Novel function of (-)-epigallocatechin-3-gallate on gut hormone secretion

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Novel function of (-)-epigallocatechin-3-gallate on gut hormone secretion

エピガロカテキンガレートの
腸管ホルモン分泌に関する新しい機能

August 2015

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CONTENTS

Abbreviations

AITC	allyl isothiocyanate
C	(-)-catechin
CaSR	calcium-sensing receptor
CCK	cholecystokinin
Cg	(-)-catechin -3-gallate
CNS	central nervous system
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyl sulfoxide
DPP IV	dipeptidyl peptidase IV
EC	(-)-epicatechin
ECg	(-)-epicatechin-3-gallate
EGC	(-)-epigallocatechin
EGCg	(-)-eigallocatechin-3-gallate
FBS	fetal bovin serum
GC	(-)-gallocatechin
GCg	(-)-gallocatechin-3-gallate
GLP-1	glucagon-like peptide 1
GPCR	G-protein coupled receptors
HBSS	Hanks’ balanced salt solution
PBS	phosphate buffered saline
PC1	prohormone convertase 1
PYY	peptide tyrosine-tyrosine
qPCR	quantitative polymerase chain reaction

General introduction

Food compounds are composed of not only nutrients, but also of non-digestive compounds that affect the body. Polyphenols are one of these chemicals; they are secondary metabolites in plants and function as protective agents against stress. When consumed, dietary polyphenols help humans to prevent various diseases, including obesity [1]. One of the causes of obesity is excessive eating, which is generally regulated by the neuroendocrine system. It is possible that polyphenols affect this endocrine system and modify appetite.

Anorexigenic gut hormones

Postprandial energy metabolism is regulated not only by the brain, but also by gut organs. Its regulation is mediated by nervous and endocrine systems [2]. Key players of these systems are peptide hormones secreted from the gut epithelia in response to food compounds. At least 10 gut hormones were identified and revealed their contribution in digestion-related functions such as motility of the stomach and intestines, secretion of digestive enzymes, blood glucose homeostasis, and appetite control in the brain (Table 0.1). Part of the gut hormones reach the hypothalamus via afferent neurons that are innervated to the gastrointestinal tract, and accordingly modify food intake behavior [3]. Among these hormones, cholecystokinin (CCK), glucagon-like peptide (GLP)-1 and peptide tyrosine-tyrosine (PYY) have been shown

to be the primary satiety signals and act as anorexigenic hormones [4] (Figure 0.1). Dietary nutrients ingested in the gut are potent inducers of these gut hormones [4]. CCK is secreted from the proximal intestine such as the duodenum and jejunum in response to dietary carbohydrates, proteins and fats [4, 5]. CCK consists of a series of peptides which are cleaved from the common precursor composed of 115 amino acids. The one which is produced in the gut consists of 33 amino acids of 1 to 33 (CCK-33). CCK-33 is an amidated carboxy-terminal pentapeptide (Gly-Trp-Asp-Met-Phe-NH_2) at their C-terminal, which confers the biological activity of CCK, and where the tyrosine residue occurs in sulfated form [6]. The secreted CCK is received by vagal afferent nerve which projects to the nucleus of the solitary tract (NST) in the brain stem. The neural network transmits the satiety input at NST to hypothalamus, where anorexigenic pathways are activated.

GLP-1 is released from the hind gut region such as the ileum and the colon in response to dietary carbohydrates, proteins, and fats [4, 7]. The secreted GLP-1 reaches to the NST directly by blood stream or via signals in the vagal nerve. The active forms of GLP-1 produced in the gut are GLP-1 (7-37) and GLP-1 (7-36) amide, which is a C-terminal α-amidated form of GLP-1 (7-36). Secreted GLP-1 (7-36) amide is rapidly metabolized to GLP-1 (9-36) by dipeptidyl peptidase IV [8,9], which make a half life time of its efficacy in less than 2 minutes [9,10]. GLP-1 (9-36) comprises about 60% of circulating GLP-1 peptides in the blood and shown to no longer possess its bioactivity.

PYY is produced in the same cell which produces GLP-1 in the gut, and secreted in response to proteins and fats [4, 11]. PYY undergoes modification by cleaving the last 2 residues at N-terminus, tyrosine-proline residues, results to form PYY (3-36) [12]. PYY (1-36) amounts to about 60%, and PYY (3-36) does 40% of circulating PYY in the blood [13]. PYY (1-36) and PYY (3-36) cross the blood-brain barrier to be received at hypothalamus and related with weight control via feeding regulation [14, 15]. The PYY peptides selectively activate neuropeptide Y receptor (NPYR) isoforms. These are G protein coupled-receptors (GPCR) and consists 5 isoforms [16]. PYY (3-36) is received by NPY2R and NPY5R, whereas PYY (1-36) activate NPY1R in addition to those 2 receptors [17]. Those 3 receptors were shown to be expressed in hypothalamus as well as the colon and the intestine. Especially, PYY (3-36) has been shown to induce anorexigenic effects through NPY2R and NPY5R [17, 18]. PYY (3-36) has been shown to have a longer duration of action than other anorexigenic peptides such as CCK and GLP-1 [3,4]. Together with other body states such as stomach distension and energy metabolism at the liver and adipocytes, anorexigenic gut hormones coordinate energy intake and expenditure through hypothalamus.

Reception of food compounds

The study of chemical reception of food compounds has revealed that some nutrients activate several kinds of GPCRs, including taste receptors, calcium-sensing receptor (CaSR), lysophosphatidic acid receptor 5 (LPAR5), G-protein coupled

receptor family C group 6 (GPRC6A), and free fatty acid receptors (FFARs) (Table 0.2). Type 1 family of taste receptors, TAS1Rs, has been shown to be activated by sugars, amino acids and oligopeptides [19, 20]. CaSR and GPRC6A have also been shown to respond to amino acids [21, 22, 23]. LPAR5 has been shown to be activated by amino acids and protein hydrolysates [24]. FFARs respond to many kinds of free fatty acids [25, 26], i.e., FFAR1 and FFAR4, which are also called GPR40 and GPR120, have been known as a receptor for unsaturated medium and long chain free fatty acids [4,27]. FFAR2 and FFAR3, which are also called GPR43 and GPR41, have been shown to be activated by unsaturated short chain free fatty acids [4, 25, 26]. Some of these receptors have been shown to relate to gut hormone secretions. For example, TAS1R2 - TAS1R3 complex is activated by sugars, such as glucose, to release GLP-1 [28, 29]. On the other hand, gut hormones have been shown to be produced in the distinct cells located differently in the epithelia of the gut. Those enteroendocrine cells have been named as I, K, L, and M cells after their characteristics and locations (Table 0.1) [4]. As a receptor for amino acids, CaSR has been shown to be involved in both CCK secretion in I cells and GLP-1 secretion in L cells [4, 30]. GPRC6A receptor was also activated by amino acids, induced GLP-1 secretion from GLUTag cell, an L-cell like model derived from mouse intestine [31]. Activation of LPAR5 by protein hydrolysate induces CCK transcription and secretion in STC-1 cell, a model enteroendocrine cell line derived from mouse small intestine [32]. For fat metabolites, FFAR1 and FFAR4 were revealed to mediate oleic acid

stimulation to induce CCK in I cells and PYY in L cells [21, 26]. Especially, FFAR4 was revealed to be involved in the secretion of GLP-1 *in vivo* as well [33].

Recently, it has been demonstrated that some non-nutrients act on the subfamily of bitter taste receptors in human, hTAS2Rs [34]. Among non-nutrients, polyphenols have been reported to activate these receptors, e.g., malvidin-3 glucoside activates hTAS2R7 [35, 36] and epigallocatechin-3-gallate (EGCg) activates hTAS2R14 and hTAS2R39 [36, 37]. On the other hand, some hTAS2Rs have been shown to act as triggers for gut hormone secretion. For example, activation of hTAS2R3, hTAS2R4, or hTAS2R46 by denatonium benzoate was shown to be involved in the GLP-1 secretion [38] and activation of hTAS2R38 by phenylthiocarbamide was involved in the CCK secretion [39]. These examples demonstrate that not only nutrients but also non-nutrients possibly induce anorexigenic gut hormone secretion.

Epigallocatechin-3-gallate

Green and oolong teas have been reported to play a role of anti-obesity [40, 41]. The anti-obesity effect of teas was mainly attributed to catechins [30]. Catechins share a flavan-3-ol structure, which contains two benzene rings, A-rings and B-rings and a heterocyclic C-ring with a hydroxyl group on third carbon, and additionally contain another hydroxyl group on A-rings, B-rings or a galloyl group on C-ring. Most catechins have hydroxyl group at the C5 and C7 of the A-ring (Figure 0.2) [42, 43]. Among catechins, (-)-epigallocatechin-3-gallate (EGCg) is the most prevalent catechin in green tea and reported to have the anti-obesity activity [44]. EGCg is

accounting for 54%-59% of total green tea catechins, followed by EGC (9-12%), ECg (9-12%) and EC (5-7%) [45, 46]. The underlying mechanism of anti-obesity activity by EGCg was demonstrated in several ways: controlling adipocyte, inhibition of fat absorption, delaying glucose absorption, regulation of peroxisome proliferator-activated receptors which mediates adipogenesis [40, 46, 47, 48]. In addition, it was shown that EGCg reduces adipogenesis through blocking of cell cycles [29]. Inhibitions of pancreatic lipase activity by EGCg reduced fat absorption from the gut [49]. EGCg was also shown to acutely reduce postprandial blood glucose levels via inhibition of α-amylase activity [50, 51]. It was also reported that EGCg affects food intake reduction, which largely participate in reduction of body weight in lean and obesity patients [44]. However, it was not fully understood how EGCg leads reduction of food intake.

This study has been performed to examine the hypothesis that polyphenols such as EGCg affect the secretion of anorexigenic gut hormones, CCK, GLP-1 or PYY, and cause reduction of food intake. In chapter 1, some polyphenols including EGCg found to induce CCK, GLP-1 and PYY secretion *in vitro*, and the effect of EGCg on secretion of anorexigenic hormones was confirmed *ex vivo* using murine intestines. In chapter 2, the molecular mechanisms of GLP-1 release by EGCg were investigated. To investigate a physiological effect of EGCg through anorexigenic gut hormones *in vivo*, food consumption was evaluated for 24 hours after EGCg administration in chapter 3.

Table 0.1. Gut hormones secreted in response to food compounds.

Hormones	Secretagogues	Secretory tissues	Producing cells	Functions
CCK	carbohydrates, proteins, fats	duodenum, jejunum	I cell	brain; postprandial satiety ↑ stomach; gastric emptying pancreas; glucagon release ↑
secretin	proteins, fats	duodenum	S cell	stomach; gastric acid release ↑
GIP	carbohydrates, proteins, fats	duodenum	K cell	stomach; gastric acid release ↑ pancreas; insulin secretion ↑
GLP-1	carbohydrates, proteins, fats	ileum, colon	L cell	brain; postprandial satiety ↑ pancreas; insulin secretion ↑ glucagon secretion ↓
enteroglucagon	carbohydrates, fats	ileum, colon	L cell	stomach; gastric acid release ↓ gastric emptying
PYY	proteins, fats	ileum, colon	L cell	brain; postprandial satiety ↑ stomach; gastric emptying
motilin	fats	duodenum, jejunum	M cell	stomach; gastric emptying intestine; gut motility
somatostatin	fats	duodenum, Stomach, pancreas	D cell	stomach; gastric acid release ↓ pancreas; insulin secretion ↓
oxyntomodulin	proteins, fats	ileum, colon	L cell	stomach; gastric acid release ↓ gastric emptying

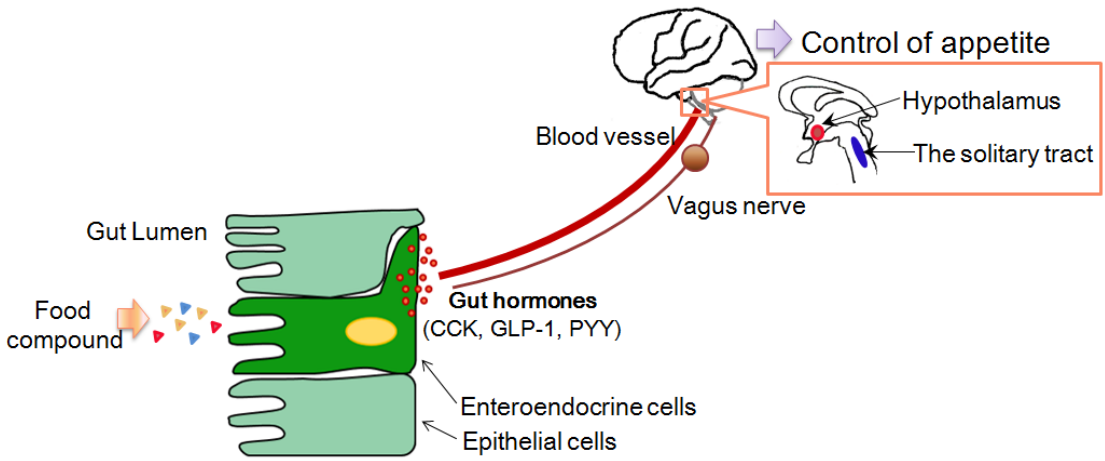

Figure 0.1. The schematic drawing of the function of anorexigenic gut hormones.

Gut hormones are produced in the enteroendocrine cells located in the gut epithelia. Anorexic gut hormones, CCK, GLP-1, and PYY are secreted in response to the food compounds passing along the gut lumen. The hormones are transmitted to the brain where they affect the regulation of food intake.

Table 0.2. GPCRs which are activated by nutrients.

Nutrients		Receptors
carbohydrates	glucose, sucrose	TAS1R2 - TAS1R3 [20, 28]
proteins	amino acids, oligopeptides	TAS1R1-TAS1R3 [20]
	amino acids, protein hydrolysates	LPAR5* [24]
	amino acids	CaSR [31], GPRC6A #[23]
fats	short-chain fatty acids	FFAR2, FFAR3 [52, 53]
	medium-chain fatty acids long-chain fatty acids	FFAR1, FFAR4 [54, 55]
	long-chain fatty acids	GPR120 † [27]

* LPAR5; lysophosphatidic acid receptor 5
# GPRC6A; G-protein coupled receptor family C group 6
† GPR120; G protein-coupled receptor 119

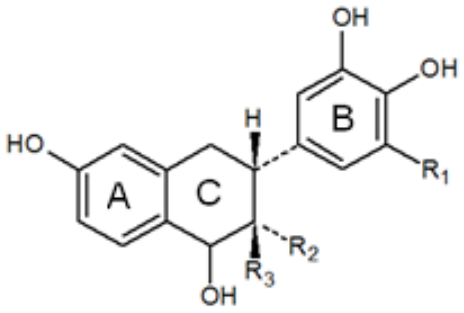

Figure 0.2. The structure of catechins.

(-) C (R1=H, R2=H, R3=OH) , (-) EC (R1=H, R2=OH, R3=H), (-) GC (R1=OH, R2=H, R3=OH), (-) EGC (R1=OH, R2=OH, R3=OH) (-) Cg (R1=H, R2=H, R3=OG), (-) ECg (R1=H, R2=OG, R3=H), (-) GCC (R1=OH, R2=H, R3=OG), (-) EGCg (R1=OH, R2=OG, R3=H). OH, hydroxy group; OG, galloyl group.

Chapter 1

Induction of secretion of anorexigenic gut hormones by polyphenols

1.1 Introduction

Gut hormones are produced and secreted from the enteroendocrine cells in response to food compounds. Enteroendocrine cells are distributed throughout the epithelial layer of gut [55]. There are more than 10 subtypes of enteroendocrine cells which release peptide hormones. Most of those cells secrete single hormone but some are able to secrete more than one hormone [4, 56]. In order to investigate the candidate inducers for these gut hormones, cultured cell lines provide useful tools. The Caco-2 cell line derived from a human colon cancer tissue is often used as an intestinal epithelial model for *in vitro* analysis of uptake of food compounds, whereas it is partially presents chromogranin A, which is widely expressed in enteroendocrine cells [57]. Therefore, it was worth evaluating to verify whether the Caco-2 cells can be used as a model for release of anorexigenic gut hormones such as CCK, GLP-1 and PYY.

The functional food compounds apart from the nutrients are mostly secondly metabolites in various plants. Polyphenols are part of them and compose a large family. The family contains several thousand molecules, and among them, several

hundred are found in edible plants [58]. They are divided into flavonoids, phenylpropanoids and anthraquinones by the degree of polymerization of the phenol structure. Flavonoids are further divided into several groups such as flavonols, flavones, isoflavones, flavanones, and flavanols (Figure 1.1) [1]. In order to search new triggers for gut hormone release among polyphenols, at least one compound from each group were selected for the experiments of anorexigenic gut hormones.

In this chapter, first, Caco-2 cell was evaluated if it secrets anorexigenic gut hormones in response to natural secretagogues, such as amino acids, fatty acids, and sugars. Then, polyphenols are applied to Caco-2 cells to search their activity on gut hormone secretion. To clarify if this effect of polyphenols on gut hormone release arise in animal tissues or not, an *ex vivo* analysis using murine intestines was performed. As a result, Caco-2 cell was shown to secrete CCK, GLP-1 and PYY in response to nutrients. Using Caco-2 cell, chlorogenic acid, EGCg, GCg, and ferulic acid were revealed to secret anorexigenic gut hormones. The fact that stereoisomers of EGCg share the functions on gut hormone release might provide electric properties of chemicals. Especially, EGCg was shown to induce all three hormones *in vitro.*

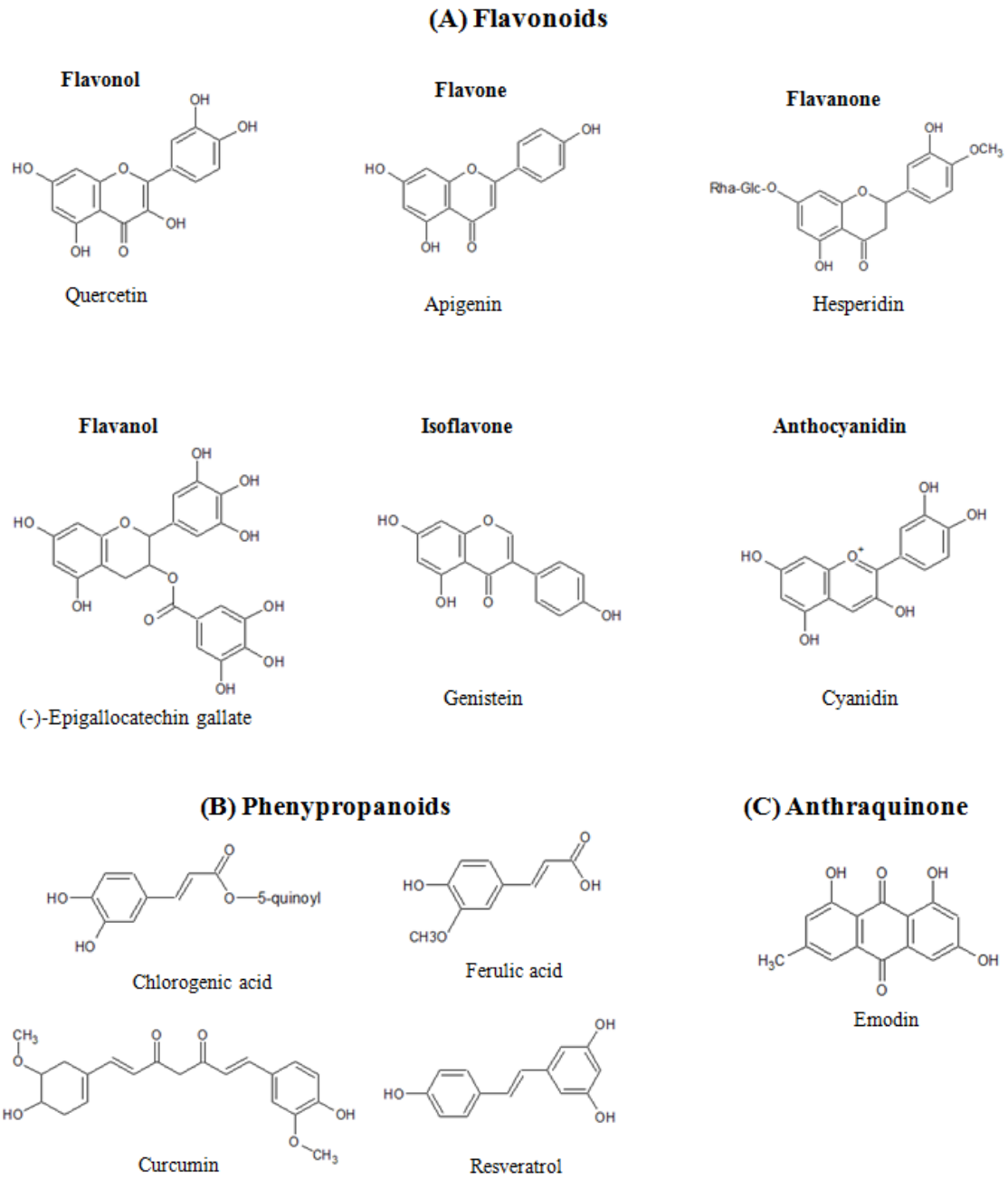

Figure 1.1. Chemical structure of polyphenols used in this study

The names written in bold at upper position of structural drawings represent the groups, and those in the lower position represent the name of chemicals.

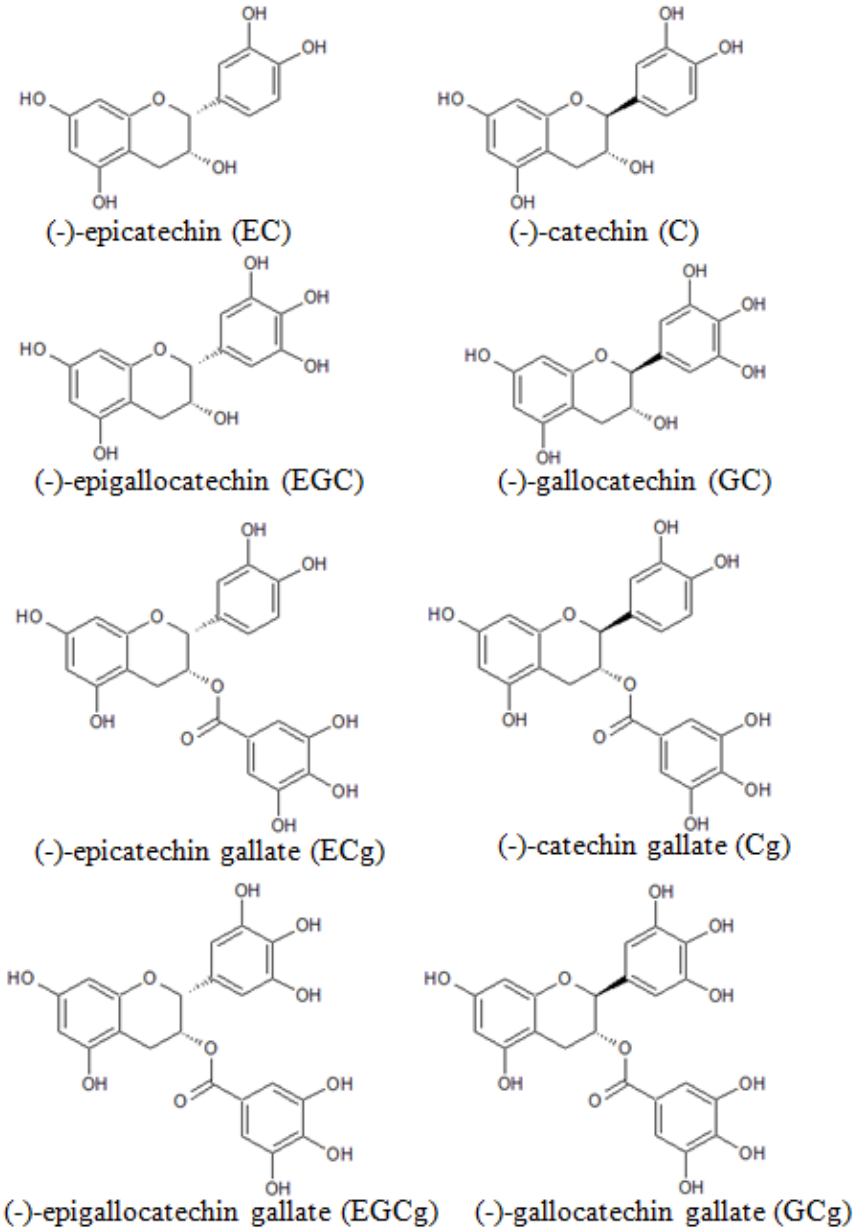

Figure 1.2. Chemical structures of catechins used in this study.

1.2 Materials and methods

1.2.1 Chemicals.

Oleic acid and resveratrol were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Phenylalanine was from Nippon Rika (Tokyo, Japan). Quercetin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Apigenin and emodin were from Tokyo Chemical Industry (Tokyo, Japan). Cyanidin chloride and hesperidin were from Extrasynthese (Genay, France). Ferulic acid and genistein were from LKT Laboratories (St. Paul, MN, USA). Dipeptidyl peptidase (DPP) IV inhibitor was from Calbiochem (San Diego, CA, USA). Palmitic acid, glutamine, glucose, sucrose, chlorogenic acid hemihydrates, (-)-epicatechin (EC), (-)-catechin (C), (-)-epigallocatechin (EGC), (-)-gallocatechin (GC), (-)-epicatechin-3-gallate (ECg), (-)-catechin-3-gallate (Cg), EGCg, (-)-gallocatechin-3-gallate (GCg), curcumin and sodium pyruvate solution were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest grade commercially available.

1.2.2 Cell culture and treatment

All the procedures were performed at 37°C unless specifically described. Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, # D6429, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 95% air-5% CO_2. Cells were used from passage 54 to 58. For assays, cells were seeded into 24-well culture plate with 2×10^5 cells per well and incubated for 24 hours. Subsequently, the medium was changed to stimulant-

containing assay buffer composed of phosphate buffered saline (PBS) containing 1 mM pyruvate and 0.1 mg/ml DPP IV inhibitor. After incubation for 2 hours, assay buffers were collected and stored at -20°C until used. According to the stimulants' solubility, the assay buffers were modified for each experiment. Oleic acid and palmitic acid were dissolved in dimethyl sulfoxide and diluted with assay buffer at 1:1000. Each tested polyphenols was dissolved in solution composed by methanol and trifluoroacetic acid as 10:1, then diluted with assay buffer at 1:200. The concentrations of stimulants for the assay are as follows: glucose and sucrose, 100 mM; glutamine, 25 mM; phenylalanine, 50 mM; oleic acid and palmitic acid, 1.5 mM; apigenin, genistein, hesperidin and quercetin, 100 μM; cyanidin, EC, C, EGC, GC, ECg, Cg, EGCg, GCg and resveratrol, 300 μM; chlorogenic acid and ferulic acid, 1 mM; curcumin and emodin, 50 μM.

1.2.3 RNA extraction and RT-PCR from Caco-2 cells.

Total RNA was extracted using Sepasol (Nacalai Tesque) with manufactural instructions. Two micrograms of RNA were used for cDNA synthesis with ReverTra Ace (Toyobo, Tokyo, Japan). The sequences of primers (Invitrogen, Carlsbad, CA, USA) for analyzed genes are shown Table 1.1. The PCR program was 30 cycles of denaturation at 98°C for 10 seconds, annealing at 56-58°C for 30 seconds and extension at 68°C for 30 seconds by iCycler (Bio-Rad, Munich, Germany). The products were detected on an agarose gel by UV irradiation with ethidium bromide.

1.2.4 Cell viability assay

Cell viability was determined using the CellTiter-Glo® luminescent cell viability assay (# G7570, Promega, Madison, WI, USA). Caco-2 cells (2×10^3) were seeded onto 384 well plates, incubated for 24 hours for adherence, and treated with stimulant for 2 hours. After rinse with PBS, CellTiter-Glo® reagent was added and mixed for 2 minutes on an orbital shaker to induce cell lysis, subsequently incubated at room temperature for 10 minutes. The luminescence was recorded by SH-9000 microplate reader (Corona Electric Co., Ibaraki, Japan). Cell viability was calculated by each luminescent value normalized with that of the well treated with no stimulants, and present as percentiles. The assay was performed in triplicate for each stimulant.

1.2.5 Treatment of murine intestines

This study was approved by the Institutional Animal Care and Use Committee (Permission number: 25-10-03) and carried out according to the Kobe University Animal Experimentation Regulations. Entire intestine of mice (ICR, male, 7 weeks, n=6) was excised and divided into duodenum, ileum and colon. Each piece of intestine was washed with Hanks' balanced salt solution (HBSS, Nacalai Tesque) and cut into length of 0.5 cm. These pieces were turned inside out and immersed in 0.25 ml of HBSS containing 0.1 mg/ml DPP IV inhibitor with or without 1 mM EGCg at 37°C for 45 minutes. Subsequently, HBSS was collected and centrifuged at 4°C for 5 minutes at 1000 *g* to remove debris. The supernatants were stored at -20°C until measured.

1.2.6 ELISA

Concentration of CCK, GLP-1 and PYY was measured by ELISA (# CEK-069-04, # EK-028-11, and # EK-059-02, Phoenix Phamaceuticals. Burlingame, CA, USA) according to the manufacturer's instructions with SH-9000 microplate reader. The primary antiserum for CCK cross reacts 100% with non-sulfated CCK (26–33) (human, rat, mouse), CCK (27-33), CCK-33 (porcine), big gastrin-1 (human), caerulein, gastrin-1 (human), 42.9% with CCK-33 (human), 12.6% with CCK (30–33), and 0% with pancreatic polypeptide (human) and vasoactive intestinal polypeptide (VIP; human, porcine, rat). The primary antiserum for GLP-1 cross reacts 100% with GLP-1 (7-36) (human, rat, mouse), GLP-1 (9-36) amide, GLP-1 (1-36) amide (human), 0.4% with GLP-1 (7-37) (human, rat, mouse), 0% with calcitonin gene related peptide (CGRP), exendin-4, glucagon (human, rat, mouse, porcine, bovine), glucose-dependent insulinotropic polypeptide, GLP-2 (human), insulin, secretin and VIP. The primary antiserum for PYY cross reacts 100% with PYY (human), PYY (3-36) (human), 5% with PYY (rat, mouse, porcine), PYY (3-36) (rat, mouse, porcine), 0% with amylin amide (human), glucagon (human), insulin (human), pancreatic polypeptide (human), porcine neuropeptide Y and substance P. Data are presented as mean ± SD of individual triplicate experiments. Data were presented as means ± SD of individual triplicate experiments.

1.2.7 Statistical Analysis.

Dunnett's test was performed to analyze the data of cultured cells. The data from the assay using murine intestines were analyzed by Student's *t*-test. Provability (*p*) values below 0.05 were considered statistically significant.

Table 1.1. Sequence information for RT-PCR primers.

Genes	Primer sequences
ACTB (β-actin)	Forward; 5'- CGTGACATTAAGGAGAAGCT-3'
	Reverse; 5'-CATACTCCTGCTTGCTGATC-3'
CCK	Forward; 5'- CGGAAACCTGGAGAACTGCG-3'
	Reverse; 5'-TATCGCAGAGAACGGATGGC-3'
GCG (proglucagon)	Forward; 5'-CCTTCAAGACACAGAGGAGAAATCC-3'
	Reverse; 5'-CCCCAACCTGTTTACATTTAGCG-3'
PC1	Forward; 5'-GGGACAGTAGTAGCTTCCCT-3'
	Reverse; 5'-CATTTCCATTCTGGCTGGGA-3'
PYY	Forward; 5'-TATGGTGTTCGTGCGCAGGC-3'
	Reverse; 5'-GAGGGCCCAGACCTGTGGTGA-3'

1.3 Results

1.3.1 Endocrine characteristics of Caco-2 cells.

The expression of genes encoding the anorexigenic gut hormones, CCK, GLP-1 and PYY, was investigated. GLP-1 is dissociated from proglucagon by prohormone convertase 1 (PC1) during posttranslational processing [59]. RT-PCR analysis demonstrated that *CCK*, *PC1*, *GCG* (proglucagon) and *PYY* were expressed in Caco-2 cells (Figure 1.3). Together with a report that has described CCK secretion from Caco-2 cells [39], these results suggest that Caco-2 cells potentially secrete GLP-1 and PYY in addition to CCK.

1.3.2 Nutrients-dependent secretion of gut hormones from Caco-2 cells.

In humans, CCK and GLP-1 are secreted in response to food intake of dietary carbohydrates, fats and proteins, whereas PYY secretion is induced by fats and proteins. The secretion of CCK, GLP-1 and PYY from Caco-2 cells was investigated by stimulating with the nutrients. Significant CCK secretion from Caco-2 cells was induced by all the tested compounds, except glutamine (Figure 1.4A). GLP-1 secretion increased by all the compounds tested (Figure 1.4B). Only two compounds, glutamine and oleic acid, stimulated PYY secretion (Figure 1.4C). The results indicate that the Caco-2 cell line was a feasible model for secretion of CCK, GLP-1 and PYY.

1.3.3 Non-nutrients which induce secretion of gut hormones from Caco-2 cells.

To investigate whether polyphenols induce anorexigenic gut hormone secretion, Caco-2 cells were directly treated with polyphenols, followed by measurement of secreted hormones in the buffer. The cell viability assay was performed to confirm that the stimulants affect no damage at the each tested concentration (Figure 1.5). Among the polyphenols tested, EGCg significantly increased the secretion of CCK, GLP-1 and PYY from Caco-2 cells in 1.7, 1.8, and 2.0 fold, respectively (Figure 1.6). Ferulic acid increased CCK and GLP-1 secretion in 1.4 and 1.9 fold, respectively (Figure 1.6A and B). Chlorogenic acid increased CCK secretion in 1.3 fold (Figure 1.6A). These result indicated that some polyphenols are able to induce CCK, GLP-1 and PYY secretion.

1.3.4 Gut hormones secretion from Caco-2 cells stimulated by catechins.

The induction of anorexigenic gut hormone release was compared among eight tea catechins listed in Figure 1.2. The catechins tested in this study are in pair of stereoisomers; C and EC, Cg and ECg, EGC and GC, and EGCg and GCg. Concentrations of catechins were determined by its solubility and cell viability. Catechins did not affect the cell viability of Caco-2 cells at 300 μM (Figure 1.7). When Caco-2 was stimulated, CCK secretion was induced by GC, EGCg and GCg in 1.4, 2.2 and 2.1 fold, respectively (Figure 1.8A). EGCg and GCg increased GLP-1 secretion in 2.0 and 1.7 fold and PYY secretion in 3.0 and 2.1 fold, respectively (Figure 1.8B and C). These results indicated that one pairs of stereoisomers, EGCg and GCg induced CCK, GLP-1 and PYY secretion.

1.3.5 Induction of anorexigenic gut hormone secretion from murine intestines by EGCg.

To show whether the candidates proved to stimulate gut hormone secretion from Caco-2 cells also induce gut hormones in the tissue level, an *ex vivo* assay was performed using the duodenum, ileum and colon from mice. EGCg was selected as the test stimulant, because it induced the secretion of all three gut hormones from Caco-2 cells (Figure 1.6). Dissected murine intestines were treated for 45 minutes with 1 mM EGCg, a concentration that is in the range of green tea [58]. The results showed that significant CCK secretion induced by EGCg was observed in the duodenum (Figure 1.9A). A slight, but not significant induction of CCK secretion was observed in the ileum and colon (Figure 1.9A). GLP-1 secretion was to be secreted from the ileum (Figure 1.9B). These results supported the notion that EGCg is able to induce anorexigenic gut hormones in animals.

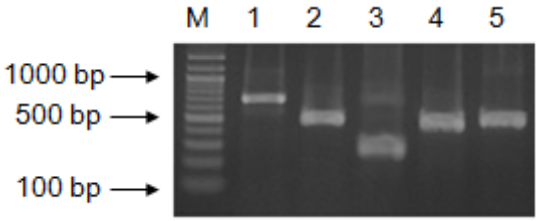

Figure 1.3. Expression of enteroendocrine genes in Caco-2 cells.

Transcripts of *GCG* (lane 1), *PC1* (lane 2), *PYY* (lane 3) and *CCK* (lane 4) were detected by RT-PCR. *ACTB* (lane 5) was used as a positive control. Lane M shows a 100 bp DNA ladder. The expected sizes of the PCR products were 1: 758 bp, 2: 472 bp, 3: 295 bp, 4: 462 bp, and 5: 470 bp.

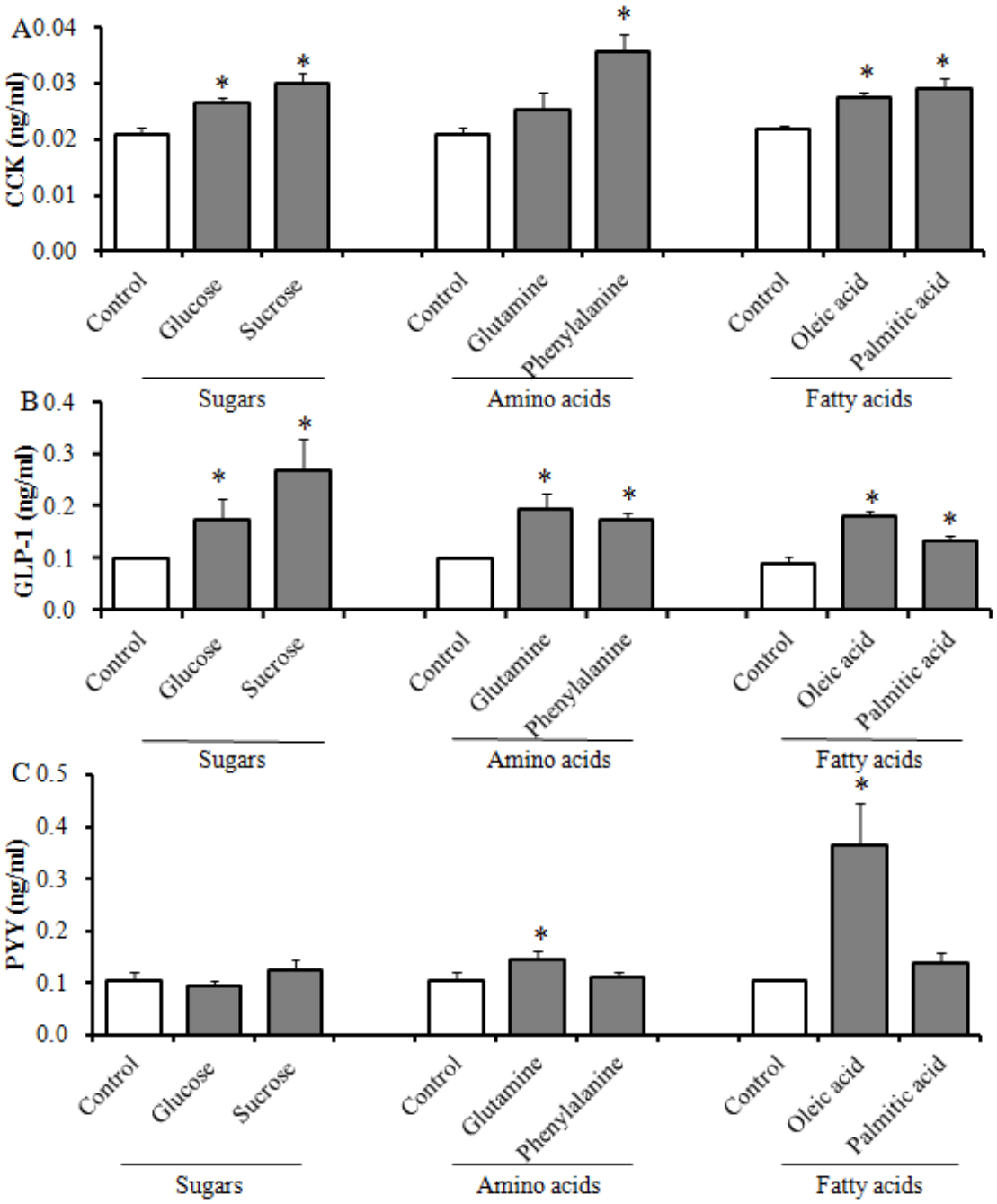

Figure 1.4. Secretion of gut hormones from Caco-2 cells by stimulation of dietary nutrients.

The secretion of CCK (A), GLP-1 (B), and PYY (C) from Caco-2 cells into the buffer was measured after incubation with nutrients for 2 hours. The stimulants' concentrations of were: glucose, 100 mM; sucrose, 100 mM; glutamine, 25 mM; phenylalanine, 50 mM; oleic acid, 1.5 mM and palmitic acid, 1.5 mM. A control value indicates the amount of the hormones in the assay buffer without the nutrients.*Statistical significance was determined by Dunnett's test at $p < 0.05$ vs. control (n=3).

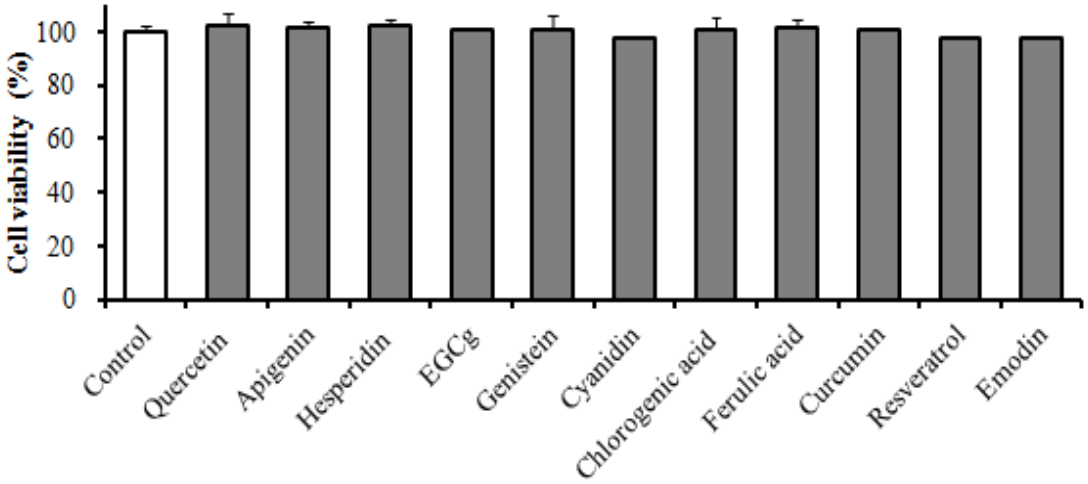

Figure 1.5. Comparative cell viabilities exposed to polyphenols.

Caco-2 cells were treated for 2 hours with polyphenols of indicated concentrations: quercetin, apigenin, hesperidin and genistein, 100 μM; EGCg, cyanidin and resveratrol, 300 μM; chlorogenic acid and ferulic acid, 1 mM; emodin and curcumin, 50 μM, and evaluated for the cell viability compare to the control, which treated without stimulants. No statistical significance was determined by Dunnett's test at $p < 0.05$ vs. control (n=3).

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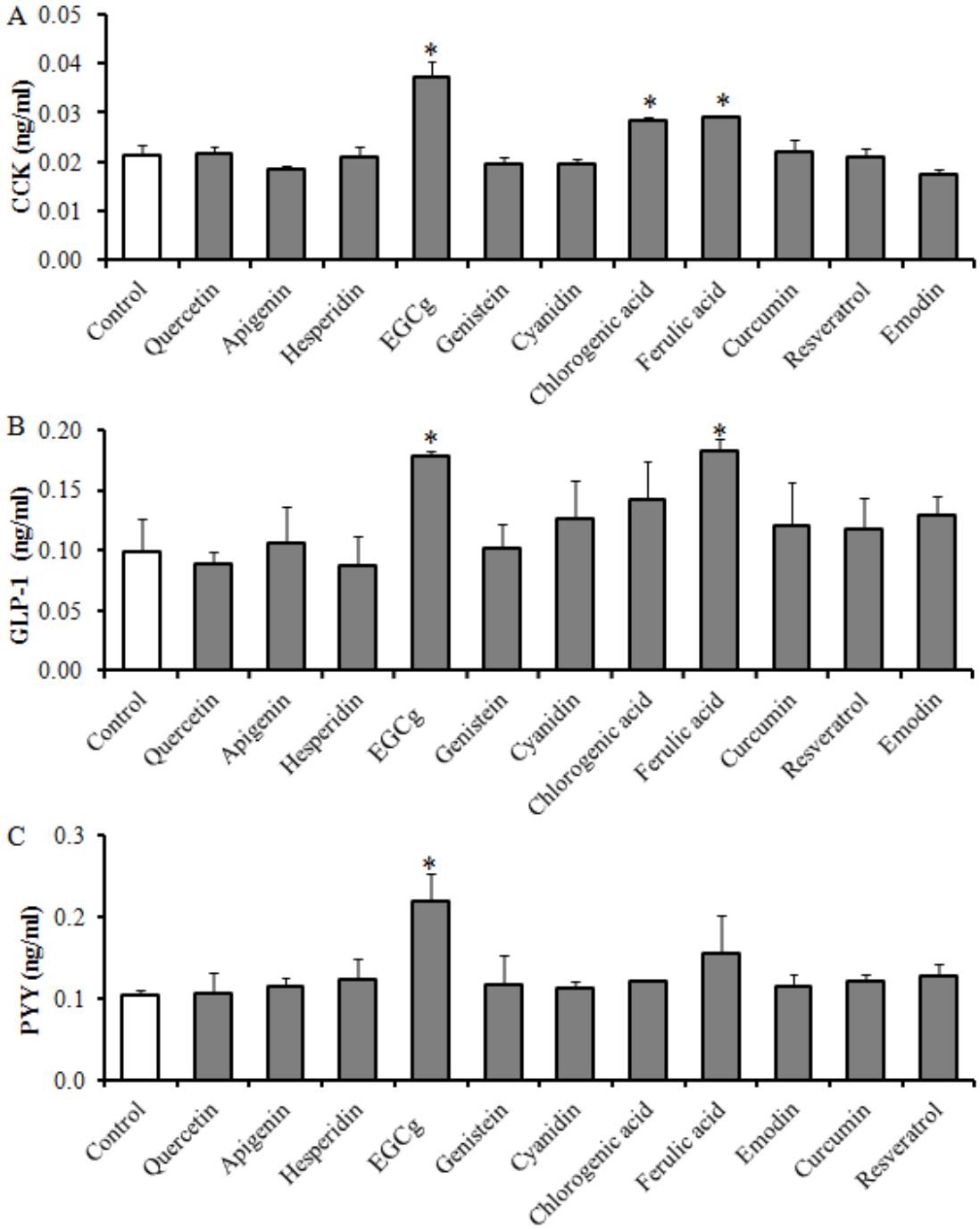

Figure 1.6. Secretion of gut hormones from Caco-2 cells stimulated by polyphenols. The secretion of CCK (A), GLP-1 (B), and PYY (C) from Caco-2 cells into the buffer was measured 2 hours after incubation with the indicated polyphenols. The concentrations of each polyphenol was: quercetin, apigenin, hesperidin and genistein, 100 μM; EGCg, cyanidin and resveratrol, 300 μM; chlorogenic acid and ferulic acid, 1 mM; emodin and curcumin, 50 μM. Control indicates a sample stimulated only with assay buffer. *Statistical significance was determined by Dunnett's test at $p < 0.05$ vs. control (n=3).

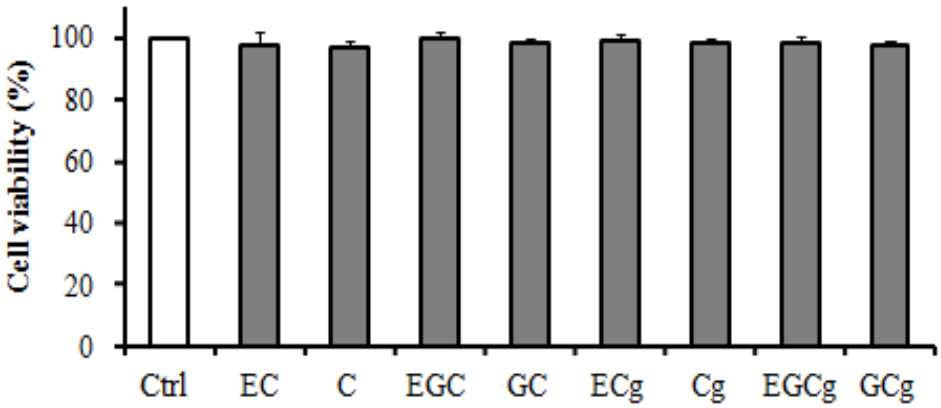

Figure 1.7. Comparative cell viabilities exposed to eight catechins.

Caco-2 cells were treated for 2 hours with 300 μM catechins. Cell viability was compared to the control, which treated without stimulants. No statistical significance was determined by Dunnett's test at $p < 0.05$ vs. control (n=3).

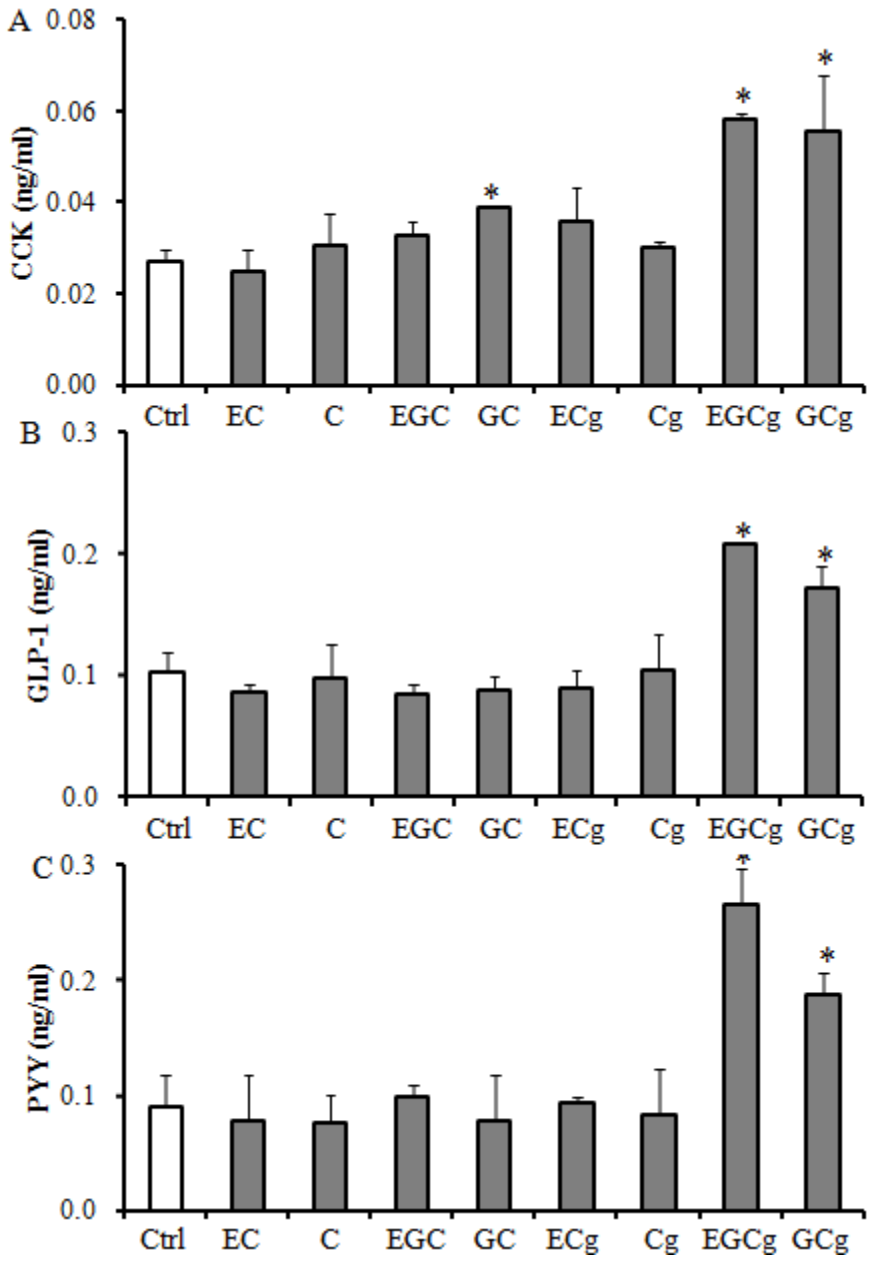

Figure 1.8. Secretion of gut hormones from Caco-2 cells stimulated by catechins.

The secretion of CCK (A), GLP-1 (B), and PYY (C) from Caco-2 cells was measured 2 hours after incubating with catechins. The concentration of each catechins were 300 μM. Control indicates a sample stimulated only with assay buffer. *Statistical significance was determined by Dunnett's test at $p < 0.05$ vs. control (n=3).

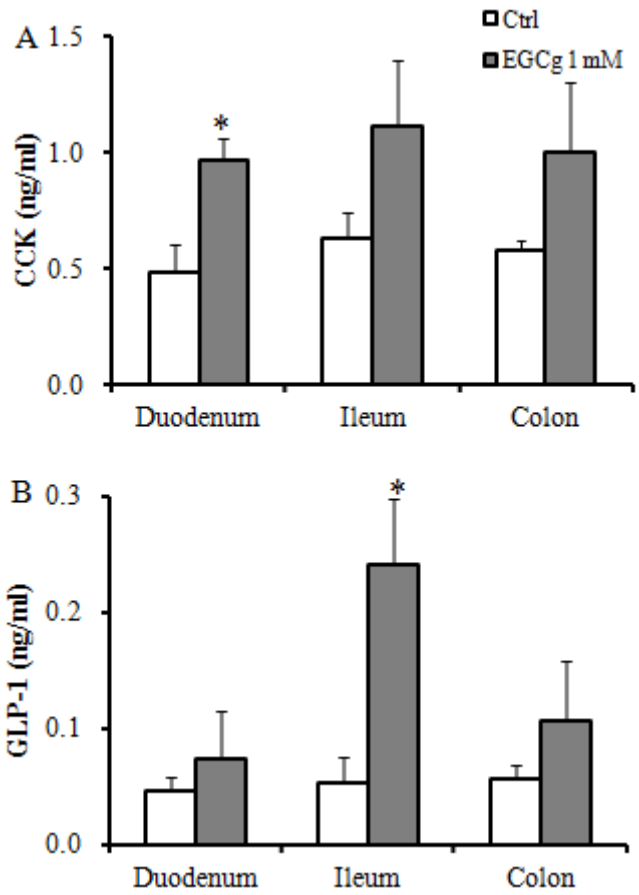

Figure 1.9. Secretion of gut hormones from murine intestines in response to EGCg.

The secretion of CCK (A) and GLP-1 (B) from inverted murine intestines was measured 45 minutes after the treatment with (■) or without (□) 1 mM EGCg. *Statistical significance was determined by and Student's *t*-test at $p < 0.05$ vs. each control (n=3).

1.4 Discussion

Caco-2 cell line as a model of gut hormone release

The Caco-2 cell line was induced to secrete CCK, GLP-1 and PYY in response to nutrients (Figure 1.4). The increase in CCK secretion by glucose, phenylalanine and oleic acid was equivalent to the experiments using humans [4, 60, 61]. Although CCK was shown to be secreted by proteins, the response to amino acids seems to be different among them, *e.g.*, phenylalanine was shown to induce CCK secretion, whereas glutamine wasn't [62]. STC-1 cell line was derived from murine intestinal cell and has been used for a model for I cells. This cell was shown to induce secretion of CCK in response to free fatty acids and amino acids; palmitic acid and oleic acid as free fatty acids whereas phenylalanine as the most strong secretagogues among amino acids [63, 64, 65]. Caco-2 cell in this study also showed response to free fatty acids and amino acids on CCK secretion. However, the intensity of responses toward each compounds seemed to differ between the cell models, STC-1 and Caco-2. It may due to the cellular characteristics including expression of genes related to the signaling cascades from receptors to hormone secretion. Further investigation of relation analysis between gene expression and response characteristics of hormone secretion may provide some strategy underlying this contradiction.

The induction of GLP-1 by sucrose and glutamine was equivalent to that of direct induction in humans [66, 68] There is a cell line named NCI-H716 derived from a human colon tissue, proved to possess characteristics of enteroendocrinal L cells. This cell was shown to release GLP-1 in response to glucose, sucrose, oleic acid

and palmitic acid [28, 29]. A murine cell line named GLUTag cell has been also often used for GLP-1 release and was shown to respond to glutamine [69]. Caco-2 cell was considered to respond to as much stimulants as the other cell models do.

The induction of PYY by oleic acid was equivalent to that of direct induction in humans [4, 61]. There is a report showing that administration of glutamine caused a trend of elevation of PYY level in blood [70], which may support the small induction of PYY by glutamine (Figure. 1.4C). There is no evidence on PYY secretion in response to palmitic acid *in vivo*, whereas an analysis using STC-1 cell line showed slight increase of PYY by palmitic acid [71]. Though STC-1 cell provides the only cell model to work on PYY secretion, there has been only small number of experiments performed. It is not clear why *in vitro* analysis for PYY has been poorly examined, but Caco-2 cell possibly provide a new tool to work with. Taken together, Caco-2 cell was shown to secrete three anorexigenic gut hormones in response to natural secretagogues in a feasible manner with *in vivo* experiments. Hence, Caco-2 cell line is expected to provide useful model for finding the stimulants which induce the secretion of anorexigenic gut hormones.

Non-nutrients as the inducer of anorexigenic gut hormones

There are few reports showing that non-nutrients induce gut hormone secretion. The steroid glycoside, H.g.-12, from *Hoodia gordonii* has been shown to induce CCK secretion in HuTu-80 cells [72]. This compound has also been shown to be associated with loss of body weight [72]. Smeets *et al.* have found that capsaicin, the pungent

compound in red peppers, increased amounts of GLP-1 in human plasma [73]. Cinnamon, *Cinnamomum*, was found to have effect to increase GLP-1 levels in humans and mediate satiety [74]. Methyl syringate, a pungent ingredient in *Kalopanax pictus*, has been shown to significantly suppress food intake and delay gastric emptying via elevated plasma PYY levels in mice [75]. These reports support the idea that some non-nutrients stimulate the secretion of anorexigenic gut hormones including CCK, GLP-1 and PYY, causing appetite or food intake suppression. The present study demonstrated that EGCg stimulated the gut hormone secretion from cultured Caco-2 cells (Figure 1.6 and 1.8), and from murine duodenum and ileum (Figure 1.9). It has been reported that EGCg contributes to energy expenditure [62], that its consumption correlates with weight-loss led by reduction in food intake [44]. These physiological functions of EGCg may be associated with anorexigenic gut hormones, CCK, GLP-1 and PYY secretion.

The present study also showed that ferulic acid induced the secretion of CCK and GLP-1 from Caco-2 cells (Figure 1.6A and B), and that chlorogenic acid induced CCK secretion (Figure 1.6A). Ferulic acid found in grains such as oats and rice has been shown to increase insulin secretion, delay intestinal glucose absorption, and inhibit gluconeogenesis [76]. Chlorogenic acid, which is a major polyphenol in coffee, reduces blood glucose level [77]. Besides the anorexigenic effect on the brain, CCK and GLP-1 are known to act on glucose homeostasis [75]. GLP-1 acts to stimulate glucose dependent insulin secretion, increased insulin sensitivity and inhibition of glucagon secretion [78]. CCK was known to induce glucagon release [79]. The

physiological effects of ferulic acid and chlorogenic acid on glucose homeostasis may be mediated by the stimulation of gut hormone secretion.

Structure-activity relationship for gut hormone release

Tea naturally contains a variety of catechins, which differ in the number of hydroxyl groups at the B-ring and the existence of a galloyl group at C-ring (Figure 1.2) [80]. Catechin is the trans isomer, and EC is the cis isomer. catechin and EC are converted to hydroxylation forms as GC and EGC, respectively. They can acylate with gallic acid to form gallate forms such as Cg, ECg, GCg and EGCg [81]. It has been reported that physiological roles of catechins are dependent on their structure [82, 83]. For example, ability to scavenge free radicals among catechins found relate with the hydroxyl groups on the B-ring and a galloyl group bound to the position 3 of the C-ring [83]. A pair of structural isomers of EGCg and GCg showed the most resembled activity each other, revealed strong induction of CCK, GLP-1, and PYY (Figure 1.8). They share a pyrogallol structure possessing three hydroxyl groups on the B-ring and galloyl group at the position 3 of the C-ring, thus these characteristics may provide stronger reactivity compare to other catechins used in this study. When the effect of green tea catechins, EC, EGC, ECg and EGCg was compared on Sprague Dawley rats, EGCg showed significant reduction of food intake, whereas other 3 catechins didn't [44]. If the structure-activity relationship would be revealed, it may offer the more active derivatives. In chapter 1, Caco-2 cell line was shown to be an *in vitro* model for CCK, GLP-1 and PYY secretion. Among several polyphenols,

chrologenic acid, EGCg, feluric acid, and GCg were revealed to induce one or several anorexigenic gut hormones secretion. EGCg induced the secretion of three anorexigenic gut hormones, CCK, GLP-1 and PYY *in vitro* and EGCg was shown to secrete CCK from the duodenum, and GLP-1 from the ileum in *ex vivo* experiments using murine intestines. Since a stereoisomer of EGCg, GCg showed similar activity on anorexigenic gut hormone release, the stimulation mechanism of polyphenol might relate to their electronic properties than steric characteristics. These findings suggest that polyphenols present appetite control by induction of secretion of anorexigenic gut hormones.

Chapter 2

Molecular mechanisms underlying GLP-1 secretion induced by (-) epigallocatechin-3-gallate

2.1 Introduction

The series of studies on molecular mechanisms of gut hormone secretion in enteroendocrine cells revealed the signaling cascades from receiving the stimulus until releasing hormones. Similar to other peptide hormones such as insulin, gut hormones are also secreted via exocytosis of secretory granules. The trigger of degranulation is often demonstrated as a topical increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [84, 85], which caused by extracellular Ca^{2+} entry and/or release of $Ca^{2+}$from intracellular stores. Using these model cells such as GLUTag cells, it is shown that glucose and amino acids, such as glutamines, trigger depolarization resulting in Ca^{2+} entry and initiation of GLP-1 secretion [69, 86]. Medium chain fatty acids are shown to trigger release of Ca^{2+} from intracellular stores and GLP-1 secretion from the STC-1 cell line [87]. Other secretagogues for GLP-1, protein hydrolysate and fatty acids also showed elevation of intracellular calcium concentration in NCI-H716 [29, 88]. The elevation of $[Ca^{2+}]_i$ in enteroendocrine cells by stimulation will provide a strong evidence for hormone release. Figure 2.1 shows inner cellular molecular mechanisms of CCK, GLP-1 and PYY secretions in enteroendocrine cells.

The binding of ligands to transmembrane receptors generally induce structural changes which arise the inner cellular signaling transduction [89]. After being stimulated, the receptors need to be recovered to non-active form. One way of this recovery is degradation of the receptor-ligand complex in the cells. The endocytosis of those receptors triggers up-regulation of transcription of receptors [90, 91]. This phenomenon is observed in reception of food compounds as well. For example, a pungent phytochemical, capsaicin, is known to activate a member of transient receptor potential channel (TRP) subfamily V, TRPV1, and up-regulate mRNA and protein levels of TRPV1 itself after reception of capsaicin [92]. Evaluating the expression of receptors would provide a basis to screening candidates which bind to the ligands.

The induction of gut hormone release by EGCg is also expected to involve an increase of inner cellular Ca^{2+} concentration. Recently, two families of receptors were shown to be activated by EGCg and induce Ca^{2+} influx. A nociceptive receptor, TRPA1 is an ankyrin repeat containing member of the TRP channels, and transfers extracellular Ca^{2+} into cytosol in response to cinnamaldehyde and allyl isothiocyanate (AITC) [93]. Identically, EGCg was shown to activate TRPA1 and triggers Ca^{2+} influx [94]. On the other hand, human bitter taste receptor family is another receptor family which also be activated by EGCg. Two molecules, hTAS2R14 and hTAS2R39, were shown to be activated by EGCg and transmit the signal to increase Ca^{2+} [36, 37]. Thus, hTAS2R14, hTAS2R39, and TRPA1 are considered to be the strong candidate for the mediator of gut hormone release by EGCg in enteroendocrine cells. On the

other hand, a non-integrin cell surface receptor, a 67 kDa laminin receptor (67LR) is famous to mediate EGCg stimulation in tumor cells and induces various functions, such as inhibition of cell growth, induction of apotosis, downregulation of inflammatory mediators, and inhibition of histamine release [47, 49, 95]. Especially the inhibitory function against histamine release is caused by reconstruction of cytoskeletal organization mediated by a phosphorylation signal under control of eukaryotic translation elongation factor 1A (eEF1a) [96, 97]. Since the signal downstream of 67LR-eEF1a seems to work another way to induction of degranulation, this reaction should have been investigated.

Caco-2 cell is feasible and useful especially to search stimulants for anorexigenic gut hormone in human, but this cell possesses an experimental difficulty in chemical loading [98]. This characteristic might be a disadvantage to research on inner cellular signaling. As described in chapter 1, NCI-H716 cell line derived from human colon cell possesses enteroendocrine characteristics of L cells [29]. This cell line is often used for analysis on GLP-1 release. Even, this cell line has evidence to demonstrate Ca^{2+} increase in response to protein hydrolysates [99]. Thus, NCI-H716 cell was considered to be a good alternative model cell to investigate signaling pathway of GLP-1 release.

In chapter 1, EGCg was shown to induce anorexigenic gut hormones. In Chapter 2, it is examined if signals from receptors of EGCg cause an increase in intracellular Ca^{2+} level, and thereby induce degranulation which allows release of the

peptide hormones. Thus, inner cellular Ca^{2+} levels were evaluated after stimulation by EGCg using NCI-H716 cells.

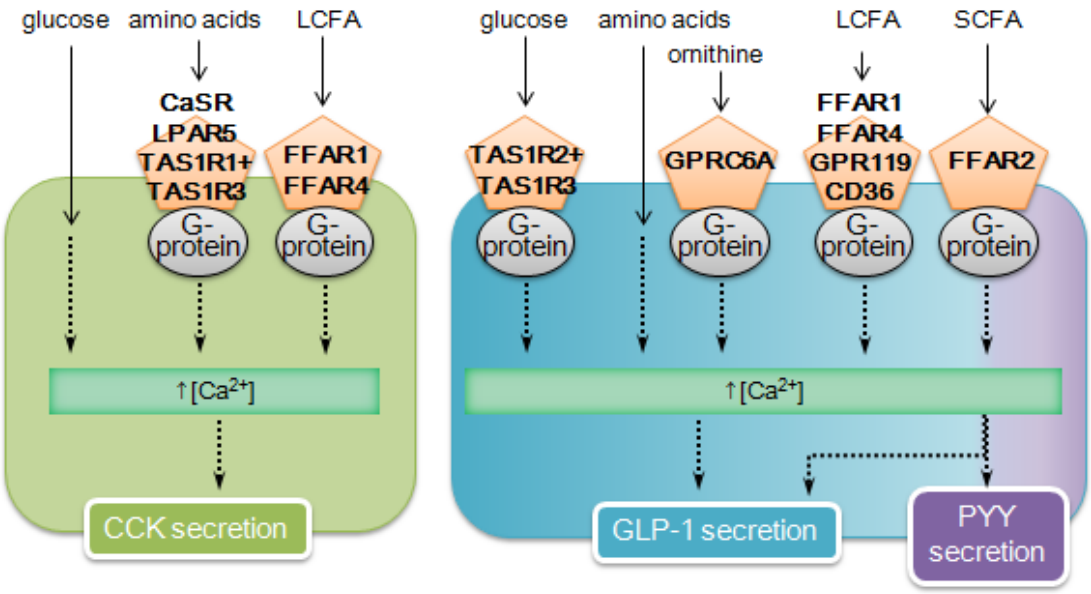

Figure 2.1 The Ca^{2+} dependent signaling pathways related to gut hormone secretion in the enteroendocrine cells.

Schematic drawings show the Ca^{2+} dependent signaling pathways underling nutrients induced gut hormone secretions in enteroendocrine cells. Left: Known pairs of secretagogues and receptors for CCK secretion demonstrated in I cells or STC-1 cells. The receptor responsible for glucose in CCK secretion is still missing. Right: Known pairs of secretagogues and receptors for GLP-1 and PYY secretion demonstrated in L cells or L cell-like cultured cells. The activation of FFAR2 by SCFA shares both GLP-1 and PYY secretion. The receptor responsible for amino acids in GLP-1 secretion is still missing. GPRC6A, G-protein coupled receptor family C group 6; GPR119, G protein-coupled receptor 119; LPAR5, lysophosphatidic acid receptor 5; SCFA, short-chain fatty acids; LCFA, long-chain fatty acids.

2.2 Materials and methods

2.2.1 Cell culture

Caco-2 cells were maintained by the same way as Chapter 1. NCI-H716 cells were cultivated in RPMI 1640 medium (Wako Pure Chemical Industries) supplemented with 5% FBS in a humidified atmosphere of 95% air-5% CO_2. Before assays, the cells were seeded on cell matrix (#637-00653, Nitta Gelatin, Osaka, Japan) and cultured in DMEM with 10% FBS for 48 hours for differentiation. NCI-H716 cells were from passage 7 to 11.

2.2.2 RNA extracttion and quantitative PCR

Caco-2 cells are incubated at 37°C for 2 hours after adding phenylalanine, oleic acid, and EGCg of final concentrations of 50 mM, 1.5 mM and 300 μM, respectively. Subsequently, total RNA of Caco-2 cells was extracted by using Sepasol (Nacalai tesque) with manufactural instructions, and then 1 μg of RNA was used for cDNA synthesis with ReverTra Ace (Toyobo). The sequences of primers (Invitrogen) for analyzed genes are shown in Table 2.1. The program for real time qPCRs was 50 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 20 seconds. The Ct values bigger than 35 were considered as too low expression to evaluate. Gene expression ratio was normalized to average of Ct values on *B2M*, *GAPDH*, *HPRT1* and *ACTB* as a housekeeping gene.

2.2.3 GLP-1 secretion stimulated by EGCg with pretreatment of inhibitors of EGCg candidate receptors

The inhibitors were prepared as follows; 10 mg/ml solution of anti-67LR antibody (#MS-259-PABX, Thermo Fisher Scientific, Waltham, MA, USA), 20 mM DMSO solution of AP18 (Enzo Life Science, Farmingdale, NY, USA), 10 mM DMSO solution of U73122 (Cayman Chemical, Ann Arbor, MI, USA). Caco-2 cells were seeded into 24-well culture plate with 2×10^5 cells per well and incubated at 37°C for 24 hours. The medium was changed to each inhibitor solution diluted with PBS containing 1 mM pyruvate at 1:1000, and incubated for 30 minutes. Then buffer was changed to 300 μM EGCg containing assay buffer containing 0.1 mg/ml DPP IV inhibitor. After incubation for 2 hours, assay buffers were collected and stored at -20°C until used. Concentration of GLP-1 was measured as described in Chapter 1. Data are presented as means ± SD obtained from triplicate wells.

2.2.4 GLP-1 secretion from Caco-2 cell and NCI-H716 cells stimulated by EGCg

Caco-2 cells were seeded into 24-well culture plate with 2×10^5 cells per well and incubated at 37°C for 24 hours. NCI-H716 cells were seeded into 24-well culture plate precoated with cell matrix (Nitta Gelatin) with 2×10^5 cells per well and incubated at 37°C for 2 days. Subsequently, the medium was changed to 1 mM EGCg containing assay buffer composed of PBS containing 1 mM pyruvate and 0.1 mg/ml DPP IV inhibitor. After incubation for 45 minutes, assay buffers were collected and

stored at -20°C until used. Concentration of GLP-1 was measured as described above. Data are presented as means ± SD of triplicate experiments.

2.2.5 Calcium imaging

Caco-2 cells and differentiated NCI-H716 cells were prepared in 96-well culture plate with 4×10^4 cells per well density. The medium was removed, and the cells were incubated with 100 μl of 4 μM Fluo-8 AM (AAT Bioquest, Sunnyvale, CA, USA) in PBS for 1 hour. The cells were washed twice with PBS and placed at inverted microscope (BIOREVO, BZ 9000; Keyence, Osaka, Japan). After adding ionomycin (Nacalai tesque) and EGCg of final concentrations 50 μM and 300 μM respectively, fluorescent images were recorded by each 8 seconds for 2 minutes. The fluorescent intensity calculated from each image was analyzed to evaluate inner cellular calcium concentrations.

2.2.6 Statistical Analysis

Dunnett's test was applied to compare GLP-1 secretion among samples treated with EGCg receptor related inhibitors. Student's *t*-test was applied for qPCR analysis and GLP-1 secretion analysis between stimulated by EGCg and buffer. Provability values below 0.05 were considered statistically significant.

Table 2.1. Sequence information for RT-PCR primers.

Genes	Primer sequences
ACTB(β-actin)	Forward; 5'-TTCTACAATGAGCTGCGTGTG-3'
	Reverse; 5'-GGGGTGTTGAAGGTCTCAAA-3'
B2M	Forward; 5'-ATGAGTATGCCTGCCGTGTGA-3'
	Reverse; 5'- GGCATCTTCAAACCTCCATG-3'
CASR	Forward; 5'- AGGAAAGGGATCATTGAGGG-3'
	Reverse; 5'-GATGCCAGTGCCTGTAACA-3'
GAPDH	Forward; 5'- TGCACCACCAACTGCTTAGC-3'
	Reverse; 5'-GGCATGGACTGTGGTCATGAG-3'
FFAR1	Forward; 5'-GCATCAACACACCGGTCAA-3'
	Reverse; 5'-GCTTCAGCCTCTCTCTCCT-3'
FFAR4	Forward; 5'-GCCAGGACTGGTCATTGTG-3'
	Reverse; 5'- GCTGTCGTGACTCACAGTG-3'
HPRT1	Forward; 5'-TGACACTGGCAAAACAATGCA-3
	Reverse; 5'-GGTCCTTTTCACCAGCAAGCT-3'
TAS2R14	Forward; 5'- GGTGATGGGAATGGCTTATC-3'
	Reverse; 5'-ACAGTAGCACTGACAGAGAG-3'
TAS2R39	Forward; 5'-GCCATCAAAGCTATCAGCTAC-3'
	Reverse; 5'- TGTCAAACATGTTGGACAGG-3'
TRPA1	Forward; 5'- GCACTGGGGAAATAAGACAAG-3'
	Reverse; 5'-AAGTAAGATCCTTCAGCCGG-3'
67LR	Forward; 5'-AGACTGGTCTGAAGGTGTAC-3'
	Reverse; 5'- AGCTGCAGACCAGTCTTC-3'

2.3 Results

2.3.1 Investigation of the up-stream pathway of GLP-1 secretion by EGCg in Caco-2 cells

To examine the involvements of EGCg receptors in induction of GLP-1 secretion, amount of GLP-1 released with selected antibody and inhibitors was measured from Caco-2 cells. Anti-67LR antibody (#MS-259-PABX) is used as a neutralizing antibody against 67LR activation. AP18 is an antagonist against TRPA1. The other inhibitor, U73122 was selected because it blocks the downstream signaling of G-proteins by inhibiting the activity of β subtype of phospholipase C enzymes (PLCβ), which regulate the downstream signaling of TAS1Rs and TAS2Rs. The addition of these inhibitors hardly affected GLP-1 secretion from Caco-2 cells compared to stimulation of EGCg alone (Figure 2.2). It was not clear if this result demonstrated the incompetency on GLP-1 release by EGCg, because the intracellular signaling was not evaluated.

To see the effect of EGCg on candidate receptors directly, the expression levels of receptors after stimulation was evaluated. First, the response of the receptors involved in the reception of the natural secretagogues were examined. The qPCR was performed to compare expressing levels of *CASR* in response to phenylalanine, or *FFAR1* and *FFAR4* in response to oleic acids. Stimulation with phenylalanine increased *CASR* expression in 2.1 fold to control. Oleic acid increased 3.8 fold expression of *FFAR4*, whereas the expression of *FFAR1* was not detected. In addition, the same method was applied to evaluate the expression of candidate EGCg receptors.

After stimulation of EGCg, expression of *TAS2R39* increased 7.2 fold, but those of *T2R14*, *67LR* and *TRPA1* were not changed (Table 2.2). This result indicated that hTAS2R39 might be related to recognition of EGCg in Caco-2 cells.

2.3.2 Changes in intracellular calcium concentration under stimulation of EGCg

Gut hormone secretion often requires increase in intracellular Ca^{2+} concentration. Therefore, the effect of EGCg on intracellular Ca^{2+} concentration was investigated. At first, Caco-2 cell was tried to be loaded with the calcium indicator, Fluo-8. However, this method applied with Caco-2 cells, an addition of an ionophore, ionomycin, did not affect inner cellular calcium increase (Figure 2.3). The other human endocrine cell line, a NCI-H716, was then analyzed for its ability of responding to EGCg. As shown in Figure 2.4, NCI-H716 was confirmed to induce GLP-1 by stimulation of 300 μM EGCg as similar level as Caco-2 does. The loading of Fluo-8 showed detection of calcium influx by ionomycin (Figure 2.4 B), which confirm the adaptation to Ca^{2+} imaging analysis. When NCI-H716 cells were stimulated by 300 μM of EGCg, the increase of intracellular Ca^{2+} was observed from 8-16 seconds. (Figure 2.4C). These results offer the possibility that increased Ca^{2+} by EGCg is one of the pathway of secretion GLP-1.

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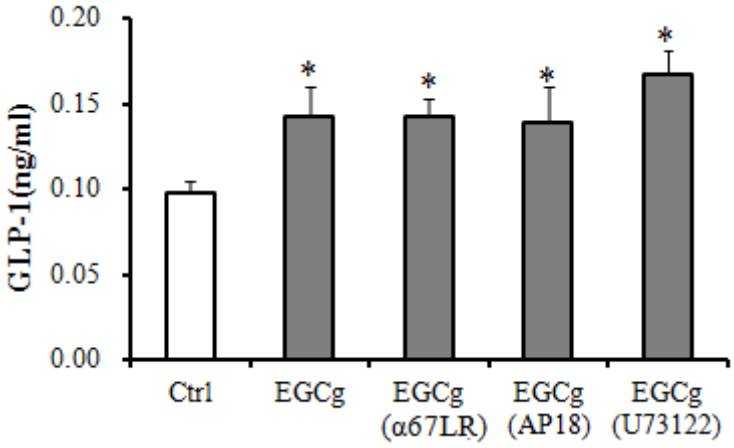

Figure 2.2. Effect of inhibitors for EGCg receptor pathway on GLP-1 secretions from Caco-2 cells stimulated by EGCg.

The secretion of GLP-1 from Caco-2 in the buffer was measured 2 hours after incubating with 300 μM EGCg, with the preincubation with anti-67LR antibody, TRPA1 antagonist (AP18) and PLC inhibitor (U73122). Control indicates a sample stimulated only with assay buffer. *Statistical significance was determined by Dunnett's test at $p < 0.05$ vs. control (n=3).

Table 2.2. mRNA expression values of binding receptors after stimulated by ligands in Caco-2 cells.

A

Target gene	Control	Phenylalanine
CASR	$5.0 \times 10^{-5} \pm 0.0$	$10.5 \times 10^{-5} \pm 0.0$*

B

Target gene	Control	Oleic acid
FFAR4	$2.3 \times 10^{-4} \pm 0.0$	$8.7 \times 10^{-4} \pm 0.1$*
FFAR1	N.D.	N.D.

C

Target gene	Control	EGCg
TAS2R14	$8.8 \times 10^{-3} \pm 0.2$	$10.1 \times 10^{-3} \pm 0.1$
TAS2R39	$6.1 \times 10^{-4} \pm 0.0$	$4.4 \times 10^{-3} \pm 0.0$*
67LR	$5.4 \times 1 \pm 2.6$	$4.1 \times 1 \pm 1.5$
TRPA1	$6.8 \times 10^{-6} \pm 0.0$	$7.1 \times 10^{-6} \pm 0.0$

Real time qPCR was performed to compare gene expressions after addition of stimulants. (A) Expression of CaSR in response to phenylalanine. (B) Expression of FFAR4 and FFAR1 in response to oleic acid. (C) Expression of candidate receptors after stimulation by EGCg. The stimulants were; 50 mM phenylalanine, 1.5 mM oleic acid, and 300 μM EGCg. The level of expression was normalized to average of Ct values of house-keeping genes; *B2M*, *GAPDH*, *HPRT1* and *ACTB*. Statistical significance determined by and Student's *t*-test at $p < 0.05$ to each control (n=3). N.D. ;couldn't evaluate because of too low expression.

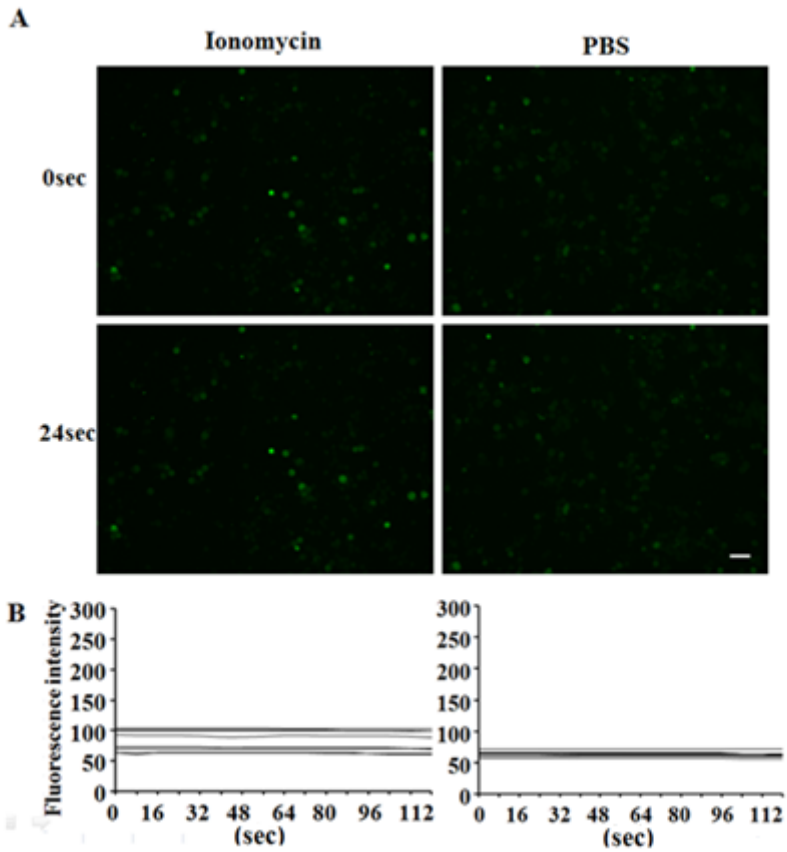

Figure 2.3. Calcium imaging on Caco-2 cells response to EGCg.

(A) Increase in intracellular Ca^{2+} concentrations after adding ionomycin (left) or PBS (right) on Caco-2 cells are introduced with Fluo-8. The images were obtained immediately after adding samples (0 second) and after 24 seconds by a fluorescence microscope. (B) The traces of fluorescent intensity in individual cells by 8 seconds intervals. Scale bar ; 50 μm.

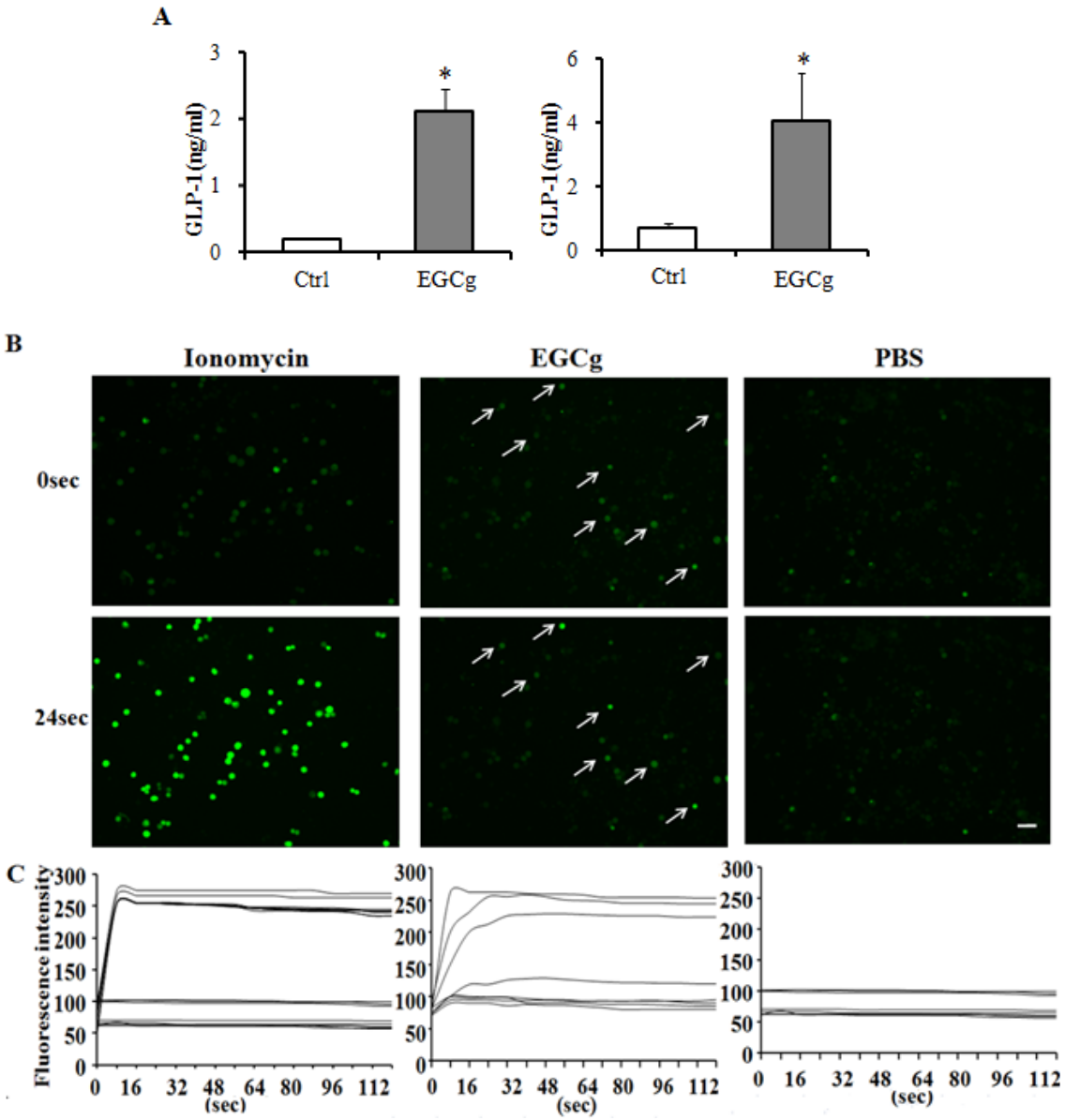

Figure 2.4.The response of NCI-H716 cells stimulated by EGCg.

(A) Alteration of GLP-1 secretions from Caco-2 cells (left) and NCI-H716 cells (right) simulated by 1 mM EGCg was evaluated. *Statistical significance was determined by and Student's *t*-test at $p < 0.05$ to each control (n=3). (B) Increase in intracellular Ca^{2+} concentrations after adding ionomycin or EGCg on NCI-H716 cells introduced with Fluo-8 was detected. The images were obtained immediately after adding samples (0 second) and after 24 seconds by a fluorescence microscope. (C) The traces of fluorescent intensity in individual cells by 8 seconds intervals. Scale bar ; 50 μm.

2.4 Discussion

Differentiation in usage between Caco-2 and NCI-H716

In this chapter, other human cell line named NCI-H716 was also used to study gut hormone secretion in response to EGCg. NCI-H716 was derived from colon and shown to have L-cell like characteristics, thus used as an *in vitro* model for GLP-1 and PYY secretion. As described in chapter 1, the response to nutrients in GLP-1 release is similar between NCI-H716 and Caco-2 [28, 29]. In addition, the increase of GLP-1 secretion in response to EGCg was also similar (Figure 2.4A). Compare to the number of researches performed with NCI-H716 on GLP-1, there are no PYY secretion by nutrients' induction from NCI-H716. The transcriptional characteristics showed NCI-H716 expressed variety of hTAS2Rs including hTAS2R14 and hTAS2R39 as well as gustducin, which works as a binding G-protein to transmit the signals from T2Rs [100, 101], whereas Caco-2 was confirmed to express gustducin and PLCβ2, which is an effector enzyme underlying TAS1Rs and TAS2Rs (data not shown). Since it is known that taste receptors and the down signaling molecules were expressed in enteroendocrine cells *in vivo*, both NCI-H716 and Caco-2 are considered to share the original characteristics of enteroendocrine cells. Regarding to the response to nutrients and the transcriptional characteristics, Caco-2 cell and NCI-H716 cell are comparable models [28, 29]. However, loading a calcium indicator into Caco-2 cell was impossible, likewise other techniques such as transfections of nucleic acids [102, 103]. Caco-2 might relatively resistant to be introduced compounds, may due to the tight connection between cells. Ionomycin is a chemical which act as

ionophoric Ca^{2+} transport traverse membrane. The addition of ionomycin clearly showed that NCI-H716, but not Caco-2, succeeded to provide the experimental basis for calcium imaging. In this sense, NCI-H716 may provide a better tool to investigate inner cellar mechanisms. However, it is not negligible that Caco-2 might be suitable to examine PYY secretion, since NCI-H716 has not been examined.

Possibility of hTAS2R39 being as the EGCg receptor in GLP-1 secretion

To clarify the molecular mechanism underling the EGCg induction on GLP-1 secretion, three experiments were performed considering candidate four receptors; 67LR, hTAS2R14, hTAS2R39, and TRPA1. The 67LR neutralizing antibody didn't affect GLP-1 secretion by EGCg nor TRPA1 antagonist didn't affect GLP-1 secretion by EGCg, nor U73122 inhibit the downstream of GPCR, such as hTAS2Rs. The gene expression analysis showed only *hTAS2R39* was up-regulated, which is similar to those of *CASR* in response to phenylalanine and *FFAR4* in response to oleic acid. When changes of $[Ca^{2+}]_i$ was detected after stimulating EGCg by fluorescent indicator, Fluo-8, NCI-H716 cells showed increase of fluorescence, which demonstrate that Ca^{2+} influx arose under stimulation of EGCg.

An up-regulation of transcripts of hTAS39 by EGCg stimulation would provide verification for its involvement of EGCg-dependent inner cellular signaling. The downstream signaling was considered to be G-protein mediated pathway (Figure 2.1). The signal downstream of GPCRs depends on the coupling G-proteins. It is well known that TAS2Rs activate PLC resulting in elevation of $[Ca^{2+}]_I$ in mainly taste receptor cells. On the other hand, there seems to be some other pathways to initiate

GLP-1 release in enteroendocrine cells. An elevation of cyclic AMP (cAMP) triggers GLP-1 release via $[Ca^{2+}]_i$ elevation in GLUTag cells [104] (Figure 2.5 A left). Phosphodiesterase (PDE) regulates the cellular levels of cAMP downstream of glucose [105]. A stimulation of denatonium benzoate, which activate TAS2Rs, induced GLP-1 secretion via PDE activation, independent to $[Ca^{2+}]_i$ in NCI-H716 cells [100] (Figure 2.5A right). The candidate receptor for EGCg in enteroendocrine cells estimated as hTAS2R39, which shares the expression profile with both TAS1Rs and TAS2Rs. It is possible that the stimulation of EGCg might mediate its signal via PLC independent pathway (Figure 2.5B). The evaluation of intracellular cAMP levels would provide more strong evidence for possible pathways for GLP-1 release under TAS2Rs. Another reason for the conflicting result with PLC activation would be caused by unsuccessful permeation of U73122 into Caco-2 cells. The proof would be provided by the direct evaluation of activity of PLC such as measuring IP_3 and diacylglycerol. As shown in Figure 2.4, $[Ca^{2+}]_i$ was elevated by stimulation of EGCg. The requirement of $[Ca^{2+}]_i$ for GLP-1 secretion by EGCg will be confirmed by measuring GLP-1 under depleting extracellular Ca^{2+} or addition of blocker of voltage dependent calcium channels. Altogether, the mechanism of EGCg stimulation on GLP-1 release in Caco-2 cells was shown to possibly involve hTAS2R39 and subsequent $[Ca^{2+}]_i$ elevation. It is shown that EGCg induced an elevation of $[Ca^{2+}]_i$ in NCI-H716 cells, which supports the idea that GLP-1 release was occurred via elevation of $[Ca^{2+}]_i$ in response to EGCg. The candidate receptor for EGCg in intestinal cells is estimated as hTAS2R39 from gene expression analysis using Caco-2.

The conflicting result showing the non-effectiveness of the inhibitor for PLC on the secretion of GLP-1 by EGCg stimulation, might due to a concurrent contribution to degranulation, such as cAMP dependent pathway (Figure 2.5B). Through the analysis in this chapter, a molecular mechanism to secrete GLP-1 stimulated by EGCg is partially revealed. The expected signaling is activation of TAS2R followed by elevation of $[Ca^{2+}]_i$ and subsequent exocytosis of secretion granules. Further experiments such as gene knock down analysis by RNA interference would be needed to confirm the whole pathway.

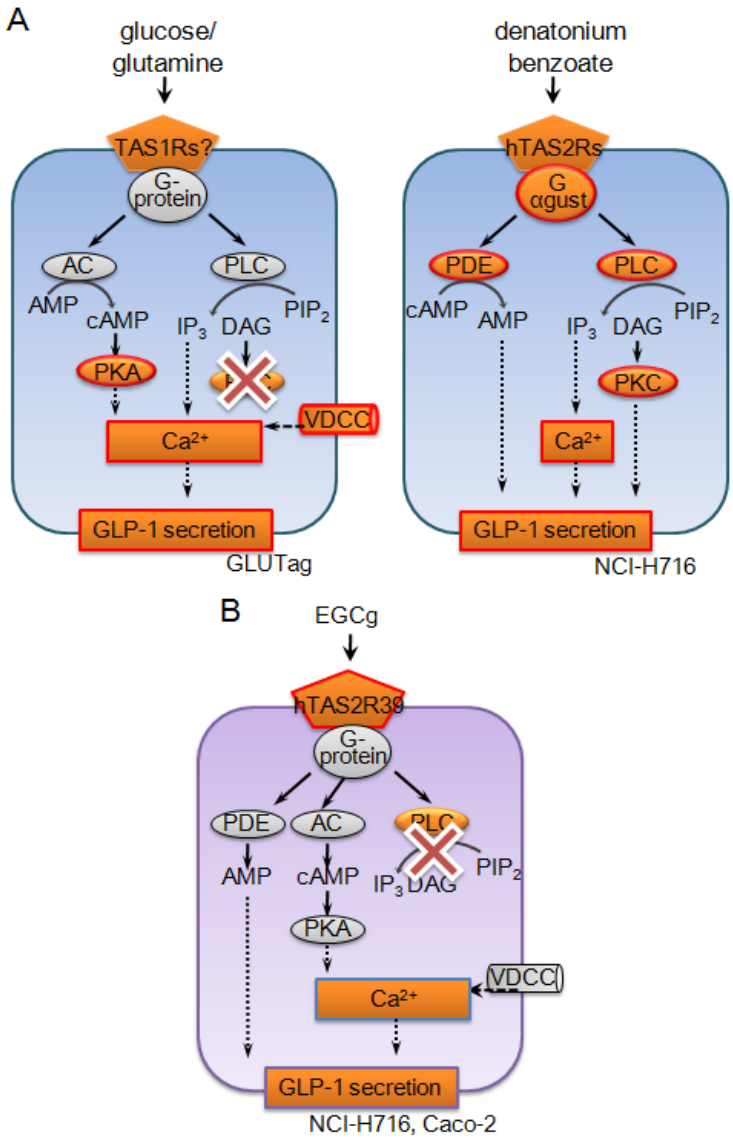

Figure 2.5 Intracellular signaling pathways underling GLP-1 secretion of taste receptors

Schematic drawings of the intracellular signaling pathways for GLP-1 secretion in cultured cells stimulated by denatonium benzoate, glucose or glutamine, and EGCg. (A) Left: The signaling cascades inducing elevation of $[Ca^{2+}]_i$ stimulated by glucose or glutamine. The molecules framed in red indicate the factors whose activity is confirmed in murine GLUTag cells [79]. Right: GLP-1 is released through both Ca^{2+} dependent and independent pathways by stimulation of denatonium benzoate. The molecules framed in red indicate the factors whose activity is confirmed in human NCI-H716 cells [22]. (B) The summary of the result obtained in this study. The involvement of hTAS2R39 was expected by PCR analysis in human Caco-2 cells, and elevation of $[Ca^{2+}]_i$ was demonstrated in human NCI-H716 cells. The involvement of PLC was disproven in Caco-2 cells. AC, adenylcyclase; cAMP, cyclic AMP; DAG, diacylglycerol; PDE, phosphodiesterase; PIP_2, phosphatidylinositol 4,5-diphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; VDCC, voltage-dependent calcium channel.

Chapter 3

Effect of (-) epigallocatechin-3-gallate on the regulation of food intake

3.1 Introduction

The control of feeding and energy expenditure is a homeostatic process. This process is taken place in several regions of brain; the arcuate nucleus (ARC), and the dorsal vagal complex in the hypothalamus [90]. The anorexigenic gut hormones are transmitted via blood circulation or vagus nerves to brain and affect hypothalamus.

CCK and PYY signals reach hypothalamus mostly via a neural pathway to the brainstem [106, 107]. GLP-1 receptors are found in large part of brain regions involving ARC and the brainstem, in addition to various peripheral tissues such as vagus nerves [108]. The fact that both central and peripheral administration of GLP-1 to rats inhibits food intake means that this hormone effect directly on brain and also from periphery through vagus nerves [109]. Peripheral administration of CCK in rodents and humans led to a decrease in meals size [110, 111]. Intravenous administration of an antagonist of CCK receptor in humans decreases satiety and increase hunger and meals size [112]. Also, central and peripheral injection of PYY in rodents results in food intake reduction [113]. It seems that PYY is more effective in obese condition; Intravenous administration of PYY in obese people resulted in a comparable decreasing in food intake when compared with normal controls [114]. It

is also demonstrated that the intraperitoneal administration of CCK, GLP-1 and PYY diminish the food intake [90, 91, 115].

It is known that non-nutritional secretagogue for gut hormones affect regulation of food intake, *e. g.*, cinnamaldehyde and methyl syringate suppress food intake via anorexigenic gut hormone [74]. Cinnamaldehyde and methyl syringate induced PYY secretion in mice [75]. Cinnamaldehyde and methyl syringate administered orally at a dose of 10 mg/kg after fasting induced reduce of food intake in mice [74].

According to chapter 1, showing that EGCg induces CCK, GLP-1 and PYY secretion from cultured intestinal cells. Furthermore, EGCg induces CCK and GLP-1 secretion in dissociated murine intestines. Because of these results, the physiological effect of EGCg was investigated by evaluating meal size during each 2 hours for successive 24 hours in mice.

3.2 Materials and methods

3.2.1 Evaluation of food intake

The study was approved by the Institutional Animal Care and Use Committee (Permission number: 25-10-03) and carried out according to the Kobe University Animal Experimentation Regulations. Male ICR mice (9 weeks old) weighing 37-44 g were used to evaluate food consumption. Mice are first acclimated to laboratory condition at 23 °C with a 12 hours light-dark cycle for 1 week. Mice were orally administrated with water, 2 mM EGCg solution, or 2 mM EC solution of 200 μl/mouse just before a dark cycle at 8 o'clock PM, and were allowed *ad libitum* access to food (Japan SLC) and drinking water. The amount of food left in the individual cage was weighed at every two hours for 24 hours. Subtraction between the left food amounts was calculated to obtain food consumption in each 2 hours. Data were presented as means ± SEM of five mice.

3.2.2 Statistical Analysis

Statistical analysis was performed using Student's *t*-test. Probability values less than 0.05 were considered statistically significant.

3.3 Result

3.3.1 Alteration of food consumption by EGCg

The food consumption was measured in every 2 hours after administration of EGCg or water. As shown in Figure 3.1A, food consumption after 2 hours is significantly less in EGCg administrated mice compare to control. When the amount of food cumulated, there was no significant difference between the groups later than 4 hours after administration. The result indicated that the effect of EGCg on food consumption was observed in 2 hours as a reduction of food intake.

3.3.2 Epicatechin doesn't induce reduction of food intake

Epicatechin (EC) shares basic structure of EGCg but doesn't induce anorexigenic gut hormones (Figure 1.8). EC was administrated in the same way to EGCg and evaluated the food consumption. As shown in Figure 3.2A, food consumption in each 2 hours after administration of EC showed no difference to control for 24 hours, while EGCg showed significant reduction in the first 2 hours. The cumulated food consumption also didn't change by EC compare to control. EC did not induce secretion of anorexigenic hormones or reduction of food intake. This result suggests that anorexigenic hormone release by EGCg affect reduction of food intake.

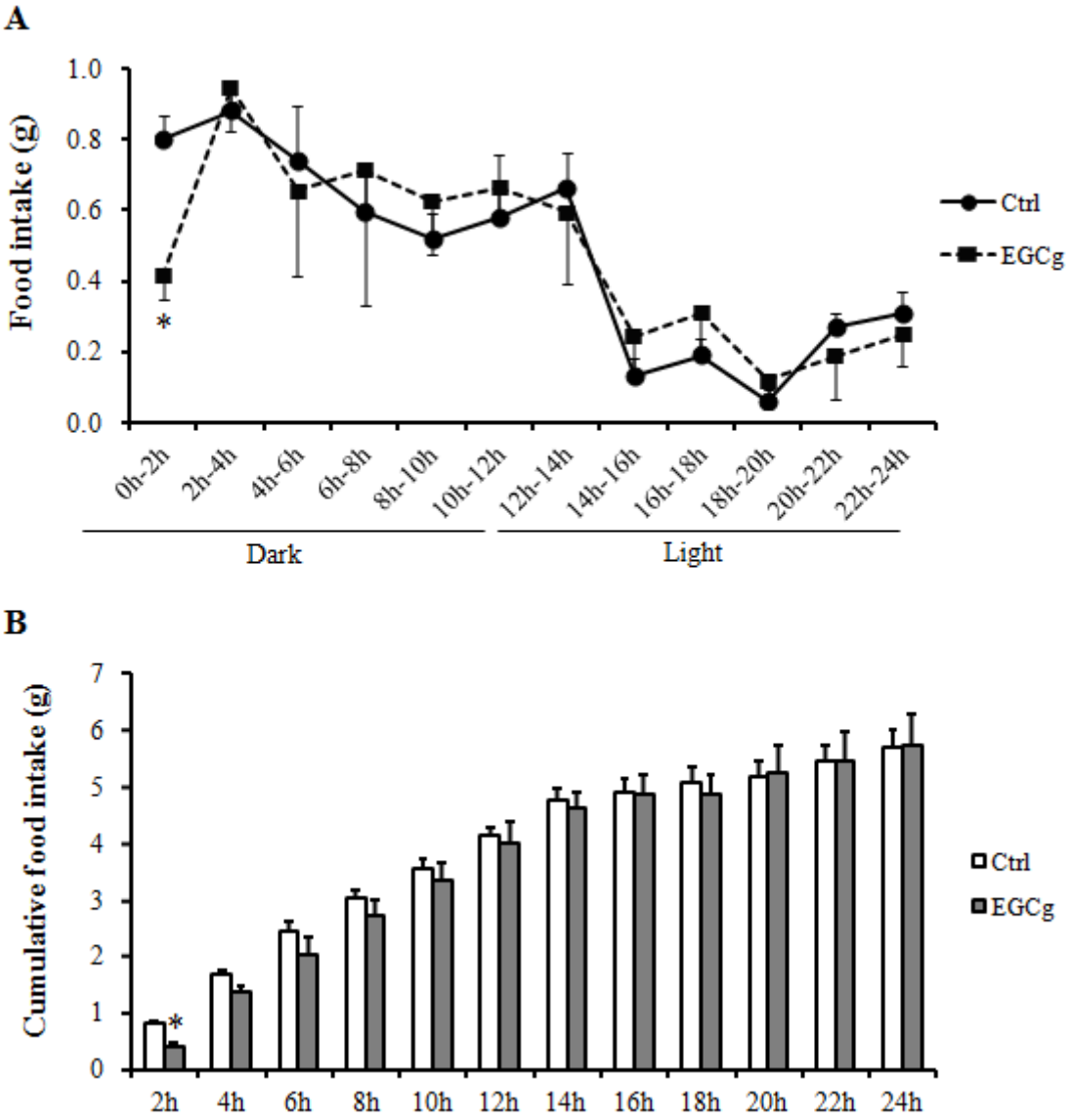

Figure 3.1. Effect of EGCg on food consumption for 24 hours.

The 24 hours records of food consumption of mice administrated 200 µl of water or EGCg (2 mM). (A) Consumed food amount for every 2 hours was shown for EGCg dosed mice (■, solid line) and water dosed mice (●, break line). (B) Cumulative food intake after administration was shown by 2 hours for EGCg dosed mice (gray bar) and water dosed mice (white bar). *Statistical significance was determined by and Student's *t*-test at $p < 0.05$ vs. each control (n=5).

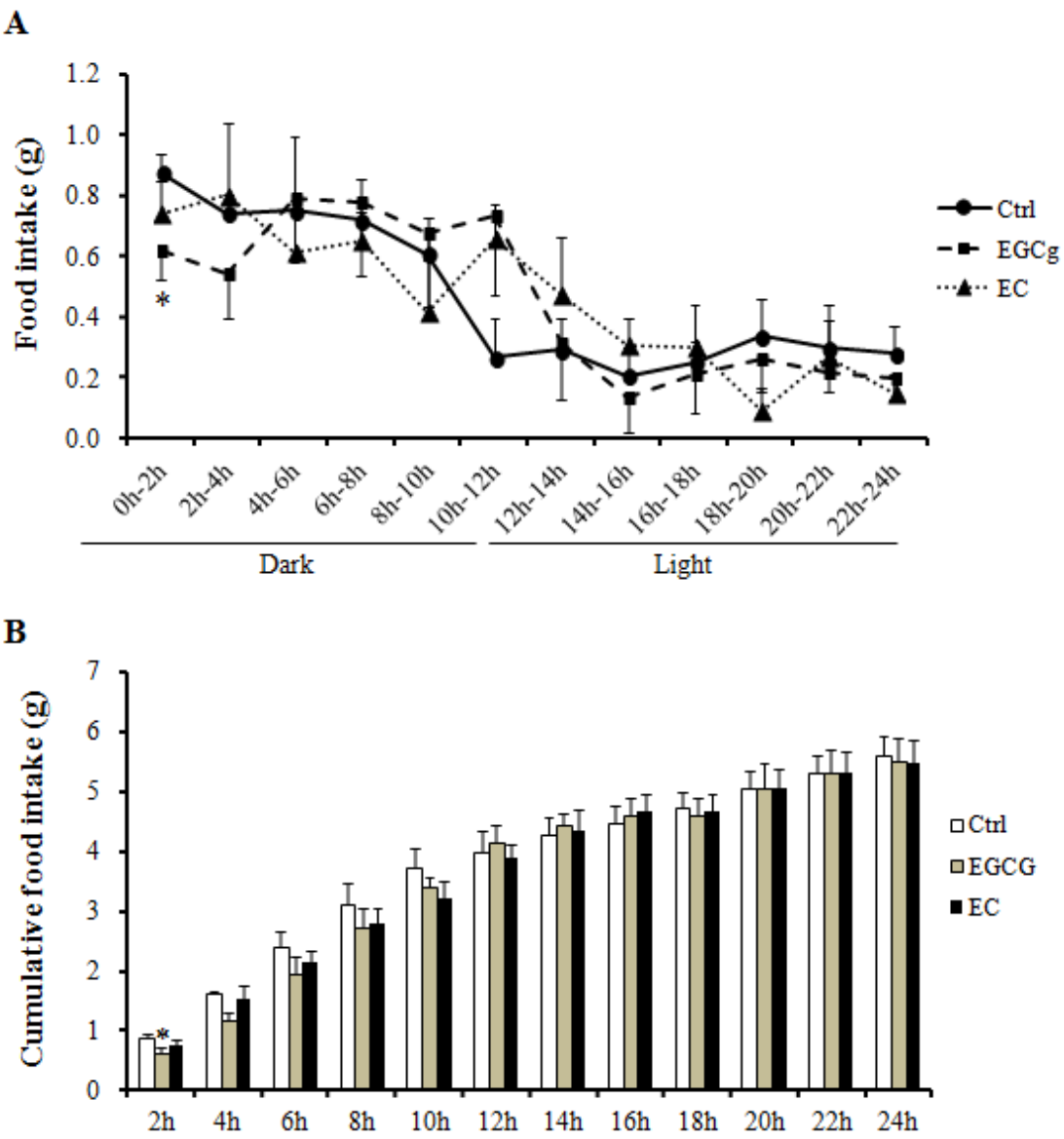

Figure 3.2. Effect of EGCg and EC on food consumption for 24 hours.

The 24 hours records of food consumption of mice administrated 200 µl of water, EGCg (2 mM) or EC (2 mM). (A) Consumed food amount for every 2 hours was shown for EC dosed mice (▲, dotted line), EGCg dosed mice (■, solid line) and water dosed mice (●, break line). (B) Cumulative food intake after administration was shown by 2 hours for EC dosed mice (black bar), EGCg dosed mice (gray bar) and water dosed mice (white bar). *Statistical significance was determined by and Student's *t*-test at $p < 0.05$ vs. each control (n=5).

3.4 Discussion

Secretion dynamics of gut hormones

The secreted GLP-1 is rapidly inactivated by DPP IV, which cleaves off the N-terminal 2 amino acids of GLP-1 [116]. This degradation occurred very quickly, so that the half-life time of intact GLP-1 is estimated as less than 2 minutes. Thus, the effect of GLP-1 is expected to continue only during the secreted period and doesn't last long. The hormones secreted in plasma have a peak time of concentration. Concentration of GLP-1 in plasma in the empty stomach state is generally between the 5-10 pmol/L, and increase rapidly to 15-50 pmol/L after eating a meal in human [4, 117]. In human, concentration of CCK in plasma is below 1 pmol/L in hungry condition to increase 5-10 pmol/L in satiety condition; whereas concentration of PYY in plasma is about 10 pmol/L in hungry condition to increase 20-25 pmol/L [118, 119]. GLP-1 is elevated in 5-30 minutes after food intake, reaching peak around 40 min [120]. PYY is increased in 15-30 minutes and CCK is 10-30 minutes after food intake [121, 122]. A range of experiments administrating anorexigenic hormones showed that the effect on food intake reduction was exhibited in 2-4 hours after administration. The observation of the reduction of food consumption during the first 2 hours after administration of EGCg (Figure 3.1A) speculates a relationship between anorexigenic effect of EGCg and anorexigenic gut hormones. As well as the result obtained by administration of EC, which had no effect on anorexigenic gut hormones, CCK, GLP-1 and PYY release *in vitro* (Figure 1.8) and on reduction of

food intake *in vivo* (Figure 3.2), it is probable that EGCg affect regulation of feeding behavior via secretion of anorexigenic gut hormones, CCK, GLP-1, and PYY.

Contribution of gut hormones on regulation of food intake and energy expenditure

One should balance the energy expenditure and supplementation in both short term and long term. To keep a good balance for total body energy, feeding behavior should be regulated correctly. Hypothalamus is considered to control food intake, since the lesion of that area decisively alters animals' feeding behavior [3]. The area is largely divided into two; ventromedial nucleus as the satiety center and lateral hypothalamus as the fasting center [123]. The area where the neurons directly receive signals is arcuate nucleus, located at the bottom of hypothalamus. The signals transmitted to this feeding center are hormones secreted from gastrointestinal tract and adipocytes [124]. Leptin, a hormone secreted from adipose tissue, is known as a long term satiety hormone. It is revealed that this gene responsible for obesity, especially related to feeding behavior by mediating energy consumption [125, 126]. On the other hand, the gut hormones, such as CCK, GLP-1 and PYY are known as a short term signal for satiation. They convey direct postprandial satiety signals to hypothalamus at ARC region, which is known to control energy and glucose metabolism [123]. It is probable that the gut hormones tightly relate to energy control.

Oral administration of EGCg for 0.4 μmol per mouse altered the food consumption in first 2 hours about half of that of control (Figure 3.1A). After reducing food consumption during the first 2 hours, the cumulated food consumption

at 4 hours after administration was recovered to the level of control (Figure 3.1B). This might mean a normal control of feeding behavior related to whole body energy balance including the information not only from gut hormones but also other signals such as leptin. There is a report showing the chronic effect on a range of biochemical markers by administration of EGCg for 7 days using Sparague Dawley rats [44]. Male rats orally given 15 mg of EGCg for 7 days exhibited reduction in food intake at 15% from control, though body weight showed no difference. The serum concentrations of hormones which correlate with food intake were shown by 30% reduction for leptin, but not insulin. The dosage of EGCg used in that report was ten times higher, and the alteration of food intake was demonstrated as a result of repeated treatment. The effect of reduction of food intake by single administration of EGCg in palatable dose provides a new function of EGCg. Further experiment such as evaluation of food consumption in EGCg receptor knock out mice would be needed to clarify the involvement of gut hormones in feeding control by EGCg.

When male Sprague Dawley rats were injected intraperitoneally with green tea catechins, EC, EGC, ECg and EGCg (each 85 mg/kg body weight) for 7 days, their food intake decreased for 50–60% by EGCg, whereas EC, EGC and ECg did not show any effect [44].

In this chapter, the effect of EGCg on food intake was investigated *in vivo*. The cumulated food consumption during after 2 hours of EGCg administration detected significantly less than control. The effect for reduction of food intake might due to the

anorexigenic hormones, CCK, GLP-1 and PYY secreted from intestine in response to EGCg. The amount of EGCg dosed to mice was practical when calculated to human. Accordingly, it is expected that about 2 cup of green tea will affect human to reduce appetite, which will be possible to help from excessive eating.

Conclusion

This study demonstrated that some polyphenols induce secretion of anorexigenic gut hormones such as CCK, GLP-1 and PYY. These findings will provide a new perspective on polyphenols as endocrine inducers and on their potential function in appetite regulation, which in turn controls energy balance.

In chapter 1, it was firstly proven that some polyphenols such as EGCg, GCg, GC, chlorogenic acid, and ferulic acid released anorexigenic gut hormones *in vitro*. Particularly, EGCg secreted all three hormones. In an *ex vivo* assay using murine intestines, EGCg also induced secretion of anorexigenic gut hormones. EGCg, the major flavonoid found in green tea, has been reported to have an effect in food intake reduction. These facts support the possibility that food intake reduction by EGCg is related in anorexigenic gut hormone release.

In chapter 2, it was shown that intracellular Ca^{2+} concentrations were increased responding to EGCg in NCI-H716 cells. Among candidates of EGCg receptors, hTAS2R39 are expected to be the receptor of EGCg for release of GLP-1 from gene expression analysis. The 67LR antibody did not affect gut hormone secretion and the TRPA1 antagonist did not affect gut hormone secretion, so these receptors seemed to be unrelated to secretion of gut hormone by EGCg. Analysis to inhibit PLC activity which is estimated to be the downstream of GPCR also showed no effect on GLP-1

release, speculated other pathway between hTAS2R39 and elevation of Ca^{2+} concentration.

In chapter 3, orally administration of EGCg to mice was performed to evaluate its effect on food intake. The food consumption during 2 hours after administration of EGCg was suppressed, and food intake during a short time *in vivo*. By contrast of administration of EC, which did not induce anorexigenic hormones, had no change in food consumption. These results suggest that food intake reduction by EGCg, may be related in anorexigenic gut hormone secretion.

This study showed that EGCg can induce anorexigenic gut hormone, which in turn supports that EGCg might affect appetite via gut hormones. Anti-obesity effect of EGCg has shown to act after being absorbed into the body, however, the bioavailability of absorbed EGCg is very little since polyphenols is hard to be absorbed and released from body in short term [1, 23]. Here, gut hormone release by reception in intestinal tract does not require a process of absorption, so is expected to have high bioavailability of EGCg. The anti-obesity effect of EGCg via anorexigenic gut hormone release has not been shown. Thus, the whole study here proposes a novel biological function of EGCg.

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