Adaptive changes in postprandial glucagon-like peptide-1 response and its role during the progression of diet-induced obesity and diabetic state in rats

（食事誘導性肥満および糖尿病モデルラットにおける食後 glucagon-like peptide-1 分泌応答の変動とその役割）

Hokkaido University, Graduate School of Agriculture,

Division of Applied Bioscience, Doctor Course

Jukkrapong Pinyo

Division of Applied Bioscience

Jukkrapong Pinyo

Abstract

Obesity positively correlates with excessive energy intake, especially from high fat and/or high sucrose diet. Gut hormones have various physiological functions under normal conditions, however, changes and roles of gut hormones in the development of obesity or diabetic condition have not been clearly identified. Glucagon-like peptide-1 (GLP-1) is a gut hormone, which is produced by enteroendocrine L-cells and secreted in response to nutrient ingestion (e.g. glucose, amino acid, fat, fiber, etc.). GLP-1 is well recognized as an “incretin hormone” that regulates postprandial glucose homeostasis through enhancing insulin secretion. Until now, alterations of postprandial GLP-1 secretion remain controversial in responses to nutrient in the ingested diet under obesity, prediabetes, and diabetes.

The purposes of overall study were to clarify (1) alteration of postprandial GLP-1 secretion and (2) role of postprandial GLP-1 secretion during progression of diet-induced obesity, and (3) mechanism underlying the adaptive changes in GLP-1 responses. Elucidation of these issues will contribute to prevention of obesity, glucose intolerance, and possibly diabetes.

The aim of experiment 1 was to clarify postprandial GLP-1 secretion in response to meal ingestion during the progression of a diet-induced obesity, and to determine whether dietary fat and/or sucrose contribute to adaptive changes in postprandial GLP-1 secretion. Male Sprague-Dawley (SD) rats were fed a control diet, a high fat (HiFat) diet (30% fat), a high sucrose diet (HiSuc) diet (40% sucrose) or a high fat and high sucrose diet (HFS) diet (30% fat and 40% sucrose) for 5 weeks. Meal tolerance tests (MTTs) were conducted at 2 and 4 weeks after feeding with the test diets to assess the concentrations of glucose, insulin, and GLP-1 in systemic circulation

In both MTTs, postprandial glycemic responses were not different among the

treatments. Postprandial GLP-1 responses were higher in HFS and HiFat group after 2 weeks of feeding period, but HiSuc group also had higher response after 4 weeks of feeding period, compared to that in the control group. The results in experiment 1 suggested that an enhanced postprandial GLP-1 secretion in the rats fed HFS diet may have a role in maintaining postprandial glycemia and retarding the establishment of glucose intolerance.

The aim of experiment 2 was to clarify the role of enhanced endogenous GLP-1 secretion in glucose intolerance and obesity development. The SD rats were given the control or the HFS diet with or without continuous administration of exendin-9 (Ex9, 100 μg/day), a GLP-1 receptor antagonist, for 5 weeks. MTTs were performed by an oral administration of a liquid diet (15 kcal/kg BW). Consistently with the result of experiment 1, postprandial glycemic response in the HFS group was maintained similarly to that in the control group, whereas postprandial secretions of GLP-1 and insulin were increased in the HFS group. In Ex9-treated group (blocking GLP-1 signal), an elevated postprandial glycemic response was observed accompanied by a lower insulin response, compared to the HFS group, which demonstrated that enhancement of the postprandial GLP-1 response during obesity development is required to maintain a normal postprandial glycemic response.

On the other hand, it has been unclear whether the increased GLP-1 responses in the diet-induced obese models were sustained or disappeared in a prolonged diet-induced obesity. Also, the meal-induced GLP-1 response in genetically diabetic models under treatment with the obesogenic diet was unknown. Hence, the aim of experiment 3 was to examine changes in adaptive enhancement of postprandial GLP-1 responses in the diet-induced obesity rats in a long-term experiment (26 weeks). Non-diabetic Wistar rat and diabetic Goto-Kakizaki (GK) rats were fed the control or the HFS diet for 26 weeks. MTTs were conducted every 4 or 8 week after the test diet feeding. In Wistar rats, postprandial

GLP-1 responses were sustained at a higher level or tended to be higher in the HFS-fed rats throughout the study with a similar postprandial glycemic response in the early period of diet-induced obesity (first 8 weeks), compared to the control group. Both of diabetic GK rats showed a similar postprandial GLP-1 response, whereas the postprandial glycemic responses in HFS-fed GK rats was markedly higher, compared to GK rats fed with the control diet throughout the experimental period. GLP-1 contents (jejunum and ileum) and mRNA expression level of free fatty acid receptor 1 (*Ffar1*) in the jejunum were increased or mildly increased by HFS diet only in Wistar rats. The failure of those adaptive changes in GK rats could be partly responsible for the development of glucose intolerance.

In summary, HiFat diet rather than HiSuc diet has a potent impact on postprandial GLP-1 response and obesity (adiposity) development in rats. Moreover, HFS diet showed much impact on them compared to the HiFat diet alone. The enhancement of GLP-1 secretion during obesity development plays an important role in maintaining normal postprandial glycemia through increasing insulin secretion. However, the adaptation was absent in the diabetic GK rats, implying that failure of adaptive enhancement of GLP-1 response accelerates the development of glucose intolerance. Increased GLP-1 production and fatty acid sensitivity in the small intestine are potent factors that are involved in enhanced GLP-1 responses under obesity.

From overall experiments, a better understanding of GLP-1 secretion and its adaptation will lead us to design therapeutics or dietary interventions that modulate GLP-1 secretion by certain materials.

List of abbreviations

ANOVA	Analysis of variance
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
BW	Body weight
cDNA	Complementary deoxyribonucleic acid
DIO	Diet-induced obesity
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidyl peptidase IV
Ex9	Exendin-9
Ffar1	Free fatty acid receptor 1
Ffar2	Free fatty acid receptor 2
Ffar3	Free fatty acid receptor 1
Ffar4	Free fatty acid receptor 1
Gcg	Proglucagon gene
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide-1
HFS	High fat and high sucrose
HiFat	High fat
HiSuc	High sucrose
HOMA-IR	Homeostasis model assessment-insulin resistance
MTT	Meal tolerance test
OGTT	Oral glucose tolerance tests
RNA	Ribonucleic acid

List of abbreviations (Cont.)

SEM	Standard error of the mean
TG	Triacylglycerol

List of tables and figures

List of tables and figures (Cont.)

List of tables and figures (Cont.)

Contents

Contents (Cont.)

Contents (Cont.)

General introduction

Obesity, metabolic syndrome, and gut hormone secretion

Excess energy intake, especially from high fat and/or high sucrose diet, promotes the development of overweight and obesity (Sheludiakova, 2012; Wang, 2015). Nowadays, obesity has become a major public health problem, and the incidence of the disease is increasing rapidly in the world population (Omar, 2012; Whiting, 2011). In addition, obesity is associated with metabolic syndrome disorders, including hyperglycemia, insulin resistance, and type 2 diabetes mellitus (Grundy, 2004; Kao, 2006; Zimmet, 2010; Hira, 2017; Sakar, 2014; Makaronidis 2019). Not only metabolic syndrome disorders, obesity has been reported to decrease gut hormone secretion (Ranganath, 1996; Lean, 2016; Makaronidis, 2019).

Incretin hormones

Incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are gut peptide hormones (Deacon, 2011; Yabe, 2011). GIP is comprised of 42 amino acids that is produced and secreted from enteroendocrine K-cells in the upper small intestine (Inagaki, 1989). GLP-1, a product of the proglucagon gene, is primarily secreted in two forms, such as GLP1-(7-37) NH_2 and GLP-1-(7-36) NH_2 (Mojsov, 1987; Vahl, 2003) from enteroendocrince L-cells in the lower small intestine and the colon (Yabe, 2011) in response to nutrients in the ingested diet including glucose, fatty acids, proteins, amino acids, or dietary fibers (Baggio, 2007; Carr, 2008; De León, 2008; Tolhurst, 2009; Hira, 2015). Incretin hormones play an important role in the regulation of blood glucose level by enhancing insulin secretion through G-protein-coupled rise in intracellular cyclic adenosine monophosphate (cAMP) (Gromada, 1998; Deacon, 2011). GLP-1 also performs various intrapancreatic and extrapancreatic functions (Abu-Hamdah, 2009; Fava, 2016; Habener, 2017). The incretin seems to play a major role in the regulation

of glucose metabolism, gastrointestinal motility, and food intake (Vilsbøll, 2004; Wang, 2015). GLP-1 and GIP are rapidly degraded by enzyme dipeptidyl peptidase-IV (DPP-IV). The peptidase inactive these incretins by cleaving the two NH_2-terminal peptides of the peptides (Carr, 2008). It had been reported that GLP-1 potently stimulated postprandial insulin secretion, whereas GIP had less or no effect in type 2 diabetes patient (Edwards, 1999; Lee, 2010)

Diet-induced obesity (DIO) and type 2 diabetes models

In diet-induced obesity (DIO) rodent models, obesogenic diets (high fat and/or high sucrose diet) are fed to mimic the pathophysiology of human obesity and metabolic syndrome (Wang, 2015; Hira, 2020). Both Sprague–Dawley (SD) rats and Wistar rats fed with obesogenic diets are commonly used as DIO model. In addition, rats are more suitable for the evaluation of metabolic parameters due to relatively larger body size of rats than that of mice such as a large volume of blood collection (Marques, 2016).

There are various rodent models of diabetes, such as Zucker diabetic fatty (ZDF) rats, diabetic (*db/db*) mice, and obese (*ob/ob*) mice (Rees, 2005; Srinivasan, 2007). The Goto-Kakizaki (GK) rat was developed by breeding of glucose intolerant Wistar rats (Goto, 1975; Amiri, 2015). GK rats, one of type 2 diabetes mellitus (T2DM) model animals, have been used to study the pathophysiology of diabetes and its complications, and to evaluate anti-diabetes drugs because they present symptoms similar to type 2 diabetic patients such as hyperglycemia, hyperinsulinemia, and insulin resistance (Ishii, 2012).

Nutrient challenge to assess postprandial metabolic responses

Both oral glucose tolerance test (OGTT) and meal tolerance test (MTT) are generally employed to assess postprandial metabolic responses such as glucose, insulin, and other hormones (Rijkelijkhuizen, 2009; Staten, 2014). Nevertheless, administration of

glucose is not always the best method to access overall physiological glucose regulation (Ahlkvist, 2012). Moreover, a study demonstrated that MTT is more suitable for postprandial metabolic responses than OGTT (Brodovicz, 2011). Also, there is a marked secreted of insulin and GLP-1 after mixed meal ingestion (Vilsbøll, 2003; Ahlkvist, 2012). Therefore, MTTs have been employed to evaluate postprandial responses in these studies. In order to mimic typical dietary habit, voluntary ingestion is more appropriate than enforced gavage feeding.

Postprandial GLP-1 responses under obese and/or impaired glucose tolerance status

Until now, direction of postprandial GLP-1 secretion (enhanced, unchanged, or decreased) is still under debate in response to nutrients ingestion in obese, pre-diabetic, and diabetic patients, including in animal models (Nauck, 2011; Smushkin, 2012; Faerch, 2015; Wölnerhanssen, 2016; Dirksen, 2019; Otten, 2019). GLP-1 secretion increased in response to oral glucose in type 2 diabetic patients (Alssema, 2013). In contrast, obese and type 2 diabetic patients showed reduced GLP-1 secretion after mixed-meal ingestion (Ranganath, 1996; Toft-Nielsen 2001). Also, no change was observed in postprandial GLP-1 secretion in response to oral glucose in type 2 diabetic patients (Calanna, 2013). In our previous study (Nakajima, 2015), postprandial GLP-1 secretion was increased in response to normal diet administration during the DIO progression in rat fed an HFS diet. In order to clarify how GLP-1 responses are changed in both non-diabetic and diabetic state and the role of GLP-1 in DIO progression, it is important to adopt animal models in which the animals are continuously fed with an obesogenic diet.

Objectives of studies

This dissertation composes of 3 chapters. In Chapter 1, GLP-1 secretion was examined in response to meal ingestion during obesity development and determine whether

dietary fat or sucrose potently impacts on adaptive changes in the postprandial GLP-1 response. In this study, the rats were fed a control, high fat (HiFat) diet (30% weight), high sucrose (HiSuc) diet (40% weight), or high fat and high sucrose (HFS) (30% fat and 40% sucrose) diet for 5 weeks.

In chapter 2, the role of endogenous GLP-1 in postprandial glycemic response was determined during the DIO progression. The SD rats were provided either a control diet or the HFS diet with or without continuous administration of the GLP-1 receptor antagonist exendin (9–39) (Ex9, 100 μg/day) by using a subcutaneously implanted osmotic pump, for 5 weeks.

Lastly, in chapter 3, it was investigated whether the adaptive enhancement of postprandial GLP-1 responses was sustained or disappeared in the DIO rats in a long-term study. Adaptive change in GLP-1 response was also investigated in the diabetic model rats fed with the HFS diet. The non-diabetic Wistar and diabetic (GK) rats were fed either with the control diet or the HFS diet for 26 weeks.

Chapter 1: Effect of high fat and/or high sucrose diet on meal-induced GLP-1 secretion during the progression of diet-induced obesity in rats

Abstract

Nowadays, obesity is one of the major health concerns. It positively correlates with excessive energy intake especially high fat and/or high sucrose consumption. GLP-1 is a gut-derived hormone, which is secreted by enteroendocrine L-cells in response to nutrient ingestion. The incretin hormone, GLP-1, normalizes postprandial glucose homeostasis through stimulating insulin secretion. To date, changes in postprandial GLP-1 response during the progression of diet-induced obesity (DIO) are not well characterized. The aims of this study were to examine GLP-1 response to meal ingestion during the DIO progression and to determine whether high fat or high sucrose diet largely attribute to obesity (adiposity) and/or glucose intolerance development.

Male Sprague-Dawley (SD) rats were fed a control diet, a high fat diet (HiFat, 30%), a high sucrose diet (HiSuc, 40%) or a high fat/high sucrose diet (HFS) totally for 5 weeks. Meal tolerance tests (MTTs) were conducted 2 and 4 weeks after feeding the test diet in order to examine postprandial glucose, insulin, and GLP-1 responses after standard (control) diet ingestion. After 5 weeks of feeding period, blood samples from portal vein and abdominal aorta were taken under sodium pentobarbital anesthesia. The rats were then sacrificed and tissue samples were collected.

Body weight gain of HiFat group and adipose tissue weight of HFS group were increased, compared to the control group, suggesting that HiFat diet had relatively high impact on obesity (adiposity) development. In both MTTs, postprandial glycemic response showed a similar level in all groups, but postprandial insulin response and HOMA-IR (index of insulin resistance) were significantly higher in HFS group rather than other groups. Postprandial GLP-1 responses were higher in HFS and HiFat group after feeding

the test diet for 2 weeks, but HiSuc group also had higher response after feeding the test diet for 4 weeks, compared to control group. These findings suggest that continuous ingestion of excessive fat rapidly enhances sensitivity of GLP-1-producing cells to luminal nutrients and the increment of postprandial GLP-1 and following insulin secretion may have a role in normalization of postprandial glycemia and slowing down the establishment of glucose intolerance.

1.1 Introduction

The previous study showed that long-term high fat and high sucrose diet feeding (8 weeks) increased postprandial GLP-1 secretion in response to diet ingestion (Nakajima, 2015). In addition, chronic high fat diet feeding in rats enhanced GLP-1 secretion in response to a mixed-meal challenge (Wang, 2015). In contrast, high fat diet fed rats showed decreased GLP-1 secretion in response to oral glucose during obesity development (Gniuli, 2010). Although the impact of high fat and/or high sucrose diet feeding in animal models on postprandial GLP-1 secretion have been previously conducted, the alteration of postprandial GLP-1 response during the DIO progression has not been clearly characterized. It still remains controversial whether GLP-1 secretion is enhanced or diminished during obesity development. The aims of this study were to clarify GLP-1 response to meal ingestion during the DIO progression and to determine whether high fat or high sucrose diet largely attribute to postprandial GLP-1 secretion and the development of obesity (adiposity) and/or glucose intolerance. To imitate the dietary exposure in normal life, voluntary oral ingestion might be more appropriate than enforced gavage feeding. Hence, the rats were fed with a standard (control) diet as a routine diet ingestion (MTT) instead of oral glucose loading (oral glucose tolerance test) in this experiment to determine postprandial glucose, insulin, and GLP-1 levels in response to “meal” ingestion.

1.2 Material and methods

1.2.1 Animal 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 an American Institute of Nutrition (AIN)-93G (control) diet for 1 week as an acclimation period (Reeves, 1997). Each rat was housed in an individual cage under 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*) except the day before experiment. The rats were divided into four groups based on body weight (BW), plasma glucose, and GLP-1 concentrations as control, HFS, HiFat, and HiSuc groups, which were given a control, a high fat/high sucrose (Nakajima, 2015), a high fat (HiFat, 30% fat), and a high sucrose (HiSuc, 40% sucrose) diet, respectively, for 5 weeks. The final compositions of 30% fat in a HiFat diet and 40% sucrose in a HiSuc diet were adjusted to be equal to those in a HFS diet (30% fat and 40% sucrose). The compositions of test diets (AIN-93G based) are shown in Table 1.1. Body weight and food intake were measured every 2 days. All animal experimental procedures used in this study were approved by Hokkaido University Animal Committee (approval no. 19-0064), and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

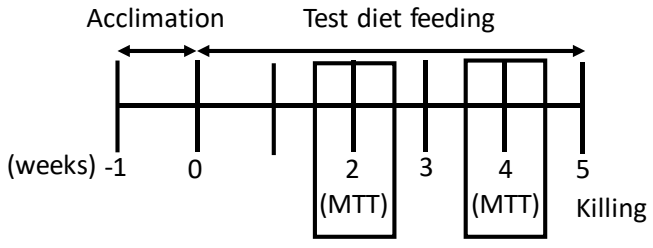


Figure 1.1. Schematic representation of experiment timeline.

After 1 week of the acclimation period, the rats were then given the test diet for totally 5 weeks.

Table 1.1 Experimental 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	–
Fiber (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
Energy density (kcal/g)	4.03	5.18	5.18	4.03

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

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

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

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

1.2.2 Meal tolerance test (MTT)

In this study, meal tolerance test (MTT) was used to determine postprandial responses in response to standard diet (control diet) ingestion. MTT was performed on rats after feeding the test diets for 2 and 4 weeks in order to determine postprandial glucose, insulin, and GLP-1 levels in responses to meal ingestion. Before conducting MTT experiment, the rats were fasted for 16 h (2 weeks) or 24 h (4 weeks) then basal blood collection (0 min) was conducted. After the basal blood collection, the rats were given control diet (10 g/kg BW) in diet container as routine diet ingestion for 30 min and blood samples from tail vein were then collected at 15, 30, 60, 90 and 120 min after feeding the control diet. In this study, only the rats that ingested >90% of the given diet were used to

assess the postprandial responses. Blood samples were transferred into chilled tubes containing heparin (final concentration 50 IU/mL; Ajinomoto Company, Inc., Tokyo, Japan) and aprotinin (final concentration 500 Kallikrein inhibitor units (kIU)/mL; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma was separated by centrifugation at 2300 x *g* for 10 min at 4°C and stored at -80°C until analysis. Plasma glucose level was determined using the Glucose CII Test Kit (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin and GLP-1 concentration were analyzed using the Rat Insulin ELISA (AKRIN-010T; Shibayagi Company Limited, Gunma, Japan) and Multi Species GLP-1 Total ELISA (EZGLP1T-36K; Merck Millipore, Darmstadt, Germany), respectively. The homeostatic model assessment of insulin resistance (HOMA-IR) was used to assess insulin resistance and calculated using the following equation with glucose and insulin values at the fasting state and values of the area under the curve (AUC) during MTT (Cacho, 2008):

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

Where: 1 mg insulin = 26 IU

1.2.3 Blood and tissue collection

After feeding the test diet for 5 weeks, the rats were fasted for overnight (16 h). Blood samples from portal vein and abdominal aorta of rats were taken under sodium pentobarbital anesthesia (50 mg/kg BW, somnopentyl injection; Kyoritsu Seiyaku Corporation, Tokyo, Japan) into chilled syringe containing heparin (final concentration 50 IU/mL), aprotinin (final concentration 500 KIU/mL), and DPP-IV inhibitors (final concentration 50 µmol/L; DPP4-010; Merck Millipore, Darmstadt, Germany). Plasma was collected and stored as described above in order to measure glucose, insulin, total GLP-1, TG, and total cholesterol levels. Plasma glucose level was measured by using the Glucose CII Test Kit (Wako Pure Chemical Industries, Osaka, Japan). Total GLP-1 and insulin

levels were assessed using Multi Species GLP-1 Total ELISA (EZGLP1T-36K; Merck Millipore, Darmstadt, Germany) and the Rat Insulin ELISA (AKRIN-010T; Shibayagi Company Limited, Gunma, Japan). Plasma TG and cholesterol concentrations were measured using the Triglyceride E Test and Cholesterol E Test kits (Wako Pure Chemical Industries, Osaka, Japan), respectively. The rats were then sacrificed by exsanguination. Intestinal segments were carefully dissected and washed with cold saline solution (0.9% NaCl) then mucosa (5 cm segment) and 2 cm segments of jejunum, and ileum, and colon were taken from the middle region of each segment to measure mRNA expression level and GLP-1 content, respectively (Appendix D). The cecal tissue was washed with cold saline solution and divided equally into 2 parts, the first half part was collected for GLP-1 measurements and the mucosa from the second half was scraped for mRNA analysis (Appendix D). All intestinal segments were rapidly frozen in liquid N_2 and stored at -80°C until GLP-1 content measurement. Intestinal mucosa samples were taken and transferred into tubes containing RLT buffer (RNeasy Mini Kit; Qiagen, Hilden, Germany) and immediately frozen in liquid N_2 then kept at -80°C until RNA extraction. The mesenteric, retroperitoneal, and epididymal adipose tissue were individually weighed and expressed as visceral adipose tissue weight.

1.2.4 Measurement of GLP-1 content in intestinal tissue

Intestinal segments (2 cm length) were immersed in an ethanol acid solution (absolute ethanol–MilliQ water–12 M HCl, 74:25:1, 5 mL/g of intestinal tissue segments) (Tourrel, 2002; Cani, 2007; Granata, 2009) and then cut into small pieces. The tissue samples were homogenized at maximum level (Ultra Turrax homogenizer T18, IKA, Staufen, Germany) for 2 min and placed at 4°C for 24 h. The homogenate samples were then centrifuged at 2000 x *g* for 20 min. The supernatant was collected for measuring GLP-

1 and protein content using Multi Species GLP-1 Total ELISA (EZGLP1T-36K; Merck Millipore, Darmstadt, Germany) and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) respectively. Before GLP-1 and protein content analysis, the supernatant was diluted 10-fold for protein content, 500-fold (jejunum, ileum, and colon segments), and 625-fold (colon segment) for GLP-1 content with normal saline solution.

1.2.5 Real-time quantitative PCR

Mucosa of each intestinal segment was scrapped and stored in RLT buffer solution (RLT buffer:2-mercaptoethanol as the ratio of 100:1) at -80°C until RNA extraction. Total RNA was prepared using the RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and immediately converted into complementary DNA (cDNA) using the ReverTra Ace qPCR Master Mix with gDNA Remover (Toyobo Company Limited, Osaka, Japan), following the manufacturer's instructions. Gene expression levels were determined by TaqMan gene expression assays (Life Technologies Company) with rat gene-specific, predesigned TaqMan primers and probe sets (Rn99999916_s1 for glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and Rn00562293_m1 for proglucagon (*Gcg*)). The relative expression level of each individual gene was compared to a reference gene (*Gapdh*) by using the standard curve method.

1.2.6 Statistical Analysis

The primary endpoint of this study was nutrient-induced postprandial GLP-1 responses in rats fed various (obesogenic) diets. The results were expressed as mean ± SEM. The sample size was calculated based on the experimental design (two-way repeated measures analysis of variance) using G*Power software (version 3.1.9.2) with effect size f

= 0.4 and power = 0.8. Significant effects of time (TI), treatment (TR), and interactions of time and treatment (TIxTR) were assessed by two-way repeated measures ANOVA in the results of MTTs. One-way ANOVA and Tukey-Kramer's tests were used to assess the significant difference among the treatments. Dunnett's test was used to determine the significant differences from baseline (0 min) within the same group. *P* values < 0.05 were considered statistically significant. The statistical analysis was performed using JMP Pro, version 13 software (SAS Institute, Inc., Cary, NC).

1.3 Results

1.3.1 BW, food intake, and fat accumulation

The initial BWs of rats were similar in all groups (167.7 ± 2.8 to 169.3 ± 2.5 g). After feeding the test diet for 5 weeks, BW gains and total energy intake of HiFat (232.3 ± 7.7 g) and HFS (228 ± 7.8 g) groups were significantly higher than those in the control group (200.7 ± 5.4 g) as shown in Table 1.2. Visceral adipose tissue weight (total weight of epididymal, mesenteric, and retroperitoneal) in the HFS and the HiFat groups were slightly higher than the control group, but significant differences were not observed. Epididymal adipose tissue weight significantly increased in the HFS group, compared to the control group (9.7 ± 0.5 g *vs.* 7.5 ± 0.6 g, Table 1.2). The HiSuc group showed similar values for these parameters as those in the control group.

Table 1.2 Initial BW, BW, visceral adipose tissue (mesenteric, epididymal, and retroperitoneal) weight, and energy intake after feeding the test diet for 5 weeks

	Control	HFS	HiFat	HiSuc
Initial BW (g)	168.7 ± 3.1^{NS}	169.3 ± 2.5	169.1 ± 4.3	167.7 ± 2.8
Final BW (g)	369.4 ± 8.2	397.3 ± 9.3	401.4 ± 10.3	388.0 ± 10.0^{NS}
BW 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}

Visceral fat weight is the sum of the mesenteric, epididymal, and retroperitoneal fat weight. The values are expressed as the mean ± SEM for n = 8-10 rats. The superscripts (a, b, c) without the same letters differed significantly between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant difference among the treatments.

1.3.2 Basal glucose, insulin, GLP-1 levels, and HOMA-IR after feeding the test diet for 2 and 4 weeks

After consuming the test diet for 2 and 4 weeks, the rats were fasted for overnight (16 or 24 h) before conducting the MTT. After 2 weeks of feeding, basal glucose (84.1 ± 4.5 to 90.0 ± 3.6 mg/dL), insulin (0.11 ± 0.01 to 0.20 ± 0.06 nM), GLP-1 (12.1 ± 1.5 to 20.4 ± 2.6), and HOMA-IR (0.54 ± 0.07 to 1.11 ± 0.34) were not significantly different among all treatment group (Table 1.3). After 4 weeks, the basal insulin level and HOMA-IR tended to be higher in the HFS group (0.26 ± 0.03 and 1.45 ± 0.20) compared to the control group (0.17 ± 0.02 nM; and 0.90 ± 0.10).

Table 1.3 Fasting glucose, insulin, GLP-1 levels and HOMA-IR

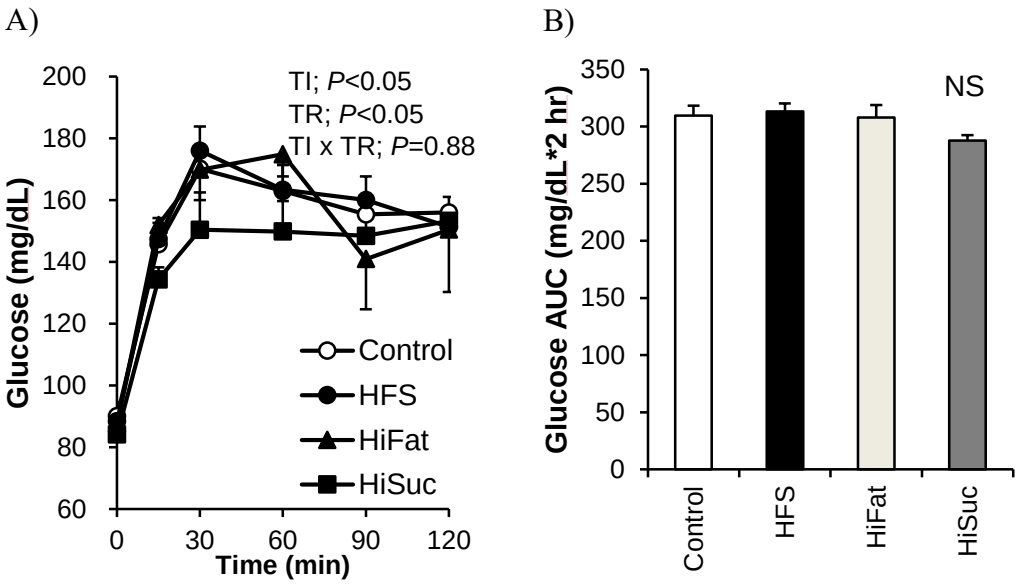
	Control	HFS	HiFat	HiSuc
2 weeks				
Glucose (mg/dL)	90.0 ± 3.6	88.4 ± 6.2	87.5 ± 2.5	84.1 ± 4.5^{NS}
Insulin (nM)	0.11 ± 0.22	0.20 ± 0.06	0.13 ± 0.05	0.11 ± 0.01^{NS}
GLP-1 (pM)	20.4 ± 2.6	12.1 ± 1.5	12.6 ± 2.3	18.2 ± 3.8^{NS}
HOMA-IR	0.63 ± 0.12	1.11 ± 0.34	0.74 ± 0.31	0.54 ± 0.07^{NS}
4 weeks				
Glucose (mg/dL)	86.6 ± 2.3	87.4 ± 2.9	87.0 ± 1.1	81.9 ± 3.2^{NS}
Insulin (nM)	0.17 ± 0.02	0.26 ± 0.03	0.15 ± 0.04	0.22 ± 0.03^{NS}
GLP-1 (pM)	15.0 ± 1.9	13.7 ± 3.7	11.8 ± 2.9	11.9 ± 2.7^{NS}
HOMA-IR	0.90 ± 0.10	1.45 ± 0.20	1.00 ± 0.20	1.11 ± 0.16^{NS}

The values are expressed as the mean ± SEM for n = 8-10 rats. The superscripts (a, b, c) without the same letters differed significantly between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant difference among the treatments.

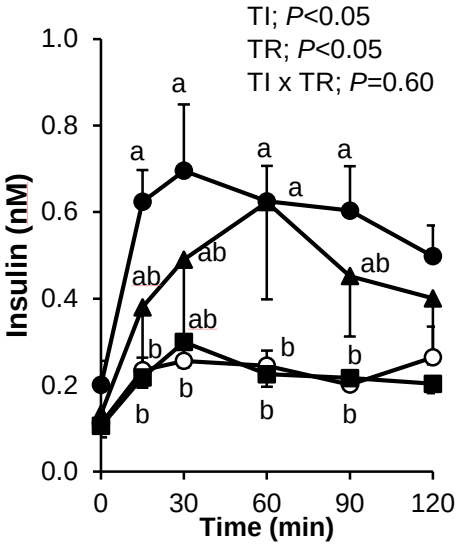
1.3.3 Postprandial glycemic, insulin, and GLP-1 responses during meal tolerance test (MTT, 2 weeks)

In order to mimic dietary exposure in normal life, meal tolerance test (MTT) was used to assess postprandial response in this study. In addition, voluntary ingestion is more relevant than enforced gavage feeding. The rats were fed with a control diet at the dose of 10 g/kg BW during MTT experiment. MTT was performed after feeding the test diet for 2 and 4 weeks to monitor the change in postprandial response during the DIO progression. After feeding the test diet for 2 weeks, the area under curve (AUC) of postprandial glycemia in all treatment groups were slightly higher than the control group, but significant difference between treatment groups and control group were not detected (Fig. 1.2A and B). The AUC of insulin largely increased in the HFS group, compared to that in the control group (1.15 ± 0.19 nM *vs.* 0.46 ± 0.04 nM). HiFat diet feeding group also showed the similar impact on insulin secretion but less than HFS group (Fig.1.2D). In addition, insulin

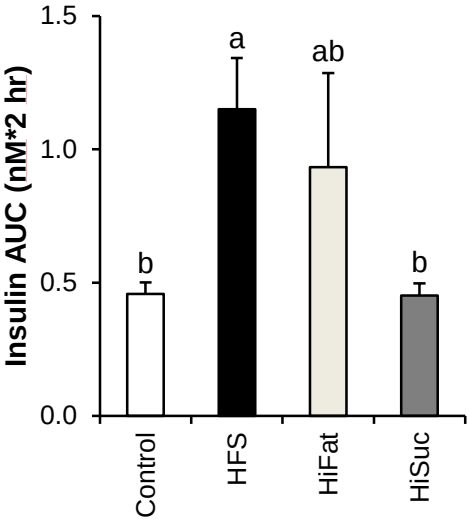
secretion at 60 min after feeding control diet significantly increased in HFS (0.62 ± 0.08 nM) and HiFat (0.62 ± 0.22 nM) groups, whereas HiSuc group (0.23 ± 0.03 nM) showed the similar level as shown in the control group (0.24 ± 0.03 nM) (Fig. 1.2C). Similarly to insulin response, GLP-1 secretion at 15 min after feeding control diet in HFS (17.0 ± 2.6 nM) and HiFat (16.6 ± 3.7 pM) group tended to increase compared to those in the control group (7.3 ± 2.3 pM), while HiSuc group (6.6 ± 4.8 pM) showed the similar level to control group as summarized in Fig. 1.2E. However, the GLP-1 level significantly increased after 15 min, compared to the basal level in both HFS and HiFat groups, while the HiSuc group was more similar to the control group (Fig. 1.2E). The AUC of HOMA-IR (AUC of HOMA-IR) was calculated by using postprandial glycemic and insulin response (0-120 min) during meal tolerance test and was used to estimate the degree of insulin resistance during MTT experiment. The results revealed that HFS group had the highest AUC of HOMA-IR followed by HiFat group, whereas HiSuc group showed the similar level as shown in the control group.



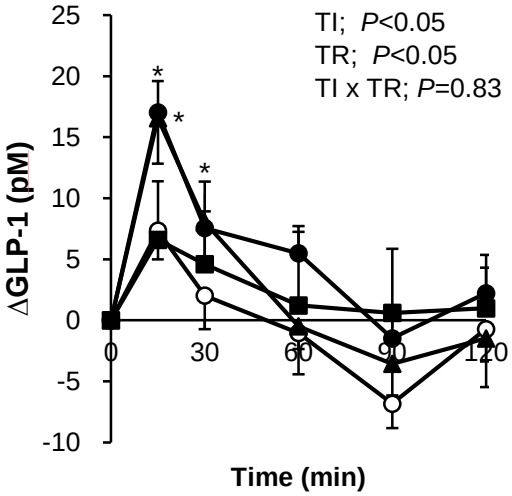
C)


TI; P<0.05
TR; P<0.05
TI x TR; P=0.60
Insulin (nM)
Time (min)

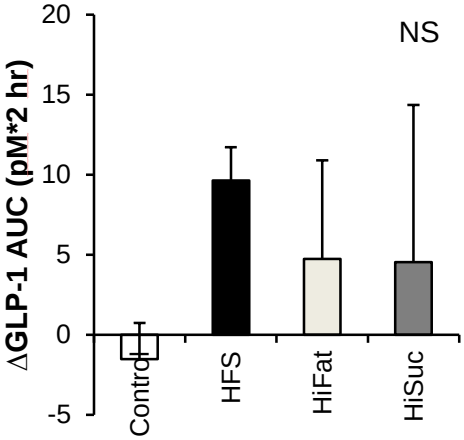
D)


Insulin AUC (nM*2 hr)
Control
HFS
HiFat
HiSuc

E)


TI; P<0.05
TR; P<0.05
TI x TR; P=0.83
ΔGLP-1 (pM)
Time (min)

F)


NS
ΔGLP-1 AUC (pM*2 hr)
Control
HFS
HiFat
HiSuc

G)

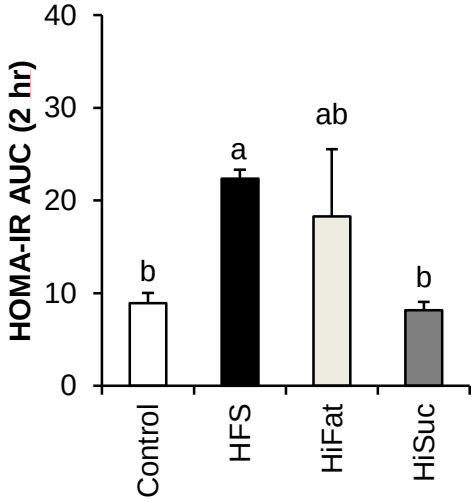
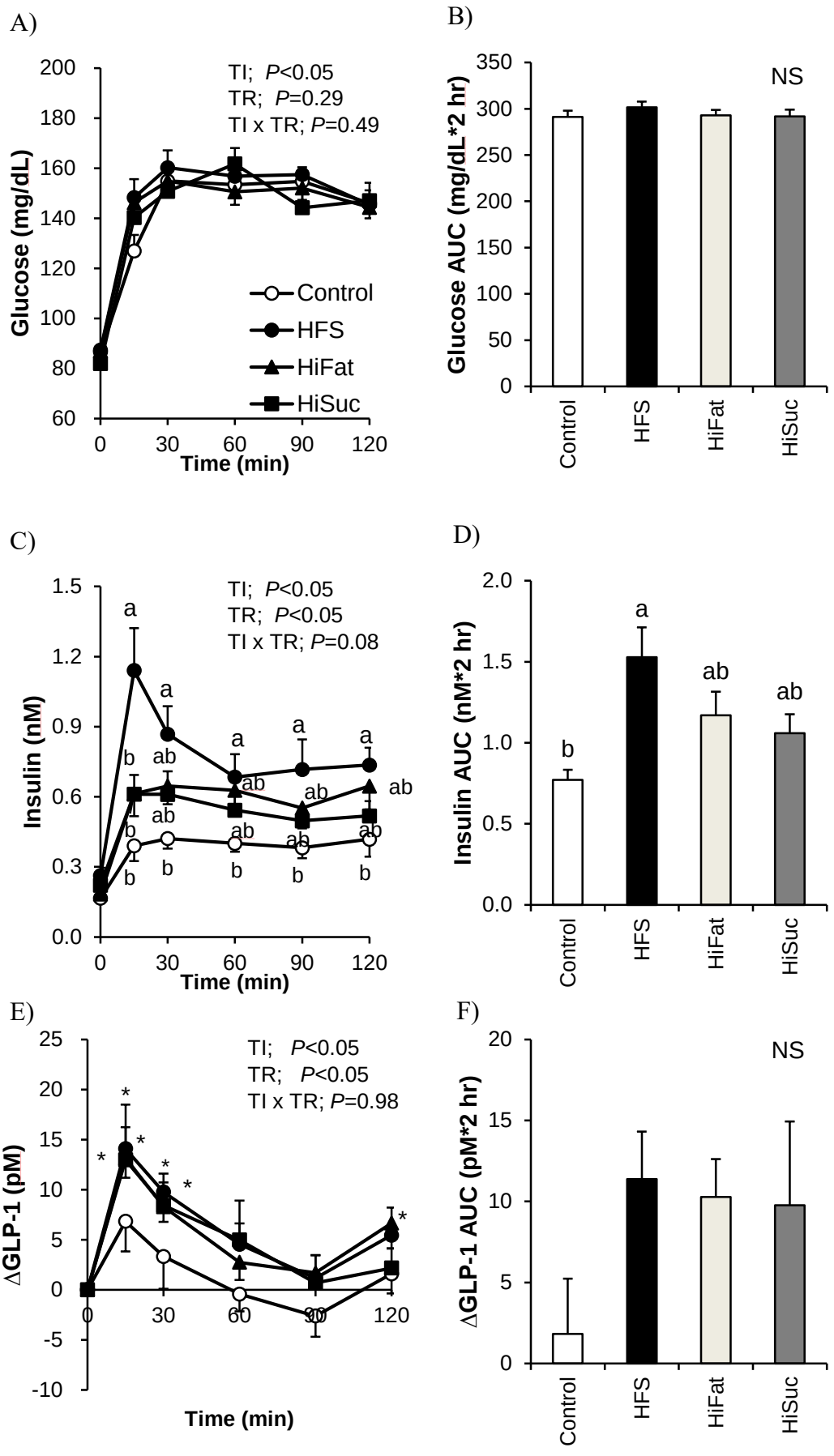

HOMA-IR AUC (2 hr)
Control
HFS
HiFat
HiSuc

Figure 1.2. Postprandial glucose, insulin, GLP-1 level, and HOMA-IR during meal tolerance test (MTT) after feeding the test diet for 2 weeks.

Rats were given the control, HFS, HiFat, and HiSuc diet (*ad libitum*) for 2 weeks Subsequently, the meal tolerance test was performed. After overnight fasting, basal blood (0 min) was taken, followed by feeding of the control diet (10 g/kg BW) for 30 min, and blood samples were collected at 15, 30, 60, 90, and 120 min. The values are expressed as mean ± SEM for n = 4 - 9 rats. Two-way repeated measures analysis of variance *P* values for time, treatment, and the interactions of time and treatment are shown in each panel. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). Asterisks (*) significant differences from the basal value (0 min) in each group ($P < 0.05$, Dunnett's test). NS indicates that there was no significant difference among the treatments.

1.3.3 Postprandial glycemic, insulin, and GLP-1 responses during meal tolerance test (MTT, 4 weeks)

The AUC of postprandial glycemic response was maintained at similar levels in all treatment groups including control group after feeding the test diet for 4 weeks as shown in Fig. 1.3A and B, but postprandial AUC of insulin secretion, HOMA-IR, and GLP-1 secretion of HiSuc diet feeding group were increased into the similar level, compared to HiFat group (Fig. 1.3D, F, and G). From 30 until 120 min after feeding control diet, HFS group had higher insulin level than control group, while HiFat and HiSuc groups showed the intermediate level between HFS and control group (Fig. 1.3C). Postprandial GLP-1 level at 15 min after feeding the control diet tended to be increased in HFS (14.1 ± 4.4 pM), HiFat (13.2 ± 2.0 pM), and HiSuc groups (13.0 ± 3.3 pM), compared to the control group (6.8 ± 3.0 pM) as shown in Fig. 1.3E. Postprandial GLP-1 levels at 15 min were also significantly higher in all of the treatment groups (HFS, HiFat, and HiSuc), compared to the basal level. Significant effects of treatment (TR) on postprandial GLP-1 secretion were also detected by two-way repeated measures ANOVA in both MTTs. Although a significant difference was not detected, AUC values of incremental postprandial GLP-1 (ΔAUC) in HFS, HiFat, and HiSuc groups slightly increased, compared to the control group (1.8 ± 3.4 pM*2 hr).


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)
NS
Glucose AUC (mg/dL*2 hr)
C)
TI; P<0.05
TR; P<0.05
TI x TR; P=0.08
Insulin (nM)
Time (min)
D)
Insulin AUC (nM*2 hr)
E)
TI; P<0.05
TR; P<0.05
TI x TR; P=0.98
ΔGLP-1 (pM)
Time (min)
F)
NS
ΔGLP-1 AUC (pM*2 hr)

G)

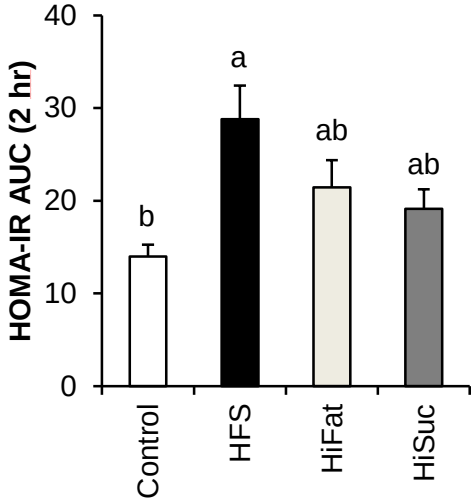


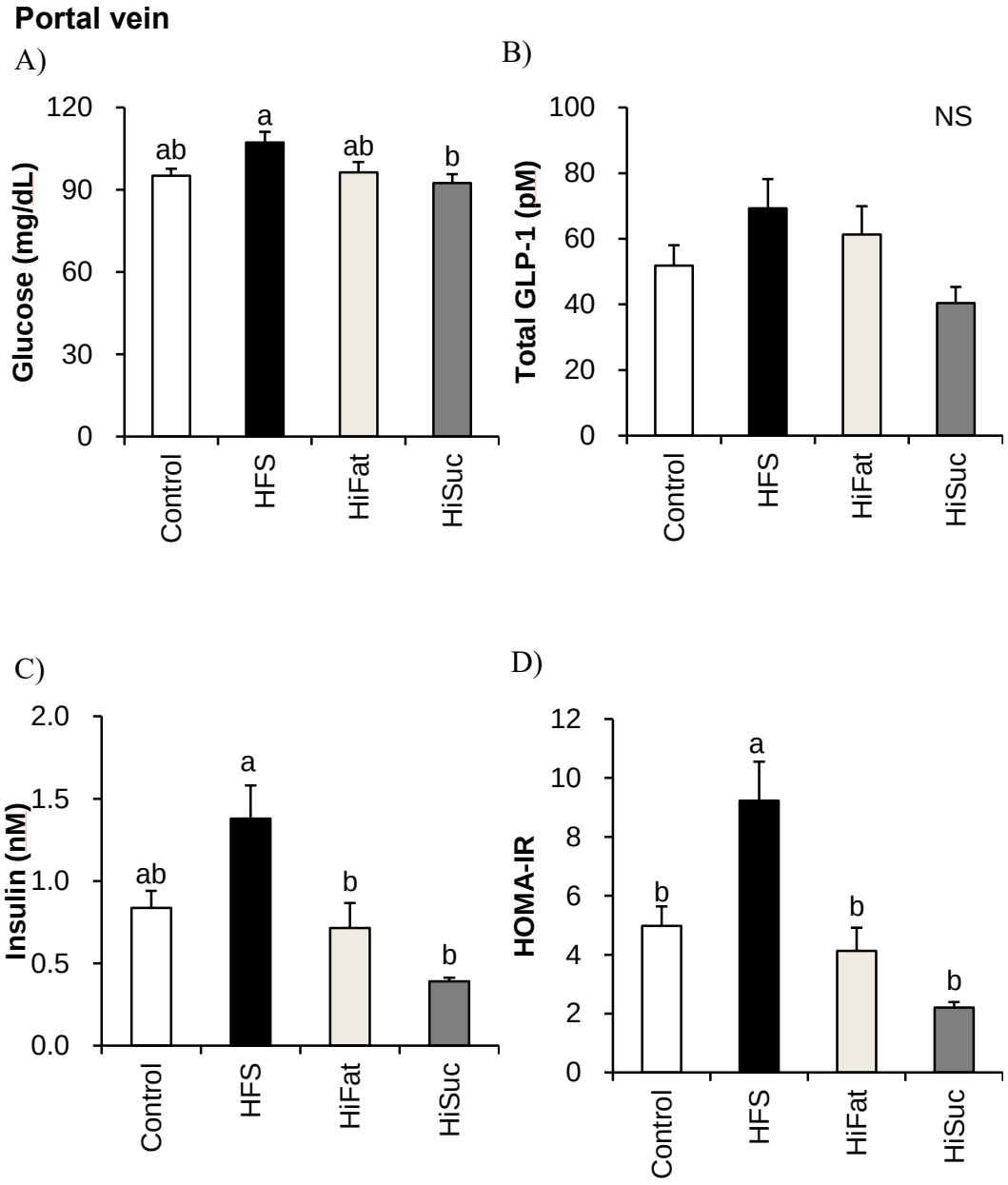
Figure 1.3. Postprandial glucose, insulin, GLP-1 level, and HOMA-IR during meal tolerance test (MTT) after feeding the test diet for 4 weeks.

Rats were given the control, HFS, HiFat, and HiSuc diet (*ad libitum*) for 4 weeks Subsequently, the meal tolerance test was performed. After overnight fasting, basal blood (0 min) was taken, followed by feeding of the control diet (10 g/kg BW) for 30 min, and blood samples were collected at 15, 30, 60, 90, and 120 min. The values are expressed as mean ± SEM for n = 5 - 9 rats. Two-way repeated measures analysis of variance *P* values for time, treatment, and the interactions of time and treatment are shown in each panel. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). Asterisks (*) significant differences from the basal value (0 min) in each group ($P < 0.05$, Dunnett's test). NS indicates that there was no significant difference among the treatments.

1.3.4 Peptide hormones, cholesterol, and TG levels in portal vein and abdominal aorta after feeding the test diet for 5 weeks.

On the final day of experiment, blood samples were collected from the portal vein and abdominal aorta after an overnight fasting in order to determine the effect of diet on basal gut hormone and obesity parameters. The results showed that HFS group had the highest impact on glucose, insulin, HOMA-IR, TG, and cholesterol levels in portal vein as shown in Fig. 1.4. HiFat diet also had similar results but slightly less impacts on these parameters, whereas HiSuc diet feeding group showed the similar level as shown in the control group. In addition, glucose, insulin, cholesterol and TG levels in abdominal aorta also showed the similar tendency with portal vein (Fig. 1.5). The GLP-1 levels in both

portal vein and abdominal aorta of HFS and HiFat groups were slightly higher than those in the control group (Fig. 1.4B and 1.5C).



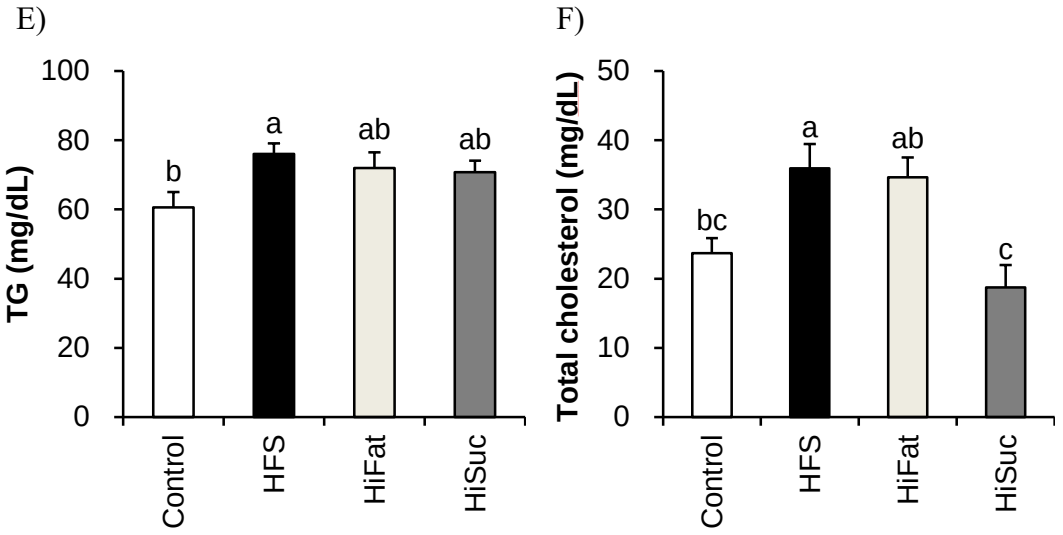
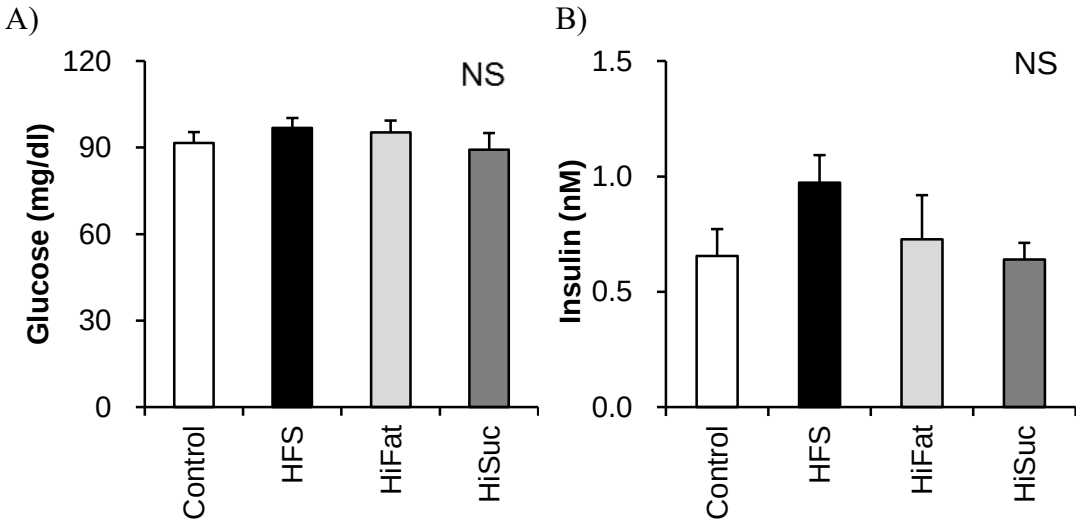


Figure 1.4. Portal glucose, insulin, GLP-1, HOMA-IR, triglyceride (TG), and cholesterol levels after feeding the test diet for 5 weeks.

Rats were given the control, HFS, HiFat, and HiSuc diet (*ad libitum*) for 5 weeks. After overnight fasting (16 h), blood sample was collected from portal vein and aorta under sodium pentobarbital anesthesia (50 mg/kg BW). The values are expressed as mean ± SEM for n = 8 - 10 rats. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant difference among the treatments.

Abdominal aorta



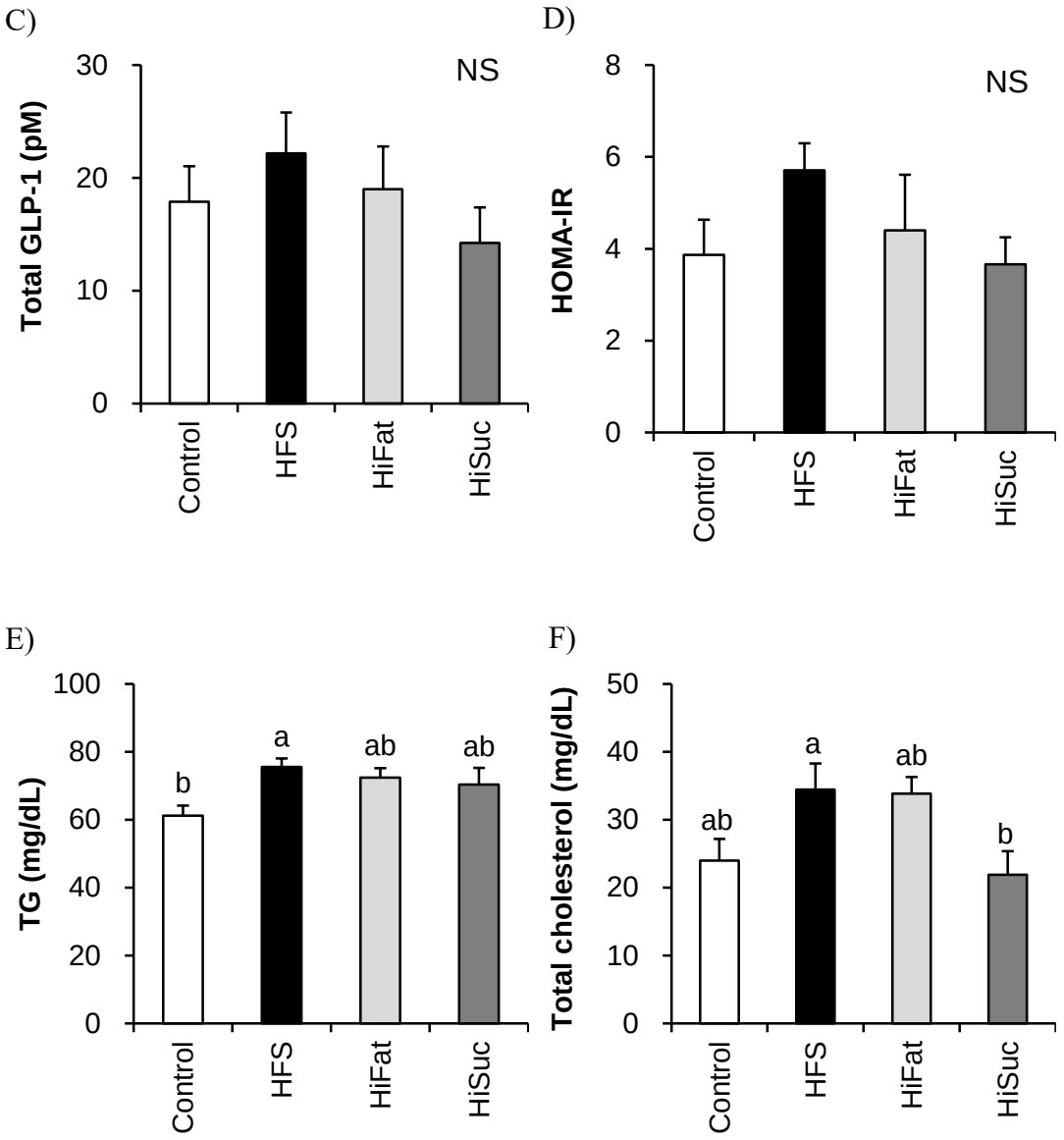


Figure 1.5. Glucose, insulin, GLP-1, HOMA-IR, triglyceride (TG), and cholesterol levels in aorta after feeding the test diet for 5 weeks.

Rats were given the control, HFS, HiFat, and HiSuc diet (*ad libitum*) for 5 weeks. After overnight fasting (16 h), blood sample was collected from portal vein and aorta under sodium pentobarbital anesthesia (50 mg/kg BW). The values are expressed as mean ± SEM for n = 8 - 10 rats. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant difference among the treatments.

1.3.5 GLP-1 content and *Gcg* mRNA expression level in intestinal tissues of rats after feeding the test diet for 5 weeks.

GLP-1 concentration in intestinal tissues was calculated based on total protein

concentration (pmol/mg protein). The results showed that HFS (4.8 ± 0.4 pmol/mg protein) and HiFat (4.4 ± 0.2 pmol/mg protein) groups had higher GLP-1 content in jejunum segment than those in the control group (3.2 ± 0.2 pmol/mg protein), while in other segments (ileum, cecum, and colon) significant differences were not observed (Fig. 1.6). *Gcg* mRNA expression in all tissue did not increase in any intestinal segments in response to any diet treatment compared to control diet feeding (Fig. 1.7A, B, and C). Generally, one microgram of extracted RNA is used to prepare cDNA, and it is subsequently subjected to real-time PCR analysis. However, in this study, amount of RNA extracted from the cecum segment were much less than 1 µg. Therefore, mRNA expressions in the cecum were not able to analyze.

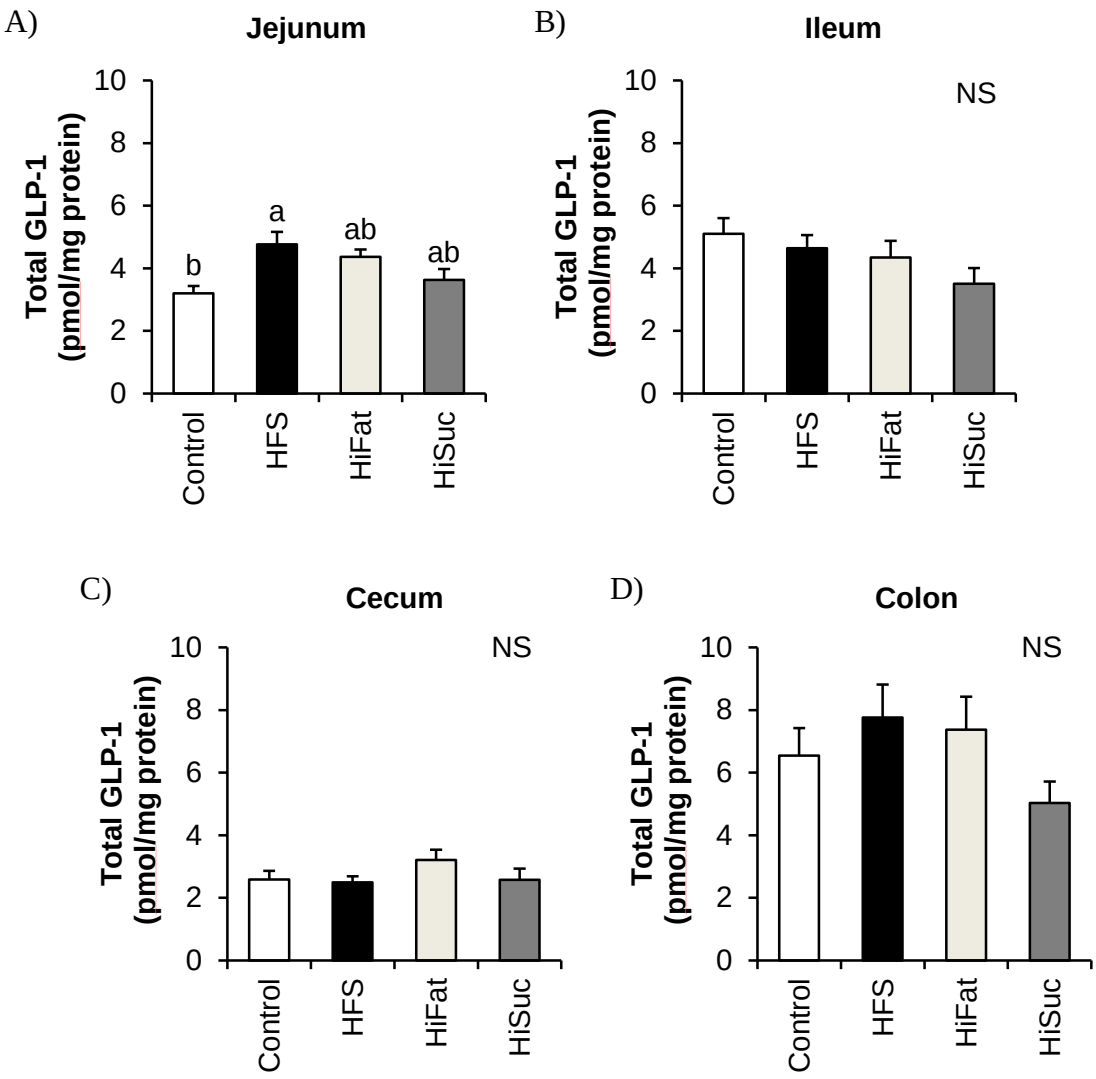


Figure 1.6. GLP-1 content in intestinal tissue after feeding the test diet for 5 weeks.

After feeding the test diet for 5 weeks, each intestinal segment A) jejunum, B) ileum, C) cecum, and D) colon were collected to measure GLP-1 content. GLP-1 content was calculated based on protein content. The values are expressed as mean ± SEM for n = 8 - 10 rats. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant difference among the treatments.

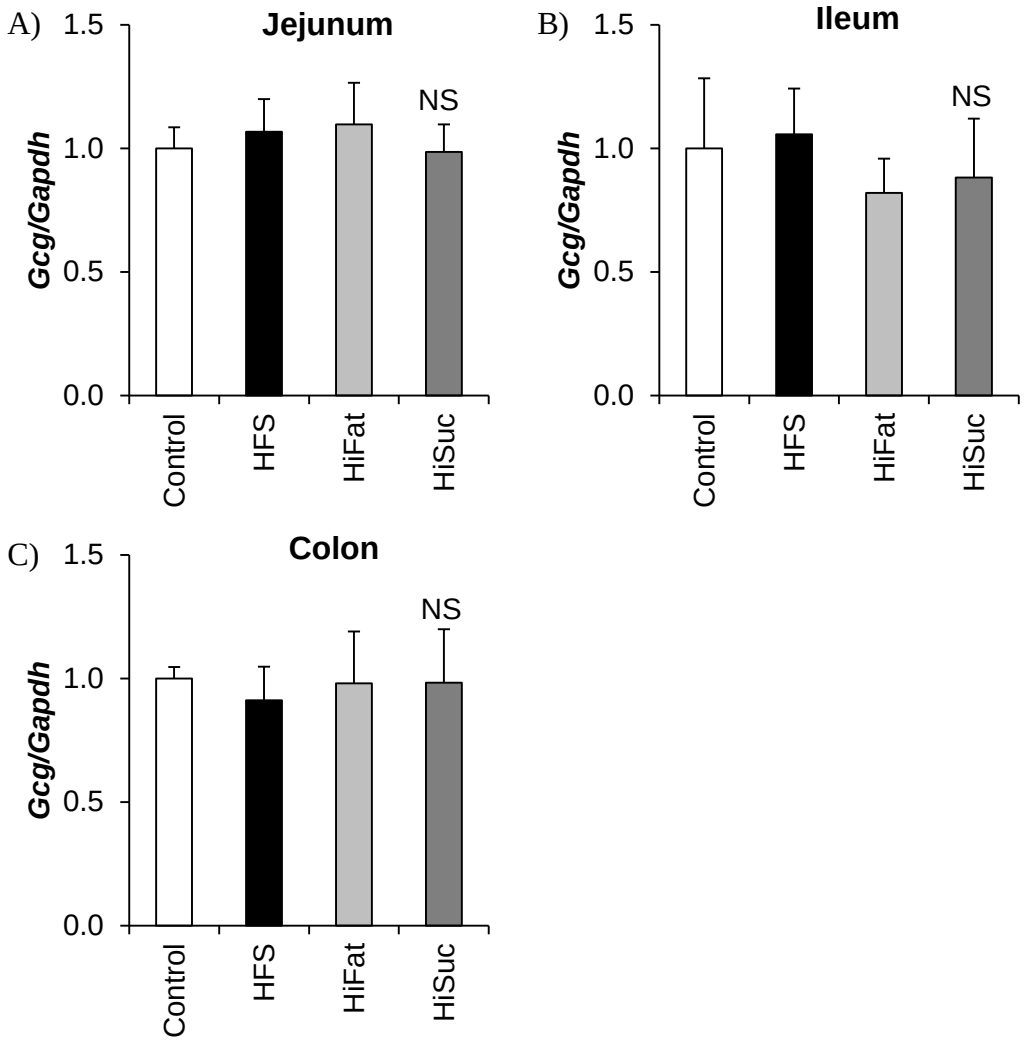


Figure 1.7. Proglucagon (*Gcg*) mRNA expression in intestinal tissue after feeding the test diet for 5 weeks.

After feeding the test diet for 5 weeks, intestinal mucosa of each intestinal segment A) jejunum, B) ileum, and C) colon respectively, were taken and subsequently extracted RNA for real-time PCR analysis. *Gapdh* housekeeping gene was used for data normalization. The values are expressed as mean ± SEM for n = 8 - 10. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant difference among the treatments.

1.4 Discussion

Excess energy consumption, especially from high fat and/or high sucrose diet, is the major risk of metabolic disorders including obesity, insulin resistance, and type 2 diabetes (Long, 2015; Pang, 2016; Avila-Nava, 2017). Therefore, diet containing high fat and/or high sucrose were commonly used to establish obesity development in animal

models (Sumiyoshi, 2006; Sato-Mito, 2009; Yang, 2012; Long, 2015). In this study, a HFS (30% fat and 40% sucrose), a HiFat (30% fat diet), or a HiSuc (40% sucrose) diet were provided to the rats for a total of 5 weeks to determine whether a combined HFS or an individual HiFat or HiSuc diet contributed to adaptive changes in postprandial GLP-1 secretion and obesity development. As expected, HFS and HiFat groups had higher BW gain and total energy intake compared with the control group, while HiSuc group was similar to the control group. The results were also consistent with the previous finding that chronic feeding of HFS and HiFat caused additional BW gain and obesity development (Sato-Mito, 2009; Yang, 2012; Dusaulcy, 2016; Pang, 2016; Qi, 2016; Hira, 2017; Hong, 2017). Consumption of high-caloric diet such as high fat and carbohydrate levels directly relates to fat accumulation in various tissues and obesity development (Sumiyoshi, 2006; Hariri, 2010; Yang, 2012). In addition, epididymal adipose tissue weight of HFS and HiFat group were largely increased, compared to HiSuc group. These results suggest that high fat diet had a potent impact on obesity (adiposity) development rather than high sucrose diet. Nevertheless, a combination of high fat and high sucrose diet had an intense impact on obesity development than that of individual high fat diet consumption alone.

Nowadays, several methodologies such as oral glucose tolerance (OGTT) and meal tolerance test (MTT) are available to assess postprandial glucose and insulin responses (Rijkelijkhuizen, 2009; Staten, 2014). However, the previous study supported that MTT is more reflective to postprandial metabolic responses than OGTT (Brodovicz, 2011). Therefore, in the present study, meal tolerance test in response to standard (control) diet ingestion at the dose of 10 g/kg BW was performed in order to assess postprandial glycemia, insulin, GLP-1, and HOMA-IR during the DIO progression. Unfortunately, some rats did not consume > 90% weight of the provided diet (20.3-89.7% of provided diet), which could be a limitation of the present study. The data from these rats were not included due to

glycemic and gut hormone responses primarily depending on the amount of food ingested. In addition, the interpretation of the data from all rats with largely varied food consumption was difficult, the data from those rats were then omitted. Moreover, the previous studies also supported the fact that GLP-1 secretion depends on the administration of caloric intake (Ma, 2012; Sakamoto, 2012).

Chronic feeding of HFS and HiFat diet for 2 weeks much highly affected to postprandial insulin response and slightly enhanced postprandial GLP-1 secretion, whereas HiSuc feeding group showed no any impact on these parameters. Likewise, the AUC of HOMA-IR was largely increased by chronic HFS diet feeding for 2 weeks. It indicated that chronic HFS diet feeding immediately triggered insulin resistance development. Interestingly, continuous consumption of HiSuc diet until 4 weeks showed that postprandial GLP-1, insulin, and HOMA-IR were similar to those observed in HiFat diet feeding group. Chronic feeding of HiSuc diet gradually increased postprandial GLP-1 and insulin secretion including HOMA-IR index suggesting that HiSuc feeding requires the time to develop metabolic impairment. These findings are supported by a previous study demonstrating that HiFat diet caused severe metabolic dysfunction faster than Hisuc (Long, 2015). In Contrast to HiFat diet, HiSuc diet gradually develop metabolic abnormality (Long, 2015).

HOMA-IR is used to evaluate insulin resistance which represents the characteristic of type 2 diabetes mellitus (Taylor, 2012; Long, 2015). HOMA-IR were slightly higher in in HFS, HiFat, and HiSuc groups, compared to control group, indicating that chronic consumption of obesogenic diet could lead to insulin resistance and type 2 diabetes development.

After 2 weeks of feeding the test diet, HFS and HFS feeding groups showed a significant elevation of the postprandial GLP-1 level at 15 min, compared to basal level

after control diet ingestion, while other groups had only small (insignificant) increment in GLP-1, suggesting that the sensitivity of L-cells in the small intestine to luminal nutrients (protein, carbohydrates, fatty acids) was enhanced by the chronic feeding of HFS and HiFat diet. GLP-1 is secreted in response to nutrient ingestion including proteins, carbohydrates, and triglycerides (Tolhurst, 2009; Sakamoto, 2012; Shannon, 2017).

Up to date, it is still controversial whether GLP-1 was increased or diminished during obesity development. In this study, postprandial GLP-1 level was slightly increased in HFS and HiFat feeding group than the control group. It appears to be consistent with the previous studies that chronic feeding of HFS and HiFat diet enhanced postprandial GLP-1 secretion (Nakajima, 2015; Dusaulcy, 2016; Hira, 2017). In contrast, other studies demonstrated that a chronic feeding of high fat diet diminished postprandial GLP-1 secretion during OGTT and impaired GLP-1 producing L-cells function (Gniuli, 2010; Richards, 2015). The different results in each research team might be caused by the difference in the animal experimental model such as treatment period, diet composition, rodent species, etc.

From overall MTTs experiment, the results revealed that postprandial glycemic responses were not apparently changed by chronic feeding of high fat and/or high sucrose diet throughout the experimental period, whereas postprandial insulin and GLP-1 secretion were progressively increased during the DIO progression. These results suggest that the increment of postprandial GLP-1 and insulin secretion might have a role in normalizing the postprandial glycemia and slowing down the establishment of glucose intolerance and obesity development. Therefore, the role of endogenous GLP-1 secretion during the DIO progression in rats should be further investigated in the next experiment. The better understanding of GLP-1 secretion and metabolic response could allow a better design of therapeutic and preventive approaches for obesity and diabetes.

After feeding the test diet for 5 weeks, the basal level of glucose and insulin in portal vein were significantly increased in HFS feeding group, whereas portal GLP-1 tended to be higher than a control group. HiFat group showed a similar tendency, but the values were slightly less than that of the HFS group, whereas the changes in GLP-1 in HiSuc group was more similar to that in the control group. It should be noted that the higher level of GLP-1 and insulin during fasting state may have a role in prevention the blood glucose elevation and obesity development as well as to slowing down the establishment of glucose intolerance development. In this experiment, basal TG and cholesterol level in portal vein were increased in HFS group. HiFat group also had similar impact on these parameters but significant differences compared to the control group were not observed. It has been previously reported that a long-term feeding of excessive energy is directly associated with hyperlipidemia development (Sumiyoshi, 2006; Pang, 2016). These findings clearly suggested that long-term feeding of an energy-dense diet (HFS and HiFat) could evoke disorder in lipid metabolism and subsequent hyperlipidemia.

Because GLP-1 is produced in enteroendocrine L-cells along with the intestinal epithelium, we also measured GLP-1 content in the intestinal tissue. GLP-1 content in jejunum segment in HFS group and HiFat group were higher than those in the control group, while significant differences were not observed in other segment, indicating that jejunum part is more sensitive to the chronic feeding of HFS and HiFat diet than other intestinal segments. It can be assumed that jejunum part is the major source of postprandial initial GLP-1 secretion. In this study, however, proglucagon mRNA expression levels were inconsistent with GLP-1 content in each intestinal segments. It might be affected by other factors which are involved in GLP-1 production, secretion, and degradation such as DPP-IV (dipeptidyl peptidase) activity and prohormone convertase 1/3 activity. The GLP-1 molecule after secretion from enteroendocrine L-cells is rapidly degraded by DPP-IV

enzyme which ubiquitously exists in plasma and the membrane of endothelial cells (Holst, 2007; Bösenberg, 2008). Another possible factor is a posttranslational mechanism regulated by prohormone convertase 1/3 which is involved in the processing of proglucagon peptide to GLP-1 production (Rouillé, 1997; Hira, 2015).

1.5 Conclusions

HFS diet had the highest impact on postprandial GLP-1 and insulin response. HiFat diet also had similar (but slightly less than HFS) impacts on these parameters. These results suggest that excessive ingestion of an HFS diet rapidly caused adaptive changes in nutrient sensitivity in GLP-1-producing cells rather than an individual HiFat or HiSuc diet alone. However, a HiFat diet likely has a relatively potent effect on GLP-1 response compared with a HiSuc diet, which may play a role in the normalizing postprandial glycemia and retarding the development of glucose intolerance. In addition, the BW gain and epididymal adipose tissue weight of the HFS and HiFat groups increased largely, compared to the HiSuc group, indicating that the HiFat diet rather than the HiSuc diet has a potent impact on obesity (adiposity) development. Nevertheless, an HFS diet had an intense impact on obesity development compared with a HiFat diet alone.

Chapter 2: The role of endogenous GLP-1 in the development of glucose intolerance in rats fed a high fat and high sucrose diet

Abstract

The results in experiment 1 demonstrated that the postprandial GLP-1 response was enhanced during the development of diet-induced obesity (DIO) in rats. However, the physiological relevance of enhanced GLP-1 response has been remained unclear. Therefore, the aim of this study was to determine the role of endogenous GLP-1 during obesity development.

Male Sprague-Dawley (SD) rats were given either a control diet or a high fat and high sucrose (HFS, 30% fat and 40% sucrose) diet with or without continuous administration of the GLP-1 receptor antagonist, exendin (9-39) (Ex9, 100 μg/day), for 5 weeks. Meal tolerance test (MTT) was performed after 2 and 4 weeks of feeding period in order to assess postprandial glucose, insulin, and GLP-1 responses to liquid diet administration (15 kcal/ kg BW) After 5 weeks of feeding period, blood samples were taken under anesthesia then tissue samples were collected after sacrifice.

Postprandial glycemic response in the HFS group was maintained similarly to that in the control group, whereas postprandial GLP-1 and insulin secretion were increased in the HFS group. In Ex9-treated group (blocking GLP-1 signal), postprandial glycemic response was elevated with lower insulin response, compared to the HFS group. These results suggest that the enhancement of the postprandial GLP-1 secretion play an important role in maintaining the normal postprandial glycemia during the DIO development. Therefore, increasing endogenous GLP-1 secretion by certain materials (not by fat or sucrose) could be a potential target for prevention of glucose intolerance and obesity development.

2.1 Introduction

Due to the incretin effect (stimulating insulin secretion), increased GLP-1 concentrations could contribute to normalizing the postprandial glucose homeostasis (Cho, 2014). Nowadays, incretin-based therapy is effectively used for type 2 diabetes treatment. Therefore, increasing GLP-1 secretion may be a promising target for prevention and treatment of glucose intolerance (Nauck, 2016).

Up to date, a consensus on how GLP-1 secretion/production changes during the DIO progression has not been established. The previous study showed that GLP-1 secretion in response to normal diet administration was increased by chronic consumption of high fat and high sucrose diet, at least in the early stage of DIO (Nakajima, 2015). This was also supported by the results in the previous experiment (Chapter 1) that postprandial GLP-1 secretion was enhanced in rats fed with an obesogenic diet during the DIO progression. In contrast, it has been reported that GLP-1 secretion was diminished during obesity development (Gniuli, 2010; Richards, 2015). Therefore, the role of endogenous GLP-1 in the development of glucose intolerance and obesity has not been clearly identified. The present study aimed to clarify the role of endogenous GLP-1 in postprandial glycemia during the DIO progression by chronic administration of a GLP-1 receptor antagonist. GLP-1 receptor antagonist, exendin (9-39) (Ex9) is a peptide having 31 amino acid (Serre, 1998; Edwards, 1999). Ex9 is a specific and competitive antagonist of the GLP-1 receptor, which has similar structure to GLP-1 as shown in (Fig. 2.1) and is widely used to block the GLP-1 signaling at its receptor in various studies (Green, 2005; Schirra, 2006; Coopman, 2011; Sathananthan, 2013; Dusaulcy, 2016). In this study, the GLP-1 receptor antagonist (Ex9) or vehicle (0.09% NaCl) was chronically delivered to the rats throughout the experimental period (35 days) by using an osmotic pump implanted subcutaneously. MTT using a liquid diet was employed to assess postprandial response (glucose, insulin, GLP-1,

and acetaminophen levels) instead of re-feeding of a powder diet (chapter 1) or oral glucose tolerance test (OGTT) during the DIO progression.

Peptide	Sequence	AA
Ex(9-39)	DLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH2	31
GLP-1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKG-NH2	30

Figure 2.1. Peptide sequence alignments of Ex9 and GLP-1 (Chepurny, 2018)

2.2 Material and methods

2.2.1 Animal and diet

Male Sprague–Dawley rats (5-week-old) were acclimatized for 1 week before conducting the experiment as previously described in Chapter 1 (Reeves, 1997). The rats were housed individually and maintained in a temperature-controlled room at 22 ± 2°C with a standard light cycle (8.00 a.m. - 8.00 p.m. light period). All rats were given free access to diet and water (*ad libitum*).

Ex9 (analytical grade, purity > 95%) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). According to a previous study (Dusaulcy, 2016), Ex9 at a daily dose of 100 µg/rat was administered to block GLP-1 signal. Ex9 (220 µL) or vehicle (0.9% NaCl) was transferred to Alzet Mini-osmotic pump (Alzet Mini-osmotic pump, Model 2006, Durect corporation, CA, USA) following the manufacturer's instructions (Appendix E). The rats were anesthetized under sodium pentobarbital (50 mg/kg BW) and were then subcutaneously implanted with the osmotic pump in the interscapular region to deliver Ex9 or vehicle at a constant rate of 0.13 µL/h throughout the experimental study (Appendix E).

All rats were divided into three groups to have comparable BW, glucose, and GLP-1 levels based on the measurement of these parameters on the day before starting the test

diet feeding and Ex9 administration, as a control diet and a high fat/high sucrose diet with or without Ex9 administration. The control group was fed with a control diet with continuous saline administration, the high fat and high sucrose (HFS) group was fed a HFS diet (30% fat and 40% sucrose) (Hira, 2017; Nakajima, 2015; Pinyo, 2019a) with continuous saline administration, and the Ex9 group was also fed the HFS diet with continuous Ex9 administration for 5 weeks. All experimental test diets were AIN-93G based diet as shown in Table. 2.1. BW and food intake (weighing uneaten food) were measured every 2 days. All experimental animal procedures were approved by the Hokkaido University Animal Committee (approval no. 19-0064), and animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Table 2.1 Experimental diet composition

Ingredient	Control	HFS
	g/kg of diet	
Cornstarch	397.486	–
Casein[1)]	200	200
Dextrinized cornstarch[2)]	132	–
Sucrose	100	399.486
Soybean oil	70	70
Lard oil	–	230
Fiber (cellulose)[3)]	50	50
Mineral mixture[4)]	35	35
Vitamin mixture[4)]	10	10
L-Cystine	3	3
Choline bitartrate	2.5	2.5
tert-Butylhydroquinone	0.014	0.014
Energy density (kcal/g)	4.03	5.18

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

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

3) Just Fiber BH200FCC (Morimura Bros., Inc, Tokyo, Japan);

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

Experimental timeline

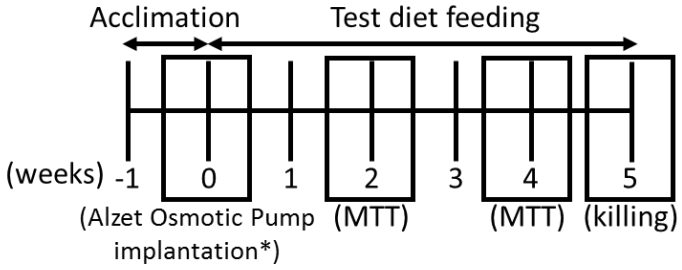


Figure 2.2. Schematic representation of experiment timeline

*After 1 week of the acclimation period, the rats were then subcutaneously implanted with an Alzet Mini-osmotic pump to deliver Ex9 (100 μg/day) or vehicle (saline).

2.2.2 Meal tolerance test (MTT)

Meal tolerance test was performed after 2 and 4 weeks of feeding period in order to determine postprandial responses. In this study, the rats were orally administrated a liquid diet (15 kcal/kg BW, Ensure H, Abbott, Tokyo, Japan) instead of re-feeding of a powder diet. Acetaminophen (100 mg/kg BW) was premixed (10 mg/mL) with a liquid diet before the oral administration. After administration of liquid diet, blood collection (0, 15, 30, 60, 90, and 120 min) was then performed. Blood samples were centrifuged at 2300 x *g* for 10 min at 4°C and plasma samples were collected and stored at −80°C until further analysis. All plasma parameters (glucose and GLP-1) were measured as described in chapter 1, whereas plasma insulin was measured using an insulin ELISA (U-E type was used instead of T-type, AKRIN-130; Shibayagi Company Limited). The Acetaminophen levels were determined using acetaminophen detection kit (Kanto Chemical Co., Inc., Tokyo, Japan). Since acetaminophen is rapidly absorbed in the small intestine (Hatanaka, 1994; Maida, 2008). The gastric emptying rate was determined by using acetaminophen (paracetamol) absorption test (Medhus, 2001).

2.2.3 Blood and tissue collection

After 5 weeks of feeding period, blood and tissue samples were collected after overnight fasting and measured biochemical parameters as previously summarized in chapter 1. For measurement of DPP-IV activity, blood samples were simultaneously collected from the portal vein and abdominal aorta into a syringe containing only heparin (final concentration 50 IU/ml). DPP-IV activity was measured based on the rate of surrogate substrate (Gly-Pro-*p*-nitroaniline; Gly-Pro-*p*NA) hydrolysis as described previously (Karl, 2003; Flock 2007; Ishikawa, 2015). One unit of DPP-IV activity was defined as the liberation of 1 μmole of *p*NA in 1 min. DPP-IV activity was calculated by

using the following formula;

$$\text{DPP-IV activity} = \frac{\text{Amount of pNA release (μmole)}}{\text{Reaction time(min) x Volume (mL)}} \quad \text{Unit/mL}$$

2.2.4 Measurement of GLP-1 content in intestinal tissue

An ethanol acid solution was used to extract GLP-1 from intestinal segment as previously described in chapter 1 (Cani, 2007; Tourrel, 2002; Granata, 2009). In brief, tissue sample were submerged in the ethanol acid solution (5 mL/g tissue) and homogenized at 25,000 rpm (Ultra-Turrax homogeniser T18, IKA) for 2 min. Homogenized samples were placed at 4°C for 24 h. After 24 h, aliquots (150 μL) of homogenate were stored separately at −30°C until protein analysis. The remaining homogenate was centrifuged at 2000 g for 20 min. The supernatant was collected and stored at −80°C until analyzed. Protein and GLP-1 content were measured using Lowry's protein assay and Multi Species GLP-1 Total ELISA kit (EZGLP1T-36K; Merck Millipore), respectively. Homogenate samples were diluted 30-fold for protein measurement, whereas the supernatant was diluted 500- or 1000-fold (colon only) for GLP-1 measurement.

2.2.5 Statistical Analysis

Sample size was calculated based on the experimental design (two-way repeated-measures ANOVA) for MTT to determine the role of endogenous GLP-1 in glucose homeostasis as the primary outcome measure using G*Power software (version 3.1.9.2) following the power = 0.8, significant level = 0.05 and effect size = 0.25. The total number of rats was calculated as 24 (8 rats/group) in the present study. Results are presented as means with their standard errors. Significant effects of time, treatment and the interactions of time and treatment were assessed by two-way repeated measures ANOVA in the MTT

results. One-way ANOVA, Tukey–Kramer's test or Student's *t*-test were used to determine significant differences among treatment groups with $P < 0.05$ considered statistically significant. Data were statistically analyzed using JMP pro version 13.0 software (SAS Institute, Inc.).

2.3 Results

2.3.1 Body weight, energy intake and tissue weights

At the beginning of the experiment, there were no differences in the initial body weight of rats in all groups (182.1 ± 2.5 to 184.0 ± 2.7 g). At the end of the feeding period (5 weeks), the BWs of HFS (407.5 ± 7.7 g) and Ex (400.5 ± 11.0 g) groups were significantly higher than those in control group (358.1 ± 11.6 g) as shown in Table 2.2. BW gain, energy intake, visceral adipose tissue weight (mesenteric, epididymal, and retroperitoneal adipose tissue), and liver weight of HFS and Ex groups were also higher than control group as summarized in Table 2.2. However, no significant differences were noted between HFS and Ex groups in any parameters.

Table 2.2 Initial BW, final BW, BW gain, visceral adipose tissue (mesenteric, epididymal, and retroperitoneal) weight, liver weight, and energy intake after 5 weeks of feeding period.

	Control	HFS	Ex
Initial BW (g)	183.2 ± 3.7 ^{NS}	184.0 ± 2.7	182.1 ± 2.5
Final BW (g)	358.1 ± 11.6^{b}	407.5 ± 7.7^{a}	400.5 ± 11.0^{a}
BW gain (g)	174.9 ± 9.0^{b}	223.5 ± 6.3^{a}	218.4 ± 9.0^{a}
Visceral fat (g)	20.7 ± 2.3^{b}	34.3 ± 2.2^{a}	31.3 ± 2.1^{a}
Mesenteric fat (g)	5.6 ± 0.9^{b}	8.9 ± 0.6^{a}	8.2 ± 0.7^{a}
Epididymal fat (g)	6.8 ± 0.6^{b}	11.5 ± 0.8^{a}	10.6 ± 0.8^{a}
Retroperitoneal fat (g)	8.3 ± 0.8^{b}	13.8 ± 0.9^{a}	12.6 ± 0.7^{a}
Liver weight	9.4 ± 0.4^{b}	12.2 ± 0.4^{a}	12.0 ± 0.5^{a}
Total energy intake (kcal)	2619.4 ± 93.3^{b}	3140.3 ± 98.0^{a}	3053.9 ± 74.3^{a}

Visceral fat weight is the sum of the mesenteric, epididymal, and retroperitoneal fat weight. The values were expressed as the mean ± SEM for n = 8-9 rats. The superscripts without the same letters differed significantly between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant difference among the treatments.

2.3.2 Basal glucose, insulin and GLP-1 levels

After 2 and 4 weeks of the feeding period, the rats were fasted overnight (16 h) before conducting meal tolerance test. Before oral administration of liquid diet ensure H, blood samples from tail vein at fasting state were collected to determine glucose, insulin, and GLP-1 levels as well as HOMA-IR. After 2 and 4 weeks of feeding period, no significant differences were observed in any of the measured parameters (Table 2.3). However, basal insulin level (0.16 ± 0.04 nM, $P = 0.07$) and HOMA-IR (5.56 ± 1.30, $P = 0.07$) tended to be higher in the HFS group compared with the control group after 4 weeks of feeding period (Table 2.3).

Table 2.3 Fasting glucose, insulin, GLP-1 levels and HOMA-IR

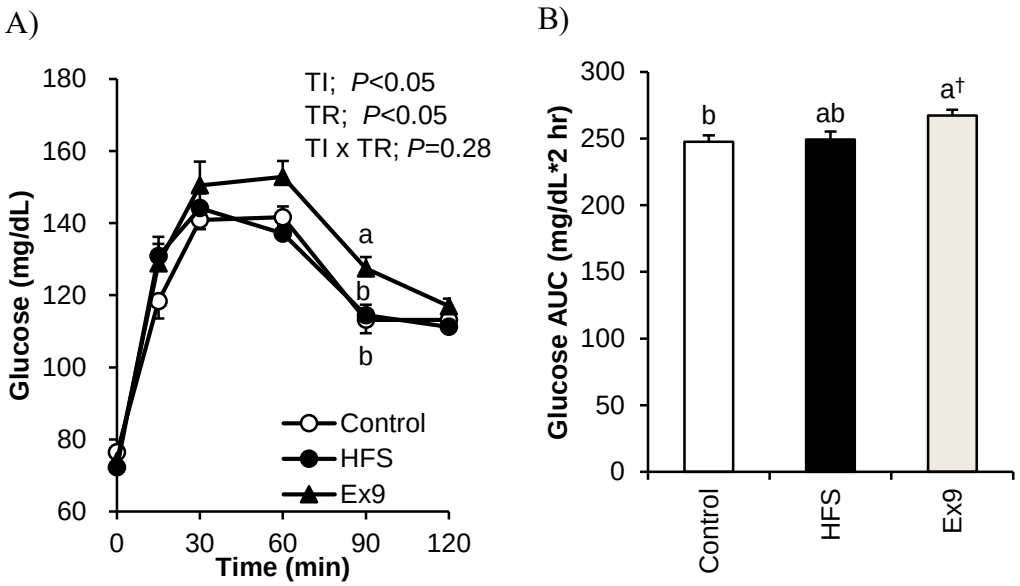
	Control	HFS	Ex9
2 weeks			
Glucose (mg/dL)	76.4 ± 2.6	72.3 ± 2.7	74.0 ± 2.2^{NS}
Insulin (nM)	0.036 ± 0.003	0.043 ± 0.014	0.021 ± 0.002^{NS}
GLP-1 (pM)	12.0 ± 2.7	14.3 ± 2.4	15.7 ± 1.8^{NS}
HOMA-IR	1.02 ± 0.09	1.17 ± 0.39	0.59 ± 0.07^{NS}
4 weeks			
Glucose (mg/dL)	89.3 ± 2.5	92.5 ± 2.3	93.8 ± 2.8^{NS}
Insulin (nM)	0.07 ± 0.02	0.16 ± 0.04	0.08 ± 0.01^{NS}
GLP-1 (pM)	8.9 ± 0.9	14.8 ± 2.6	12.8 ± 1.9^{NS}
HOMA-IR	2.70 ± 0.67	5.56 ± 1.30	2.75 ± 0.47^{NS}

The values are expressed as the mean ± SEM for n = 8-10 rats. The superscripts (a, b, c) without the same letters differed significantly between treatments ($P < 0.05$, Tukey–Kramer's test). NS indicates that there was no significant difference among the treatments.

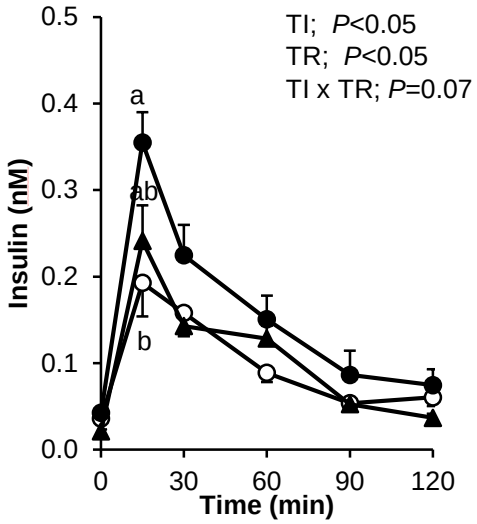
2.3.3 Postprandial glycemic, insulin, and GLP-1 responses and gastric emptying during meal tolerance tests after 2 week of feeding period

In this experiment, meal tolerance test (MTT) was designed to access the changes in postprandial glucose, insulin, GLP-1, and gastric emptying in response to oral administration of a liquid diet (Ensure H) during the DIO progression (after 2 and 4 weeks of feeding period). Liquid diet was premixed with acetaminophen (0.1 g/ kg BW) in order to determine gastric emptying rate and then orally administered to the rats (15 kcal/kg BW). After 2 weeks of feeding period, postprandial glucose level of Ex9 group gradually increased, compared to the HFS and the control groups at 30 until 120 min. Especially, postprandial glycemic response at 90 min after diet administration of Ex group (127.4 ± 3.2 mg/dL) was significantly higher than those in HFS (114.5 ± 2.9 mg/dL) and control (113.2 ± 3.7 mg/dL) groups, respectively (Fig 2.3A). The AUC of postprandial glucose response was also significantly increased in the Ex9 group, compared to HFS (assessed by

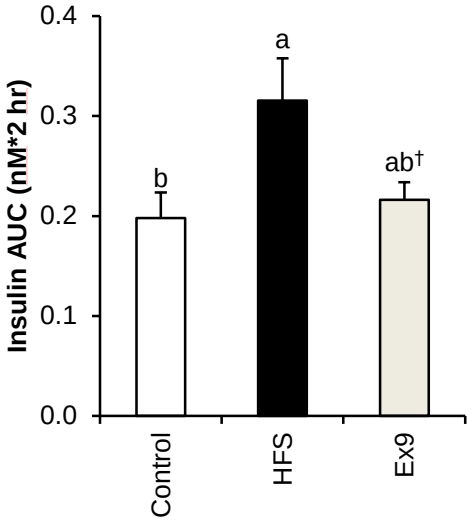
Student's *t*-test) and control groups (Fig. 2.3B). Postprandial insulin level at 15 min of HFS groups (0.35 ± 0.04 nM) was significantly higher than the control group (0.19 ± 0.04 nM), whereas Ex9 (0.24 ± 0.04 nM) group showed the intermediate level between HFS and control groups (Fig. 2.3C). Furthermore, the AUC of insulin in HFS group was increased compared to those in Ex9 and control groups (Fig. 2.3D). The results showed that basal GLP-1 level in HFS and Ex9 groups were similar to control group (Fig. 2.3E), but postprandial GLP-1 secretion after liquid diet administration (AUC of GLP-1) in HFS and Ex9 groups slightly higher than those in the control group after 2 weeks of feeding period (Fig 2.3F). In this study, changes in plasma acetaminophen concentrations were not different between treatments (Fig 2.3G).



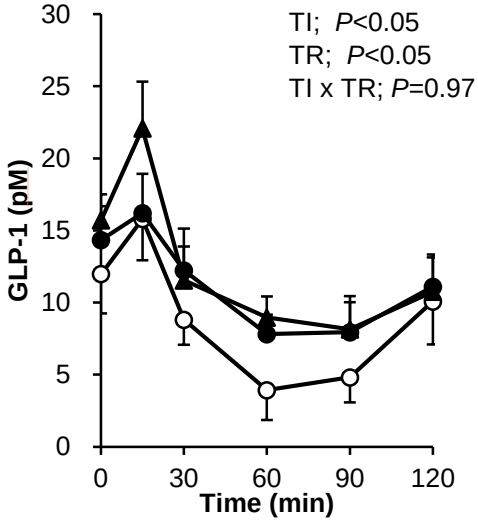
C)


TI; P<0.05
TR; P<0.05
TI x TR; P=0.07
Insulin (nM)
Time (min)
a
ab
b

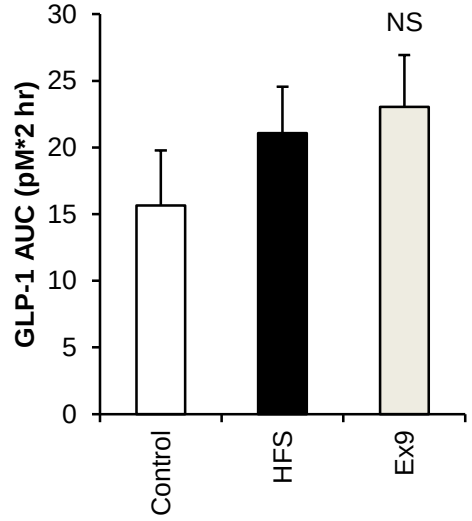
D)


Insulin AUC (nM*2 hr)
a
b
ab†
Control
HFS
Ex9

E)


TI; P<0.05
TR; P<0.05
TI x TR; P=0.97
GLP-1 (pM)
Time (min)

F)


GLP-1 AUC (pM*2 hr)
NS
Control
HFS
Ex9

G)

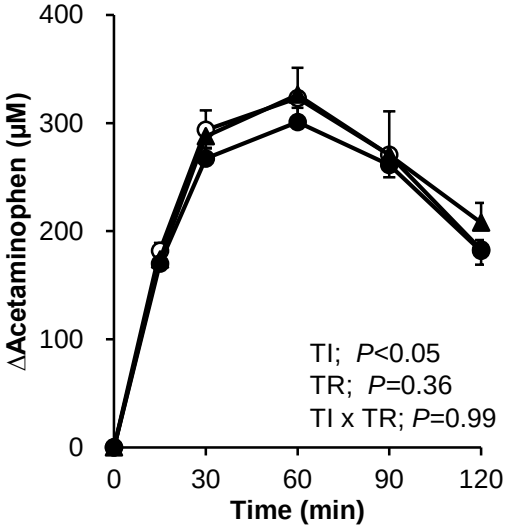

ΔAcetaminophen (μM)
TI; P<0.05
TR; P=0.36
TI x TR; P=0.99
Time (min)

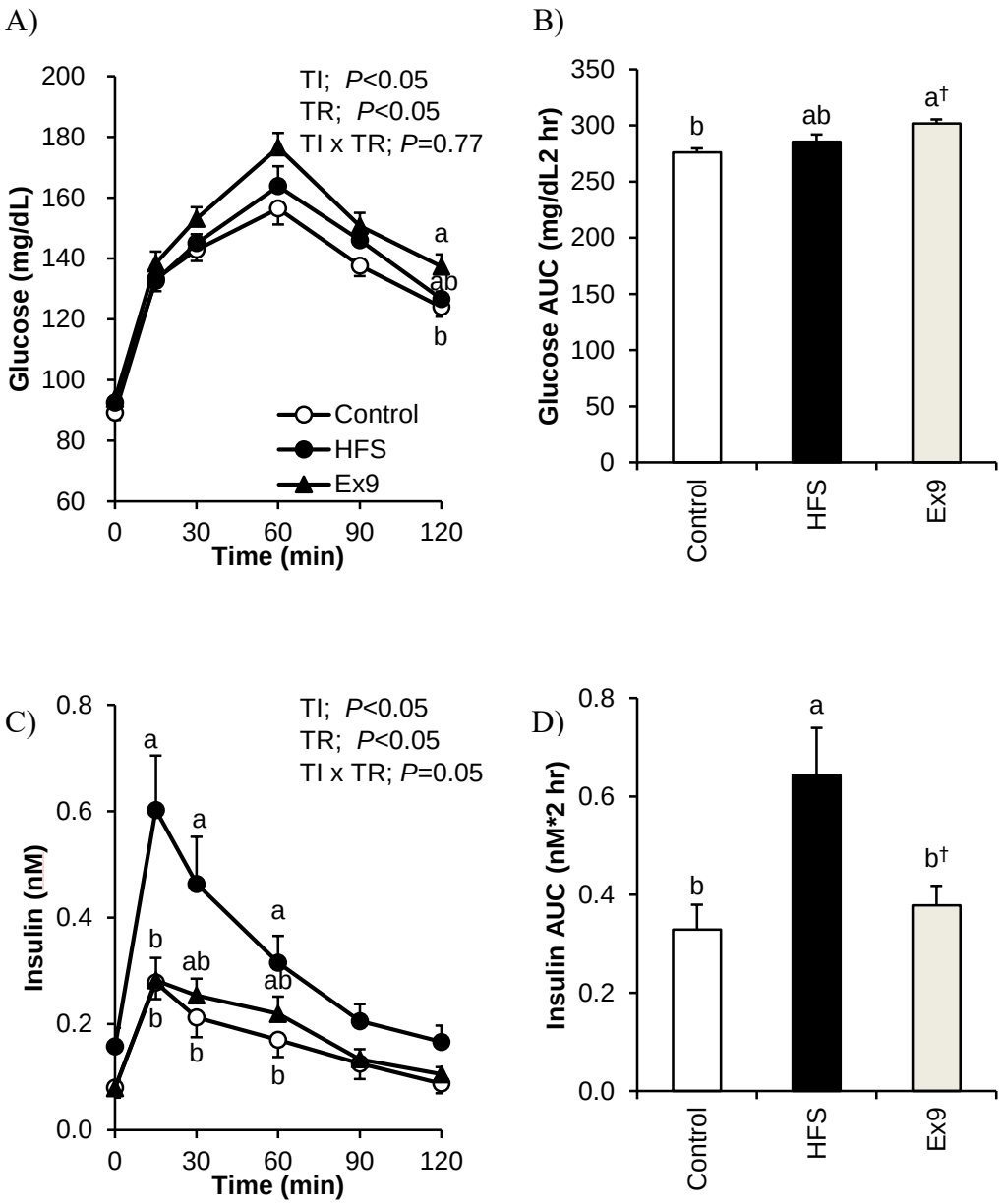
Figure 2.3. Postprandial glucose, insulin, GLP-1 level, and HOMA-IR during meal tolerance test (MTT) after feeding the test diet for 2 weeks.

Rats were provided a control or HFS diet with either saline or Ex9 administration (100 μg/day) for 2 weeks before conducting MTT. After overnight fasting, basal blood (0 min) was taken, followed by administration of a liquid diet (15 kcal/kg BW), and blood samples were collected at 15, 30, 60, 90, and 120 min. The values are expressed as mean ± SEM for n = 8-9 rats. Two-way repeated measures analysis of variance *P* values for time, treatment, and the interactions of time and treatment are shown in each panel. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). †Significant differences between mean values of the HFS and Ex9 groups ($P < 0.05$, Student's *t*-test). NS, no significant difference among the treatments.

2.3.4 Postprandial glycemic, insulin, and GLP-1 responses and gastric emptying during meal tolerance tests after 4 week of feeding period

Postprandial glucose levels in the Ex9 group were slightly higher than the control group (15–90 min) and significantly (120 min) higher than the control group following administration of the liquid diet (Fig. 2.4A). The AUC of postprandial glycemia clearly showed that postprandial glucose in Ex9 (301.7 ± 3.7 mg/dL*2 hr) group was significantly higher than that in the HFS (285.7 ± 6.3 mg/dL*2 hr, assessed by Student's *t*-test) and control (276.1 ± 3.6 mg/dL*2 hr) groups as shown in Fig. 2.4B. Throughout the MTT experiment, HFS group had higher insulin secretion than the other groups (Fig. 2.4C). At 15 min after diet administration, postprandial insulin secretion in HFS (0.60 ± 0.10 nM) group was significantly higher than those in control (0.28 ± 0.03 nM) and Ex (0.28 ± 0.04 nM) groups as shown in Fig. 2.4C. The AUC of postprandial insulin secretion also confirmed that HFS group (0.64 ± 0.10 nM*2 hr) had highest postprandial insulin secretion, while Ex9 group (0.38 ± 0.04 nM*2 hr) had a similar response to that in the control group (0.33 ± 0.05 nM*2 hr), as summarized in Fig. 2.4D. Interestingly, GLP-1 levels at 60 and 90 min in the HFS group and at 90 min in Ex9 group became significantly higher than the control group, demonstrating that the postprandial GLP-1 response was larger in the HFS and Ex9 groups than the control group (Fig. 2.4E). Significant effects of treatment on

postprandial glycemia, insulin and GLP-1 secretion were detected by two-way repeated measures ANOVA in both MTT. Plasma acetaminophen responses in all time points of HFS and Ex groups were similar to that in the control group (Fig. 2.4G).



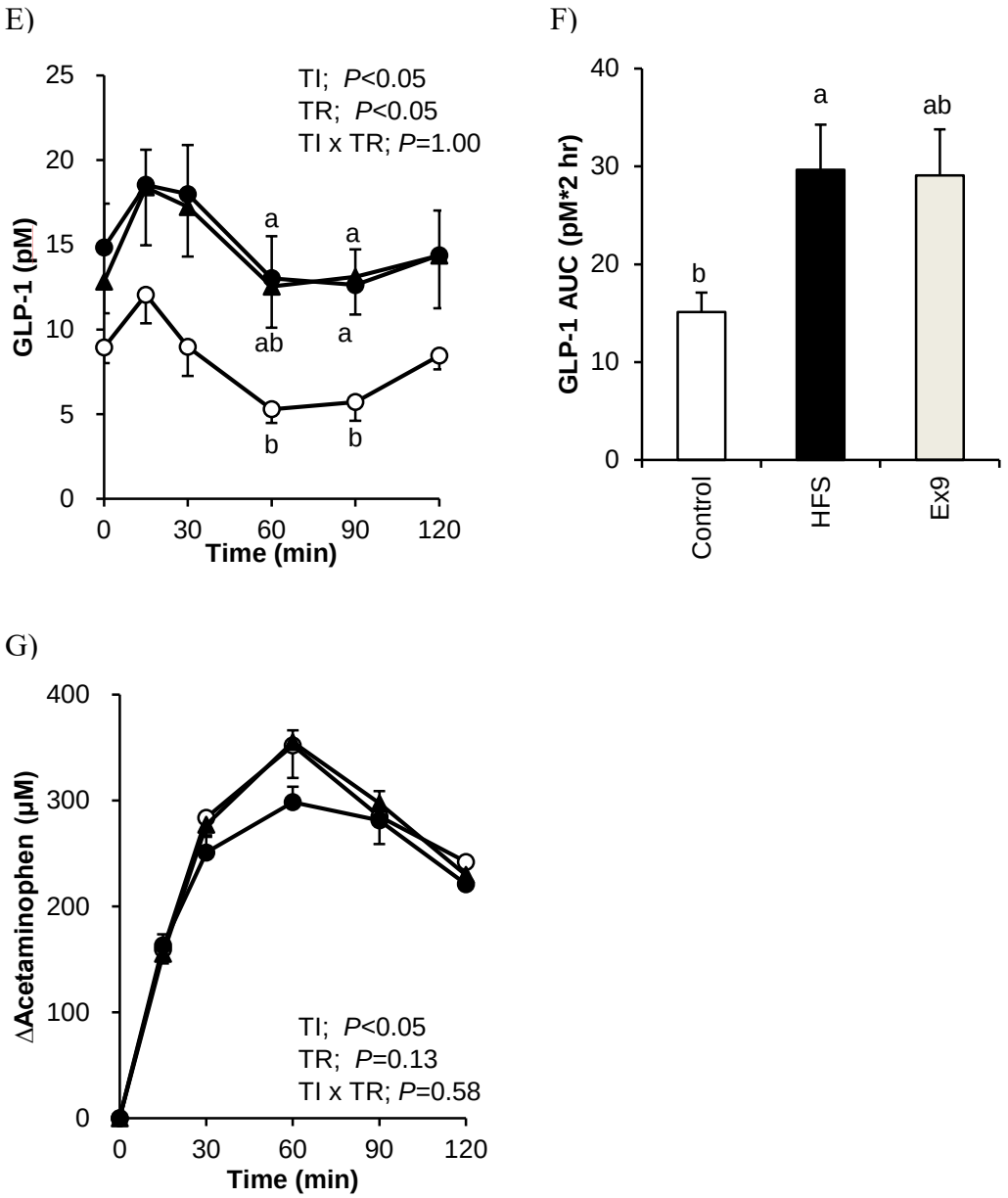


Figure 2.4. Postprandial glucose, insulin, GLP-1 level, and HOMA-IR during meal tolerance test (MTT) after feeding the test diet for 4 weeks.

Rats were provided a control or HFS diet with either saline or Ex9 administration (100 μg/day) for 2 weeks before conducting MTT. After overnight fasting, basal blood (0 min) was taken, followed by administration of a liquid diet (15 kcal/kg BW), and blood samples were collected at 15, 30, 60, 90, and 120 min. The values are expressed as mean ± SEM for n = 8-9 rats. Two-way repeated measures analysis of variance *P* values for time, treatment, and the interactions of time and treatment are shown in each panel. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). †Significant differences between mean values of the HFS and Ex9 groups ($P < 0.05$, Student's *t*-test). NS, no significant difference among the treatments.

2.3.5 Plasma parameters after 5 week of feeding period

In order to evaluate the effect of chronic HFS feeding with or without continuous Ex9 administration on gut hormone and obesity parameters, blood sample from portal vein and abdominal aorta at fasting state were collected. Consistent with postprandial GLP-1 secretion after feeding the test diet for 4 weeks, both the HFS and Ex9 groups showed significantly higher GLP-1 concentrations compared with the control group (Fig. 2.5C). DPP-IV activity in both the portal vein and abdominal aorta was not different among the groups (Fig. 2.5D and 2.5E). Glucose levels were significantly higher in the Ex9 group compared with the HFS and control groups, both in the portal vein and abdominal aorta Fig. 2.5A and 2.6A. Similar to the postprandial insulin response, portal/aorta insulin levels in the HFS group were higher than in the control group, while the Ex9 group showed an intermediate value between the HFS and control groups (Fig. 2.5B and 2.6B). TG and cholesterol levels in the HFS and Ex9 groups were higher than in the control group (Fig. 2.6C and D).

Portal vein

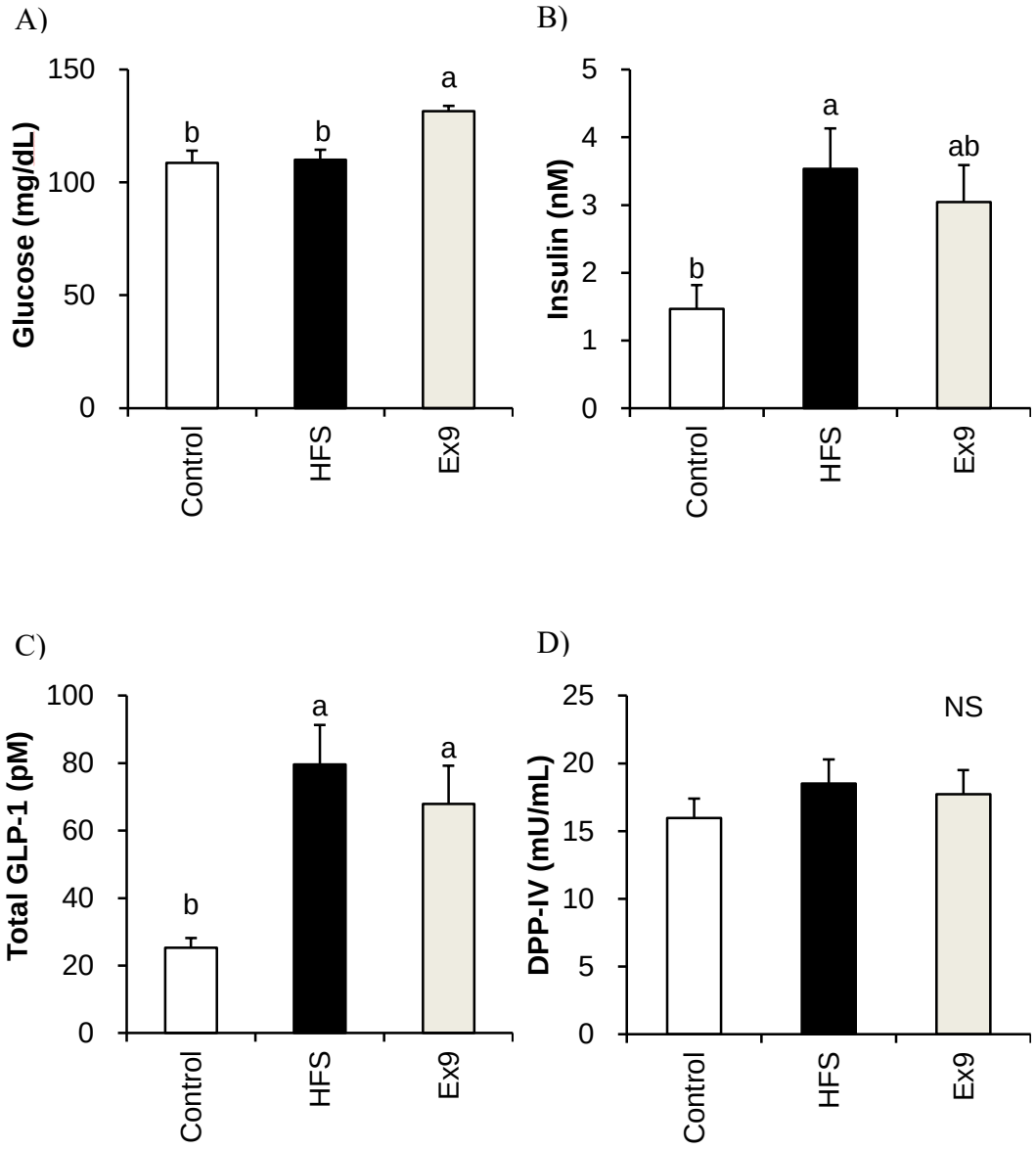
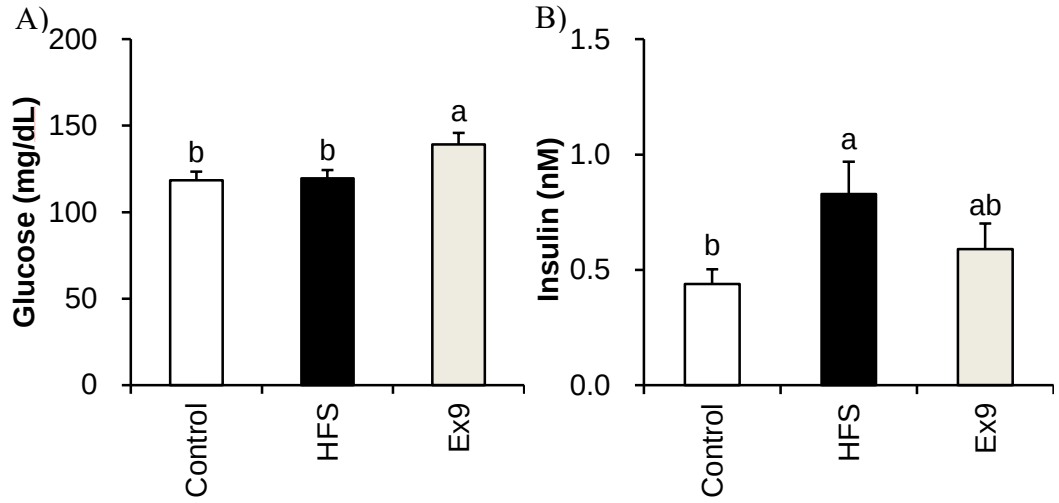
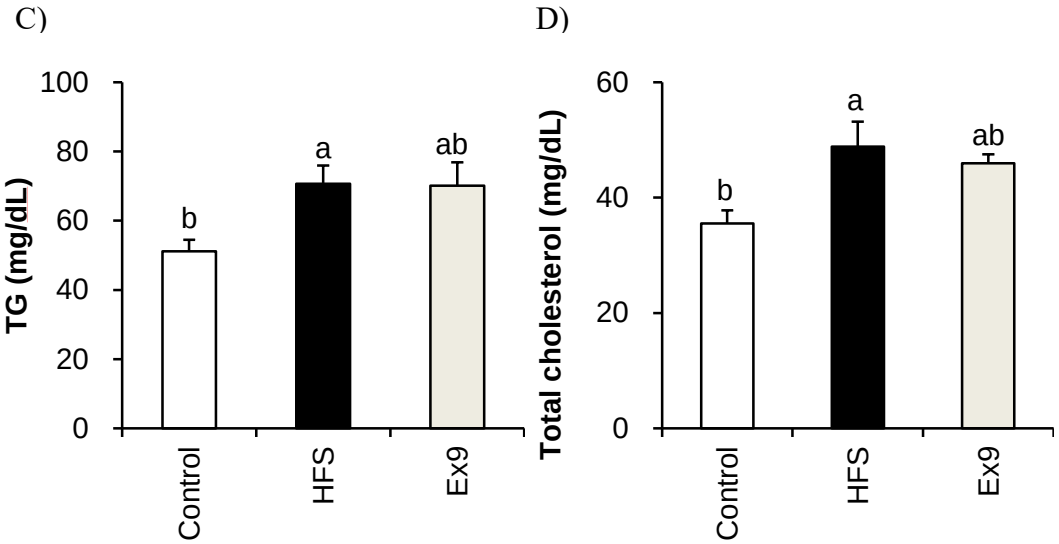


Figure 2.5. Portal glucose, insulin, GLP-1 levels and dipeptidyl peptidase (DPP)-IV activity after a 5-week feeding period of a control or high-fat/high-sucrose (HFS) diet with or without continuous exendin (9–39) (Ex9) administration.

Rats were provided a control or high-fat/high-sucrose(HFS) diet with either saline or Ex9 administration (100 µg/day) for 5 weeks. Blood samples from the portal vein and abdominal aorta were collected under sodium pentobarbital anaesthesia (50mg/kg BW) after overnight fasting (16 h). The values are expressed as mean ± SEM for n = 8-9 rats. The superscripts (a, b, c) with different letters show significant differences between treatments (P < 0.05, Tukey–Kramer's test). NS, no significant difference among the treatments.

Abdominal aorta





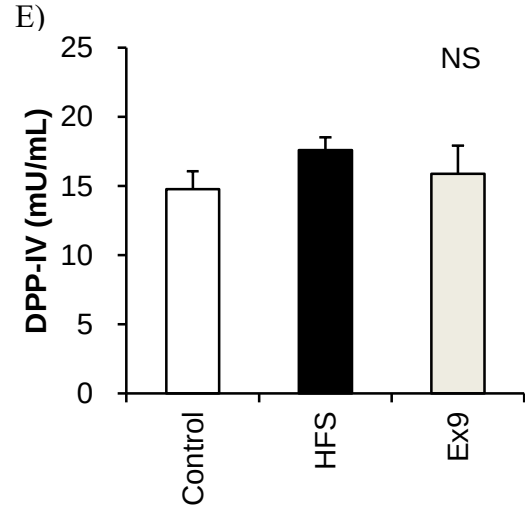
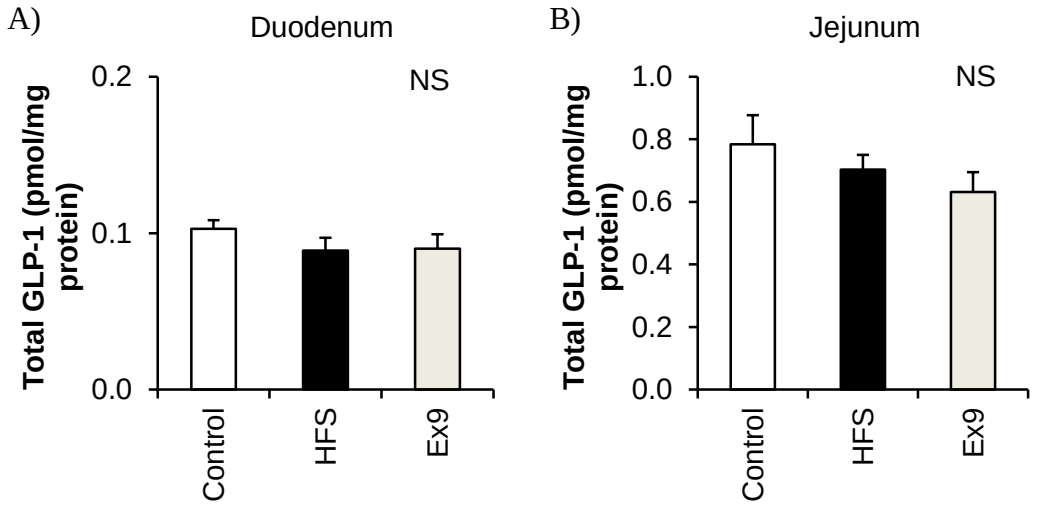


Figure 2.6. Aortic glucose, insulin, TAG, cholesterol levels and dipeptidyl peptidase-IV (DPP-IV) activity after 5 week of feeding period.

Rats were provided a control or high-fat/high-sucrose(HFS) diet with either saline or Ex9 administration (100 µg/day) for 5weeks. Blood samples from the portal vein and abdominal aorta were collected under sodium pentobarbital anaesthesia (50mg/kg BW) after overnight fasting (16 h). The values are expressed as mean ± SEM for n = 8-9 rats. The superscripts (a, b, c) with different letters show significant differences between treatments (P < 0.05, Tukey–Kramer's test). NS, no significant difference among the treatments.

2.3.6 GLP-1 content in the intestinal tissues

As expected, GLP-1 content gradually increased along the proximal-distal axis of intestinal regions. In the ileal segment, the GLP-1 content of the HFS group was significantly higher than in the control group (Fig. 2.7C). The Ex9 group also showed a similar tendency, but slightly less than the HFS group. Conversely, there were no significant differences in GLP-1 concentration in the other intestinal segments (Fig. 2.7).



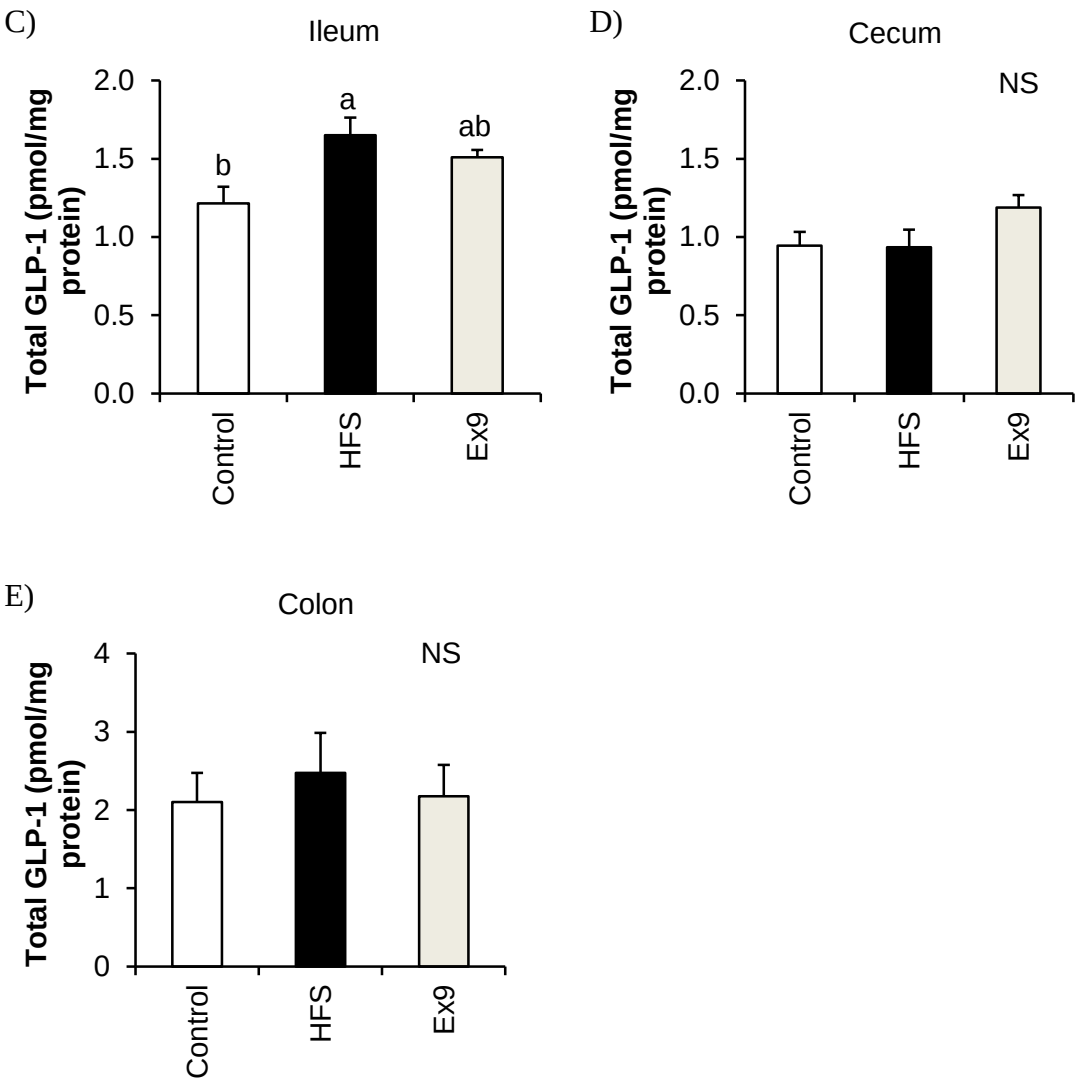


Figure 2.7. GLP-1 content in intestinal tissues after 5 week of feeding period.

Rats were provided a control or high-fat/high-sucrose(HFS) diet with either saline or Ex9 administration (100 μg/day) for 5weeks. Each intestinal segment: (A) duodenum, (B) jejunum, (C) ileum, (D) cecum and (E) colon were collected after killing. The values are expressed as mean ± SEM for n = 8-9 rats. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). NS, no significant difference among the treatments.

2.4 Discussion

This study aimed to determine the physiological relevance of increased/decreased postprandial GLP-1 secretion during the DIO progression and/or glucose intolerance

development as the primary outcome. The rats were fed an HFS diet (30% fat and 40% sucrose) for 5 weeks with or without continuous exendin (9–39) (Ex9) administration in order to monitor chronic changes in postprandial glucose, insulin, and GLP-1 responses. The results showed that normalization of the postprandial glycemic response was accompanied by an increase in insulin and GLP-1 secretion in rats fed an HFS diet, whereas continuous GLP-1 receptor antagonist administration caused glucose intolerance. These results indicate that enhancement of the postprandial GLP-1 response plays an important role in prevention of glucose intolerance during DIO.

In general, a high fat and/or high sucrose diet has been used to establish obesity and metabolic disorder animal models (Long, 2015; Sato-Mito, 2009; Sumiyoshi, 2006; Yang, 2012). In this experiment, the results suggest that a diet containing high fat and high sucrose establishes obesity in an animal model, even in a shorter period (5 weeks) than generally employed (8–12 weeks or longer). However, BW, BW gain and visceral adipose tissue weight in the HFS and Ex9 groups were similar levels. It can be assumed that longer experimental periods are required to observe the effect of blocking endogenous GLP-1 signal on these parameters.

Until now, postprandial glycemic and insulin responses are generally tested using the classical models such as oral glucose tolerance test (OGTT) and MTT (Rijkelijkhuizen, 2009; Staten, 2014). However, MTT is more appropriate methodology for investigating postprandial metabolic responses, compared to OGTT (Brodovicz, 2011). In order to mimic ordinary dietary habit, voluntary ingestion might be more appropriate than enforced gavage feeding. Nevertheless, some of the rats did not consume the provided diet (10 g/kg BW, that is enough to consume within 30 min after overnight or 16 hour fasting according to preliminary experiment) during MTT in experiment 1 (chapter 1). It might be due to acute stress response after basal blood collection. Hence, oral administration of a liquid

diet (Ensure H at 15 kcal/kg BW) was employed instead of voluntary ingestion in this study.

Postprandial GLP-1 levels in the HFS and Ex9 groups were slightly higher after 2 weeks of feeding period and significantly higher after 4 weeks of feeding period than the control group. The results confirmed that chronic consumption of an HFS diet enhances postprandial GLP-1 secretion in response to the diet administration.

Increased GLP-1 production in the ileum may contribute to the increased GLP-1 secretion. It is also speculated that the sensitivity of L-cells to luminal nutrients was increased following chronic consumption of a high fat and high sucrose diet. No significant differences were observed in GLP-1 concentration in the other intestinal segments, suggesting that L-cells located in the ileum segment are more susceptible to chronic obesogenic diet feeding than other intestinal segments. It could be implied that the ileum plays an important role in postprandial GLP-1 release. This was supported by the findings that postprandial GLP-1 secretion is stimulated by luminal contents in L-cells of the proximal ileum (Singh, 2015; Wang, 2015). However, the major source of postprandial GLP-1 secretion has not been not clearly identified in my studies (chapter 1 and 2). Although the L-cells are predominantly located in the ileum and colon (Lim, 2006), GLP-1 is secreted primarily from enteroendocrine L-cells located in the distal jejunum and ileum (Lean, 2016). In chapter 1, the results suggest that L-cells in the jejunum is the major source of enhanced postprandial GLP-1 secretion in obesogenic diet-fed rats, which was consistent with recent studies demonstrate GLP-1-producing cells exists in the jejunum part (Eissele, 1992; Cordier-Bussat, 1998; Damholt, 1999). Therefore, the source of GLP-1 during obesity development needs to be clarified in the future study.

In the present study, gastric emptying rates were unchanged by the chronic HFS diet with or without Ex9 compared with the control group; therefore, enhanced GLP-1 responses in the HFS and Ex9 groups cannot be explained by differences in the rate of

nutrient load into the small intestine from the stomach. In line with this study, acute infusion of Ex9 before meal ingestion had no effect on gastric emptying in humans (Witte, 2010; Nicolaus, 2011). Gastric emptying rate is regulated by various factors such as ghrelin, serotonin, CCK, PYY, not only on GLP-1 (Hellström, 2006; Nakajima, 2015). In the present study, compensatory elevation of GLP-1 secretion by Ex9 treatment was not observed compared to the HFS group. It can be assumed that GLP-1 secretion in both HFS and Ex9 groups is the maximum level from the L-cells in this experimental model. In addition, compensatory elevations of GLP-1 by Ex9 have not necessarily been found in the previous studies (Schirra, 2006; Calabria, 2012).

Although both the HFS and Ex9 groups showed elevated postprandial GLP-1 secretion compared with the control group, postprandial insulin secretion was diminished in the Ex9 group compared with the HFS group. These results clearly demonstrate that postprandial GLP-1 was effectively blocked by Ex9 treatment, resulting in insufficient insulin secretion. Accordingly, the results illustrate that chronic HFS feeding with continuous Ex9 administration rapidly established postprandial hyperglycemia, while HFS alone maintained a glycemic response similar to the control group in both the 2 and 4 weeks of feeding periods. Previous studies demonstrated that Ex9 treatment for 14 days accelerated glucose intolerance in rats assessed by OGTT (Dusaulcy, 2016) and that intraperitoneal injection of Ex9 prior to OGTT induced glucose intolerance in rats (Tseng, 1999). According to the MTT experiments, the results clearly demonstrated that elevation of postprandial GLP-1 plays an important role in the prevention and amelioration of glucose intolerance during DIO through increasing insulin responses. Hence, enhancing endogenous GLP-1 secretion could be a potential target for prevention of glucose intolerance and obesity development. A better understanding of GLP-1 secretion mechanism could lead us to design therapeutics or dietary interventions for obesity and

diabetes treatment.

After 5 weeks of feeding period, the fasting glucose level in the HFS group was similar to the level of the control group, whereas fasting GLP-1 and insulin levels were elevated in the HFS group. In the Ex9 group, the fasting glucose level was higher with a partially lower insulin level compared with the HFS group. These results demonstrate that not only postprandial GLP-1 but also fasting GLP-1 plays an important role in the prevention of blood glucose elevation. Fasting TG and cholesterol level in abdominal aorta were increased in HFS group suggesting that diet containing fat and sucrose could induce disorder in lipid metabolism which results in hyperlipidemia development (Pang, 2016). In addition, similar elevations in fasting TG and cholesterol levels in the HFS and Ex9 groups suggest that endogenous GLP-1 does not directly affect dyslipidemia under the experimental conditions.

However, it is still unclear whether the increased GLP-1 responses in the diet-induced obese models were sustained or disappeared after prolonged treatment with an obesogenic diet. Moreover, the meal-induced GLP-1 responses in genetically diabetic models under treatment with the obesogenic diet are still unknown. Therefore, investigating direction of postprandial GLP-1 responses (enhanced, reduced, or unchanged) during prolong DIO in both diabetic and non-diabetic rats are needed to clarify in the further studies.

2.5 Conclusions

The postprandial glycemic response in the HFS group was similar to the control group, whereas postprandial GLP-1 and insulin secretion were increased in the HFS group. In the Ex9 group, whose GLP-1 signal was blocked, the postprandial glycemic response was elevated with a lower insulin response compared with the HFS group. It could be

concluded that enhancement of postprandial GLP-1 induction by chronic feeding of an obesogenic diet has a role in maintaining normal postprandial glycemia. These results support the notion that increasing endogenous GLP-1 secretion by dietary interventions is a potential target for prevention of glucose intolerance and obesity development.

Chapter 3: Long-term effects of high fat and high sucrose diet feeding on postprandial GLP-1 response in Wistar and GK rats

Abstract

To explore mechanisms underlying the adaptive changes in GLP-1 responses under diet-induced obesity (DIO) and glucose intolerance status, postprandial GLP-1 responses in normal rats and in a diabetic model rats fed an obesogenic diet were monitored for more than 6 months.

The non-diabetic Wistar and diabetic Goto-Kakizaki (GK) rats (5-week-old) were fed either a control diet or a high fat/high sucrose (HFS, 30% fat and 40% sucrose w/w) diet for 26 weeks. Meal tolerance tests (MTTs) were performed for monitoring postprandial glucose, insulin, and GLP-1 responses after a liquid diet administration (15 kcal/kg BW) every 4 or 8 weeks after feeding the obesogenic diet.

Postprandial glycemic responses were higher in Wistar rats fed an HFS diet (WH), compared to Wistar rats fed the control diet (WC) after 16 weeks. The postprandial GLP-1 and insulin responses in the WH group were higher or tended to be higher than those in the WC group throughout the study. Although GK rats fed with an HFS diet (GH) had higher glycemic responses, compared to GK rats fed with the control diet (GC), GH and GC groups had similar postprandial GLP-1 and insulin responses throughout the study. Jejunal GLP-1 contents and *Ffar1* mRNA expression level were increased and tended to be increased by HFS diet in Wistar rats, but not in GK rats. These results demonstrate that the postprandial GLP-1 responses were enhanced and sustained, but not attenuated in the DIO in normal rats after a long experimental period. Moreover, failure of adaptive enhancement of GLP-1 response in GK rats could be responsible for exacerbating glucose impairment.

3.1 Introduction

Goto-Kakizaki (GK) rats are recognized as non-obese diabetic rodent model and widely used to investigate the development of type 2 diabetes and its complications (Akash, 2013; Kuwabara, 2017). GK rats, developed by selective breeding of glucose intolerant Wistar rats over many generations, are spontaneously polygenic model (Goto, 1975; Wang, 2014). Glucose intolerance is developed within the first 3 weeks after birth, which can be considered as a prediabetes stage (Movassat, 2008). The adult GK rats are characterized by hyperglycemia, impaired glucose-induced insulin secretion (insulin resistance), decreased β-cell mass, and decreased insulin sensitivity (Movassat, 2008). GK rats are considered as one of the best type 2 diabetic animal models because they present many similarities with type 2 diabetic patients, (Akash, 2013; Amiri, 2015).

Nowadays, many of rodent models (both obese and non-obese type 2 diabetic models) can be used to study type 2 diabetes such as GK rats, Zucker diabetic fatty (ZDF) rats, diabetic (*db/db*) mice, and obese (*ob/ob*) mice (Rees, 2005; Srinivasan, 2007). The *ob/ob* mice, *db/db* mice, and ZDF rats are monogenic models, which exhibit a mutation in the leptin (*ob/ob*) or leptin receptor gene (ZDF, *db/db*). They are useful for studying particular disease characteristics, but do not necessarily represent the human disease state (Wang, 2013). In addition, the symptom of type 2 diabetes of *ob/ob* mice attenuates with age. Also, *db/db* mouse seems not compatible to investigate the complication of type 2 diabetes due to the short lifespan. The ZDF rat requires time to develop hyperglycemia (about 7 weeks of age and fully diabetic at 12 weeks) and also require insulin treatment in the later stage for survival (Akash, 2013; Wang, 2013). In contrast to obese model, GK rats showed an early and relatively stable hyperglycemia and insulin resistance with a long lifespan and can be used to study the diabetic complications in humans (Miralles, 2001; Ishii, 2012; Akash, 2013; Amiri, 2015). Furthermore, polygenic models represent the

human condition more closely, compared to monogenic model (Srinivasan, 2007).

Up to date, the impact of obesogenic diet in animal models has been widely investigated; however, changes in postprandial GLP-1 response during obesity development in both non-diabetic and diabetic rat models remain controversial. The previous studies reported that the postprandial GLP-1 responses were increased in rats during obesity development (Nakajima, 2015; Pinyo, 2019a; Pinyo, 2019b). However, it is still unclear whether the increased GLP-1 responses in the diet-induced obese models were sustained or disappeared after prolonged treatment with an obesogenic diet. Moreover, the meal-induced GLP-1 responses in genetically diabetic models under obesogenic diet feeding are unknown. In the present study, whether the adaptive enhancement of postprandial GLP-1 responses sustains or disappears in the diet-induced obesity (DIO) model rats after a long experimental period (26 weeks) were also examined. To explore potential mechanisms involved in the adaptive changes in postprandial GLP-1 responses in DIO and glucose intolerance status, the GLP-1 responses in diabetic model rats treated with an obesogenic diet was also investigated.

3.2 Materials and methods

3.2.1 Animals

Non-diabetic Wistar rats (not Wistar ST rats) and non-obese diabetic Goto-Kakizaki (GK) rats (5 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Prior to the experiment, the rats were acclimatized for 1 week. The rats were housed individually in environmentally controlled rooms and provided ad libitum access to food and water except the day before experiment as earlier described in chapter 1 and 2.

After an acclimation period, both Wistar and GK rats were subdivided into control and high fat and high sucrose (HFS, 30% fat and 40% sucrose) (Nakajima, 2015; Pinyo,

2019a; Pinyo, 2019b) groups with each group consisting of 8-9 rats that were selected based on BW, glucose, and GLP-1 levels after an overnight fasting. The rats were assigned to different groups as follows: Wistar rats fed a control diet (WC group), Wistar rats fed an HFS diet (WH group), GK rats fed a control diet (GC group), and GK rats fed an HFS diet (GH group). All procedures related to animal experiments in this study were approved by the Hokkaido University Animal Committee (approval no. 19-0064) and the rats were maintained according to the guidelines for the care and use of laboratory animals.

Experimental timeline

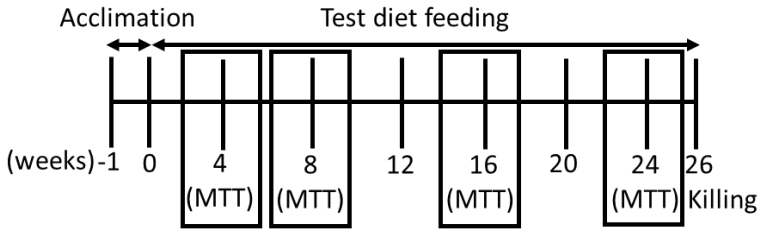


Figure 3.1. Schematic representation of experiment timeline.

3.2.2 Meal tolerance test (MTT)

The meal tolerance test has previously been described in detail in Chapter 2. In this study, however, MTTs were performed after 4, 8, 16, and 24 weeks of feeding period. In brief, the rats were fasted for 16 h and then orally administered with a liquid diet (15 kcal/kg BW; Ensure H, Abbott, Tokyo, Japan). The blood samples were collected at basal level and 15, 30, 60, 90, and 120 min after the diet administration. Blood plasma was separated at 2300 x *g* for 10 min at 4 °C. Then the plasma samples were immediately collected and stored at -80°C until analysis (glucose, insulin, GLP-1, and acetaminophen levels). Glucose levels were measured using the Glucose CII Test Kit (Wako Pure Chemical Industries, Osaka, Japan). Insulin and GLP-1 concentrations were determined using Rat Insulin ELISA

kit (U-E type, AKRIN-130; Shibayagi Company Limited, Gunma, Japan) and GLP-1 ELISA kit (High sensitive; Wako, Osaka, Japan), respectively.

3.2.3 Blood and tissue collection

After 26 weeks of test diet feeding, blood and tissue samples were collected after overnight fasting as previously described in chapter 1 and 2. The blood samples were then measured biochemical parameters (glucose, insulin, GLP-1, TG, cholesterol, and DPP-IV activity). The intestinal mucosa and tissue were collected to measure mRNA expression and GLP-1 content, respectively. In this study, pancreatic tissues were also collected in order to measure insulin content.

3.2.4 GLP-1 content in intestinal tissue

An ethanol acid solution was used to extract GLP-1 from intestinal segment as earlier mentioned in chapter 1 and 2. However, the homogenate samples were diluted 40 times for measurement of protein concentration, whereas the supernatant was diluted 500 times for the measurement of GLP-1 content in this current study.

3.2.5 Pancreatic insulin content measurement

Pancreatic tissue was minced in ethanol acid solution (10 mL/g tissue; 75% absolute ethanol, 23.5 % water, and 1.5% HCl 12 N) (Tourrel, 2002; Granata, 2009), and then homogenized at 24,000 rpm (Ultra Turrax homogenizer T18, IKA, Staufen, Germany) for 2 min. The pancreatic homogenate samples were stored at 4℃ for 24 h to extract insulin. Aliquots of the homogenate samples (150 µL) were stored for the measurement of protein concentration. The remaining homogenate samples were centrifuged (5000 x *g*, 10 min, 4°C). The supernatants were collected and stored at -80 ℃ for the estimation of insulin

concentration. The homogenate samples were diluted 50 times for protein quantification using Lowry's protein assay. The supernatant was diluted 6000 times for assessment of insulin content using Rat Insulin ELISA kit (AKRIN-010T; Shibayagi Company Limited, Gunma, Japan).

3.2.6 Real-time quantitative PCR

Mucosa of each intestinal segment was scraped and stored in RLT buffer solution (RLT buffer:2-mercaptoethanol at a ratio of 100:1) at -80 °C for RNA extraction. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany), and subsequently, complementary DNA (cDNA) was synthesized using the ReverTra Ace qPCR Master Mix with gDNA Remover (Toyobo Company Limited, Osaka, Japan) according to the manufacturer's instructions. Gene expression levels were analyzed by TaqMan gene expression assays (Life Technologies Company) using the rat gene-specific, predesigned TaqMan primers, as follows:

[Rn99999916_s1 for glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)

Rn00562293_m1 for proglucagon (*Gcg*)

Rn00824686_s1 for free fatty acid receptor 1 (*Ffar1* or *Gpr40*)

Rn02345824_s1 for free fatty acid receptor 2 (*Ffar2* or *Gpr43*)

Rn01457614_g1 for free fatty acid receptor 3 (*Ffar3* or *Gpr41*)

Rn01759772_m1 for free fatty acid receptor 4 (*Ffar4* or *Gpr120*)]

3.2.7 Statistical Analysis

In the present study, the total sample size was calculated based on the experimental design (analysis of variance, ANOVA repeated measures) for MTTs to assess postprandial GLP-1 responses in both non-diabetic and diabetic rats fed either control or HFS diet as

the primary outcome measure using G*Power software (version 3.1.9.2). The parameters used for the analysis were as follows: power = 0.8, significance level = 0.05, and effect size (medium) = 0.25. The total number of rats was calculated as 28 (7 rats/group). The data are expressed as the mean ± standard error of the mean (SEM). Significant effects of diet, strain, and strain-by-diet interactions were assessed using two-way ANOVA. Statistically significant difference was determined using one-way ANOVA followed by an appropriate posthoc test (Tukey-Kramer's or Student's *t*-test). The data were statistically analyzed using JMP pro version 12.2.0 software (SAS Institute, Inc., Cary, USA). All *P* values were considered to be significant at $P < 0.05$.

3.3 Results

3.3.1 BW, energy intake, and tissue weights

The final BW, BW gain, and total energy intake of rats fed an HFS diet were significantly higher than those of rats fed control diet both in Wistar and GK rats (Table 3.1). HFS feeding groups (WH and GH) showed significantly higher liver weight and visceral fat accumulation (sum of mesenteric, epididymal, and retroperitoneal fat) as compared to the control diet feeding groups (WC and GC) as shown in Table 3.1.

Table 3.1 BW, food and energy intake, tissue weights

	WC	WH	GC	GH
Initial BW (g)	143.2 ± 1.6	142.8 ± 2.1	143.9±3.8	144.4 ± 2.9^{NS}
Final BW (g)	422.6 ± 5.0^{c}	465.7 ± 7.3^{b§}	446.3 ± 10.7^{bc}	501.9 ± 5.1^{a†}
BW gain (g)	279.3 ± 5.2^{c}	322.9 ± 6.2^{b§}	302.4 ± 10.7^{bc}	357.5 ± 5.6^{a†}
Visceral fat (g)	39.8 ± 1.9^{b}	59.2 ± 3.0^{a§}	37.6 ± 1.4^{b}	56.5 ± 1.1^{a†}
Mesenteric fat (g)	11.5 ± 0.8^{b}	16.9 ± 0.9^{a§}	11.2 ± 0.3^{b}	13.1 ± 0.1^{b†}
Epididymal fat (g)	13.9 ± 0.6^{b}	18.9 ± 1.1^{a§}	8.5 ± 0.6^{d}	11.2 ± 0.2^{c†}
Retroperitoneal fat (g)	14.4 ± 0.7^{c}	23.4 ± 1.3^{b§}	17.9 ± 0.7^{c}	32.3 ± 0.8^{a†}
Liver weight (g)	10.0 ± 0.3^{c}	11.1 ± 0.2^{b§}	12.9 ± 0.2^{a}	13.5 ± 0.2^{a†}
Total energy intake (kcal)	11284.1 ± 127.2^{c}	12489.3 ± 208.1^{b§}	12467.8 ± 247.9^{b}	13247.7 ± 115.3^{a†}

Visceral fat weight is the sum of the mesenteric, epididymal, and retroperitoneal fat weight. The values are expressed as the mean ± SEM for n = 7-9 rats. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). §Significant differences between mean values of the WC and WH groups and †Significant differences between mean values of the GC and GH groups ($P < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between the treatments.

Two-way ANOVA	Diet	Strain	Diet*Strain
Initial BW	$P = 0.63$	$P < 0.05$	$P = 0.94$
Final BW	$P < 0.05$	$P < 0.05$	$P = 0.41$
BW gain	$P < 0.05$	$P < 0.05$	$P = 0.44$
Visceral fat	$P < 0.05$	$P < 0.23$	$P = 0.89$
Mesenteric fat	$P < 0.05$	$P < 0.05$	$P < 0.05$
Epididymal fat	$P < 0.05$	$P < 0.05$	$P = 0.10$
Retroperitoneal fat	$P < 0.05$	$P < 0.05$	$P < 0.05$
Liver weight	$P < 0.05$	$P < 0.05$	$P = 0.23$
Total energy intake	$P < 0.05$	$P < 0.05$	$P = 0.26$

Two-way ANOVA results of BW, food and energy intake, tissue weights

3.3.2 Fasting glucose, insulin, GLP-1 levels and HOMA-IR

Fasting glucose levels in GK rats (GC and GH) were higher than those of the Wistar rats (WC and WH) throughout the monitoring period (Table 3.2). After 16 weeks of feeding,

the WH (87.9 ± 0.9 mg/dL) and GH groups (162.2 ± 6.7 mg/dL) had higher fasting glucose levels compared to the respective control diet feeding groups (80.0 ± 1.5 mg/dL and 145.5 ± 2.6 mg/dL) as shown in Table 3.2. Fasting GLP-1 and insulin levels were higher or tended to be higher in the WH group compared to the WC group; however, such trends were not observed between GC and GH groups. Both the HFS diet groups had higher HOMA-IR at fasting state compared to the control diet group after 16 weeks of feeding period (Table 3.2).

Table 3.2 Fasting glucose, insulin, GLP-1 levels and HOMA-IR

	WC	WH	GC	GH
4 weeks				
Glucose (mg/dL)	90.3 ± 2.7^{b}	91.7 ± 3.0^{b}	141.0 ± 3.7^{a}	138.9 ± 3.6^{a}
Insulin (nM)	0.04 ± 0.01^{b}	$0.13 \pm 0.02^{a\S}$	0.12 ± 0.01^{a}	0.12 ± 0.03^{a}
GLP-1 (pM)	4.38 ± 0.63^{ab}	5.69 ± 0.41^{a}	3.12 ± 0.35^{b}	3.76 ± 0.57^{ab}
HOMA-IR	0.22 ± 0.05^{b}	$0.76 \pm 0.14^{ab\S}$	1.02 ± 0.14^{a}	1.09 ± 0.26^{a}
8 weeks				
Glucose (mg/dL)	84.9 ± 2.1^{b}	82.1 ± 2.8^{b}	145.1 ± 3.1^{a}	146.1 ± 3.5^{a}
Insulin (nM)	0.15 ± 0.01	0.18 ± 0.03	0.19 ± 0.03	0.19 ± 0.04^{NS}
GLP-1 (pM)	4.83 ± 0.96	5.86 ± 0.90	3.25 ± 0.80	3.47 ± 0.79^{NS}
HOMA-IR	0.78 ± 0.07^{b}	0.92 ± 0.18^{ab}	1.71 ± 0.27^{ab}	1.76 ± 0.3^{a}
16 weeks				
Glucose (mg/dL)	80.0 ± 1.5^{c}	$87.9 \pm 0.9^{c\S}$	145.5 ± 2.6^{b}	$162.2 \pm 6.7^{a\dagger}$
Insulin (nM)	0.15 ± 0.035	0.20 ± 0.02	0.19 ± 0.02	0.24 ± 0.03^{NS}
GLP-1 (pM)	5.36 ± 1.07	6.80 ± 1.46	4.78 ± 1.04	5.65 ± 0.75^{NS}
HOMA-IR	0.75 ± 0.11^{c}	$1.12 \pm 0.11^{bc\S}$	1.74 ± 0.16^{b}	$2.40 \pm 0.24^{a\dagger}$
24 weeks				
Glucose (mg/dL)	81.4 ± 1.8^{b}	$86.9 \pm 1.0^{b\S}$	145.7 ± 5.2^{a}	151.3 ± 4.5^{a}
Insulin (nM)	0.13 ± 0.02	0.21 ± 0.02	0.16 ± 0.03	0.19 ± 0.04^{NS}
GLP-1 (pM)	2.61 ± 0.34	3.43 ± 0.27	2.79 ± 0.52	2.77 ± 0.26^{NS}
HOMA-IR	0.69 ± 0.12	$1.13 \pm 0.15^{\S}$	1.42 ± 0.30	1.75 ± 0.41

The values are expressed as the mean ± SEM for n = 6-9 rats. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). §Significant differences between mean values of the WC and WH groups and †Significant differences between mean values of the GC and GH groups ($P < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between the treatments.

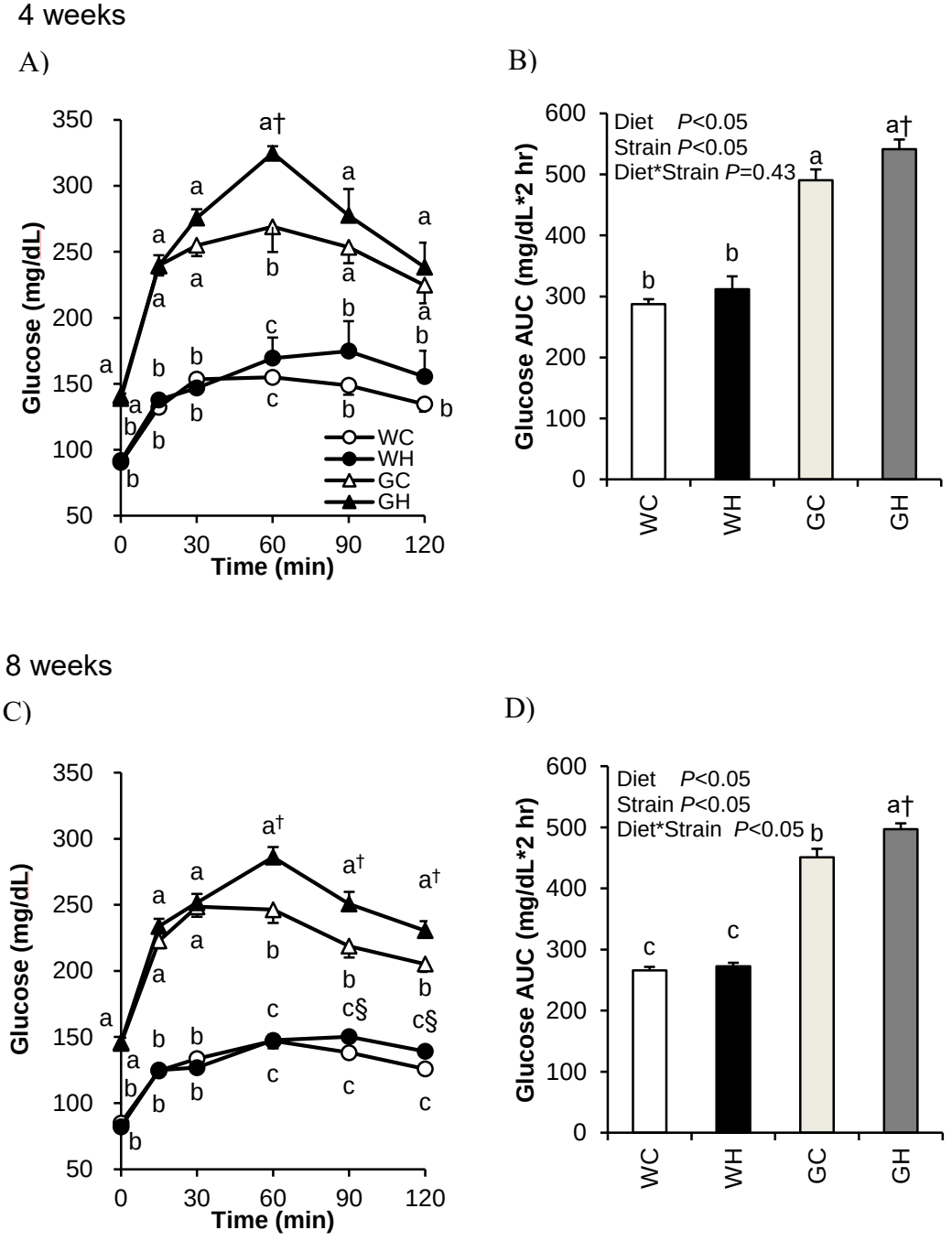
Two-way ANOVA	Diet	Strain	Diet*Strain
4 weeks			
Glucose	$P = 0.92$	$P < 0.05$	$P = 0.60$
Insulin	$P < 0.05$	$P = 0.19$	$P < 0.05$
GLP-1	$P = 0.06$	$P < 0.05$	$P = 0.52$
HOMA-IR	$P = 0.07$	$P < 0.05$	$P = 0.15$
8 weeks			
Glucose	$P = 0.75$	$P < 0.05$	$P = 0.53$
Insulin	$P = 0.57$	$P = 0.35$	$P = 0.69$
GLP-1	$P = 0.47$	$P < 0.05$	$P = 0.65$
HOMA-IR	$P = 0.08$	$P < 0.05$	$P = 0.11$
16 weeks			
Glucose	$P < 0.05$	$P < 0.05$	$P =0.28$
Insulin	$P < 0.05$	$P = 0.11$	$P = 0.98$
GLP-1	$P = 0.27$	$P = 0.43$	$P = 0.78$
HOMA-IR	$P < 0.05$	$P < 0.05$	$P =0.34$
24 weeks			
Glucose	$P = 0.15$	$P < 0.05$	$P = 0.99$
Insulin	$P = 0.11$	$P = 0.99$	$P = 0.54$
GLP-1	$P = 0.29$	$P = 0.51$	$P = 0.28$
HOMA-IR	$P = 0.20$	$P < 0.05$	$P = 0.86$

Two-way ANOVA results of fasting glucose, insulin, GLP-1 levels and HOMA-IR

3.3.3 Postprandial glycemic response

After an overnight fasting, the liquid diet (15 kcal/kg BW) was administered orally to rats to determine responses to a meal. WH group had higher glycemic responses than the WC group at multiple time points after 16 and 24 weeks of feeding periods as shown in Fig. 3.2E and G, and significant differences were observed in the area under curve (AUC) (Fig. 3.2F and H). In GK rats, glucose levels and the AUC in the GH group were significantly higher compared to those in the GC group throughout the experimental period

(Fig. 3.2A-H). Significant effects of diet and strain on postprandial glycemia AUC were detected by two-way ANOVA in all MTTs.



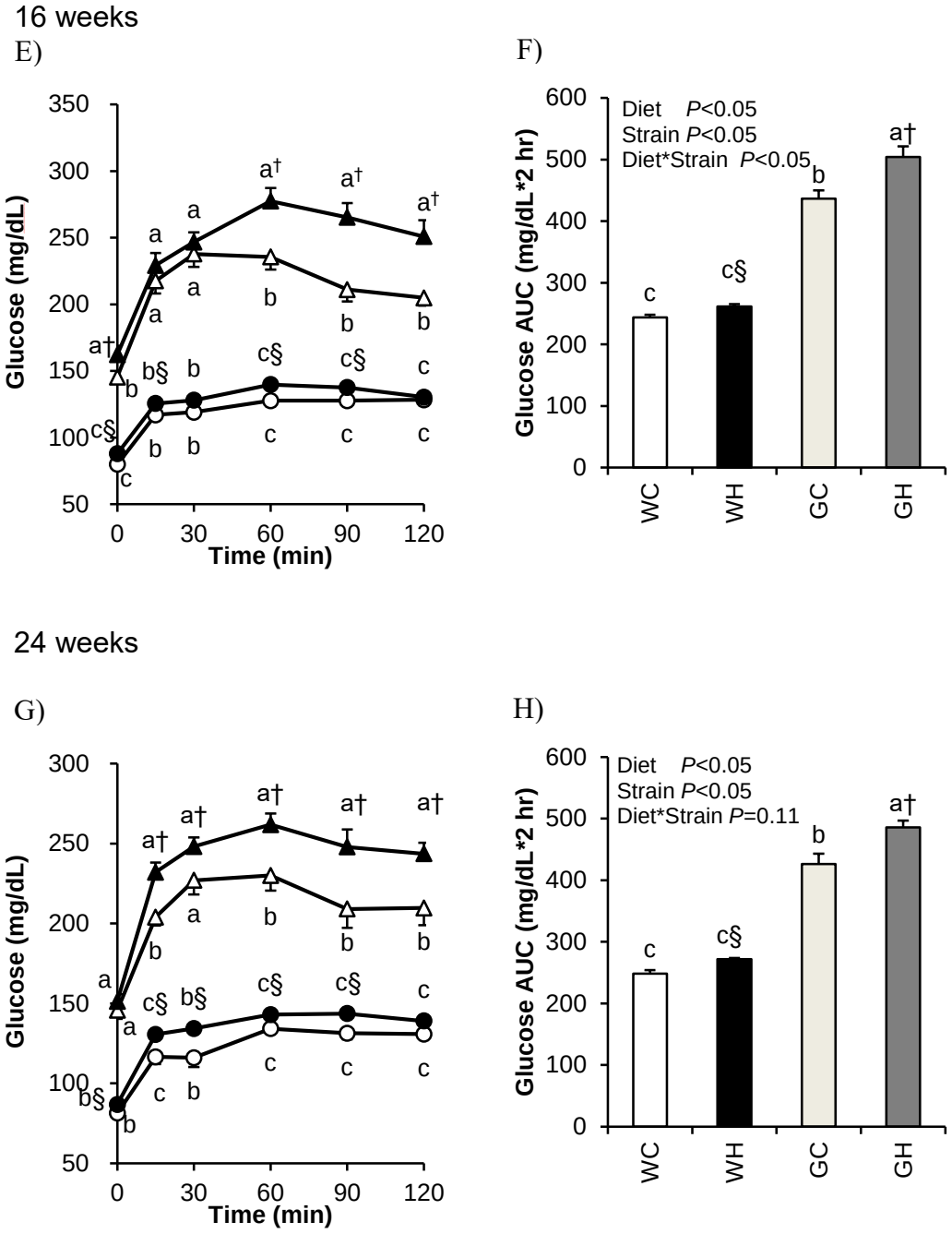
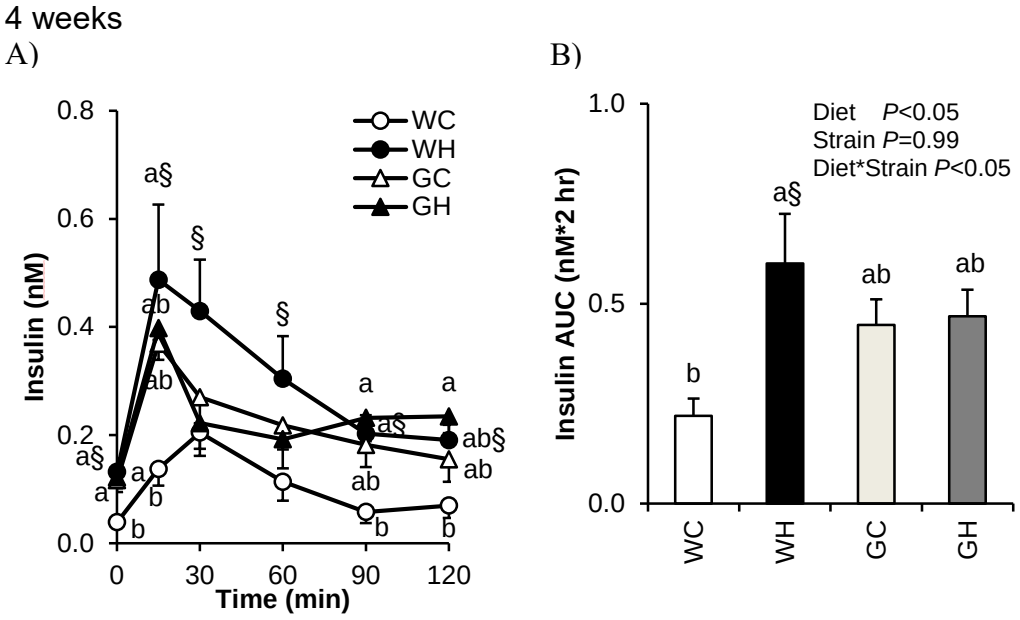


Figure 3.2. Postprandial glycemic responses under MTTs after 4, 8, 16, and 24 weeks of feeding period

Wistar rats and diabetic GK rats were fed either control diet or HFS diet for 4, 8, 16, and 24 weeks before conducting MTT. After overnight fasting, basal blood (0 min) was taken, followed by administration of a liquid diet (15 kcal/kg BW), and blood samples were collected at 15, 30, 60, 90, and 120 min. The values are expressed as mean ± SEM for n = 6-9 rats. *P* values of two-way ANOVA for diet, strain, and strain by diet interactions are shown in each panel. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). §Significant differences between mean values of the WC and WH groups and †Significant differences between mean values of the GC and GH groups ($P < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between the treatments.

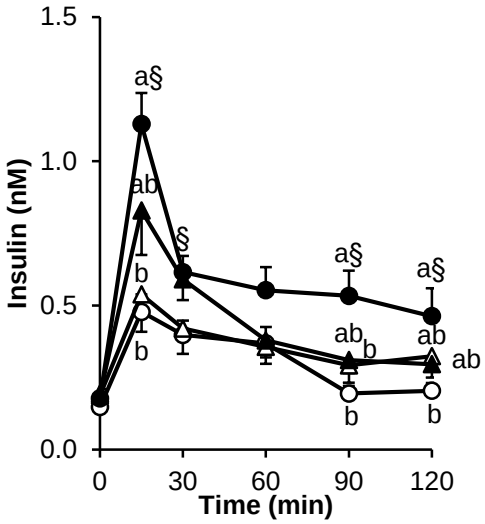
3.3.4 Postprandial insulin response

Insulin secretion was immediately increased (peaked at 15 or 30 min) after the administration of liquid diet in all groups. In the WH group, insulin concentrations at multiple time points and the AUC were higher compared to those in the WC group throughout experimental period (Fig. 3.3). In contrast, the GH group had higher insulin concentrations only at 15 min after 16 weeks, compared to those of the GC group (Fig 3.3E, F, G and H). However, no significant difference was observed in the AUC between the GC and GK groups throughout the experimental period (Fig. 3.3B, D, F, and H).

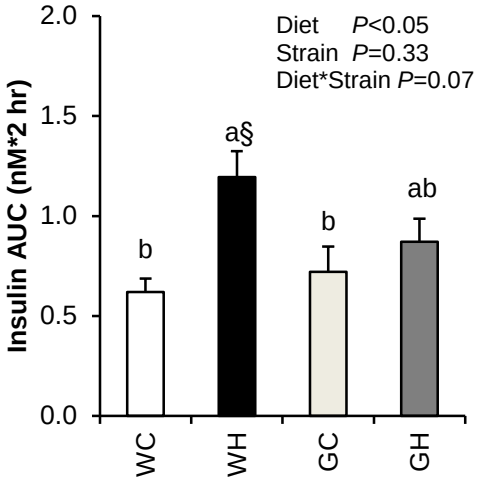


8 weeks

C)

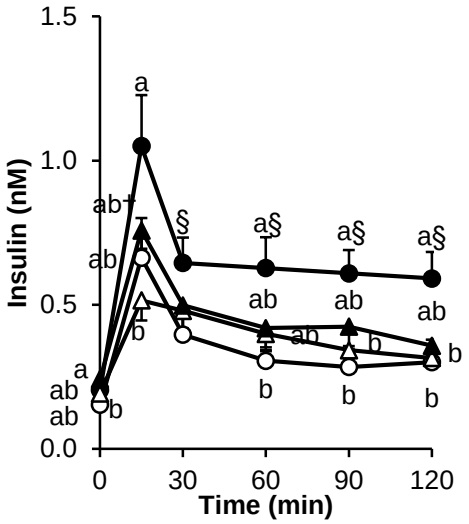


D)

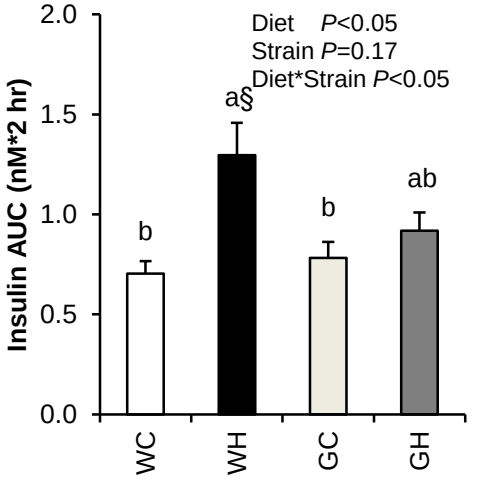


16 weeks

E)



F)



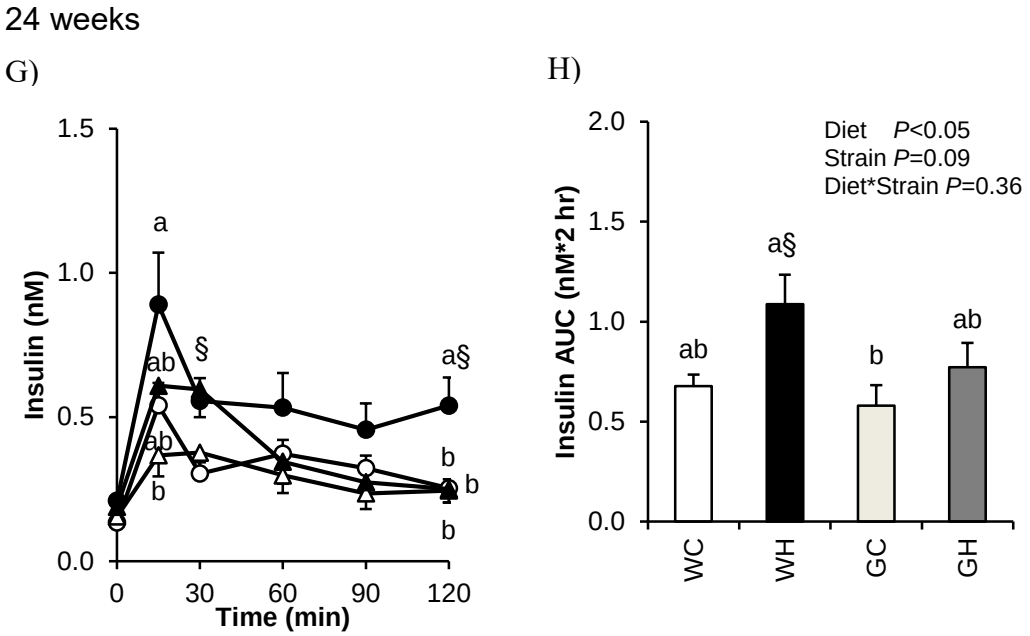


Figure 3.3. Postprandial insulin responses under MTTs after 4, 8, 16, and 24 weeks of feeding period

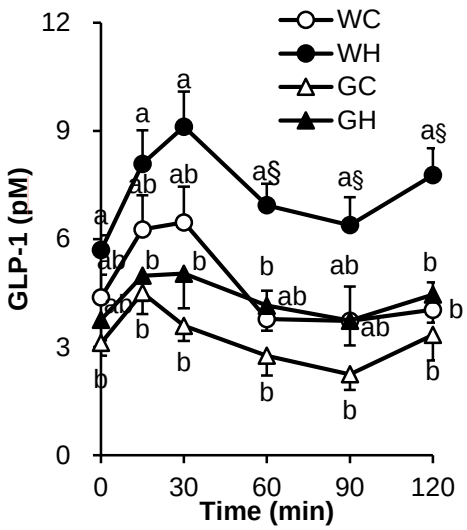
Wistar rats and diabetic GK rats were fed either control diet or HFS diet for 4, 8, 16, and 24 weeks before conducting MTT. After overnight fasting, basal blood (0 min) was taken, followed by administration of a liquid diet (15 kcal/kg BW), and blood samples were collected at 15, 30, 60, 90, and 120 min. The values are expressed as mean ± SEM for n = 6-9 rats. *P* values of two-way ANOVA for diet, strain, and strain by diet interactions are shown in each panel. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). §Significant differences between mean values of the WC and WH groups and †Significant differences between mean values of the GC and GH groups ($P < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between the treatments.

3.3.5 Postprandial GLP-1 response

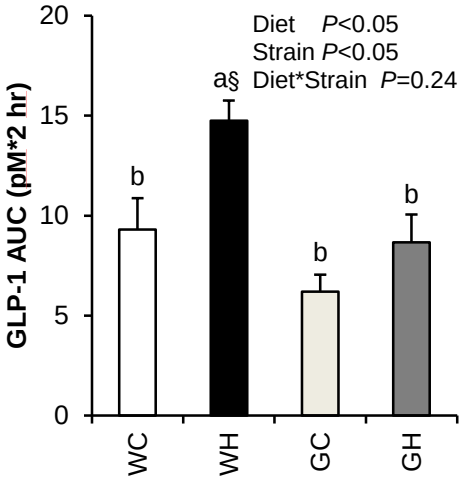
GLP-1 responses were higher in the WH group compared to those of the WC group (at 60-120 min and the AUC after 4 weeks, as well as at 15 min after 8 and 16 weeks), whereas GLP-1 responses and the AUC were observed to be similar in the GH and GC groups throughout the experimental period (Fig. 3.4A-H). Likewise, two-way ANOVA showed significant effects of diet and of strain on postprandial GLP-1 AUC in all MTTs.

4 weeks

A)

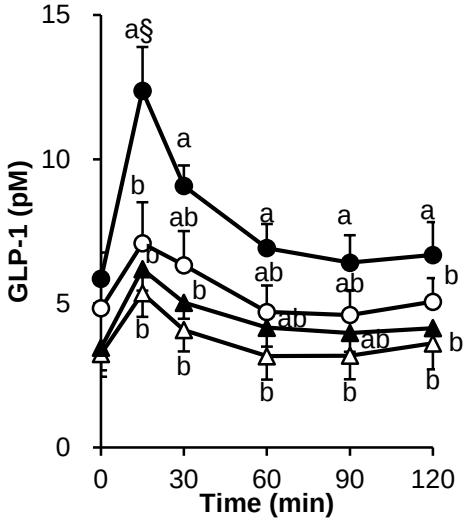


B)

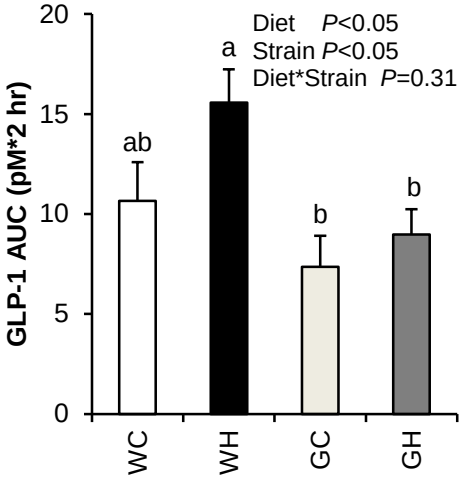


8 weeks

C)



D)



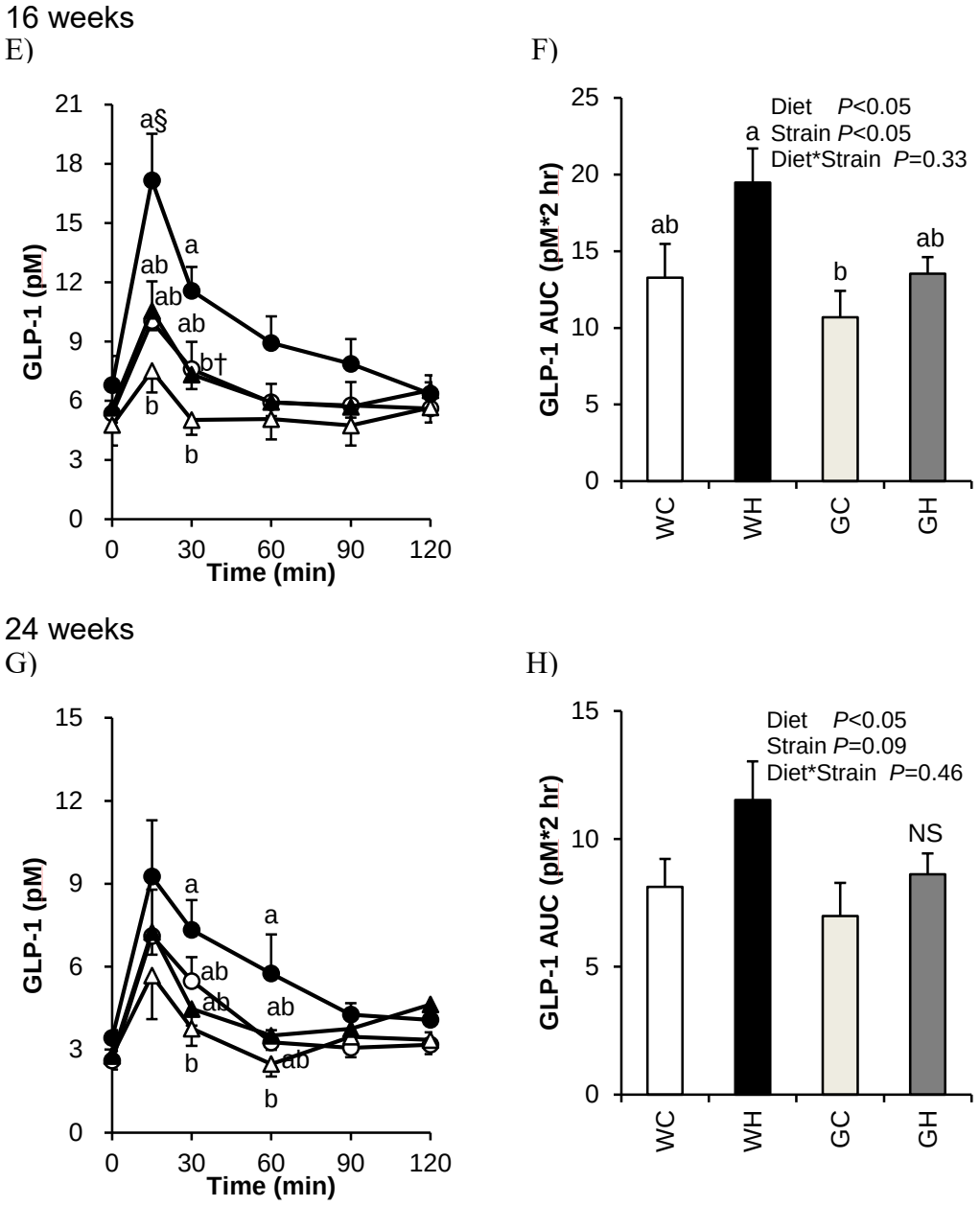


Figure 3.4. Postprandial GLP-1 responses under MTTs after 4, 8, 16, and 24 weeks of feeding period

Wistar rats and diabetic GK rats were fed either control diet or HFS diet for 4, 8, 16, and 24 weeks before conducting MTT. After overnight fasting, basal blood (0 min) was taken, followed by administration of a liquid diet (15 kcal/kg BW), and blood samples were collected at 15, 30, 60, 90, and 120 min. The values are expressed as mean ± SEM for n = 6-9 rats. *P* values of two-way ANOVA for diet, strain, and strain by diet interactions are shown in each panel. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). §Significant differences between mean values of the WC and WH groups and †Significant differences between mean values of the GC and GH groups ($P < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between the treatments.

3.3.6 Gastric emptying rate

Gastric emptying rate assessed by the acetaminophen test was used to estimate nutrient delivery into the intestinal lumen. In this study, changes in acetaminophen levels were similar in all groups regardless of differences in strain or diet treatment throughout the experimental period (Fig. 3.5).

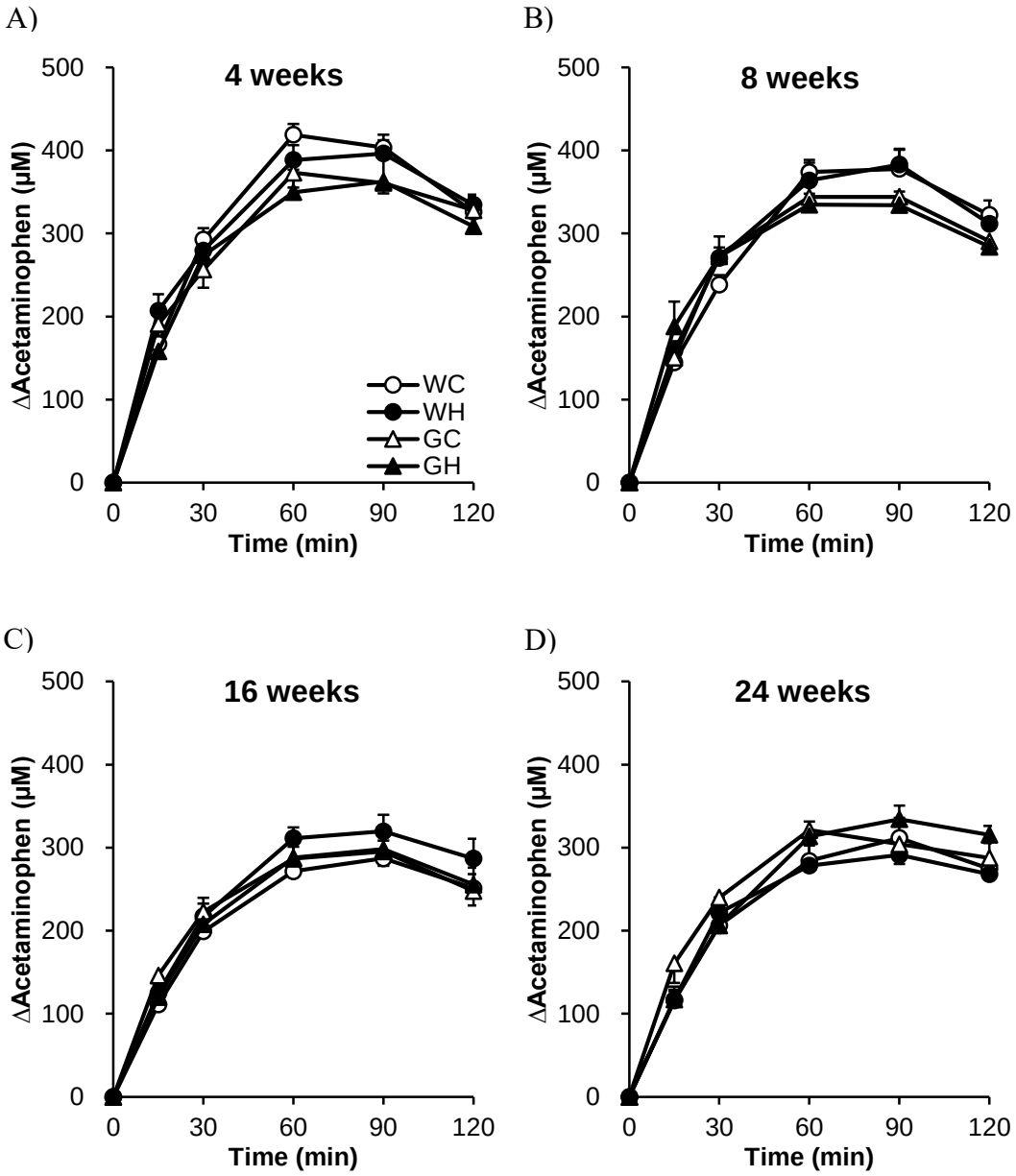


Figure 3-5. Gastric emptying under MTTs after 4, 8, 16, and 24 weeks of feeding period

Wistar rats and diabetic GK rats were fed either control diet or HFS diet for 4, 8, 16, and 24 weeks before conducting MTT. After overnight fasting, basal blood (0 min) was taken, followed by administration of a liquid diet (15 kcal/kg BW), and blood samples were collected at 15, 30, 60, 90, and 120 min. The values are expressed as mean ± SEM for n = 6-9 rats. NS indicates that there was no significant difference between the treatments.

3.3.6 Plasma parameters after 26 weeks feeding period

After the feeding period for 26 weeks, fasting glucose levels in HFS diet feeding groups (WH and GH) were higher than those in the respective control diet feeding groups (WC and GC) both in portal vein and abdominal aorta blood samples (Table 3.3). Regardless of the diet consumption, the GK rats showed higher fasting glucose levels compared to the Wistar rats. WH group showed significantly higher or such tendency of fasting insulin and GLP-1, respectively in comparison to the WC group, whereas GH group showed similar levels to those of the GC group (Table 3.3). These results were consistent with postprandial glucose, insulin, and GLP responses after feeding the test diet for 24 weeks. Fasting TG and cholesterol levels in the abdominal aorta had similar trends with fasting glucose concentrations (Table 3.3). DPP-IV activity in GK rats was significantly higher in the portal vein compared to that of Wistar rats. Diet did not affect DPP-IV activity both in Wistar and GK rats (Table 3.3).

Table 3.3 Plasma parameters after 26-week feeding period

	WC	WH	GC	GH
Portal vein				
Glucose (mg/dL)	108.0 ± 4.2^{b}	$122.9 \pm 3.7^{b\S}$	172.1 ± 3.1^{a}	$189.5 \pm 6.9^{a\dagger}$
Insulin (nM)	2.23 ± 0.20^{b}	$3.94 \pm 0.23^{a\S}$	2.32 ± 0.23^{b}	2.46 ± 0.20^{b}
Total GLP-1 (pM)	9.6 ± 0.9	15.1 ± 2.6	10.1 ± 0.8	12.7 ± 1.4^{NS}
HOMA-IR	14.9 ± 1.3^{b}	$30.0 \pm 1.9^{a\S}$	24.8 ± 2.4^{a}	28.8 ± 2.5^{a}
DPP-IV (mU/mL)	23.0 ± 1.8^{b}	24.7 ± 1.0^{b}	31.3 ± 1.6^{a}	32.0 ± 0.9^{a}
Abdominal aorta				
Glucose (mg/dL)	123.4 ± 3.0^{c}	$136.8 \pm 3.6^{c\S}$	175.8 ± 4.7^{b}	$192.9 \pm 5.3^{a\dagger}$
TG (mg/dL)	72.9 ± 4.6^{c}	$112.9 \pm 10.8^{ab\S}$	100.8 ± 4.1^{b}	$132.2 \pm 3.8^{a\dagger}$
Cholesterol (mg/dL)	66.2 ± 3.8^{c}	$85.5 \pm 6.4^{b\S}$	79.5 ± 2.4^{bc}	$105.7 \pm 4.4^{a\dagger}$
DPP-IV (mU/mL)	22.8 ± 1.6	23.2 ± 1.2	27.2 ± 1.1	27.5 ± 1.4^{NS}

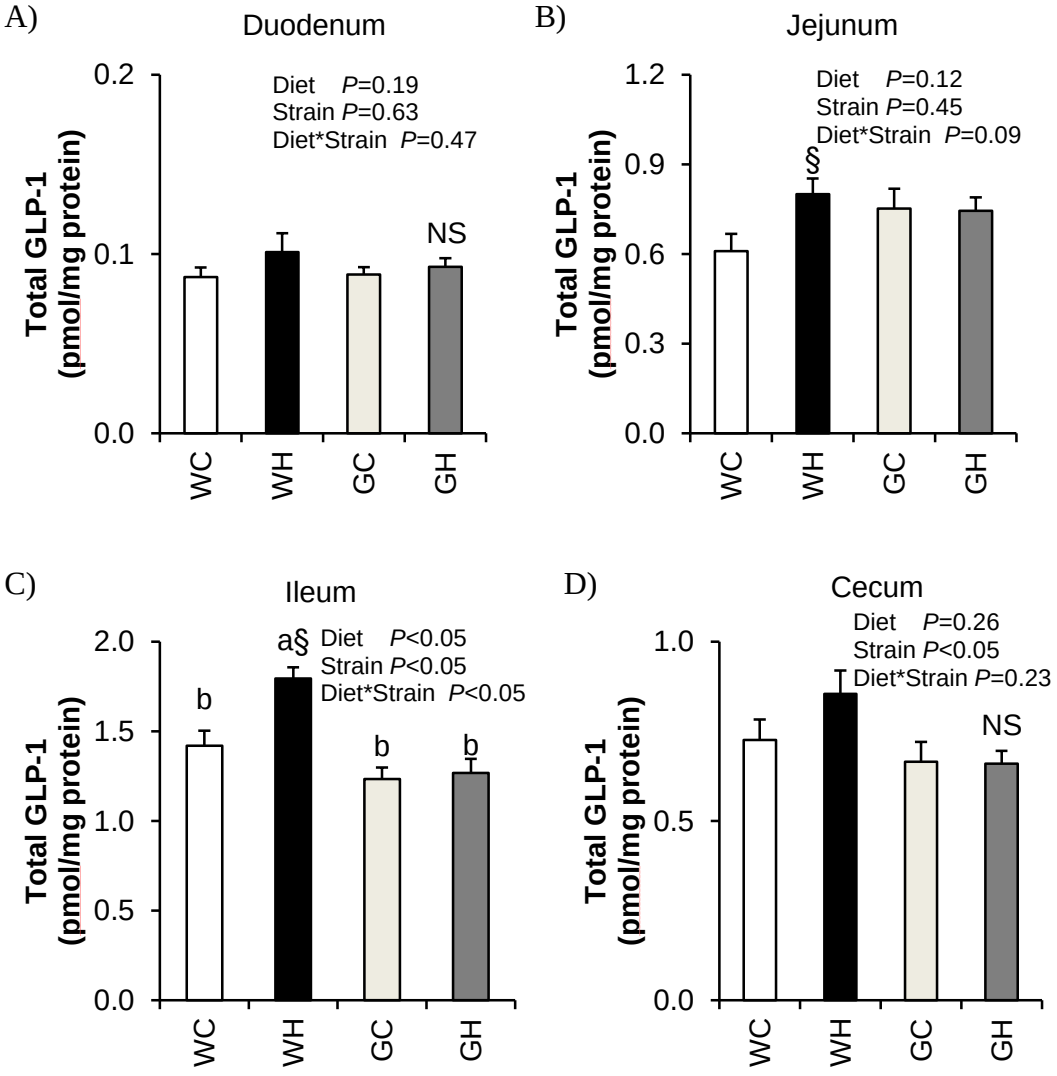
Wistar and diabetic GK rats were fed either control diet or HFS diet for 26 weeks. Blood samples from the portal vein and abdominal aorta were collected under sodium pentobarbital anaesthesia (50 mg/kg BW) after an overnight fasting. The values are expressed as the mean ± SEM for n = 7-9 rats. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). §Significant differences between mean values of the WC and WH groups and †Significant differences between mean values of the GC and GH groups ($P < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between the treatments.

Two-way ANOVA	Diet	Strain	Diet*Strain
Portal vein			
Glucose	$P < 0.05$	$P < 0.05$	$P = 0.80$
Insulin	$P < 0.05$	$P < 0.05$	$P < 0.05$
Total GLP-1	$P < 0.05$	$P = 0.56$	$P = 0.34$
HOMA-IR	$P < 0.05$	$P = 0.05$	$P < 0.05$
DPP-IV	$P = 0.71$	$P < 0.05$	$P = 0.92$
Abdominal aorta			
Glucose	$P < 0.05$	$P < 0.05$	$P = 0.43$
TG	$P < 0.05$	$P < 0.05$	$P = 0.51$
Cholesterol	$P < 0.05$	$P < 0.05$	$P = 0.45$
DPP-IV	$P = 0.79$	$P < 0.05$	$P = 0.98$

Two-way ANOVA result of plasma parameters after 26-week feeding period

3.3.7 GLP-1 content in the intestinal tissues and insulin content in the pancreatic tissue

WH group had higher GLP-1 concentrations than those of the WC group in the jejunal and ileal segments, whereas no difference was observed in the intestinal GLP-1 concentrations between the GC and GH groups (Fig. 3.6B, C). Significant effects of diet, strain, and strain by diet interaction on GLP-1 content were detected by two-way ANOVA in the ileum. In the pancreas, WH group had higher insulin content compared to that of the WC group, whereas GC and GH groups showed similar insulin content (Fig. 3.6F). Significant effects of diet and strain on pancreatic insulin content were detected by two-way ANOVA.



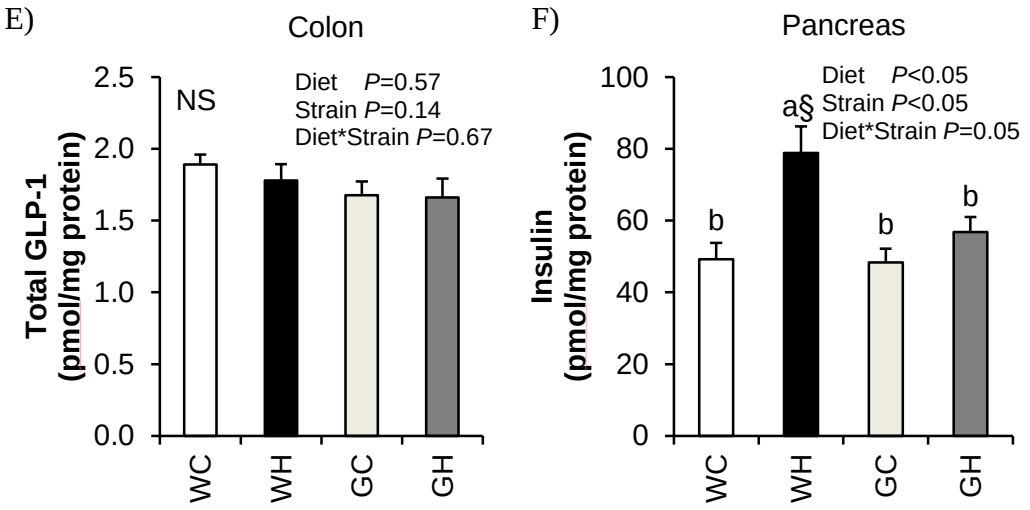


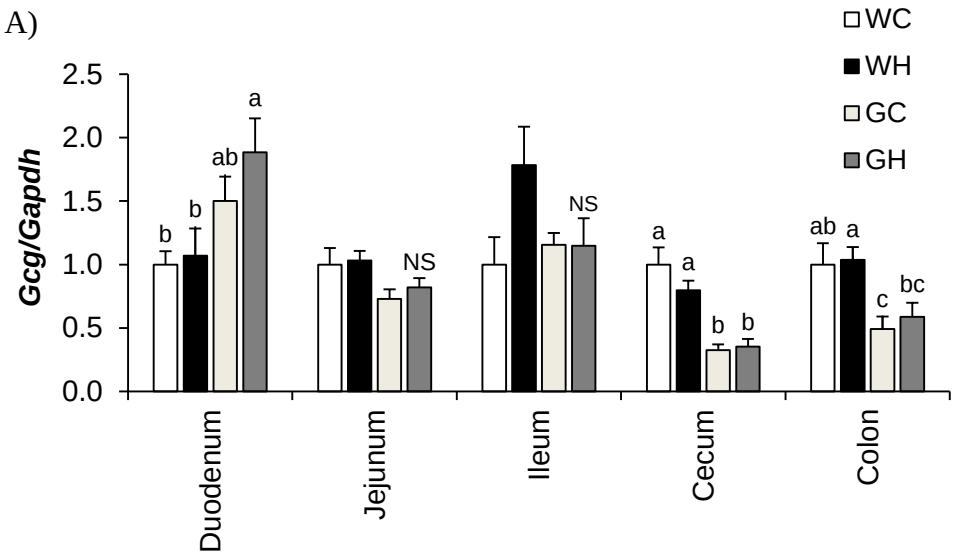
Figure 3-6. GLP-1 content in the intestinal tissues and insulin content in the pancreas after 26 weeks of feeding period

Wistar rats and diabetic GK rats were fed either control diet or HFS diet for 26 weeks. Each intestinal segment: (A) duodenum, (B) jejunum, (C) ileum, (D) cecum, and (E) colon and (F) rat pancreas were collected after killing. Data are presented as the mean ± SEM (n = 7-9 rats in each group). *P* values of two-way ANOVA for diet, strain, and strain by diet interactions are shown in each panel. The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). §Significant differences between mean values of the WC and WH groups and †Significant differences between mean values of the GC and GH groups ($P < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between the treatments.

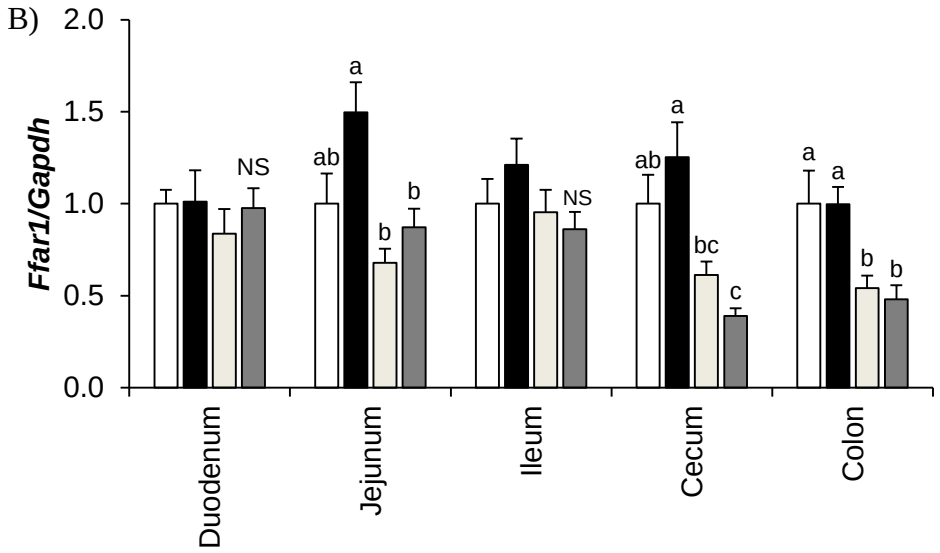
3.3.8 mRNA expression analysis of Proglucagon (*Gcg*) and free fatty acid receptors (*Ffar1*, *Ffar2*, *Ffar3*, and *Ffar4*) in each intestinal segment and pancreas

In the duodenum segment, *Gcg* mRNA expression level tended to be higher in GK rats than in Wistar rats. In contrast, GK rats had lower *Gcg* mRNA expression levels in the cecum and colon segments. Significant effects of diet were not seen observed in *Gcg* mRNA expression levels of any intestinal regions Fig. 3.7A. High fat rather than high sucrose had higher impact on enhancing GLP-1 responses during DIO (experiment I), and fatty acid receptors function in enteroendocrine cells as sensors for fatty acids trigger gut hormone secretions (Hirasawa, 2005; Ekberg, 2016). In the jejunum of WH group, *Ffar1*

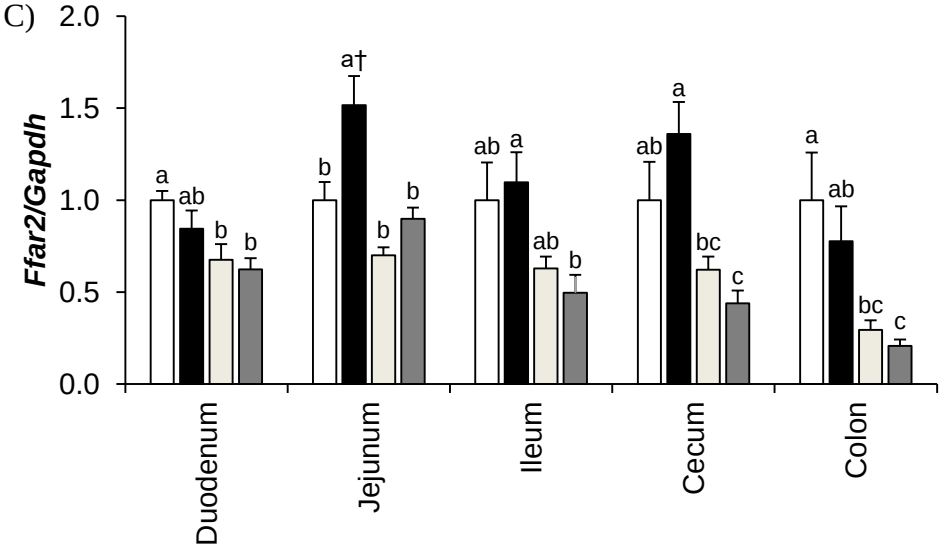
mRNA level tended to be higher ($P = 0.0534$, Student's *t*-test) and *Ffar2* mRNA level was significantly higher than those in the WC group (Fig. 3.7B and C). Although diet treatment had no effect, *Ffar4* mRNA expression was almost undetectable in the quantitative PCR in GK rats; however, it was normally detectable in pancreas of GK rat and in all intestinal segments of Wistar rats (Fig. 3.7E).



Two-way ANOVA	Duodenum	Jejunum	Ileum	Cecum	Colon
Diet	$P = 0.30$	$P = 0.49$	$P = 0.09$	$P = 0.30$	$P = 0.58$
Strain	$P < 0.05$	$P < 0.05$	$P = 0.29$	$P < 0.05$	$P < 0.05$
Diet*Strain	$P = 0.47$	$P = 0.74$	$P = 0.09$	$P = 0.17$	$P = 0.80$

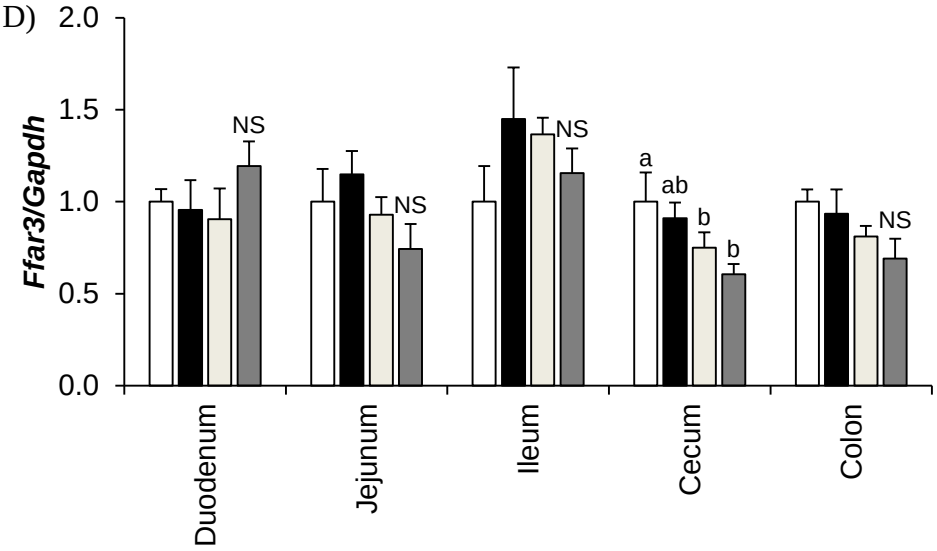


Two-way ANOVA	Duodenum	Jejunum	Ileum	Cecum	Colon
Diet	$P = 0.57$	$P < 0.05$	$P = 0.63$	$P = 0.90$	$P = 0.77$
Strain	$P = 0.45$	$P < 0.05$	$P = 0.12$	$P < 0.05$	$P < 0.05$
Diet*Strain	$P = 0.62$	$P = 0.25$	$P = 0.23$	$P = 0.07$	$P = 0.79$



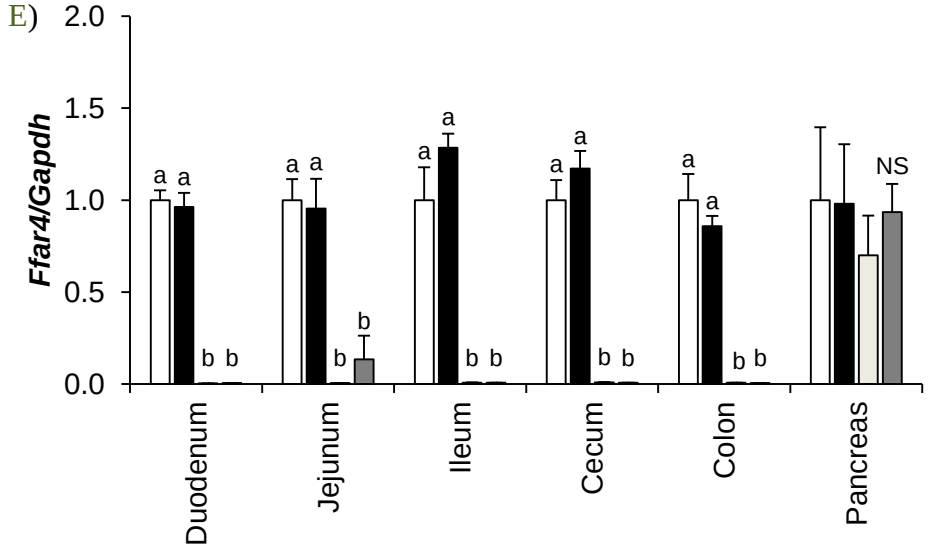
Two-way ANOVA	Duodenum	Jejunum	Ileum	Cecum	Colon
Diet	$P = 0.19$	$P < 0.05$	$P = 0.90$	$P = 0.52$	$P = 0.31$
Strain	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Diet*Strain	$P = 0.51$	$P = 0.12$	$P = 0.41$	$P = 0.06$	$P = 0.66$

D)



Two-way ANOVA	Duodenum	Jejunum	Ileum	Cecum	Colon
Diet	$P = 0.40$	$P = 0.40$	$P = 0.53$	$P = 0.24$	$P = 0.36$
Strain	$P = 0.62$	$P = 0.62$	$P = 0.85$	$P < 0.05$	$P < 0.05$
Diet*Strain	$P = 0.25$	$P = 0.25$	$P = 0.09$	$P = 0.77$	$P = 0.79$

E)



Two-way ANOVA	Duodenum	Jejunum	Ileum	Cecum	Colon	Pancreas
Diet	$P = 0.71$	$P = 0.73$	$P = 0.13$	$P = 0.22$	$P = 0.30$	$P = 0.70$
Strain	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P = 0.54$
Diet*Strain	$P = 0.69$	$P = 0.47$	$P = 0.12$	$P = 0.21$	$P = 0.31$	$P = 0.65$

Figure 3.7. mRNA expression levels of Proglucagon (*Gcg*) and fatty acid receptors (*Ffar1*, *Ffar2*, *Ffar3*, and *Ffar4*) in the intestinal mucosa of rats after a 26-week feeding period in either control or HFS diet group

Wistar rats and diabetic GK rats were fed either control diet or HFS diet for 26 weeks. Intestinal mucosa was collected from each intestinal segment after sacrificing the animals. The mRNA expression levels were determined by real-time quantitative PCR (qPCR). Data are presented as the relative expression level to that of the control group normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA expression level. Data are presented as the mean ± SEM (n = 7-9 rats in each group). The superscripts (a, b, c) with different letters show significant differences between treatments ($P < 0.05$, Tukey–Kramer's test). §Significant differences between mean values of the WC and WH groups and †Significant differences between mean values of the GC and GH groups ($P < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between the treatments.

3.4 Discussion

The aims of this study were to investigate whether the adaptive enhancement of postprandial GLP-1 responses sustains or disappears during long-term DIO. In addition, whether the adaptive change in GLP-1 response occurs or not in the diabetic model rats fed an HFS diet were also investigated. The results showed that the postprandial glycemic responses in WH group were higher than those in the WC group after 16 weeks of feeding period. However, the postprandial insulin, and GLP-1 responses were significantly higher or slightly but not significantly higher in the WH group than in the WC group throughout the study. Although GK rats showed higher glycemic responses than Wistar rats regardless of the dietary intervention, GK rats fed with GH had higher glycemic responses compared to the rats fed with GC. In contrast to Wistar rats (WC *vs*. WH), both the GK groups (GC and GH) had similar postprandial insulin and GLP-1 responses in all MTTs. Small intestinal GLP-1 production and *Ffar* mRNA expression levels were enhanced by HFS feeding in Wistar rats; however, such alteration was not observed in GK rats in response to HFS diet. These results demonstrate that the enhanced postprandial GLP-1 response was sustained but not impaired after prolonged treatment with an obesogenic diet. Furthermore, adaptive changes of the postprandial GLP-1 response did not occur in the genetic diabetic

model rats. The difference in the GLP-1 responses between Wistar and GK rats could be involved in the development of glucose intolerance.

After 26 weeks of HFS feeding, both strains had higher BW and visceral adipose tissue weight, compared to the respective control group, confirming the potent impact of HFS diet even on diabetic model rats. Wistar rats had higher glycemic response after 16 weeks of HFS diet intake; however, GK rats had higher glycemic response already after 4 weeks of HFS diet consumption. Even after 24 weeks of HFS diet feeding, WH rats still had lower fasting and postprandial glucose levels than those in GC rats. Consistent with the results in a previous study, although Wistar rats fed a high fat diet (10-month) developed glucose intolerance, they did not develop diabetes (Chalkley, 2015). Although it is not universally authorized, a previous study used following criteria for diabetes in rats; a peak of plasma glucose concentration > 16.8 mM (302.9 mg/dL) or 120-min postload plasma glucose concentration > 11.2 mM (201.6 mg/dL) (Wang, 2003).

In the present study, postprandial GLP-1 response increased after treatment with the HFS diet in non-diabetic Wistar rats, which was consistent with the results of the previous studies using non-diabetic Sprague–Dawley rats (experiment 1 and 2). In GK rats, both control and HFS diet feeding showed a similar postprandial GLP-1 response. Likewise, the GLP-1 levels were not observed to be different between the control subjects and patients with type 2 diabetes (Laferrère, 2007).

Acetaminophen appearances in MTTs remained unchanged among treatments throughout the experimental period, indicating that the nutrient delivery to the small intestine (gastric emptying rate) does not explain the differences in GLP-1 responses. Accordingly, the sensitivity of GLP-1-producing L-cells to luminal nutrients (e.g., proteins, peptides, carbohydrates, or fatty acids) seems to be enhanced by chronic HFS diet feeding in Wistar rats, but not in GK rats. This further suggests that the increased jejunal and ileal

GLP-1 contents in response to HFS feeding in Wistar rats, and not in GK rats, are responsible for the increased GLP-1 secretion. Based on these results, it is likely that L-cells in the jejunum and ileum are the major sources of postprandial GLP-1 secretion. These findings are consistent with the results of the previous studies in which postprandial GLP-1 secretion was found to be a direct action of the luminal contents on L-cells in the distal jejunum and ileum (Singh, 2015; Wang, 2015).

In the present study, the *Gcg* mRNA expression levels were not correlated with GLP-1 contents in intestinal segments. This might be due to some other factors involved in GLP-1 production, secretion, and degradation, such as DPP-IV (dipeptidyl peptidase) and prohormone convertase 1/3 activity (Rouillé, 1997; Morimoto, 2016). In experiment 1, HFS and HiFat diet had a potent effect on postprandial GLP-1 response rather than HiSuc diet feeding (Pinyo, 2019a). Hence, the mRNA expression of free fatty acid receptors (*Ffar1*, *Ffar2*, *Ffar3*, and *Ffar4*) was investigated in the present study, and *Ffar1* mRNA expression level in the jejunum of WH rats tended to be higher than that of WC rats. The results suggest that chronic HFS diet feeding enhanced the sensitivity to long chain fatty acids possibly in GLP-1 producing cells (Beloqui, 2016; Thombare, 2017). Although specific reasons are not entirely clear, Ffar4 mRNA was almost undetectable in GK rats regardless of diet consumption. In contrast to intestinal *Ffar4* mRNA expression, pancreatic *Ffar4* mRNA expression showed a similar level in both diabetic and non-diabetic rats. Thus, the development of glucose intolerance in GK rats might be partly due to *Ffar4* deficiency in the intestine. In support our hypothesis, dysfunction of lipid sensor *Ffar4* has been found to cause glucose intolerance in mice (Ichimura, 2012).

GK rats are well recognized as type 2 diabetes models, with reduced β-cell number, and impaired glucose-induced insulin secretion (Kindel, 2009; Amiri, 2015; Shirai, 2016). Although impaired insulin secretion was not observed in both the GK groups, GH rats

failed to elicit an enhanced insulin response compared to GC rats. Similar trend was observed in pancreatic insulin content, suggesting that the β-cells in Wistar rats fed an HFS diet were capable of producing compensatory insulin to maintain a normal blood glucose level. Genetic defects in GK rats, such as impaired development of pancreatic β-cell or abnormal β-cell insulin secretory machinery (Movassat, 1997; Shang, 2002) may be partially explained by a lack of enhanced GLP-1 secretion response as observed in GH rats.

The enhancement of fasting GLP-1 and insulin may play a role in the prevention of blood glucose elevation in non-diabetic Wistar rats. However, these adaptations were absent in diabetic GK rats. Interestingly, GK rats had higher plasma DPP-IV activity than Wistar rats regardless of diet composition. In addition, a previous study showed a higher basal DPP-4 activity in the GK rats relative to the Wistar rats (Keller, 2015). Although it does not seem to be universal, an elevated plasma DPP-IV activity has been previously demonstrated in type 2 diabetic patients (Mannucci, 2005; Ryskjaer, 2006). Possibly, higher DPP-IV activity in GK rats is involved in unchanged basal insulin level. However, the mechanisms that underlie an increase in plasma DPP-IV activity remain unclear. A previous study reported that the chronic exposure to high glucose (1 week) can determine an increase in endothelial DPP-IV mRNA expression and protein secretion in human glomerular endothelial cells (Pala, 2003).

3.5 Conclusions

After a long-term (24 weeks) feeding of HFS diet, in Wistar rats, postprandial GLP-1 responses were sustained higher or tended to be higher compared to the control group throughout the study, whereas diabetic GK rats had similar postprandial GLP-1 responses regardless of diet feeding. Additionally, GLP-1 content and *Ffar1* mRNA expression level in the jejunum segment were elevated and tended to be higher in Wistar rats after HFS diet

feeding, but not in GK rats. The results suggest that the sensitivity of GLP-1 producing L-cells to long-chain fatty acids was enhanced by chronic HFS diet feeding in Wistar rats. Failure of adaptive enhancement of GLP-1 response in GK rats could be responsible for glucose intolerance development.

General discussion

Understanding the relationship between GLP-1 secretory responses and metabolic status during the progression of diet-induced obesity (DIO) may provide valuable information applicable to the management or prevention of glucose intolerance, obesity, and type 2 diabetes in the future. It is difficult to conduct DIO in human study in a designed experiment. Therefore, animal experiments are advantageous to explore the mechanisms involved in the development of metabolic diseases including obesity and diabetes. In order to determine adaptive changes in postprandial GLP-1 during the progression of DIO and diabetic state, animal models continuously fed obesogenic diets were employed in the present studies in this thesis. Accordingly, it was possible to monitor the changes in postprandial response (glucose, insulin, GLP-1, and gastric emptying) during obesity development.

Throughout the study in this thesis, a high fat diet (HiFat, 30% w/w), a high sucrose diet (HiSuc, 40% w/w), or a high fat and high sucrose diet (HFS, 30% fat and 40% sucrose w/w) were used to establish obesity in the animal models (Nakajima, 2015). The results confirmed that consumption of HFS diet as an obesogenic diet induced fat accumulation in various tissues and obesity development in both non-diabetic and diabetic rats. In contrast to previous studies demonstrating attenuation of GLP-1 secretion in obesity and diabetes (Meyer-gerspac, 2014; Faerch, 2015), the results in this thesis demonstrated that postprandial GLP-1 response was enhanced during the progression of DIO. The enhanced GLP-1 response appeared to play a role in maintaining a normal postprandial glycemic response in normal rat (both SD and Wistar rats). However, the protective adaptation was absent in the diabetic GK rats. It was implied that enhancing the GLP-1 secretion by certain materials (not by fat or sucrose) could be a promising target for prevention and treatment

of glucose intolerance and obesity.

Not only incretin effect and insulin hormone, gastric emptying rate also plays a role in controlling glycemic response (Marathe, 2013). Gastric emptying rate is regulated by various gut hormones such as ghrelin, serotonin, CCK, PYY, not only on GLP-1(Hellström, 2006; Nakajima, 2015). In overall study, the results showed that gastric emptying rates were unchanged by the chronic HFS diet or Ex9 treatment, compared to the control group, suggesting that the role of endogenous GLP-1 as incretin hormone, independently of effect on gastric emptying (Witte, 2010). In human studies showed no significant changes in gastric emptying rate by acute infusion of Ex9 before meal ingestion (Witte, 2010; Nicolaus, 2011). Likewise, the prevalence of impaired gastric emptying in obesity and diabetes is still controversial. The previous studies reported that gastric emptying in human with obesity and diabetes were normal, accelerated, or slow, compared to normal subjects (Loo, 1984; Bertin, 2001; Buchholz, 2013; Acosta, 2015; Nguyen, 2018). In accordance with my study, obesity and diabetes had no effect on gastric emptying (Bertin, 2001; Buchholz, 2013).

Gastrointestinal hormones such as GLP-1 and GIP play an important role in the regulation of postprandial glucose homeostasis under normal condition (Drucker, 2007; Holst, 2016). However, obesity and diabetes mellitus have been reported to have detrimental effects on gut hormone secretion (Farilla, 2002; Gribble, 2019). Not only GLP-1 secretion, the secretion of GIP, CCK, and PYY are also altered in obesity and diabetes (Steinert, 2017; Zakrzewski-Fruer, 2017; Dirksen, 2019). Basal PYY was significantly higher in overweight/obese subjects than in lean subjects, but there were no significant differences in basal CCK as well as postprandial PYY and CCK levels between overweight/obese and lean subjects (Damgaard, 2013; Dirksen, 2019). Fasting CCK and postprandial CCK concentration were reduced in patients with type 2 diabetes, compared

to normal subjects (Bucceri, 2002). Both fasting PYY and postprandial PYY concentrations were lower in obese rats rather than the control rats (Feng, 2019). In type 2 diabetic patients, the circulating levels of GIP are normal (Knop, 2007) or increased (Jones, 1989) in basal and postprandial conditions. Although the existing data suggest that both GIP and GLP-1 have potent effect on insulinotropic properties under normal condition, the insulinotropic effects of GIP are virtually absent and seems less effective than GLP-1 in diabetes (Kinde, 2009; Nauck, 2016). From the previous studies, it can be concluded that the secretion of gut hormones in obese and diabetes remains unclear. Also, several gut hormones, such as CCK and PYY, also exhibit actions important for glucoregulation. Therefore, not only GLP-1, but GIP, CCK, and PYY should be further investigated to determine the relation of gut hormones secretion in the regulation of postprandial glucose homeostasis during the progression of DIO.

Free fatty acid receptors (*Ffars*) are highly enriched in enteroendocrine cells and provides the relation between dietary fats and gut hormones secretion (Lu, 2018). The results in experiment 3 showed that small intestinal *Ffar1* mRNA expression levels tended to be increased by HFS feeding in Wistar rats, but not in GK rats, suggesting that the sensitivity of GLP-1 producing L-cells to long chain fatty acid seems to be enhanced by chronic HFS diet feeding (Beloqui, 2016; Thombare, 2017). In addition, GLP-1 production (jejunum and/or ileum) was increased by HFS feeding in non-diabetic rats (both Wistar and SD rats). This may be the consequence of increased fatty acid sensitivity, and also may be one of causes of enhanced postprandial GLP-1 response under obesity. In contrast to mRNA expression of *Ffar4* in pancreas, *Ffar4* mRNA expression in all intestinal segments was almost undetectable in GK rats, suggesting that glucose intolerance development in GK rats might be due to *Ffar4* deficiency in the intestinal segments (experiment 3). Consistently, dysfunction of lipid sensor *Ffar4* has been found to cause glucose intolerance

in mice (Ichimura, 2012). Sweet taste receptors and bitter taste receptors might be involved in regulating GLP-1 release (Kok, 2018; Feng, 2019). In addition, nutrient transporters such as sodium glucose transporters (SGLTs) and glucose transporter are also involved in GLP-1 secretion (Sun, 2017). Overall, mRNA and protein expression of sweet taste receptors such as Tas1r2, and Tas1r3 were not analyzed throughout the study. Since HFS diet composes of both fat and sucrose, mRNA expression and protein expression of both free fatty acid receptors and sweet taste receptor as well as nutrient transporters (e.g. SGLT1, GLUT5, and GLUT2) need to be investigated in DIO models to identify the pathways that may underlie the L-cells response to food ingestion.

The major source of postprandial GLP-1 secretion is still not clearly elucidated. Although the L-cells are predominantly located in the ileum and colon (Lim, 2006), GLP-1 is reportedly secreted primarily from enteroendocrine L-cells located in the distal jejunum and ileum (Lean, 2016). The results in experiment 1 suggest that jejunum segment might be the major source of postprandial GLP-1 secretion, whereas experiment 2 indicates the ileum segment, since GLP-1 content increased by HFS diet in these regions. Therefore, it is necessary to analyze total L-cell number and L-cell distribution throughout the rat intestinal tract in order to clarify the major source of postprandial GLP-1 secretion during obesity development in both diabetic and non-diabetic rats.

The underlying mechanisms involved in the increment of postprandial GLP-1 secretion are still not clearly elucidated in the current study. Although possible involvement of increased fatty acid receptor expression and/or GLP-1 production in the small intestine were suggested, upstream pathways and other possible pathways need to be clarified. However, understanding the relationship between GLP-1 secretory responses and metabolic status described in the study could contribute to a better design of therapeutic and preventive approaches for obesity and diabetes. Enhancing GLP-1 secretion may hold

the key for management of obesity and type 2 diabetes. Manipulating the diet, supplementary ingredients, and/or therapeutics in a way to promote the interactions with free fatty acid receptors could increase GLP-1 secretion and enhance its beneficial effects for obesity and diabetes. Finally, the potential side effects of any treatments should be further considered before applying to humans.

Conclusion

HiFat diet rather than HiSuc diet has a potent impact on postprandial GLP-1 response and obesity (adiposity) development in rats. Moreover, HFS diet showed much impact on them compared to the HiFat diet alone. The enhancement of GLP-1 secretion during obesity development plays an important role in maintaining normal postprandial glycemia through increasing insulin secretion. However, the adaptation was absent in the diabetic GK rats, implying that failure of adaptive enhancement of GLP-1 response accelerates the development of glucose intolerance. Increased GLP-1 production and fatty acid sensitivity in the small intestine are potent factors that are involved in enhanced GLP-1 responses under obesity. A better understanding of GLP-1 secretion and its adaptation will lead us to design therapeutics or dietary interventions that modulate GLP-1 secretion by certain materials.

Appendices

Appendix A: Composition of AIN-93G Vitamin mixture

Ingredients	g/kg Diet
Nicotinic acid	3.000
Calcium Pantothenic acid	1.600
Pyridoxine hydrochloride	0.700
Thiamin hydrochloride	0.600
Riboflavin	0.600
Folic acid	0.200
D-Biotin	0.020
Cyanocobalamin[1]	2.500
All-rac-α-tocopherylacetate	75.000
All-trans-retinyl acetate[2]	0.842
Cholecalciferol	0.250
Phylloquinone	7.500
Sucrose	907.188
Total	1000

[1] Cyanocobalamin 10 mg mixed with Sucrose 9.99 g

[2] Sigma 475000 IU

Appendix B: Composition of AIN-93G Mineral mixture

Ingredients	g/kg Diet
Calcium carbonate ($CaCO_3$)	357
Potassium phosphate monobasic (KH_2PO_4)	196
Tripotassium citrate monohydrate ($KOCOCH_2C(OH)(COOK)CH_2COOK \cdot H_2O$	70.78
Sodium chloride (NaCl)	74
Potassium sulfate (K_2SO_4)	46.6
Magnesium oxide (MgO)	24
Ferric citrate ($FeC_6H_5O_7 \cdot nH_2O$)	6.06
Zinc carbonate ($ZnCO_3$)	1.65
Manganous carbonate ($MnCO_3$)	0.63
Cupric carbonate ($CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$)	0.3
Potassium iodate (KIO_3)	0.01
Sodium selenate (Na_2SeO_4)	0.01025
Ammonium molybdate ($(NH_4)Mo_7O_{24} \cdot 4H_2O$)	0.00795
Sodium metasilicate nanohydrate ($Na_2SiO_3 \cdot 9H_2O$)	1.45
Chromium potassium sulfate ($CrK(SO_4)_2 \cdot 12H_2O$)	0.275
Lithium chloride (LiCl)	0.0174
Boric acid (H_3BO_3)	0.0815
Sodium fluoride (NaF)	0.0635
Nickel (II) carbonate ($NiCO_3 \cdot 2Ni(OH)_2 \cdot 4H_2O$)	0.0318
Ammonium vanadate (NH_4VO_3)	0.0066
Sucrose	221.026
Total	1000

Appendix C: Experimental diet compositions

Ingredients	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	–
Fiber (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
Energy density (kcal/g)	4.03	5.18	5.18	4.03

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

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

3) Avicel PH102 in experiment 1 (Asahi Kasei Chemicals Corporation, Tokyo, Japan) or Just Fiber BH200FCC in experiment 2 and 3 *(*Morimura Bros., Inc, Tokyo, Japan);

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

Appendix D: Intestinal mucosa and tissue collection

After killing, intestinal segments were carefully dissected and washed with cold saline solution (0.9% NaCl) before collecting intestinal mucosa and tissue samples.

- Intestinal mucosa (30-50 mg) was scrapped by using sterilize glass slide from each intestinal segment for mRNA expression analysis.
- Intestinal tissue samples (2 cm) were collected from each intestinal segment in order to measure GLP-1 content.

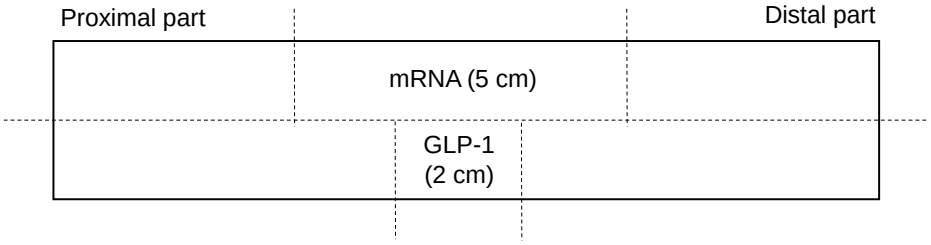


Figure A1. Intestinal mucosa and tissue collection from duodenum segment.

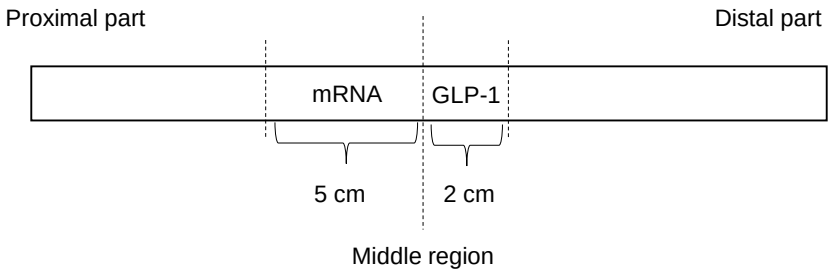


Figure A2. Intestinal mucosa and tissue collection from jejunum segment.

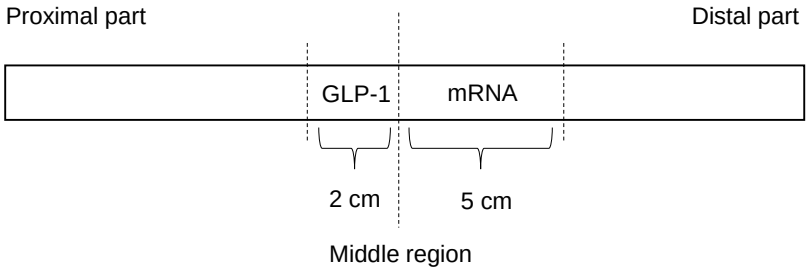


Figure A3. Intestinal mucosa and tissue collection from ileum segment.

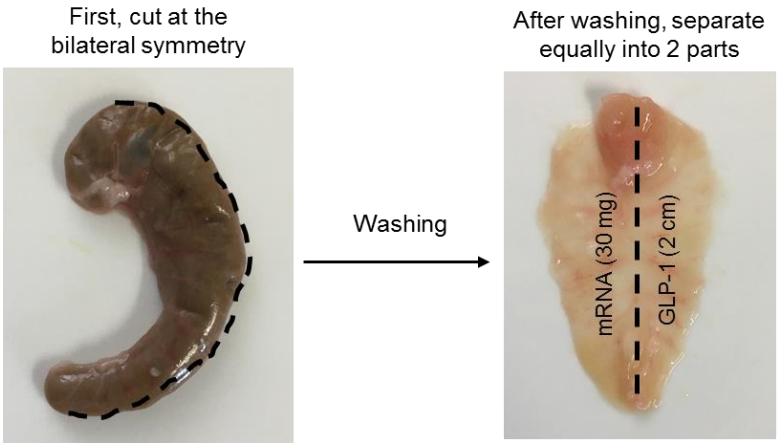


Figure A4. Intestinal mucosa and tissue collection from cecum.

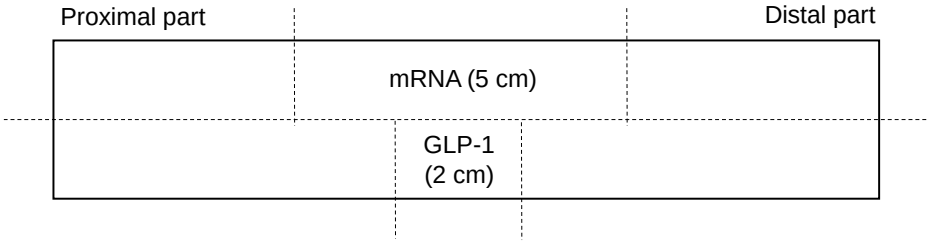


Figure A5. Intestinal mucosa and tissue collection from colon segment.

Appendix E: GLP-1 receptor antagonist exendin (9-39) preparation

1. Exendin 9 (Ex9, analytical grade, purity > 95%; Thermo Fisher Scientific Inc., Waltham, MA, USA) was prepared at a dose of 100 µg/day/rat (9.5 mM or 32 mg/mL) by dissolving in sterile saline.

2. Ex9 solution (220 µL) or vehicle (0.9% NaCl) was transferred to Alzet Mini-osmotic pump (Alzet Mini-osmotic pump, Model 2006, Durect corporation, CA, USA) following the manufacturer's instructions.

3. The rats were anesthetized under sodium pentobarbital (50 mg/kg body weight) before operation.

4. The osmotic pump was then subcutaneously implanted in the interscapular region to deliver Ex9 or vehicle at a constant rate of 0.13 µL/h throughout the experimental study (Figure A6).

Figure A6. Implantation of the osmotic pump

Appendix F: Glucose measurement (Glucose CII Test Kit, 439-90901; Wako Pure Chemical Industries, Osaka, Japan)

1. Five microliters of each plasma sample or standard solution (0, 50, 100, 200, and 300 mg/dL) was transferred to 48-well plate (Costar, Corning, New York, USA).
2. Color reagent solution (Glucose CII Test Kit, 750 µL) was transferred to each well.
3. The plate was then incubated with shaking at 300 rpm for 15 min.
4. The absorbance was detected at 505 nm (reference 600 nm) by using microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

Appendix G: Insulin measurement (Rat insulin ELISA Kit TMB or AKRIN-010T Shibayagi, 637-01471 Wako Pure Chemical Industries, Osaka, Japan)

Preparation step

I. Washing buffer was prepared by mixing 50 mL of washing buffer concentrate (10x) and 450 mL of MilliQ water.

Reaction step

1. The anti-insulin-coated plate was washed with washing buffer for 4 times (300 µL of washing buffer/time)*.
2. Biotin conjugated anti-insulin (100 µL) was pipetted to the plate and shaken at 600-1200 rpm for 3 times (10 s/time). Biotin conjugated anti-insulin was diluted 4000 times with buffer solution before using.
3. Ten microliters of each sample** or standard solution (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0 ng/mL) were transferred to each well. The reaction mixture was shaken at 600-1200 rpm for 10 seconds (3 times) and was then sealed with plate sealer. The solution was incubated for 2 h at 20-25ºC. After incubation, mixed solution was discarded and washed with washing buffer for 4 times (300 µL of wash buffer/time)*.
4. One hundred microliters of HRP conjugated streptavidin was added and mixed thoroughly by shaking the plate at 600-1200 rpm for 10 seconds (3 times) then the plate was sealed with plate sealer. The reaction was incubated for 30 min at 20-25ºC. HRP conjugated streptavidin was diluted 2000 times with buffer solution before using. After incubation, HRP conjugated streptavidin solution was discarded and washed with washing buffer for 4 times (300 µL of wash buffer/time)*.
5. After that, 100 µL of substrate chromogen reagent (TMB) was added and shaken on the plate at 600-1200 rpm for 10 seconds (3 times) then the plate was sealed with plate sealer. The reaction was then incubated again for 30 min at 20-25ºC.

6. Finally, stop solution was immediately added to the well.
7. The plate was measured the absorbance (main 450 nm, reference 620 nm) by using microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

   * Washing buffer in the plate was discarded then the plate was struck on paper towel several times in order to remove remained wash buffer in the well.

   ** Blood samples from abdominal aorta and tail vein were diluted 2 times with buffer solution, whereas portal blood samples were diluted 5 times before measurement (depended on experiment).

Appendix H: Insulin measurement (U-E type, AKRIN-130; Shibayagi Company Limited, Gunma, Japan)

Preparation step

I. Washing buffer was made by mixing 50 mL of washing buffer concentrate (10x) and 450 mL of MilliQ water.

Reaction step

1. The anti-insulin-coated plate was washed with washing buffer 4 times (300 µL of wash buffer/time)*.
2. Biotin conjugated anti-insulin (100 µL) was immediately added into the plate after washing process. Then the plate was shaken at 600-1200 rpm for 3 times (10 s/time). Biotin conjugated anti-insulin was diluted 100 times with buffer solution before using.
3. 10 µL of sample** and standard solution (2500, 1250, 625, 313, 156, 78, 39, and 0 pg/mL) were transferred to each well then reaction mixture was blended by shaking the plate at 600-1200 rpm for 10 second (3 times) then plate was sealed with plate sealer. The reaction mixture was incubated for 2 h at 20-25ºC. After incubation, the reaction mixture was discarded and washed with washing buffer 4 times (300 µL of wash buffer/time)*.
4. One hundred microliters of Peroxidase-avidin conjugate was added and shaken on a plate shaker at 600-1200 rpm for 10 seconds (3 times) then the plate was sealed with plate sealer. The reaction was incubated for 30 min at 20-25ºC. HRP conjugated streptavidin was diluted 100 times with buffer solution before using. After incubation, the solution was discarded and washed with washing buffer 4 times (300 µL of wash buffer/time)*.
5. Then 100 µL of coloring solution (TMB) was added and mixed by shaking the plate at 600-1200 rpm for 10 seconds (3 times) then the plate was sealed with plate sealer. The reaction was incubated for 30 min at 20-25ºC.

6. Lastly, stop solution was immediately transferred to the well and shake at 600-1200 rpm for 10 seconds (3 times).
7. The plate was detected the absorbance (main 450 nm, reference 620 nm) by using microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

   *Washing buffer in the plate was discarded then plate was struck on paper towel several times in order to remove remained wash buffer in the well.

   **Blood samples from tail vein were used without dilution (depended on experiment).

Appendix I: Total GLP-1 measurement (Multi Species GLP-1 Total ELISA, EZGLP1T-36K; Merck Millipore, Darmstadt, Germany)

Preparation step

I. Diluted wash buffer was prepared by mixing 50 mL of 10X concentrated HRP wash buffer and 450 mL of MilliQ water.

II. Standard was made by mixing 500 µL of MilliQ water and GLP-1 (1000 pM, please check before using) standard powder then the standard solution was mixed thoroughly. Assay buffer (AB buffer) was used to dilute other standard concentrations instead of MilliQ water.

Reaction step

1. First, microtiter assay plate was washed with diluted washing buffer 3 times (300 µL of diluted washing buffer/time) then the plate was inverted on paper towel several times in order to discard the diluted washing buffer.
2. Then assay buffer (50 µL) or matrix solution (70 µL) was immediately added into the well.
3. Each standard concentration (200, 100, 50, 20, 10, 5 and 0 pM, 50 µL) and samples (30 µL) were transferred to the well. Then the plate was sealed with plate sealer and incubated at room temperature on a plate shaker (400-500 rpm) for 1.5 h. After incubation, the microtiter assay plate was washed with diluted wash buffer 3 times (300 µL of diluted washing buffer/time) then residual diluted wash buffer was removed by inverting the plate on paper towel several times.
4. 100 µL of detection antibody solution was added to the wells. After that, the microtiter assay plate was sealed with plate sealer and incubated at room temperature on a plate shaker (400-500 rpm) for 1 h. Then the microtiter assay plate was washed with diluted wash buffer 3 times (300 µL of diluted washing buffer/time) and residual diluted wash

buffer was removed by inverting the plate on paper towel several times.

5. Enzyme solution (100 µL) was transferred to each well. The microtiter assay plate was then sealed with plate sealer and incubated at room temperature on plate shaker (300 rpm) for 30 min. After the reaction, microtiter assay plate was washed with diluted washing buffer 3 times (300 µL of diluted wash buffer/time) then residual diluted wash buffer was removed by inverting the plate on paper towel several times.
6. 100 µL of substrate solution was added to the wells and the plate was sealed with plate sealer and incubated at room temperature on a plate shaker (300 rpm) for 20 min.
7. After incubation, stop solution was immediately transferred to each well.
8. The absorbance was measured at 450 nm (reference 590 nm) by using microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

Appendix J: Total GLP-1 measurement (GLP-1 ELISA Kit, High Sensitive, 299-75501; Wako Pure Chemical Industries, Osaka, Japan)

Preparation step

I. Diluted wash solution was prepared by mixing 50 mL of wash solution (10X) and 450 mL of MilliQ water.

II. GLP-1 Standard Solution (30 pM) was diluted with buffer solution in order to prepare each standard solution.

Reaction step

1. Antibody-coated Plate was washed with diluted wash solution 3 times (300 µL of diluted washing buffer/time) then the plate was inverted on paper towel several times in order to discarded the diluted washing buffer.
2. 50 µL of standards (30, 15, 7.5, 3.75, 1.88, and 0 pM) or samples were transferred to the well and were then sealed and shaken at 600-1200 rpm for 10 s (3 times). The plate was incubated at 20-25°C for 2 hours.
3. After incubation, the plate was washed with diluted wash solution 3 times (300 µL of diluted wash solution/time) then residual diluted wash buffer was removed by inverting the plate on paper towel several times.
4. Biotinylated Anti GLP-1 Antibody Solution (50 µl) was transferred to the wells. The plate was sealed with plate sealer and was shaken at 600-1200 rpm for 10 s (3 times) and then incubated at 20-25°C for 1 hour.
5. The plate was then washed with diluted wash solution 3 times (300 µL of diluted wash solution/time) and residual diluted wash buffer was removed by inverting the plate on paper towel several.
6. Peroxidase-conjugate Streptavidin Solution (50 µl) was transferred to each well. The plate was then sealed with plate sealer and was shaken at 600-1200 rpm for 10 s (3

times) incubated at room temperature for 30 min. After the reaction, microtiter assay plate was washed with diluted washing buffer 3 times (300 µL of diluted wash buffer/time) then residual diluted wash buffer was removed by inverting the plate on paper towel several times.

7. 50 µL of TMB Solution 50 µl was added to the wells and the plate was sealed with plate sealer and incubated at 20-25℃ for 20 min.
8. Stop solution was immediately transferred to each well after incubation.
9. The absorbance was measured at 450 nm (reference 620 nm) by using microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

Appendix K: Total triglyceride measurement (Triglyceride E-Test kit, 432-40201; Wako Pure Chemical Industries, Osaka, Japan)

1. Two microliters of standard solution (0, 100, 200, 300, and 596.1 mg/dL) and plasma samples (without dilution) were transferred to 96-well plate (Greiner, Frickenhausen, Germany).
2. Then 300 μL of color reagent was added into the wells and mixed by using plate shaker at 600 rpm for 10 s (3 times).
3. The reaction was then incubated at 37°C for 5 min.
4. The reaction plate was detected the absorbance at 600 nm (reference 700 nm) by using microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

Appendix L: Total cholesterol measurement (Cholesterol E Test kits, 439-17501; Wako Pure Chemical Industries, Osaka, Japan)

1. Three microliters of standard solution (200, 100, 50, 25, and 0 mg/dL) and plasma samples (without dilution) were transferred to 96-well plate (Greiner, Frickenhausen, Germany).
2. Then 300 μL of color reagent was added into the wells and mixed by using plate shaker at 600 rpm for 10 s (3 times).
3. After mixing well, the reaction plate was exactly incubated at 37°C for 5 min.
4. The reaction plate was measured the absorbance (main 600 nm, reference 700 nm).

Appendix M: Protein measurement (BCA Protein Assay kit, 23228; Pierce, Rockford, IL, USA)

Preparation step

I. BCA working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B).

Reaction step

1. 25 µL of each standard concentration or samples was added into each well of 96-well plate (Greiner, Frickenhausen, Germany).
2. BCA working reagent was transferred to each well and mixed plate thoroughly on a plate shaker 600 rpm for 3 times (10 sec/time).
3. The plate was sealed or covered and was then incubated at 37°C for 30 min.
4. After incubation, the plate was cooled down at room temperature for 10 min.
5. The absorbance was monitored at 562 nm by using microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

Appendix N: Protein measurement (Lowry's assay)

Preparation step

I. Reagent A (Alkaline solution) (1 L)

Na_2CO_3 (Wako 196-01595, Mw. 105.99) 0.188 M (20 g/L)

NaOH (Wako, 198-13765, Mw. 40) 0.1 M (4 g/L)

II. Reagent B (50 mL)

$CuSO_4 \cdot 5H_20$ 0.25 g.

Sodium Pottasium Tartrate Tetrahydrate (Wako, 192-02991, $C_4H_4KNaO_6 \cdot 4H_2O$) 0.5 g

III. Mixed reagent A and B was freshly prepared as a ratio of 50:1.

IV. Phenol Reagent Solution (Folin-Ciocalteu's Reagent Solution) (10 mL)

Phenol reagent (Nacalai tesque, 37205-45): H_2O = 1: 1

V. BSA standard (1 mL)

BSA (Sigma, A7030) 500 µg/ml

Reaction step

1. 50 µL of each standard concentration (protein conc. 100-1000 µg/ml, depend on experiment) or samples was transferred to 48-well plate (Costar, Corning, New York, USA).
2. Mixed reagent A and B solution (500 µL) was then added to the well.
3. The plate was then shaken at 750 rpm for 10 s (3 times) and incubated at 30 °C for 10 min.
4. Diluted Folin-Ciocalteu's Reagent Solution (50 µL) was transferred to the well.
5. The plate was shaken at 750 rpm for 10 s (3 times) again and incubated at 30 °C for 30 min.
6. After incubation, the plate was measured the absorbance at 500 nm (770 nm is also available).

Appendix O: **DPP-IV Activity measurement** (Karl, 2003; Flock, 2007; Ishikawa, 2015)

Reagent

1. Standard solution: *p*-nitroaniline (4-nitroaniline, *p*NA)

2. Substrate: 1.6 mM Gly-Pro-*p*-nitroaniline (Gly-Pro-*p*NA) (G2901; Sigma, Mw. 464.49)

3. Assay buffer: 0.25 M Tris-HCl buffer (pH 8)

4. Stop solution: 1 M sodium acetate buffer (pH 4.2)

5. Sample: 5 µL (2 wells, activity and blank)

Reagent preparation

1. Standard solution

    1.1) *p*-nitroaniline (4-nitroaniline, *p*NA) (N2128; Sigma, Mw. 138.13) was dissolved in MilliQ to get 1 mM solution.

    1.2) Each concentration was adjusted and used as a standard.

    *4-nitroaniline is difficult to dissolve (vortex and ultrasonic were needed).

    *The solution should be stored at -20°C (It can be reused).

2. Substrate

    2.1) Gly-Pro-*p*-nitroaniline (Gly-Pro-*p*NA) was dissolved in MilliQ to get 1.6 mM solution (1.6 mM = 743.184 mg/L → 7.43 mg dissolved in 10 mL of MilliQ).

3. Assay buffer

    3.1) 15.14 g of 2-amino-2-hydroxymethyl-1,3-propanedial (Tris (hydroxymethyl) aminomethane) (207-06275; Wako, Mw. 121.14) was weighed and mixed with 400 mL of MilliQ. Stirrer bar was needed to completely mix.

    3.2) Assay buffer was adjusted the pH to pH 8 by using 4 M HCl (6 M or 1 M HCl might be used).

    3.3) MilliQ water was transferred to the prepared solution until 500 mL. (Final concentration of Tris base: 0.25 M).

4. Stop solution

4.1) Acetic acid (017-00256; Wako, Mw. 60.05) was diluted with MilliQ to get 1 M concentration (A).

4.2) Sodium acetate (192-01075; Wako, Mw. 82.03) was diluted with MilliQ to get 1 M concentration (B).

4.3) 1 M sodium acetate was mixed with 1 M acetate and adjusted the pH to 4.2 (C).

(A) Acetic acid (99.7%; 17.43 M) was diluted to get 1 M

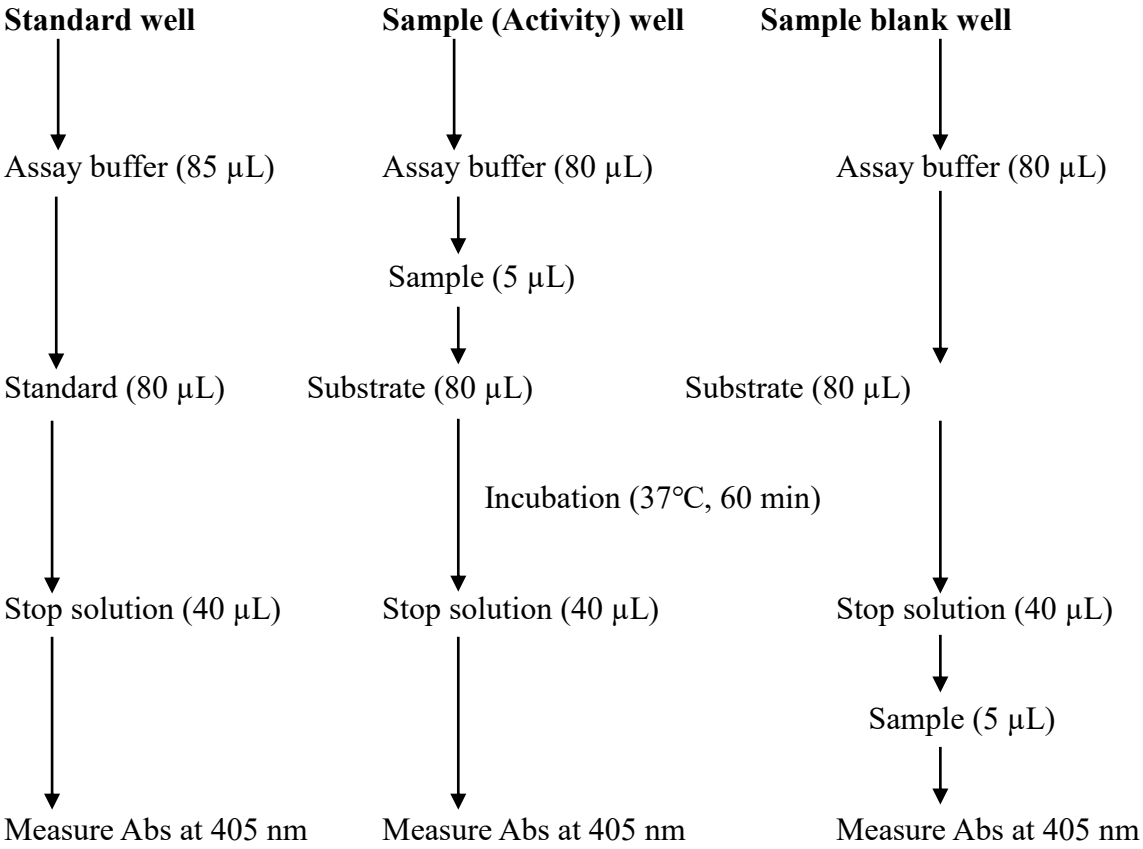
(B) Sodium acetate (purity 98.5%, Mw. 82.03) was weighted 4.165 g and dissolve in 50 mL MilliQ.

(C) The solution was prepared by mixing of (A) and (B) and adjusted pH to 4.2.

Reaction step

1. Assay buffer (85 µL for standard well, 80 µL of sample and blank well) was transferred to 96-well plate (Greiner, Frickenhausen, Germany).
2. Sample (5 µL) was added into the well.
3. Standard solution of each concentration (1, 0.8, 0.6, 0.4, 0.2, and 0 mM, 80 µL) or substrate (80 µL) was transferred to well and incubated at 37°C for 60 min.
4. Then, stop solution was immediately added to well.
5. After that, sample (5 µL) was transferred into the sample blank well.
6. The absorbance was read at 405 nm using microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

Experimental flow of DPP-IV activity measurement



Appendix P: RNA extraction

The mucosa of each intestinal segment was scrapped and stored in RLT buffer solution (RLT buffer:2-mercaptoethanol as the ratio of 100:1) at -80°C until RNA extraction. Total RNA was prepared using the RNeasy Mini kit (74106; Qiagen, Hilden, Germany), according to the manufacturer's instructions.

1. Intestinal mucosa samples were homogenized for 2 min and centrifuged at 15000 rpm for 3 min.
2. The supernatant (400 µL) was completely mixed with 70% ethanol as a ratio of 1:1. The mixed solution (400 µL) was transferred to Rneasy Mini spin column tube and centrifuged for 24 s at 15000 rpm then the filtrated solution was discarded (2 times for this step).
3. Buffer RW1 (700 µL) was added into Rneasy Mini spin column tube and centrifuged for 24 s at 15000 rpm. RPE buffer (500 µL, 2 times) was transferred to Rneasy Mini spin column tube and centrifuged for 24 s and 1 min respectively, at 15000 rpm then the filtrated solution was discarded.
4. Rneasy Mini spin column was placed into the new tube. RNase-free water was directly added into Rneasy Mini spin column about 30-50 µL and centrifuged at 15000 rpm for 1 min.
5. Finally, RNA concentration of each sample was determined using NanoDrop Lite (Thermo Fisher Scientific Inc., Wilmington, DE, USA) by using 1.5 µL of extracted RNA solution.

Appendix Q: Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesized from RNA using the ReverTra Ace qPCR Master Mix with gDNA Remover (FSQ-301; Toyobo Company Limited), following the manufacturer's instructions.

1. RNA solution, total volume 12 µL, was prepared by mixing one microgram (1 µg) of RNA concentration and RNase-free water.
2. Then 4x DN Master Mix (4 µL) was transferred to RNA solution. Genomic DNA removal (DNase I treatment, total volume 16 µL) was performed at 37°C for 5 min using T100 Thermo Cycler (Biorad, Hercules, CA, USA).
3. After that, 5xRT Master Mix II (4 µL) was added into RNA solution (total volume 20 µL) in order to reverse transcription (cDNA synthesis). The condition was programmed as 37°C for 5 min, 50°C for 5 min, and 98°C for 5 min respectively.
4. cDNA was kept at -30°C until further measurement.

Appendix R: Agarose gel preparation

1. 1.5 g of GenePure LE Agarose (E-3120-125; BMBio, Tokyo, Japan) was weighed and stirred in TAE buffer (100 mL) then mixed thoroughly in a conical flask.
2. After mixing, agarose gel solution was heated in microwave (30 s intermittent time) with hand shaking until it boiled.
3. Conical flask was placed at room temperature until the temperature down to 60°C then Midori green (4 µl/100 mL, MG01; Nippon Genetics Europe GmbH, Düren, Germany) was added into agarose gel solution and mixed thoroughly.
4. Agarose gel solution was poured into a gel tray and covered with aluminum foil at room temperature for 30 min.
5. Agarose gel was stored in TAE buffer and covered with aluminum foil at 4°C until use (Not more than 1 week after preparation, freshly prepare is the best).

Appendix S: Polymerase chain reaction (PCR)

1. Mixed PCR solution→ Nuclease free water (Promega, Madison, WI, USA), GoTaq Green Master Mix (Promega, Madison, WI, USA), primer forward, and primer reverse were transferred to eppendorp tube and mixed thoroughly.

Reagents	Volume/sample (µL)
1. GoTaq Green Master Mix	12.5
2. Primer forward (10 µM)	1
3. Primer reverse (10 µM)	1
4. Nuclease free water	9.5
5. cDNA	1
Total	25

2. Then mixed PCR solution was transferred to PCR tube.

3. cDNA (DNA template) was added and mixed thoroughly with mixed PCR solution.

4. PCR tubes were placed into T100 Thermo Cycler (Biorad, Hercules, CA, USA) and programmed as follow:

35 cycles of convectional PCR
95°C : 2.00 min
95°C : 30 s
60°C* : 30 s } 35X
72°C : 30 s
72°C : 5.00 min
4°C: ∞
*The temperature was varied depending on annealing temperature of each primer (58-65°C)

5. All PCR products were detected by gel electrophoresis using 100 V for 30 min.

6. Then agarose gel was photographed using Lumivision Pro 400EX.

Appendix T: Standard real-time PCR (qPCR) preparation

1. Nuclease free water (19 µL) was transferred to PCR tube.

2. After that, PCR master mix (25 µL) was added to PCR tube

3. cDNA (pool cDNA, 2 µL) was then added to PCR tube.

4. Forward primer (2 µL) and reverse primer (2 µL) were transferred to PCR tube respectively.

List of primer sequences for standard qPCR

Gene	Forward primer	Reverse primer	T_a (°C)
Gapdh	AACGACCCCTTCATTGACCTC	GAAGGGGCGGAGATGATGAC	58
Gcg	CTCAGCTCAGTCCCACAAGG	TGGTGAATGTGCCCTGTGAA	58
Ffar1	TGGGCATCAACATACCCGTG	TACTTCTGAATTGTCCCTCTTGGAG	61.4
Ffar2	GGCTGTGGTGACGCTTCTTA	TCACTCGGTGACAAAGTCAG	58.8
Ffar3	GTAGGAGCTAGCTGGGTGCTG	TGAGCTGATGCCAAGAACCA	58.8
Ffar4	CTGCCCCTCTGCATCTTGTT	ACAGAATGGGGTTTAGGGCG	58.8

Reagents	Volume/sample (µL)
1. Primer forward	2.0
2. Primer reverse	2.0
3. Nuclease free water	19.0
4. PCR master mix	25.0
5. cDNA	2.0
Total	50

4. PCR tubes were placed into T100 Thermo Cycler (Biorad, Hercules, CA, USA) and programmed as follow:

20 cycles of Standard real time PCR
95°C : 2.00 min
95°C : 30 s
64°C* : 30 s } 20X
72°C : 30 s
72°C : 5.00 min
4°C : ∞
*The temperature was varied depending on annealing temperature of each primer (58-65°C)

Appendix U: Real time PCR (qPCR) analysis (Taqman)

1. Nuclease free water, Taqman primer probe* (Applied Biosystems, Foster City, CA, USA), and Taqman gene expression master mix (Applied Biosystems, Foster City, CA, USA) were mixed thoroughly and moved stored at 4°C before adding cDNA.

List of TaqMan primers and probe set

TaqMan primers and probe set	
Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)	Rn99999916_s1
Sequence: GGGAAACCCATCACCATCTTCCAGG	
Proglucagon (Gcg)	Rn00562293_m1
Sequence: GGAGGAGAACGCCAGATCATTCCCA	
Free fatty acid receptor 1 (Ffar1 or Gpr40)	Rn00824686_s1
Sequence: TTGGGAACAGGTCCTGGACAGGGGA	
Free fatty acid receptor 2 (Ffar2 or Gpr43)	Rn02345824_s1
Sequence: CAAGACAGACAGGGGTGGGAGTCAA	
Free fatty acid receptor 3 (Ffar3 or Gpr41)	Rn01457614_g1
Sequence: GCTCTCCAACACTCTGCATCTGTGA	
Free fatty acid receptor 4 (Ffar4 or Gpr120)	Rn01759772_m1
Sequence: CCAAGATTTTACAGATCACGAAAGC	

Reagents	Volume/sample (µL)
1. Taqman primer probe	0.5
2. Nuclease free water	2.5
3. Taqman gene expression master mix	6.0
4. Sample/Standard/Negative control	3.0
Total	12.0

2. Mixed solution was transferred to FrameStar 96 Fast plate (4titude, Surrey, UK).

3. After transferring mixed solution, sample, standard**, and negative control were added into MicroAmp Optical 96-Well Reaction Plate.

** Standard after PCR were diluted (10-folds dilution as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}).

4. The plate was covered by plastic seal and then centrifuged for 10 s about 3 times at 2000 rpm.
5. MicroAmp Optical 96-Well Reaction Plate was inserted into real time PCR machine (Mx3000P Real-Time PCR System, Stratagene) and started running real time PCR (qPCR).
6. Real time PCR was programmed as follow:

40 cycles for real time PCR (qPCR)

50°C	: 2 min	
95°C	: 10 min	
95°C	: 15 s	} 40X
60°C	: 20 s	
72°C	: 10 s	
95°C	: 15 s	
58°C	: 1 min	
95°C	: 15 s	

7. Relative expression levels were calculated for each sample after normalization to those of *Gapdh* as a reference gene using the standard curve method.

References

Abu-Hamdah R, Rabiee A, Meneilly GS, Shannon RP, Andersen DK, & Elahi D. (2009) Clinical review: The extrapancreatic effects of glucagon-like peptide-1 and related peptides. Journal of Clinical Endocrinology and Metabolism, 94(6), 1843–1852. PMID: 19336511 https://doi.org/10.1210/jc.2008-1296

Acosta A, Camilleri M, Burton D, O'Neill J, Eckert D, Carlson P, & Zinsmeister AR. (2015) Exenatide in obesity with accelerated gastric emptying: a randomized, pharmacodynamics study. Physiological Reports, 3(11), 1–10. PMID: 26542264 https://doi.org/10.14814/phy2.12610

Ahlkvist L, Vikman J, Pacini G, & Ahrén B. (2012) Synergism by individual macronutrients explains the marked early GLP-1 and islet hormone responses to mixed meal challenge in mice. Regulatory Peptides, 178(1–3), 29–35. PMID: 22750278 https://doi.org/10.1016/j.regpep.2012.06.004

Akash MS, Rehman K, & Chen S. (2015) Goto-Kakizaki rats: its suitability as non-obese diabetic animal model for spontaneous type 2 diabetes mellitus. Current Diabetes Reviews, 9(5), 387–396. PMID: 23855509 https://doi.org/10.2174/15733998113099990069

Alssema M, Rijkelijkhuizen JM, Holst JJ, Teerlink T, Scheffer PG, Eekhoff EM, Gastaldelli A, Mari A, Hart LM, Nijpels G, & Dekker JM. (2013) Preserved GLP-1 and exaggerated GIP secretion in type 2 diabetes and relationships with triglycerides and ALT. European Journal of Endocrinology, 169(4), 421–430. PMID: 23864340 https://doi.org/10.1530/EJE-13-0487

Amiri L, John A, Shafarin J, Adeghate E, Jayaprakash P, Yasin J, Howarth FC, & Raza H. (2015) Enhanced glucose tolerance and pancreatic beta cell function by low dose aspirin in hyperglycemic insulin-resistant type 2 diabetic Goto-Kakizaki (GK) rats. Cellular Physiology and Biochemistry, 36(5), 1939–1950. PMID: 26202354 https://doi.org/10.1159/000430162

Avila-Nava A, Noriega LG, Tovar AR, Granados O, Perez-Cruz C, Pedraza-Chaverri J, & Torres N. (2017) Food combination based on a pre-hispanic Mexican diet decreases metabolic and cognitive abnormalities and gut microbiota dysbiosis caused by a sucrose-enriched high-fat diet in rats. Molecular Nutrition and Food Research, 61(1), 1–13. PMID: 27352915 https://doi.org/10.1002/mnfr.201501023

Baggio LL & Drucker DJ. (2007) Biology of incretins: GLP-1 and GIP. Gastroenterology, 132(6), 2131–2157. PMID: 17498508 https://doi.org/10.1053/j.gastro.2007.03.054

Beloqui A, Alhouayek M, Carradori D, Vanvarenberg K, Muccioli GG, Cani PD, & Préat V. (2016) A mechanistic study on nanoparticle-mediated glucagon-like peptide-1 (GLP-1) secretion from enteroendocrine L cells. Molecular Pharmaceutics, 13(12), 4222–4230. PMID: 27934480 https://doi.org/10.1021/acs.molpharmaceut.6b00871

Bertin E, Schneider N, Abdelli N, Wampach H, Cadiot G, Loboguerrero A, Leutenegger M, Liehn JC, & Thiefin G. (2001) Gastric emptying is accelerated in obese type 2 diabetic patients without autonomic neuropathy. Diabetes and Metabolism, 27(3), 357–364. PMID: 11431601

Bösenberg, LH, & Van Zyl, DG. (2008) The mechanism of action of oral antidiabetic drugs: A review of recent literature. Journal of Endocrinology, Metabolism and Diabetes of South Africa, 13(3), 80–89. https://doi.org/10.1080/22201009.2008.10872177

Brodovicz KG, Girman CJ, Simonis-Bik AM, Rijkelijkhuizen JM, Zelis M, Bunck MC, Mari A, Nijpels G, Eekhoff EM, & Dekker JM. (2011) Postprandial metabolic responses to mixed versus liquid meal tests in healthy men and men with type 2 diabetes. Diabetes Research and Clinical Practice, 94(3), 449–455. PMID: 21955958 https://doi.org/10.1016/j.diabres.2011.09.002

Bucceri AM, Calogero AE, & Brogna A. (2002) Gallbladder and gastric emptying: relationship to cholecystokininemia in diabetics. European Journal of Internal Medicine, 13(2):123-128. PMID: 11893470 https://doi.org/10.1016/s0953-6205(02)00003-1

Buchholz V, Berkenstadt H, Goitein D, Dickman R, Bernstine H, & Rubin M. (2013) Gastric emptying is not prolonged in obese patients. Surgery for Obesity and Related Diseases, 9(5), 714–717. PMID: 22571886 https://doi.org/10.1016/j.soard.2012.03.008

Cacho J, Sevillano J, de Castro J, Herrera E, & Ramos MP. (2008) Validation of simple indexes to assess insulin sensitivity during pregnancy in Wistar and Sprague-Dawley rats. American Journal of Physiology-Endocrinology and Metabolism, 295(5), E1269-E1276. PMID: 18796548 https://doi.org/10.1152/ajpendo.90207.2008

Calabria AC, Li C, Gallagher PR, Stanley CA, & De León DD. (2012) GLP-1 receptor antagonist exendin-(9-39) elevates fasting blood glucose levels in congenital hyperinsulinism owing to inactivating mutations in the ATP-sensitive K+ channel. Diabetes, 61(10), 2585–2591. PMID: 22855730 https://doi.org/10.2337/db12-0166

Calanna S, Christensen M, Holst JJ, Laferrère B, Gluud LL, Vilsbøll T, & Knop FK. (2013) Secretion of glucagon-like peptide-1 in patients with type 2 diabetes mellitus: systematic review and meta-analyses of clinical studies. Diabetologia, 56(5), 965–972. PMID: 23377698 https://doi.org/10.1007/s00125-013-2841-0

Cani PD, Hoste S, Guiot Y, & Delzenne NM. (2007) Dietary non-digestible carbohydrates promote L-cell differentiation in the proximal colon of rats. British Journal of Nutrition, 98(1), 32–37. PMID: 17367575 https://doi.org/10.1017/S0007114507691648

Carr RD, Larsen MO, Winzell MS, Jelic K, Lindgren O, Deacon CF, & Ahrén B. (2008) Incretin and islet hormonal responses to fat and protein ingestion in healthy men. American Journal of Physiology-Endocrinology and Metabolism, 295(4), E779–E784. PMID: 18612044 https://doi.org/10.1152/ajpendo.90233.2008

Chalkley SM, Hettiarachchi M, Chisholm DJ, & Kraegen EW. (2002) Long-term high-fat feeding leads to severe insulin resistance but not diabetes in Wistar rats. American Journal of Physiology-Endocrinology and Metabolism, 282(6), E1231–E1238. PMID: 12006352 https://doi.org/10.1152/ajpendo.00173.

Chepurny OG, Bonaccorso RL, Leech CA, Wöllert T, Langford GM, Schwede F, Roth CL, Doyle RP, & Holz GG. (2018) Chimeric peptide EP45 as a dual agonist at GLP-1 and NPY2R receptors. Scientific Reports, 8(1), 3749. PMID: 29491394 https://doi.org/10.1038/s41598-018-22106-1

Cho YM, Fujita Y, & Kieffer TJ. (2014) Glucagon-Like Peptide-1: Glucose Homeostasis and Beyond. Annual Review of Physiology, 76(1), 535–559. PMID: 24245943 https://doi.org/10.1146/annurev-physiol-021113-170315

Coopman K, Wallis R, Robb G, Brown AJ, Wilkinson GF, Timms D, & Willars GB. (2011) Residues within the transmembrane domain of the Glucagon-Like Peptide-1 receptor involved in ligand binding and receptor activation: modelling the ligand-bound receptor. Molecular Endocrinology, 25(10), 1804–1818. PMID: 21868452 https://doi.org/10.1210/me.2011-1160

Cordier-Bussat M, Bernard C, Levenez F, Klages N, Laser-Ritz B, Philippe J, Chayvialle JA, & Cuber JC. (1998) Peptones stimulate both the secretion of the incretin hormone glucagon- like peptide 1 and the transcription of the proglucagon gene. Diabetes, 47(7), 1038–1045. PMID: 9648826 https://doi.org/10.2337/diabetes.47.7.1038

Damgaard M, Graff J, Fuglsang S, Holst JJ, Rehfeld JF, & Madsen JL. (2013) Effects of oleic acid and olive oil on gastric emptying, Gut hormone secretion and appetite in lean and overweight or obese males. e-SPEN Journal, 8(1), e8-e14. https://doi.org/10.1016/j.clnme.2012.10.004

Damholt AB, Kofod H, & Buchan AM. (1999) Immunocytochemical evidence for a paracrine interaction between GIP and GLP-1-producing cells in canine small intestine. Cell and Tissue Research, 298(2):287-93. PMID: 10571117 https://doi.org/10.1007/s004419900093

De León DD, Li C, Delson MI, Matschinsky FM, Stanley CA, & Stoffers DA. (2008) Exendin-(9-39) corrects fasting hypoglycemia in SUR-1-/- mice by lowering camp in pancreatic β-cells and inhibiting insulin secretion. Journal of Biological Chemistry, 283(38), 25786–25793. PMID: 18635551 https://doi.org/10.1074/jbc.M804372200

Deacon CF & Ahrén B. (2011) Physiology of incretins in health and disease. The Review of Diabetic Studies, 8(3), 293–306. PMID: 22262068 https://doi.org/10.1900/RDS.2011.8.293

Dirksen C, Graff J, Fuglsang S, Rehfeld JF, Holst JJ, & Madsen JL. (2019) Energy intake, gastrointestinal transit, and gut hormone release in response to oral triglycerides and fatty acids in men with and without severe obesity. American Journal of Physiology-Gastrointestinal and Liver Physiology, 316(3), G332–G337. PMID: 30520691 https://doi.org/10.1152/ajpgi.00310.2018

Drucker DJ. (2007) The role of gut hormones in glucose homeostasis. Pancreas, 117(1), 24–32. PMID: 17200703 https://doi.org/10.1172/JCI30076.24

Dusaulcy R, Handgraaf S, Skarupelova S, Visentin F, Vesin C, Heddad-Masson M, Reimann F, Gribble F, Philippe J, & Gosmain Y. (2016) Functional and molecular adaptations of enteroendocrine L-cells in male obese mice are associated with preservation of pancreatic alpha-cells function and prevention of hyperglycemia. Endocrinology, 157, 3832–3843. PMID: 27547850 https://doi.org/10.1210/en.2016-1433

Edwards CM, Todd JF, Mahmoudi M, Wang Z, Wang RM, Ghatei MA, & Bloom SR. (1999) Glucagon-like peptide 1 has a physiological role in the control of postprandial glucose in humans: studies with the antagonist exendin 9-39. Diabetes, 48(1), 86-93. PMID: 9892226 https://doi.org/10.2337/diabetes.48.1.86

Eissele R, Göke R, Willemer S, Harthus HP, Vermeer H, Arnold R, & Göke B. (1992) Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. European Journal of Clinical Investigation, 22(4), 283–291. PMID: 1499644 https://doi.org/10.1111/j.1365-2362.1992.tb01464.x

Ekberg JH, Hauge M, Kristensen LV, Madsen AN, Engelstoft MS, Husted AS, Sichlau R, Egerod KL, Timshel P, Kowalski TJ, Gribble FM, Reiman F, Hansen HS, Howard AD, Holst B, & Schwartz TW. (2016) GPR119, a major enteroendocrine sensor of dietary triglyceride metabolites coacting in synergy with FFA1 (GPR40). Endocrinology, 157(12), 4561-4569. PMID: 27779915 https://doi.org/10.1210/en.2016-1334

Færch K, Torekov SS, Vistisen D, Johansen NB, Witte DR, Jonsson A, Pedersen O, Hansen T, Lauritzen T, Sandbæk A, Holst JJ, & Jørgensen ME. GLP-1 response to oral glucose is reduced in prediabetes, screen-detected type 2 diabetes, and obesity and influenced by sex. Diabetes, 64(7), 2513–2525. PMID: 25677912 https://doi.org/10.2337/db14-1751

Farilla L, Hui H, Bertolotto C, Kang E, Bulotta A, Di Mario U, & Perfetti R. (2002) Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in Zucker diabetic rats. Endocrinology, 143(11), 4397-4408. PMID: 12399437 https://doi.org/10.1210/en.2002-220405

Fava GE, Dong EW, & Wu H. (2016) Intra-islet glucagon-like peptide 1. Journal of Diabetes and Its Complications, 30(8), 1651–1658. PMID: 27267264 https://doi.org/10.1016/j.jdiacomp.2016.05.016

Feng RL, Qian C, Liu LY, Liu QJ, Jin YQ, Li SX, Liu W, Rayner CK, & Ma J. (2019) Secretion of gut hormones and expression of sweet taste receptors and glucose transporters in a rat model of obesity. Obesity Fact, 12(2):190-198. PMID: 30928977 https://doi.org/10.1159/000497122

Flock G, Baggio LL, Longuet C, & Drucker DJ. (2007) Essential for the sustained metabolic actions of vildagliptin in mice. Diabetes, 56(12), 3006–3013. PMID: 17717280 https://doi.org/10.2337/db07-0697.D.D.

Gniuli D, Calcagno A, Dalla Libera L, Calvani R, Leccesi L, Caristo ME, Vettor R, Castagneto M, Ghirlanda G, & Mingrone G. (2010) High-fat feeding stimulates endocrine, glucose-dependent insulinotropic polypeptide (GlP)-expressing cell hyperplasia in the duodenum of Wistar rats. Diabetologia, 53(10), 2233–2240. PMID: 20585935 https://doi.org/10.1007/s00125-010-1830-9

Goto Y, Kakizaki M, & Masaki N. (1975) Spontaneous Diabetes Produced by Selective Breeding of Normal Wistar Rats. Proceedings of the Japan Academy, 51(1), 80–85. https://doi.org/10.2183/PJAB1945.51.80

Granata R, Volante M, Settanni F, Gauna C, Ghé C, Annunziata M, Deidda B, Gesmundo I, Abribat T, van der Lely AJ, Muccioli G, Ghigo E, & Papotti M. (2010) Unacylated ghrelin and obestatin increase islet cell mass and prevent diabetes in streptozotocin-treated newborn rats. Journal of Molecular Endocrinology, 45(1), 9-17. PMID: 20382773 https://doi.org/10.1677/JME-09-0141

Green BD, Irwin N, Gault VA, Bailey CJ, O'Harte FP, & Flatt PR. (2005) Chronic treatment with exendin(9-39)amide indicates a minor role for endogenous glucagon-like peptide-1 in metabolic abnormalities of obesity-related diabetes in ob/ob mice. Journal of Endocrinology, 185(2), 307–317. PMID: 15845923 https://doi.org/10.1677/joe.1.05876

Gribble FM & Reimann F. (2019) Function and mechanisms of enteroendocrine cells and gut hormones in metabolism. Nature Reviews Endocrinology, 15(4), 226–237. PMID: 30760847 https://doi.org/10.1038/s41574-019-0168-8

Gromada J, Holst JJ, & Rorsman P. (1998) Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide 1. Pflügers Archiv: European Journal of Physiology, 435(5), 583–594. PMID: 9479010 https://doi.org/10.1007/s004240050558

Grundy SM. (2004) Obesity, metabolic syndrome, and cardiovascular disease. Journal of Clinical Endocrinology & Metabolism, 89(6), 2595–2600. PMID: 15181029 https://doi.org/10.1210/jc.2004-0372

Habener JF & Stanojevic V. (2017) Pancreas and not gut mediates the GLP-1-induced glucoincretin effect. Cell Metabolism, 25(4), 757–758. PMID: 28380367 https://doi.org/10.1016/j.cmet.2017.03.020

Hariri N & Thibault L. (2010) High-fat diet-induced obesity in animal models. Nutrition Research Reviews, 23(2), 270–299. PMID: 20977819 https://doi.org/10.1017/S0954422410000168

Hatanaka S, Kondoh M, Kawarabayashi K, & Furuhama K. (1994) The measurement of gastric emptying in conscious rats by monitoring serial changes in serum acetaminophen level. Journal of Pharmacological and Toxicological Methods, 31(3), 161–165. PMID: 8068978 https://doi.org/10.1016/1056-8719(94)90079-5

Hellström PM, Grybäck P, & Jacobsson H. (2006) The physiology of gastric emptying. Best Practice & Research Clinical Anaesthesiology, 20(3), 397–407. https://doi.org/10.1016/j.bpa.2006.02.002

Hira T, Ikee A, Kishimoto Y, Kanahori S, & Hara H. (2015) Resistant maltodextrin promotes fasting glucagon-like peptide-1 secretion and production together with glucose tolerance in rats. British Journal of Nutrition, 114, 34–42. PMID: 25671387 https://doi.org/10.1017/S0007114514004322

Hira T, Koga T, Sasaki K, & Hara H. (2017) Canagliflozin potentiates GLP-1 secretion and lowers the peak of GIP secretion in rats fed a high-fat high-sucrose diet. Biochemical and Biophysical Research Communications, 492(2), 161–165. PMID: 28803984 https://doi.org/10.1016/j.bbrc.2017.08.031

Hira T, Pinyo J, & Hara H. (2020) What Is GLP-1 Really Doing in Obesity? Trends in Endocrinology and Metabolism, 31(2), 71-80. PMID: 31636017 https://doi.org/10.1016/j.tem.2019.09.003

Hira T, Suto R, Kishimoto Y, Kanahori S, & Hara H. (2017) Resistant maltodextrin or fructooligosaccharides promotes GLP-1 production in male rats fed a high-fat and high-sucrose diet, and partially reduces energy intake and adiposity. European Journal of Nutrition, 57(3), 965-979. PMID: 28161724 https://doi.org/10.1007/s00394-017-1381-7

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, & Tsujimoto G. (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nature Medicine, 11(1), 90–94. PMID: 15619630 https://doi.org/10.1038/nm1168

Holst JJ. (2007) The physiology of glucagon-like peptide 1. Physiological Review, 87(4), 1409–1439. https://doi.org/10.1152/physrev.00034.2006.

Holst JJ, Gribble F, Horowitz M, & Rayner CK. (2016) Roles of the gut in glucose homeostasis. Diabetes Care, 39(6), 884–892. https://doi.org/10.2337/dc16-0351

Hong SJ, Lee JH, Kim EJ, Yang HJ, Park JS, & Hong SK. (2017) Anti-obesity and anti-diabetic effect of neoagarooligosaccharides on high-fat diet-induced obesity in mice. Marine Drugs, 15(4), 90. PMID: 28333098 https://doi.org/10.3390/md15040090

Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L, Kimura I, Leloire A, Liu N, Iida K, Choquet H, Besnard P, Lecoeur C, Vivequin S, Ayukawa K, Takeuchi M, Ozawa K, Tauber M, Maffeis C, Morandi A, Buzzetti R, Elliott P, Pouta A, Jarvelin MR, Körner A, Kiess W, Pigeyre M, Caiazzo R, Van Hul W, Van Gaal L, Horber F, Balkau B, Lévy-Marchal C, Rouskas K, Kouvatsi A, Hebebrand J, Hinney A, Scherag A, Pattou F, Meyre D, Koshimizu TA, Wolowczuk I, Tsujimoto G, & Froguel P. (2012) Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. Nature, 483(7389), 350–354. PMID: 22343897 https://doi.org/10.1038/nature10798

Inagaki N, Seino Y, Takeda J, Yano H, Yamada Y, Bell GI, Eddy RL, Fukushima Y, Byers MG, Shows TB, & Imura, H. (1989) Gastric inhibitory polypeptide: Structure and chromosomal localization of the human gene. Molecular Endocrinology, 3(6), 1014–1021. PMID: 2739653 https://doi.org/10.1210/mend-3-6-1014

Ishii Y, Ohta T, & Sasase T. (2012) Non-obese type 2 diabetes animal models. Glucose Tolerance, 223–242. https://doi.org/10.5772/52712

Ishikawa Y, Hira T, Inoue D, Harada Y, Hashimoto H, Fujii M, Kadowaki M, & Hara H. (2015) Rice protein hydrolysates stimulate GLP-1 secretion, reduce GLP-1 degradation, and lower the glycemic response in rats. Food & Function, 6(8), 2525–2534. PMID: 26107658 https://doi.org/10.1039/c4fo01054j

Witte AB, Grybäck P, Jacobsson H, Näslund E, Hellström PM, Holst JJ, Hilsted L, & Schmidt PT. 2010) Involvement of endogenous glucagon-like peptide-1 in regulation of gastric motility and pancreatic endocrine secretion. Scandinavian Journal of Gastroenterology, 46(4), 428–435. PMID: 21114428 https://doi.org/10.3109/00365521.2010.537680

Jones IR, Owens DR, Luzio S, Williams S, & Hayes TM. (1989) The glucose dependent insulinotropic polypeptide response to oral glucose and mixed meals is increased in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia, 32(9), 668–677. PMID: 2676668 https://doi.org/10.1007/BF00274255

Kao YH, Chang HH, Lee MJ, & Chen CL. (2006) Tea, obesity, and diabetes. Molecular Nutrition and Food Research, 50, 188–210. https://doi.org/10.1002/mnfr.200500109

Karl T, Chwalisz WT, Wedekind D, Hedrich HJ, Hoffmann T, Jacobs R, Pabst R, & von Hörsten S. (2003) Localization, transmission, spontaneous mutations, and variation of function of the Dpp4 (Dipeptidyl-peptidase IV; CD26) gene in rats. Regulatory Peptides, 115(2), 81–90. PMID: 12972323 https://doi.org/10.1016/S0167-0115(03)00149-6

Keller AC, Knaub LA, Miller MW, Birdsey N, Klemm DJ, & Reusch JE. (2015) Saxagliptin restores vascular mitochondrial exercise response in the Goto-Kakizaki rat. Journal of Cardiovascular Pharmacology, 65(2), 137–147. PMID: 25264749 https://doi.org/10.1097/FJC.0000000000000170

Kindel TL, Yang Q, Yoder SM, & Tso P. (2009) Nutrient-driven incretin secretion into intestinal lymph is different between diabetic Goto-Kakizaki rats and Wistar rats. American Journal of Physiology-Gastrointestinal and Liver Physiology, 296(2), G168-G174. PMID: 19056762 https://doi.org/10.1152/ajpgi.90506.2008

Knop FK, Vilsbøll T, Højberg PV, Larsen S, Madsbad S, Vølund A, Holst JJ, & Krarup T. (2007) Reduced incretin effect in type 2 diabetes: cause or consequence of the diabetic state? Diabetes, 56(8), 1951-1959. PMID: 17513701 https://doi.org/10.2337/db07-0100

Kok BP, Galmozzi A, Littlejohn NK, Albert V, Godio C, Kim W, Kim SM, Bland JS, Grayson N, Fang M, Meyerhof W, Siuzdak G, Srinivasan S, Behrens M, & Saez E. (2018) Intestinal bitter taste receptor activation alters hormone secretion and imparts metabolic benefits. Molecular Metabolism, 16, 76-87. PMID: 30120064 https://doi.org/10.1016/j.molmet.2018.07.013

Kuwabara WMT, Panveloski-Costa AC, Yokota CNF1, Pereira JNB, Filho JM, Torres RP, Hirabara SM, Curi R, & Alba-Loureiro T. (2017) Comparison of Goto-Kakizaki rats and high fat diet-induced obese rats: Are they reliable models to study type 2 diabetes mellitus? PLoS One, 12, e0189622. PMID: 29220408 https://doi.org/10.1371/journal.pone.0189622

Laferrère B, Heshka S, Wang K, Khan Y, McGinty J, Teixeira J, Hart AB, & Olivan B. (2007) Incretin levels and effect are markedly enhanced 1 month after Roux-en-Y gastric bypass surgery in obese patients with type 2 diabetes. Diabetes Care, 30(7), 1709-1716. PMID: 17416796 https://doi.org/10.2337/dc06-1549.

Lean ME & Malkova D. (2016) Altered gut and adipose tissue hormones in overweight and obese individuals: Cause or consequence. International Journal of Obesity, 40(4), 622–632. PMID: 26499438 https://doi.org/10.1038/ijo.2015.220

Lee S, Yabe D, Nohtomi K, Takada M, Morita R, Seino Y, & Hirano T. (2010) Intact glucagon-like peptide-1 levels are not decreased in Japanese patients with type 2 diabetes. Endocrine Journal, 57(2), 119–126. PMID: 19881250 https://doi.org/10.1507/endocrj.K09E-269

Lim GE & Brubaker PL (2006) Glucagon-like peptide 1 secretion by the L-cell: The view from within. Diabetes, 55, S70–S77. https://doi.org/10.2337/db06-S020

Long Z, Zhang X, Sun Q, Liu Y, Liao N, Wu H, Wang X, Hai C. (2015) Evolution of metabolic disorder in rats fed high sucrose or high fat diet: Focus on redox state and mitochondrial function. General and Comparative Endocrinology, 242, 92–100. PMID: 26497252 https://doi.org/10.1016/j.ygcen.2015.10.012

Loo FD, Palmer DW, Soergel KH, Kalbfleisch JH, & Wood CM. (1984) Gastric emptying in patients with diabetes mellitus. Gastroenterology, 86(3), 485–494. https://doi.org/10.1016/S0016-5085(84)80019-0

Lu VB, Gribble FM, & Reimann F. (2018) Free Fatty Acid Receptors in enteroendocrine cells. Endocrinology, 159(7), 2826–2835. PMID: 29688303 https://doi.org/10.1210/en.2018-00261

Ma J, Pilichiewicz AN, Feinle-Bisset C, Wishart JM, Jones KL, Horowitz M, & Rayner CK. (2012) Effects of variations in duodenal glucose load on glycaemic, insulin, and incretin responses in type 2 diabetes. Diabetic Medicine, 29(5), 604–608. PMID: 22004512 https://doi.org/10.1111/j.1464-5491.2011.03496.x

Maida A, Lovshin JA, Baggio LL, & Drucker DJ. (2008) The glucagon-like peptide-1 receptor agonist oxyntomodulin enhances β-cell function but does not inhibit gastric emptying in mice. Endocrinology, 149(11), 5670–5678. PMID: 18669601 https://doi.org/10.1210/en.2008-0336

Makaronidis JM & Batterham RL. (2019) The role of gut hormones in the pathogenesis and management of obesity. Current Opinion in Physiology, 12, 1–11. https://doi.org/10.1016/j.cophys.2019.04.007

Mannucci E, Pala L, Ciani S, Bardini G, Pezzatini A, Sposato I, Cremasco F, Ognibene A, & Rotella CM. (2005) Hyperglycaemia increases dipeptidyl peptidase IV activity in diabetes mellitus. Diabetologia, 48(6), 1168–1172. PMID: 15864535 https://doi.org/10.1007/s00125-005-1749-8

Marathe CS, Rayner CK, Jones KL, & Horowitz M. (2013) Relationships between gastric emptying, postprandial glycemia, and incretin hormones. Diabetes Care, 36(5), 1396–1405. PMID: 23613599 https://doi.org/10.2337/dc12-1609

Marques C, Meireles M, Norberto S, Leite J, Freitas J, Pestana D, Faria A, & Calhau C. (2016) High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte, 5(1), 11–21. PMID: 27144092 https://doi.org/10.1080/21623945.2015.1061723

Medhus AW, Lofthus CM, Bredesen J, & Husebye E. (2001) Gastric emptying: The validity of the paracetamol absorption test adjusted for individual pharmacokinetics. Neurogastroenterology and Motility, 13(3), 179–185. PMID: 11437980 https://doi.org/10.1046/j.1365-2982.2001.00249.x

Meyer-Gerspach AC, Wölnerhanssen B, Beglinger B, Nessenius F, Napitupulu M, Schulte FH, Steinert RE, & Beglinger C. (2014) Gastric and intestinal satiation in obese and normal weight healthy people. Physiology & Behavior, 129, 265–271. PMID: 24582673 https://doi.org/10.1016/j.physbeh.2014.02.043

Miralles F & Portha B. (2001) Early development of beta-cells is impaired in the GK rat model of type 2 diabetes. Diabetes, 50, S84–S88. PMID: 11272209 https://doi.org/10.2337/diabetes.50.2007.s84

Mojsov S, Weir GC, & Habener JF. (1987) Insulinotropin: Glucagon-like peptide I (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. Journal of Clinical Investigation, 79(2), 616–619. PMID: 3543057 https://doi.org/10.1172/JCI112855

Morimoto K, Watanabe M, Sugizaki T, Irie J, & Itoh H. Intestinal bile acid composition modulates prohormone convertase 1/3 (PC1/3) expression and consequent GLP-1 production in male mice. Endocrinology, 157(3), 1071-1081. PMID: 26789236 https://doi.org/10.1210/en.2015-1551

Movassat J, Saulnier C, Serradas P, & Portha B. (1997) Impaired development of pancreatic beta-cell mass is a primary event during the progression to diabetes in the GK rat. Diabetologia, 40(8), 916–925. PMID: 9267986 https://doi.org/10.1007/s001250050768

Movassat J, Bailbé D, Lubrano-Berthelier C, Picarel-Blanchot F, Bertin E, Mourot J, & Portha B. (2008) Follow-up of GK rats during prediabetes highlights increased insulin action and fat deposition despite low insulin secretion. American Journal of Physiology-Endocrinology and Metabolism, 294(1), E168–E175. PMID: 17986628 https://doi.org/10.1152/ajpendo.00501.2007

Nakajima S, Hira T, & Hara H. (2015) Postprandial glucagon-like peptide-1 secretion is increased during the progression of glucose intolerance and obesity in high-fat/high-sucrose diet-fed rats. British Journal of Nutrition, 113, 1477–1488. PMID: 25827219 https://doi.org/10.1017/S0007114515000550

Nauck MA, Vardarli I, Deacon CF, Holst JJ, & Meier JJ. (2011) Secretion of glucagon-like peptide-1 (GLP-1) in type 2 diabetes: What is up, what is down? Diabetologia, 54(1), 10–18. PMID: 20871975 https://doi.org/10.1007/s00125-010-1896-4

Nauck, M. (2016) Incretin therapies: Highlighting common features and differences in the modes of action of glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors. Diabetes, Obesity and Metabolism, 18(3), 203–216. PMID: 26489970 https://doi.org/10.1111/dom.12591

Nauck MA, Kind J, Köthe LD, Holst JJ, Deacon CF, Broschag M, He YL, Kjems L, & Foley J. (2016) Quantification of the contribution of GLP-1 to mediating insulinotropic effects of DPP-4 inhibition with vildagliptin in healthy subjects and patients with type 2 diabetes using exendin [9-39] as a GLP-1 receptor antagonist. Diabetes, 65(8), 2440–2447. PMID: 27207543 https://doi.org/10.2337/db16-0107

Nauck MA & Meier JJ. (2018) Incretin hormones: Their role in health and disease. Diabetes, Obesity and Metabolism, 20, 5–21. PMID: 29364588 https://doi.org/10.1111/dom.13129

Nguyen NQ, Debreceni TL, Burgess JE, Bellon M, Wishart J, Standfield S, Malbert CH, & Horowitz M. (2018) Impact of gastric emptying and small intestinal transit on blood glucose, intestinal hormones, glucose absorption in the morbidly obese. International Journal of Obesity, 42(9), 1556–1564. PMID: 29453463 https://doi.org/10.1038/s41366-018-0012-6

Nicolaus M, Brödl J, Linke R, Woerle HJ, Göke B, & Schirra J. (2011) Endogenous GLP-1 regulates postprandial glycemia in humans: relative contributions of insulin, glucagon, and gastric emptying. Journal of Clinical Endocrinology and Metabolism, 96(1), 229–236. PMID: 21047924 https://doi.org/10.1210/jc.2010-0841

Omar B, Pacini G, & Ahrén B. (2012) Differential development of glucose intolerance and pancreatic islet adaptation in multiple diet induced obesity models. Nutrients, 4(10), 1367–1381. PMID: 23201760 https://doi.org/10.3390/nu4101367

Otten J, Ryberg M, Mellberg C, Andersson T, Chorell E, Lindahl B, Larsson C, Holst JJ, & Olsson T. (2019) Postprandial levels of GLP-1, GIP and glucagon after 2 years of weight loss with a Paleolithic diet: a randomised controlled trial in healthy obese women. European Journal of Endocrinology, 180(6), 417–427. PMID: 31042670 https://doi.org/10.1530/EJE-19-0082

Pala L, Mannucci E, Pezzatini A, Ciani S, Sardi J, Raimondi L, Ognibene A, Cappadona A, Vannelli BG, & Rotella CM. (2003) Dipeptidyl peptidase-IV expression and activity in human glomerular endothelial cells. Biochemical and Biophysical Research Communications, 310, 28–31. PMID: 14511643 https://doi.org/10.1016/j.bbrc.2003.08.111

Pang J, Xi C, Huang X, Cui J, Gong H, & Zhang T. (2016) Effects of excess energy intake on glucose and lipid metabolism in C57BL/6 mice. Plos One, 11(1), e0146675. PMID: 26745179 https://doi.org/10.1371/journal.pone.0146675

Pinyo J, Hira T, & Hara H. (2019a) 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. Nutrition, 62, 122–130. PMID: 30878816 https://doi.org/10.1016/j.nut.2019.01.004

Pinyo J, Hira T, & Hara H. (2019a) Enhanced postprandial glucagon-like peptide-1 secretion during obesity development has a protective role against glucose intolerance induction in rats. British Journal of Nutrition, 122(4), 411-422. PMID: 31352909 https://doi.org/10.1017/S0007114519001223

Qi Z, Xia J, Xue X, He Q, Ji L, & Ding S. (2016) Long-term treatment with nicotinamide induces glucose intolerance and skeletal muscle lipotoxicity in normal chow-fed mice: compared to diet-induced obesity. Journal of Nutritional Biochemistry, 36, 31–41. PMID: 27567590 https://doi.org/10.1016/j.jnutbio.2016.07.005

Ranganath LR, Beety JM, Morgan LM, Wright JW, Howland R, & Marks V. (1996) Attenuated GLP-1 secretion in obesity: cause or consequence? Gut, 38(6), 916–919. PMID: 8984033 https://doi.org/10.1136/gut.38.6.916

Rees DA & Alcolado JC. (2005) Animal models of diabetes mellitus. Diabetic Medicine, 22(4), 359–370. PMID: 15787657 https://doi.org/10.1111/j.1464-5491.2005.01499.x

Reeves PG (1997) Components of the AIN-93 diets as improvements in the AIN-76A diet. Journal of Nutrition, 127, 838S-841S. PMID: 9164249 https://doi.org/10.1093/jn/127.5.838S

Richards P, Pais R, Habib AM, Brighton CA, Yeo GS, Reimann F, & Gribble FM. (2015) High fat diet impairs the function of glucagon-like peptide-1 producing L-cells. Peptides, 77, 21-27. PMID: 26145551 https://doi.org/10.1016/j.peptides.2015.06.006

Rijkelijkhuizen JM, Girman CJ, Mari A, Alssema M, Rhodes T, Nijpels G, Kostense PJ, Stein PP, Eekhoff EM, Heine RJ, & Dekker JM. (2009) Classical and model-based estimates of beta-cell function during a mixed meal vs. an OGTT in a population-based cohort. Diabetes Research and Clinical Practice, 83, 280–288. PMID: 19097663 https://doi.org/10.1016/j.diabres.2008.11.017

Rouillé Y, Kantengwa S, Irminger JC, & Halban PA. (1997) Role of the prohormone convertase PC3 in the processing of proglucagon to glucagon-like peptide 1. Journal of Biological Chemistry, 272(52), 32810–32816. PMID: 9407057 https://doi.org/10.1074/jbc.272.52.32810

Ryskjaer J, Deacon CF, Carr RD, Krarup T, Madsbad S, Holst J, & Vilsbøll T. (2006) Plasma dipeptidyl peptidase-IV activity in patients with type-2 diabetes mellitus correlates positively with HbAlc levels, but is not acutely affected by food intake. European Journal of Endocrinology, 155(3), 485-93. PMID: 1691460 https://doi.org/10.1530/eje.1.02221

Sakamoto E, Seino Y, Fukami A, Mizutani N, Tsunekawa S, Ishikawa K, Ogata H, Uenishi E, Kamiya H, Hamada Y, Sato H, Harada N, Toyoda Y, Miwa I, Nakamura J, Inagaki N, Oiso Y, & Ozaki N. (2012) Ingestion of a moderate high-sucrose diet results in glucose intolerance with reduced liver glucokinase activity and impaired glucagon-like peptide-1 secretion. Journal of Diabetes Investigation, 3(5), 432–440. PMID: 24843603 https://doi.org/10.1111/j.2040-1124.2012.00208.x

Sakar Y, Duca FA, Langelier B, Devime F, Blottiere H, Delorme C, Renault P, & Covasa M. (2014) Impact of high-fat feeding on basic helix-loop-helix transcription factors controlling enteroendocrine cell differentiation. International Journal of Obesity, 2, 1440–1448. PMID: 24480860 https://doi.org/10.1038/ijo.2014.20

Sathananthan M, Farrugia LP, Miles JM, Piccinini F, Dalla Man C, Zinsmeister AR, Cobelli C, Rizza RA, & Vella A. (2013) Direct effects of exendin-(9,39) and GLP-1-(9,36)amide on insulin action, β-cell function, and glucose metabolism in nondiabetic subjects. Diabetes, 62(8), 2752-2756. PMID: 23545708 https://doi.org/10.2337/db13-0140.

Sato-Mito N, Suzui M, Yoshino H, Kaburagi T, & Sato K. (2009) Long term effects of high fat and sucrose diets on obesity and lymphocyte proliferation in mice. Journal of Nutrition, 13(7), 602–606. PMID: 19621195 https://doi.org/10.1007/s12603-009-0170-2

Schirra J, Nicolaus M, Roggel R, Katschinski M, Storr M, Woerle HJ, & Göke B. (2006) Endogenous glucagon-like peptide 1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans. Gut, 55(2), 243–251. PMID: 15985560 https://doi.org/10.1136/gut.2004.059741

Beglinger S, Drewe J, Schirra J, Göke B, D'Amato M, & Beglinger C. (2010) Role of fat hydrolysis in regulating glucagon-like Peptide-1 secretion. The Journal of Clinical Endocrinology and Metabolism, 95(2), 879-986. PMID: 19837920 https://doi.org/10.1210/jc.2009-1062

Serre V, Dolci W, Schaerer E, Scrocchi L, Drucker D, Efrat S, & Thorens B. (1998) Exendin-(9–39) is an inverse agonist of the murine Glucagon-Like Peptide-1 receptor: Implications for basal intracellular cyclic adenosine 3',5'- monophosphate levels and beta cell glucose competence. Endocrinology, 139(11), 4448–4454. PMID: 9794451 https://doi.org/10.1210/en.139.11.4448

Shang W, Yasuda K, Takahashi A, Hamasaki A, Takehiro M, Nabe K, Zhou H, Naito R, Fujiwara H, Shimono D, Ueno H, Ikeda H, Toyoda K, Yamada Y, & Kurose T. (2002) Effect of high dietary fat on insulin secretion in genetically diabetic Goto-Kakizaki rats. Pancreas, 25(4), 393–399. PMID: 12409835 https://doi.org/10.1097/00006676-200211000-00012

Shannon M, Green B, Willars G, Wilson J, Matthews N, Lamb J, Gillespie A, & Connolly L. 2017) The endocrine disrupting potential of monosodium glutamate (MSG) on secretion of the glucagon-like peptide-1 (GLP-1) gut hormone and GLP-1 receptor interaction. Toxicology Letters, 265, 97–105. PMID: 27890806 https://doi.org/10.1016/j.toxlet.2016.11.015

Sheludiakova A, Rooney K, & Boakes RA. (2012) Metabolic and behavioural effects of sucrose and fructose/glucose drinks in the rat. European Journal of Nutrition, 51(4), 445–454. PMID: 21800086 https://doi.org/10.1007/s00394-011-0228-x

Shirai T, Shichi Y, Sato M, Tanioka Y, Furusho T, Ota T, Tadokoro T, Suzuki T, Kobayashi K, & Yamamoto Y. (2015) High dietary fat-induced obesity in Wistar rats and type 2 diabetes in nonobese Goto-Kakizaki rats differentially affect retinol binding protein 4 expression and vitamin A metabolism. Nutrition Research, 36, 262–270. PMID: 26923513 https://doi.org/10.1016/j.nutres.2015.11.018

Singh AK. (2015) Glucagon-like peptide 1 and dysglycemia: Conflict in incretin science. Indian Journal of Endocrinology and Metabolism, 19(1), 182–187. PMID: 25593851 https://doi.org/10.4103/2230-8210.146881

Smushkin G, Sathananthan A, Man CD, Zinsmeister AR, Camilleri M, Cobelli C, Rizza RA, & Vella A. (2012) Defects in GLP-1 response to an oral challenge do not play a significant role in the pathogenesis of prediabetes. Journal of Clinical Endocrinology and Metabolism, 97(2), 589–598. PMID: 22090278 https://doi.org/10.1210/jc.2011-2561

Srinivasan K & Ramarao P. (2007) Animal models in type 2 diabetes research: an overview. Indian Journal of Medical Research, 125(3), 451-472. PMID: 17496368

Staten MA & Kelley DE. (2014) Using oral challenge testing to assess insulin action and secretion with mathematical modeling. Diabetes, 63, 1188–1190. PMID: 24651802 https://doi.org/10.2337/db13-1839

Steinert RE, Feinle-Bisset C, Asarian L, Horowitz M, Beglinger C, & Geary N. (2017) Ghrelin, CCK, GLP-1, and PYY(3-36): Secretory Controls and Physiological Roles in Eating and Glycemia in Health, Obesity, and After RYGB. Physiological Reviews, 97(1), 411–463. PMID: 28003328 https://doi.org/10.1152/physrev.00031.2014

Sumiyoshi M, Sakanaka M, & Kimura Y. (2006) Chronic intake of high-fat and high-sucrose diets differentially affects glucose intolerance in mice. Journal of Nutrition, 136, 582–587. PMID: 16484528 https://doi.org/10.1093/jn/136.3.582

Sun EW, de Fontgalland D, Rabbitt P, Hollington P, Sposato L, Due SL, Wattchow DA, Rayner CK, Deane AM, Young RL, & Keating DJ. (2017) Mechanisms controlling glucose-induced GLP-1 secretion in human small intestine. Diabetes, 66(8), 2144-2149. PMID: 28385801 https://doi.org/10.2337/db17-0058

Taylor R. (2012) Insulin resistance and type 2 diabetes. Diabetes, 61, 778–779. https://doi.org/10.2337/db12-0073

Thombare K, Ntika S, Wang X, & Krizhanovskii C. (2017) Long chain saturated and unsaturated fatty acids exert opposing effects on viability and function of GLP-1-producing cells: Mechanisms of lipotoxicity. PLoS One, 12(5), e0177605. PMID: 28520810 https://doi.org/10.1371/journal.pone.0177605

Toft-Nielsen MB, Damholt MB, Madsbad S, Hilsted LM, Hughes TE, Michelsen BK, & Holst JJ. (2001) Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients. The Journal of Clinical Endocrinology and Metabolism, 86(8), 371737-23. PMID: 11502801 https://doi.org/10.1210/jcem.86.8.7750

Tolhurst G, Reimann F, & Gribble FM. (2009) Nutritional regulation of glucagon-like peptide-1 secretion. Journal of Physiology, 587(1), 27–32. PMID: 19001044 https://doi.org/10.1113/jphysiol.2008.164012

Tourrel C, Bailbe D, Lacorne M, Meile MJ, Kergoat M, & Portha B. (2002) Persistent improvement of type 2 diabetes in the Goto-Kakizaki rat model by expansion of the beta-cell mass during the prediabetic period with glucagon-like peptide-1 or exendin-4. Diabetes, 51, 1443–1452. PMID: 11978641 https://doi.org/10.2337/diabetes.51.5.1443

Tseng CC, Zhang XY, & Wolfe MM. (1999) Effect of GIP and GLP-1 antagonists on insulin release in the rat. American Journal of Physiology, 276(6):E1049-54. PMID: 10362617 https://doi.org/10.1152/ajpendo.1999.276.6.E1049

Vahl TP, Paty BW, Fuller BD, Prigeon RL, & D'Alessio DA. (2003) Effects of GLP-1-(7-36)NH2, GLP-1-(7-37), and GLP-1-(9-36)NH2 on intravenous glucose tolerance and glucose-induced insulin secretion in healthy humans. Journal of Clinical Endocrinology and Metabolism, 88(4), 1772–1779. PMID: 12679472 https://doi.org/10.1210/jc.2002-021479

Vilsbøll T & Holst JJ. (2004) Incretins, insulin secretion and type 2 diabetes mellitus. Diabetologia, 47(3), 357–366. PMID: 14968296 https://doi.org/10.1007/s00125-004-1342-6

Vilsbøll T, Krarup T, Madsbad S, & Holst JJ. (2003) Both GLP-1 and GIP are insulinotropic at basal and postprandial glucose levels and contribute nearly equally to the incretin effect of a meal in healthy subjects. Regulatory Peptides, 114(2–3), 115–121. PMID: 12832099 https://doi.org/10.1016/S0167-0115(03)00111-3

Wang F, Yoder SM, Yang Q, Kohan AB, Kindel TL, Wang J, & Tso P. (2015) Chronic high-fat feeding increases GIP and GLP-1 secretion without altering body weight. American Journal of Physiology-Gastrointestinal and Liver Physiology, 309(10), 807–815. PMID: 26336929 https://doi.org/10.1152/ajpgi.00351.2013

Wang X, DuBois DC, Cao Y, Jusko WJ, & Almon RR. (2014) Diabetes disease progression in Goto-Kakizaki rats: effects of salsalate treatment. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy, 7, 381–389. PMID: 25120374 https://doi.org/10.2147/DMSO.S65818

Wang X, Liu H, Chen J, Li Y, & Qu S. (2015) Multiple factors related to the secretion of glucagon-like peptide-1. International Journal of Endocrinology, 2015, 651757 PMID: 26366173 https://doi.org/10.1155/2015/651757

Wang YW, Sun GD, Sun J, Liu SJ, Wang J, Xu XH, & Miao LN. (2013) Spontaneous type 2 diabetic rodent models. Journal of Diabetes Research, 2013, 401723. PMID: 23671868 https://doi.org/10.1155/2013/401723

Wang Y, Wang PY, Qin LQ, Davaasambuu G, Kaneko T, Xu J, Murata S, Katoh R, & Sato A. (2003) The development of diabetes mellitus in Wistar rats kept on a high-fat/low-carbohydrate diet for long periods. Endocrine, 22(2), 85–92. PMID: 14665711 https://doi.org/10.1385/ENDO:22:2:85

Whiting DR, Guariguata L, Weil C, & Shaw J. (2011) IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Research and Clinical Practice, 94(3), 311–321. PMID: 22079683 https://doi.org/10.1016/j.diabres.2011.10.029

Wölnerhanssen BK, Cajacob L, Keller N, Doody A, Rehfeld JF, Drewe J, Peterli R, Beglinger C, & Meyer-Gerspach AC. (2016) Gut hormone secretion, gastric emptying, and glycemic responses to erythritol and xylitol in lean and obese subjects. American Journal of Physiology-Endocrinology and Metabolism, 310(11), E1053–E1061. PMID: 27117004https://doi.org/10.1152/ajpendo.00037.2016

Yabe D & Seino Y. (2011) Two incretin hormones GLP-1 and GIP: Comparison of their actions in insulin secretion and b cell preservation. Progress in Biophysics and Molecular Biology, 107(2), 248–256. PMID: 21820006 https://doi.org/10.1016/j.pbiomolbio.2011.07.010

Yang ZH, Miyahara H, Takeo J, & Katayama M. (2012) Diet high in fat and sucrose induces rapid onset of obesity-related metabolic syndrome partly through rapid response of genes involved in lipogenesis, insulin signalling and inflammation in mice. Diabetology & Metabolic Syndrome, 4, 32. PMID: 22762794 https://doi.org/10.1186/1758-5996-4-32

Zakrzewski-Fruer JK, Plekhanova T, Mandila D, Lekatis Y, & Tolfrey K. (2017) Effect of breakfast omission and consumption on energy intake and physical activity in adolescent girls: a randomised controlled trial. British Journal of Nutrition, 118(5), 392–400. PMID: 28901889 https://doi.org/10.1017/S0007114517002148

Zimmet P, Alberti KG, & Shaw J. (2010) Global and societal implications of the diabetes epidemic. Nature, 414, 782-787. PMID: 11742409 https://doi.org/10.1038/414782a

Academic conferences

2016 *Poster presentation* in the title of "Effect of high fat and/or high sucrose diet on meal-induced GLP-1 secretion during the progression of diet induced obesity in rats" in the Annual conference of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, Hokkaido branch" in Sapporo, Japan.

2017 *Oral presentation* in the title of "Impact of chronic high fat and/or high sucrose feeding on meal-induced GLP-1 secretion" in the Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry", Kyoto, Japan

Poster presentation in the title of "The role of endogenous GLP-1 against the development of glucose intolerance in rats fed a high fat and high sucrose diet" in the Annual conference of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, Hokkaido branch", Obihiro, Japan

2018 *Poster presentation* in the title of "Postprandial GLP-1 response in Wistar and Goto-Kakizaki rats during diet-induced obesity" in the Asia-Pacific Diabetes and Obesity Study Group (APDO) Symposium 2018, Kobe, Japan

2019 *Poster presentation* in a title of "Long-term effects of high fat/high sucrose diet feeding on postprandial GLP-1 response in Wistar and Goto-Kakizaki rats" in Hokkaido University Internal Symposium, Sapporo, Japan

Poster presentation in the title of "Postprandial GLP-1 response was enhanced in non-diabetic rats, but not in diabetic rats during obesity development" in the International Diabetes Federation (IDF) Congress 2019, Busan, Korea

Peer-reviewed publication

Pinyo, J., Hira, T., and Hara, H. (2019). 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. Nutrition, 62, 122-130.

Pinyo, J., Hira, T., and Hara, H. (2019). Enhanced postprandial glucagon-like peptide-1 secretion during obesity development has a protective role against glucose intolerance induction in rats. British Journal of Nutrition, 122, 411–422.

Hira, T., Pinyo, J., and Hara, H. (2020). What Is GLP-1 Really Doing in Obesity? Trends in Endocrinology & Metabolism, 31(2), 71-80.

Pinyo, J., Hara, H., and Hira T. (2020). Diet-induced obesity enhances postprandial GLP-1 secretion in Wistar rats, but not in diabetic Goto-Kakizaki rats (Under submission)

Acknowledgements

Throughout the writing of this dissertation I have received a great deal of support and assistance. First and foremost, I would like to express my special appreciation and thanks to my advisor, Associate professor Dr. Tohru Hira, for accepting me as your advisee, encouraging the research, and allowing me to grow as a research scientist. You have been a tremendous mentor for me. Your advice and suggestion on both research and my livelihood during my study at Hokkaido University have been invaluable. Your valuable advice and wonderful supports made me possible to achieve my goal in academic field.

This is also a great opportunity to express my respect to Professor Dr. Satoshi Ishizuka for your kindly review my dissertation. Also, I am really thankful for your time and effort in suggestion and insightful comments through my research for improvement.

I sincerely thanks to Professor Dr. Hiroshi Hara for giving me the opportunity to study at Hokkaido University. Without your supervision, help, suggestion, and encouragement throughout my study, this thesis could not have been possibly finished. I will keep on trusting you for my future.

I owe my profound gratitude to Professor Dr. Kei Sonoyama, who kindly reviewed my dissertation and gave me the valuable suggestions as well as constructive comments.

I am so much indebted to all members in Laboratory of Nutritional Biochemistry since I first came here until now for their kind help, support, advice, and sharing every unforgettable moment and being my family here in Sapporo.

I also would like to thank Dr. Pairoj Luangpituksa and Associate Professor Dr. Rungtiwa Wongsagonsup at Mahidol University, Thailand for your support since my study in Thailand up to this point.

My appreciation is also extended to Thai students in Hokkaido University for always being beside me in both good and bad times.

I gratefully thank Monbukagakusho (MEXT) Scholarship for providing me an excellent opportunity to study in Laboratory of Nutritional Biochemistry, Graduate School of Agriculture, Faculty of Agriculture, Hokkaido University.

Special thanks to Mrs. Yoko Iwatani, Ms. Asami Igarashi, Ms. Hiromi Kanahashi, Ms. Natsumi Kudo, and Ms. Haruka Imada for always helping me in academic services throughout my study at Hokkaido University.

Last but not least, I would like to thank my family for their love, understanding, and all supporting during my study.

This accomplishment would not have been possible without all these people.

Jukkrapong Pinyo (Hongtae)

Hokkaido University, Sapporo, Japan