Title

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

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Statement of Author's Contributions to Manuscript

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

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Abstract

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

Introduction

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

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

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

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

Materials and Methods

Animals

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

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

Experimental protocol for meal tolerance test (MTT)

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

Blood and tissue collection at final day

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

Plasma hormone measurement

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

assay buffers, plasma concentration of CCK (50 μL) and gastrin (100 μl) were measured according to the manufacturer protocols.

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

Real-time quantitative polymerase chain reaction

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

expression level compared with the control group.

Statistical analysis

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

Results

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

The body weight was increased in HF/HS groups, the significant differences to control group were observed from day 30 (Fig. 1). At the end of the experiment (day 56), the body weight of HF/HS rats was significantly higher than the body weight of control and food-restricted groups. Total food intake of HF/HS group was significantly lower than control, while total energy intake was significantly higher in the HF/HS group than in other groups. To confirm the effect of micronutrient deficiency caused by HF/HS-decreased food intake, a food-restricted group was added to the experiment. The 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 glycemic response (Δglucose shown in Fig. 2B). On day 6, significantly
210 higher glycemic 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

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

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

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

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

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

Postprandial gastric emptying rate under MTT

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

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

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

Proglucagon and cholecystokinin mRNA expression in the gastrointestinal tract

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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Author disclosure

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

Statement of Author's Contributions to Manuscript

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

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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

Table 2. Body weight, total food intake, waist, visceral adipose tissue weight, and liver 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}

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

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 Tr 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

Legend to Figures

Fig. 1. Daily changes in body weight

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

Fig. 2. Postprandial glycemic responses under MTT

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

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

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

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

Fig. 4. Postprandial GLP-1 secretion under MTT

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

Fig. 5. Changes in plasma acetaminophen concentration under MTT

Acetaminophen (100 mg/kg body weight) was orally administered with the control diet (3 g/kg body weight) in the MTT to assess gastric emptying rate after 6-hour fasting on days 6 (A), 13 (B), 20 (C), 27 (D), 34 (E), 41 (F), and 50 (G). Rats were fed the control diet ad lib (open circle), restricted amount of control diet (open triangle), and HF/HS diet ad lib (filled square), except for the day of the MTT. Changes in plasma acetaminophen levels were presented. Values are means ± SEM of 6-9 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 food-restricted (Tukey-Krammer's post-hoc test).

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

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

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

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

post-hoc test).

Figure 1

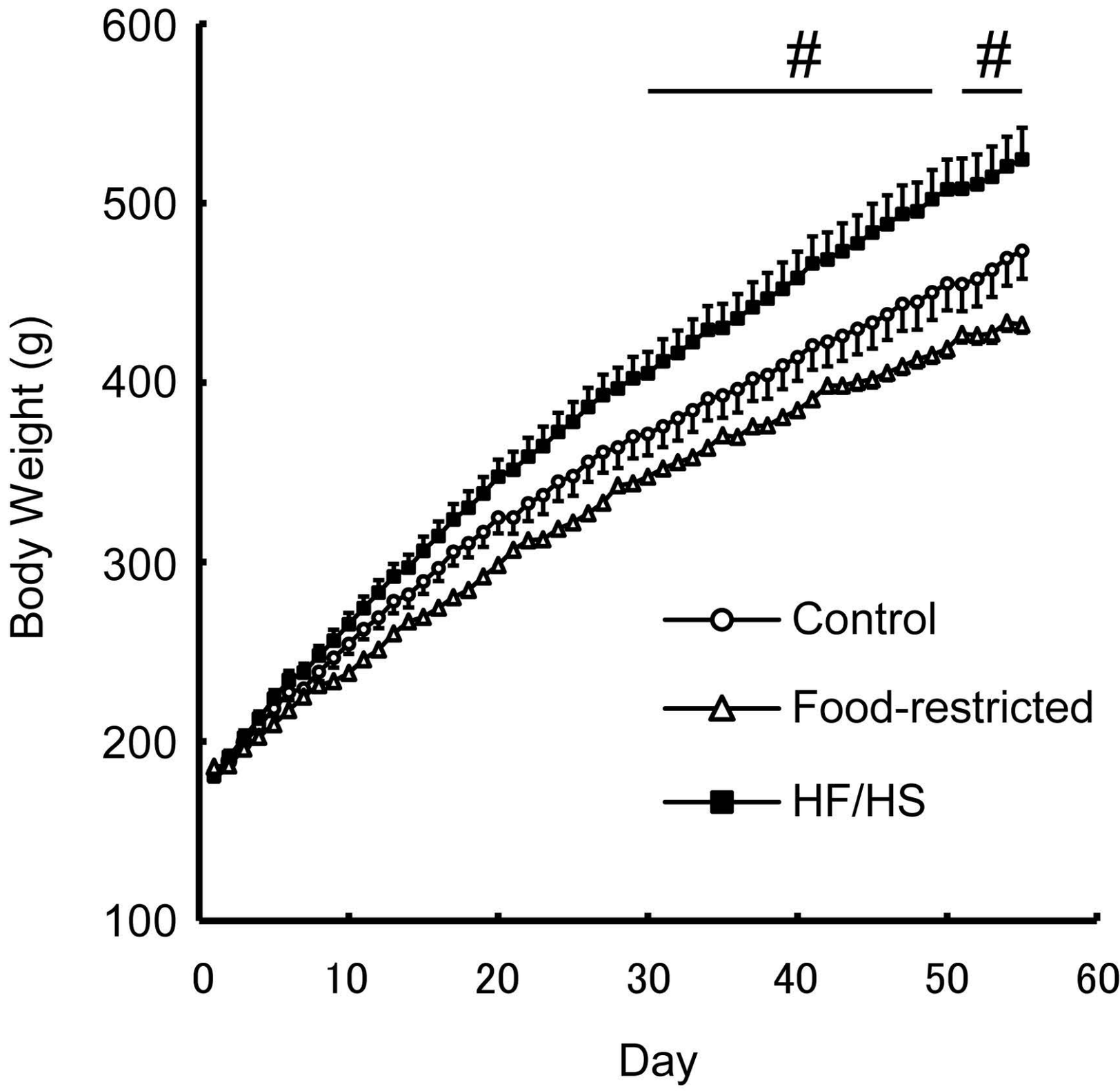

600
500
400
300
200
100
Body Weight (g)
0
10
20
30
40
50
60
Day
#
#
Control
Food-restricted
HF/HS

Figure 2

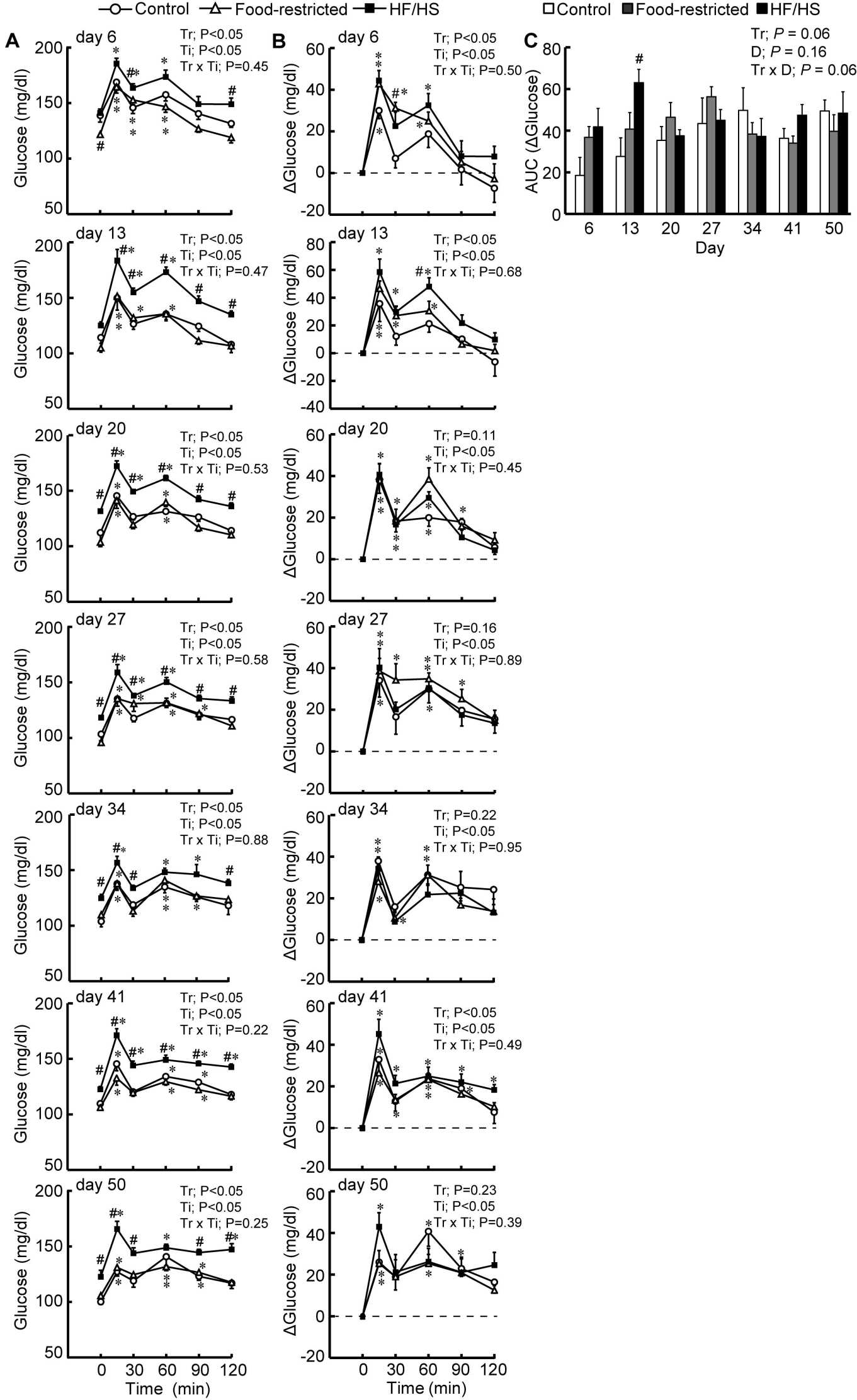

Control
Food-restricted
HF/HS
A
B
C
day 6
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.45
day 13
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.47
day 20
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.53
day 27
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.58
day 34
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.88
day 41
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.22
day 50
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.25
Glucose (mg/dl)
Time (min)
0 30 60 90 120
day 6
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.50
day 13
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.68
day 20
Tr; P=0.11
Ti; P<0.05
Tr x Ti; P=0.45
day 27
Tr; P=0.16
Ti; P<0.05
Tr x Ti; P=0.89
day 34
Tr; P=0.22
Ti; P<0.05
Tr x Ti; P=0.95
day 41
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P=0.49
day 50
Tr; P=0.23
Ti; P<0.05
Tr x Ti; P=0.39
ΔGlucose (mg/dl)
Time (min)
0 30 60 90 120
Tr; P = 0.06
D; P = 0.16
Tr x D; P = 0.06
AUC (ΔGlucose)
6 13 20 27 34 41 50
Day

Figure 3

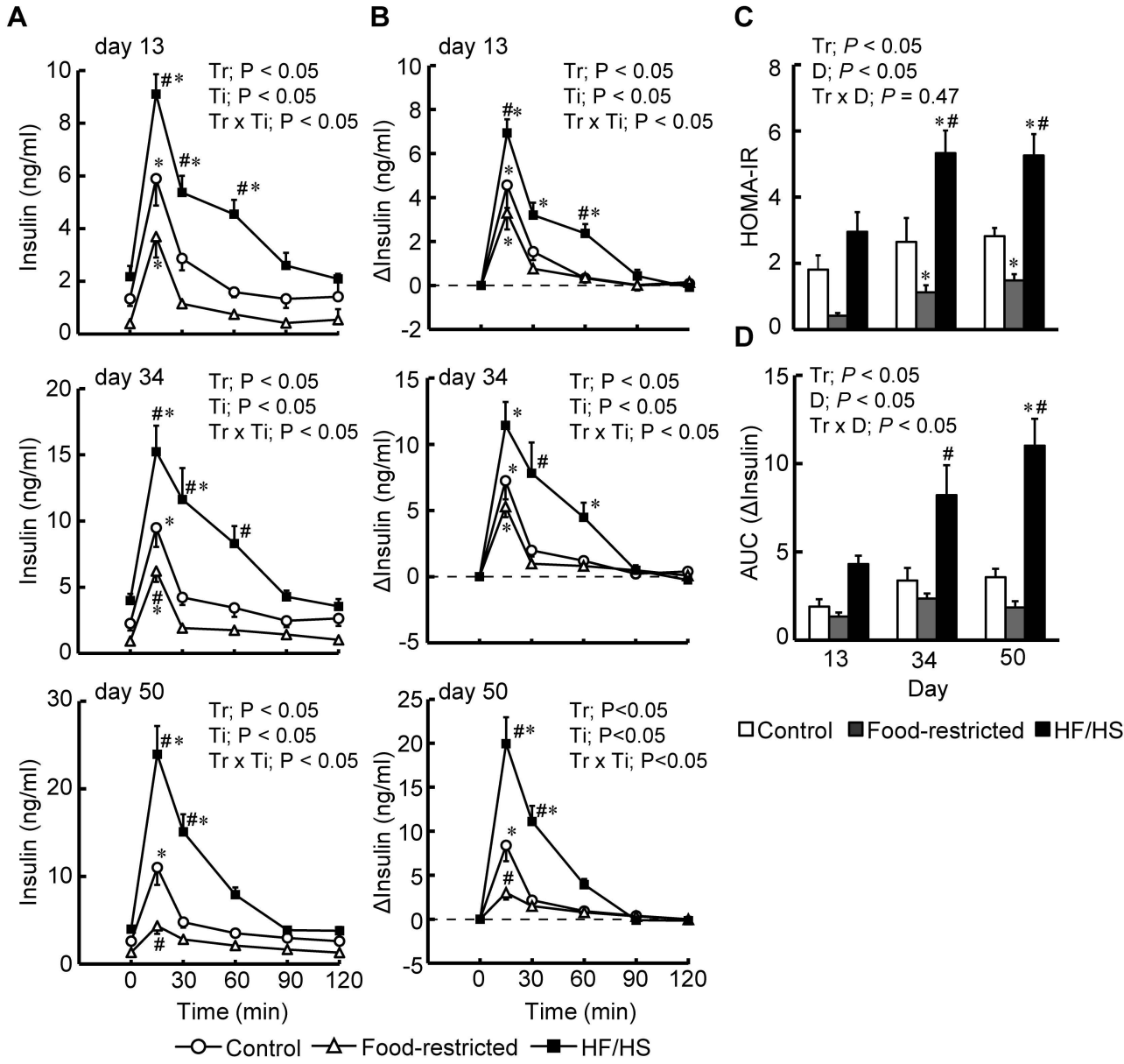

A
day 13
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P < 0.05
Insulin (ng/ml)
day 34
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P < 0.05
Insulin (ng/ml)
day 50
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P < 0.05
Insulin (ng/ml)
Time (min)
B
day 13
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P < 0.05
ΔInsulin (ng/ml)
day 34
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P < 0.05
ΔInsulin (ng/ml)
day 50
Tr; P<0.05
Ti; P<0.05
Tr x Ti; P<0.05
ΔInsulin (ng/ml)
Time (min)
C
Tr; P < 0.05
D; P < 0.05
Tr x D; P = 0.47
HOMA-IR
D
Tr; P < 0.05
D; P < 0.05
Tr x D; P < 0.05
AUC (ΔInsulin)
13
34
50
Day
Control
Food-restricted
HF/HS
Control
Food-restricted
HF/HS

Figure 4

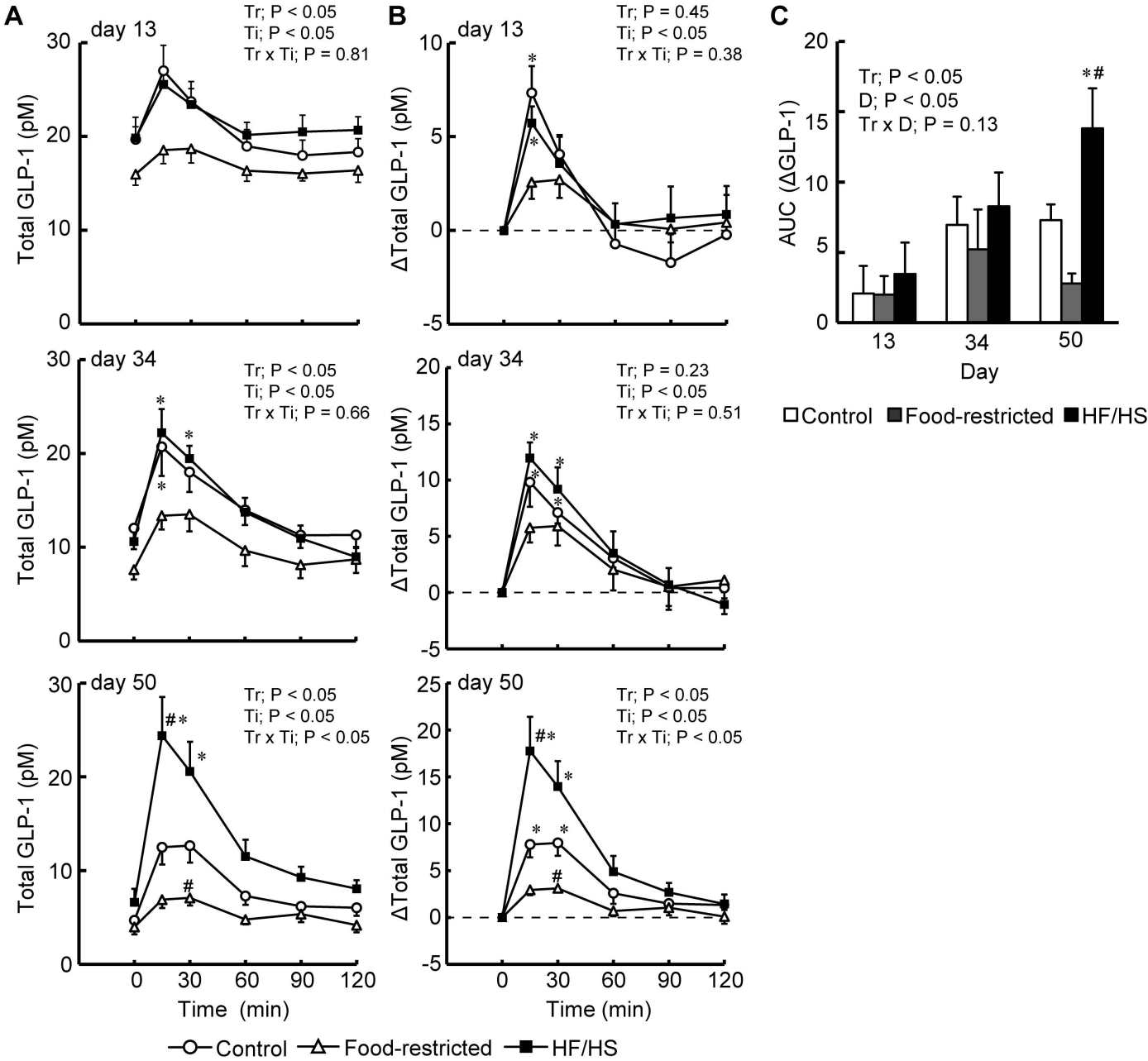

A
day 13
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P = 0.81
Total GLP-1 (pM)
day 34
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P = 0.66
day 50
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P < 0.05
B
day 13
Tr; P = 0.45
Ti; P < 0.05
Tr x Ti; P = 0.38
ΔTotal GLP-1 (pM)
day 34
Tr; P = 0.23
Ti; P < 0.05
Tr x Ti; P = 0.51
day 50
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P < 0.05
Time (min)
C
Tr; P < 0.05
D; P < 0.05
Tr x D; P = 0.13
AUC (ΔGLP-1)
13
34
50
Day
Control
Food-restricted
HF/HS

Figure 5

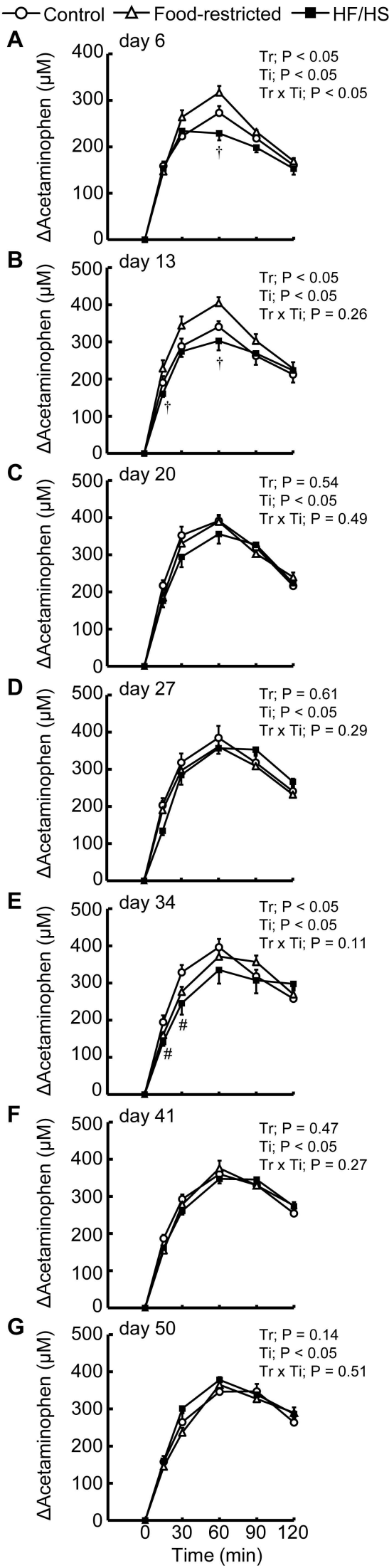

Control
Food-restricted
HF/HS
A
day 6
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P < 0.05
B
day 13
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P = 0.26
C
day 20
Tr; P = 0.54
Ti; P < 0.05
Tr x Ti; P = 0.49
D
day 27
Tr; P = 0.61
Ti; P < 0.05
Tr x Ti; P = 0.29
E
day 34
Tr; P < 0.05
Ti; P < 0.05
Tr x Ti; P = 0.11
F
day 41
Tr; P = 0.47
Ti; P < 0.05
Tr x Ti; P = 0.27
G
day 50
Tr; P = 0.14
Ti; P < 0.05
Tr x Ti; P = 0.51
ΔAcetaminophen (μM)
0
100
200
300
400
500
†
#
0
30
60
90
120
Time (min)

Figure 6

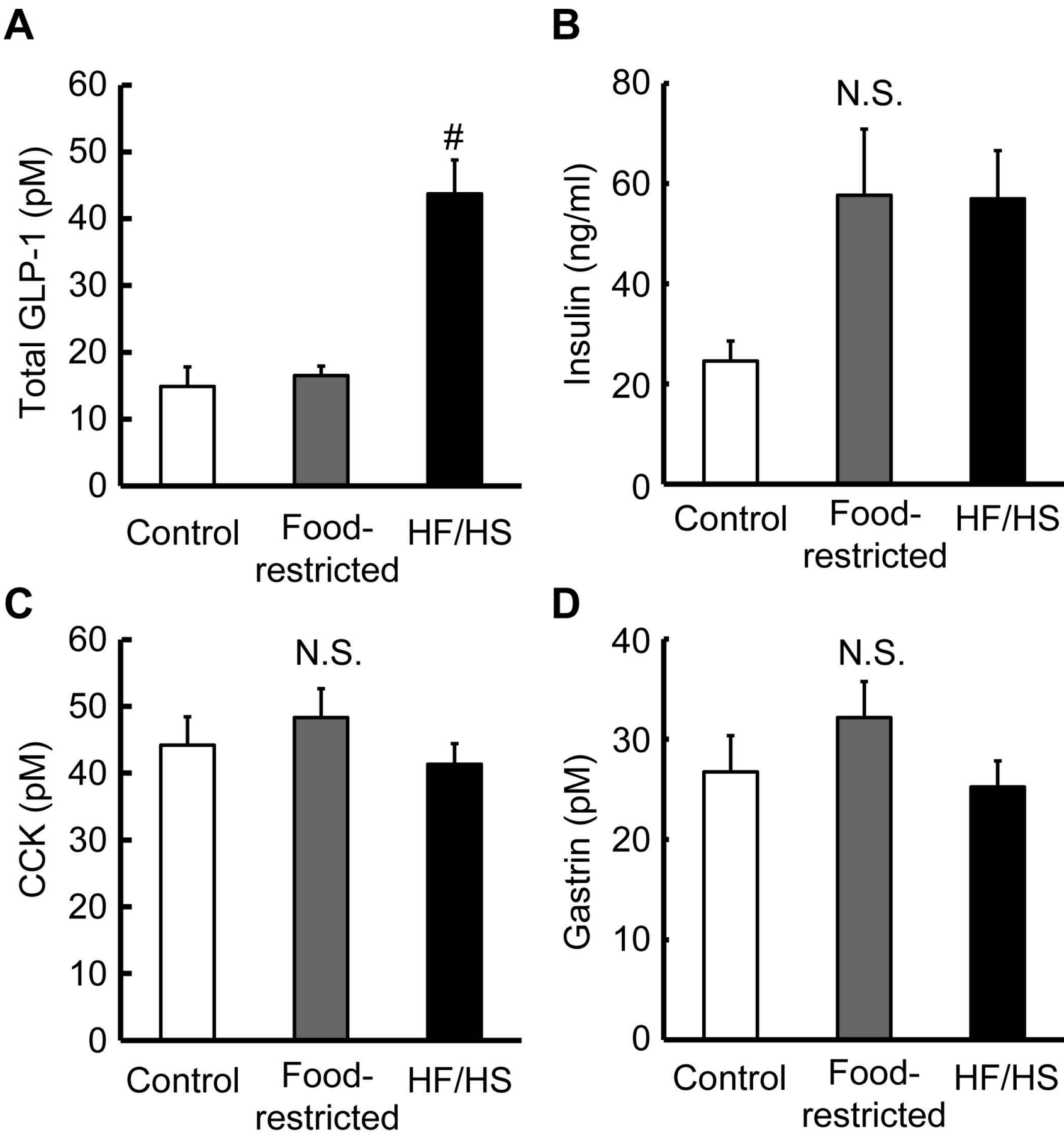
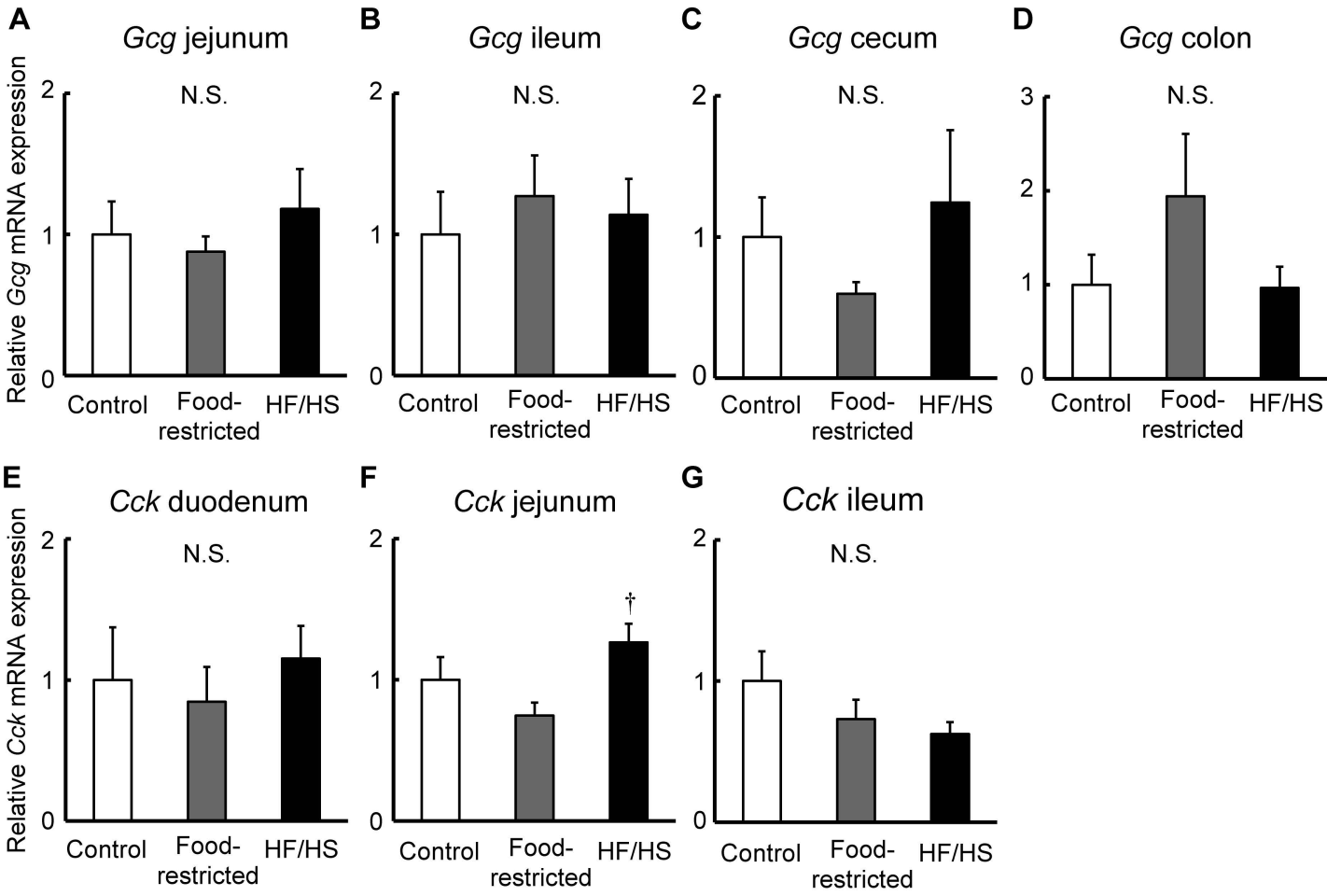
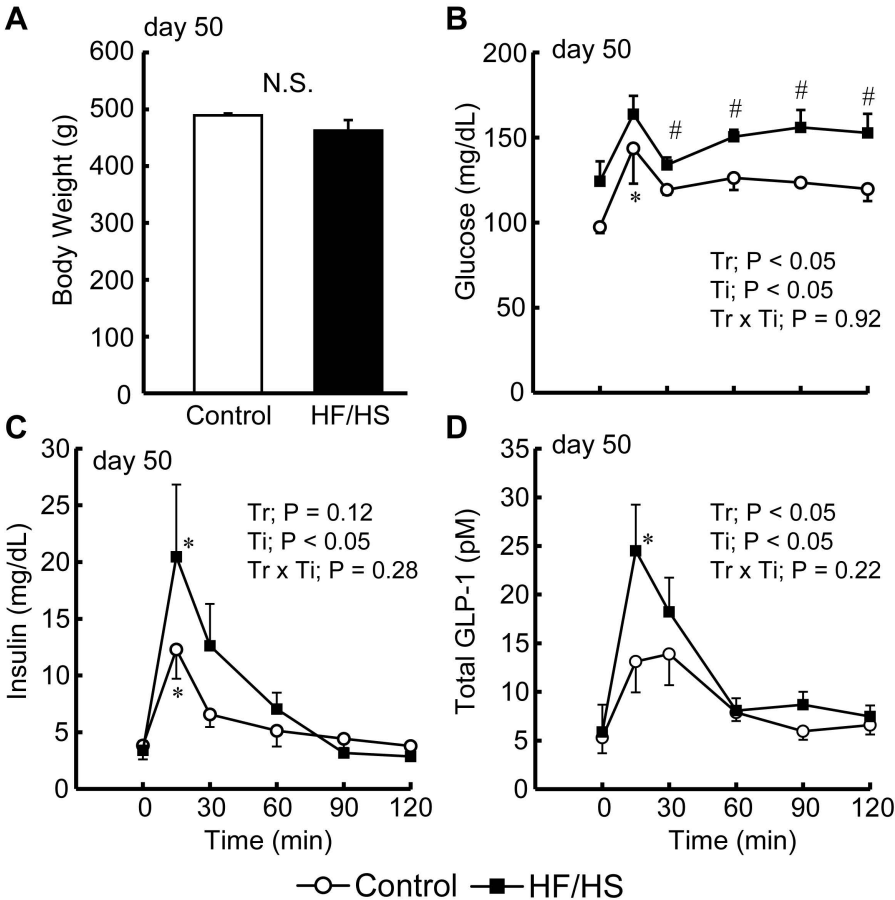

A
Total GLP-1 (pM)
60
50
40
30
20
10
0
#
Control
Food-restricted
HF/HS
B
Insulin (ng/ml)
80
60
40
20
0
N.S.
Control
Food-restricted
HF/HS
C
CCK (pM)
60
50
40
30
20
10
0
N.S.
Control
Food-restricted
HF/HS
D
Gastrin (pM)
40
30
20
10
0
N.S.
Control
Food-restricted
HF/HS

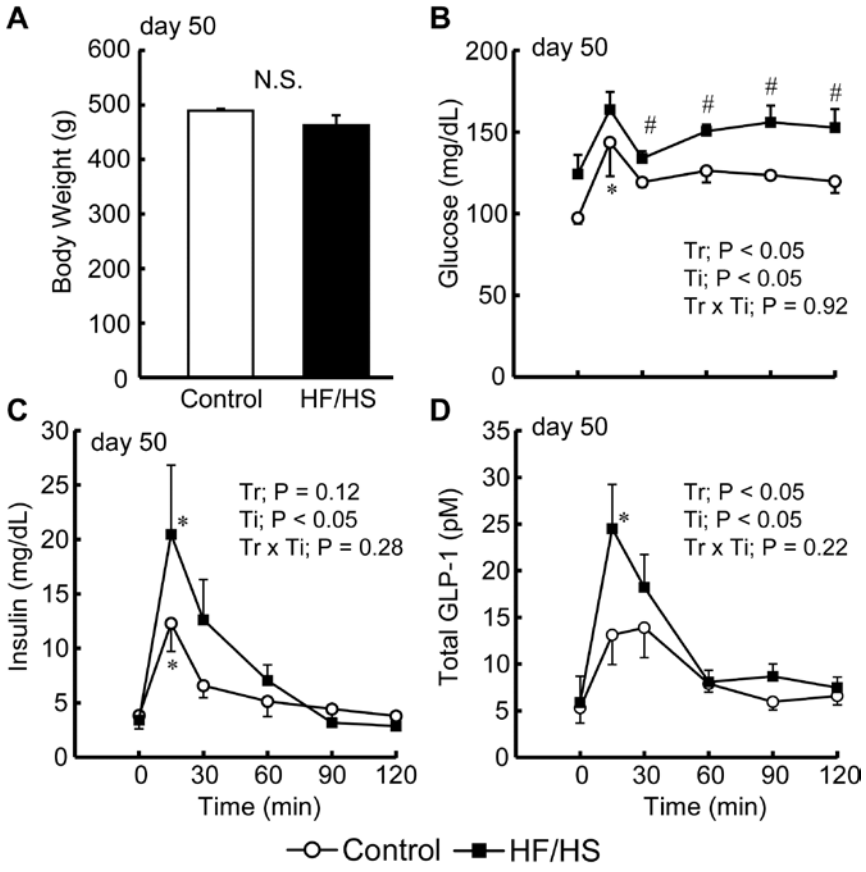
Figure 7


A
Gcg jejunum
N.S.
Relative Gcg mRNA expression
2
1
0
Control
Food-restricted
HF/HS
B
Gcg ileum
N.S.
2
1
0
Control
Food-restricted
HF/HS
C
Gcg cecum
N.S.
2
1
0
Control
Food-restricted
HF/HS
D
Gcg colon
N.S.
3
2
1
0
Control
Food-restricted
HF/HS
E
Cck duodenum
N.S.
Relative Cck mRNA expression
2
1
0
Control
Food-restricted
HF/HS
F
Cck jejunum
†
2
1
0
Control
Food-restricted
HF/HS
G
Cck ileum
N.S.
2
1
0
Control
Food-restricted
HF/HS

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 ± 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-Krammer's post-hoc test).