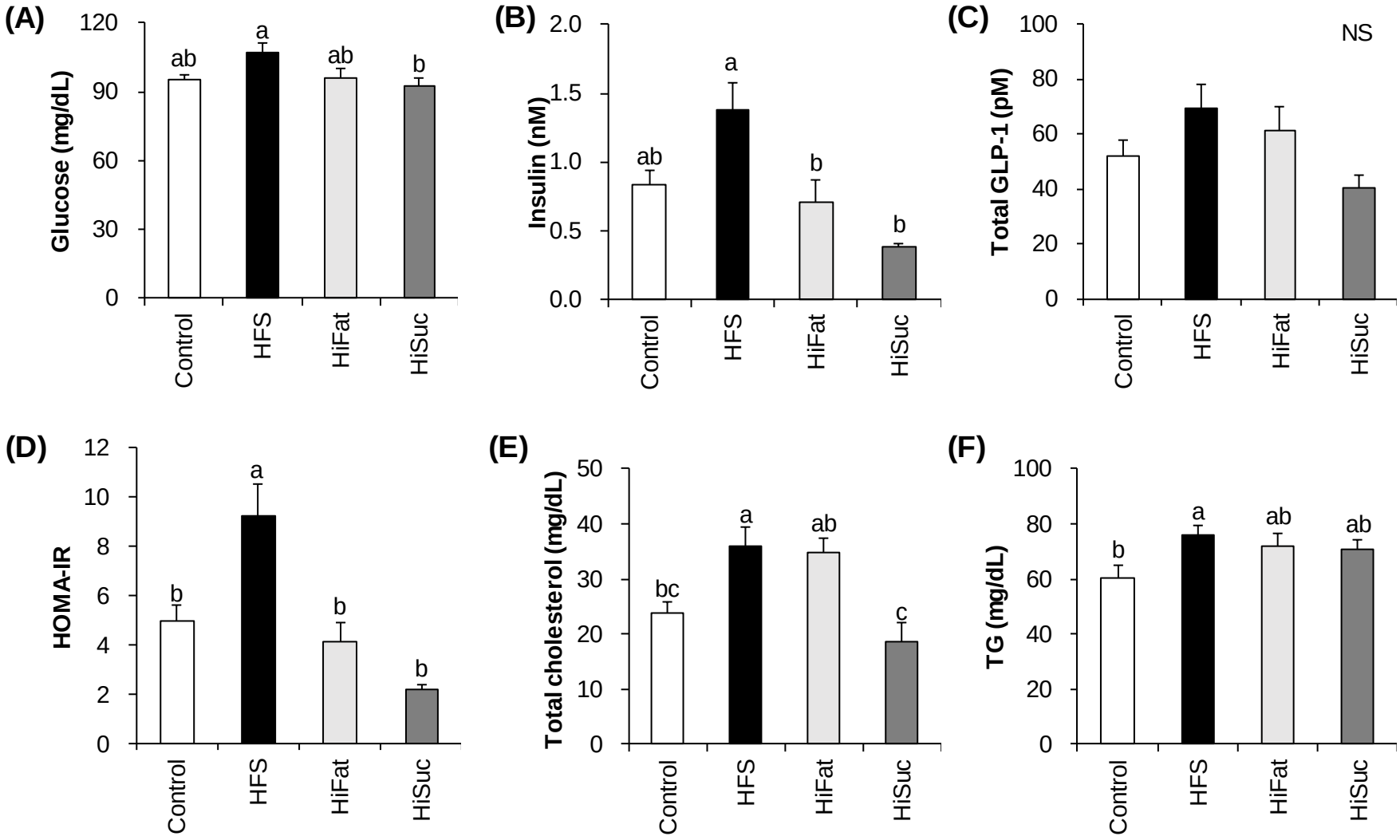

(A)
Glucose (mg/dL)
120
90
60
30
0
ab
a
ab
b
Control
HFS
HiFat
HiSuc
(B)
Insulin (nM)
2.0
1.5
1.0
0.5
0.0
ab
a
b
b
Control
HFS
HiFat
HiSuc
(C)
Total GLP-1 (pM)
100
80
60
40
20
0
NS
Control
HFS
HiFat
HiSuc
(D)
HOMA-IR
12
10
8
6
4
2
0
b
a
b
b
Control
HFS
HiFat
HiSuc
(E)
Total cholesterol (mg/dL)
50
40
30
20
10
0
bc
a
ab
c
Control
HFS
HiFat
HiSuc
(F)
TG (mg/dL)
100
80
60
40
20
0
b
a
ab
ab
Control
HFS
HiFat
HiSuc

Fig. 4.

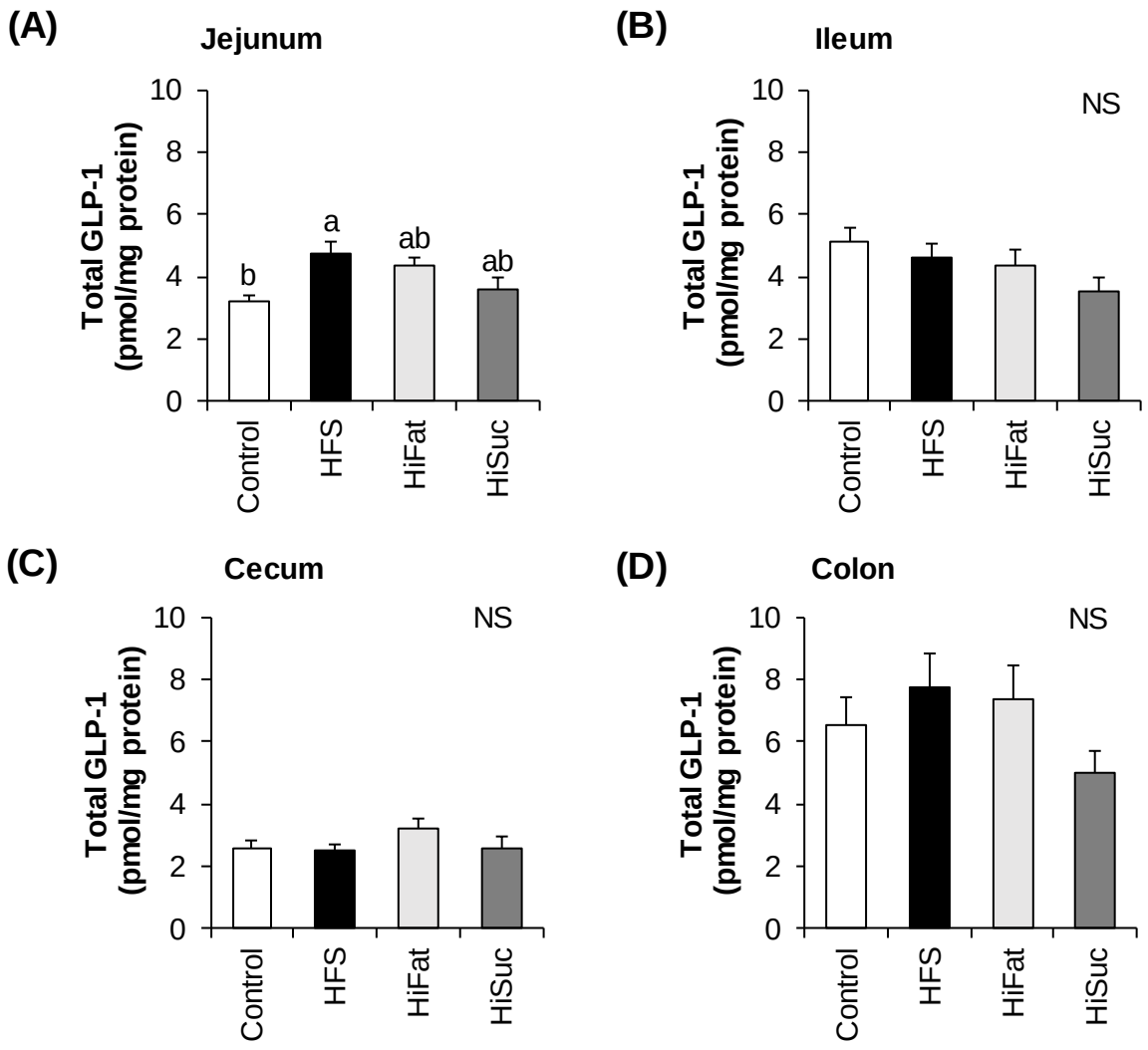

(A)
Jejunum
Total GLP-1 (pmol/mg protein)
10
8
6
4
2
0
b
a
ab
ab
Control
HFS
HiFat
HiSuc
(B)
Ileum
NS
(C)
Cecum
NS
(D)
Colon
NS

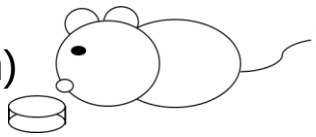
Fig. 5.

Continuous feeding <u>obesogenic</u> diets
(HFS, HiFat, or HiSuc diet, vs control diet)

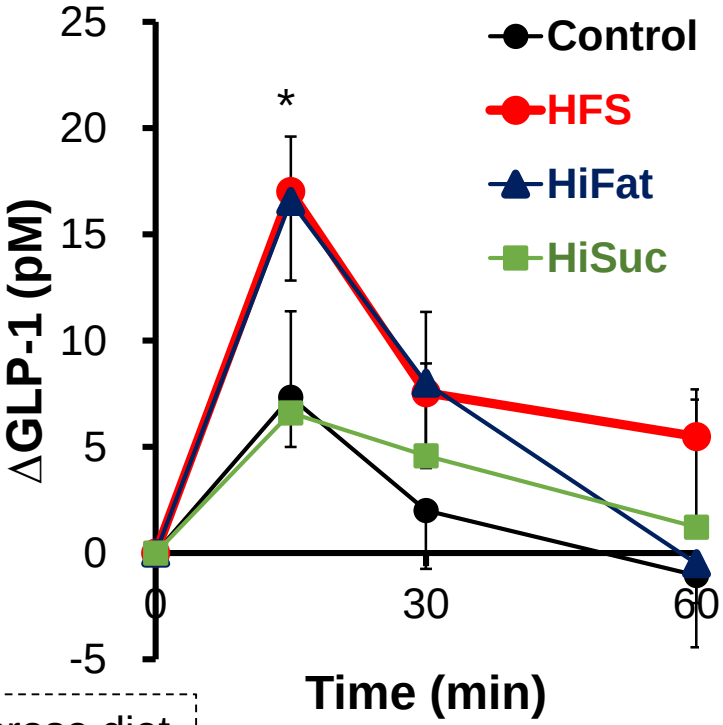
↓ after 2 and 4 weeks

MTTs to assess postprandial GLP-1 response

Voluntary ingestion
Control diet (30 min)



Rats fed **HFS** diet had enhanced GLP-1 response to luminal nutrients after 2 weeks



Combination of **fat and sucrose** rapidly enhanced GLP-1 secretory response during obesity development

MTT; Meal tolerance test
HiFat; High fat diet
HFS; High fat/high sucrose diet
HiSuc; High sucrose diet