



Title	Postprandial glucagon-like peptide-1 secretion is increased during the progression of glucose intolerance and obesity in high-fat/high-sucrose diet-fed rats
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1 **Title**

2 Postprandial GLP-1 secretion is increased during the progression of glucose intolerance
3 and obesity in high-fat/high-sucrose diet fed rats

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17 **Statement of Author's Contributions to Manuscript**

18 S. N., T. H., and H. H. designed research; S.N. conducted research and analyzed data;
19 S.N. and T. H. wrote the paper. T. H. had primary responsibility for final content. All
20 authors read and approved the final manuscript.

21 **RUNNING TITLE:** Postprandial GLP-1 in the progress of obesity

22 **Keywords:** Obesity, GLP-1 (Glucagon-like peptide-1), HF/HS diet (high fat and high
23 sucrose diet), MTT (Meal tolerance test).

24

25 **Abstract**

26 Glucagon-like peptide-1 (GLP-1) is secreted from distal enteroendocrine cells in
27 response to luminal nutrients, and exerts insulinotropic and anorexigenic effects.
28 Although GLP-1 secretory responses under established obese or diabetic conditions
29 have been studied, it has not been investigated whether or how postprandial GLP-1
30 responses were affected during the progression of diet-induced obesity. In the present
31 study, a meal tolerance test (MTT) was performed every week in rats fed a high fat and
32 high sucrose diet (HF/HS diet) to evaluate the postprandial glycemic, insulin, and
33 GLP-1 responses. In addition, gastric emptying was assessed by the acetaminophen
34 method. After 8 weeks of HF/HS diet treatment, portal vein and intestinal mucosa were
35 collected to examine GLP-1 production. Postprandial glucose in response to normal
36 meal ingestion was increased in the HF/HS diet group within 2 weeks, and its elevation
37 gradually returned close to control group until day 50. Slower postprandial gastric
38 emptying was observed in the HF/HS diet group at days 6, 13, and 34. Postprandial
39 GLP-1 and insulin response were increased in HF/HS group at 7 weeks. Higher portal
40 GLP-1 and insulin levels were observed in the HF/HS diet group, but mucosal gut
41 hormone mRNA levels were unchanged. These results revealed that the postprandial
42 GLP-1 response to meal ingestion is enhanced during the progression of diet-induced
43 glucose intolerance and obesity in rats. The boosted postprandial GLP-1 secretion by
44 chronic HF/HS diet treatment suggests increased sensitivity to luminal nutrients in the
45 gut, and this may slow the establishment of glucose intolerance and obesity.

46

47 **Introduction**

48 Obesity and glucose intolerance are major risk factors for various diseases, such as
49 cancer, depression, diabetes, and cardiovascular disease (1-3). Excessive energy (food)
50 intake is a critical cause of obesity. In response to every meal ingestion, various gut
51 hormones are immediately released from enteroendocrine cells to regulate postprandial
52 responses, including gut motility, pancreatic endocrine and exocrine secretions, and
53 satiety induction (4, 5). Since some gut hormones have anorexigenic and insulinotropic
54 action, enteroendocrine hormone mimetics is thought to be a new therapy for obesity
55 and or diabetes (5, 6).

56 Postprandial glycemia is tightly regulated not only by insulin action but also by the
57 gastric emptying rate (7). Glucagon-like peptide-1 (GLP-1) has critical roles in
58 maintaining postprandial glycemia through its insulinotropic effect and gastric
59 inhibitory effect (8). Secretion of GLP-1 is stimulated by luminal nutrients, including
60 glucose, fatty acids, proteins, protein hydrolysates, and amino acids (9, 10), indicating
61 that postprandial GLP-1 release represents the sensitivity to luminal nutrients in the gut.
62 Because of these physiological functions of GLP-1, incretin-based therapy using GLP-1
63 receptor agonists or dipeptidyl peptidase-IV inhibitors is increasingly used for treatment
64 of diabetes (11, 12).

65 Although the insulinotropic effect of GLP-1 under normal condition and
66 improvement of glucose tolerance under diabetic condition by GLP-1-based therapies
67 are well recognized, changes (reduced, enhanced or unchanged) in nutrient-induced
68 GLP-1 secretion in type 2 diabetes patients are still controversial (13-15). In high fat
69 (HF) diet-induced obesity animal model, GLP-1 secretory response was decreased to
70 glucose (16, 17), but unchanged to fatty acids (18). However, it has not been

71 characterized yet whether the GLP-1 secretory response to ‘meal’ is decreased or
72 increased during the progression of diet-induced obesity. In the present study, rats were
73 fed with a high-fat and high-sucrose diet (HF/HS diet) to induce obesity. To examine the
74 physiological response to meal ingestion during the progression of obesity, a “normal
75 diet” was orally given to rats every week for measurement of postprandial plasma
76 glucose, insulin, and GLP-1 levels as meal tolerance test (MTT) rather than loading a
77 glucose solution (oral glucose tolerance test).

78

79 **Materials and Methods**

80 **Animals**

81 Male Sprague-Dawley rats (5 weeks old) were purchased from Japan SLC
82 (Hamamatsu, Japan). The experiments were performed in a temperature-controlled
83 room maintained at $23 \pm 2^\circ\text{C}$ with a 12 h light-dark cycle (8:00-20:00, light period).
84 Rats were fed AIN-93G (control) diet for 1 week as an acclimation period, and then
85 divided into 3 groups based on body weight. Control and HF/HS groups were
86 respectively fed AIN-93G diet or a fat/sucrose rich diet ad libitum (see Table 1 for
87 composition of each diet). Because the food intake (in grams) is generally lower in
88 HF/HS diet compared to control diet due to high energy density of HF/HS diet, this
89 results in relatively lower protein, mineral and vitamin intake in HF/HS group compared
90 to control group, and the deficient in these nutrients affects the expression of nutrient
91 transporters and receptors (19-21). To compensate the effect of lower protein /mineral
92 /vitamin intake in HF/HS group, the food-restricted group was included in the present
93 study. Rats in the food-restricted group were fed the control diet with the same amount
94 in grams as that consumed by the HF/HS group in the previous day to examine the

95 effects of reduced intake of nutrients, such as protein, minerals and vitamins. All rats
96 had free access to water throughout the experiment. The study was approved by the
97 Hokkaido University Animal Committee, and the animals were maintained in
98 accordance with the guidelines for care and use of laboratory animals at Hokkaido
99 University.

100 *Experimental protocol for meal tolerance test (MTT)*

101 A MTT was conducted every week to examine postprandial glycemc and GLP-1
102 responses after single meal (control diet) ingestion throughout the experiment. Rats
103 were fasted for 6 h (9:00-15:00) (22, 23, 24), and then orally administrated AIN-93G (3
104 g/kg body weight) diet suspended in deionized water (0.167 g/mL, 18 mL/kg body
105 weight) by a feeding tube (Fr.6, Atom Medical Co., Tokyo, Japan). The suspension
106 contained acetaminophen (100 mg/kg body weight) to evaluate gastric emptying rate
107 (25, 26). Tail vein blood samples (120 μ L) were collected just before (0 min), and 15,
108 30, 60, 90, and 120 min after the oral meal administration. Blood samples were
109 immediately mixed with aprotinin (final concentration at 500 KIU/mL, Wako Pure
110 Chemical Industries, Ltd. Osaka, Japan) and heparin (final concentration at 25 IU/mL,
111 Nacalai Tesque, Inc., Kyoto, Japan) on ice. Plasma was separated from blood samples
112 by centrifugation at $2,300 \times g$ for 10 min at 4°C, and then frozen at -80°C until
113 measurements were taken. Plasma glucose and acetaminophen were measured using
114 Glucose CII-test kit (Wako) and acetaminophen detection kit (Kanto Chemical Co., Inc.,
115 Tokyo, Japan), respectively. Homeostasis model assessment of insulin resistance
116 (HOMA-IR) was calculated using the following formula was as follows HOMA-IR =
117 {Fasting plasma glucose (mg/dL) \times Fasting plasma insulin (μ U/mL)}/2,430.

118 *Blood and tissue collection at final day*

119 After overnight fasting, rats were anesthetized using sodium pentobarbital
120 (Somnopentyl, Kyoritsu Seiyaku Co., Tokyo, Japan) on day 56. The waist
121 circumference length (mid-line girth) of individual rat was measured as an obesity
122 parameter which reflects the amount of adipose tissue (27, 28). Portal blood was
123 collected into a syringe containing heparin (final concentration 25 IU/mL), aprotinin
124 (final concentration 540 KIU/mL), and DPP-IV inhibitor (final concentration 50 μ M,
125 Millipore, MA, USA). Mucosa samples were collected from middle (approximately 10
126 cm) duodenum, jejunum, ileum and colon, respectively, after washing out the luminal
127 content with cold saline. Cecal mucosa was collected from the whole cecal tissue after
128 washing out the cecal content with cold saline. These samples were immediately frozen
129 with liquid nitrogen, and stored at -80°C until RNA extraction was taken.

130 *Plasma hormone measurement*

131 Plasma GLP-1 concentrations (25 μ l) were measured with Total GLP-1 EIA kit
132 (intra- and inter-assay variation were $< 5\%$ and $< 12\%$, respectively; Millipore)
133 according to manufacturer instructions. Plasma insulin concentrations (10 μ l) were
134 measured with the insulin-ELISA kit (intra- and inter-assay variation were $< 5\%$ and $<$
135 5% , respectively; AKRIN-010T, Shibayagi, Gunma, Japan) according to manufacturer
136 protocols. The collected plasma at day 50 was diluted 2-times to adjust for standard
137 curve. For measurement of plasma cholecystokinin (CCK) and gastrin, plasma was
138 extracted as described in a previous paper (29). In brief, one volume of plasma sample
139 was mixed with two volumes of 99.5% ethanol. The mixture was incubated on ice for
140 30 min, and then centrifuged at $9,300 \times g$ for 10 min at 4°C . The supernatant was
141 transferred to a new tube and evaporated in a vacuum centrifuge. The dried extracts
142 were stored at -80°C until analysis. After reconstituted into equivalent volume by the

143 assay buffers, plasma concentration of CCK (50 μ L) and gastrin (100 μ l) were measured
144 according to the manufacturer protocols.

145 Because the primary antiserum in CCK EIA-kit (intra- and inter-assay variation of
146 were < 5% and < 14%, respectively; Phoenix Pharmaceuticals Inc., Belmont, CA)
147 cross-reacts (100%) not only with sulfated and non-sulfated CCK-8 (26-33), but also
148 with gastrin-1, we measured plasma gastrin concentration using human gastrin 1
149 EIA-kit (intra- and inter-assay variation were < 9% and < 7%, respectively; Assay
150 designs, Inc. Ann Arbor, MI). The primary antiserum in human gastrin 1 EIA-kit has
151 high reactivity with rat gastrin-1 (70.7%), human gastrin-1 (100%), and human mini
152 gastrin (74.6%), but it slightly reacts with CCK-8 (2.67%).

153 *Real-time quantitative polymerase chain reaction*

154 Total RNA was extracted by using the RNeasy Mini kit (Qiagen, Hilden, Germany)
155 according to the manufacturer's protocol. RNA concentrations were determined by
156 optical densitometry at 260 nm; RNA quality was assessed by the ratio of 260 nm/280
157 nm (> 1.8). cDNA was synthesized using the ReverTra Ace qPCR with genome DNA
158 remover (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol.
159 Gene expression levels were determined by TaqMan gene expression assays (Life
160 Technologies Co., Carlsbad, CA, USA) with rat gene-specific, predesigned TaqMan
161 primers and probe sets (proglucagon: Rn00562293_m1, cck: Rn00563215_m1). PCR
162 amplification and fluorescence data collection were performed with the Mx3000P
163 real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The mRNA
164 expression level was calculated with a standard curve determined from several
165 concentrations of cDNA. The concentration of samples was corrected with *Gapdh*
166 (Rn99999916_s1) mRNA as a reference gene. The data were shown as relative

167 expression level compared with the control group.

168 *Statistical analysis*

169 All results are expressed as mean \pm SEM. In MTT, data were analyzed by three-way
170 ANOVA with treatment, time, and day (SPSS Japan, Tokyo, Japan). When there were
171 significant main effects or interaction, two-way ANOVA (treatment and time) was
172 performed to identify the both main effects on each day. Data on area under the curve
173 (AUC), HOMA-IR, mRNA expression, and portal hormone levels were analyzed by
174 one-way ANOVA (treatment) or two-way ANOVA (treatment and day). Significant
175 differences among the groups or time points were determined with Student's t-test,
176 Tukey-Kramer's or Dunnett's post-hoc test ($P < 0.05$) as described in figure legends.
177 AUC of plasma glucose, insulin GLP-1 levels during the MTT was calculated by the
178 trapezoidal rule.

179

180 **Results**

181 *The effect of HF/HS diet on body weight (Fig. 1), food intake, waist circumference, fat*
182 *accumulation, and liver weight (Table 2)*

183 The body weight was increased in HF/HS groups, the significant differences to
184 control group were observed from day 30 (Fig. 1). At the end of the experiment (day 56),
185 the body weight of HF/HS rats was significantly higher than the body weight of control
186 and food-restricted groups. Total food intake of HF/HS group was significantly lower
187 than control, while total energy intake was significantly higher in the HF/HS group than
188 in other groups. To confirm the effect of micronutrient deficiency caused by
189 HF/HS-decreased food intake, a food-restricted group was added to the experiment. The
190 energy intake of the food-restricted group was significantly lower than energy intake in

191 the control and HF/HS groups, but the weight of total food intake in the restricted group
192 was similar to that in HF/HS group. This indicates that the total intake of protein,
193 vitamins and minerals did not differ between the food-restricted and HF/HS groups.
194 Similar to the results reported for a HF diet (30, 31), the chronic HF/HS diet in this
195 study significantly increased body weight, waist circumference, visceral fat, and liver
196 weight.

197

198 *Basal and postprandial glycemia during the meal tolerance test (MTT)*

199 In the present study, we used the MTT rather than the oral/intraperitoneal glucose
200 tolerance test to evaluate postprandial glucose tolerance and GLP-1 secretion (32). It
201 should be noted that the control diet was orally administrated in all the groups during
202 the MTT after 6-hour deprivation of the respective experimental diets. The MTT was
203 conducted every week to monitor 8-week changes in postprandial responses during the
204 establishment of obesity or glucose intolerance.

205 Basal glucose levels were significantly higher in the HF/HS group than in the other
206 groups after day 20 (Fig. 2A). Postprandial glucose levels were higher in HF/HS group
207 than in the other two groups throughout the experimental period due to increased basal
208 glucose level (Fig. 2A). Significant treatment effects were observed at days 6 and 13 for
209 postprandial glycemc response (Δ glucose shown in Fig. 2B). On day 6, significantly
210 higher glycemc responses compared with basal level (0 min) were observed at 15 and
211 60 min in HF/HS group, but only at 15 min in the control group. Similarly, the control
212 group showed significant increment from basal level only at 15 min, but HF/HS group
213 showed the increment at 15, 30, and 60 min at day 13. Although a significant effect was
214 not detected by the two-way ANOVA with treatments and days, the one-way ANOVA

215 and post-hoc test demonstrated the significant effect of HF/HS diet treatment on the
216 AUC of Δ glucose on day 13 compared with control group (Fig. 2C).

217

218 *Basal insulin, homeostasis model assessment of insulin resistance and postprandial*
219 *insulin secretion during the meal tolerance test*

220 Basal insulin levels in the HF/HS group gradually increased from day 13 to day 50
221 (Fig. 3A), and were significantly higher than those in the other groups on days 34 and
222 50. HOMA-IR was also significantly higher in the HF/HS group than in the other
223 groups (Fig. 3C) after day 34. Postprandial insulin levels in the HF/HS group were
224 significantly higher than those in the control at 15, 30, and 60 min in each MTT (Fig.
225 3B). Further, a significant difference in the AUC of Δ insulin levels between HF/HS
226 group and control group was observed at day 34 and 50, and its levels were increased by
227 the chronic intake of HF/HS diet (Fig. 3D).

228

229 *Basal and postprandial glucagon-like peptide-1 levels during the meal tolerance test*

230 Postprandial GLP-1 secretions in the HF/HS group and control group were
231 significantly higher than its basal lines but not in the food-restricted group on day 13
232 and day 34 (Figs. 4A and 4B). GLP-1 levels at 15 min were significantly higher in the
233 HF/HS group than in the control and food-restricted groups on day 50 (Figs. 4A and 4B).
234 Furthermore, the AUC of GLP-1 levels in HF/HS groups on day 50 was significantly
235 increased from day 13, which was significantly higher than that in the control group
236 (Fig. 4C). The food-restricted group had the lowest basal and postprandial GLP-1 levels
237 among all groups in each MTT (Figs. 4A and 4B).

238

239 *Postprandial gastric emptying rate under MTT*

240 The rate of gastric emptying affects postprandial glycemia, and dysregulation of
241 gastric emptying has been reported in obese patients (33) and diet-induced obese
242 rodents (34). The acetaminophen (paracetamol) absorption test is used to assess the
243 gastric emptying rate because acetaminophen is absorbed in the small intestine (25, 26).
244 On day 6 and day 13, acetaminophen concentrations at 15 and 60 min after preload of
245 the control diet suspension were significantly lower in the HF/HS group than in the
246 food-restricted group (Figs. 5A and 5B). On day 34, acetaminophen concentrations in
247 the HF/HS group at 15 and 30 min were significantly lower than in the control group
248 (Fig. 5E). However, on days 41 and 50, the significant differences among treatments
249 were not observed (Figs. 5F and 5G).

250

251 *Portal peptide hormones levels after 8 weeks high-fat and high-sucrose diet treatment*

252 On day 56, we collected portal vein samples from overnight fasted rats to evaluate
253 the effect of HF/HS diet on basal gut hormone levels. Portal GLP-1 concentration was
254 significantly higher in the HF/HS group than in the control and food-restricted groups
255 (Fig. 6A). Although significant difference between portal insulin concentrations in the
256 HF/HS and the control groups was determined with student's *t-test* ($p=0.010$), there are
257 insignificant changes of insulin levels among the all groups (Fig. 6B). Because the CCK
258 EIA kit is able to detect both CCK and gastrin, we measured both CCK and gastrin
259 levels. Portal CCK and gastrin levels did not differ among the three groups (Figs. 6C
260 and 6D).

261

262 *Proglucagon and cholecystokinin mRNA expression in the gastrointestinal tract*

263 To examine the effect of HF/HS diet on gut hormone mRNA expression, intestinal
264 mucosa was collected from various regions. Although the GLP-1 level in the portal vein
265 was higher in the HF/HS group (Fig. 6A), *Gcg* mRNA expression did not differ by
266 dietary treatment group for any of the regions (Figs. 7A-7D). *Cck* mRNA expression
267 was significantly increased in the jejunum dependent on energy intake (Fig. 7F).

268

269 **Discussion**

270 In the present study, we monitored postprandial GLP-1, insulin, glycaemia, and
271 gastric emptying in rats during the progression of diet-induced obesity in rats. Daily
272 intake of a HF/HS diet increased postprandial glycemic and insulin responses to
273 “normal diet” (AIN-93G) under the MTT from the early period of experiment (day13).
274 After day 20, the HF/HS diet increased fasting glucose and insulin levels compared with
275 the control group, indicating that HF/HS-feeding induced glucose intolerance
276 accompanied by insulin resistance within 3 weeks in rats. Importantly, postprandial
277 glucose response was not further impaired by the HF/HS diet, and postprandial GLP-1
278 and insulin responses to the meal in the HF/HS group gradually increased until the end
279 of the experimental period. The present study revealed that the postprandial GLP-1
280 response to meal ingestion is increased during the progression of glucose intolerance
281 and obesity, which may slow the establishment of diet-induced obesity.

282 Epidemiological studies have provided evidence that dietary fat intake is closely
283 related to obesity (35, 36). Therefore, HF diets have been widely used and recognized to
284 induce diet-related obesity in animal experiments (37, 38). Long-term feeding of a
285 sucrose-rich diet has been shown to induce higher glucose levels compared with a high
286 fat diet as measured by oral glucose tolerance test (30). The combination of HF diet and

287 HS diet has also been used to induce obesity as a model of the western diet (39, 40).
288 Sucrose consists of glucose and fructose equally, and fructose is known as a highly
289 lipogenic sugar. It has been reported that excessive consumption of commercial
290 beverages containing glucose and fructose (high-fructose corn syrup: 50% glucose, 50%
291 fructose) has been linked to development of the metabolic syndrome (41).

292 As shown in Fig. 2 and Fig. 3, weekly monitoring of postprandial glycemia and
293 insulin response revealed that glucose intolerance was induced in rats just after 2 weeks
294 on the HF/HS diet. Significant differences in body weight between control and HF/HS
295 groups was observed from day 30 (Fig. 1), indicating that impairment of glucose
296 homeostasis occurs in advance of body weight increase. Generally, diet-induced
297 obesity-model animals are studied after feeding with high-energy diets for 8 weeks or
298 longer. However, the present result suggests that postprandial glucose intolerance is
299 immediately caused by daily intake of a high-energy diet rich in fat and sucrose as is the
300 case in the intravenous glucose tolerance test (42). The food-restricted group fed control
301 diet with the same amount (in g) as that consumed by the HF/HS group (Table 2), so
302 that both groups consumed the same amounts of protein, vitamin and mineral with
303 HF/HS group, and finally both groups had lower protein/vitamin/mineral intake
304 compared to control group. However, the food-restricted group did not show the similar
305 phenotype to the HF/HS groups on postprandial response, suggesting that the excessive
306 energy intake, rather than the reduced intake of protein, vitamin, and mineral, has a
307 large impact on impairment of postprandial glycaemia. The food-restricted group
308 showed almost similar postprandial glycaemia overall but relatively smaller responses
309 in insulin and GLP-1 secretion compared to control group (Figs. 2-4), suggesting
310 restricting (90%) food consumption is beneficial for improvement of glucose tolerance.

311 However, it is possible that these results were observed due to the lower body weight
312 and lower energy load in the food-restricted group than the control group. Another
313 limitation is that the food-restricted group had a longer fasting period because they
314 finished the diet every day before they were given fresh diet.

315 The effects of each macronutrient (carbohydrate, fat, and protein) on gut hormone
316 secretion have been reported, and the ratio of fat to protein is closely related to GLP-1
317 secretion in healthy subjects (43, 44). The intake of a mixed meal has a potent effect on
318 GLP-1 secretion compared with solo administration of each macronutrient (31). It has
319 been previously reported that the MTT represents a better indication of normal
320 postprandial glucose and insulin responses compared to the oral glucose tolerance test
321 in a population-based cohort (45). In the present study, the MTT was conducted (rather
322 than the widely-used oral glucose tolerance test), to evaluate ‘postprandial’ glycaemic
323 and gastrointestinal responses under a more physiological condition reflecting the
324 dietary exposure in normal life. As equivalent dietary components are used to compare
325 the effect of diet on obesity as shown in the clinical study (46, 47), all rats received
326 normal diet rather than respective test diets in the MTT. For HF/HS group, control diet
327 administered was different from usual diet (high fat / high sucrose diet). However, all
328 rats received the control diet during the acclimation period before feeding respective test
329 diets, therefore, having control diet in the MTT was not for the first time even for the
330 HF/HS group. In addition, all rats were subjected to oral administration of
331 water-suspended diet in the MTT. Although the composition of diet was unchanged for
332 control group, the form and way of ingestion were changed from usual ‘meal’ for all of
333 groups. Therefore, we assume the impact of changing diet composition from daily
334 consuming HF/HS diet on postprandial responses in the HF/HS group was smaller than

335 chronic effect of high fat / high sucrose diet. Because daily postprandial responses
336 would be an important factor that would affect metabolic status, it is interesting to know
337 the daily glycaemic, insulin and GLP-1 responses in each group after having the
338 respective test diet. However, if the MTT had been performed in such a way,
339 interpretations to the observed result would be complicated with respect to nutrient
340 sensing because both of chronic and acute effect of respective diet compositions could
341 affect the postprandial responses. It would be interesting to examine the postprandial
342 response to the HF/HS diet or a single nutrients load in the control and HF/HS group in
343 the future.

344 Previous reports demonstrated that the peak of GLP-1 secretion after oral glucose
345 administration was decreased in diet-induced obesity (16, 17). In contrast, it has been
346 reported that GLP-1 secretion in response to oral fatty acid administration was
347 unchanged between diet-induced obesity rats and diet resistant rats (18). Interestingly,
348 the present study showed that postprandial GLP-1 response (to normal diet) was
349 gradually increased, but not decreased, by chronic intake of HF/HS diet compared with
350 the control diet, and a significant difference was observed after 7 weeks (Fig. 4). The
351 result suggests that chronic intake of HF/HS diet altered the nutrient-sensing function of
352 the gastrointestinal tract to be more sensitive to the mixed meal. Possibly, the different
353 postprandial responses arose from different amount of energy load, because the meal
354 was given depending on the body weight of individual rats (3 g/kg) in MTT of the
355 present study. Indeed, the body weight of HF/HS group was around 50 g (12%) higher
356 than control group, and the energy load in the HF/HS group was 12% higher than that in
357 the control group. Although a similar difference in body weight (10%) was already
358 observed at day 34, postprandial GLP-1 response was 2-fold higher in HF/HS group

359 than in control group (Figs. 4B and 4C). Furthermore, the data (supplemental figure 1)
360 comparing selected rats having higher body weight in control group and those having
361 lower body weight in HF/HS group demonstrated GLP-1 and insulin responses are
362 apparently higher in HF/HS group than in control group, although there was no
363 significant difference in body weight between the two groups.

364 The present results also demonstrated that fasting GLP-1 levels in the portal vein
365 were increased in the HF/HS group (Fig. 6), but *Gcg* mRNA expression did not differ
366 by dietary treatments in any of the intestinal regions (Fig. 7), which implies that GLP-1
367 secretion, but not mucosal GLP-1 production, was changed by the HF/HS diet. Despite
368 the delta change in postprandial plasma glucose were not increased from day 20 to day
369 50 (Fig. 2B), enhancement of postprandial and fasting GLP-1 levels with increased
370 insulin secretion was observed (Figs. 3, 4, and 6). Although gut hormones, such as
371 GLP-1, are immediately secreted in response to meal ingestion, adaptive changes to a
372 chronic high-energy diet develop over time in the peripheral insulin-targeting tissues
373 such as adipose, and liver and skeletal muscles. The physiological relevance of
374 increased GLP-1 and nutrient sensitivity needs to be further studied in the future; it,
375 which may contribute to prevention of excessive plasma glucose elevation and slow the
376 establishment of glucose intolerance and obesity with the enhancement of insulin
377 secretion.

378 Changes in acetaminophen concentration were smaller in the HF/HS group
379 compared with the control group during the MTT on day 34 (Fig. 5E), suggesting
380 delayed gastric emptying in the HF/HS group. Such an effect might prevent excessive
381 loading of nutrients in the small intestine in the HF/HS group. Several reports have
382 demonstrated that the dosage of luminal nutrients, including fat and protein, is an

383 important factor on GLP-1 secretion (48, 49). On day 34, postprandial GLP-1 levels in
384 the HF/HS group were similar to those in the control group, although gastric emptying
385 was delayed in the HF/HS group (Figs.4B and 5E). In contrast, increased postprandial
386 GLP-1 secretion and unchanged postprandial gastric emptying were observed on day 50
387 (Figs. 4B and 5G). GLP-1 secretion depends on luminal nutrients that are emptied from
388 the stomach, but gastric emptying is regulated by various factors, such as CCK,
389 serotonin and GLP-1. Although significant treatment effects were detected on days 6, 13,
390 and 34 by the two-way ANOVA, it is unclear how such changes in gastric emptying rate
391 appeared and contribute to postprandial hormone and glycaemic responses in the
392 present study.

393 In summary, feeding rats with a HF/HS diet rapidly impaired postprandial glycaemic
394 responses (i.e., within 2 weeks) in advance of increased weight gain. Postprandial
395 GLP-1 secretion during the MTT was increased by HF/HS diet treatment after 7 weeks.
396 Food restriction demonstrates that the habitual excessive energy (fat and sucrose) intake
397 is the main factor that contributes to changes in postprandial GLP-1 secretion. Although
398 mRNA expression levels of gut hormones were unchanged, fasting GLP-1 and insulin in
399 portal blood were increased by the HF/HS diet after 8 weeks. The present study
400 revealed that chronic ingestion of high-energy diet elevates the postprandial GLP-1 and
401 insulin responses to meal ingestion in rats. The boosted postprandial GLP-1 secretion by
402 chronic high energy diet treatment suggests enhanced sensitivity to luminal nutrients in
403 the gut, which may slow the establishment of glucose intolerance and obesity.

404

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407

408 **Author disclosure**

409 S Nakajima, T Hira, and H Hara have no conflicts of interest

410

411 **Statement of Author's Contributions to Manuscript**

412 S. N., T. H., and H. H. designed research; S.N. conducted research and analyzed data;

413 S.N. and T. H. wrote the paper. T. H. had primary responsibility for final content. All

414 authors read and approved the final manuscript.

415

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550 dietary lipid: is it dose dependent? *Am J Physiol Gastrointest Liver Physio* **297**,

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552

553

554 Table 1. The composition of experimental diets.

	g/kg	
	Control	HF/HS
Cornstarch	397.486	0
Casein	200	200
Dextrinized cornstarch ¹	132	0
Sucrose	100	399.486
Soybean oil	70	70
Lard oil	0	230
Fiber ²	50	50
Mineral mix (AIN-93G-MX)	35	35
Vitamin mix (AIN-93-VX)	10	10
L-Cystine	3	3
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.014	0.014
Total	1000	1000

555 ¹ TK-16 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan)

556 ² Just Fiber (Morimura Bros., Inc., Tokyo, Japan)

557

558 Table 2. Body weight, total food intake, waist, visceral adipose tissue weight, and liver
 559 weight at day 56 after chronic intake of HF/HS diet

	Control	Food-restricted	HF/HS
Initial body weight (g)	178.5 ± 3.6	177.4 ± 3.1	179.9 ± 2.9
Final body weight (g)	453.7 ± 14.8 ^b	424.0 ± 4.6 ^b	508.3 ± 16.8 ^a
Total Food intake (g)	1161 ± 32 ^a	1030 ± 1 ^b	1002 ± 34 ^b
Total Energy intake (kcal)	4588 ± 128 ^b	4067 ± 5 ^c	5110 ± 173.2 ^a
Waist circumference (cm)	18.3 ± 0.3 ^b	18.1 ± 0.2 ^b	19.6 ± 0.4 ^a
Mesenteric fat (g)	5.9 ± 0.6 ^b	5.1 ± 0.3 ^b	9.7 ± 0.8 ^a
Epididymal fat (g)	8.6 ± 0.7 ^b	9.4 ± 1.3 ^b	15.4 ± 1.2 ^a
Retroperitoneal fat (g)	12.4 ± 1.3 ^b	11.4 ± 1.0 ^b	19.0 ± 1.2 ^a
Liver weight (g)	13.5 ± 0.8 ^b	12.2 ± 0.3 ^b	16.6 ± 0.9 ^a

560 Values are means ± SEM of 8-9 rats. Bars not sharing the same alphabets represent
 561 significant difference between treatments (P < 0.05 by Tukey-Krammer's post-hoc test).

562

563 Table 3. *P* values for effects of diet, time, and day in MTT, evaluated by three-way
 564 ANOVA.

	Tr	Ti	D	Tr x Ti	Tr x D	Ti x D	Tr x Ti x D
Glucose	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.93
Δ Glucose	< 0.05	< 0.05	< 0.05	0.13	< 0.05	< 0.05	0.99
Insulin	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Δ Insulin	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Total GLP-1	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.28	0.55
Δ Total GLP-1	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.16
Acetaminophen	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.40

565 Data obtained from MTT were analyzed by three-way ANOVA. Main factors were
 566 abbreviated as Tr; Treatment, Ti; Time, and D; Day.

567

568 **Legend to Figures**

569 **Fig. 1. Daily changes in body weight**

570 Rats were fed the control diet ad lib (open circle), restricted amount of control diet
571 (open triangle), and HF/HS diet ad lib (filled square), except for the day of the MTT.
572 Body weight was measured every morning. Values are means \pm SEM of 8-9 rats. [#] $P <$
573 0.05 vs control (Tukey-Krammer's post-hoc test).

574

575 **Fig. 2. Postprandial glycemic responses under MTT**

576 The control diet (AIN-93G) suspended in water was gavaged in rats (3 g/kg body
577 weight) after 6-hour fasting on days 6, 13, 20, 27, 34, 41, and 50. Rats were fed the
578 control diet ad lib (open circle), restricted amount of control diet (open triangle), and
579 HF/HS diet ad lib (filled square), except for on the day of the MTT. Tail vein blood was
580 collected before (0 min) and after (15, 30, 60, 90, and 120 min) the meal load, and
581 plasma glucose levels were measured. Absolute glucose levels (A) and changes from
582 basal levels (Δ glucose) (B) were presented. AUC of Δ glucose was shown in (C). Values
583 are means \pm SEM of 6-9 rats. P values for effects of treatment (Tr), time (Ti), Day (D)
584 and the interaction of treatment and time (Tr x Ti) or day (Tr x D) calculated by
585 two-way ANOVA was represented in each panels. [#] $P <$ 0.05 vs control, * $P <$ 0.05 vs
586 basal level (Tukey-Krammer's post-hoc test).

587

588 **Fig. 3. Postprandial insulin secretion under MTT and fasting HOMA-IR**

589 The control diet (AIN-93G) suspended in water was gavaged in rats (3 g/kg body
590 weight) after 6-hour fasting on days 13, 34, and 50. Rats were fed the control diet ad lib
591 (open circle), restricted amount of control diet (open triangle), and HF/HS diet ad lib

592 (filled square), except for the day of the MTT. Tail vein blood was collected before (0
593 min) and after (15, 30, 60, 90, and 120 min) the meal load, and plasma insulin levels
594 were measured. Absolute insulin levels (A) and changes from basal levels (Δ insulin) (B)
595 were presented. HOMA-IR was calculated as described in the materials and methods
596 section (C). AUC of Δ insulin was shown in (D). Values are means \pm SEM of 7-9 rats. *P*
597 values for effects of treatment (Tr), time (Ti), Day (D) and the interaction of treatment
598 and time (Tr x Ti) or day (Tr x D) calculated by two-way ANOVA was represented in
599 each panels. [#] *P* < 0.05 vs control, * *P* < 0.05 vs basal level (Tukey-Kramer's post-hoc
600 test).

601

602 **Fig. 4. Postprandial GLP-1 secretion under MTT**

603 The control diet (AIN-93G) suspended in water was gavaged in rats (3 g/kg body
604 weight) after 6-hour fasting on days 13, 34, and 50. Rats were fed the control diet ad lib
605 (open circle), restricted amount of control diet (open triangle), and HF/HS diet ad lib
606 (filled square), except for the day of the MTT. Tail vein blood was collected before (0
607 min) and after (15, 30, 60, 90, and 120 min) the meal load, and plasma total GLP-1
608 levels were measured. Absolute GLP-1 levels (A) and changes from basal levels
609 (Δ GLP-1) (B) were presented. AUC of Δ total GLP-1 was shown in (C). Values are
610 means \pm SEM of 7-9 rats. *P* values for effects of treatment (Tr), time (Ti), Day (D) and
611 the interaction of treatment and time (Tr x Ti) or day (Tr x D) calculated by two-way
612 ANOVA was represented in each panels. [#] *P* < 0.05 vs control, * *P* < 0.05 vs basal level
613 (Tukey-Kramer's or Dunnett's post-hoc test).

614

615 **Fig. 5. Changes in plasma acetaminophen concentration under MTT**

616 Acetaminophen (100 mg/kg body weight) was orally administered with the control diet
617 (3 g/kg body weight) in the MTT to assess gastric emptying rate after 6-hour fasting on
618 days 6 (A), 13 (B), 20 (C), 27 (D), 34 (E), 41 (F), and 50 (G). Rats were fed the control
619 diet ad lib (open circle), restricted amount of control diet (open triangle), and HF/HS
620 diet ad lib (filled square), except for the day of the MTT. Changes in plasma
621 acetaminophen levels were presented. Values are means \pm SEM of 6-9 rats. *P* values for
622 effects of treatment (Tr), time (Ti) and the interaction of treatment and time (Tr x Ti)
623 calculated by two-way ANOVA was represented in each panels. [#] *P* < 0.05 vs control, [†]
624 *P* < 0.05 vs food-restricted (Tukey-Krammer's post-hoc test).

625

626 **Fig. 6. Fasting peptide hormone levels in the portal vein of rats fed respective test**
627 **diets for 8 weeks**

628 Portal blood was collected from the rats after overnight fasting on day 56. The levels of
629 total GLP-1 (A), insulin (B), CCK (C), and gastrin (D) were measured by respective
630 EIA kits. Values are means \pm SEM of 8-9 rats. [#] *P* < 0.05 vs control (Tukey-Krammer's
631 post-hoc test).

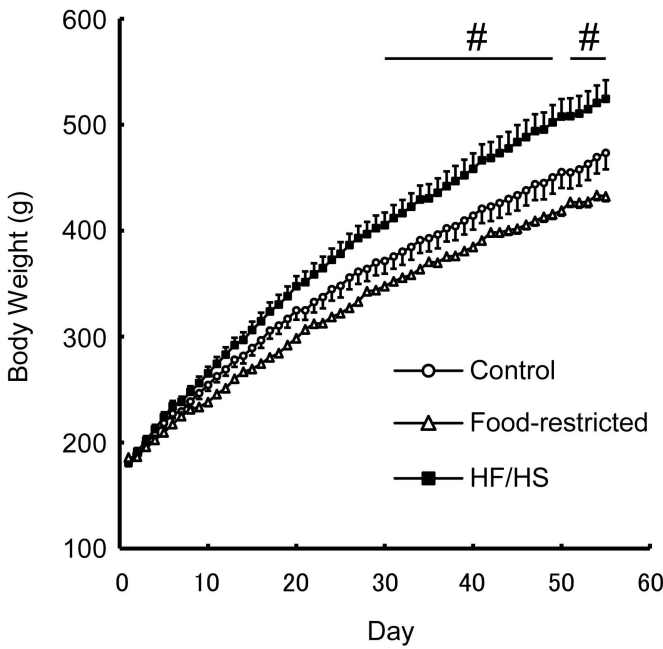
632

633 **Fig. 7. Proglucagon (gcg) and cck mRNA expression in intestinal mucosa of rats**
634 **fed respective test diets for 8 weeks.**

635 Mucosa was collected from the jejunum (A, F), ileum (B, G), cecum (C), colon (D), and
636 duodenum (E) of rats after overnight fasting on day 56. The expressions of *Gcg* (A-D)
637 and *Cck* mRNA (E-F) were determined by quantitative real-time PCR. Data are
638 presented as relative value to control group normalized to *Gapdh* mRNA expression,
639 and are means \pm SEM of 8-9 rats. [†] *P* < 0.05 vs food-restricted (Tukey-Krammer's

640 post-hoc test).

Figure 1



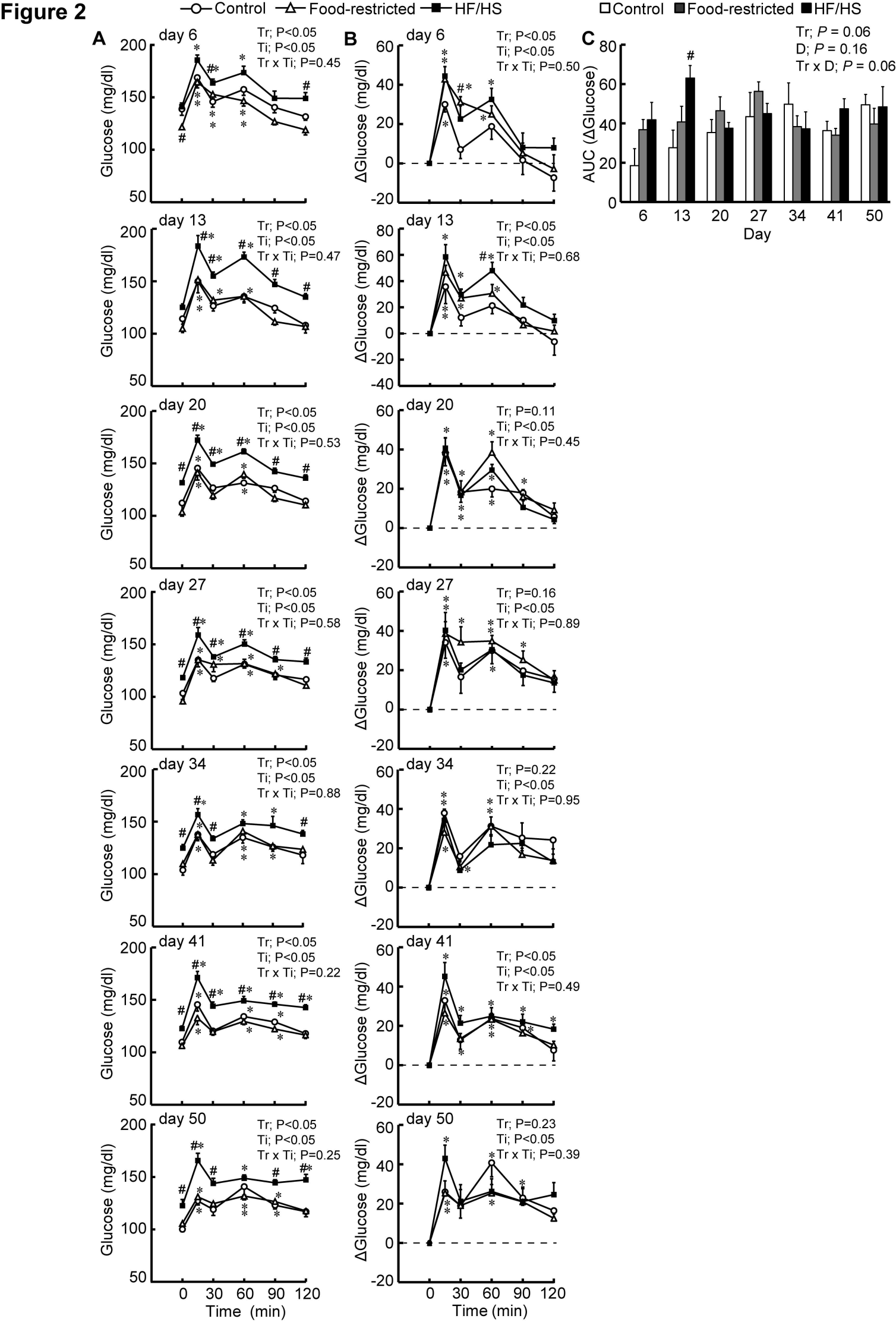
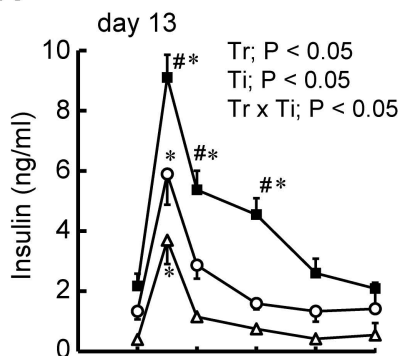
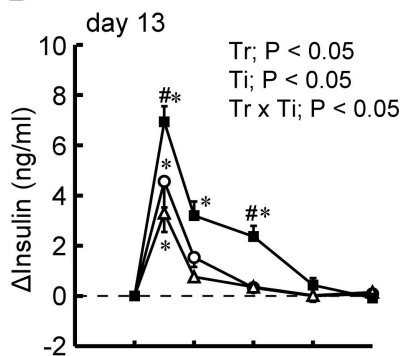
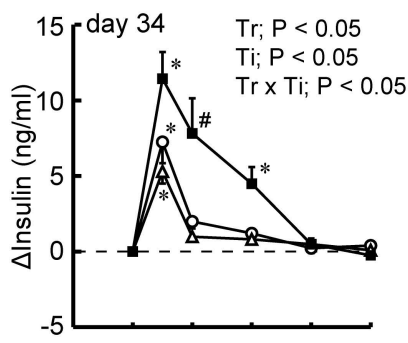
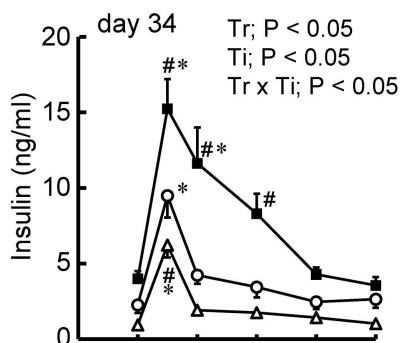
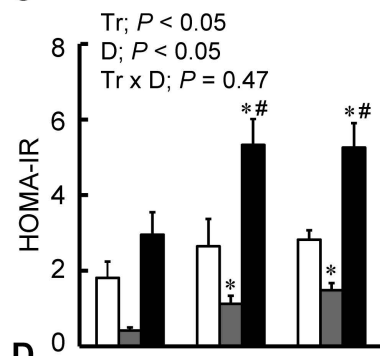
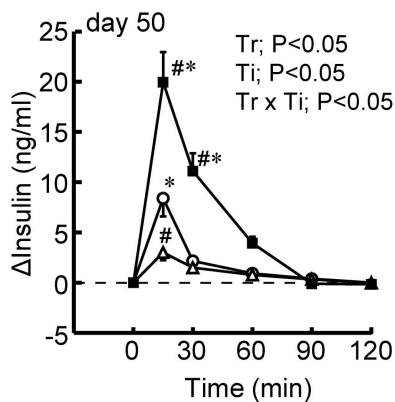
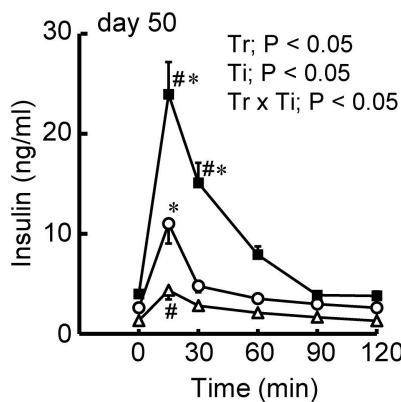
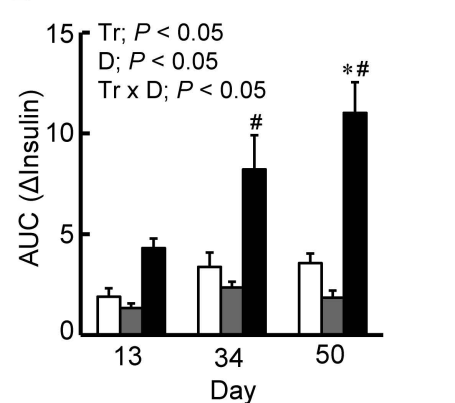


Figure 3**A****B****C****D**

○ Control △ Food-restricted ■ HF/HS

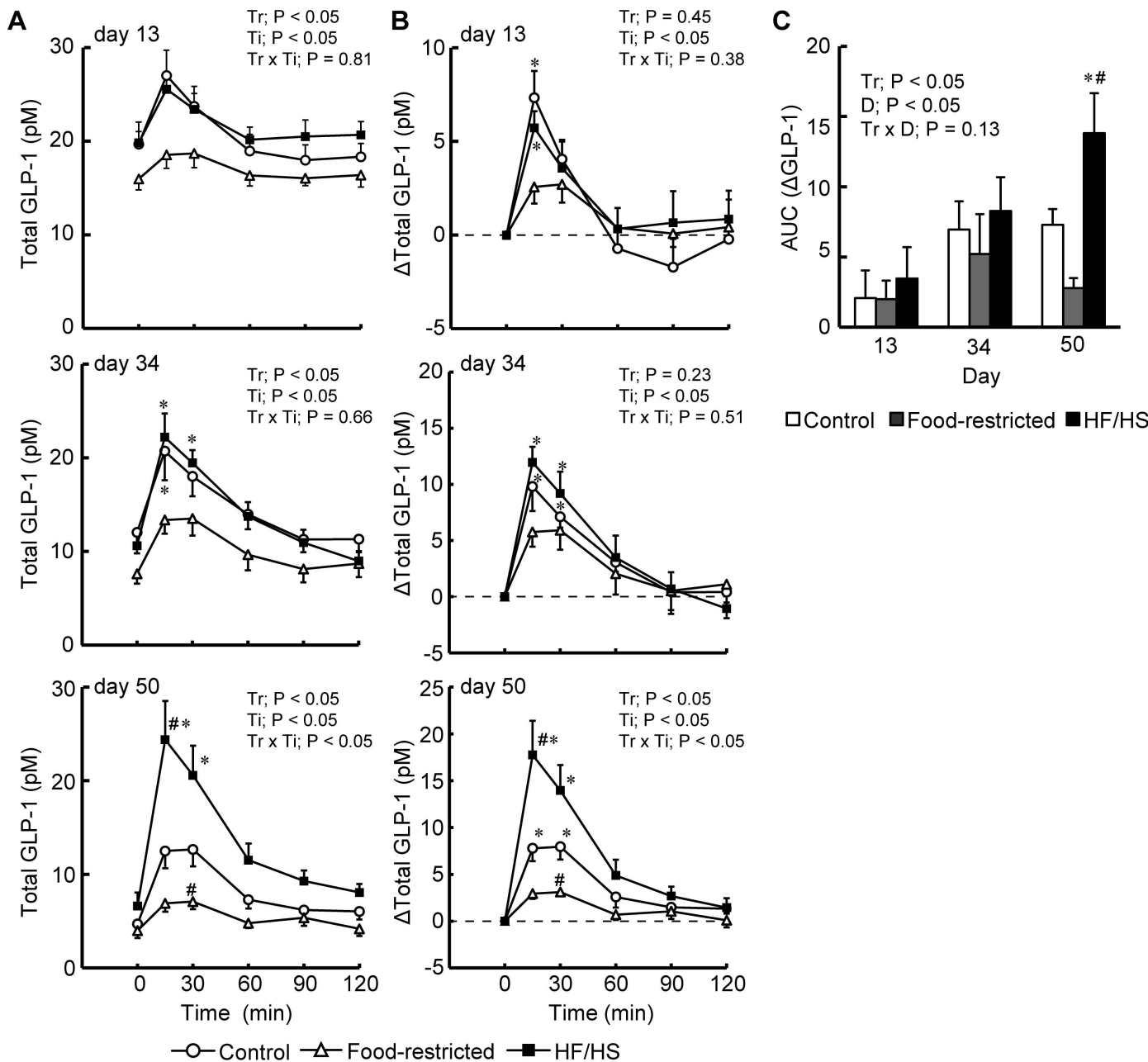
Figure 4

Figure 5

—○— Control —△— Food-restricted —■— HF/HS

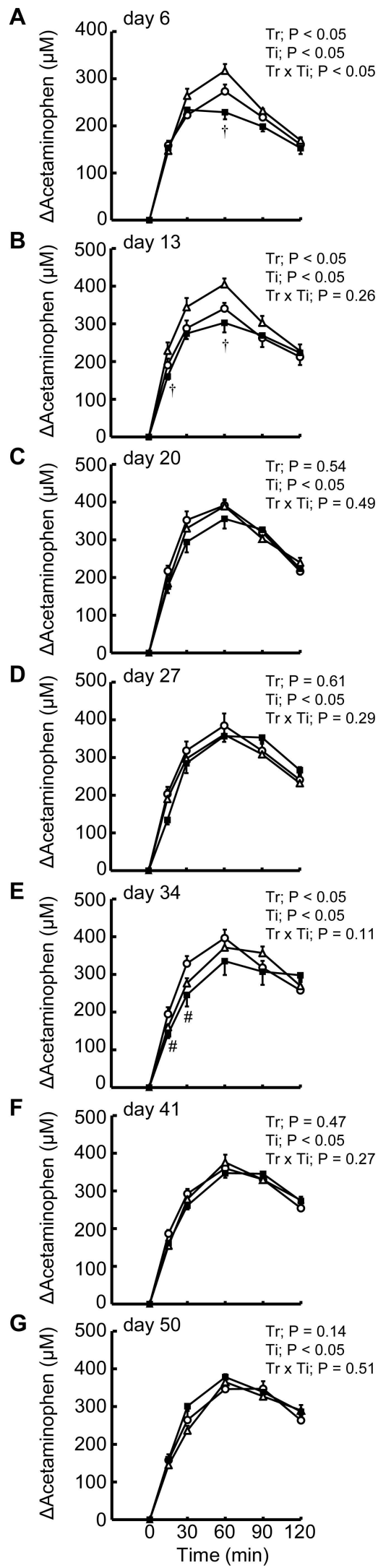
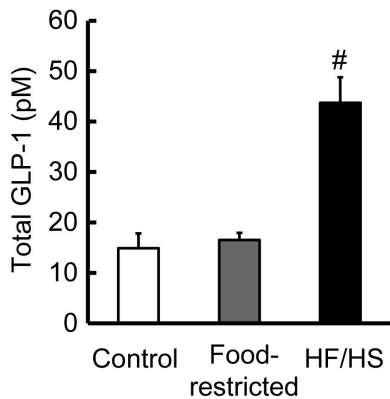
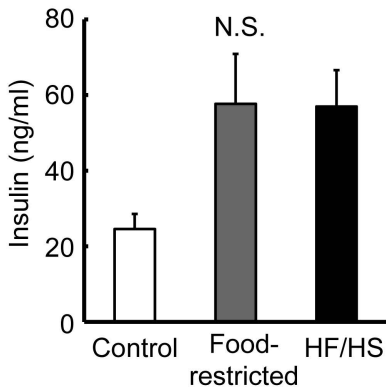


Figure 6

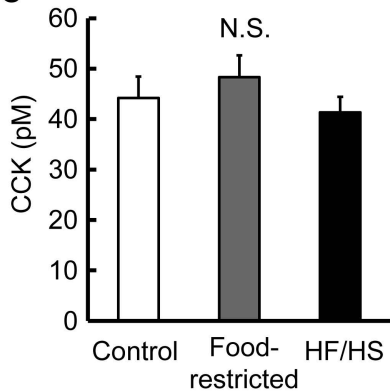
A



B



C



D

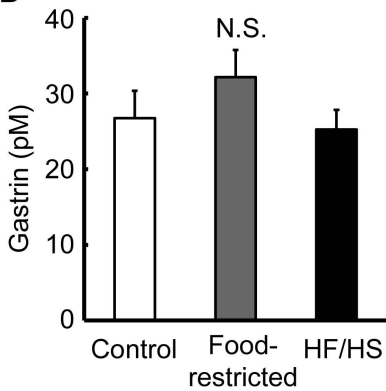
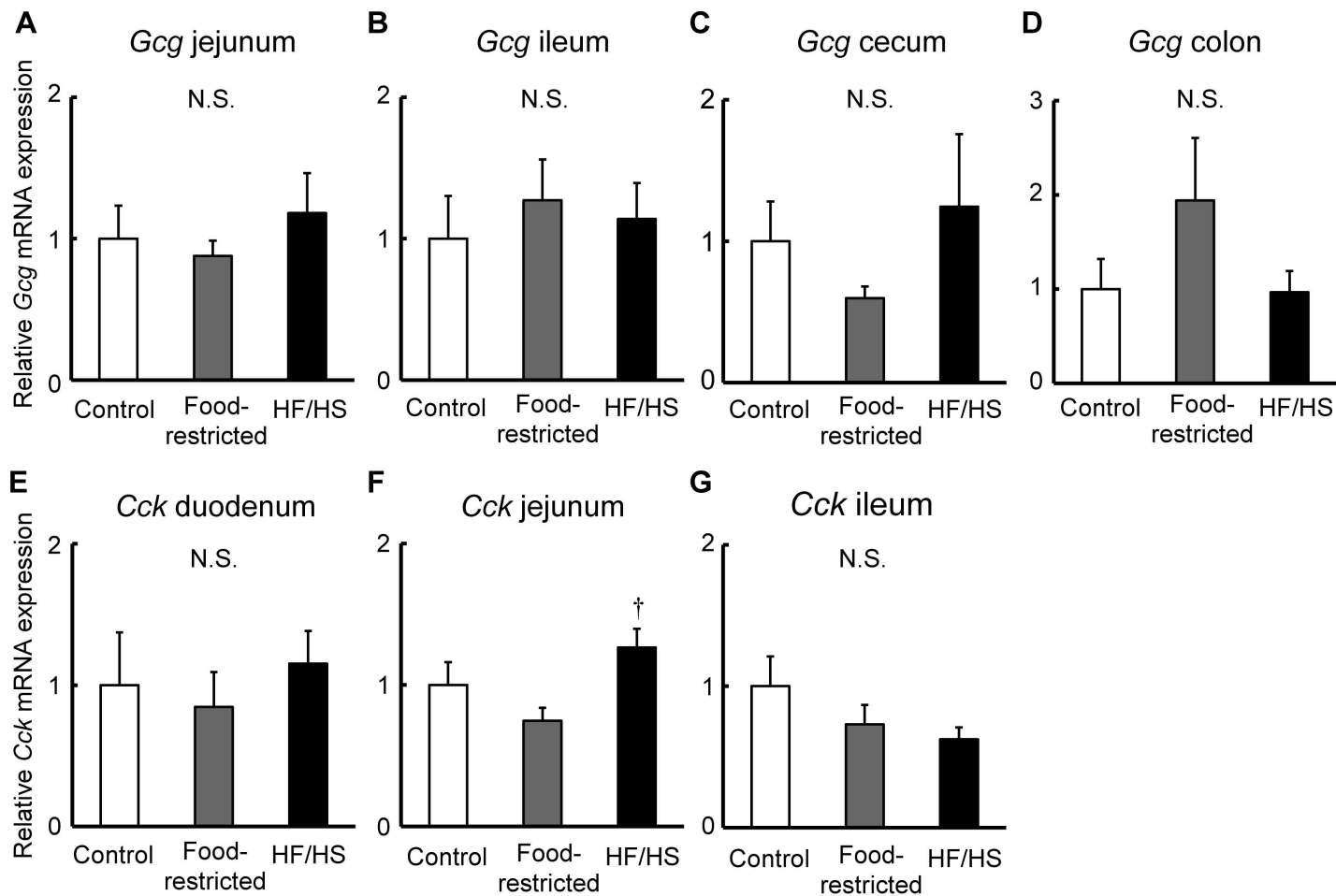
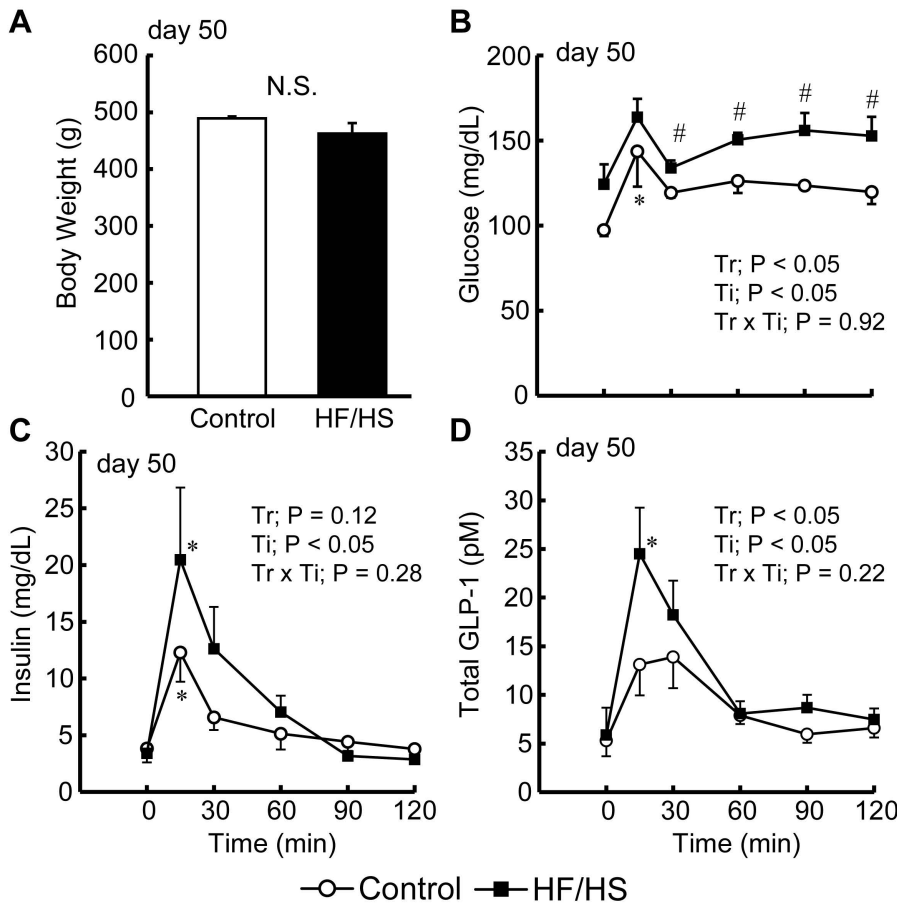
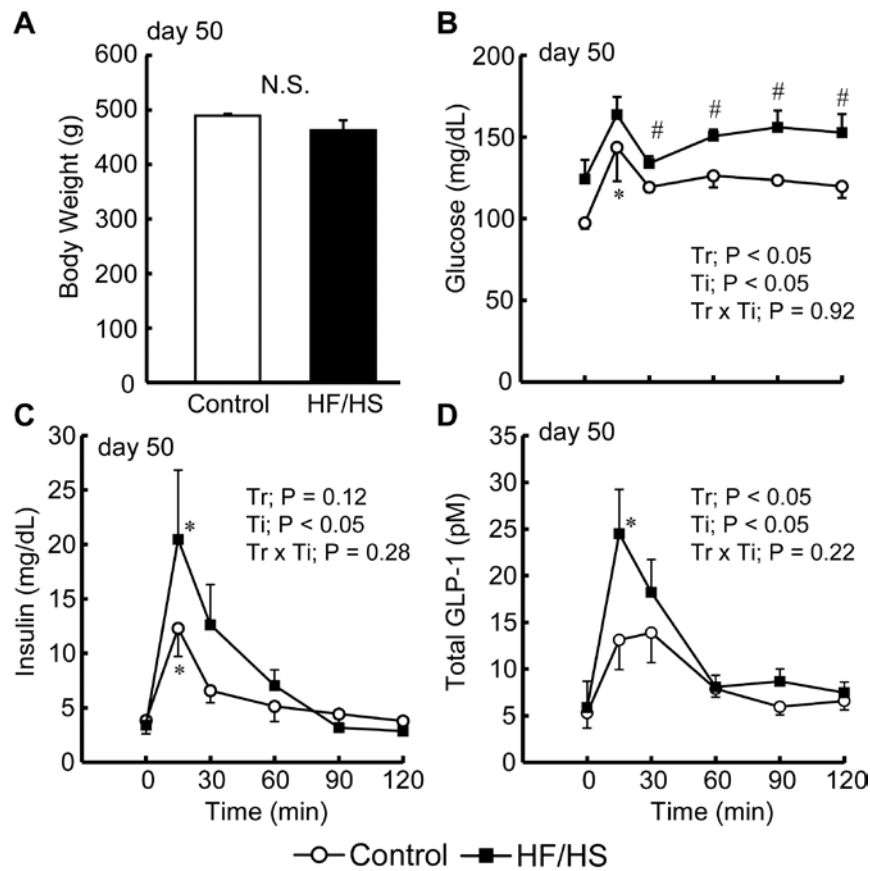


Figure 7

Supplemental Figure 1



Supplemental Figure 1



Supplemental Fig. 1. Postprandial glycemia and hormone levels in rats having similar body weights in control and HF/HS group at day 50

Data from 4 rats with higher body weight in control and 4 rats with lower body weight in HF/HS were selected. Rats were fed the control diet ad lib (open circle) and HF/HS diet ad lib (filled square), except for the day of the MTT. Average body weight of selected rats (A), changes in plasma glucose (B), insulin (C), and total GLP-1 (D) levels at day 50 were presented. Values are means \pm SEM of 4 rats. *P* values for effects of treatment (Tr), time (Ti) and the interaction of treatment and time (Tr x Ti) calculated by two-way ANOVA was represented in each panels. # *P* < 0.05 vs control, * *P* < 0.05 vs basal level (Student's *t*-test and Tukey-Kramer's post-hoc test).