

Study on the regulation of intestinal absorption by theaflavins in Caco-2 cells

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<https://doi.org/10.15017/1470633>

出版情報：九州大学, 2014, 博士（農学）, 課程博士
バージョン：
権利関係：全文ファイル公表済



**Study on the regulation of intestinal absorption
by theaflavins in Caco-2 cells**

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2014

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Abbreviation

- ADME, absorption, distribution, metabolism and excretion
- AMPK, AMP-activated protein kinase
- CaMKK, Ca^{2+} /camodulin-dependent protein kinase kinase
- cPKC, conventional isoforms
- CYPs, cytochrome P540 enzymes
- DAG, diacylglycerol
- DMEM, dulbecco's modified eagle's medium
- DMSO, dimethylsulfoxide
- EC, (-)-epicatechin
- ECG, (-)-epicatechin gallate
- ECL, enhanced chemiluminescence
- EDTA, ethylenediamine tetraacetic acid
- EGC, (-)-epigallocatechin
- EGCG, (-)-epigallocatechin gallate
- ELISA, enzyme-linked immunosorbent assay
- FBS, fetal bovine serum
- HBSS, hanks' balanced salt solution
- IBD, inflammatory bowel disease
- IBS, irritable bowel syndrome
- IECs, intestinal epithelial cells
- IP_3 , inositol (1,4,5)-trisphosphate
- JAMs, junctional adhesion molecules
- LC-ESI-TOF-MS, liquid chromatography–electrospray ionization–time-of-flight–mass spectrometry
- LKB1, liver kinase B1
- MDCK, Madin-Darby canine kidney Cells
- MDR, multidrug resistance

- MRPS, multidrug-associated proteins
- NDA,
naphthalene-2,3-dicarboxaldehyde
- P_{app} , apparent permeability coefficient
- PBS, phosphate buffered saline
- PEPT1, peptide transporter 1
- PIP_2 , phosphatidylinositol (4,5)-
bisphosphate
- PIP_3 , phosphatidylinositol (3,4,5)-
trisphosphate
- PKC, protein kinase C
- PLC, phospholipase C
- PVDF, polyvinylidene difluoride
- RIPA,
radioimmunoprecipitation assay
- RT-PCR,
real-time reverse transcription PCR
- SDS-PAGE, SDS-polyacrylamide gel
electrophoresis
- SGLT1, sodium-dependent
glucose transporter 1
- SIRT1, sirtuin 1
- TAK-1, transforming growth factor- β -
activated kinase
- TBS-T, Tris-buffered saline
containing 0.05% Tween 20
- TEER,
transepithelial electrical resistance
- TF, theaflavin
- TF3G, theaflavin-3-O-gallate
- TF3'G, theaflavin-3'-O-gallate
- TFdG, theaflavin-3,3'-O-digallate
- TJ, tight junction
- TLR, toll-like receptor
- TMB, tetramethylbenzidine
- TR, thearubigin
- ZO, zonula occluden

Chapter I

Introduction

In the modern society, lifestyle-related diseases in company with chronic diseases are growing rapidly and have emerged as a critical public health issue in the world.⁽¹⁾ Such diseases including atherosclerosis, heart disease, stroke, obesity and type 2 diabetes are associated with the daily habits of people. The risk factors contributing to these diseases include poor food habits, physical inactivity, and/or wrong body posture.⁽²⁾

Nowadays, foods are known to be not only a supplier for nutrients and energies to human, but also to prevent lifestyle-related diseases and to enhance physical and mental well-being of the consumers.^(3, 4) The term “functional food” is the best word to express these outstanding roles of food ingredient. This concept was first used in Japan in the 1980s and defined as “any food or ingredient that has a positive impact on an individual’s health, physical performance, or state of mind, in addition to its nutritive value.”⁽⁵⁾ Their health-claim involves pre- and pro-biotics and reduced risks such as cholesterol-lowering, anti-hypertension and anti-diabetic effects.⁽⁶⁾

To date, many studies on functional food are addressing the physiological effects and health benefits of foods to clarify the body modulating functions of food-derived compounds.

In particular, polyphenols are the most abundant group in the diet including fruits, plant-derived beverages (e.g. fruit juices, tea, coffee, and red wine), vegetables, cereals, chocolate and dry legumes. *In vivo* studies have revealed that they could improve endothelial function, to modulate inflammation and lipid metabolism and to contribute to the prevention of cardio-vascular diseases, cancers, osteoporosis and diabetes mellitus.⁽⁷⁾ However, knowledge still appears too limited for evidence on bioavailability of polyphenols.

It is important to know the absorption mechanism of bioactive food-derived compounds regarding their absorption, distribution, metabolism and excretion (ADME) (Fig. 1-1). Many food-derived compounds such as polyphenols have been shown to have specific and potent physiological activities in *in vitro* test with higher throughput treatment, but conflicting results are often obtained in animals or human when they are orally taken.⁽⁸⁾

To propose the behavioral pharmacological movement, most of the bioavailability and ADME studies of food-derived compounds have been investigated by focusing a target substance in food so far. However, most of foods contain not only target compounds, but also other compounds, so that they might affect the ADME activity of target compounds. Researches on the combination effect of target with food contaminants on bioavailability have not been investigated in much detail. Direct interactions between food contaminants and polyphenols, such as binding to proteins and polysaccharides, can occur and these interactions may affect the absorption target compounds. Furthermore, parameters of gut physiology including pH, biliary excretion, intestinal fermentations and transit time may have an effect on the absorption of polyphenols indirectly.

For example, when teas (green and black tea) were consumed with milk in *in vivo*, their antioxidant activity were totally inhibited due to the complexation of tea polyphenols by milk proteins, whereas teas have a strong antioxidant activity both *in vitro* and *in vivo* by

exertion of polyphenols moiety without milk.⁽⁹⁾ Some results have shown that the combination of lipids and emulsifiers or ethanol (more than 30%) significantly enhanced the intestinal absorption of quercetin in rats due to improved quercetin's solubility in the vehicle.⁽¹⁰⁾ Tartaric acid that is a major organic acid in wine was also shown to enhance the absorption of catechin in rats.⁽¹¹⁾ In humans, catechin metabolites were excreted in urine after red wine drinking, compared with de-alcoholized red wine, indicating the possible role of ethanol in the increased elimination of catechins into urine.⁽¹²⁾ These reports suggest that the intake of polyphenols in foods may affect their absorption.

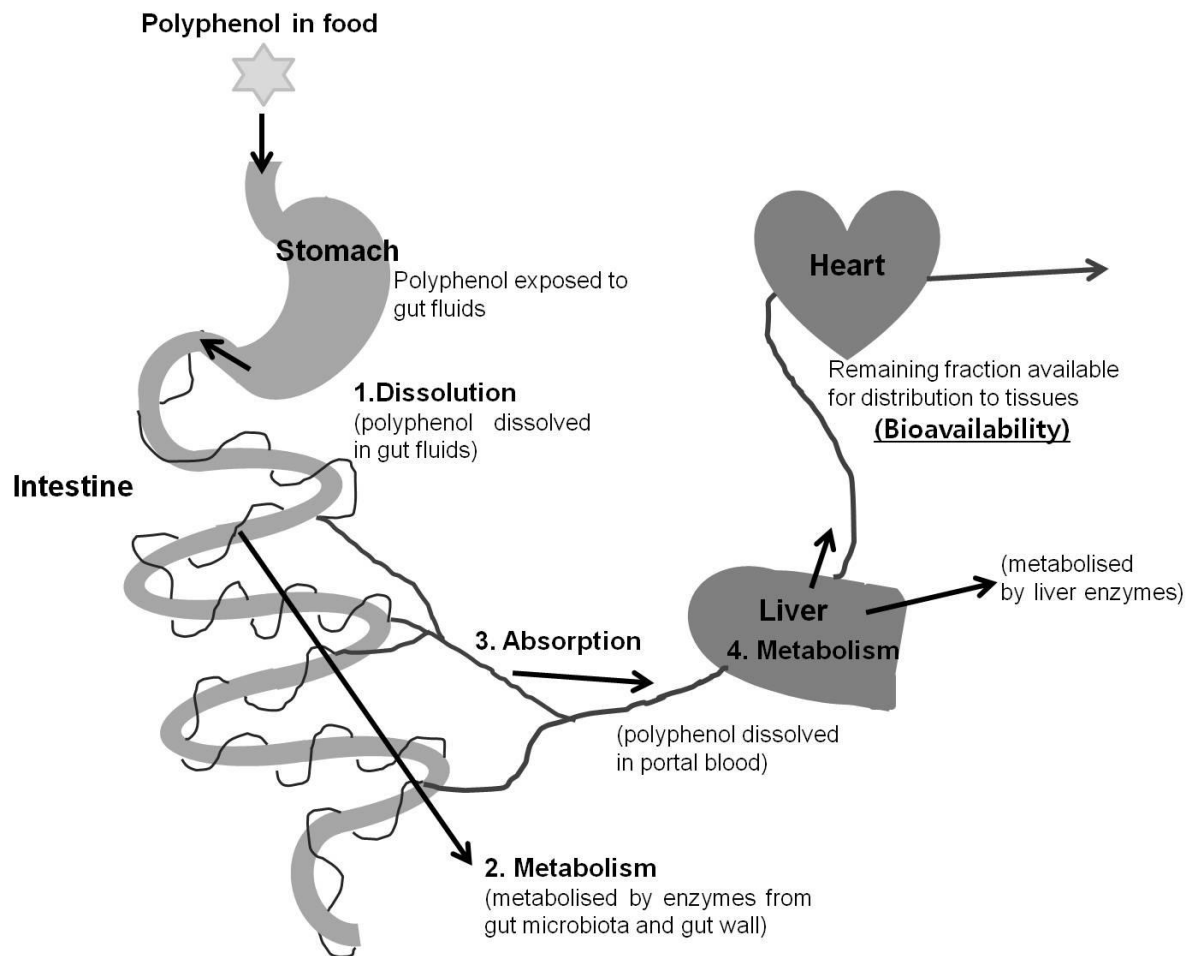


Fig. 1-1. Schematic representation of major processes governing polyphenol bioavailability in mammals ⁽⁸⁾

The small intestinal surface was composed of many folds and villi, the total surface area being estimated to be more than 200 m². Using this huge area, large amounts of nutrients derived from diet are quickly and efficiently absorbed.⁽¹³⁾ Intestinal epithelial cells (IECs), covering the internal surface of the intestines, have many functions for nutrient absorption. IECs are exposed to high concentrations of components such as nutrients, non-nutrients, microbes and xenobiotic chemicals. This indicates that the functions of IECs may be affected or regulated by external factors including food components, although IEC functions are known to be generally regulated by internal factors such as hormones and cytokines.⁽¹³⁾

As depicted in Fig. 1-2, IECs have three major functions: absorption, barrier, signal recognition and transduction function. Compounds in foods can be absorbed in multiple routes. Carbohydrates, proteins and lipids are broken down into monosaccharides, amino acids and fatty acids, respectively, by diverse gut hydrolases.⁽¹⁴⁾ The apical cell membrane (apical side) of IECs is involved in the transport of nutrients such as monosaccharides, amino acids, peptides, fatty acids, vitamins, minerals, and non-nutrient food compounds or xenobiotics by nutrient transporter (carrier-mediated active transport) and/or passive diffusion. The nutrients, apically taken by these transporters into IECs, are then excreted from the cells to the blood circulation via the same or different transporters located in the basolateral membrane (basolateral side).^(13,15) Also, hydrophobic or lipophilic substances that can easily penetrate cell membranes are in general transported via transcellular passive diffusion.^(13,16) Paracellular transport is a passive diffusion pathway through a space or gap at intercellular junctions between IECs. This is regulated by the permeability of tight junction (TJ), which provides another absorption of small molecular compounds including minerals.^(13,17) In contrast, indigestible macromolecules are incorporated by M cells (microfold cells) present in the follicle-associated envelope of Peyer's patches or through endocytosis.^(13,14)

The second function is a barrier function, which may be divided into physical and biological barrier function. The epithelial TJ barrier that is essential for the integrity of IEC monolayers acts as physical barrier to larger particulate matters and substances such as microbes are met substances, particles and allergenic proteins. In contrast, biological barrier includes a detoxification system constructed by detoxification enzymes (e.g., cytochrome P450 enzymes (CYPs)) and efflux transporters (e.g., multidrug resistance (MDR), and multidrug-associated proteins (MRPS)), at which the transport of toxic compounds into the body is inhibited.^(13,18)

The third function is a signal recognition and transduction. Enteroendocrine cells located in the IEC monolayers are known to recognize food-derived nutrients reaching the intestinal tract via certain receptors including toll-like receptors (TLRs), thereby secreting gut hormones such as cholecystokinin (CCK) to the basolateral side.^(13,19,20) In other words, IECs act as a converter, by which food signals are converted to internal signals. The IEC monolayer is a unique tissue directly contacting with various external substances, including food and its digests.^(13,19,20) Studying the interaction between food and IECs is, therefore, important to understand how dietary compounds regulate the absorption pathway of our body and keep us healthy.

The intestinal absorption of food compounds can be determined by *in vitro* systems using IEC-mimic on Caco-2 cell lines.⁽²¹⁾ Caco-2 cell lines originated from human colorectal carcinoma and have been used as an *in vitro* model of human small intestinal epithelial system.⁽²⁰⁻²³⁾ In long-term cell culture, it could spontaneously differentiate into monolayers of polarized enterocytes under conventional cell culture conditions. Completely differentiated and polarized Caco-2 cell monolayers express various enterocyte characteristics including the expression of brush border enzymes, nutrient transporters, and the formation of intercellular

TJs (Fig.1-3).⁽²⁰⁻²³⁾

Tea is one of the ancient and most widely consumed beverages in the world. *Camellia sinensis*, an evergreen shrub originally from south China is famous for the utilization of its shoots to produce more than 300 different kinds of tea. These are subdivided into three major types according to the different manufacturing process: unfermented green tea, partially fermented oolong tea and fully fermented black tea.⁽²⁴⁻²⁶⁾ Among types of tea, black tea is consumed about 78% of the tea production worldwide. More research has been done on green tea, but in recent years black tea has attracted significant attention for its extensive health promotional effect.⁽²⁶⁾ Black tea contains 30-40% polyphenols including theaflavins (TFs), thearubigins (TRs) and bisflavonols, all of which are formed by the oxidation and polymerization of catechins during fermentation. TFs including theaflavin (TF), theaflavin-3-*O*-gallate (TF3G), theaflavin-3'-*O*-gallate (TF3'G) and theaflavin-3,3'-*O*-digallate (TFdG) are orange or orange-red in color and possess a benzotropolone skeleton that is formed from co-oxidation of selected pairs of catechins, one with a *vic*-trihydroxyphenyl moiety, and the other with an *ortho*-dihydroxyphenyl structure (Fig. 1-4).^(24,26)

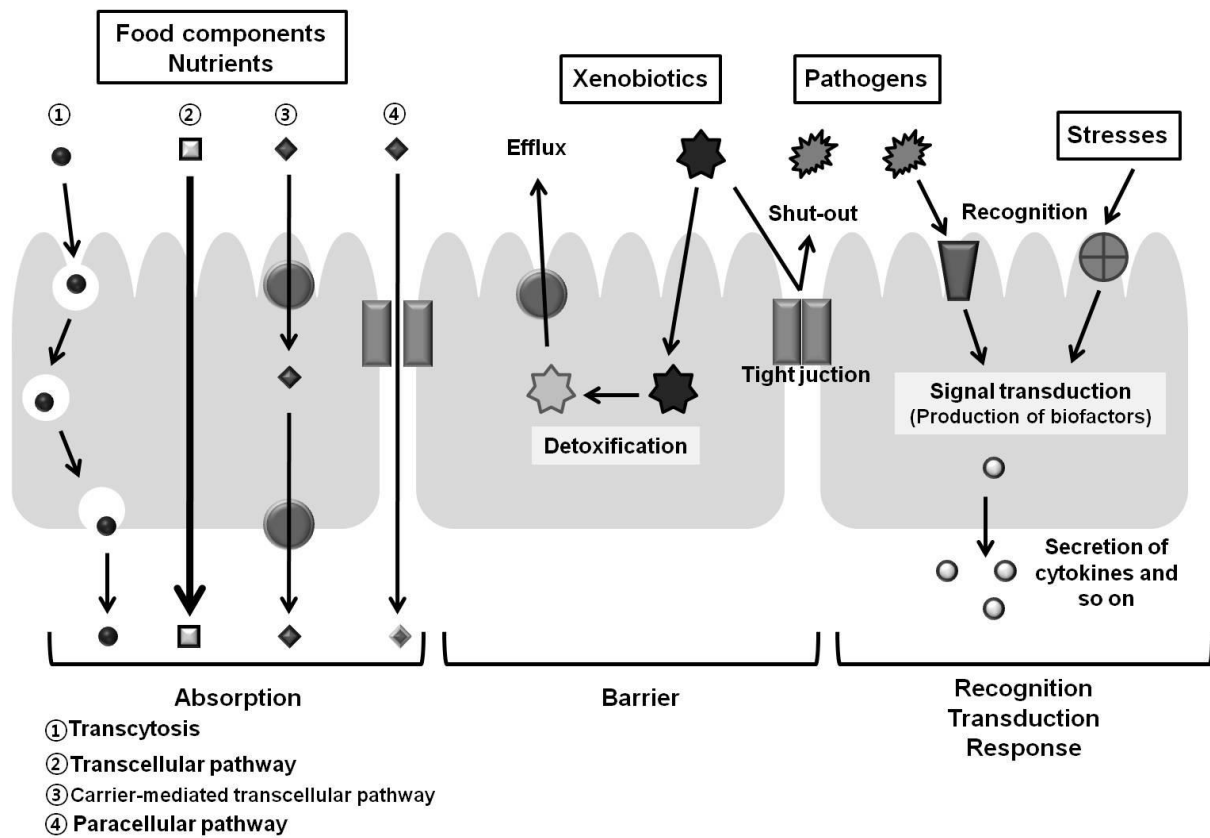


Fig. 1-2. Major functions of the intestinal epithelial cell (IEC) monolayer ^(13, 14)

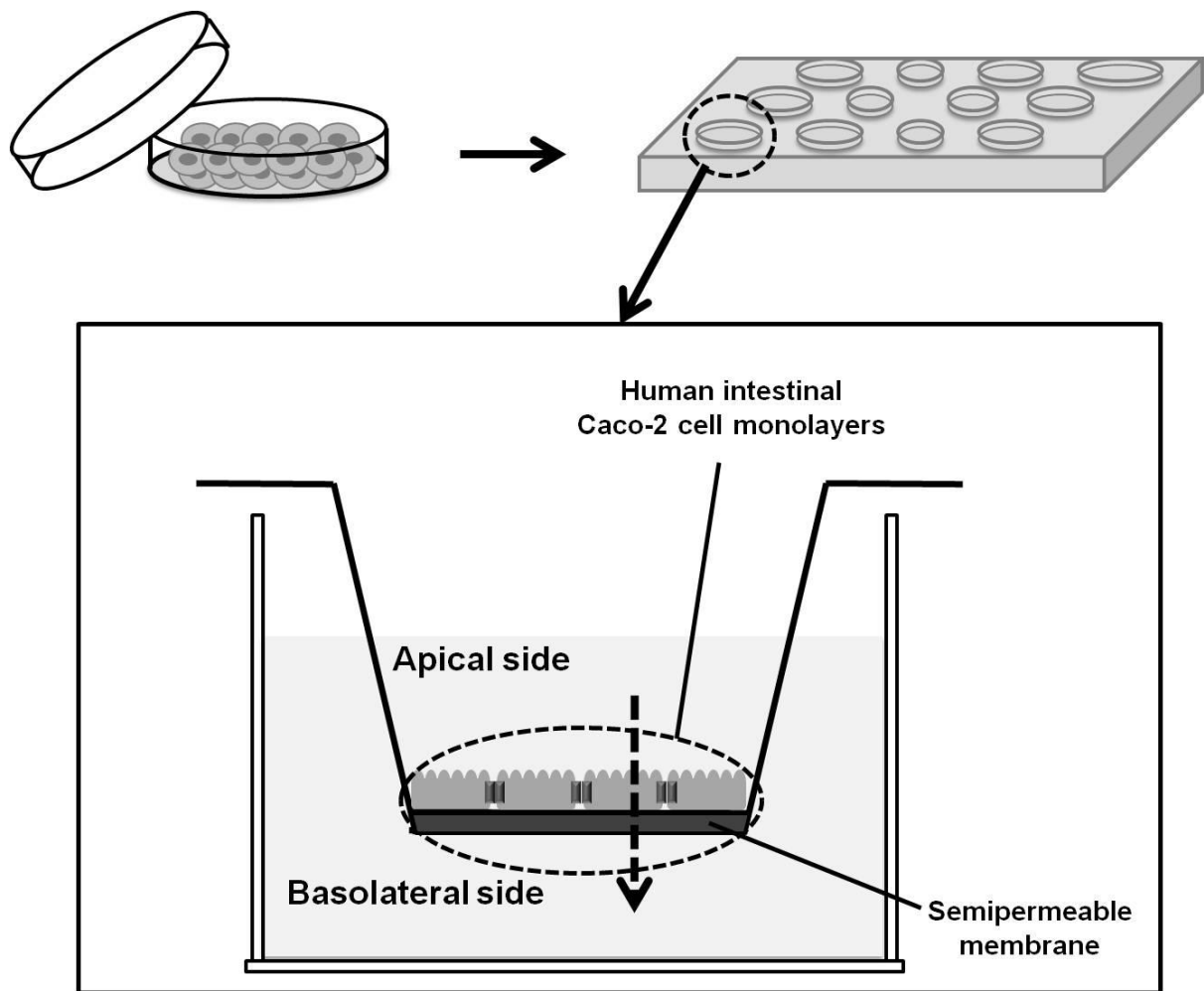
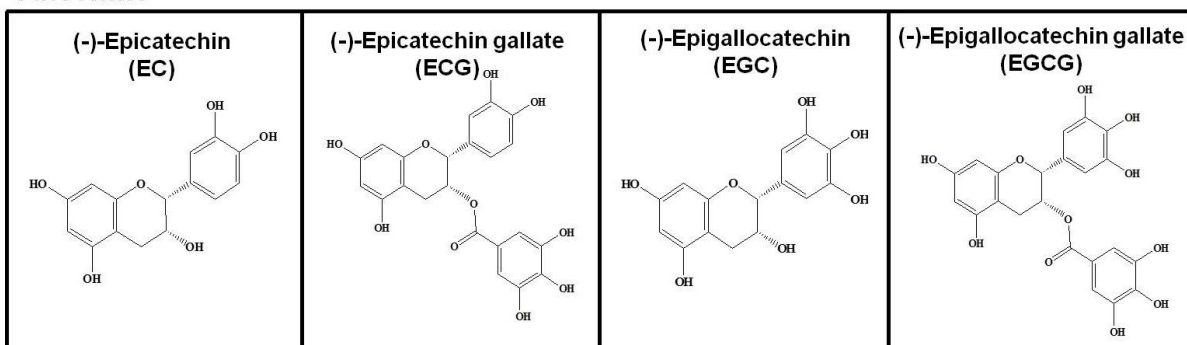


Fig. 1-3. Characteristics of human intestinal Caco-2 cell monolayer

Caco-2 cells are spontaneously differentiated by culturing them for one week to form functional monolayers on semipermeable membranes.

Catechins



Theaflavins

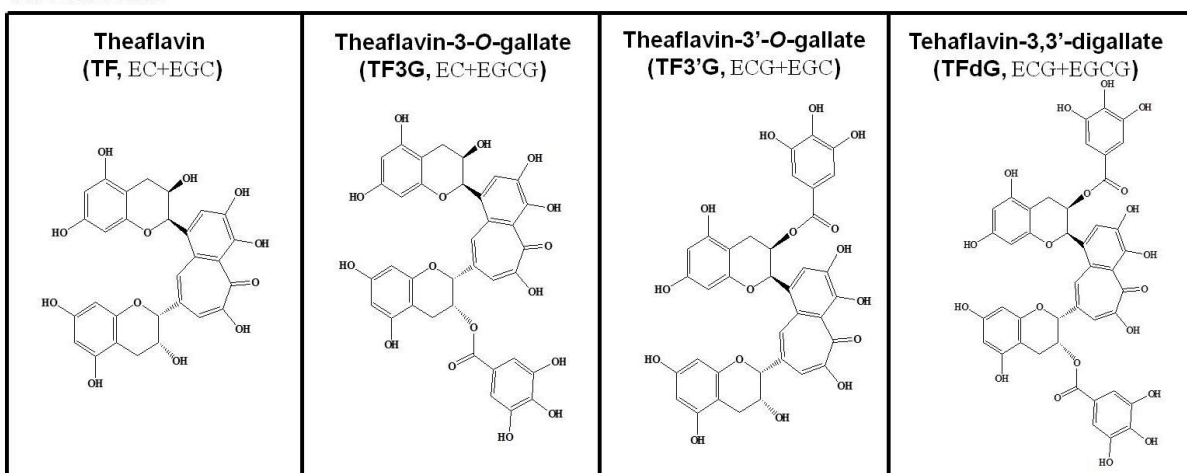


Fig. 1-4. Chemical structures of catechins and theaflavins

In recent years, attention has been paid to health beneficial function of TFs of black tea including antioxidant, anti-inflammation, anti-hyperglycemic activity, antimutagenic, anticlastogenic, anticancer, antipathogenic and anticarcinogenic effects.⁽²⁷⁻³¹⁾ However, the underlying mechanisms responsible for the health benefits of TFs on absorption of food substances in IECs are largely unknown. Most of polyphenols showed low activity within the body due to low intrinsic activity or poor absorption from intestine, high metabolism or rapid elimination.⁽³²⁾ Therefore, the metabolites found in blood and target organs may differ from the native substances with respect to biological activity. Thus, it is important to know extensive bioavailability and their mechanism(s) of polyphenols for further understanding of their health effects.⁽³²⁾

To date, some studies have reported that TFs have poor and limited bioavailability.^(33,34) Very small amounts of TFdG (< 1 nmol/g tissue) were detected in tissue sample collected from mice treated with decaffeinated black tea (50 mg/g diet) for two weeks.⁽³³⁾ Also, the C_{max} of TF in human plasma and urine was only 1 ng/ml and 4.2 ng/ml, respectively, after the consumption of 700 mg of TFs.⁽³⁴⁾ However, it is still unclear how TFs can exert their biological functions at the intestinal membrane. In this regard, transport route and the mechanism(s) of TFs on intestine will be considered in this study.

Recent studies have demonstrated that polyphenols stimulate AMP-activated protein kinase (AMPK) and protein kinase C (PKC) activity in a variety of cell types including IECs, which are involved in the expression of multiple routes of intestine such as nutrient transporter and TJ.^(35,36) AMPK, a serine-threonine kinase protein complex, is a key player in regulating energy balance at both cellular and whole-body levels, and also centre stage in studies of lifestyle-related diseases such as obesity, diabetes and metabolic syndrome. It exists as heterotrimeric complexes comprising a catalytic α -subunit and regulatory β - and γ -

subunits. Activation of AMPK requires phosphorylation at Thr-172, which resides in the activation loop of the $\alpha 1$ and $\alpha 2$ subunits, and this activation is related to up-stream kinases including liver kinase B1 (LKB1) and Ca^{2+} /calmodulin-dependent protein kinase kinase- β (CaMKK β).⁽³⁵⁻³⁸⁾ PKC consists of a family of Ser/Thr-specific kinases which includes 12 known isozymes that can be divided into three subfamilies following as conventional (α , $\beta 1$, $\beta 2$ and γ), novel (δ , ϵ , θ , η and μ) and atypical (λ , τ and ζ). These isoforms have isoform-specific dependent mechanism for their action. Conventional isoforms (cPKC) are both Ca^{2+} and diacylglycerol (DAG), novel isoforms (nPKC) are DAG dependent, not Ca^{2+} , while the atypical PKC isoenzymes (aPKC) are neither Ca^{2+} nor DAG dependent.^(35,39)

Polyphenol derived from diet holds promise in the prevention and treatment of various illnesses such as cancer, hypertension and inflammation, and AMPK and/or PKC is one of the major pathways activated by many polyphenols. Not only do polyphenols activate AMPK and/or PKC to affect their absorption mechanism, but also they have other up-stream mechanisms associated with absorption pathway such as nutrient transporter and TJ.^(35,36,38)

According to all of the above-mentioned points, the aim of this study is to clarify the intestinal absorption and their underlying mechanism(s) of TFs, dimeric catechins, in human intestinal Caco-2 cells. The detailed objectives and results for each Chapter will be seen as follows:

1) Chapter II investigated whether theaflavins (TFs) in black tea, i.e., oxidatively coupled dimers of catechins, affect the absorption of small peptides across human intestinal Caco-2 cells. The results clearly indicate that TFs inhibit peptide transport across Caco-2 cell monolayers, likely through the suppression of AMPK-mediated peptide transporter 1 (PEPT1) expression.

2) To clarify the structural requirement of TFs for the effect and the mechanism(s) underlying TF-induced AMPK activation in Caco-2 cells, transport experiment and Western blot analysis were performed. Chapter III showed that the benzotropolone moiety in TFs was a crucial structural requirement for exerting the inhibition of intestinal peptide-transport, and CaMKK/AMPK pathway is not involved in the suppression of PEPT1 expression by TFs.

3) Based on the findings obtained above, the effect of TFs on enhancement of tight junction (TJ), AMPK-related intestinal transport systems, was illustrated. Chapter IV showed that TFs enhance the intestinal barrier by increasing the expression of TJ-related proteins (occludin, claudin-1 and ZO-1) through the activation of AMPK in Caco-2 cell monolayers.

Chapter II

Effect of theaflavins on peptide transport across Caco-2 cell monolayers

1. Introduction

To date, much attention has been paid to the function of peptides in our body, since clinical and animal evidences strongly support the preventive effect of peptides for life-style related diseases, such as hypertension ⁽⁴⁰⁾ and hypercholesterolemia.⁽⁴¹⁾ In a randomized double-blind placebo study of mild hypertensive subjects, a 4-wk daily intake of a peptide drink was successful in eliciting a significant reduction of ca. 10 mmHg in systolic blood pressure via inhibition of the renin-angiotensin system.⁽⁴²⁾ Clinical reports on anti-hypertensive effects of other small peptides ^(43,44) also support the health-benefit of peptide intake. In addition, a recent finding that ingestion of di-peptide, Trp-His, could ameliorate the onset of atherosclerosis in apolipoprotein E-deficient mice after 3 months ⁽⁴⁵⁾ strongly suggests the possible physiological roles of peptides after their intact absorption.

In the gut, dietary proteins are digested to produce short chain peptides and free amino acids in epithelial cells (Fig. 2-1A). Mammalian small intestine, thus, expresses a transport system for digested protein.^(46,47)

Intestinal transport of amino acids is mediated by several classes of amino acid transporters.^(46,48) Transport of di- and tri-peptides across the brush border of intestine is a major mechanism, by which the digestion products of proteins are absorbed.⁽⁴⁶⁻⁴⁹⁾ Transport of di- and tri-peptides (or small peptides) occurs by H⁺-dependent peptide cotransport system, in which protons are co-transported with peptides across the epithelial membrane (Fig. 2-1B). The proton gradient as well as membrane potential provides the driving force for peptide uptake into intestinal epithelial cells through the proton-dependent peptide transporter located in the apical membrane.

Fei *et al.*⁽⁵⁰⁾ have demonstrated the expression of a peptide transporter in the intestinal membrane, named it as PEPT1. The peptide transporter PEPT1 (SLC15A1; SLC, solute carrier) belongs to a SLC15 family of membrane transporters, which includes renal peptide transporter isoform PEPT2 (SLC15A2) and two peptide/histidine transporters (PHT) 1/2 which transport histidine and selected peptides.^(46,51) PEPT1 is composed of 708 amino acids with 12 membrane-spanning domains.⁽⁴⁷⁾ To date, it has been clarified that the PEPT1 transporter can recognize substrates with 1 and 2 peptide bonding sites, namely di- and tri-peptide lengths, although the details on substrate specificity for a number of peptides and their absorption behavior remain unclear.

Contrary to the few studies on peptide absorption, studies on polyphenol absorption have been extensively performed to understand their potential roles in the body.

Recent studies have also claimed alternative potentials of polyphenols towards intestinal function, including anti-inflammatory effects and regulation of the absorption of

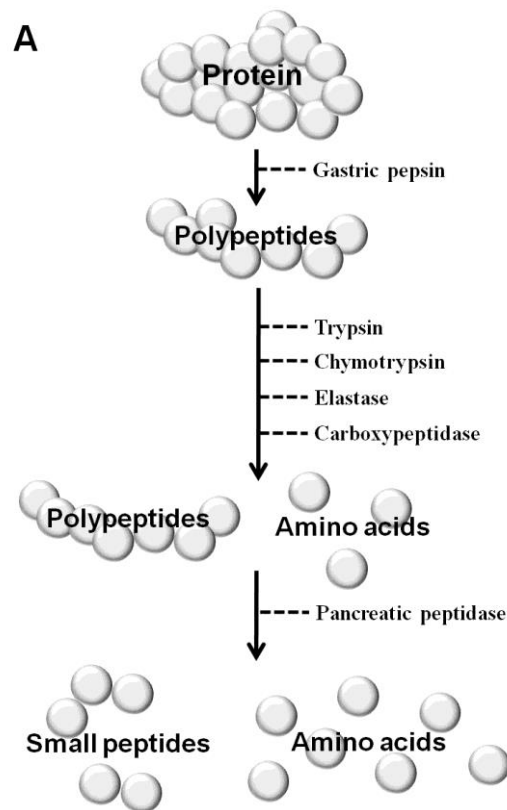


Fig. 2-1A. Protein digestion

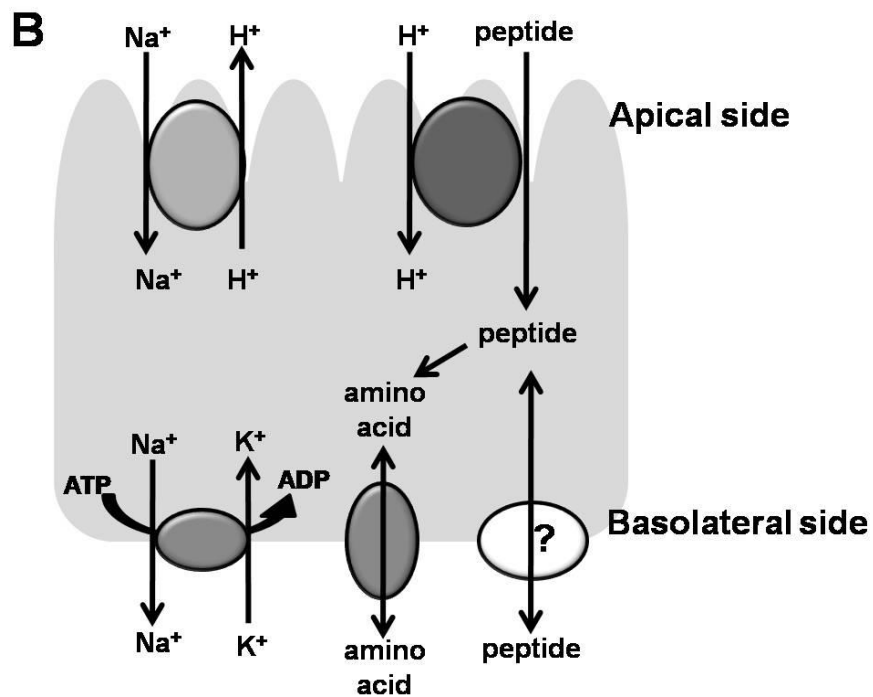


Fig. 2-1B. Transport at the intestinal epithelial cell

drugs and food compounds.⁽⁵²⁾ One good example is that green tea polyphenols inhibit expression of the sodium-dependent glucose transporter (SGLT1) in a competitive manner and quercetin closed the TJ through the assembly and expression of TJ.⁽⁵²⁻⁵⁴⁾ However, no detailed study has been conducted on the effect of TFs on peptide absorption. TFs are oxidatively coupled dimers of catechins and are major constituents in black tea, possessing diverse bioactivities, such as anti-hyperglycemic, anti-inflammatory and cytotoxic effects.⁽²⁷⁻²⁹⁾

In this Chapter II, thus, we primarily focused on the absorption of small peptides that can be recognized by PEPT1 to elucidate the potential role(s) of TFs on overall intestinal absorption using human intestinal Caco-2 cell monolayers. The four major TFs in black tea (i.e., TF, TF-3-*O*-gallate (TF3G), TF-3'-*O*-gallate (TF3'G), TF-3,3'-*O*-digallate (TFdG), Fig. 1-4) were used to evaluate the effect of TFs on glycyl-sarcosine (Gly-Sar, a di-peptidic model for PEPT1 transport,⁽⁵⁵⁾ together with Val-Tyr and Ile-Phe, which have been reported to be able to penetrate the monolayer.^(56,57)

2. Materials and Methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO Life Technologies (Grand Island, NY, USA). Nonessential amino acid was purchased from MP Biomedicals (Irvine, CA, USA). Penicillin was purchased from Meiji Seika Co. (Tokyo, Japan). Insulin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Gly-Sar, Val-Tyr and Ile-Phe were obtained from Kokusan Chemical Co. (Tokyo, Japan). TFs (TF, TF3G, TF3'G and TFdG, purity > 90%) used in this study were purchased from Nacalai Tesque Co. (Kyoto, Japan). Compound C, an AMP-activated protein kinase (AMPK) inhibitor, and bisindolylmaleimide I (GF109203X), a non-specific PKC inhibitor were obtained from Merck (Darmstadt, Germany). Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Molecular Probes Inc. (Eugene, OR, USA). All other chemicals from Nacalai Tesque were of analytical grade and were used without purification.

2.2. Cell culture

Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acid, 2 mM L-glutamine (Nacalai Tesque), 100 U/ml penicillin, 100 µg/ml streptomycin (Nacalai Tesque), and 1.7 µM insulin. The cells were incubated at 37°C, in a humidified atmosphere of 5% CO₂ in air. The monolayers became confluent 4 - 5 days after seeding at a number of 1.0×10^6 cells per 100 mm dish and the cells were passaged at a split ratio of 4 - 8 by trypsinization with 0.25% trypsin and 0.02% di-sodium salt of ethy-

lenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS, Cosmo Bio Co., Tokyo, Japan). Caco-2 cells used in this study were between passages 50 and 60.

For transport study, the cells seeded at a density of 4.0×10^5 cells/ml were grown in BD Falcon cell culture inserts (polyethylene terephthalate membrane, 0.9 cm^2 , $1.0 \mu\text{m}$ pore size; BD Biosciences, Bedford, MA, USA) with a membrane coated with type I collagen (collagen gel culturing kit, Cellmatrix type I-A, Nitta Gelatin, Osaka, Japan). The cells were cultured using a BD BioCoatTM intestinal epithelium differentiation kit (BD Biosciences), by which monolayers were formed after 6 days.⁽⁵⁸⁾ The medium was replaced with a fresh one every day.

The integrity of the monolayers was evaluated by measuring transepithelial electrical resistance (TEER) using a Multi-channel Voltage/Current EVC-4000 system (World Precision Instruments, Sarasota, FL, USA). Monolayers with TEER value of $> 100 \Omega \cdot \text{cm}^2$ were used for transport experiments.

2.3. Cell treatment

Treatment of Caco-2 cell monolayers with TFs was performed by replacing 10% FBS-containing DMEM with medium containing TFs (5, 10 or 20 μM), allowing them to incubate for 1, 6 or 24 h at 37°C at 5% CO_2 in air. Cells incubated in TF-free medium were used as control.

For inhibition experiments, Caco-2 cells were pretreated with or without 20 μM TF3'G in the presence or absence of inhibitor (10 μM GF109203X as PKC inhibitor or 10 μM compound C as AMPK inhibitor). The medium containing TF3'G was prepared by diluting a stock solution of TF3'G, dissolved in dimethylsulfoxide (DMSO), with 10% FBS-

containing DMEM. The final concentration of DMSO was 0.5% throughout the experiments to avoid any cell damage by DMSO.⁽⁵⁹⁾

2.4. LC-ESI-TOF-MS analysis

Transport studies were started by replacing the apical buffer with fresh Hanks' balanced salt solution (HBSS) buffer (pH 6.0, 7.5 ml) containing 1 mM TF3'G. After the 60 min-incubation, total volume of the basolateral aliquots was collected and applied to a Sep-Pak Plus C₁₈ cartridge (Waters, Milford, MA, USA), followed by an elution with 3 ml of 40% acetonitrile containing 0.1% trifluoroacetic acid. The extract was then evaporated to dryness under vacuum. The dried extract was dissolved in super-pure water and then separated using an Agilent 1200 series (Agilent, Germany) equipped with a microdegasser (G1379B), a binary pump (G1312A), a high-performance autosampler SL (G1367B), and a thermostatically controlled oven apartment (G1316A). Chromatographic separation was carried out at 40°C on Cosmosil 5C₁₈-MS-II column (2.0 mm × 150 mm, Nacalai Tesque). The mobile phase consisted of 0.1% aqueous formic acid (A) and methanol with 0.1% formic acid (B) using a 20 min-linear gradient from 0 to 100% of solvent B. The flow rate was 0.2 ml/min, and the injection volume was 20 µl.

MS analysis was performed by a microTOF II (Bruker Daltonics, Bremen, Germany). The LC-ESI-TOF-MS analysis worked in negative mode, and the mass range was set at 100 - 1000 m/z. The conditions of ESI source were as follows: drying gas, N₂; flow rate, 8.0 l/min; drying gas temperature, 200°C; nebulizing gas pressure, 1.6 bar; and capillary voltage, 3800 V. All of the acquisitions and analyses of data were controlled by a Bruker Data Analysis 3.2 Software.

To tune the detector to optimal conditions, calibration was performed with sodium formate clusters [10 mM sodium hydroxide in water:acetonitrile (1:1, v/v)]. The calibration solution was injected at the beginning of the run, and all of the spectra were calibrated prior to identification.

2.5. Cell viability assay

Cytotoxicity was evaluated using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay.

Caco-2 cells (4.0×10^5 cells/ml) were seeded onto 96 well-plates in 100 μ l of 10% FBS-containing DMEM medium and cultured for 24 h. Caco-2 cells were pretreated with TF3'G (5, 10, 20 and 50 μ M) for 24 h. After incubation, 10 μ l of WST-8 reagent solution (Cell Counting Kit, Dojindo Co., Kumamoto, Japan) was added and incubated for 4 h at 37°C. Cell viability was determined by measuring absorbance at 450 nm. (Wallac ARVO™ SX 1420 Multilabel Counter, Perkin-Elmer Life Sciences, Tokyo, Japan).

2.6. Transport study

Transport experiments in Caco-2 cell monolayers were performed using an Ussing Chamber system (Model U-2500; Warner Instrument Co., Hamden, CT, USA). Caco-2 cell monolayers grown in transwell inserts were gently rinsed with HBSS buffer (pH 6.0) to wash or remove any free TFs in the medium, and carefully mounted in the Ussing Chamber using blunt forceps. An aliquot (7.5 ml) of HBSS buffer (adjusted to pH 6.0 with 10 mM MES [2-(N-morpholino) ethanesulfonic acid]) or HBSS buffer (adjusted to pH 7.4 with 10 mM

HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid]) was added to the apical or basolateral side, respectively. After equilibration for 15 min at 37°C, the transport experiments were started by replacing the apical buffer with fresh HBSS buffer (pH 6.0) containing peptides (1, 3, 5 or 10 mM) for Caco-2 cells pretreated with or without TFs. During the experiments, solutions on both sides were bubbled continuously with a mixture of O₂:CO₂ (95:5).

An aliquots (0.2 ml) of solution were drawn from the basolateral side at time intervals of 15, 30, 45 and 60 min to determine the amount of transported peptide. The same volume of fresh buffer was added to the basolateral side at each time point. The amount of peptide transported to the basolateral side was determined simultaneously for all samples by a fluorescent NDA-derivatization method ⁽⁶⁰⁾ to avoid any degradation of peptide during all scheduled assays by e.g., LC-ESI-TOF-MS. Briefly, 10 µl of 20 mM sodium cyanide in borate buffer (pH 9.5) was added to 100 µl of basolateral solution taken at each time point in a 96-well microplate, followed by the addition of 50 µl of 0.1 mM NDA solution in methanol. After incubating at 25°C for 60 min, the fluorescence intensity of the NDA-derivatized peptide was measured using a fluorescence spectrophotometer at an excitation wavelength of 405 nm and emission wavelength of 460 nm. The fluorescence intensity of basolateral solution taken at each time point for blank (no peptide) transport experiments was used to cancel an increase in intensity by any contaminants from Caco-2 cells.

The apparent permeability coefficient (P_{app}) was calculated from the following equation:

$$P_{app} \text{ (cm/s)} = \frac{V}{AC_0} \frac{dC}{dt}$$

where dC/dt is the change in concentration at the basolateral side over time (mM/s), V is the volume of solution in the basolateral compartment (7.5 ml), A is the surface area of

the membrane (0.2826 cm^2) and C_0 is the initial concentration at the apical side (mM). The effect of inhibitor on P_{app} was expressed relative to control (%).

The Lineweaver-Burk plot of Gly-Sar uptake (1-10 mM) was performed based on a 60 min-transport at 37°C in the presence or absence of $20 \mu\text{M}$ TF3'G.

2.7. Real-time reverse transcription PCR

Caco-2 cells were cultured on a 6 well-plate under the same cell culture conditions for transport experiments as described above. Total RNA was isolated from Caco-2 cells treated for 1 h with or without TF3'G ($20 \mu\text{M}$) by using an SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). The complementary DNA was produced from $1 \mu\text{g}$ of total cellular RNA by Reverse Transcription System (AMV reverse transcriptase, Promega Corporation) following the manufacturer's instructions.

Mx3000P QPCR system (Stratagene Inc., Tokyo, Japan) and SYBR *Premix Ex Taq* II (Takara bio Inc., Otsu, Japan) were used for real-time reverse transcription PCR (RT-PCR) amplification of PEPT1 and β -actin with the following cycle profile: 1 cycle at 95°C for 10 s and then run for 47 cycles at 95°C for 30 s (denaturation step), at 60°C for 30 s (annealing step) and at 72°C for 1 min (extension step). At the end of each extension step, observed fluorescence was used for quantitative analyses. A melting point analysis was carried out by heating the amplicon from 55°C to 95°C , and a characteristic melting point curve was obtained for each product. Sequences of used primers were PEPT1, forward primer, 5'-TCACCTGTGGCGAAGTGGT- C-3' and reverse primer, 5'-GCCACGATGAGCACAATG-ATG-3'; β -actin, forward primer, 5'-TGGCACCCAGCACAATGAA-3' and reverse primer, 5'-CTAAGTCATAGTCCGCCTA- GAAGCA-3'.

As an internal control, β -actin primers were used for RNA template normalization. Quantification was reported by proprietary software, as cycle threshold (Ct). The expression of the target genes relative to the housekeeping genes was calculated as the difference between the threshold values for the two groups. All PCR reactions were done in triplicates. Amplification products were separated using a 4% polyacrylamide gel and stained with ethidium bromide. Predicted sizes are 139 bp for PEPT1.

2.8. Western blot analysis

Western blot analysis was carried out to determine the expression of PEPT1 in Caco-2 cells. Briefly, Caco-2 cells were treated with or without TF3'G (20 μ M) for 24 h at 37°C. Protein (1.3 mg/ml) extracted from Caco-2 cells using radioimmunoprecipitation assay (RIPA) buffer (150 mM/l NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium sulfate, 1% (w/v) NP-40 and 50 mM/l Tris-HCl, pH 8.0) was mixed with an equal volume of sample buffer (20% glycerol, 4% sodium dodecylsulfate, 3% dithiothreitol, 0.002% bromophenol blue and 0.125 M Tris-HCl, pH 6.8) and incubated overnight at 4°C. An aliquot (19.5 μ g) of the extracted sample was applied to 10% SDS-polyacrylamide gel electrophoresis (PAGE) for 1 h at 20 mA and transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond-P, GE Healthcare, Piscataway, NJ, USA) for 1.5 h at 40 mA. The membrane was blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and then probed with the rabbit polyclonal antibody against PEPT1 (1:1000, Abbiotec, San Diego, CA, USA) overnight at 4°C. The membrane was then reprobed with HRP-conjugated donkey anti-rabbit IgG antibody (1:1000, GE Healthcare, Buckinghamshire, UK) for 1 h at room temperature. The

membrane was detected with enhanced chemiluminescence (ECL) plus detection reagent using an ImageQuant LAS 4000 (GE Healthcare). Quantitation of PEPT1 was performed with ImageQuant TL 7.0 software (GE Healthcare), and the PEPT1 protein levels in Caco-2 cells treated with TF3'G were expressed relative to a control (TF3'G-untreated cells).

2.9. PKC activity assay

The PKC kinase activity assay was obtained from Enzo Life Sciences (Plymouth Meeting, PA, USA). Caco-2 cells were treated with or without TF3'G (20 μ M) for 6 h at 37°C and protein was extracted under the same conditions for Western blot assay as described above. All of the steps were performed following the manufacturer's instructions. (Enzo Life Sciences, Plymouth Meeting, PA, USA). The PKC activity was measured by solid phase enzyme-linked immunosorbent assay (ELISA) utilizing a specific synthetic peptide as a substrate for PKC and a rabbit polyclonal antibody specific for phosphorylated PKC substrate. The assay is developed with tetramethylbenzidine (TMB) substrate and a color develops in proportion to PKC phosphotransferase activity. The intensity of the color was measured by fluorescence spectrophotometer at 450 nm.

2.10. Statistical analysis

Results are expressed as the mean $\pm$ SEM. The statistical significance between groups was analysed using one-way ANOVA followed by Tukey-Kramer's *t*-test for *post hoc* analysis. Statistical differences between curves were analysed by two-way ANOVA, and the differences between two groups were analysed by unpaired Student's *t*-test. *P*-values < 0.05

were considered to be significant. All analyses were conducted with StatView J5.0 (SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1. Effect of theaflavins on Gly-Sar transport across Caco-2 cell monolayers

TFs, which are characteristic polyphenols found in black tea that are formed by the oxidative condensation of catechins⁽²⁸⁾ have been proven to exhibit physiological effects in the gut, including an anti-hyperglycaemic effect by inhibiting intestinal α -glucosidase.⁽⁶¹⁾ Although some functionalities of TFs including anti-cancer and anti-inflammatory effects have been reported after absorption, absorption of the intact form seemed to be negligible.^(62,63) In fact, Mulder *et al.*⁽³⁴⁾ revealed that the absorption of TFs in healthy volunteers resulted in plasma concentration of <1 ng/ml at 24 h after consumption of 700 mg of TFs (equivalent to ca. 30 cups of black tea). Thus, firstly, transport ability of TF3'G (one of the TFs) across Caco-2 cell monolayers was demonstrated by LC-ESI-TOF-MS. As shown in Fig. 2-2, no significant transport of TFs (1 mM) across Caco-2 cell monolayer was observed during 60 min-transport experiment, allowing to consider TFs as less absorbable polyphenols.

Four TFs (TF, TF3G, TF3'G and TFdG) were evaluated to determine whether they affect the transport of Gly-Sar, a model substrate of PEPT1 transporter, across Caco-2 cell monolayers. Cells that were pretreated individually with 20 μ M TFs for 24 h before the transport experiments of Gly-Sar were used. As shown in Fig. 2-3, the P_{app} of Gly-Sar in unpretreated Caco-2 cells (control: $7.4 \pm 0.5 \times 10^{-6}$ cm/s) was significantly reduced by TF pretreatment in the descending order of TF3'G ($1.8 \pm 0.3 \times 10^{-6}$ cm/s) > TF3G ($2.7 \pm 0.01 \times 10^{-6}$ cm/s) > TFdG ($4.3 \pm 0.7 \times 10^{-6}$ cm/s) > TF ($5.1 \pm 0.5 \times 10^{-6}$ cm/s). During the TFs, in particular TF3'G showed the most potent inhibition effect of Gly-Sar uptake compared to

untreated group (almost 80%). TF3'G was, thus, used throughout the experiments in Chapter II.

To determine the cytotoxicity of TF3'G in Caco-2 cells, Caco-2 cells treated with TF3'G from 5 μ M to 50 μ M for 24 h were evaluated by WST-8. As a result, TF3'G treatment did not show any toxicity to the cells at all concentrations (Fig. 2-4, > 95% of living cells after the 24 h-treatment).

Under our experimental conditions, the mono-galloyl group attached to TF skeleton was responsible for TF-induced retardation of peptide transport. The decrease in Gly-Sar uptake in 20 μ M TF3'G-pretreated Caco-2 cells during the 60 min-transporting experiment (Fig. 2-5) and the significant decrease in P_{app} for > 6 h-pretreatment (Fig. 2-6) suggested that any sustainable modification or suppression of the peptide transport signaling system(s) by TF3'G in Caco-2 cells was completed within 6 h-pretreatment time. In addition, concentration (5, 10 and 20 μ M TF3'G)-independent reduction in the P_{app} for Gly-Sar (Fig. 2-6) revealed that TF3'G had a potent modulator at 5 μ M level for the peptide transport systems in Caco-2 cells, to which TF3'G and Gly-Sar showed no cell-toxicity as afore-mentioned. However, further experiments on Caco-2 transport of peptides by TFs were performed at 20 μ M for 24 h-pretreatment time in Caco-2 cells to make clear the mechanism(s) of TF-induced reduction of peptide transports.

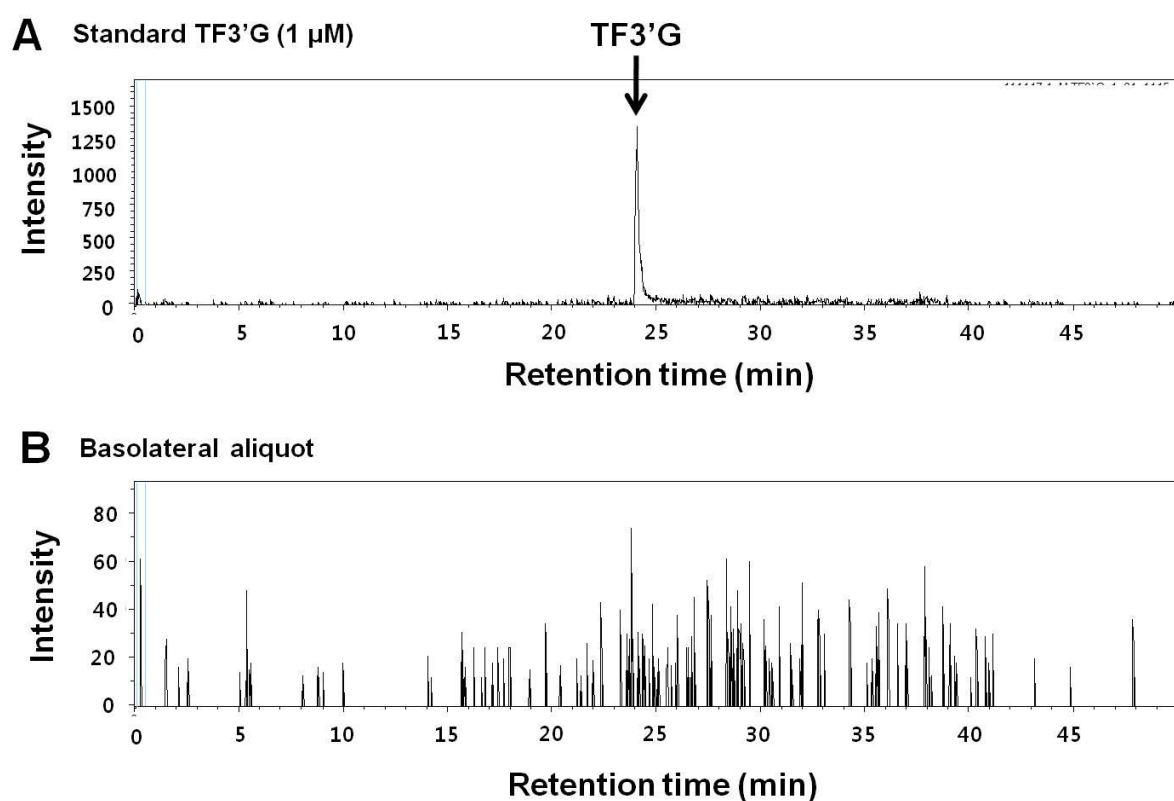


Fig. 2-2. LC-ESI-TOF-MS chromatograms

(A) standard TF3'G (1 μ M) and (B) basolateral solution obtained after the 60 min-transport experiment of TF3'G (1 μ M) in Caco-2 cell monolayers. No peak corresponding to the retention time of standard TF3'G was observed in the basolateral solution. The conditions of LC-ESI-TOF-MS are described in the section of 2.4. in Chapter II.

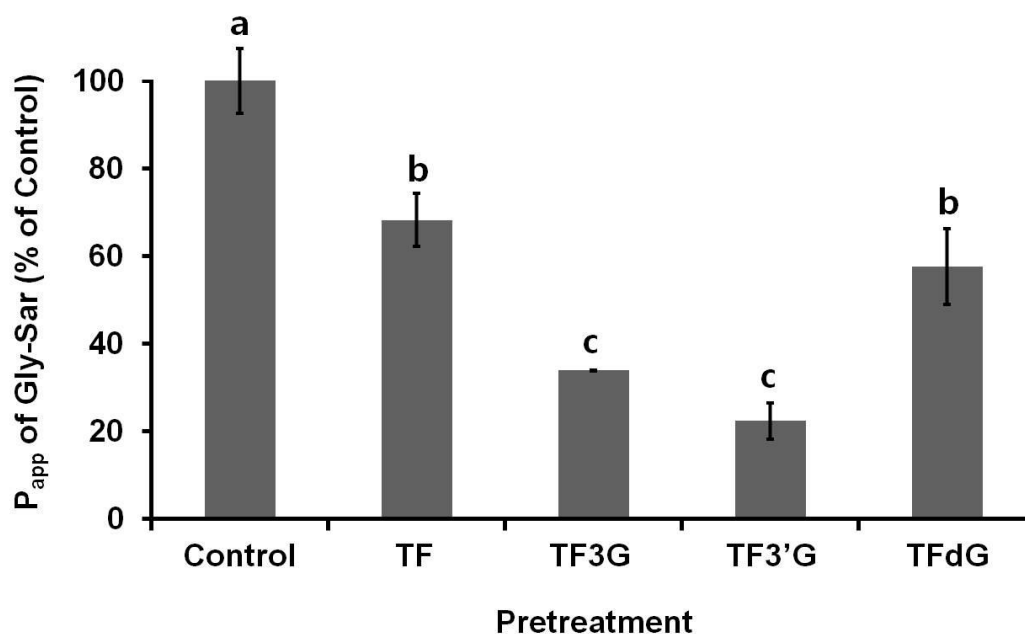


Fig. 2-3. Effects of theaflavins on P_{app} values of Gly-Sar transport in Caco-2 cells

Transport experiments of 20 μ M Gly-Sar were performed in Caco-2 cells pretreated with 20 μ M theaflavins for 24 h at 37°C. The theaflavins used for this experiment were theaflavin (TF), theaflavin-3-*O*-gallate (TF3G), theaflavin-3'-*O*-gallate (TF3'G) and theaflavin-3,3'-*O*-digallate (TFdG). Values are expressed as the mean $\pm$ SEM ($n = 3 - 5$). Means without a common letter are significantly different; $P < 0.05$. Significant differences between each group were evaluated by Tukey–Kramer's *t*-test.

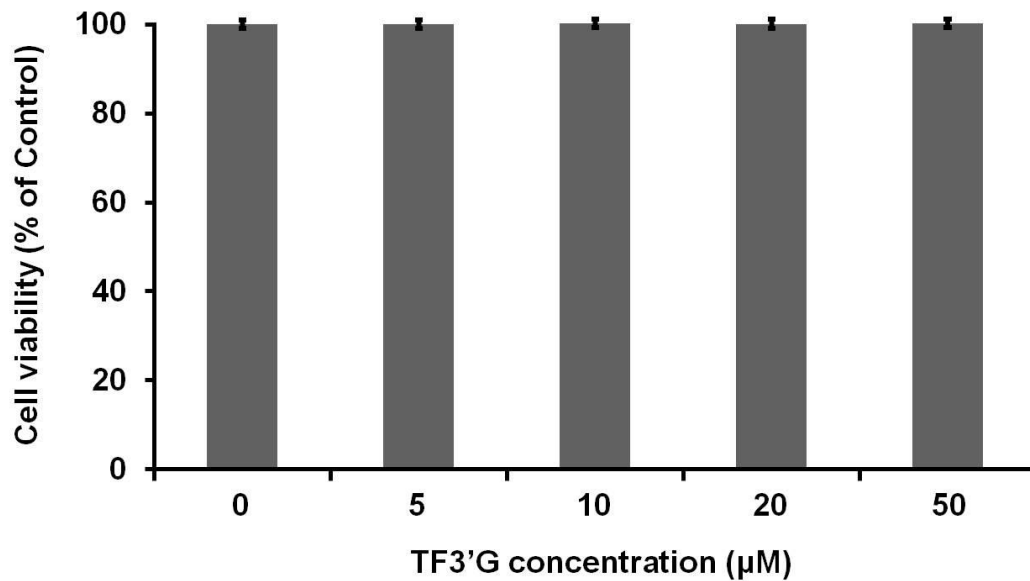


Fig. 2-4. Viability of Caco-2 cells treated with TF3'G

Viability of Caco-2 cells were determined by WST-8 assay after pretreatment with TF3'G at 5, 10, 20 and 50 μM for 24 h at 37°C. Values are expressed as the mean $\pm$ SEM (n=10). There was no significant difference between groups at $P = 0.05$, indicating that TF treatment did not show any toxicity effect to the cells.

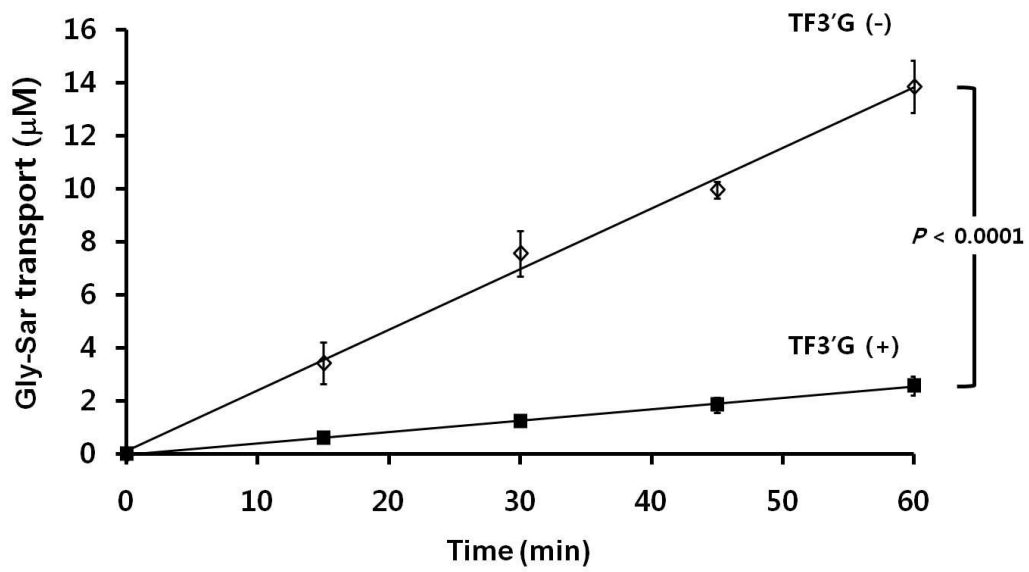


Fig. 2-5. Effect of TF3'G on Gly-Sar transport

Transport experiments of Gly-Sar were performed at 10 mM in Caco-2 cells pretreated with or without 20 μM TF3'G for 24 h at 37°C. Values are expressed as the mean ± SEM (n=3 - 5). Significant differences between TF3'G (+) and TF3'G (-) groups were evaluated by two-way ANOVA.

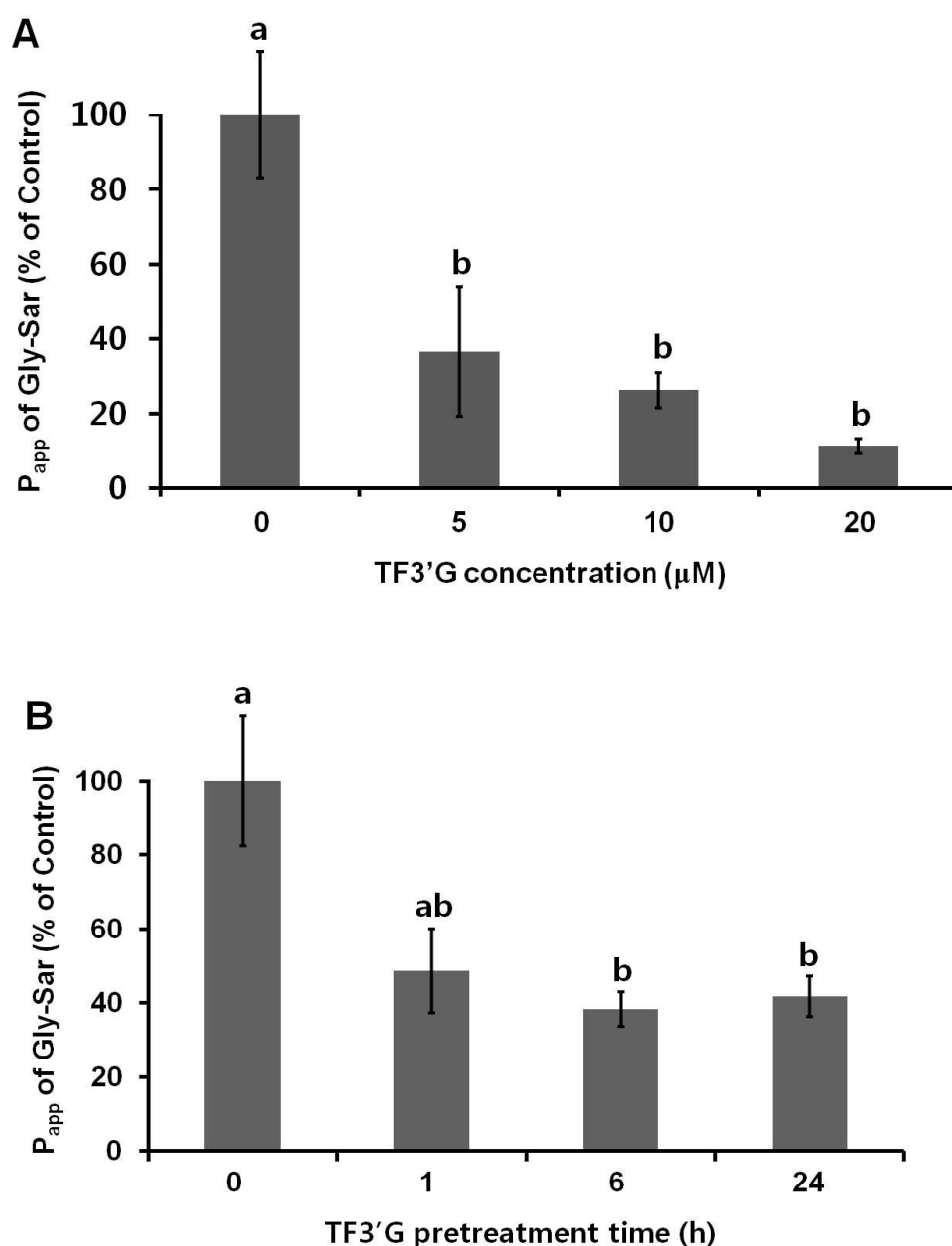


Fig. 2-6. Effect of TF3'G on Gly-Sar transport

In experiments for concentration dependency of TF3'G on 10 mM Gly-Sar transport (A), Caco-2 cells were pretreated with TF3'G at concentrations of 5, 10 and 20 μM for 24 h at 37°C. In experiments for pretreatment time of TF3'G on Gly-Sar transport (B), Caco-2 cells were pretreated with 20 μM TF3'G for 1, 6 or 24 h at 37°C. Values are expressed as the mean ± SEM (n=3). Means without a common letter are significantly different; $P < 0.05$. Significant differences between each group were evaluated by Tukey-Kramer's *t*-test.

3.2. Effect of theaflavins on di-peptide transport across Caco-2 cell monolayers

The *in vivo* evidences that the intake of small peptides prevented the promotion of hypertension in mild hypertensive subjects ⁽⁴²⁾ and that long-term administration of di-peptide ameliorated the onset of atherosclerosis in apo-E deficient mice ⁽⁴⁵⁾ also supported the importance of peptide intake in maintaining homeostasis, although highly selective and sensitive analytical assays for absorbed peptides are still required to fully understand their absorption and distribution behaviors.⁽⁶⁴⁾

Table 2-1 summarizes the P_{app} values of Val-Tyr and Ile-Phe in Caco-2 cells with or without 20 μ M TF3'G pretreatment. Both di-peptides used in this study have already been clarified as penetrant.^(56,57) As shown in Table 2-1, the magnitudes of P_{app} reduction in TF3'G-pretreated Caco-2 cells were 31 and 49%, respectively, for Ile-Phe and Val-Tyr, similar to that (43%) of Gly-Sar. This indicated that TF3'G (or more likely TFs) may have a uniform potential to regulate the peptide-related transport (signaling) system in Caco-2 cells. In addition, no peaks corresponding to ions of hydrolyzed residues (Sar from Gly-Sar and Val from Val-Tyr) were observed in basolateral solution after 60 min-transport experiments of Gly-Sar (10 mM) or Val-Tyr (10 mM) using LC-ESI-TOF-MS (Fig. 2-7). This suggested that the di-peptides could transport without hydrolytic degradation during the 60 min-transport experiments.

Di-peptide	$P_{app} \times 10^{-6}$ (cm/s)	
	TF3'G (-)	TF3'G (+)
Gly-Sar	21.8 ± 1.8	$12.6 \pm 1.9^{**}$
Val-Tyr	6.8 ± 0.7	$3.5 \pm 0.3^{**}$
Ile-Phe	2.9 ± 0.2	$2.0 \pm 0.2^*$

Table 2-1. The apparent permeability coefficients (P_{app}) of di-peptides across Caco-2 cell monolayers in the presence or absence of 20 μ M TF3'G

Significant differences between the TF3'G (+) and TF3'G (-) groups were evaluated by unpaired Student's *t*-test. Data are expressed as the means $\pm$ SEM (n = 3 - 4). * $P < 0.05$, ** $P < 0.01$; significantly different from untreated cells (TF3'G(-)).

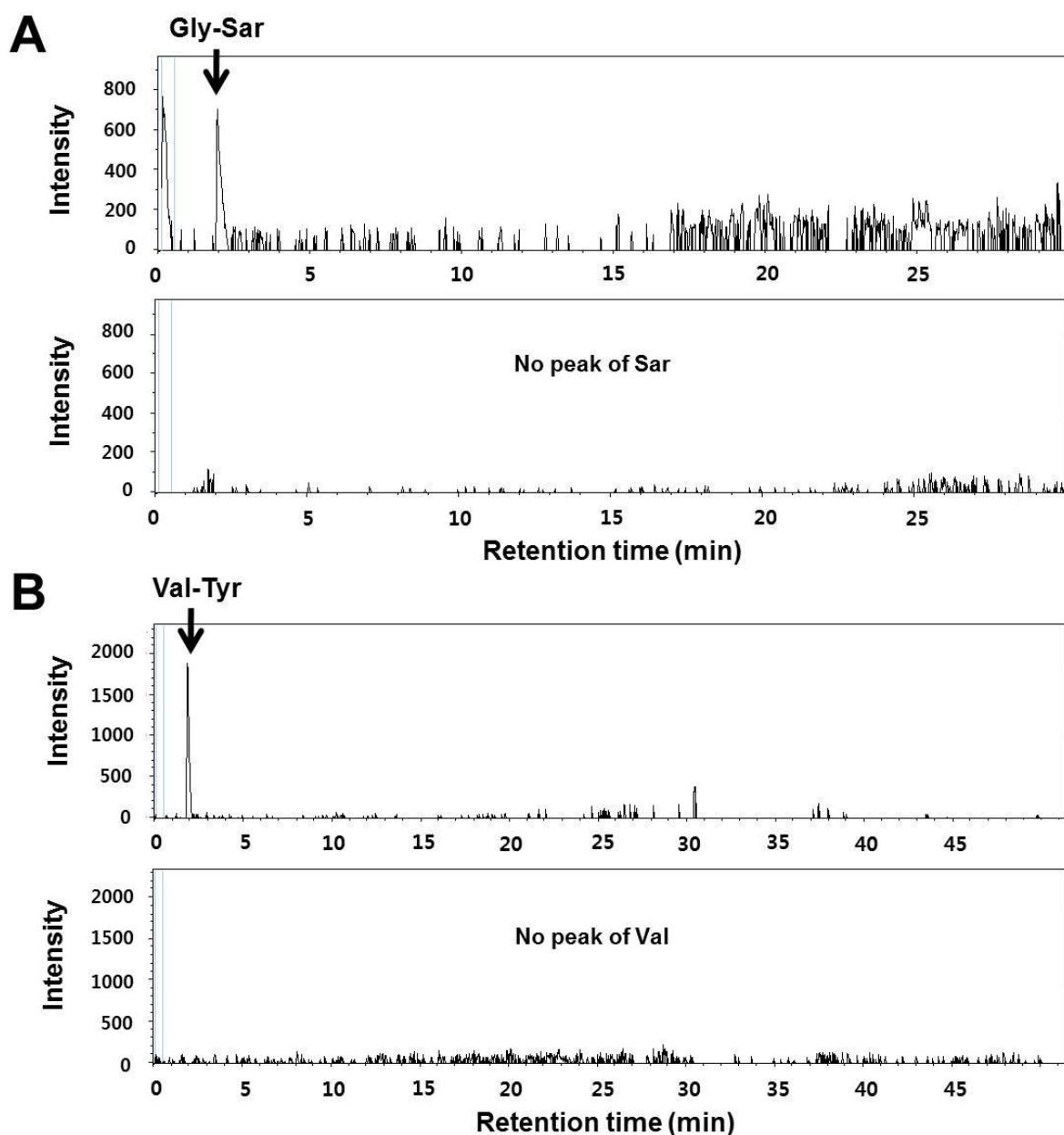
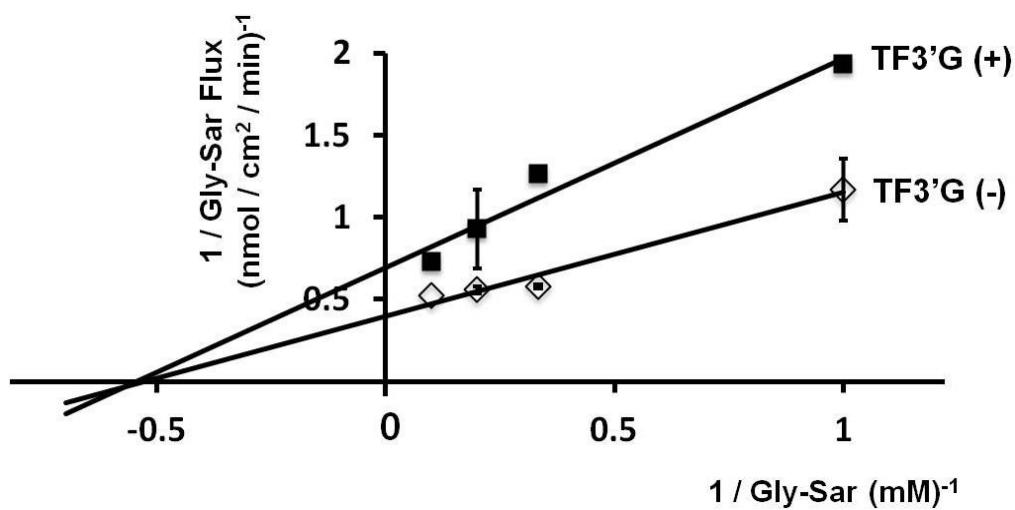


Fig. 2-7. LC-ESI-TOF-MS chromatograms of 10 mM Gly-Sar (A) and 10 mM Val-Tyr (B) in basolateral solution from 60 min-transport experiments

No peaks corresponding to Sar from Gly-Sar and Val from Val-Tyr were observed in basolateral solution. LC-ESI-TOF-MS conditions are described in the section of 2.4. in Chapter II.

3.3. Lineweaver-Burk plots of Gly-Sar transport in Caco-2 cell monolayers

The Lineweaver-Burk plot was performed to determine whether the reduction in P_{app} for Gly-Sar in TF-pretreated Caco-2 cell monolayers was caused by the decrease in PEPT1 expression or affinity. As shown in Fig. 2-8, 20 μ M TF3'G-pretreated cells resulted in an apparent reduction in Gly-Sar flux at concentrations ranging from 1 to 10 mM with no change in K_m (2.0 mM), indicating that TF3'G may inhibit the expression of PEPT1 proteins. Therefore, RT-PCR and Western blot of analysis of PEPT1 were then performed in TF3'G-pretreated Caco-2 cells.



	K_m (mM)	V_{max} (nmol/cm ² /min)
TF3'G (-)	2.0 ± 0.2	2.5 ± 0.1
TF3'G (+)	2.1 ± 0.2	$1.0 \pm 0.04^{**}$

Fig. 2-8. The Lineweaver-Burk plots of Gly-Sar transport

The Lineweaver–Burk plots of Gly-Sar transport were performed in Caco-2 cell monolayers pretreated or untreated with 20 μ M TF3'G. Gly-Sar flux was measured at various concentrations (1, 3, 5, 10 mM) for 1 h at 37°C. Caco-2 cells were pretreated with or without 20 μ M TF3'G for 24 h. Values are expressed as the mean $\pm$ SEM (n=3). ** $P < 0.01$ vs. TF3'G (-) by unpaired Student's t -test.

3.4. Effect of theaflavins on expression of PEPT1 in Caco-2 cells

As a result of accumulating evidence that a proton-coupled peptide transporter, PEPT1, is expressed in rabbit and human intestines, ^(47,50) small peptides or peptidic drugs having di- and tri-peptide lengths have been recognized as potential penetrants for intestinal absorption.⁽⁶⁴⁾

The expression of PEPT1 protein in Caco-2 cells pretreated with or without 20 μ M TF3'G for 24 h was determined by Western blot analysis. As shown in Fig. 2-10, the TF3'G-pretreated cells significantly ($P < 0.05$) decreased the expression of PEPT1 protein compared to control cells, as well as the transcription ($P < 0.01$, Fig. 2-9).

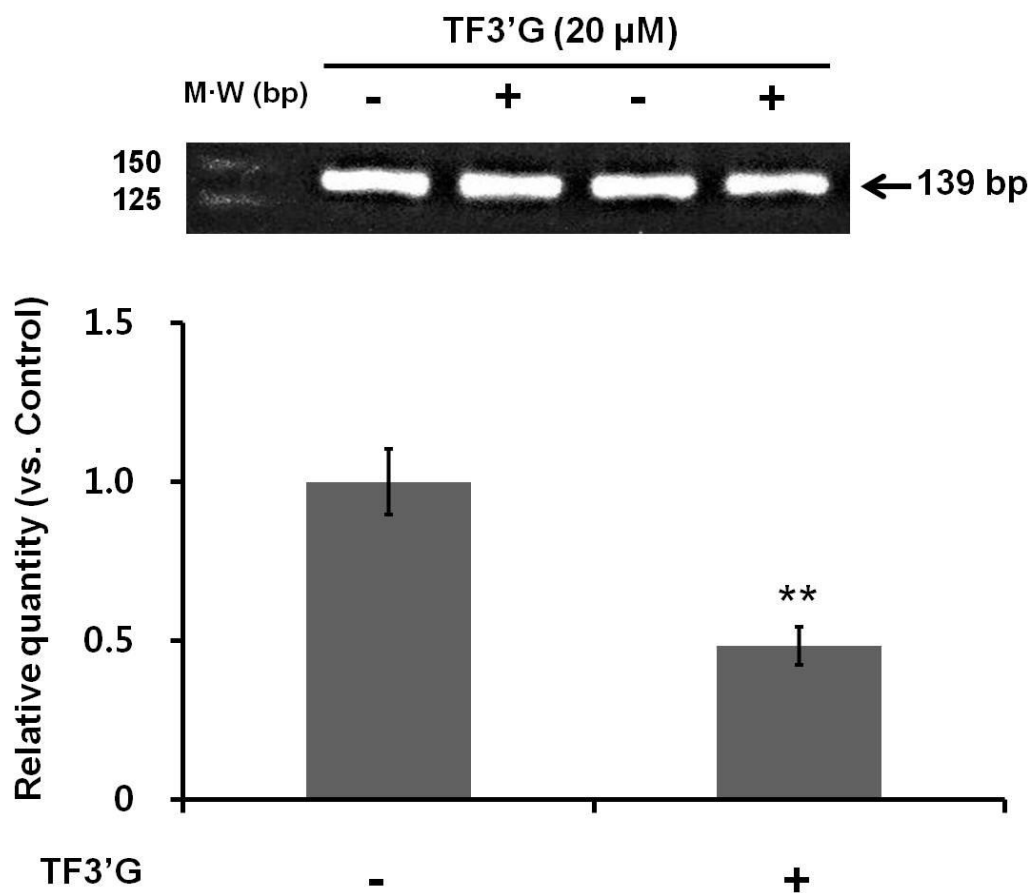


Fig. 2-9. Effect of TF3'G on transcription of PEPT1 gene in Caco-2 cells

Caco-2 cells were treated with or without 20 μ M TF3'G for 1 h at 37°C. Total RNA was isolated, and the expression level of mRNA was measured by RT-PCR using specific primers for PEPT1 gene. Values are expressed as the mean $\pm$ SEM (n=3). ** $P < 0.01$, significantly different from TF3'G (-) by unpaired Student's *t*-test.

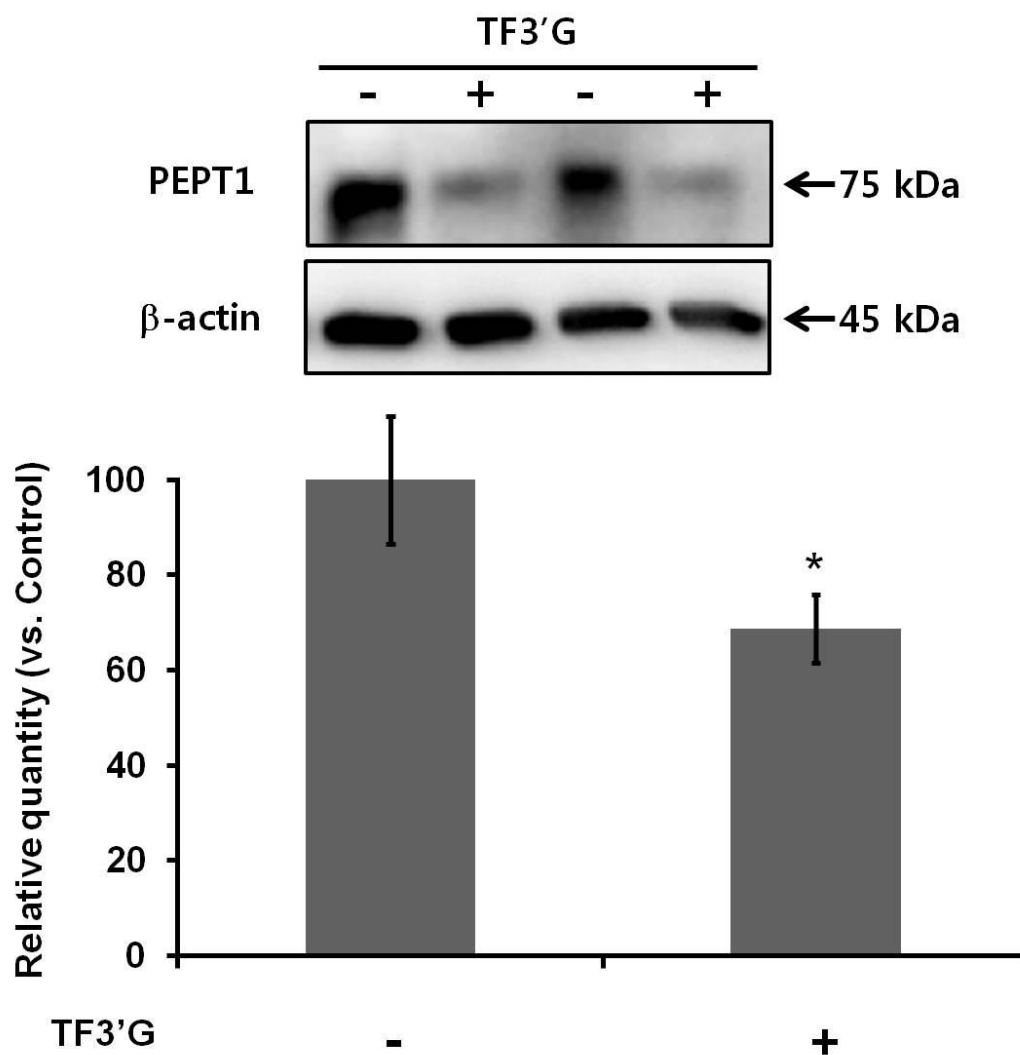


Fig. 2-10. Effect of TF3'G on the expression of PEPT1 protein in Caco-2 cells

Caco-2 cells were treated with or without 20 μ M TF3'G for 24 h at 37°C. Specific bands for PEPT1 and β -actin were quantitated by western blot analysis. Values are expressed as the mean $\pm$ SEM (n=3). * $P < 0.05$, significantly different from TF3'G (-) by unpaired Student's *t*-test.

3.5. PKC and AMPK on reduced Gly-Sar uptake in TF3'G-pretreated Caco-2 cells

Brandsch *et al.* ⁽⁶⁴⁾ reported that phorbol esters, activators of PKCs, inhibited Gly-Sar transport in Caco-2 cells, suggesting the involvement of PKCs in the intestinal peptide transport system. AMPK has also been reported to be diversely associated with transport signaling systems in Caco-2 cells.⁽⁶⁵⁻⁶⁷⁾ In fact, AMPK activation by 5-aminoimidazole-4-carboxiamide ribonucleoside in Caco-2 cells markedly inhibited apical [³H]-D-Phe-L-Gln uptake and caused a decrease in brush-border human PEPT1 protein expression.⁽⁶⁶⁾ Therefore, AMPK and PKC pathway were primarily investigated for clarifying the mechanism on reduced PEPT1 expression by TFs (Fig. 2-10).

Effects of GF109203X (10 μ M), a non-specific inhibitor of PKC and compound C (10 μ M), a specific inhibitor of AMPK, on Gly-Sar uptake were investigated in Caco-2 cells pretreated with 20 μ M TF3'G. Non-specific PKC inhibitor (GF109203X) was used for the study to clarify the involvement of all the PKC signaling pathways in the TF3'G-induced suppression of PEPT1 expression (Fig. 2-11), since Caco-2 cells expressed some PKC subtypes (i.e., PKC α , β , ϵ , ζ).⁽⁶⁸⁾ As shown in Fig. 2-11, no change in reduced P_{app} by TF3'G was observed in GF109203X-pretreated Caco-2 cells compared with that in untreated cells ($1.3 \pm 0.2 \times 10^{-6}$ cm/s), as well as no reduction of PKC activity in Caco-2 cells pretreated with 20 μ M TF3'G for 6 h; the PKC activity in pretreated Caco-2 cells showed 93% of the control (Fig. 2-12). Therefore, the PKC-mediated PEPT1 transporter expression-signaling pathways can be excluded from the mechanism(s) underlying the suppression of PEPT1 expression by TFs. In contrast, the result that the reduced P_{app} value of Gly-Sar by TFs was significantly recovered to control level by an AMPK inhibitor (compound C) (Fig. 2-13), indicated that AMPK activation during the pretreatment of Caco-2 cells with TFs could play a

key role in the reduced peptide transport.

These findings demonstrated that TF3'G was a potent natural modulator of peptide transport probably through the suppression of AMPK-mediated PEPT1 transporter expression-signaling pathways, but not PKC pathways.

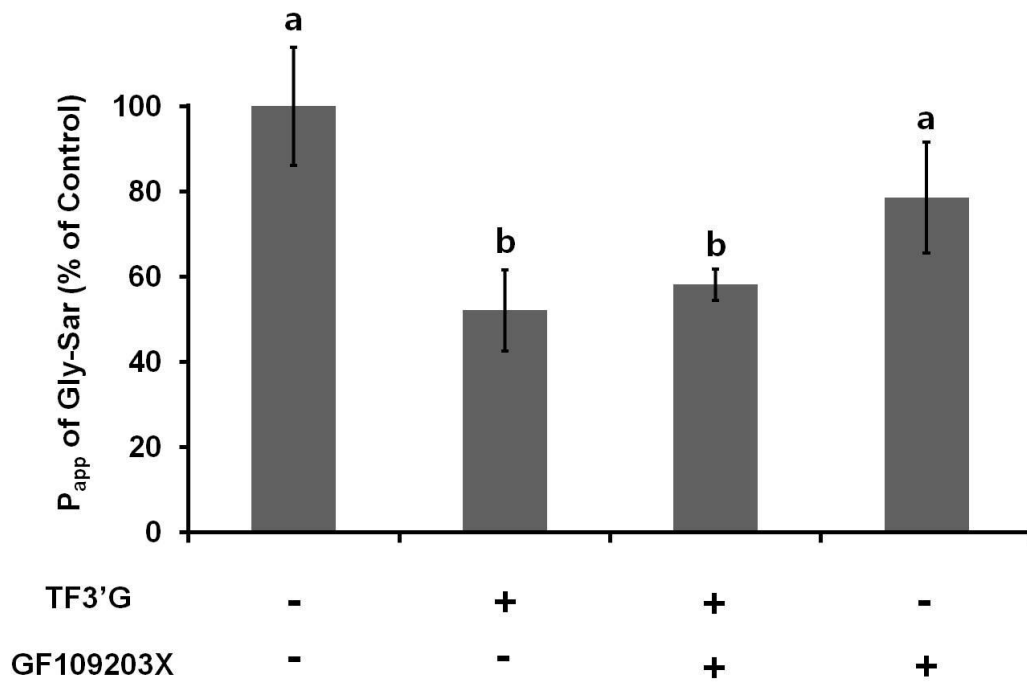


Fig. 2-11. Effect of PKC inhibition on Gly-Sar transport

Caco-2 cells for Gly-Sar transport were treated with or without 20 μ M TF3'G in the presence or absence of 10 μ M GF109203X (PKC inhibitor) for 24 h at 37°C. Values are expressed as the mean $\pm$ SEM (n=3 - 5). Means without a common letter are significantly different; $P < 0.05$. Significant differences between each group were evaluated by Tukey–Kramer's t -test.

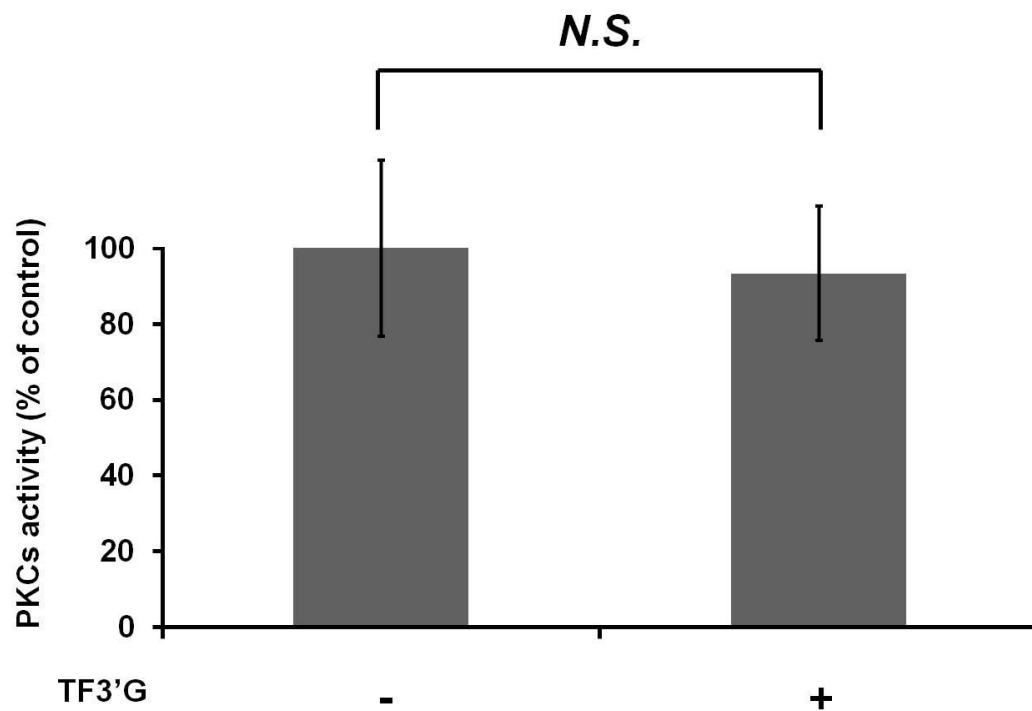


Fig. 2-12. Effect of TF3'G on PKC activity in Caco-2 cells

Caco-2 cells were treated with or without 20 μ M TF3'G for 6 h at 37°C. Values are expressed as the mean $\pm$ SEM (n=3). There was no significant difference between groups at $P = 0.05$, indicating that TFs treatment did not affect any PKC activity in Caco-2 cells; *N.S.*: not significant.

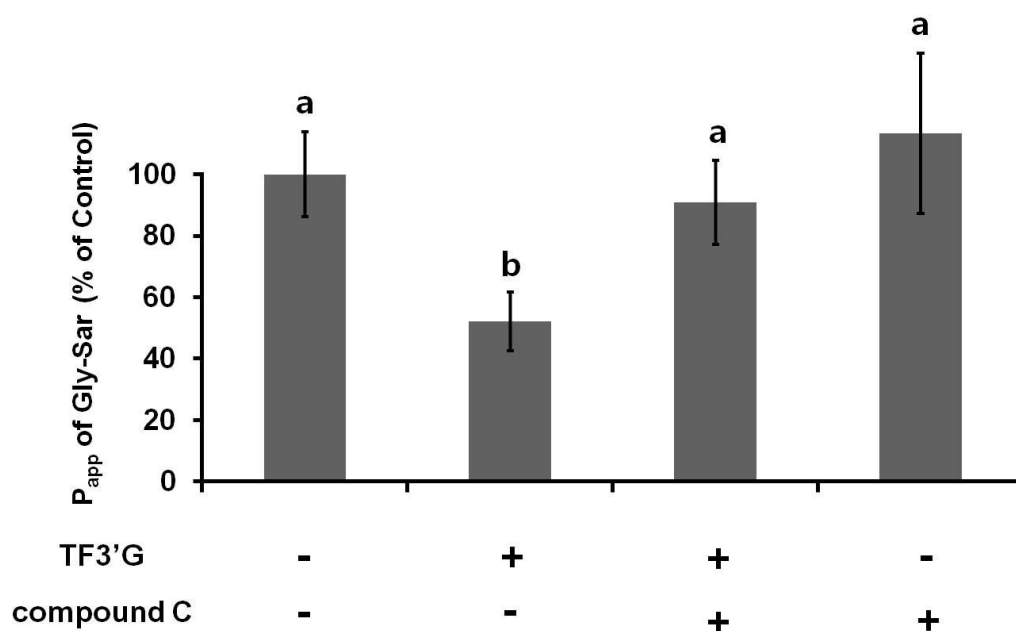


Fig. 2-13. Effect of AMPK inhibition on Gly-Sar transport

Caco-2 cells for Gly-Sar transport were treated with or without 20 μ M TF3'G in the presence or absence of 10 μ M compound C (AMPK inhibitor) for 24 h at 37°C. Values are expressed as the mean $\pm$ SEM (n=3 - 5). Means without a common letter are significantly different; $P < 0.05$. Significant differences between each group were evaluated by Tukey–Kramer's t -test.

4. Summary

In the small intestine, PEPT1 plays a role in the transport of di- and tri-peptides. Chapter II examined whether TFs could affect the absorption of small peptides in human intestinal Caco-2 cells, since TFs were far from the penetrant through the cells and might be involved in intestinal transport systems. In this study, it was found for the first time that TFs (TF, TF3G, TF3'G and TFdG), in particular TF3'G, inhibited the transport of small peptides (Gly-Sar, Val-Tyr and Ile-Phe) across Caco-2 cell monolayers, regardless of TF structure. In addition, mechanism underlying the TF-induced inhibition of peptide transport is likely to be through the activation of AMPK to down-regulate cellular PEPT1 transporter expression (Fig. 2-14). However, relationship between peptide transport and TF structure remains unknown. Furthermore, it still remains unclear how TFs activate AMPK or are involved in the triggering of up-stream signaling pathways including CaMKK. Further studies were, thus, attempted to clarify the underlying mechanisms responsible for TF-induced inhibition of peptide transport in the next Chapter III.

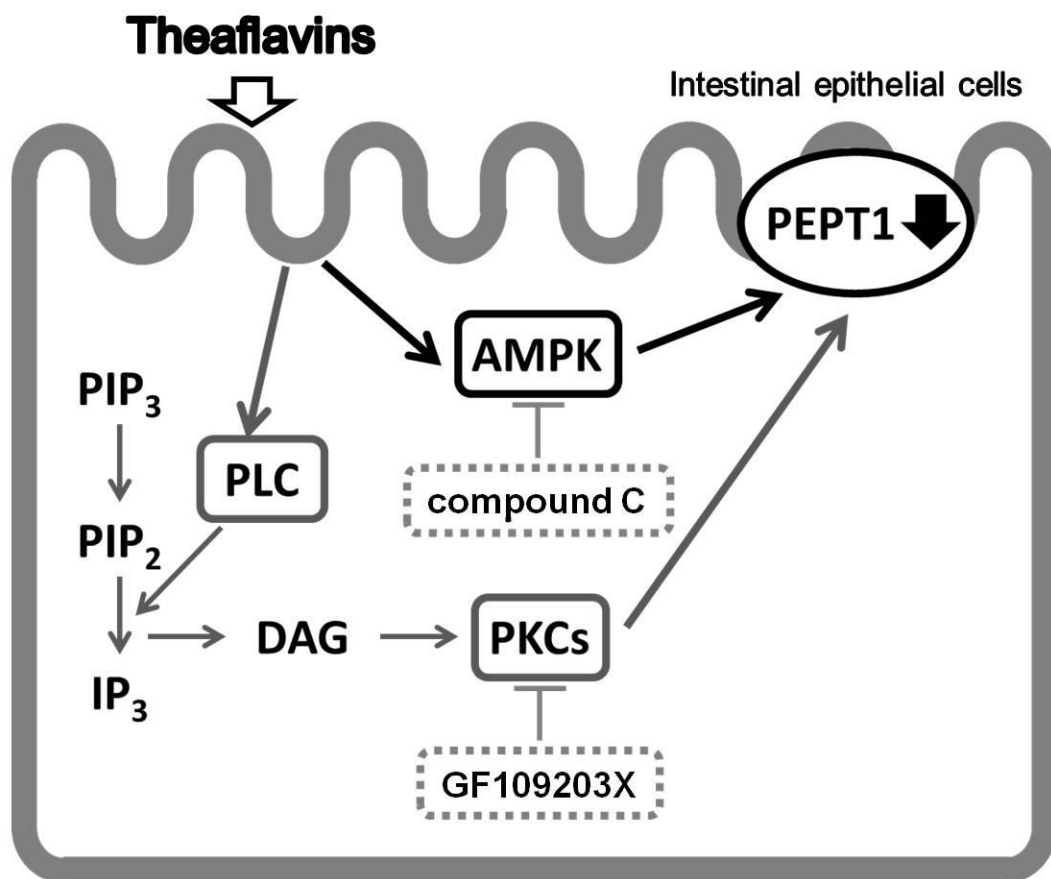


Fig. 2-14. Proposed mechanism(s) of theaflavins on signaling pathways in Caco-2 cells
 AMP-activated protein kinase (AMPK), diacylglycerol (DAG), inositol (1,4,5)-trisphosphate (IP₃), peptide transporter 1 (PEPT1), phosphatidylinositol (4,5)-biphosphate (PIP₂), phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), protein kinase C (PKC), phospholipase C (PLC)

Chapter III

Structural requirement of theaflavins and their signal regulation for reduced peptide transport in Caco-2 cells

1. Introduction

In intestinal membranes it has been reported that the expression of PEPT1 was closely associated with AMPK activation. Pieri *et al.* ⁽⁶⁶⁾ demonstrated that the activated AMPK resulted in a decrease of peptide absorption, along with the suppression of expression of PEPT1 proteins in Caco-2 cells. Chapter II also demonstrated that the transport of dipeptides (Gly-Sar, Val-Tyr and Ile-Phe) across Caco-2 monolayers was markedly inhibited by TF-induced AMPK activation. So far, although it has been reported that catechins could stimulate AMPK activity in the liver, ⁽⁶⁹⁾ no study was found for AMPK activation by TFs in the intestine. In particular, the study demonstrated non-absorbable TFs had potency in inhibiting intestinal peptide transporter, but relationship between peptide transport and TF structure remains unclear. Thus, the investigation on the underlying mechanisms responsible for peptide transport inhibition by chemically modified TFs was performed in this Chapter III.

In addition, investigation about involvement(s) of up-stream kinases on the TFs-

induced AMPK activation such as LKB1 and CaMKK is still required.

In Chapter III, we, thus, investigated the structural requirement of TFs for the inhibition of intestinal peptide-transport in Caco-2 cells, and clarified whether LKB1/ or CaMKK/AMPK was involved in TF-induced AMPK activation. All of the experiments throughout Chapter III were performed using TF3'G, since it exerted the most strongest inhibition on peptide-transport among TFs as shown in the section 3.1 in Chapter II.

2. Materials and Methods

2.1. Reagents

DMEM and FBS were purchased from GIBCO Life Technologies (Grand Island, NY, USA). DMSO was obtained from Sigma-Aldrich (St. Louis, MO, USA). Seeding basal medium, enterocyte differentiation medium and MITO+™ serum extender for Caco-2 cell culture were purchased from BD Biosciences (Franklin Lakes, NJ, USA). (-)-Epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin (EC) and TF3'G were products of Nacalai Tesque Co. (Kyoto, Japan). Purpurogallin having benzotropolone ring was purchased from Pfaltz & Bauer Inc. (Waterbury, CT, USA). Glycyl-sarcosine (Gly-Sar, a model peptide for PEPT1 transport⁽⁵⁵⁾) was obtained from Kokusan Chemical Co. (Tokyo, Japan). NDA, compound C and STO-609 were purchased from Nacalai Tesque. All other reagents were of analytical grade and used without further purification.

2.2. Cell culture

Caco-2 cells were cultured under standard cell culture conditions as described in Chapter II. Caco-2 cells used in this study were between passages 50 and 60. For transport studies, cells were grown on a BD Falcon™ cell culture insert (PET membrane, 0.9 cm², 1.0 µm pore size; BD Biosciences, Bedford, MA, USA) coated with type I collagen (collagen gel culturing kit, Cellmatrix type I-A, Nitta Gelatin, Osaka, Japan). The cells were seeded at a density of 4.0×10^5 cells/ml and cultured in a seeding basal medium containing MITO+™

serum extender for 48 h. After the medium was replaced with an enterocyte differentiation medium containing MITO+™ serum extender, cells were incubated for another 72 h to form monolayers. The medium was freshly changed every 24 h. The integrity of Caco-2 monolayers was evaluated by measuring the TEER with a multi-channel EVC-4000 system (World Precision Instruments, FL, USA). Monolayers with TEER values of $>100 \Omega \cdot \text{cm}^2$ were used for peptide-transport experiments.

2.3. Cell treatment

Treatment of Caco-2 cell monolayers with sample (TF3'G or its related compounds) were performed by replacing 10% FBS in DMEM with the medium containing 20 μM sample and allowing them to incubate for 24 h at 37°C at O₂:CO₂ (95:5).

For inhibition experiments, Caco-2 cells were pretreated with or without 20 μM TF3'G in the presence or absence of 25 μM STO-609 as CaMKK inhibitor. Cells incubated in sample-free medium were used as a control.

The medium containing the sample was prepared by diluting a stock solution of each sample, dissolved in DMSO, with 10% FBS in DMEM. The final concentration of DMSO was 0.5% throughout the experiments to avoid any cell damage by DMSO.⁽⁵⁹⁾

2.4. Transport study

Transport experiments in Caco-2 cell monolayers were performed using an Ussing Chamber system (Model U-2500; Warner Instrument Co., Hamden, CT, USA). Caco-2 cell monolayers grown in transwell inserts were gently rinsed with HBSS buffer (pH 6.0) to wash

or remove any free samples in the medium, and carefully mounted in the Ussing Chamber using blunt forceps. An aliquot (7.5 ml) of HBSS buffer (adjusted to pH 6.0 with 10 mM MES [2-(*N*-morpholino) ethanesulfonic acid]) or HBSS buffer (adjusted to pH 7.4 with 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid]) was added to the apical or basolateral side, respectively. After equilibration for 15 min at 37°C, the transport experiments were started by replacing the apical buffer with fresh HBSS buffer (pH 6.0) containing 10 mM Gly-Sar for Caco-2 cells treated with or without 20 µM sample. During the experiments, solutions on both sides were bubbled continuously with a mixture of O₂:CO₂ (95:5). Aliquots (0.2 ml each) of solution were drawn from the basolateral side at time intervals of 15, 30, 45 and 60 min to determine the amount of transported Gly-Sar. The same volume of fresh buffer was added to the basolateral side at each time point. The membrane barrier and transport ability of cells were maintained during the 60 min-transport experiments without any reduction of TEER value ($>100 \Omega \cdot \text{cm}^2$) by Gly-Sar.

The amount of Gly-Sar transported to the basolateral side was determined by a fluorescent NDA-derivatization method.⁽⁶⁰⁾ Briefly, 10 µl of 20 mM sodium cyanide in borate buffer (pH 9.5) was added to the basolateral solution taken at each time point (100 µl) in a 96-well microplate, followed by the addition of 50 µl of 0.1 mM NDA solution in methanol. After incubating at 25°C for 60 min, the fluorescence intensity of NDA-Gly-Sar was measured using a fluorescence spectrophotometer (Wallac ARVOTMSX 1420 Multilabel Counter, Perkin-Elmer Life Sciences, Tokyo, Japan) at an excitation wavelength of 405 nm and emission wavelength of 460 nm.

In addition, the fluorescence intensity of basolateral solution taken at each time point for blank (no Gly-Sar) transport experiments was used to cancel an increase in intensity by any contaminants from Caco-2 cells.

The apparent permeability coefficient (P_{app}) was calculated from the following equation:

$$P_{app} \text{ (cm/s)} = \frac{V}{AC_0} \frac{dC}{dt}$$

dC/dt is the change in concentration at the basolateral side over time (mM/s), V is the volume of solution in the basolateral compartment (7.5 ml), A is the surface area of the membrane (0.2826 cm²) and C_0 is the initial concentration at the apical side (mM). The effect of sample (TF3'G or its related compounds) on P_{app} was expressed relative to control (%).

2.5. Western blot analysis

Expression of PEPT1 protein in Caco-2 cells treated with or without TF3'G (20 μ M) in the presence or absence of inhibitor (10 μ M compound C as AMPK inhibitor or 25 μ M STO-609 as CaMKK inhibitor) for 24 h at 37°C was evaluated by Western blot analysis. Briefly, protein (1.3 mg/ml) extracted from Caco-2 cells using radioimmunoprecipitation assay buffer was mixed with an equal volume of sample buffer (20% glycerol, 4% sodium dodecylsulfate, 3% dithiothreitol, 0.002% bromophenol blue and 0.125 M Tris-HCl, pH 6.8) and boiling in water for 5 min. An aliquot (9.75 μ g) of the prepared sample was applied to 10% SDS-PAGE for 2 h at 20 mA and transferred onto PVDF membrane (Hybond-P, GE Healthcare, Piscataway, NJ, USA) for 1.5 h at 40 mA. The membrane was blocked with 5% non-fat dried milk in TBS-T for 1 h at room temperature and then probed with the rabbit polyclonal antibody against PEPT1 (1:1000, Abbiotec, San Diego, CA, USA) or the mouse monoclonal antibody against β -actin (1:1000, Applied Biological Materials Inc, Richmond, BC, Canada) overnight at 4°C. The membrane was then reprobed with HRP-conjugated donkey anti-rabbit IgG antibody or HRP-conjugated sheep anti-mouse IgG antibody (1:1000,

GE Healthcare, Buckinghamshire, UK) for 1 h at room temperature. The membrane was detected with ECL plus detection reagent using an ImageQuant LAS 4000 (GE Healthcare, Piscataway, NY, USA). Quantitation of PEPT1 and β -actin were performed with ImageQuant TL 7.0 software (GE Healthcare), and the PEPT1 protein levels in Caco-2 cells treated with TF3'G or TF3'G in the presence or absence of inhibitor were expressed relative to the control (or TF3'G-untreated cells).

2.6. Statistical analysis

Results are expressed as the mean $\pm$ SEM. The statistical significance between groups was analysed using one-way ANOVA followed by Tukey-Kramer's *t*-test for *post hoc* analysis. $P < 0.05$ was considered to be significant. Statistical analyses were conducted with StatView J5.0 (SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

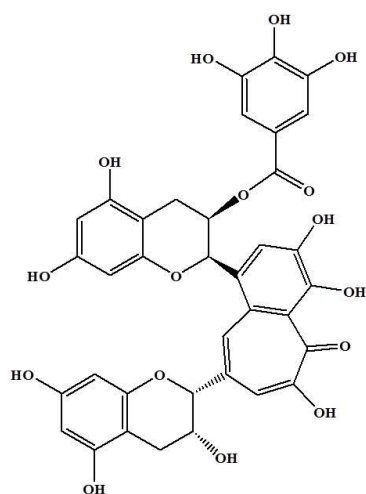
3.1. Effect of theaflavins and its analogues on Gly-Sar transport across Caco-2 cell monolayers

In the intestinal absorption system, a new physiological function of TFs that inhibited the transport of small peptides across Caco-2 cell monolayers, regardless of TF structures, was obtained in the Chapter II. Although the relationship between peptide transport and TF structure is unknown, the TF skeleton bearing a benzotropolone ring may play a crucial role in the inhibition of peptide transport in Caco-2 cells.

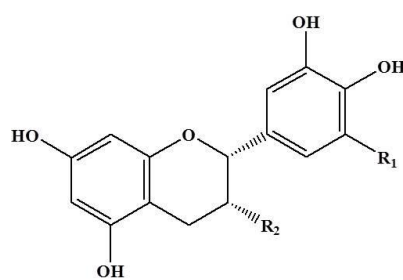
Therefore, in order to get information on structural requirement of TFs exerting peptide-transport inhibition, Gly-Sar transport experiments across Caco-2 monolayers were performed using compounds related to TF3'G skeleton, i.e., catechins and purpurogallin (Fig. 3-1).

As shown in Fig 3-2, four catechin monomers did not show any significant P_{app} reduction in Caco-2 cells, while the P_{app} of Gly-Sar was significantly ($P < 0.05$) reduced in 20 μ M purpurogallin-treated cells, as similar to TF3'G-treated cells (TF3'G, 41%; purpurogallin, 30% of control in P_{app}). This clearly demonstrated that the benzotropolone moiety in TFs, which has a characteristic 7-membered ring structure,⁽⁷⁰⁾ was a crucial structural requirement for exerting the inhibition of intestinal peptide-transport by TFs in Caco-2 cells.

Theaflavin-3'-O-gallate (TF3'G)



Catechins



	R ₁	R ₂
EC	H	H
EGC	OH	H
ECG	H	galloyl
EGCG	OH	galloyl

Purpurogallin

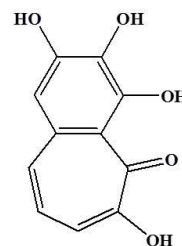


Fig. 3-1. Chemical structures of theaflavin-3'-O-gallate (TF3'G), catechins and purpurogallin

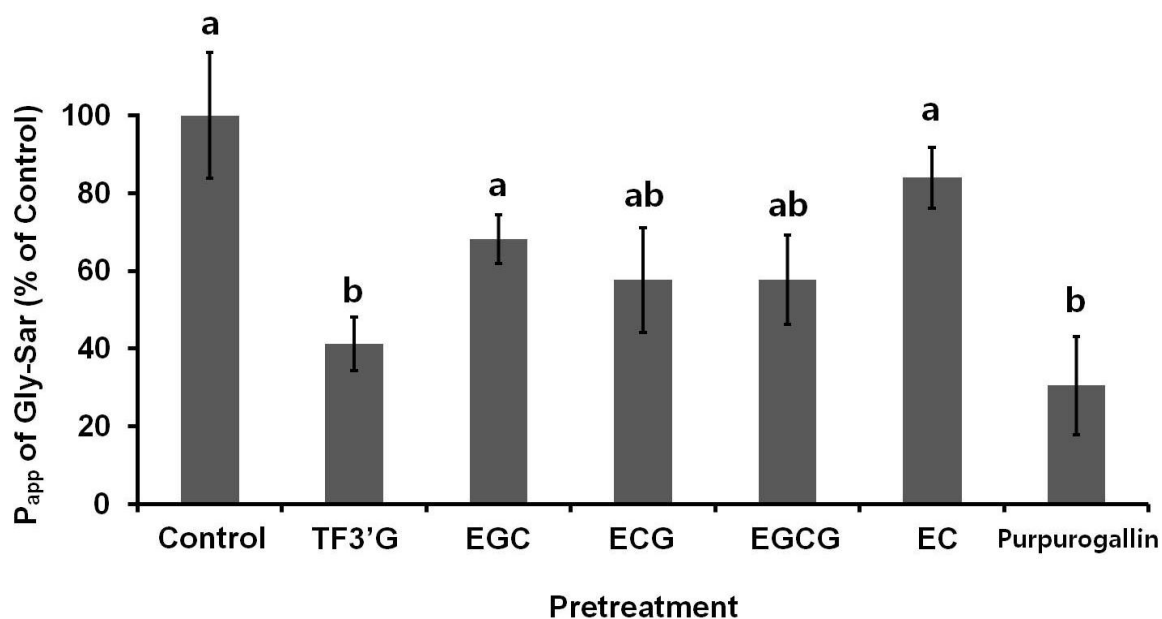


Fig. 3-2. Effect of TF3'G and its related compounds on Gly-Sar transport in Caco-2 cells

Caco-2 cells were treated with 20 μ M test compound for 24 h at 37°C. Theaflavin-3'-O-gallate (TF3'G), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin (EC), purpurogallin were used for this study. Transport of Gly-Sar in Caco-2 cells without sample treatment was used as a control. The values (relative P_{app} value vs. control, %) are expressed as the mean $\pm$ SEM (n=3 - 5). Means without a common letter are significantly different; $P < 0.05$. Significant difference between groups was evaluated by Tukey–Kramer's *t*-test.

3.2. Effect of TF3'G on AMPK/PEPT1 signaling pathways in Caco-2 cells

Effect of compound C, a specific inhibitor of AMPK, on PEPT1 protein expression in Caco-2 cells treated with 20 μ M TF3'G was evaluated by Western blot analysis. As shown in Fig. 3-3, 20 μ M TF3'G treatment resulted in a significant reduction of PEPT1 expression in Caco-2 cells. A complete recovery of the expression by 10 μ M compound C in TF3'G-treated Caco-2 cells also revealed that the reduction of PEPT1 expression by TF3'G was responsible for AMPK activation, which was in good agreement with the transport results shown in Fig. 2-11, in which reduced transport of Gly-Sar in TF3'G-pretreated Caco-2 cells was abolished by compound C. These results clearly indicated that TF3'G (or TFs) can reduce peptide transport by the suppression of PEPT1 expression pathways mediated with activated AMPK.

There have been some reports on the potential of polyphenols in the activation of CaMKK/AMPK signaling pathway.⁽⁷⁰⁻⁷²⁾ Collins *et al.*⁽⁷³⁾ reported that EGCG reduced hepatic gluconeogenesis by activating CaMKK/AMPK pathway. Thus, firstly, CaMKK kinase is received attention among up-stream kinases of AMPK activation.

Effect of STO-609, a selective inhibitor of CaMKK, on Gly-Sar transport and PEPT1 protein expression in Caco-2 cells treated with 20 μ M TF3'G was evaluated by transport experiment and Western blot analysis, respectively. As a result, no change in reduced P_{app} by TF3'G was observed in STO-609-pretreated Caco-2 cells (Fig. 3-4). In addition, as shown in Fig. 3-5, no significant recovery of the reduced PEPT1 expression by STO-609 was obtained in the TF3'G-treated Caco-2 cells. These results demonstrated that the CaMKK-activated AMPK pathway⁽⁷²⁻⁷⁴⁾ could be excluded from the mechanism(s) underlying TF3'G-induced PEPT1 suppression.

It has been reported that AMPK phosphorylation in tissues mainly concerned either the activation of CaMKK or LKB1 signaling pathway.⁽⁷¹⁻⁷³⁾ Therefore, findings in both Figs. 3-4 and 3-5 suggested the possible involvement of TFs in the activation of other up-stream kinases such as LKB1/AMPK signaling pathway by either transforming growth factor- β -activated kinase (TAK-1)⁽⁷⁵⁻⁷⁷⁾ or Sirtuin 1 (SIRT1).^(78,79)

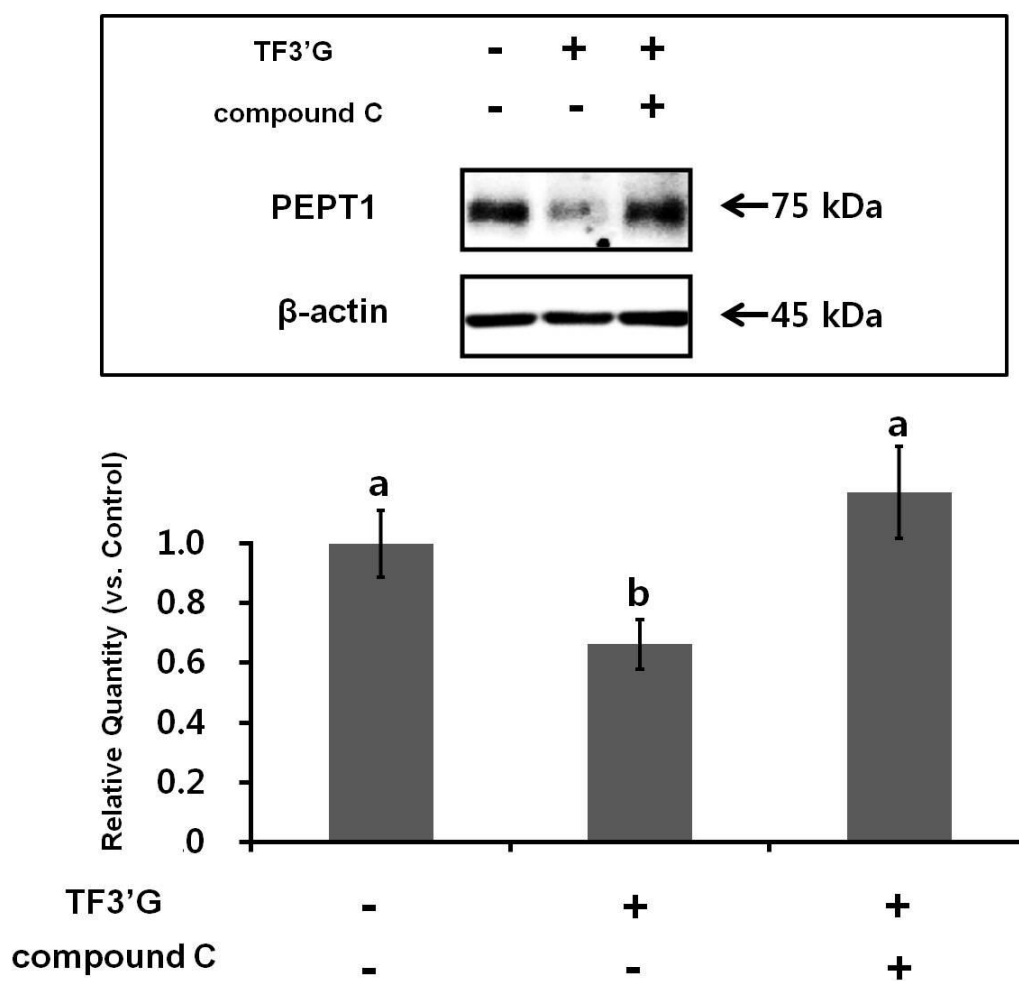


Fig. 3-3. Effect of AMPK inhibition on PEPT1 protein expression in Caco-2 cells treated with TF3'G

Caco-2 cells were treated with 20 μ M TF3'G in the presence or absence of 10 μ M compound C. Values are expressed as the mean $\pm$ SEM (n=3). Means without a common letter are significantly different; $P < 0.05$. Significant difference between groups was evaluated by Tukey–Kramer's *t*-test.

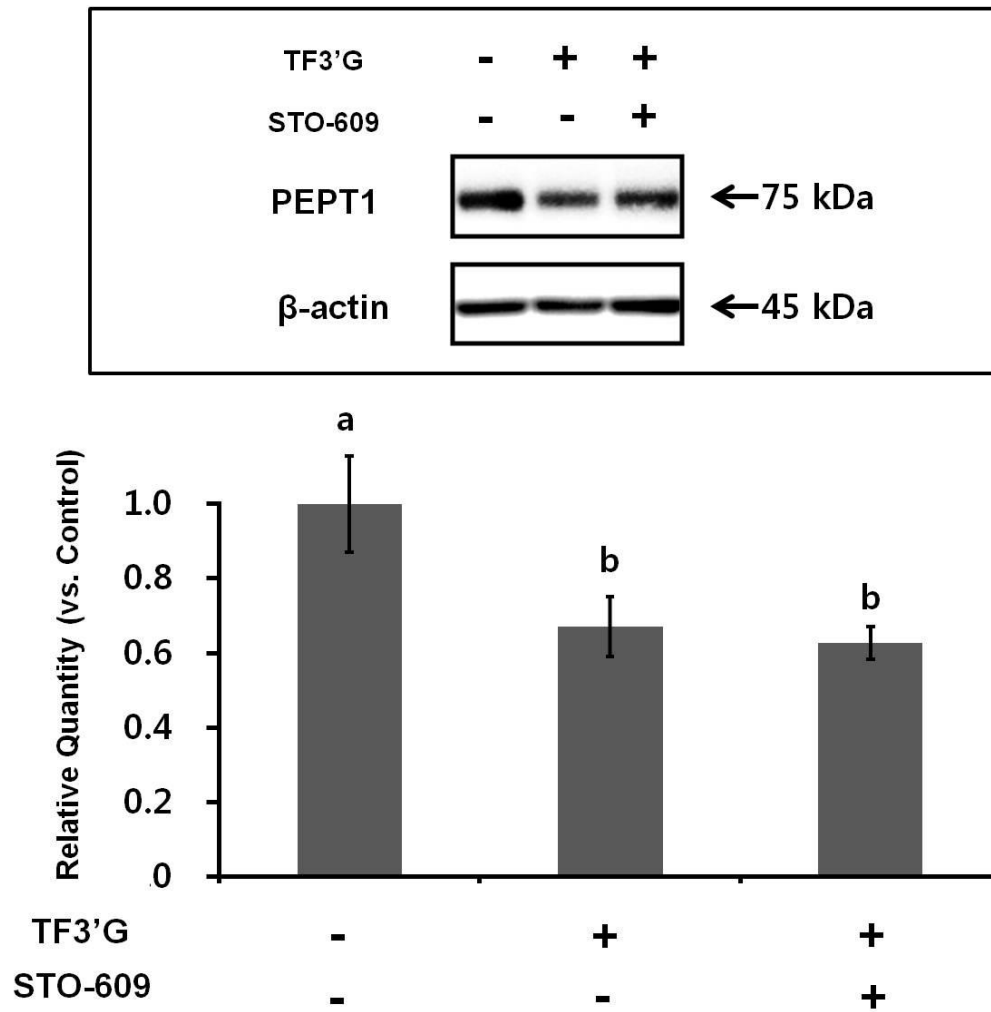


Fig. 3-4. Effect of CaMKK inhibition on PEPT1 protein expression in Caco-2 cells treated with TF3'G

Caco-2 cells were treated with 20 μ M TF3'G in the presence or absence of 25 μ M STO-609. Values are expressed as the mean $\pm$ SEM (n=3). Means without a common letter are significantly different; $P < 0.05$. Significant difference between groups was evaluated by Tukey–Kramer's *t*-test.

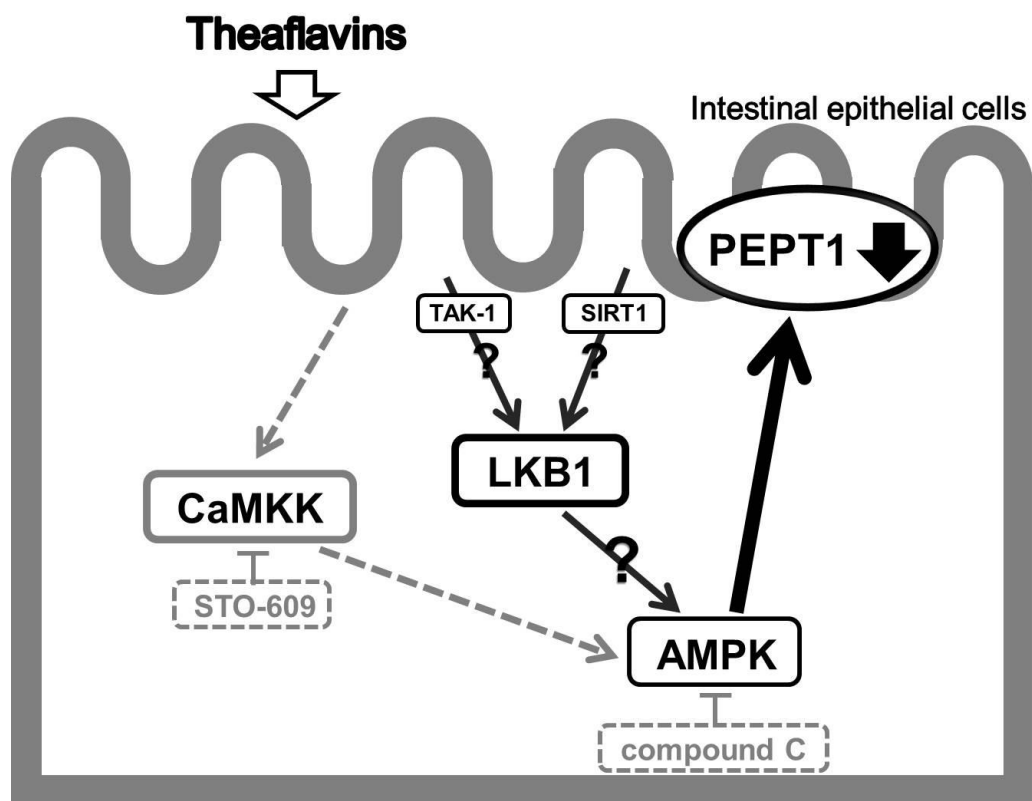


Fig. 3-5. Proposed up-stream mechanism(s) of theaflavins on signaling pathways in Caco-2 cells

4. Summary

In intestinal membranes it has been reported that the expression of PEPT1 was closely associated with AMPK activation, and also Chapter II showed that the transport of di-peptides across Caco-2 monolayers was markedly inhibited by TF-induced AMPK activation.

Chapter III, thus, investigated the structural requirement of TFs for the effect, and the mechanism underling TF-induced AMPK activation. The result showed the benzotropolone moiety in TFs, which has a characteristic 7-membered ring structure, was a crucial structural requirement for exerting the inhibition of intestinal peptide-transport and also demonstrated that TF3'G was not involved in the CaMKK/AMPK pathway, since a CaMKK inhibitor (STO-609) did not alter the reduced PEPT1 expression by TF3'G. Therefore, further experiments must be required to make clear the up-stream route activated by TFs in Caco-2 cells.

Chapter IV

Enhancement of intestinal barrier by theaflavins in Caco-2 cell monolayers

1. Introduction

The human intestine can accept the absorption of dietary nutrients and protect the absorption of noxious molecules via the intestinal barrier systems.^(35,80) One of protective intestinal barrier systems involves the TJ-closing effect that is crucial for the maintenance of barrier integrity.^(35,65,81)

In previous Chapters II and III, an alternative physiological role of TFs on the intestinal transport system as well as on reduced absorption across the membrane was demonstrated. TFs suppressed intestinal transport of small peptides via the down-regulation of AMPK-mediated PEPT1 in Caco-2 cell monolayers.

The result clearly showed that TFs induced AMPK activation in Caco-2 cells. Thus, TFs may influence other AMPK-related intestinal transport systems, since Zheng and Cantley have found that epithelial AMPK activation was closely associated with enhanced TJ integrity in Madin-Darby canine kidney (MDCK) cells.⁽⁸²⁾ In addition, Scharl *et al.*⁽⁸³⁾

supported the relation of epithelial barrier function with AMPK activation. These reports provide additional rationale for study on the effect of TFs on the paracellular transport system.

TJs localized at the junction of the apical-lateral membrane are a major determinant of intestinal barrier function and paracellular permeability.^(80,84) TJs are composed of a complex combination of integral transmembrane proteins, including occludin, claudins and junctional adhesion molecules (JAMs) as well as cytosolic proteins, such as the zonula occludens (ZO) protein family.⁽⁸⁴⁾ Although information is still limited, recent studies found intestinal TJ regulation by food-derived factors, including polyphenols.^(35,84) Suzuki and co-workers reported that kaempferol, a natural flavonoid, showed a protective effect on TJ barrier integrity via ZO-2 and claudin-4 expression and a redistribution of occludin and claudin-1/-3.⁽⁸⁵⁾ Thus, it is important to know how TJs may regulate the opening or closing of TJs.

In this Chapter IV, thus, the effect of TFs on the paracellular transport system was investigated by using fluorescein as a TJ transport model marker⁽⁸⁷⁾ in Caco-2 cells.

2. Materials and Methods

2.1. Reagents

TF, TF3G, TF3'G, TFdG, EC, EGC, ECG, and EGCG were purchased from Nacalai Tesque Co. (Kyoto, Japan). Purpurogallin was purchased from Pfaltz & Bauer Inc. (Waterbury, CT). DMEM and FBS were purchased from GIBCO Life Technologies (Grand Island, NY). Penicillin was purchased from Meiji Seika Co. (Tokyo, Japan). Insulin, fluorescein sodium salt, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Seeding basal medium, enterocyte differentiation medium, and MITO+™ serum extender for Caco-2 cell culture were purchased from BD Biosciences (Franklin Lakes, NJ). Compound C, an AMPK inhibitor, and GF109203X, a non-specific PKC inhibitor, were obtained from Merck Millipore (Darmstadt, Germany). Other reagents were of analytical grade and were used without further purification.

2.2. Cell culture

Caco-2 cells between passages 50 and 60 were propagated and maintained under standard cell culture conditions as described in Chapters II and III. For Caco-2 transport experiments, cells were seeded into BD Falcon™ cell culture insert (PET membrane, 0.9 cm², 1.0 μm pore size; BD Biosciences) coated with type I collagen (collagen gel culturing kit, Cellmatrix type I-A, Nitta Gelatin, Osaka, Japan) at a density of 4.0×10^5 cell/ml. Seeded cells were cultured in a seeding basal medium containing MITO+™ serum extender for 48 h. The medium was replaced with an enterocyte differentiation medium containing MITO+™

serum extender every day for three days to allow the cells to form monolayers. The integrity of the monolayers was evaluated by measuring TEER using a Multi-channel Voltage/Current EVC-4000 system (World Precision Instruments, Sarasota, FL). Monolayers with TEER values of $>100 \Omega \cdot \text{cm}^2$ were used for Caco-2 transport experiments.

2.3. Cell treatment

Pretreatment of Caco-2 cell monolayers in the BD Falcon™ cell culture inserts with test samples (TFs, catechins or purpurogallin) was performed by replacing 10% FBS in DMEM with medium containing sample (5, 10 or 20 μM) in the presence or absence of inhibitor (10 μM compound C or 10 μM GF109203X) and incubated for 1, 3, 6 or 24 h at 37°C, in an humidified atmosphere of 5% CO_2 in air. Cells incubated in the medium without test samples were used as a control. The Caco-2 cell monolayers were used for fluorescein transport studies. The medium containing test samples was prepared by diluting a stock solution of each sample dissolved in DMSO with 10% FBS in DMEM. The final concentration of DMSO was 0.5% throughout the experiments to avoid any cell damage by DMSO.⁽⁵⁹⁾

2.4. Fluorescein transport study

Fluorescein transport experiments were performed with an Ussing Chamber system (Model U-2500; Warner Instrument Co., Hamden, CT) using Caco-2 cell monolayers pretreated as described above. Prior to the transport experiments, Caco-2 cell monolayers in the transwell inserts were gently rinsed with HBSS buffer (pH 7.4, adjusted with 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid]) to wash out any free sample from the

insert. The insert was then carefully mounted in the Ussing Chamber using blunt forceps. An aliquot (7.5 ml) of HBSS buffer (pH 7.4) was added to both the apical and basolateral sides, since fluorescein restrictively transports through the paracellular TJ route in Caco-2 cells when both sides had no proton gradient (in this study, pH 7.4).⁽⁸⁷⁾ After equilibration for 15 min at 37°C, transport experiments were started by replacing the apical buffer with fresh HBSS buffer containing 100 µM fluorescein. During 30 min-transport experiments, solutions on both sides were bubbled continuously with a mixture of O₂:CO₂ (95:5), and an aliquot of the solution was taken from the basolateral side every 5 min. Next, fresh HBSS buffer (100 µl) was added to the basolateral side at each time point. The fluorescence intensity of the collected solutions was measured with a fluorescence spectrophotometer (Wallac ARVO SX 1420 multilabel counter; Perkin-Elmer Life Sciences, Tokyo, Japan) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.5. Apparent permeability coefficient

The apparent permeability coefficient (P_{app}) was calculated from the following equation:

$$P_{app} \text{ (cm/s)} = \frac{V}{AC_0} \frac{dC}{dt}$$

where dC/dt is the change in concentration at the basolateral side over time (mM/s), V is the volume of solution in the basolateral compartment (7.5 ml), A is the surface area of the membrane (0.2826 cm²), and C_0 is the initial concentration at the apical side (mM). The P_{app} of fluorescein in Caco-2 cells pretreated with test samples was expressed as a percentage of P_{app} of control (%).

2.6. Real-time reverse transcription PCR

Caco-2 cells were cultured on a 6 well-plate under the same cell culture conditions as described above. Total RNA was isolated from Caco-2 cells pretreated with or without 10 μ M TF3'G for 3 h using an SV Total RNA Isolation System (Promega Corporation, Madison, WI). The complementary DNA was produced from 1 μ g of total cellular RNA by Reverse Transcription System (AMV reverse transcriptase, Promega Corporation) according to the manufacturer's instructions. The Mx3000P QPCR system (Stratagene Inc., Tokyo, Japan) and SYBR *Premix Ex Taq* II (Takara bio Inc., Otsu, Japan) were used for RT-PCR amplification of transcripts derived from TJ-related proteins (occludin, claudin-1/-3/-4, ZO-1/-2 and JAM-1) and β -actin with the following cycle profile: 1 cycle at 95°C for 10 s, 47 cycles at 95°C for 30 s (denaturation step), at 60°C for 30 s (annealing step), and at 72°C for 1 min (extension step). At the end of each extension step, observed fluorescence was used for quantitative analyses. A melting point analysis was carried out by heating the amplicon from 55°C to 95°C, and a characteristic melting point curve was obtained for each product. Primer sequences were as follows: occludin, forward primer, 5'-TCCTATAAATCCACGCCGGTTC-3' and reverse primer, 5'-CTCAAAGTTACCACCGCTGCTG-3'; claudin-1 forward primer, 5'-GCACATA-CCTTCATGTGGCTCAG-3' and reverse primer, 5'-TGGAACAGAGCACAA ACATGTCA-3'; claudin-3 forward primer, 5'-ACATCATCACGTTCGAGAACAT-C-3' and reverse primer, 5'-AGTGCCAGCAGCGAGTCGTA-3'; claudin-4 forward primer, 5'-CATCGGCAGCAAC-ATTGTCAC-3' and reverse primer, 5'-GCACCACGCAGTTCATCC-ATAG-3'; ZO-1 forward primer, 5'-CGGGACTGTTGGTATTGGCTAGA-3' and reverse primer, 5'-GGCCA-GGGCCATAGTAAAGTTTG-3'; ZO-2 forward primer, 5'-CTAGCAGCGATCAACTTAG-GGACAA-3' and reverse primer, 5'-CCCAGGAGTTTCATTACCAGCAA -3'; JAM-1

forward primer, 5'-CAGAGGTGATTCATGGCTCTGTG-3' and reverse primer, 5'-TTCCAGGCTGGCAATAACTGAC-3'; β -actin forward primer, 5'-TGGCACCCAGCACAATGAA-3' and reverse primer, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'. As an internal control, β -actin primers were used for RNA template normalization. Quantification was reported by proprietary software, as cycle threshold (Ct). The expression of target genes relative to the housekeeping gene was calculated as the difference between Ct values for the two groups. All PCR reactions were done in triplicates.

2.7. Western blot analysis

The relative amounts of TJ-related proteins (occludin, claudin-1, and ZO-1) in Caco-2 cells treated with or without 10 μ M TF3'G for 1 and 3 h were detected by Western blot analysis. Proteins extracted from Caco-2 cells with RIPA buffer were mixed with an equal volume of sample buffer (20% glycerol, 4% sodium dodecylsulfate, 3% dithiothreitol, 0.002% bromophenol blue, and 0.125 M Tris-HCl, pH 6.8). The supernatant of the lysate was collected by centrifugation at $20,800 \times g$ for 10 min at 4°C, and the amount of protein was quantified with a DC protein assay kit (Bio-Rad Laboratories, Inc., Berkeley, CA). The supernatant was mixed with an equal volume of SDS-PAGE sample buffer and boiled in water for 5 min. An aliquot (15 μ g/lane) was applied to 10% SDS-PAGE for 1 h at 20 mA and transferred onto a PVDF membrane (Hybond-P, GE Healthcare, MJ) for 1.5 h at 40 mA. The membrane was blocked with 5% (w/v) ECL membrane-blocking reagent in TBS-T for 1 h at room temperature, and then probed with rabbit polyclonal antibodies against occludin (1:1000 dilution, Santa Cruz Biotechnology, Inc., Dallas, TX), claudin-1 (1:50 dilution, Thermo Fisher Scientific Inc, Runcorn, UK) and ZO-1 (1:200 dilution, Santa Cruz Biotechnology) as well

as the mouse monoclonal antibody against β -actin (1:1000 dilution, Applied Biological Materials Inc., Richmond, BC, Canada) overnight at 4°C. Finally, the membrane was incubated with the secondary antibody (HRP-conjugated donkey anti-rabbit IgG antibody at a 1:1000 dilution, GE Healthcare) for 1 h at room temperature. The immunoreactive proteins on the membrane were detected with ECL plus detection reagent using an ImageQuant LAS 4000 (GE Healthcare). Quantitation of the occludin, claudin-1 and ZO-1 amounts was determined by ImageQuant TL 7.0 software (GE Healthcare). The levels of occludin, claudin-1 and ZO-1 in Caco-2 cells were calculated by the ratio of their levels to β -actin, and the data were expressed as quantity relative to the control level.

2.8. Assay for phosphorylation of AMPK

Caco-2 cell monolayers in a 6-well microplate were treated with or without 10 μ M TF3'G in the presence or absence of 10 μ M compound C for 3 h at 37°C. Proteins were extracted from Caco-2 cells using RIPA buffer containing PhosSTOP (Roche, Indianapolis, IN), and the supernatant was obtained as mentioned above. The amounts of phospho-AMPK (p-AMPK) and AMPK proteins were evaluated by Western blot analysis. An aliquot (15 μ g) of the prepared sample was applied to 10% SDS-PAGE for 50 min at 20 mA and transferred onto PVDF membrane for 1.5 h at 40 mA. The membrane was incubated with 5% ECL membrane-blocking reagent for 1 h at room temperature. The membrane was probed with a rabbit polyclonal antibody against phospho (Thr¹⁷²)-AMPK α 1 (1:1000 dilution, Merck Millipore, Bedford, MA, USA) overnight at 4°C, and then incubated with the secondary antibody (HRP-conjugated donkey anti-rabbit IgG antibody) for 1 h at room temperature. The immunoreactive proteins on the membrane were detected with ECL detection reagents and

Image Quant LAS 4000. For removing the p-AMPK, the membrane was incubated in stripping buffer (2% SDS, 0.1 M mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8) for 30 min, and then washed with TBS-T for 1 h. The membrane was blocked with 5% ECL-blocking reagent for 1 h at room temperature and reprobed with the rabbit polyclonal antibody against AMPK α 1 (1:1000 dilution, Merck Millipore) overnight at 4°C, and then incubated with the secondary antibody (HRP-conjugated donkey anti-rabbit IgG antibody) for 1 h at room temperature, as described above. Quantification of p-AMPK and AMPK was performed by an Image Quant TL 7.0 software (GE Healthcare). Relative band intensities of p-AMPK and AMPK were expressed as a percentage of control (or of TF3'G-untreated cells).

2.9. Statistical analysis

Results are expressed as the mean $\pm$ SEM. The statistical significance between groups was analysed using one-way ANOVA followed by the Tukey-Kramer's *t*-test for *post hoc* analysis. Other statistical evaluations were performed by the Student's *t*-test. All analyses were conducted with the Star View J 5.0 (SAS Institute Inc., Cary, NC).

3. Results and Discussion

3.1. Effect of theaflavins on fluorescein transport across Caco-2 cell monolayers

To investigate the effect of TFs (TF, TF3G, TF3'G and TFdG) on intestinal barrier function in Caco-2 cell monolayers, TEER and fluxes of fluorescein were measured. Caco-2 cell monolayers were pretreated individually with 20 μ M TFs for 24 h before the transport experiments. As shown in Fig. 4-1, the P_{app} of fluorescein in control Caco-2 cells (control: $3.7 \pm 0.1 \times 10^{-6}$ cm/s) was significantly reduced by TF pretreatment in the descending order of TF3'G ($1.2 \pm 0.1 \times 10^{-6}$ cm/s) > TF3G, TFdG ($1.4 \pm 0.2 \times 10^{-6}$ cm/s) > TF ($1.7 \pm 0.1 \times 10^{-6}$ cm/s). This result revealed that the TFs used in this study significantly inhibited paracellular TJ permeability regardless of TF structures. TF3'G, which showed a potent P_{app} reduction effect, was used throughout the experiments.

As shown in Fig. 4-2, the TEER value of Caco-2 cell monolayers (control: $110 \pm 1.5 \Omega \cdot \text{cm}^2$) was significantly increased by TF3'G-pretreatment (e.g., 50 μ M: $145 \pm 16.1 \Omega \cdot \text{cm}^2$) for 24 h, suggesting that the P_{app} reduction by TF may be caused by increased barrier function of intestine induced by TF.

The effect of 20 μ M TF3'G pretreatment time on the P_{app} of fluorescein was examined (Fig. 4-3A). As a result, P_{app} decreased with time (1 - 24 h) and a significant reduction was obtained for > 3 h-pretreatment. As shown in Fig. 4-3B, the 3 h-and 5 μ M TF3'G-pretreatment reduced fluorescein transport across Caco-2 cell monolayers. Thus, further experiments were performed in Caco-2 cells pretreated with 10 μ M TF3'G for 3 h, showing reduction ratio in the P_{app} of fluorescein below 50%.

Under experimental conditions of < 24 h-pretreatment with < 100 μ M TF3'G, no toxicity was found in Caco-2 cells by WST-8 assay (Fig. 2-4A in Chapter II), suggesting that the transport inhibition by TFs may result from a change in signaling pathways towards the TJ closing route.

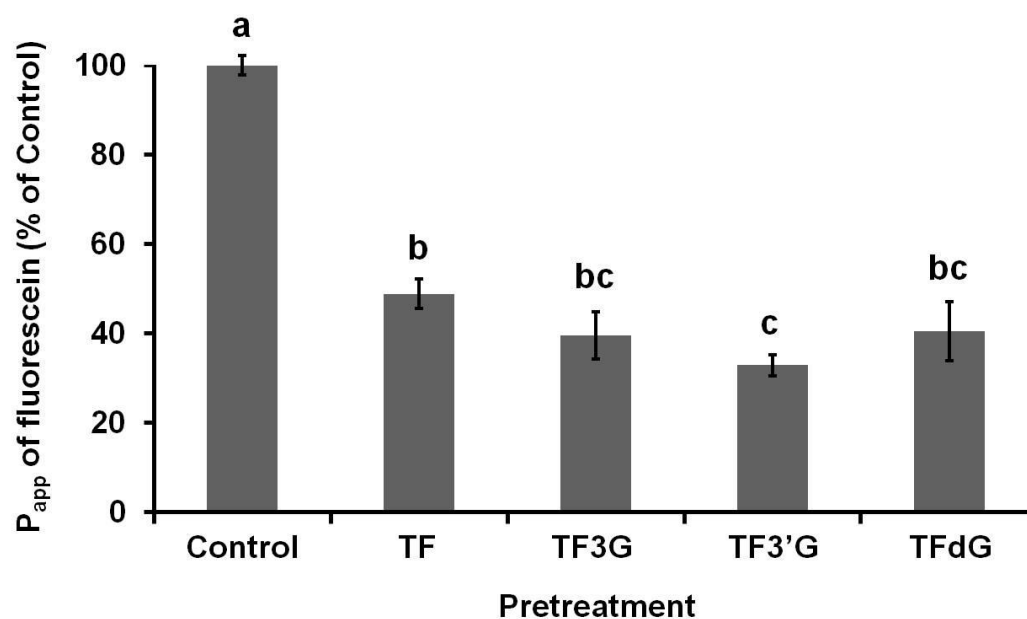


Fig. 4-1. Effect of theaflavins on P_{app} values of fluorescein transport in Caco-2 cells

Caco-2 cells pretreated with 20 μ M theaflavins for 24 h at 37°C. The theaflavins used in this experiment were theaflavin (TF), theaflavin-3-*O*-gallate (TF3G), theaflavin-3'-*O*-gallate and theaflavin-3,3'-*O*-digallate (TFdG). P_{app} values (in $\text{cm/s} \times 10^{-6}$) are expressed as the mean $\pm$ SEM ($n=3 - 5$). Different letters represent the statistical differences at $P < 0.05$ among the groups by the Tukey-Kramer's *t*-test.

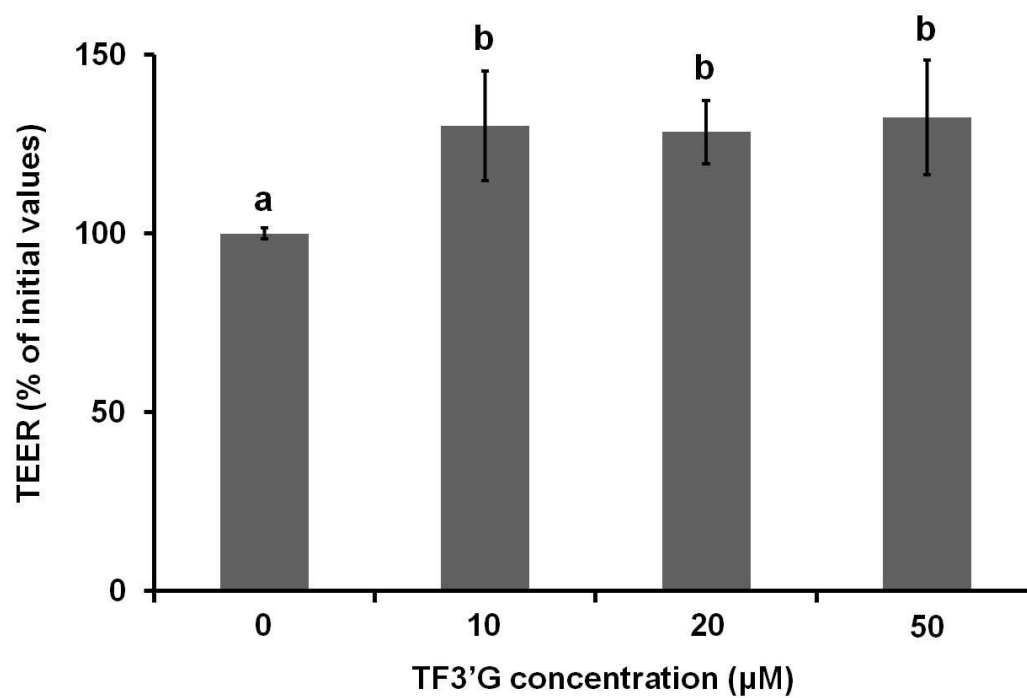


Fig. 4-2. Effect of TF3'G concentration on TEER in Caco-2 cells

Caco-2 cells were pretreated with either 10, 20 or 50 μM TF3'G for 24 h. Values are expressed as the mean $\pm$ SEM (n=3 - 5). Different letters represent the statistical differences at $P < 0.05$ among the groups by the Tukey-Kramer's t -test.

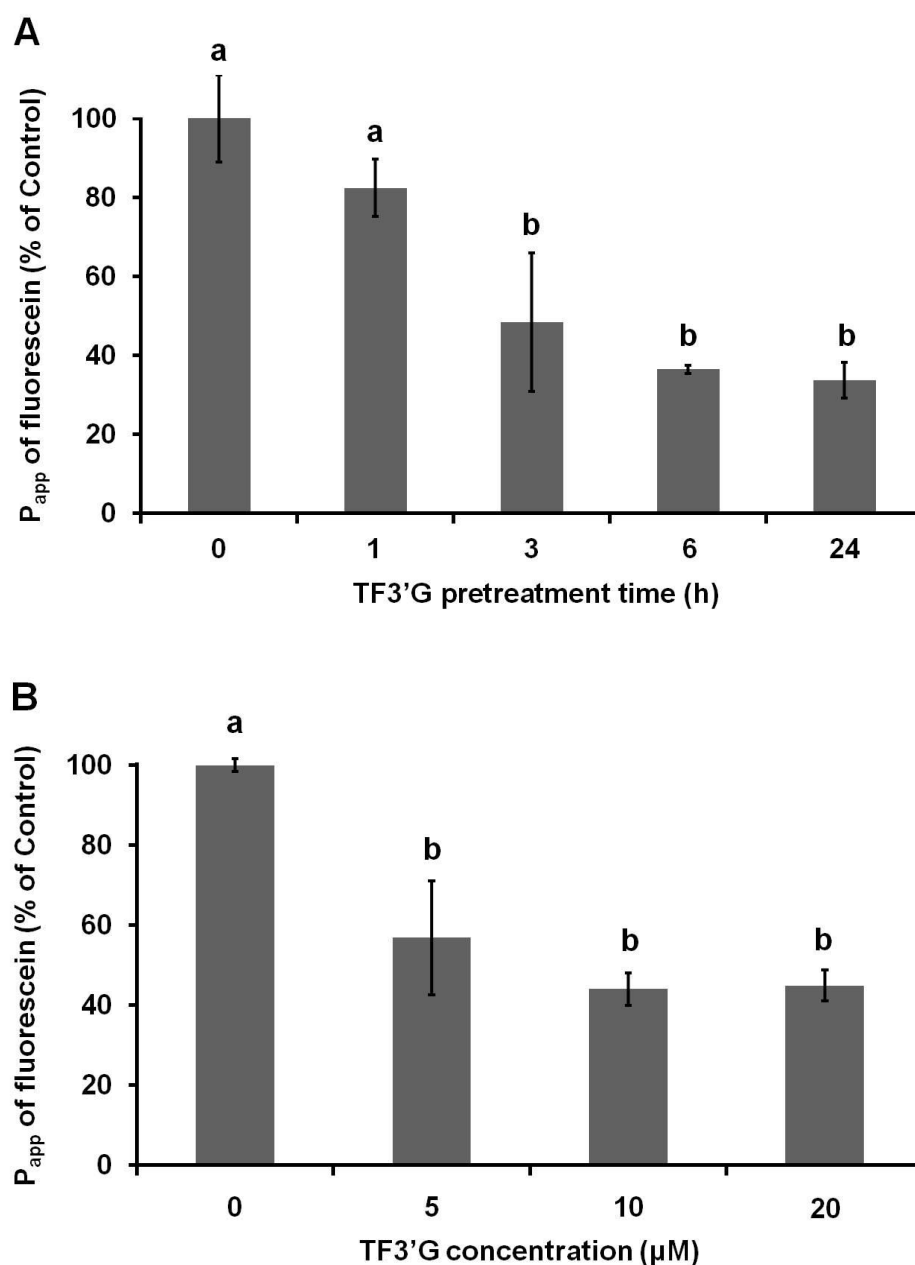


Fig. 4-3A, B. Effect of TF3'G on fluorescein transport

Time-dependent effect of TF3'G on fluorescein transport (A). Caco-2 cells were pretreated with or without 20 μ M TF3'G. Concentration-dependent effect of TF3'G on fluorescein transport (B). Caco-2 cells were incubated for 3 h. Values are expressed as the mean $\pm$ SEM (n=3 - 5). Different letters represent the statistical differences at $P < 0.05$ among the groups by the Tukey-Kramer's t -test.

3.2. Effect of theaflavins and its related compounds on fluorescein transport

Catechins and purpurogallin with benzotropolone moiety (Fig. 3-1 in Chapter III) were examined for fluorescein transport across Caco-2 monolayers to obtain insight on the structural requirements of TFs for paracellular permeability inhibition.

As shown in Fig. 4-4, four catechins did not show any significant P_{app} reduction, while the P_{app} of fluorescein was significantly reduced in Caco-2 cells pretreated with 10 μ M purpurogallin (51% of control in P_{app}), which was similar to the level obtained for TF3'G pretreated cells (46% of control in P_{app}).

The benzotropolone moiety, but not monomeric catechin moieties, was required for inhibiting fluorescein paracellular transport through the TJ route. This result also indicated that the benzotropolone moiety having a characteristic 7-membered ring structure in TFs was a crucial structural factor responsible for TFs-induced intestinal barrier in Caco-2 cells.

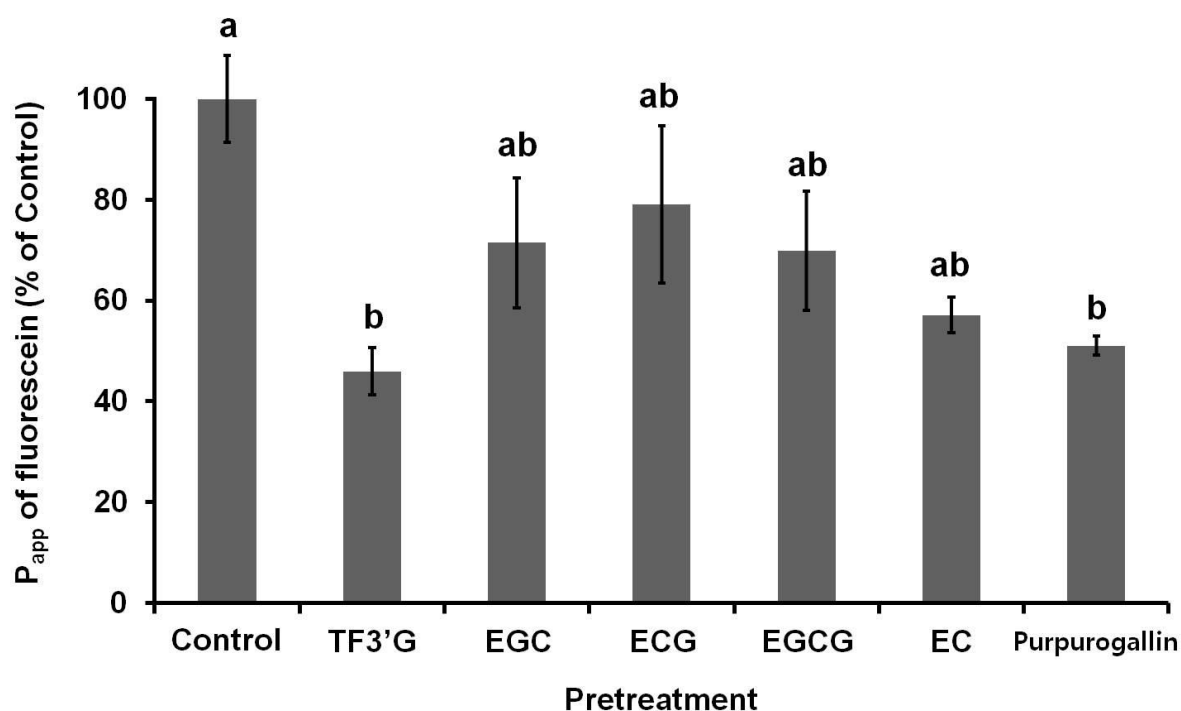


Fig. 4-4. Effect of TF3'G and its related compounds on fluorescein transport in Caco-2 cells

Caco-2 cells were treated with or without 10 μ M test compound for 3 h at 37°C. Theaflavin-3'-O-gallate (TF3'G), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin (EC). Control: transport of fluorescein in Caco-2 cells without TF3'G treatment. Values are expressed as the mean $\pm$ SEM (n=3 - 5). Means without a common letter are significantly different; $P < 0.05$. Significant differences between each group were evaluated by Tukey-Kramer's *t*-test.

3.3. Effect of AMPK or PKC inhibitor on fluorescein transport in theaflavins-pretreated Caco-2 cells

As reported by Ulluwishewa *et al.*,⁽³⁵⁾ TJ-related protein expression was induced by certain signaling pathways such as PKC. Cario *et al.*⁽⁸⁴⁾ reported that PKC stimulation induced TJ proteins, including ZO-1. AMPK activation pathway was also involved in alternative signaling for TJ protein expression, including occludin and ZO-1.⁽⁶⁶⁾ Thus, these two pathways (AMPK and PKC) were considered for investigation of mechanism underlying the inhibition of fluorescein transport by TF3'G.

Fluorescein transport in 10 μ M TF3'G-pretreated Caco-2 cells was studied in the presence of either 10 μ M GF109203X, a non-specific inhibitor of PKC or 10 μ M compound C, a specific inhibitor of AMPK.

As shown in Fig. 4-6, the P_{app} value of fluorescein was restored by 10 μ M compound C ($3.6 \pm 0.9 \times 10^{-6}$ cm/s), while GF109203X did not affect on fluorescein transport in the TF3'G-pretreated Caco-2 cells ($1.2 \pm 0.2 \times 10^{-6}$ cm/s, Fig. 4-5).

As expected from the results by compound C (Fig. 4-6), the enhanced phosphorylation of AMPK by 10 μ M TF3'G was blocked by compound C (Fig. 4-7). This clearly suggested that the possible involvement of TF3'G in the activation of AMPK, but not in the PKC pathway.

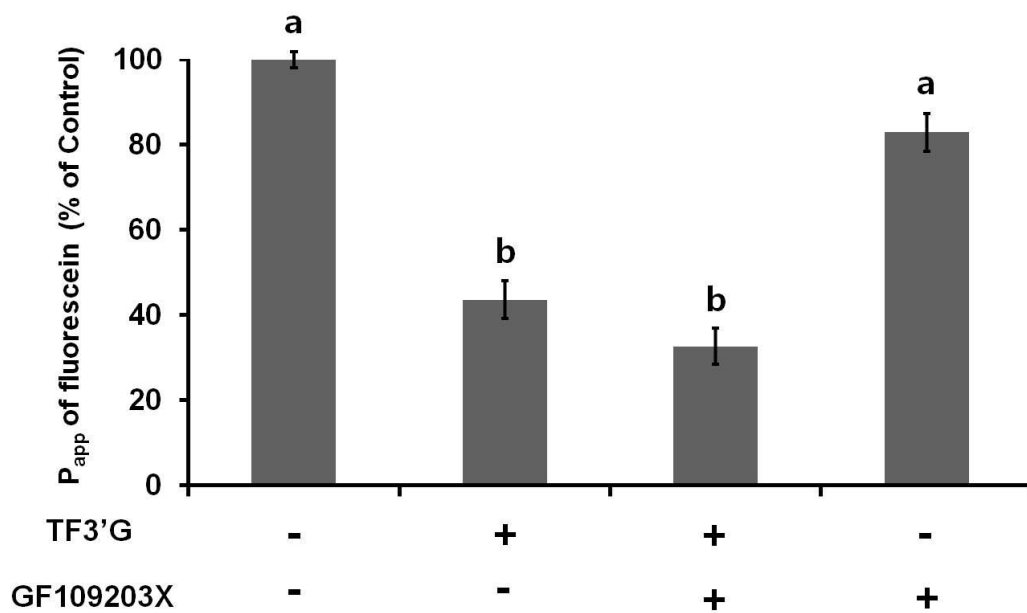


Fig. 4-5. Effect of PKC inhibition on fluorescein transport

Caco-2 cells for fluorescein transport were pretreated with or without 10 μ M TF3'G in the presence or absence of 10 μ M GF109203X (PKC inhibitor) for 3 h at 37°C. Values are expressed as the mean $\pm$ SEM (n=3 - 5). Means without a common letter are significantly different; $P < 0.05$. Significant differences between each group were evaluated by Tukey-Kramer's *t*-test.

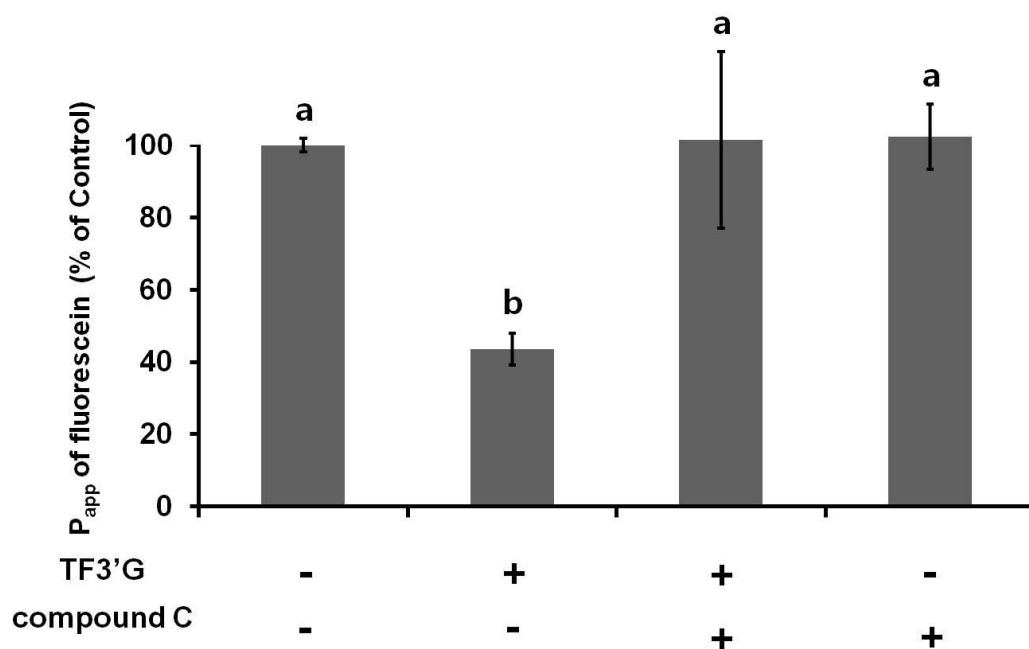


Fig. 4-6. Effect of AMPK inhibition on fluorescein transport

Caco-2 cells were pretreated with or without 10 μ M TF3'G in the presence or absence of 10 μ M compound C (AMPK inhibitor) for 3 h at 37°C. Values are expressed as the mean $\pm$ SEM (n=3 - 5). Means without a common letter are significantly different; $P < 0.05$. Significant differences between each group were evaluated by Tukey-Kramer's *t*-test.

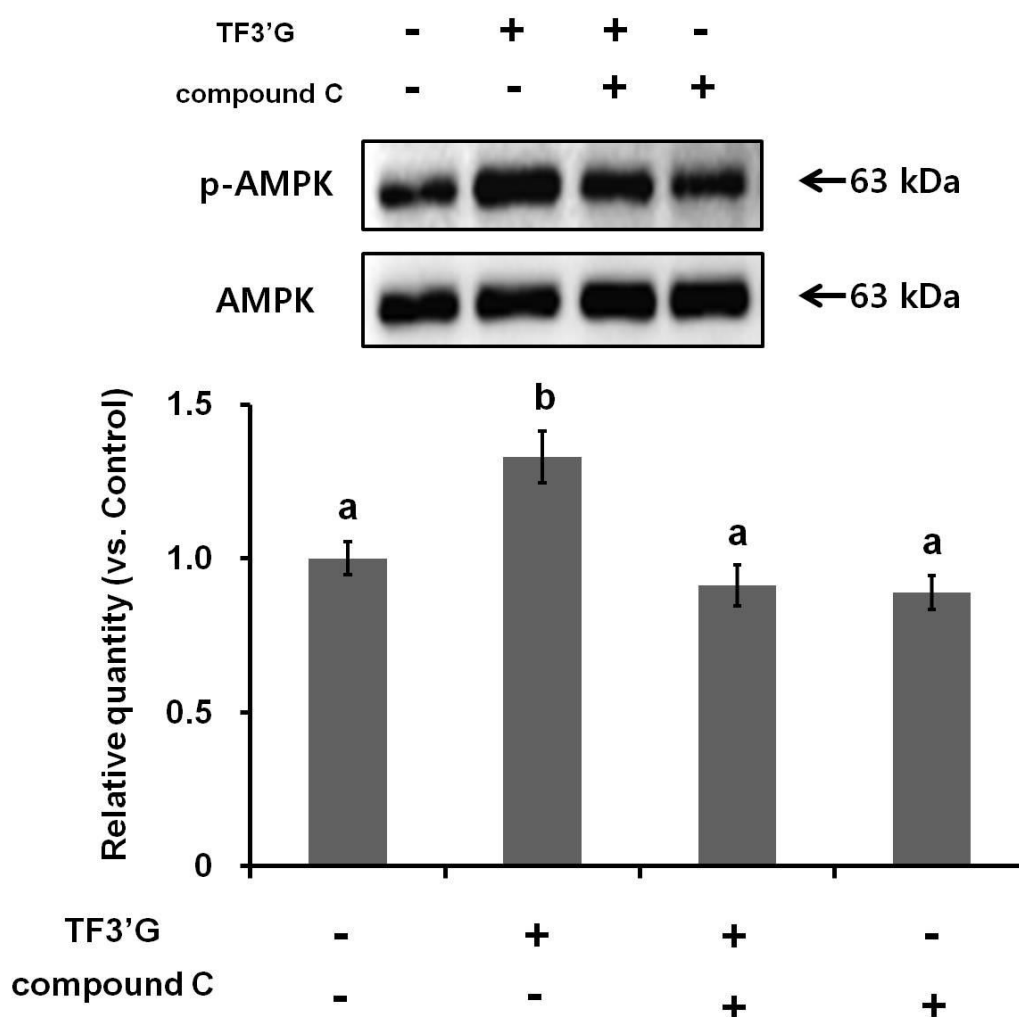


Fig. 4-7. Effect of AMPK inhibition on p-AMPK expression in Caco-2 cells treated with TF3'G

Caco-2 cells were incubated with or without 10 μ M TF3'G in the presence or absence of 10 μ M compound C (AMPK inhibitor) for 3 h at 37°C. Specific bands for p-AMPK and AMPK were quantitated by densitometric analysis. Values are expressed as the mean $\pm$ SEM (n=3 - 5). Means without a common letter are significantly different; $P < 0.05$. Significant differences between each group were evaluated by Tukey-Kramer's *t*-test.

3.4. Effect of theaflavins on the expression of TJ-related proteins in Caco-2 cells

Several groups have reported that natural compounds enhanced the TJ-closing effect and increased intestinal barrier function by expression of TJ-related protein.^(85,86) Suzuki and Hara reported that quercetin, a flavonoid, promoted the assembly of TJ proteins (occludin, claudin-1 and ZO-2)⁽⁸⁵⁾ as well as the expression of claudin-4 and ZO-2 by kaempferol.⁽⁸⁶⁾ Thus, to clarify the mechanism underlying the inhibition of paracellular transport across TF3'G-pretreated Caco-2 cell monolayers, expression of TJ-related proteins was investigated at the mRNA and protein levels. As shown in Fig. 4-8, mRNA levels for occludin, claudin-1 and ZO-1 were significantly ($P < 0.05$) increased at 3 h in 10 μ M TF3'G-treated Caco-2 cells, compared to control (0 h-incubation), whereas claudin-3/4, ZO-2 and JAM-1 mRNA levels were not affected.

Although TJ-related protein expressions are not regulated only by mRNAs,^(88,89) further Western-blot experiments were conducted for TJ-related proteins (occludin, claudin-1 and ZO-1), since they play a crucial role to adjust permeability characteristics and tightness of epithelium.⁽⁸⁹⁾ As a result of Western blot analysis, we found significant enhancement of occludin, claudin-1 and ZO-1 protein expression in 10 μ M TF3'G-treated cells, as shown in Fig. 4-9. It has been reported that occludin, claudin-1 and ZO-1 are important for both regulating TJ structure and paracellular permeability.^(35,86,90,91) Both claudin-1 and occludin are considered to be part of the structural backbone of TJ system, and are involved in reduced paracellular permeability by tightening the TJ.^(35,86,90) ZO-1 acts as an adaptor that connects transmembrane proteins to the perijunctional actomyosin ring.^(35,86)

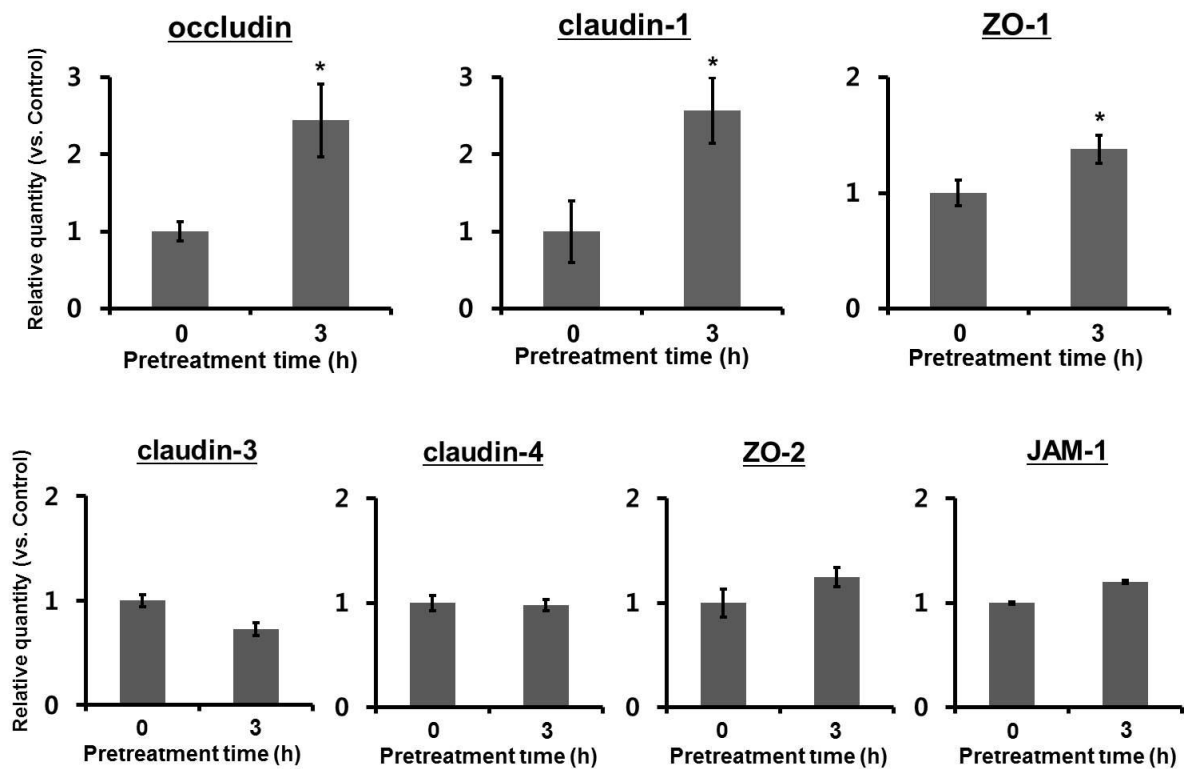


Fig. 4-8. Effect of TF3'G on transcription of TJ-related genes in Caco-2 cells

Caco-2 cells were cultured with or without 10 μ M TF3'G for 3 h at 37°C. Total RNA was isolated, and the expression level of mRNA was measured by RT-PCR using specific primers for occludin, claudin-1/3/4, ZO-1/2 and JAM-1. Values are expressed as the mean $\pm$ SEM (n=3 - 5). * $P < 0.05$, significantly different from untreated cells. Significant differences between control (0 h) and TF3'G groups were evaluated by unpaired Student's *t*-test.

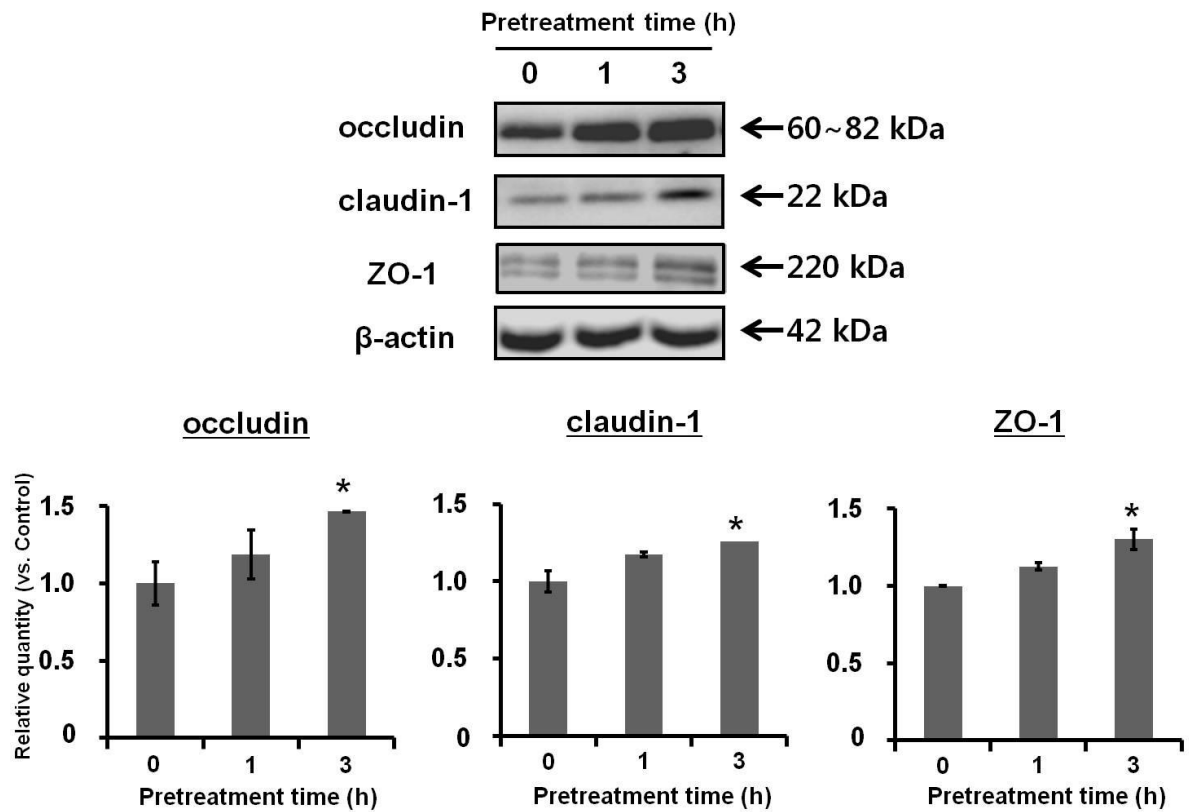


Fig. 4-9. Effect of TF3'G on the expression of TJ-related protein in Caco-2 cells

Caco-2 cells were treated with or without 10 μ M TF3'G for 1 and 3 h at 37°C. Specific bands for occludin, claudin-1, ZO-1 and β -actin were quantitated by densitometric analysis. Values are expressed as the mean $\pm$ SEM (n=3 - 5). * $P < 0.05$, significantly different from untreated cells. Significant difference between control (0 h) and TF3'G groups were evaluated by unpaired Student's t -test.

4. Summary

The human intestine allows the absorption of dietary nutrients and defenses noxious molecules by intestinal barrier systems. Increased intestinal permeability by a leak or breakdown of barrier is causative of several human diseases such as inflammatory bowel disease (IBD), celiac disease and irritable bowel syndrome (IBS). One of defended intestinal barrier systems concerns TJ-closing effect that is crucial for the maintenance of barrier integrity. Although recent studies revealed intestinal TJ regulation by food factors including polyphenols, information is still limited.

The Chapter IV demonstrated for the first time the key role of the benzotropolone ring in TFs in AMPK activation, while it showed no role in PKC inhibition for TJ-closing effect. In addition, TF-stimulated TJ proteins were occludin, claudin-1 and ZO-1, although the levels of claudin-3/4, ZO-2 and JAM-1 were not affected by TFs.

In conclusion, this Chapter IV investigated that TFs did in fact have the ability to close the TJ route in Caco-2 cells, likely through the expression of AMPK-mediated occludin, claudin-1 and ZO-1 proteins (Fig. 4-10). Thus, the ingestion of TFs seems to improve intestinal barrier function by e.g., IBD in future animal experiments.

However, it still remains unclear which up-stream signaling mechanisms such as LKB1, CaMKK and TAK-1 are involved in the AMPK phosphorylation by TFs in Caco-2 cells. In addition, the mechanism of TJ for activation of AMPK and TJ-related protein expression pathways also remains unclear. Furthermore, it might be speculated that specific receptor(s) of Caco-2 cells recognize(s) the TFs or TFs enhance the intestinal barrier directly. Therefore, further experiments must be needed to elucidate the regulation of TJ-closing.

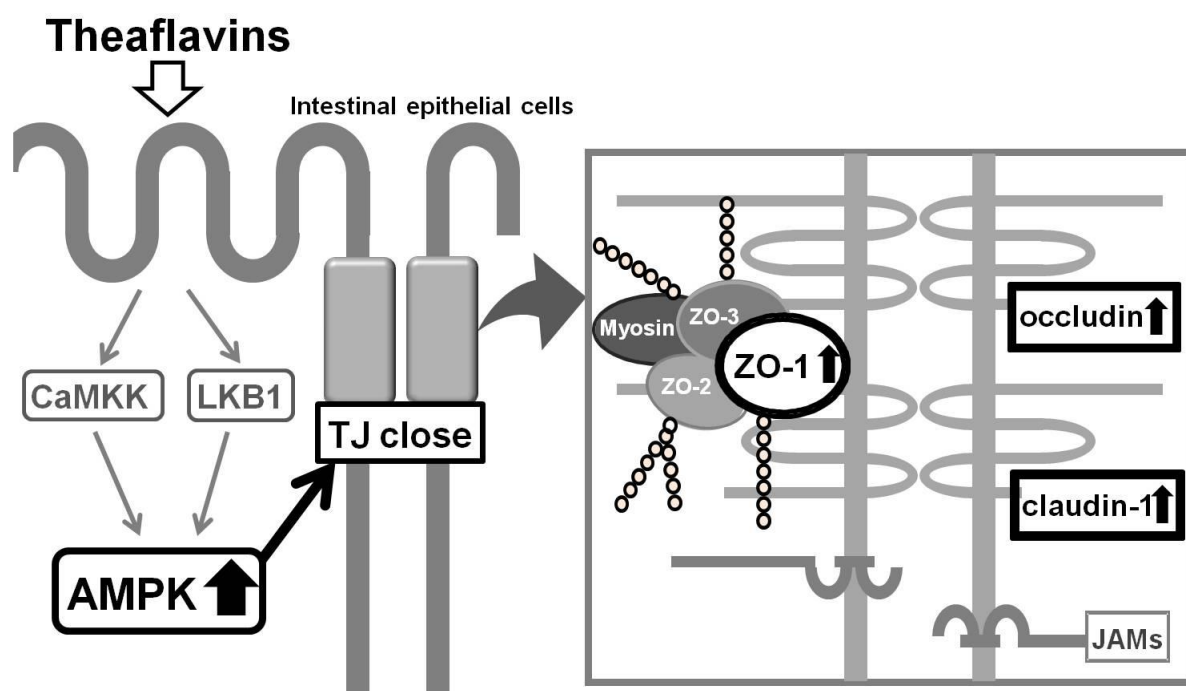


Fig. 4-10. Proposed mechanism of TFs on TJ-related signaling pathways in Caco-2 cells

Chapter V

Conclusion

Foods providing nutrients are known to prevent lifestyle-related diseases and enhance physical and mental well-being of the consumers.^(3, 4) To date, many researches on functional food are addressing the physiological effects and health benefits of food components to clarify the body modulating functions of food-derived substances. Among the diets, polyphenols have been shown to have specific and potent physiological activities in *in vitro* test with higher throughput treatment, but conflicting results are often obtained in animals or human when they are orally taken.⁽⁸⁾ That's because most of physiological researches of food-derived substances have been investigated by focusing single substances of food so far. However, most of foods contain not only target compound, but also other compounds, so that they may influence the physiological activity of the target. Researches on the combination effect of target with food contaminants or on bioavailability have not been investigated in much detail. Direct interactions between some components of food and polyphenols, such as binding to proteins and polysaccharides, can occur and these interactions may affect absorption. Thus, researches on interactions between food

components must be required.

In recent years, attention has been paid to health beneficial function of theaflavins (TFs), dimeric catechins of black tea, including antioxidant, anti-inflammation, anti-hyperglycemic activity, antimutagenic, anticlastogenic, anticancer, antipathogenic, and anticarcinogenic effects.⁽²⁷⁻³¹⁾

However, the underlying mechanisms responsible for physiological role of TFs on absorption of food substances on intestine, which can be explained about discrepancy of food components absorption between *in vitro* and *in vivo*,⁽¹³⁾ are unknown.

The aim of the present study was to clarify the regulation of intestinal absorption and their underlying mechanism by TFs, dimeric catechins, in human intestinal Caco-2 cells.

Chapter II Effect of theaflavins on peptide transport across Caco-2 cell monolayers

In the small intestine, peptide transporter 1 (PEPT1) plays a role in the transport of di- and tri-peptides. Whether TFs in black tea, oxidatively coupled dimmers of catechins, could affect the absorption of small peptides in human intestinal Caco-2 cells were investigated in Chapter II, since TFs were far from the penetrant through the cells and might be involved in intestinal transport systems. Absorption ability was measured with Caco-2 cell monolayers in an Ussing Chamber. Peptide concentration at fixed time point to 60 min was determined by measuring fluorescence spectrophotometer after derivatization of peptides with NDA at an excitation wavelength of 405 nm and emission wavelength of 460 nm. The apparent permeability coefficients (P_{app}) were used to evaluate the permeability. In transport experiments, the transport of Gly-Sar (a model molecule for PEPT1 transport) and other di-

peptides (Val-Tyr and Ile-Phe) were significantly reduced ($P < 0.05$) in TFs-pretreated cells. The reduction of the P_{app} by TFs was in the descending order of TF-3'-O-gallate (TF3'G), TF-3-O-gallate (TF3G) > >TF-3,3'-O-digallate (TFdG) > TF. Kinetic analysis indicated that TF3'G treatment significantly decreased the maximum uptake rate (V_{max}) for Gly-Sar, but had no effect on the Michaelis constants (K_m), indicating that TF3'G may reduce the number of peptide transport-related routes, but not lower or alter the affinities of peptides with any of the routes. PEPT1 mRNA and protein expressions were decreased significantly in TF3'G-pretreated Caco-2 cells. In TF3'G-pretreated Caco-2 cells, Gly-Sar transport was completely ameliorated by 10 μ M compound C, an AMPK inhibitor, but not by the GF109203X (PKC inhibitor), it seems likely that TFs activated AMPK pathways so as to inhibit PEPT1 transporter expression.

Consequently, it was the first time demonstrated that TFs inhibit peptide transport across Caco-2 cell monolayers, likely through suppression of AMPK-mediated PEPT1 expression.

Chapter III Structural requirement of theaflavins and their signal regulation for reduced peptide transport in Caco-2 cells

Previous researches have proven that TFs inhibit de-peptide transport via down regulation of AMPK-mediated peptide transporter PEPT1 in Caco-2 cells, while the relationship between peptide transport and TF structure remain unclear. Therefore, in Chapter III, the structural requirement of TFs for the effect and the mechanism(s) underling TF-induced AMPK activation in Caco-2 cells were observed. TF3'G was used for this study, since it is the strongest inhibitor of peptide-transport among TFs. Absorption ability was

measured with Caco-2 cell monolayers pretreated with or without 20 μ M sample (TF3'G, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin (EC), and purpurogallin) in an Ussing Chamber. The amount of Gly-Sar transport at fixed time-points to 60 min was determined by fluorescent NDA-derivatized assay (Ex/Em: 405 nm/460 nm). The P_{app} value of Gly-Sar significantly ($P < 0.05$) decreased in 20 μ M purpurogallin-pretreated Caco-2 cells as well as in TF3'G-pretreated cells, together with the reduction of PEPT1 expression, while their monomeric catechins did not show any P_{app} reduction. Expression of PEPT1 protein in Caco-2 cells pretreated with or without 20 μ M TF3'G in the presence or absence of inhibitor (10 μ M compound C as AMPK inhibitor or 25 μ M STO-609 as CaMKK inhibitor) was evaluated by Western blot analysis. In TF3'G-treated Caco-2 cells, the recovery of the reduced PEPT1 expression was found by 10 μ M compound C, but not by STO-609.

Consequently, these results suggested that the benzotropolone moiety in TFs was a crucial structural requirement for exerting the inhibition of intestinal peptide-transport, and CaMKK/AMPK pathway is not involved in the suppression of PEPT1 expression by TFs.

Chapter IV Enhancement of intestinal barrier by theaflavins in Caco-2 cell monolayers

The formation of tight junction (TJ) between epithelial cells is one major component of the intestinal barrier. It regulates the passage of ions, water and various macromolecules through paracellular spaces and thus relevant to several different human disease such as inflammatory bowel disease (IBD), celiac disease and irritable bowel syndrome (IBS) ⁽³⁵⁾. In previous studies (Chapters II and III) clearly showed TFs induced AMPK activation in Caco-2 cells. Thus, TFs may influence other AMPK-related intestinal transport systems such as TJ.

Permeability experiments using a fluorescein as paracellular marker were performed in Ussing chamber mounted with Caco-2 cell monolayers. Transported fluorescein at a fixed-time point to 30 min was determined with a fluorescence spectrophotometer at an excitation wavelength of 485 nm and emission wavelength of 535 nm. TFs having benzotropolone moiety significantly inhibited the paracellular permeability in the descending order of TF3'G ($1.2 \pm 0.1 \times 10^{-6}$ cm/s) > TF3G, TFdG ($1.4 \pm 0.2 \times 10^{-6}$ cm/s) > TF ($1.7 \pm 0.1 \times 10^{-6}$ cm/s) compared with untreated cells (Control: $3.7 \pm 0.1 \times 10^{-6}$ cm/s). Although the respective monomeric catechins did not show any P_{app} reduction, purpurogallin pretreatment resulted in a significant P_{app} reduction similar to that of TF3'G pretreatment. This finding indicates that the benzotropolone moiety may play a crucial role in the P_{app} reduction or TJ-closing effect induced by TFs. In TF3'G-pretreated Caco-2 cells, fluorescein transport was completely ameliorated by 10 μ M compound C, an AMPK inhibitor and also phosphorylation of AMPK was significantly increased ($P < 0.01$) compared with untreated cells. In addition, TF3'G significantly increased both the mRNA and protein expression of TJ-related proteins (occludin, claudin-1 and ZO-1) as well as the phosphorylation of AMPK.

Consequently, Chapter IV demonstrated firstly that TFs enhance the intestinal barrier by increasing the expression of TJ-related proteins occludin, claudin-1 and ZO-1 through the activation of AMPK in Caco-2 cell monolayers.

In conclusion, the present study demonstrated firstly inhibition effect on peptide transport across Caco-2 cell monolayers by TFs. TFs inhibit peptide transport across Caco-2 cell monolayers, likely through suppression of AMPK-mediated PEPT1 expression. Secondly, the benzotropolone moiety in TFs was a crucial structural requirement for exerting the inhibition of intestinal peptide-transport, and CaMKK/AMPK pathway is not involved in the suppression of PEPT1 expression by TFs. Finally, enhancement of intestinal barrier by TFs

was illustrated. TFs enhance the intestinal barrier by increasing expression of TJ-related proteins occludin, claudin-1 and ZO-1 through the activation of AMPK in Caco-2 cell monolayers.

The present finding on the potential role of TFs in intestinal absorption should, thus, be used to address new insight into food functionalities in the gut, and furthermore support awareness of the intake of TFs or black tea with bioactive peptides, peptidic drugs and other good diet. The present study also contributes to the future design and refinement of therapeutic treatment.

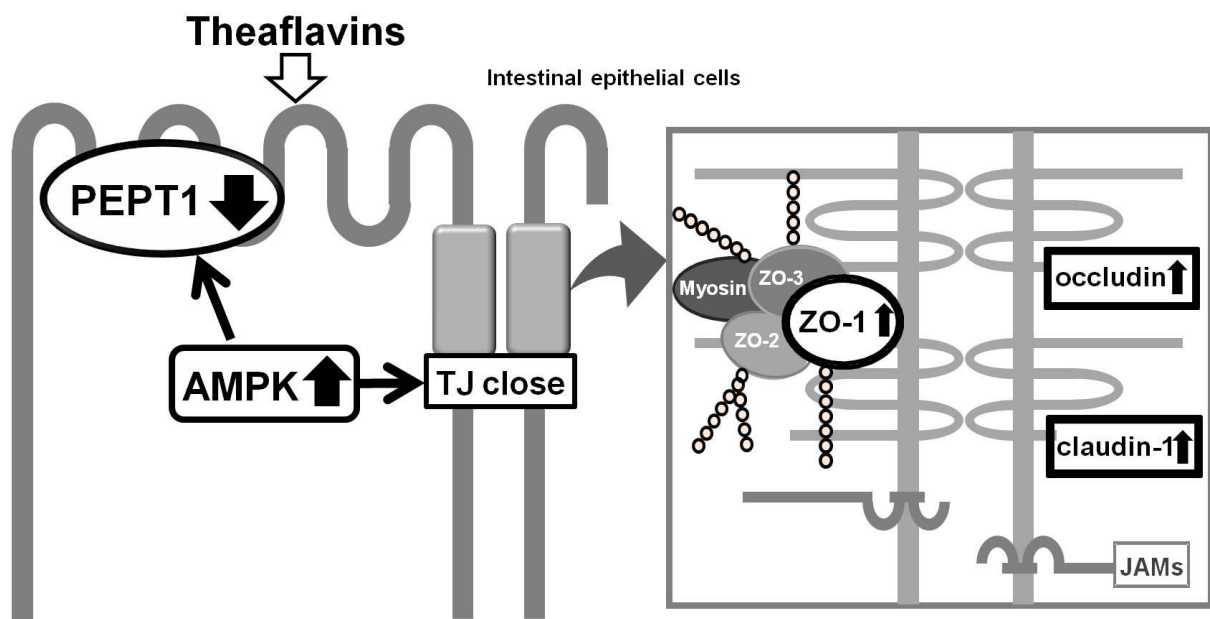


Fig. 5-1. Proposed mechanisms of TFs on intestinal membrane (Caco-2 cells) in this study.

References

- 1) Yach D, Hawkes C, Gould CL, Hofman KJ. The global burden of chronic diseases: overcoming impediments to prevention and control. *JAMA* 2004, **291(21)**, 2616-2622.
- 2) Sharma M, Majumdar PK. Occupational lifestyle diseases: An emerging issue. *Indian J Occup Environ Med* 2009, **13(3)**, 109-112
- 3) Klaus Menrad. Market and marketing of functional food in Europe. *J Food Eng* 2003, **56(2-3)**, 181–188
- 4) Roberfroid MB. A European consensus of scientific concepts of functional foods. *Nutrition* 2000, **16(7-8)**, 689-691.
- 5) Israel Goldberg. Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals. *Israel Goldberg (ed)* 1994, 454-457
- 6) Siró I, Kápolna E, Kápolna B, Lugasi A. Functional food. Product development, marketing and consumer acceptance--a review. *Appetite* 2008, **51(3)**, 456-467
- 7) Scalbert A, Johnson IT, Saltmarsh M. Polyphenols: antioxidants and beyond. *Am J Clin Nutr* 2005, **81(1 Suppl)**, 215S-217S
- 8) Arjan Scheepens, Kee Tan, James W. Paxton. Improving the oral bioavailability of beneficial polyphenols through designed synergies. *Genes Nutr* 2010, **5(1)**, 75–87
- 9) Serafini M, Ghiselli A, Ferro-Luzzi A. In vivo antioxidant effect of green and black tea in man. *Eur J Clin Nutr* 1996, **50(1)**, 28-32
- 10) Azuma K, Ippoushi K, Ito H, Higashio H, Terao J. Combination of lipids and emulsifiers enhances the absorption of orally administered quercetin in rats. *J Agric Food Chem* 2002, **50(6)**, 1706-1712

- 11) Yamashita S, Sakane T, Harada M, Sugiura N, Koda H, Kiso Y, Sezaki H. Absorption and metabolism of antioxidative polyphenolic compounds in red wine. *Ann NY Acad Sci* 2002, **957**, 325-328
- 12) Donovan JL, Kasim-Karakas S, German JB, Waterhouse AL. Urinary excretion of catechin metabolites by human subjects after red wine consumption. *Br J Nutr* 2002, **87(1)**, 31-37
- 13) Shimizu M. Interaction between food substances and the intestinal epithelium. *Biosci Biotechnol Biochem* 2010, **74(2)**, 232-241
- 14) Kaminogawa S, Nanno M. Modulation of immune functions by foods. *Evid Based Complement Alternat Med* 2004, **1(3)**, 241-250
- 15) Tsuji A, Tamai I. Carrier-mediated intestinal transport of drugs. *Pharm Res* 1996, **13(7)**, 963-977
- 16) Barthe L, Woodley J, Houin G. Gastrointestinal absorption of drugs: methods and studies. *Fundam Clin Pharmacol* 1999, **13(2)**, 154-168
- 17) Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* 2001, **2(4)**, 285-293.
- 18) Benet LZ, Cummins CL. The drug efflux-metabolism alliance: biochemical aspects. *Adv Drug Deliv Rev* 2001, **50 Suppl 1**, S3-S11.
- 19) Dockray GJ. Luminal sensing in the gut: an overview. *J Physiol Pharmacol* 2003, **54 Suppl 4**, 9-17
- 20) Abreu MT, Fukata M, Arditi M. TLR signaling in the gut in health and disease. *J Immunol* 2005, **174(8)**, 4453-4460
- 21) Broeders JJ, van Eijkeren JC, Blaauboer BJ, Hermens JL. Transport of chlorpromazine in the Caco-2 cell permeability assay: a kinetic study. *Chem Res Toxicol* 2012, **25(7)**,

1442-1451

- 22) Artursson P, Karlsson J. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem Biophys Res Commun* 1991, **175(3)**, 880-885
- 23) Yamamoto S, Tomiyama M, Nemoto R, Naganuma T, Ogawa T, Muramoto K. Effects of food lectins on the transport system of human intestinal Caco-2 cell monolayers. *Biosci Biotechnol Biochem* 2013, **77(9)**, 1917-1924
- 24) Vermeer MA, Mulder TP, Molhuizen HO. Theaflavins from black tea, especially theaflavin-3-gallate, reduce the incorporation of cholesterol into mixed micelles. *J Agric Food Chem* 2008, **56(24)**, 12031-12036
- 25) Chen H, Shurlknight K, Leung T, Sang S. Structural identification of theaflavin trigallate and tetragallate from black tea using liquid chromatography/electrospray ionization tandem mass spectrometry. *J Agric Food Chem* 2012, **60(43)**, 10850-10857
- 26) Sang S, Lambert JD, Ho CT, Yang CS. The chemistry and biotransformation of tea constituents. *Pharmacol Res* 2011, **64(2)**, 87-99
- 27) Fatima M, Kesharwani RK, Misra K, Rizvi SI. Protective effect of theaflavin on erythrocytes subjected to in vitro oxidative stress. *Biochem Res Int* 2013, **2013**, 649759
- 28) Tanaka T, Inoue K, Betsumiya Y, Mine C, Kouno I. Two types of oxidative dimerization of the black tea polyphenol theaflavin. *J Agric Food Chem* 2001, **49(12)**, 5785-5789
- 29) Sang S, Lambert JD, Tian S, Hong J, Hou Z, Ryu JH, Stark RE, Rosen RT, Huang MT, Yang CS, Ho CT. Enzymatic synthesis of tea theaflavin derivatives and their anti-inflammatory and cytotoxic activities. *Bioorg Med Chem* 2004, **12(2)**, 459-467
- 30) Gupta S, Saha B, Giri AK. Comparative antimutagenic and anticlastogenic effects of green tea and black tea: a review. *Mutat Res* 2002, **12(1)**, 37-65

- 31) Khan N, Mukhtar H. Tea polyphenols for health promotion. *Life Sci* 2007, **81(7)**, 519-533
- 32) Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004, **79(5)**, 727-747
- 33) Henning SM, Aronson W, Niu Y, Conde F, Lee NH, Seeram NP, Lee RP, Lu J, Harris DM, Moro A, Hong J, Pak-Shan L, Barnard RJ, Ziaee HG, Csathy G, Go VL, Wang H, Heber D. Tea polyphenols and theaflavins are present in prostate tissue of humans and mice after green and black tea consumption. *J Nutr* 2006, **136(7)**, 1839-1843
- 34) Mulder TP, van Platerink CJ, Wijnand Schuyt PJ, van Amelsvoort JM. Analysis of theaflavins in biological fluids using liquid chromatography-electrospray mass spectrometry. *J Chromatogr B Biomed Sci* 2001, **760(2)**, 271-279
- 35) Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. *J Nutr* 2011, **141(5)**, 769-776
- 36) Steinberg GR, Kemp BE. AMPK in health and disease. *Physiol Rev* 2009, **89(3)**, 1025-1578
- 37) Hardie DG. AMPK: a key regulator of energy balance in the single cell and the whole organism. *Int J Obes (Lond)* 2008, **32 Suppl 4**, S7-S12
- 38) Kim I, He YY. Targeting the AMP-activated protein kinase for cancer prevention and therapy. *Front Oncol* 2013, **3**, 175
- 39) González-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. *Biochim Biophys Acta* 2008, **1778(3)**, 729-756
- 40) Matsui T, Matsumoto K. Antihypertensive peptides from natural resources. *Advances in Phytomedicine* 2006, **2**, 255-271

- 41) Nagaoka S, Futamura Y, Miwa K, Awano T, Yamauchi K, Kanamaru Y, Tadashi K, Kuwata T. Identification of novel hypocholesterolemic peptides derived from bovine milk beta-lactoglobulin. *Biochem Biophys Res Commun* 2001, **281(1)**, 11-17
- 42) Kawasaki T, Seki E, Osajima K, Yoshida M, Asada K, Matsui T, Osajima Y. Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. *J Hum Hypertens* 2000, **14(8)**, 519-523
- 43) Hata Y, Yamamoto M, Ohni M, Nakajima K, Nakamura Y, Takano T. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am J Clin Nutr* 1996, **64(5)**, 767-771
- 44) Fujita H, Yoshikawa M. LKPNM: A prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology* 1999, **44(1-2)**, 123-127
- 45) Matsui T, Sato M, Tanaka M, Yamada Y, Watanabe S, Fujimoto Y, Imaizumi K, Matsumoto K. Vasodilating dipeptide Trp-His can prevent atherosclerosis in apo E-deficient mice. *Br J Nutr* 2010, **103(3)**, 309-313
- 46) Spanier B. Transcriptional and functional regulation of the intestinal peptide transporter PEPT1. *J Physiol* 2014, **592(Pt 5)**, 871-879
- 47) Liang R, Fei YJ, Prasad PD, Ramamoorthy S, Han H, Yang-Feng TL, Hediger MA, Ganapathy V, Leibach FH. Human intestinal H⁺/peptide cotransporter. Cloning, functional expression, and chromosomal localization. *J Biol Chem* 1995, **270(12)**, 6456-6463
- 48) Nässl AM, Rubio-Aliaga I, Sailer M, Daniel H. The intestinal peptide transporter PEPT1 is involved in food intake regulation in mice fed a high-protein diet. *Plos One* 2011, **6(10)**, e26407

- 49) Ziegler TR, Fernández-Estívariz C, Gu LH, Bazargan N, Umeakunne K, Wallace TM, Diaz EE, Rosado KE, Pascal RR, Galloway JR, Wilcox JN, Leader LM. Distribution of the H⁺/peptide transporter PepT1 in human intestine: up-regulated expression in the colonic mucosa of patients with short-bowel syndrome. *Am J Clin Nutr* 2002, **75(5)**, 922-930
- 50) Fei YJ, Kanai Y, Nussberger S, Ganapathy V, Leibach FH, Romero MF, Singh SK, Boron WF, Hediger MA. Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* 1994, **368(6471)**, 563-566
- 51) Daniel H, Kottra G. The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. *Pflugers Arch* 2004, **447(5)**, 610-618
- 52) Kobayashi Y, Suzuki M, Satsu H, Arai S, Hara Y, Suzuki K, Miyamoto Y, Shimizu M. Green tea polyphenols inhibit the sodium-dependent glucose transporter of intestinal epithelial cells by a competitive mechanism. *J Agric Food Chem* 2000, **48(11)**, 5618-5623
- 53) Kwon O, Eck P, Chen S, Corpe CP, Lee JH, Kruhlak M, Levine M. Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *FASEB J* 2007, **21(2)**, 366-377
- 54) Suzuki T, Hara H. Role of flavonoids in intestinal tight junction regulation. *J Nutr Biochem* 2011, **22(5)**, 401-408
- 55) Adibi SA. Regulation of expression of the intestinal oligopeptide transporter (pept-1) in health and disease. *Am J Physiol Gastrointest Liver Physiol* 2003, **285(5)**, G779-G788.
- 56) Matsui T, Tamaya K, Seki E, Osajima K, Matsumoto K, Kawasaki T. Val-Tyr as a natural antihypertensive dipeptide can be absorbed into the human circulatory blood system. *Clin Exp Pharmacol Physiol* 2002, **29 (3)**, 204-208
- 57) Zhu XL, Watanabe K, Shiraishi K, Ueki T, Noda Y, Matsui T, Matsumoto K.

- Identification of ACE-inhibitory peptides in salt-free soy sauce that are transportable across caco-2 cell monolayers. *Peptides* 2008, **29**, 338-344
- 58) Qiu J, Kitamura Y, Miyata Y, Tamaru S, Tanaka K, Tanaka T, Matsui T. Transepithelial transport of theasinensins through Caco-2 cell monolayers and their absorption in Sprague-Dawley rats after oral administration. *J Agric Food Chem* 2012, **60(32)**, 8036-8043
 - 59) Da Violante G, Zerrouk N, Richard I, Provot G, Chaumeil JC, Arnaud P. Evaluation of the cytotoxicity effect of dimethyl sulfoxide (DMSO) on Caco2/TC7 colon tumor cell cultures. *Biol Pharm Bull* 2002, **29(3)**, 1600-1603
 - 60) Ueno T, Tanaka M, Matsui T, Matsumoto K. Determination of antihypertensive small peptides, Val-Tyr and Ile-Val-Tyr, by fluorometric high-performance liquid chromatography combined with a double heart-cut column-switching technique. *Anal Sci* 2005, **21(8)**, 997-1000
 - 61) Matsui T, Tanaka T, Tamura S, Toshima A, Tamaya K, Miyata Y, Tanaka K, Matsumoto K. Alpha-glucosidase inhibitory profile of catechins and theaflavins. *J Agric Food Chem* 2007, **55(1)**, 99-105
 - 62) Way TD, Lee HH, Kao MC, Lin JK. Black tea polyphenol theaflavins inhibit aromatase activity and attenuate tamoxifen resistance in HER2/neu-transfected human breast cancer cells through tyrosine kinase suppression. *Eur J Cancer* 2004, **40(14)**, 2165-2174
 - 63) Ukil A, Maity S, Das PK. Protection from experimental colitis by theaflavin-3,3'-digallate correlates with inhibition of IKK and NF-kappaB activation. *Br J Pharmacol* 2006, **149 (1)**, 121-131
 - 64) Brandsch M, Miyamoto Y, Ganapathy V, Leibach, FH. Expression and protein kinase

- C-dependent regulation of peptide/H⁺ co-transport system in the Caco-2 human colon carcinoma cell line. *Br J Pharmacol* 2006, **149(1)**, 121-131
- 65) Peng L, Li Z, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase. *J Nutr* 2009, **139(9)**, 1619-1625
- 66) Pieri M, Christian HC, Wilkins RJ, Boyd CA, Meredith D. The apical (hPepT1) and basolateral peptide transport systems of caco-2 cells are regulated by AMP-activated protein kinase. *American Journal of Physiology, Am J Physiol Gastrointest Liver Physiol* 2010, **299**, G136-G143
- 67) Sopjani M, Bhavsar SK, Fraser S, Kemp BE, Föller M, Lang F. Regulation of Na⁺-coupled glucose carrier SGLT1 by AMP-activated protein kinase. *Mol Membr Biol* 2010, **27(2-3)**, 137-144
- 68) Bissonnette M, Tien XY, Niedziela SM, Hartmann SC, Frawley BP Jr, Roy HK, Sitrin MD, Perlman RL, Brasitus TA. 1,25(OH)₂ vitamin D₃ activates PKC- α in Caco-2 cells: a mechanism to limit secosteroid-induced rise in [Ca²⁺]_i. *Am J Physiol* 1994, **267(3 Pt 1)**, G465-G475
- 69) Murase T, Misawa K, Haramizu S, Hase T. Catechin-induced activation of the LKB1/AMP-activated protein kinase pathway. *Biochem Pharmacol* 2009, **78(1)**, 78-84
- 70) Wang C, Li Y. Research progress on property and application of theaflavins. *Am J Biotechnol* 2006, **5(3)**, 213-218
- 71) Hallows KR. Emerging role of AMP-activated protein kinase in coupling membrane transport to cellular metabolism. *Curr Opin Nephrol Hypertens* 2005, **14(5)**, 464-471
- 72) Collins QF, Liu HY, Pi J, Liu Z, Quon MJ, Cao W. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5'-AMP-activated

- protein kinase. *J Biol Chem* 2007, **282(41)**, 30143-30149
- 73) Gormand A, Henriksson E, Ström K, Jensen TE, Sakamoto K, Göransson O. Regulation of AMP-activated protein kinase by LKB1 and CaMKK in adipocytes. *J Cell Biochem* 2011, **112(5)**, 1364-1375
 - 74) Towler MC, Hardie DG. AMP-activated protein kinase in metabolic control and insulin signaling. *Circ Res* 2007, **100(3)**, 328-341
 - 75) Momcilovic M, Hong SP, Carlson M. Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. *J Biol Chem* 2006, **281(35)**, 25336-25343
 - 76) Chen A, Davis BH, Sitrin MD, Brasitus TA, Bissonnette M. Transforming growth factor-beta 1 signaling contributes to Caco-2 cell growth inhibition induced by 1,25(OH)₂D₃. *Am J Physiol Gastrointest Liver Physiol* 2002, **283(4)**, G864-G874
 - 77) Xie M, Zhang D, Dyck JR, Li Y, Zhang H, Morishima M, Mann DL, Taffet GE, Baldini A, Khoury DS, Schneider MD. A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proc Natl Acad Sci U.S.A.* 2006, **103**, 17378-17383
 - 78) Chung S, Yao H, Caito S, Hwang JW, Arunachalam G, Rahman I. Regulation of SIRT1 in cellular functions: role of polyphenols. *Arch Biochem Biophys* 2010, **501(1)**, 79-90
 - 79) Hou X, Xu S, Maitland-Toolan KA, Sato K, Jiang B, Ido Y, Lan F, Walsh K, Wierzbicki M, Verbeuren TJ, Cohen RA, Zang M. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem* 2008, **283(29)**, 20015-20026
 - 80) Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci* 2013, **70(4)**, 631-659

- 81) Camilleri M, Madsen K, Spiller R, Greenwood-Ban Meerveld B, Verne GN. Intestinal barrier function in health and gastrointestinal disease. *Neurogastroenterol Motil* 2012, **24(6)**, 503-512
- 82) Zheng B, Cantley LC. Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase. *Proc Natl Acad Sci U.S.A* 2007, **104(3)**, 819-822
- 83) Scharl M, Paul G, Barrett KE, McCole DF. AMP-activated protein kinase mediates the interferon- γ -induced decrease in intestinal epithelial barrier function, *J Biol Chem* 2009, **284(41)**, 27952-27963
- 84) Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. *Gastroenterology* 2004, **127(1)**, 224-238
- 85) Suzuki T, Hara H. Quercetin enhances intestinal barrier function through the assembly of zonula occludens-2, occluding, and claudin-1 and the expression of claudin-4 in Caco-2 cells. *J Nutr* 2009, **139(5)**, 965-974
- 86) Suzuki T, Tanabe S, Hara H. Kaempferol enhances intestinal barrier function through the cytoskeletal association and expression of tight junction proteins in Caco-2 cells. *J Nutr* 2011, **141(1)**, 87-94
- 87) Konishi Y, Hagiwara K, Shimizu M. Transepithelial transport of fluorescein in Caco-2 cell monolayers and use of such transport in in vitro evaluation of phenolic acid availability. *Biosci Biotechnol Biochem* 2002, **66(11)**, 2449-2457
- 88) Koizumi J, Kojima T, Ogasawara N, Kamekura R, Kurose M, Go M, Harimaya A, Murata M, Osanai M, Chiba H, Himi T, Sawada N. Protein kinase C enhances tight junction barrier function of human nasal epithelial cells in primary culture by transcriptional regulation. *Mol Pharmacol* 2008, **74(2)**, 432-442

- 89) De Walle JV, Sergent T, Piront N, Toussaint O, Schneider YJ, Larondelle Y. Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. *Toxicol Appl Pharmacol* 2010, **245(3)**, 291-298
- 90) Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or 2, reconstitutes tight junction strands and recruits occluding in fibroblasts. *J Cell Biol* 1998, **143(2)**, 391-401
- 91) Balda MS, Whitney JA, Flores C, González S, Cereijido M, Matter K. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol* 1996, **134(4)**, 1031-1049

Acknowledgements

I would never have been able to finish my doctoral dissertation without the help and support of great many people around me. I owe a real debt of my gratitude to all those people who have made this dissertation possible and give me the graduate experience that I will forever cherish.

First of all, I am really grateful to Prof. Toshiro Matsui who supported me enormously over these past three and a half years with the abiding and deep interest in any given situation. I have been extremely lucky to have a supervisor who taught so much about my research with his erudition and profession and also who cared my life of studying in Japan. His passion, guidance, and discipline have been indispensable to my growth as a scientist and as a person during my doctoral degree term.

Furthermore, I would like to express my gratitude to Prof. Mitsuya Shimoda and Prof. Takahisa Miyamoto for giving me a professional review of the doctoral thesis and giving me many good suggestions.

I would like to express my sincere appreciation to Asst. Prof. Mitsuru Tanaka. Thanks to his help and support, I was able to finish my course and enjoy successful life in Japan. I would like to express my gratitude to Kaori Miyazaki for taking care of all official matters to keep studying without any official problems.

In addition, I have to thank my partners and juniors, Yuri Kunitake, Naoto Hirasaki, Boonyapichest Chutinan and Junko Takeda. I was so happy to work with them. Specially, I would like to appreciate other members of Food Analysis Laboratory for kindness and good memories that make me happy. Although, I didn't mention all members' names of laboratory

and cannot express everything how much I thank all of you, but I believe that all members will realize my true feelings. Thank you all!

Most of all, I express my appreciation to my loving parents for their unconditional support until now. Without their deep love, encouragement and absolute faith to me, I would not have achieved this glory and happy day. I love so much and thank you.

I would like to thank to all of my acquaintances in Korea. First, I thank Dr. Myung-Woo Byun, who led me to the field of food science and support me. I also thank Dr. Eui-Hong Byun and Dr. Eui-Baek Byun who always give advice and support me like as my own brother. I thank Dr. Il-Yun Jeong for taking care of my work despite his busy business, and also show my appreciation to Prof. Seung-Taik Lim who directed my master degree. I really thank Prof. Hong-Sun Yook, Prof. Young-Jin Jeong, Prof. Sun-Yung Ly, Prof. Mee-Ree Kim, Prof. Seong-Ai Kim and Prof. Cheon-Bae Shon for their steadfast concern until now.

I also want to thank “Iwatani naoji foundation” and “Otsuka toshimi scholarship foundation” for their financial support during my doctoral study.

Finally, I thank all of my family, friends and acquaintances who always cheer me up. I pray for good fortune of all the people who had helped and read this thesis.

I am most grateful to you all.