Doctoral Thesis

Studies on intestinal barrier regulation by dietary polyphenols

YUNIKA MAYANGSARI

Department of Biofunctional Science and Technology
Graduate School of Biosphere Science
Hiroshima University

March 2019

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AU: arbitrary unit

5-ASA: 5-amino salisylic acid

CD: Crohn's disease

CXCL: chemokine motif ligand

DAI: disease activity index

DSS: dextran sodium sulfate

EGCG: epigallocatechin gallate

EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal-regulated kinase

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HE: hematoxylin and eosin

HED: human equivalent dose

HRP: horseradish peroxidase

IBD: inflammatory bowel diseases

IFN: interferon

Ig: immunoglobulin

IL: interleukin

IP: immunoprecipitation

JAM: junctional adhesion molecule

JNK/SAPK: stress activated-phospo kinase/c-Jun N-terminal protein kinase

LBP: lipopolysaccharide binding protein

MAPK: mitogen-activated protein kinase

MCP: monocyte chemotactic protein

MLCK: myosin light chain kinase

NF-κB: nuclear factor-κB

NOAEL: no observed adverse effect levels

PBS: phosphate buffer saline

PFA: paraformaldehyde

qRT-PCR: quantitative reversed transcriptase polymerase chain reaction

Res: resveratrol

ROS: reactive oxygen species

SDS: sodium dodecyl sulfate

SEM: standard error of the mean

Syk: spleen tyrosine kinase

TER: transepithelial electrical resistance

TJ: tight junction

TNF: tumor necrosis factor

TRAF: tumor necrosis factor receptor TNF-R-associated factor

Tyr: tyrosine

Tris: tris(hydroxymethyl)aminomethane

UC: ulcerative colitis

ZO: zonula occludens

CHAPTER 1

INTRODUCTION

1.1 Background and purpose

Dietary polyphenols provide various beneficial effects for our health. Polyphenols are bioactive substances (as secondary metabolites) widely distributed in plants and are important constituents of the human diet, they are known having antioxidant, antimicrobial, antiinflammatory, antiproliferative, and mitochondrial protective activities. Polyphenols are classified into several subgroups according to the number of phenol rings that they contain and to the structural elements that bind these rings to each other. The main groups of polyphenols are flavonoids, phenolic acids, stilbenes, and lignans (Manach et al., 2004). Recent studies demonstrate that supplemental feeding with polyphenols provides us various beneficial effects, including anti-oxidative and anti-carcinogenic effects for our health. However, their effects on intestinal mucosal functions and homeostasis are poorly understood.

The gastrointestinal epithelium forms the body's largest interface between the internal milieu of the organism and the external environment. The epithelial cells allow the individual to maintain nutrient, ions and water absorptions while establishing physical barrier from permeation of proinflammatory molecules such as microorganisms, pathogens, toxins, and allergens, from the luminal environment into the mucosal tissues and circulatory system (Suzuki, 2013; Gonzalesmariscal, 2003). Both basic and clinical studies have demonstrated that several conditions of intestinal and systemic inflammatory disorders such as

inflammatory bowel diseases (IBDs), celiac disease, food allergy, metabolic syndrome, and alcoholic liver diseases are closely related with the impaired barrier function. Accordingly, it is fundamental to maintain the intestinal barrier integrity for our health, and the modulation of the intestinal barrier by food factors could become therapeutic and preventive tools against impaired barrier related diseases.

Intestinal barrier is organized by interactions among several components, including physical, biochemical, and immune elements. The physical elements are mucous gel layer, epithelial layer, and underlying lamina propria. Tight junction (TJ) proteins connect the intestinal epithelial cells and regulate the paracellular permeability. The TJ is a multiple protein complex consisting of transmembrane and cytosolic proteins. It forms a circumferential belt that seals the paracellular space, thereby preventing the permeation of macromolecules across the epithelium (Turner, 2009; Konig et al., 2016). The TJs regulate the passage of ions and molecules through the paracellular pathway in epithelial and endothelial cells. TJs are highly dynamic structures whose degree of sealing varies according to external stimuli, physiological and pathological conditions. Four integral transmembrane proteins, occludin, claudins, junctional adhesion molecule (JAM), and tricellulin, have been identified, with the claudin family consisting of at least 24 members (Gonzalezmariscal, 2003). The intracellular domains of these transmembrane proteins interact with cytosolic scaffold proteins, such as zonula occludens (ZO) proteins. It has been reported that the TJ function and structure are dynamically regulated by cytokines and cellular signaling.

Because of the essential roles in intestinal homeostasis described above, the TJs have been attracting much attention in basic and clinical sciences in recent days. However, information about effects of food factors, including polyphenols, on the intestinal TJ regulation is scarce. Previous studies by our research group have demonstrated that some of dietary polyphenols such as quercetin, kaempferol, and naringenin strengthen and protect the intestinal TJ barrier regulation and reduce intestinal inflammation (Azuma et al., 2013; Noda et al., 2012, 2014; Suzuki et al., 2011). However, information about the roles of polyphenols in intestinal barrier function is limited. Here, we present knowledge about the biological effects of dietary polyphenols in the context of relevance to intestinal barrier protection.

The purpose of this study was to survey and select polyphenols having potentials to promote intestinal TJ barrier integrity, to examine alleviative effects of selected polyphenol on intestinal barrier defects and inflammation in both *in vitro* and *in vivo* studies, and to unveil molecular mechanisms underlying the polyphenol-mediated protection in intestinal TJ barrier.

1.2 Outline

This dissertation is organized into eight chapters as follows:

Chapter 1: Introduction, providing a brief introduction to the subject and purpose of this dissertation.

Chapter 2: Fundamentals and literature review. This chapter provides basic information about intestinal barrier function, clinical implication with impairment

of intestinal barrier function (inflammatory bowel disease), intestinal TJ regulation by cytokines, and polyphenols.

Chapter 3: Promotion of intestinal barrier function by dietary polyphenols. The objective of this chapter was to survey and to select different polyphenols including resveratrol, epigalocathecin gallate (EGCG), and delphinidin having potentials to promote intestinal TJ barrier integrity, and to investigate the promotive effect of selected polyphenol (resveratrol) in the TJ protein expression and localization in Caco-2 cell monolayers.

Chapter 4: Resveratrol prevents intestinal barrier defect and inflammation in colitic mice. The objective of this chapter was to investigate the ameliorative effect of supplemental resveratrol in a murine model of dextran sodium sulfate (DSS)-induced colitic mice, focusing on neutrophil infiltration and tight junction (TJ) barriers.

Chapter 5: Resveratrol restores tumor necrosis factor (TNF)-α-induced-interleukin (IL)-8 production in intestinal Caco-2 cell monolayers. The objective of this chapter was to examine the effect of resveratrol in the molecular mechanisms for the resveratrol-mediated suppression of neutrophil infiltration using Caco-2 cell monolayers.

Chapter 6: Resveratrol ameliorates the hydrogen peroxide (H_2O_2)-induced intestinal barrier disruption. The objective of this chapter was to investigate the resveratrol-mediated effect in Caco-2 cell monolayers with a particular focus on the modulation of TJ structure and its effect on impaired-barrier models with administration of H_2O_2.

Chapter 7: Resveratrol ameliorates the IL-6-induced intestinal barrier disruption. The objective of this chapter was to investigate the resveratrol-mediated effect in Caco-2 cell monolayers with a particular focus on the modulation of TJ structure and its effect on inflamed-barrier models with administration of IL-6.

Chapter 8: Overall conclusions, this chapter summarizes the findings of this study and subjects for future investigation.

CHAPTER 2

FUNDAMENTALS AND LITERATURE REVIEW

2.1 Intestinal barrier function

Epithelia are boundary between the internal milieu of the organism and the external environment constituted either by the air or water that surrounds the individual in case of the skin, or the content of an internal cavity or duct. Intestinal epithelium mediates selective permeability through transcellular and paracellular pathways. Transcelullar permeability is generally associated with solute transport through the epithelial cells and predominantly regulated by selective transporters for amino acids, electrolites, short chain fatty acid and sugar. Paracellular permeability is associated with transport in the space between epithelial cells and is regulated by intercellular complexes localized at the apical-lateral membrane junction and along the lateral membrane (Broer, 2008; Kunzelmann and Mall, 2002).

The intestinal barrier, primarily formed by the epithelium, is impermeable to hydrophilic solutes except where specific transporters exist (Gonzalezmariscal et al., 2008). Intestinal barrier is organized by interactions among several components, including the adhesive mucous gel layer, antibacterial peptides, and intercellular TJs. Among these components, the TJ structures constitute the major determinant of the intestinal physical barrier. The TJ is a multi-protein complex that forms a selectively permeable seal between adjacent epithelial cells and demarcates the boundary between apical and basolateral membrane domains

(Turner, 2006). TJs are composed of integral proteins that associate with cytoplasmic plaque proteins. The former function is mediating cell-cell adhesion, while the later function as a bridge beween the TJ and actin cytoskeleton (Gonzalesmariscal, 2003). Here are the main characteristics of integral transmembrane and cytosolic scaffold TJ proteins including ZO proteins, occludin, claudins and JAMs in the context of structures and functions.

2.1.1 Zonula occludens

The ZO proteins were the first TJ-specific proteins identified and 3 ZO proteins, ZO-1 (~220 kDa), ZO-2 (~160 kDa), and ZO-3 (~160 kDa) are members of the membrane-associated guanylate kinase protein family. ZOs are peripherally associated membrane proteins whose number reflects the order in which they were originally identified (Gonzalesmariscal, 2003). These proteins interact together and anchor membrane proteins like claudins, occludin and JAMs to the actin cytoskeleton. ZO proteins are scaffolds that establish numerous protein-protein interactions that cluster at the diverse kinases, phosphatases, small G proteins and nuclear and transcription factor (Lopez-baygen et al., 2006). Among the ZO proteins, the biochemical function and property of ZO-1 have been well-examined. ZO-1 localizes to the nascent cell-cell contacts in both cell cultures and animal model. Therefore, it has been proposed that ZO proteins may mediate the early assembly of TJ proteins into cell-cell contact (Suzuki, 2013). To date, intensive efforts have been made to clarify the functional role of ZO proteins, but it has been difficult to obtain clear evidence showing the importance of ZO proteins in TJ regulation. Previous studies (Balda, 2000; Umeda et al., 2004)

reported that the exogenous expression of full-length or truncated ZO-1 has only slight effect on the distribution of other TJs and it is suggested due to the redundancy in ZO proteins. However, an obvious delay in the assembly of other TJ proteins including occludin and claudins into the TJ is observed, indicating that ZO proteins have an important role in the regulation of TJ assembly.

2.1.2 Occludin

Occludin (~65 kDa) was the first integral membrane protein identified localized at TJ both in epithelial and endhotelial cells (Suzuki, 2013; Furuse et al., 1993). Occludin is a tetraspanin membrane protein with 4 transmembrane domains and 2 extracelullar loops: a short N-terminal and a long C-terminal domain project into the cytoplasm. The long C-terminal domains interact with several intracellular TJ proteins, such as ZO proteins, which are required to link occludin to the actin cytoskeleton (Al Sadi et al., 2011). The function of occludin is not yet fully understood, but numerous studies using animal and cell cultures indicate that it has crucial roles in the TJ structure and permeability in the intestinal epithelia (Al Sadi et al., 2011; Wong and Gumbiner, 2007). *In vitro* studies demonstrate that phosphorylation of occludin regulates occludin localization and TJ permeability. This phosphorylation is regulated by the balance between kinases and phosphatases responsible for the phosphorylation sites. In the epithelium, occludin is highly phosphorylated on the Ser and Thr residues and the phosphorylation has a role in the maintenance and assembly of TJ structure (Sakakibara et al., 1997; Rao, 2009).

2.1.3 Claudins

Claudins (20-27 kDa) are tetraspin membrane proteins with 1 intracellular and 2 extracelullar loops, and C-terminal and N-terminal cytoplasmic domain (Suzuki, 2013; Furuse et al., 1998). The extracellular loops of claudin molecules make hemophilic and heterophilic interactions with adjacent cells, and the interaction creates either barriers against or pores for the passage of selective molecules in the paracellular pathways (Van Itallie and Anderson, 2006). Numerous studies have demonstrated that claudins are the key component and backbone of TJs. When claudins are expressed in fibroblast, they are incorporated into TJ strands and form paired strands at the cell-cell contacts (Furuse et al., 1998). Claudins are a multigene family at least 24 members in human and mice, and each isoform shows a unique expression pattern in tissues and cell lines. In mouse intestines, claudin-1, -2, -3, -4, -5, -6, -7, -8, -10, -12, -13, -14, -15, -17, and, -18 are detected at the gene expression level, but the relative expression of each isoform varies throughout the segments of the intestine (Holmes et al., 2006). In the intestines, claudin-1, -3, -4, -5, -8, -9, -11, and -14 can be categorized as barrier-forming claudins, while claudin -2, -7, -12 and -15 are pore-forming claudins (Suzuki, 2013; Amasheh, 2002). As in the case with occludin, some claudin isoform are phosphorylated in the cells, and this phosphorylation is associated with localization and paracelullar permeability.

2.1.4 Junctional adhesion molecules

The JAM family belongs to the immunoglobulin (Ig) superfamily and characterized by 2 extracelullar Ig domains, one transmembrane domain, and one

intracellular C-terminal domain. Based on sequence similarities in the cytoplasmic domains, JAMs are divided into 2 subfamilies, first subfamily are JAM-B, and JAM-C (or JAM-1, -2, and -3), and second subfamily are JAM-4, coxsackievirus and adenovirus receptor, endothelial selective adhesion molecule, and the brain- and testis-specific immunoglobulin superfamily (Suzuki, 2013). The extracellular N-terminal domains of the JAM family members bind to various ligands through homophilic and heterophilic interactions (Bazzoni, 2003). The hemophilic interaction involving the JAM members have a role in the formation of TJs and the cell-cell border. In contrast, the heterophilic interaction function in cell-cell adhesion, associated between leucocytes and epithelial cells, platelet activation and virus recognition. JAM members are expressed in various cell types including epithelial, endothelial, and immune cells, and exhibit different expression patterns in both tissue- and cell type-specific manner. In epithelial cells, JAM-A, JAM-4, and CAR are expressed and involved in TJ regulation. JAM-A (~43 kDa) participates in the regulation and maintenance of the TJ barrier. Treatment of intestinal T84 cells with monoclonal JAM-A antibodies inhibits the resealing of the TJs, indicated by delays in TER recovery and occludin assembly (Liu et al., 2000). Animal studies using JAM-A knockout mice have also shown the importance of JAM-A in intestinal barrier function (Laukoetter et al., 2007). The JAM-A knockout mice exhibit higher permeability to dextran and myeloperoxidase activity in the colon compared to wild-type mice. Further, the colonic injury and inflammation induced by dextran sodium sulfate (DSS) are more severe in the JAM-A knockout mice than in wild-type mice.

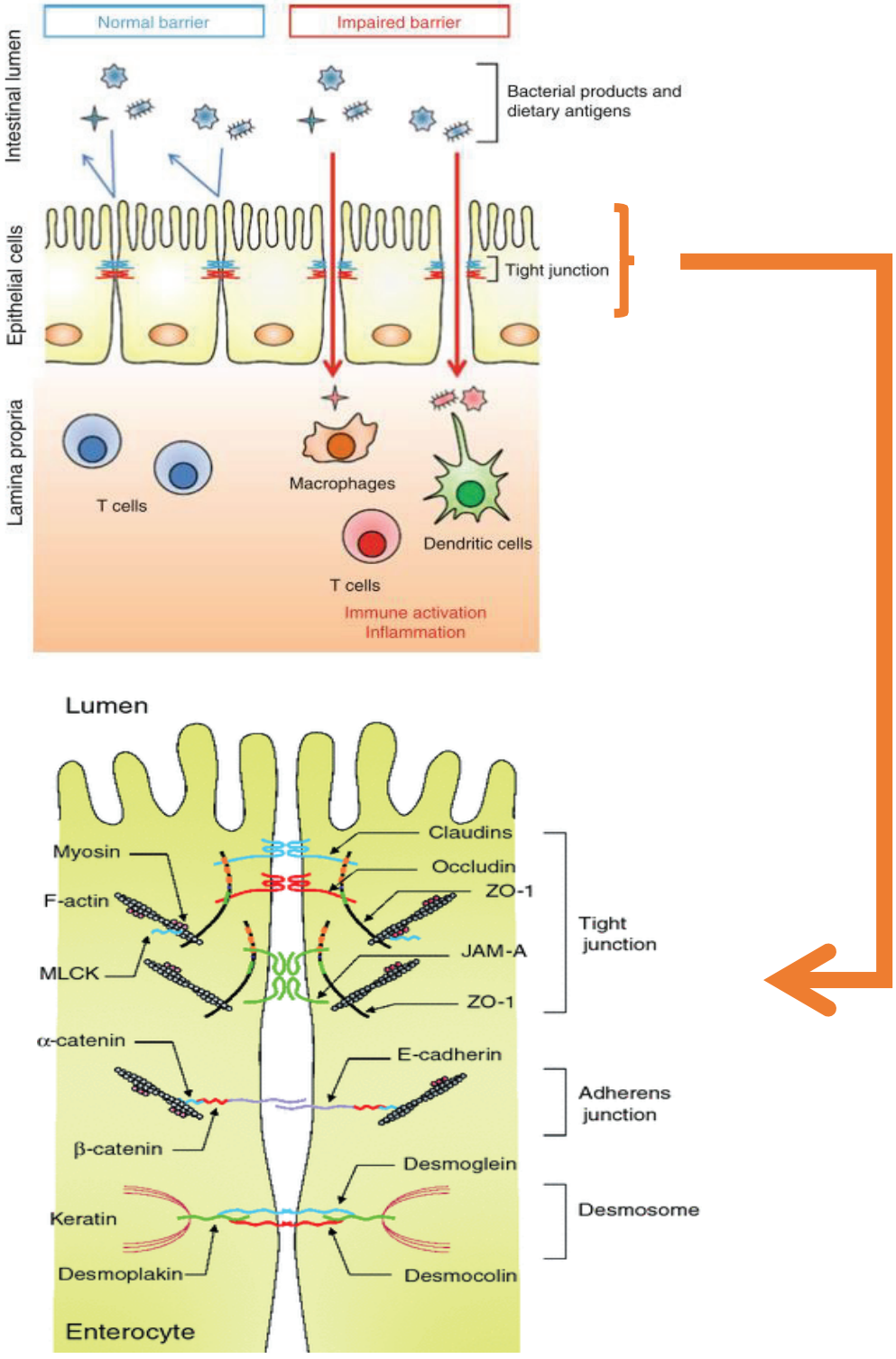


Figure 2.1. Molecular structure of the intercellular junction of intestinal epithelial cells (Suzuki, 2013)

2.2 Clinical implication with impairment of intestinal barrier function: inflammatory bowel disease

Inflammatory bowel diseases comprise a group of conditions characterized by uncontrolled inflammation in gastrointestinal tracts (Abouaf Tabet, 2014). They encompass two major forms—ulcerative colitis and Crohn's disease—and the patients suffer from diarrhea, abdominal pain, gastrointestinal bleeding and weight loss (Szigety et al., 2010; Kang et al., 2017). Crohn's disease (CD) is an IBD that causes inflammation anywhere along the lining of the digestive tract, while ulcerative colitis (UC) causes long-lasting inflammation in some part of the digestive tract (mainly the colon). UC is associated with more frequent anemia, bloody stool, and mucous and bloody stool compared with CD, for which the frequency of bloody stool is less frequent. This is attributed to the characteristics of CD, in which lesions do not easily bleed and the rectum is less frequently affected (Tontini et al., 2015). Patients with UC tend to develop intestinal hemorrhage on a daily basis during flare-ups, and many of them visited a specialist and received a definitive diagnosis at a relatively early phase. As described above, this is due to the pathological characteristics of UC, with patients having bloody stool and mucous and bloody stool more often compared with CD. On the other hand, patients with CD tend to have more adverse influences in their daily lives, such as absence from work, which seems to be due to malaise during flare-ups. However, the duration of a flare-up was not long in many patients. UC affects areas near the intestinal lumen, from the mucosal layer to the submucosal layer, representing shallow inflammation. In contrast, CD causes deeper inflammation, often reaching the muscle layer. UC, with shallower

inflammation, is generally acknowledged as a milder enterocolitis than CD, which causes deeper inflammation. Thus, CD has a greater impact on labor productivity compared with UC, from a pathological point of view (Ueno et al., 2017).

The exact etiology of IBD is not well known. There are several factors that have been postulated to have an effect on the development of this group of diseases, which include but are not limited to bacterial contamination, a change in the immune system, and genetic variations. For instance, a mutation in the NOD2 gene is associated with an increase susceptibility to IBD via production of proinflammatory cytokines (Ogura et al., 2001). While genetic tendency plays a key role in immune-mediated diseases, the major in factors appears caused by environment (Ponder and Long, 2013). Recent studies have also reported that environmental factors such as smoking, psychological stress, childhood immunological events and food factors are involved in the development of IBD (Niewiadomski et al., 2016; Cabré and Domènech, 2012).

The prevalence and incidence of these chronic diseases have recently increased and diverged widely among geographic regions. The major subtypes of IBD, including CD and UC, have a high prevalence rate in the world, with North America noting the highest frequency of people suffering with CD. In addition, statistics show that an estimated 129,000 people live with the disease in Canada (Molodecky et al., 2012). Although the onset of the disease usually occurs during adulthood, children are increasingly being diagnosed with IBD. The incidence and prevalence of IBD, including UC and CD, has been increasing rapidly in Asia including Japan, Korea, China, Singapore and other Asian countries over the last two decades (Yang et al., 2001). Preliminary studies have found that a Western

style diet, hygiene, and childhood immunological factors are associated with IBD in Asia, all of which are the results of urbanization (Tang et al., 2013). The IBD-susceptible genes that have been firmly associated with CD in the Caucasia population such as NOD2*,* IL-23R*, and* ATG16L1 showed no correlation with CD in Japanese, Korean, and Chinese Han patients with CD, except IL-23R had a weak association with Korean CD patients. However, single nucleotide polymorphisms (rs3810936, rs6478108, and rs7848647) on TNFSF15 have been identified to be more strongly associated with Asian CD patients than with Caucasian CD patients (Cheon, 2013).

Treating IBD often involves use of medications that can diminish the symptoms and decrease the inflammation in the colon lining. Group of drugs including 5-aminosalicylic acid (5-ASA), corticosteroids, thiopurines, methotrexate, cyclosporin, and anti-TNF therapy are commonly used to treat IBD (Ordas et al., 2012). Sulfasalazine and mesalazine, moiety of 5-ASA group, are the most commonly prescribed anti-inflammatory drugs in IBD and have been used successfully for the treatment of UC and CD. The mode of action of 5-ASA is unclear, but a local effect by a variety of mechanism is proposed, including the modulation of inflammation mediators such as oxygenase metabolism, platelet activating factors, cytokines, and interleukins (Kopylov et al., 2014; Van Staa et al., 2005). However, there are serious side effects associated with the long-term use of these agents such as increased risk of infection, malignancy, and autoimmunity. Therefore, there is an urgent need for novel, efficient and safe strategies for IBD management. Understanding the properties and characteristics of the gastrointestinal tract can assist in the identification of the biomolecular

mechanisms involved in IBD and can bring new ideas for the development of therapies for such chronic disorders. A growing body of evidence in murine and human studies suggests that IBDs are initiated and developed by a defect in the epithelial barrier and altered mucus production, leading to an increase in intestinal permeability and toxins adherence in the intestinal cells (Swidsinski et al., 2002). Due to the crucial roles of intestinal barrier in the IBDs progression, much attention has been attracted to intestinal barrier regulation for therapeutic and preventive approaches to the disorders.

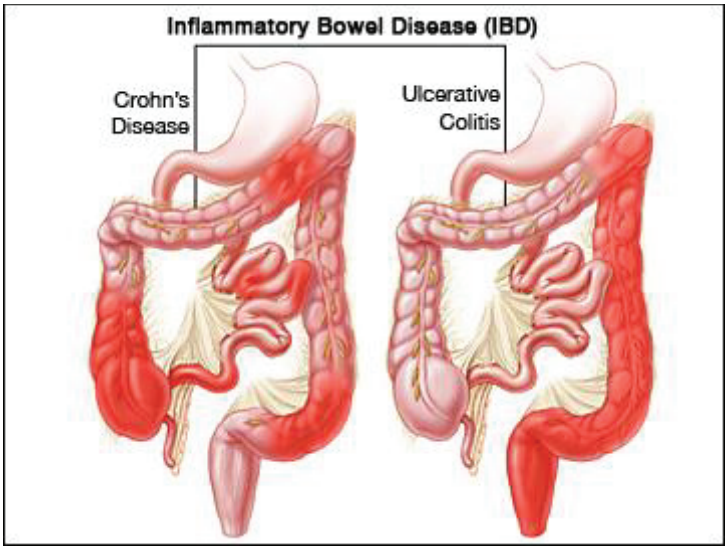


Figure 2.2. Anatomic distribution of Crohn's disease and ulcerative colitis (Johns Hopkins Medicine, 2003).

2.3 Intestinal TJ regulation by cytokines

In IBD, the immune defense against intestinal microbes fails at two levels: (1) the epithelial mucosal barrier is impaired and (2) the innate and acquired host immune responses are altered. The immunopathogenesis of IBD occurs in three temporally distinct stages: (1) penetration of luminal contents into underlying tissues which may be facilitated by environmental factors such as infection or

inherent defects in mucosal barrier, (2) impaired clearance of foreign material from the bowel wall which may be due to defective secretion of proinflammatory cytokines by macrophages and (3) a compensatory acquired immune response which leads to a chronic inflammatory response and gives rise to characteristic IBD lesions (Matricon, 2010). Moreover, epithelial cells are the first line of defense against invading pathogens.

Cytokines are abundantly produced by the cells of the gut-associated immune system maintaining lymphocyte homeostasis under both steady-state and inflammatory condition. The roles of cytokines in intestinal TJ regulation under pathophysiological conditions have been well investigated using cell cultures and animal model. Suzuki (2013) summarized recent knowledge regarding the cytokine-mediated regulation of intestinal TJ barrier function, and several cytokines associated with intestinal TJ regulation are interferon (IFN)-γ, TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-13 and IL-17.

Interferon-gamma is a pleiotrophic cytokine produced predominantly by natural kill cells and CD4+ T cells that under normal circumstances, and particularly during infection or inflammation, will be a component of the intestinal milieu. Use of colon-derived epithelial cell lines and, to a less extent, murine in vivo analyses, have revealed that IFN-γ can increase epithelial permeability as gauged by markers of paracellular permeability and bacterial transcytosis, with at least a portion of the bacteria using the transcellular permeation pathway (Beaurepaire et al., 2009). TNF- α are commonly used to treat IBDs, the intestinal barrier damaged-related diseases, instead of non-steroidal anti-inflammatory drugs. Al-Sadi (2013) reported that TNF-α causes a

rapid activation of extracellular signal-regulated kinase (ERK)1/2, which in turn leads to the activation of serum response element on the myosin light chain kinase (MLCK) promoter, leading to MLCK gene activation, transcription, protein expression, and MLCK-dependent opening of the intestinal TJ barrier. Their studies also demonstrated the feasibility of therapeutic targeting of ERK1/2 to prevent the TNF-α–induced increase in intestinal TJ permeability *in vivo*. IL-6 is a pleiotropic cytokine whose expression is important for the host response to a number of infections, exerts antigen-specific immune responses, and has both pro- as well as anti-inflammatory effects (Alonzi et al., 1998). Suzuki et al. (2011) showed that IL-6 increases TJ permeability by stimulating the expression of channel-forming claudin-2. IL-6 increases the cation-selective TJ permeability without any changes to uncharged dextran flux or cell viability in Caco-2 cells. In addition, the colonic mucosa of mice injected with IL-6 also exhibits an increase in claudin-2 expression. IL-6 is a pleiotropic cytokine whose expression is important for the host response to a number of infections, exerts antigen-specific immune responses, and has both pro- as well as anti-inflammatory effects.

In IBDs, genomic studies have shown that IL-23 relates with the progression of CD. IL-23 is a proinflammatory cytokine that results in activation of signal transducers and activators of transcription factors, which are important downstream mediators of inflammation (Yen et al., 2006). Furthermore, IL-17 expression is increased in colons from patients with UC and CD and other members of IL-23R regulated pathways are linked to both UC and CD (Fujino et al., 2003). IL-10 has also been postulated to play a role in IBD. One of the first

mouse models of IBD resulted from the generation of the IL-10 knockout mouse, which develops spontaneous inflammation (Kühn et al., 1993).

2.4 Polyphenols

Polyphenols are phytochemicals, found largely in fruits, vegetables, tea, coffee, chocolates, legumes, cereals, and beverages. There are over 8000 polyphenols identified in nature and their main functions are as antioxidant. Polyphenols protect tissues from free radical damage, UV radiation and pathogen aggression. In the last two decades, there has been more interest in the potential health benefits of dietary polyphenols as antioxidant. The average 100 grams fresh weight of fruits (grapes, apple, pear, cherries, and berries) contain up to 300 mg of polyphenols. Typically, a cup of tea or coffee contains more than 100 mg of polyphenols. In addition, cereals, vegetables, dry legumes and chocolate also contribute to the polyphenolic intake and thereby protect our body from chronic diseases (Pandey and Rizvi, 2009). Dietary polyphenols are the subject of enhancing scientific interest due to their possible beneficial effects on human health. They are usually provided to the food as color, flavor, bitter, and astringent, and maintain stability from oxidation. Several epidemiological studies and associated meta-analyses strongly showed that the consumption of these polyphenols offered better protection against chronic diseases such as cancers, cardiovascular diseases, cerebrovascular diseases, diabetes, ageing and neurodegenerative diseases (Ganesan and Xu, 2017).

Polyphenols are divided into four different categories based on the presence of number of phenolic groups and structural elements (Manach et al., 2004):

1. Flavonoids: Have a potential effect on radical scavenging and inflammatory reactions. They are predominantly found in fruits, vegetables, legumes, and green tea. They are further divided into a number of subgroups namely, flavones, flavonols, flavanones, isoflavones, anthocyanidins, chalcones, and catechins.
2. Stilbenes: Found in product of grapes, melinjo and peanuts. Resveratrol is the most well-known compound among the group.
3. Lignans: Found in seeds like flax, linseed, legumes, cereals, grains, fruits, algae, and certain vegetables.
4. Phenolic acids: Found in coffee, tea, cinnamon, blueberries, kiwis, plums, apples, and cherries and have two subgroups, namely hydroxybenzoic acids, and hydroxycinnamic acids.

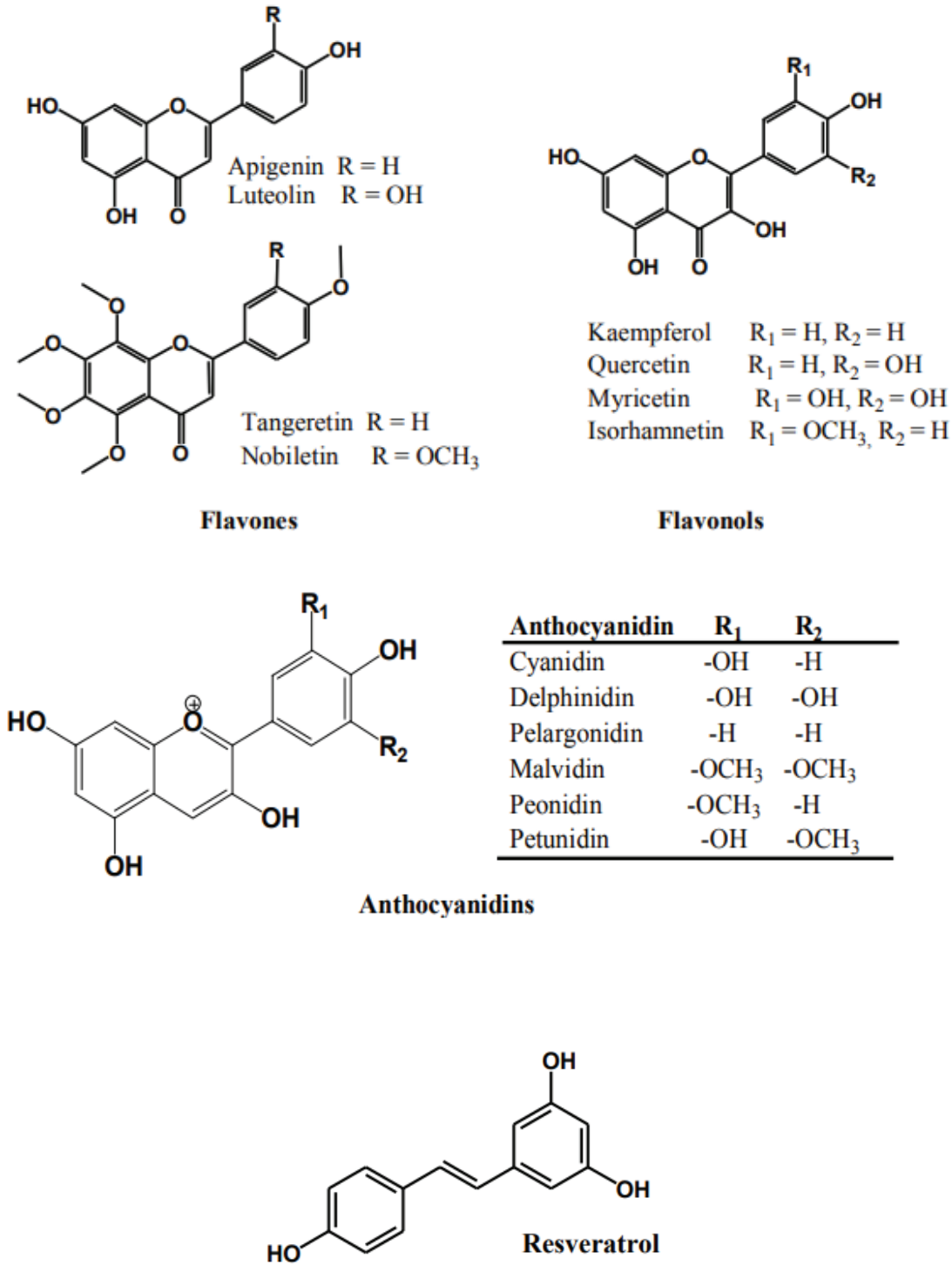
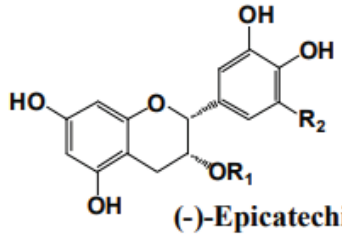


Figure 2.3. Chemical structures of polyphenols (Tsao, 2010)



(-)-Epicatechin: $R_1 = R_2 = H$;
(-)-Epicatechin gallate: R_1 = gallyl, $R_2 = H$;
(-)-Epigallocatechin: $R_1 = H$, $R_2 = OH$;
(-)-Epiallocatechin gallate: R_1 = gallyl, $R_2 = OH$;

Figure 2.3. Chemical structures of polyphenols (Tsao, 2010) (continued)

Fruit and beverages such as tea, coffee and fruit juices constitute the main sources of polyphenols. Certain polyphenols such as quercetin are found in all plant products (fruit, vegetables, cereals, leguminous plants, fruit juices, tea, infusions, etc), whereas others are specific to particular foods (flavanones in citrus fruit, isoflavones in soya, phloridzin in apples). In most cases, foods contain complex mixtures of polyphenols, which are often poorly characterized. Furthermore, numerous factors other than variety may affect the polyphenol content of plants; these factors include ripeness at the time of harvest, environmental factors, processing, and storage.

2.4.1 Resveratrol

Resveratrol (*trans*-3,5,4′-trihydroxystilbene or 5-[(E)-2-(4-hydroxyphenyl)-ethenyl]benzene-1,3-diol; $C_{14}H_{12}O_3$) was first isolated from the roots of white hellebore (*Veratrum grandiflorum*) in 1940, and later, in 1963 from the roots of *Polygonum Cupsidatum*, a plant used in traditional Chinese and Japanese medicine (Timmers et al., 2012). Resveratrol has been found in plant and plant products such as grapes, peanuts, and red wines (Szekeres et al, 2011).

The concentration of resveratrol which varies among the different sources is presented in the table below.

Table 2.1. Resveratrol concentration found in different sources

Source	Resveratrol concentration
Red wine	Non-detectable levels to 14.3 μg/g (average of 1.9 ± 1.7 μg/g) (Stervbo et al., 2007)
Grape products	0.26-270.46 μg/g (Timmers et al., 2012)
Peanuts	0.022 to 1.792 ug/g (Timmers et al., 2012)

Numerous *in vivo* animal studies and *in vitro* studies indicate that resveratrol has beneficial effects in the insulin sensitivity and resistance, neurodegenerative diseases, inflammation, aging, cardiovascular diseases and cancer. However, the low bioavailability of resveratrol still becomes an issue to optimize the potency and effectiveness of therapeutic properties of resveratrol. The effects of resveratrol are observed as a result of resveratrol alone or often a result of resveratrol in combination with other polyphenols, which is also relevant since we often come across resveratrol in combination with other polyphenols in our diet. The clinical trials addressing the possible antioxidant and antiinflammatory effect of resveratrol reports that resveratrol reduces the oxidative and inflammatory responses by increasing antioxidant transcription factor (Nrf-2 DNA binding activity) and decreasing lipopolysaccharide concentrations. The responses are seen in the consumption of 100 mg resveratrol + 75 mg polyphenols before meal in 10 healthy people (Ghanim et al., 2011). Another study presents results showing that a year daily consumption of 350 mg grape extract with 8.1 mg resveratrol supplementation decrease the level of some transcription factors related to inflammation (Tomé-Carneiro et al., 2013).

Resveratrol's ability to decrease oxidative stress and inflammation is beneficial since reactive oxygen species (ROS) normally generated by oxidative stress and inflammation, are involved in the progression of many diseases such as atherosclerosis, diabetes mellitus, chronic obstructive pulmonary disease, and cancer (Alfadda and Sallam, 2012). Resveratrol is also able to cause a decrease in plasma glucose and insulin concentration in subjects with impaired glucose tolerance or type 2 diabetes receiving resveratrol in doses of 1-2 g or 2x5 mg/day for 4 weeks indicating that resveratrol supplementation is improving insulin sensitivity (Crandall et al., 2012). Moreover, indication of cardiovascular protective effect of resveratrol is also reported in a clinical trial using resveratrol doses of 10 mg/day in 3 months resulting in improved flow-mediated dilation in obese or post infarct subjects indicating an improvement in cardiovascular health (Magyar et al., 2012).

2.4.2 Delphinidin

Delphinidin (2-(3, 4, 5-trihydroxyphenyl) chromenylium-3, 5, 7-triol) is an anthocyanin, a water-soluble flavonoid phenolic compound, commonly found in higher plants as a primary plant pigment and also an antioxidant. Delphinidins represent the most potent antioxidant anthocyanin species owed to largest number of hydroxyl groups in the B-ring. Delphinidin is found in many brightly colored fruits, vegetables and in dietary supplements that are currently consuming as complementary cancer medicine (Meiers et al., 2001).

Delphinidin has been shown to have antioxidant, antimutagenesis, anti-inflammatory and anti- angiogenic properties. In spinal cord injury rat model,

delphinidin showed to suppress the level of inflammatory factors including TNF-α, IL-6, cyclooxygenase-2 and caspase-3, as well as nuclear factor kappa B (NFκB) protein expression (Wang et al., 2017). In human skin model of psoriasis, a chronic inflammatory disorder of skin and joints, delphinidin showed to decrease the expression of markers proliferation and inflammation including proliferating cell nuclear antigen, inducible nitric oxide synthase and antimicrobial peptides S100A7-psoriasin and S100A15-koebnerisin which are often induced in psoriatic skin (Chamcheu et al., 2015). In the colon carcinoma cells model, delphinidin was found to reduce intracellular oxidative stress levels and indicated to play a role in the protection of gut from mold-induced genotoxicity (Aichinger et al., 2018).

2.4.3 Epigallocatethin gallate

Epigallocatechin gallate is a member of catechin, a polyphenol contained in green and black teas. Research findings suggest that the epigallocatechin-3-gallate found primarily in green tea, and theaflavin-3,3′-digallate, a major component of black tea, are the two most effective anti-cancer factors found in tea. Among tea catechin, EGCG is the most effective in reacting with most reactive oxygen species and may account for 50–80% of the total catechin in tea (Khan and Mukhtar, 2007). The possible beneficial health effects of tea are being extensively investigated and have received a great deal of attention in recent times. Furthermore, EGCG has shown an efficient ability in scavenging free radicals species. One hypothesis to explain these properties is a low reduction potential of EGCG due to its high capacity for giving an electron. Electron delocalization in the molecular structure is described as a property of polyphenolic compounds

which could in part be responsible for their antioxidant activity. In the catechin skeleton, the saturation of the heterocyclic ring prevents electron delocalization between the A and the B ring. Thus, for green tea catechins, the antioxidant potential mainly comes from the strong presence of hydroxyl groups in their molecular structures. EGCG, with 8 hydroxyl groups and with a gallate moiety in C-3 is a better electron donor than the others catechins and thus the best scavenger of free radical species (Higdon and Frei, 2003).

Recently, it has been reported that green tea consumed within a balanced controlled diet improve the overall antioxidative status and protect against oxidative damage in humans. Many epidemiological studies have been conducted to investigate the effects of EGCG on human cancer incidence. Most of the studies showing an inverse relationship between tea consumption and development of cancer were conducted on gastrointestinal cancers in Japan and China where green tea is the main form of tea consumed. Studies in northern Italy have suggested a protective effect of EGCG against oral, pharyngeal and laryngeal cancer. In a case-control study in Shanghai, frequent consumption of green tea has been shown to be associated with a lower incidence of esophageal cancer. A protective effect against gastric cancer by EGCG has also been suggested from studies in Japan, northern Turkey and central Sweden (Lambert and Yang, 2003). Several studies have also demonstrated that EGCG shows therapeutic benefit in numerous inflammatory diseases such as atherosclerosis, arthritis, dry eyes disease, and spinal cord injury (Tipoe et al., 2007; Lee et al., 2011).

CHAPTER 3

PROMOTION OF INTESTINAL BARRIER FUNCTION BY DIETARY POLYPHENOLS

3.1 Introduction

Intestinal barrier function is closely related to intestinal health and diseases. Moreover, it is regulated by the mucous layer of epithelial cells, antibacterial peptides, and tight junction (TJ) structure (Suzuki, 2013). Intestinal TJ structures are organized by multiple integral proteins such as transmembrane and cytosolic proteins, which provide a physical barrier to the permeation of luminal pro-inflammatory molecules such as pathogens, toxins, and dietary antigens (Denker and Sabath, 2011). Some basic scientific and recent clinical studies (Landy et al., 2016; Turner, 2009) have shown that there is a close association between intestinal barrier defect and the pathogenesis of different inflammatory diseases. Intestinal barrier defects that result in the penetration of pro-inflammatory molecules induce abnormally robust inflammatory responses, as in the case of inflammatory bowel disease (IBD). Due to the crucial roles of intestinal barrier regulation in health, much attention is being paid to the involvement of TJ in therapeutic and preventive approaches against some disorders. Previous studies by our research group have demonstrated that dietary polyphenols such as quercetin, kaempferol, and naringenin strengthen and protect the intestinal TJ barrier and reduce intestinal inflammation (Azuma, et al., 2013;

Noda et al., 2012, 2014; Suzuki et al., 2011). However, information on the roles of polyphenols in intestinal barrier function is limited.

The aims of study of this chapter was 1) to survey and to select polyphenols including resveratrol, EGCG, and delphinidin having potentials to promote intestinal TJ barrier integrity, and 2) to investigate the promotive effect of a selected polyphenol (resveratrol) in the TJ protein expression and localization in Caco-2 cell monolayers.

3.2 Materials and methods

3.2.1 Chemicals

Rabbit anti- ZO-1, ZO-2, JAM-A, occludin; claudin-1, -3, and -4; p-44/42 mitogen-activated protein kinase (MAPK)/ ERK 1/2; and goat Alexa Flour 488-conjugated anti-rabbit Ig G were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG was purchased from Sigma-Aldrich (St. Louis, MO, USA). Resveratrol and EGCG were purchased from Tokyo Chemical Industry (Tokyo, Japan) and delphinidin was purchased from Extrasynthese (Lyon, France). Cell culture reagents and supplies were purchased from Thermo Fisher Scientific. All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

3.2.2 Cell culture

Human intestinal Caco-2 cells (HTB-37; American Type Culture Collection, Manassas, VA, USA) were propagated and maintained under standard cell culture conditions as described previously (Suzuki and Hara, 2010; Suzuki et

al., 2011). The cells were seeded into permeable polyester membrane filter supports (Transwell, 12-mm diameter, 0.4-μm pore size; Corning Inc., NY, USA) at a density of 0.25×10^6 cells/cm^2. All experiments were conducted on days 12-14 post-seeding. Cultures were used between passage 48 and 65 and the medium was refreshed every 3 days.

3.2.3 Treatment of Caco-2 cells with polyphenols

Caco-2 cell monolayers were incubated with or without 100 μmol/L of resveratrol, delphinidin, and EGCG at their apical sides. Transepithelial electrical resistance (TER) was measured before and at 1, 3, 6, 12, 24 and 48 h after the treatment with polyphenols as described below.

3.2.4 Treatment of Caco-2 cells with resveratrol

Caco-2 cell monolayers were incubated with or without resveratrol (50, 100, and 200 μmol/L) at their apical or basal sides. Transepithelial electrical resistance (TER) was measured before and at 1, 3, 6, and 12 h after the treatment with resveratrol as described below. Caco-2 cell extracts were collected after 12 h of incubation for immunoblot analysis. The cell monolayers were fixed for immunostaining as described below.

3.2.5 Measurement of TER across Caco-2 cell monolayers

Intestinal TJ barrier integrity was evaluated by measuring TER across Caco-2 cell monolayers grown on Transwell filter supports. TER was measured using a Millicell-ERS system (Millipore, Darmstadt, Germany). The cell monolayers showed a TER of 1000-1500 Ω·cm^2 without any treatment (data not shown).

3.2.6 Preparation of detergent-insoluble fractions and whole-cell extracts

Detergent-insoluble fractions, which correspond to actin-cytoskeleton-associated protein content, and whole-cell extracts were prepared as described previously (Suzuki and Hara, 2010). Caco-2 cell monolayers were washed with ice-cold phosphate-buffered saline and incubated for 5 min at 4 °C with 200 μL of lysis buffer-CS [1% Triton X-100, 5 mmol/L egtazic acid (EGTA) in 50 mmol/L 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) containing protease inhibitors (5 μg/L aprotinin, 3 μg/L leupeptin hemisulfate, 5 mmol/L benzamidine hydrochloride, and 1 mmol/L phenylmethylsulfonyl fluoride) and phosphatase inhibitors (25 mmol/L glycerol-2-phosphate, 2 mmol/L sodium orthovanadate, and 10 mmol/L sodium fluoride); pH 7.4]. Cell lysates were centrifuged at 15,600 g for 10 min at 4 °C to sediment the high-density actin-rich fraction. The precipitate obtained was suspended in 100 μL of lysis buffer F [1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) Triton X-100, and 1% (w/v) sodium deoxycholate in 30 mmol/L Tris solution containing protease and phosphatase inhibitors; pH 7.4]. Protein concentrations were measured using the bicinchoninic acid method (Thermo Fisher Scientific).

3.2.7 Immunoblot analysis

The protein expression levels of ZO-1 and -2; occludin; JAM-A; and claudin-1, -3, and -4 in Caco-2 cells were determined by immunoblot analysis as described previously (Murakami et al., 2016; Oyama et al., 2017; Suzuki and Hara, 2010). Protein extracts were mixed with a half volume of Laemmli sample buffer (3× concentrated) containing 6% (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) β-mercaptoethanol, and 0.02% (w/v) bromophenol blue in 188 mmol/L Tris

solution (pH 6.8), and heated to 100 °C for 5 min. Proteins (20 μg) were separated by SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blotted for the proteins using the corresponding primary antibodies in combination with an HRP-conjugated anti-rabbit IgG antibody. Protein quantification was done by densitometric analysis of specific bands on the immunoblots using Image J software (NIH, Bethesda, Maryland, USA).

3.2.8 Statistical analysis

All data have been expressed as mean ± standard error of measurement (SEM). Statistical analyses were performed using one-way analysis of variance followed by Tukey-Kramer post-hoc test. *P* values < 0.05 were considered statistically significant. All analyses were performed using SPSS software (version 19; IBM Corporation, Armonk, NY, USA).

3.3 Results

3.3.1 TER

The degree of tightness of a TJ can be evaluated by measuring TER across cells. In order to evaluate the effect of resveratrol on TJ integrity in Caco-2 cell monolayers, the cell monolayers were incubated with or without resveratrol and studied. Among three polyphenols tested including resveratrol, EGCG and delphinidin, only resveratrol showed to promote the TER of Caco 2 cell monolayers and maintained until 24 h after administration (Fig. 3.1)

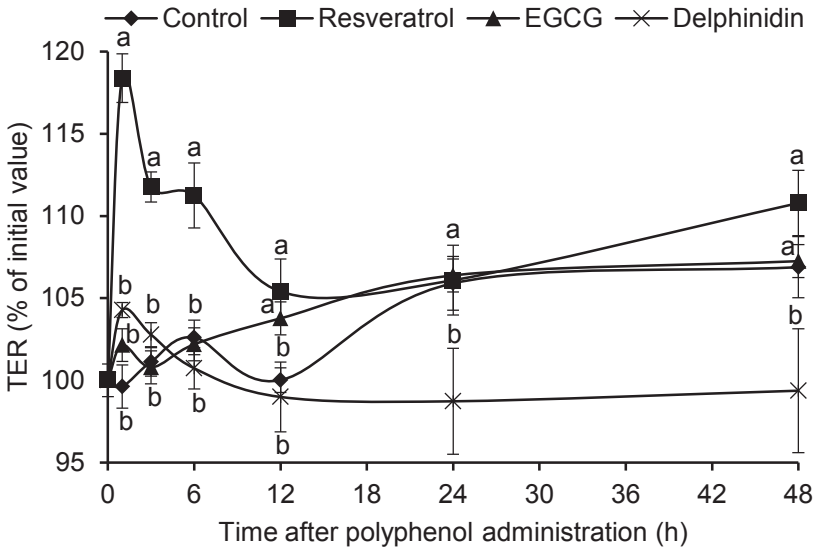


Figure 3.1. TER measurement of different polyphenols. All the values are expressed as mean ± standard error of measurement (n = 3). Means without a common letter differ significantly from each other ($P < 0.05$).

TER values for Caco-2 cell monolayers incubated with 50, 100, and 200 μmol/L resveratrol at the apical side rapidly increased in a dose-dependent manner in the first 1 h after the treatment and were almost maintained until 12 h. TER was higher after treatment with 100 and 200 μmol/L resveratrol than after treatment with 0 and 50 μmol/L resveratrol at and after 1 h of incubation (Fig. 3.2.A). Resveratrol was injected at the apical or basal sides of the cell monolayers in order to examine its effects at different sides of the monolayers. Basal application of resveratrol increased TER in a similar manner as apical application did; however, TER was higher with apical application than with basal application after 1 h of incubation (Fig. 3.2.B).

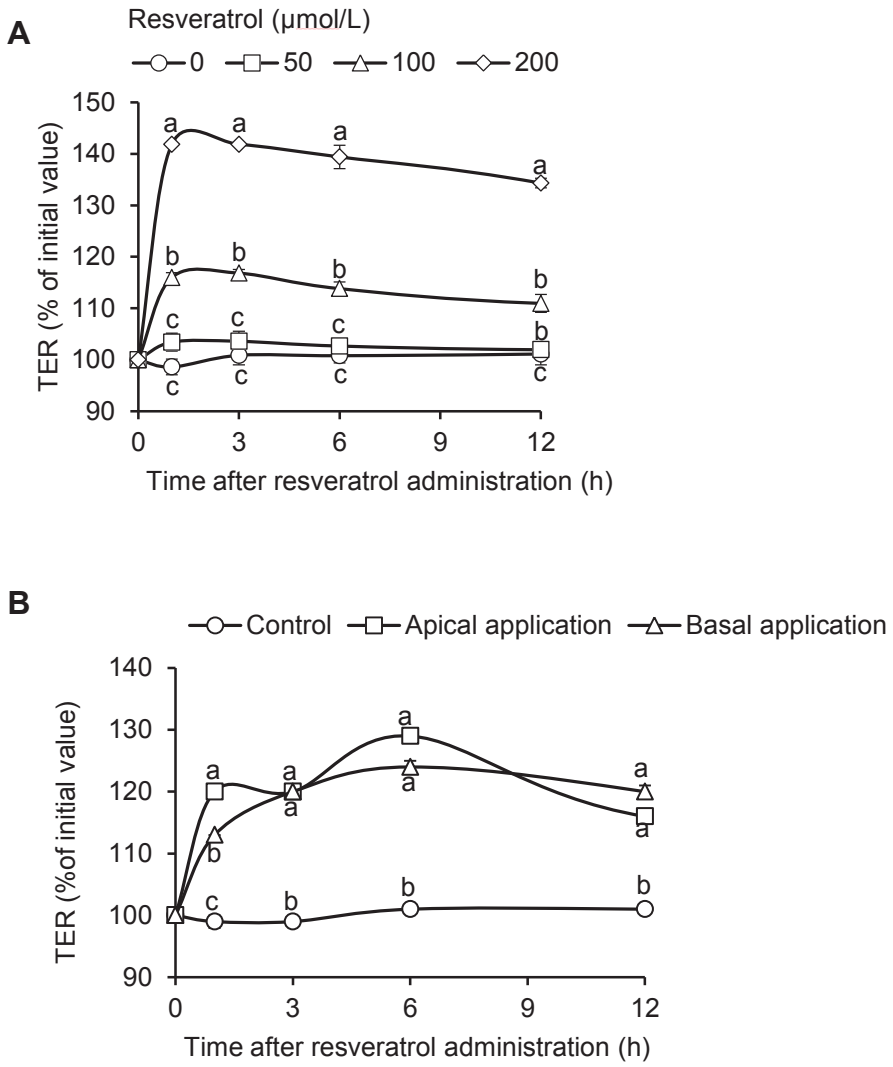


Figure 3.2. Resveratrol enhances tight junction integrity in Caco-2 cells. Transepithelial electrical resistance (TER) was measured in Caco-2 cell monolayers incubated with or without resveratrol. A, TER across cell monolayers incubated with 0-200 μmol/L resveratrol. B, TER across cell monolayers incubated with or without 100 μmol/L resveratrol injected at the apical or basolateral sides of the cell monolayers. All the values are expressed as mean ± standard error of measurement (n = 3). Means without a common letter differ significantly from each other ($P < 0.05$).

3.3.2 TJ protein expression

Immunoblot analysis of the expression levels of TJ proteins in whole-cell extracts and detergent-insoluble fractions of Caco-2 cell monolayers was performed to investigate the mechanisms underlying resveratrol-mediated improvement of intestinal barrier integrity. The total expression of ZO-2, occludin, and JAM-A was increased by 100 μmol/L resveratrol (Fig. 3.3.A). Furthermore, the total expression levels of ZO-2 at 6 and 12 h, as well as those of occludin and JAM-A at and after 1 h were higher than the respective initial expression levels. Additionally, the total expression levels of claudin-1 at 3, 6, and 12 h were slightly but significantly lower than the initial expression level. The protein levels of ZO-1, ZO-2, occludin, claudin-1, claudin-3, and claudin-4 in the detergent-insoluble fractions, which indicate the protein bound to actin cytoskeleton, were increased by 100 μmol/L resveratrol (Fig. 3.3.B). The protein levels of ZO-2 and occludin in resveratrol-treated cells at and after 1 h of incubation were higher than the respective initial protein levels. Moreover, ZO-1 and claudin-1 levels after treatment with resveratrol for 1, 3, and 6 h were higher than the respective initial levels. Claudin-3 level at 6 h and claudin-4 levels at 6 and 12 h in resveratrol-treated cells were also higher the respective initial levels.

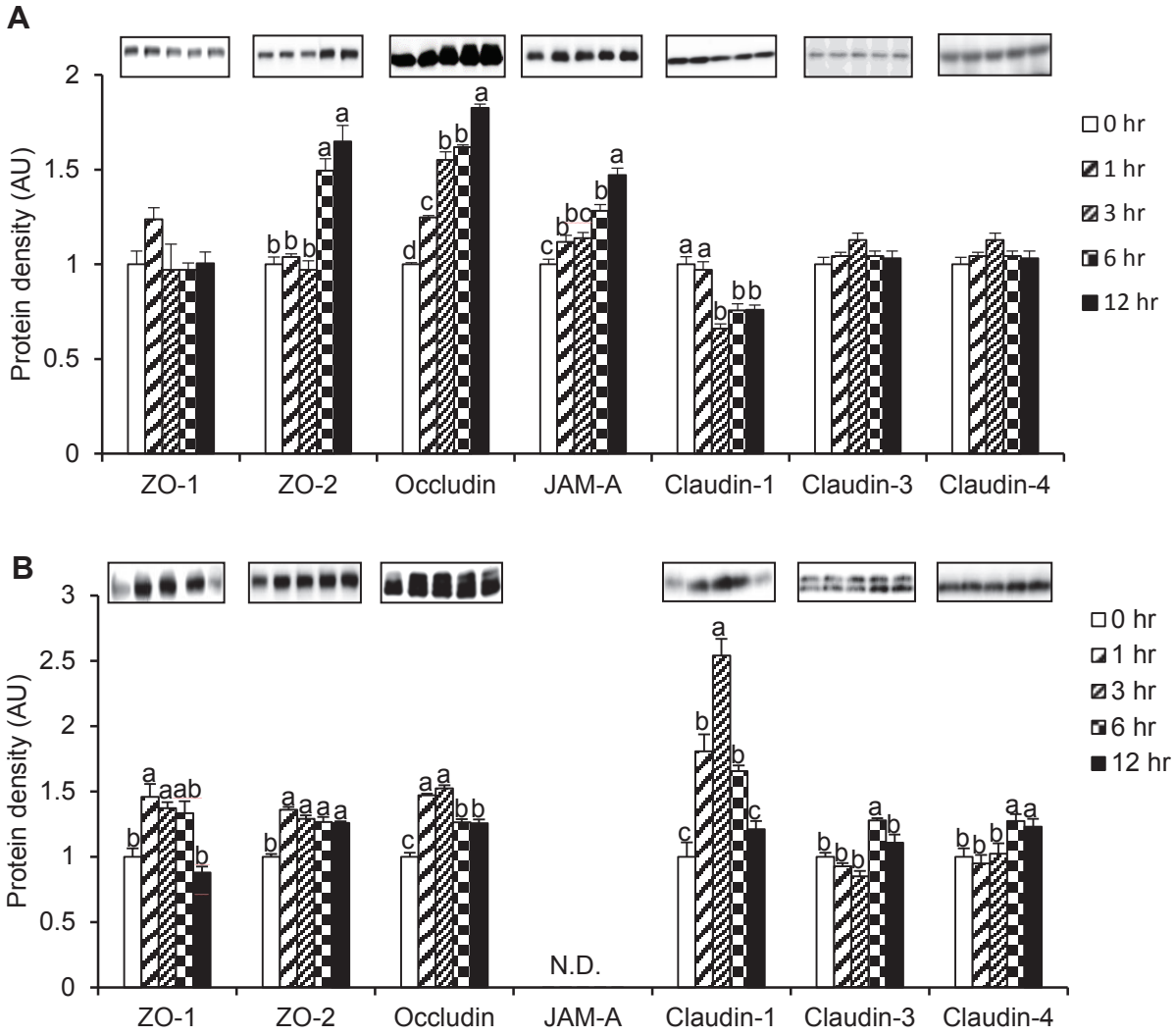


Figure 3.3. Resveratrol induces the expression and cytoskeletal association of tight junction (TJ) proteins in Caco-2 cell monolayers. A, Immunoblot analysis of TJ proteins in whole Caco-2 cell extracts incubated with or without 100 μmol/L resveratrol. B, Immunoblot analysis of TJ proteins in the detergent-insoluble fractions of the cell monolayers incubated with or without 100 μmol/L resveratrol. All the values are expressed as mean ± standard error of measurement (n = 6). Means without a common letter differ significantly from each other ($P < 0.05$). AU, arbitrary unit.

Confocal fluorescence microscopy revealed that ZO-1 and occludin were preferably localized at the intercellular junctions of the control cells. Claudin-1 was observed in the nuclei as well as in the junctional region. Cell monolayers incubated with resveratrol for 12 h showed higher immunofluorescence intensity for these TJ proteins at the intercellular junctions than the control monolayers did (Fig. 3.4).

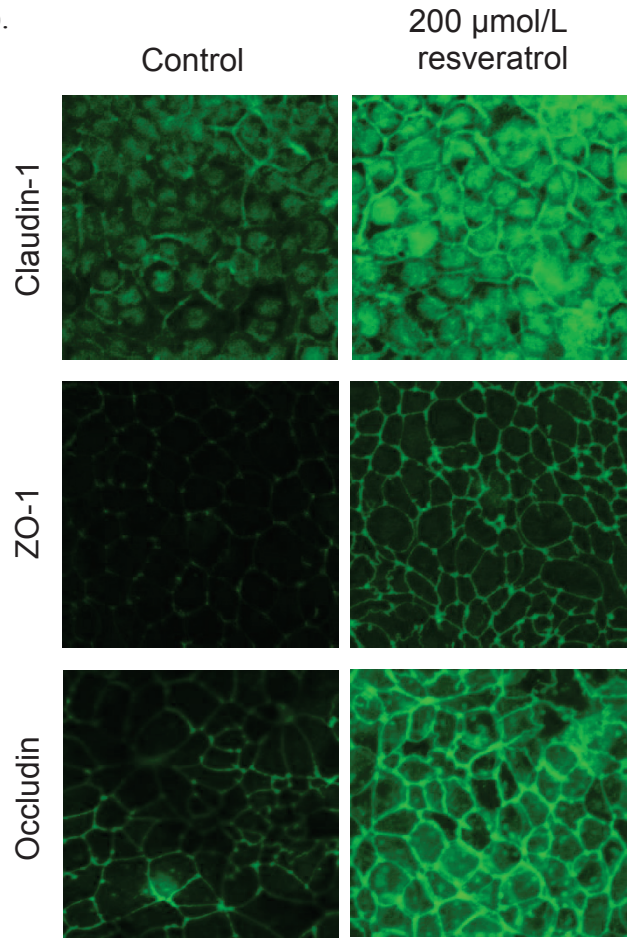


Figure 3.4. Resveratrol induces the expression of tight junction (TJ) proteins in Caco-2 cell monolayers. Immunofluorescence analysis of TJ proteins in cell monolayers incubated with or without 200 µmol/L resveratrol was done. Images were collected using a confocal microscope. Each image is representative of three monolayers.

3.4 Discussion and conclusion

Maintenance of intestinal homeostasis is critically important for human health. Functional defects in TJ and increased paracellular permeability in the intestine result in the infiltration of luminal pro-inflammatory molecules and activation of the mucosal immune system, leading to chronic intestinal inflammation (Suzuki, 2013). Based on the survey study, among three polyphenols tested including resveratrol, EGCG and delphinidin, only resveratrol showed to increase the intestinal integrity in Caco-2 cell monolayers evidenced by TER measurement. Therefore, the following studies will focus on the protective effect of resveratrol in the intestinal barrier function *in vitro* (chapter 3, 5, 6 and 7) and *in vivo* (chapter 4).

Our research group has reported that kaempferol, quercetin, and naringenin promote intestinal TJ integrity across Caco-2 cell monolayers by increasing TER in the absence of harmful agents such as inflammatory cytokines or oxidative stress (Azuma et al., 2013; Noda et al., 2014; Suzuki et al., 2011). However, alterations in TJ protein expression and cytoskeletal association that are mediated by resveratrol vary compared to those mediated by other polyphenols. The TJ complex consists of transmembrane and cytosolic proteins that directly interact with actin cytoskeleton and regulate paracellular permeability through the intestinal epithelium. Previous studies have demonstrated that binding of TJ proteins to the actin cytoskeleton has a critical role in maintaining TJ integrity (Gonzalezmariscal, 2003) and, therefore, correlates to the integrity of TJ in the epithelium (Basuroy et al., 2006).

In the present study, resveratrol induced an increase in TER at 1 h that was mostly sustained until 24 h. Although the molecular mechanisms underlying resveratrol-mediated promotion of TJ barrier integrity are unclear, it seems that assembly of ZO-1, ZO-2, occludin, and claudin-1 is responsible for the primary increase in TER. Moreover, claudin-3 and -4 supported barrier integrity at and after 6 h. The immunoblot analysis using whole-cell extracts demonstrated that resveratrol increased the total expression of the TJ proteins ZO-2, occludin, and JAM-A; however, the trends of the increases were different from those for cytoskeletal association, which suggests separate underlying mechanisms. Interestingly, comparable increases in TER were observed after apical and basal treatment of Caco-2 cells with resveratrol. This observation suggests that resveratrol permeates the membrane of Caco-2 cells and influences the regulation of assembly and expression of TJ proteins.

The results of the present study show that resveratrol has the potential to protect the TJ barrier and promote TJ barrier integrity in intestinal Caco-2 cells. In the absence of harmful substances, resveratrol-mediated promotion of TJ barrier integrity occurs through increased expression and assembly of TJ proteins in intestinal epithelial cells.

CHAPTER 4

RESVERATROL PREVENTS INTESTINAL BARRIER DEFECT AND INFLAMMATION IN COLITIC MICE

4.1 Introduction

In Chapter 3, this study has showed the effect of resveratrol in the promotion of intestinal barrier function, which has been reported in some studies to have a close association with the prevention of inflammatory disease progression. Accumulating evidence from basic and clinical studies suggests that abnormal immune responses and impaired barrier integrity in the intestines converge to provoke the initiation and progression of IBDs (Atreya and Neurath, 2014; Suzuki, 2013). Recent studies have also reported that environmental and food factors are involved in the development of IBD (Niewiadomski et al., 2016). Food factors and nutrients such as polyunsaturated fatty acids, dietary fiber, vitamins, polyphenols, probiotics, and prebiotics participate in maintaining intestinal homeostasis by their complex interaction with microbiota and the immune system (Statovci et al., 2017). These components seem to act through a variety of mechanisms in reducing inflammatory signaling and cytokines and intestinal barrier defects (Murakami et al., 2016; Ogita et al., 2011).

Resveratrol was capable of decreasing the degree of colonic damage, inflammation, and mortality of mice, and possibly influenced multiple pathways, including cytokine expression, redox status, and cellular signaling in the intestines (Sánchez-Fidalgo et al., 2010; Cui et al., 2010; Yao et al., 2010; Singh et al.,

2010). However, the underlying mechanisms for the resveratrol-mediated protection of intestinal homeostasis remain unclear.

Although the IBD pathogenesis is complicated, a growing body of evidence suggests that IBDs are initiated and developed through the interaction between excess immune reaction and barrier defects in intestines. In inflamed tissues, the accumulation of activated immune cells—such as monocytes, macrophages, neutrophils, basophils, dendritic cells, mast cells, T-cells, and B-cells—occurs and mediates chronic tissue injury through robust production of inflammatory cytokines. In particular, neutrophils play an essential role in acute mucosal inflammation because of their potent ability to produce toxic mediators such as reactive oxygen and nitrogen intermediates as well as cytokines (Jaeschke, 2011). The TJ barrier defects and the subsequent penetration of luminal pro-inflammatory substances into the intestinal mucosa lead to the robust and chronic activation of immune cells. A clinical study has demonstrated that the reduced expression and localization of TJ proteins is observed in the inflamed tissue from patients with ulcerative coliltis (Landy et al., 2016).

Based on above-mentioned evidence, the protection of the TJ structure could be effective approaches to preventing intestinal inflammation and treating IBDs, moreover, mitigation of neutrophil infiltration could be a target for disease prevention. This chapter was aimed to examine the ameliorative effect of dietary resveratrol in a murine model of colitis induced by DSS administration, focusing on neutrophil infiltration and TJ structure.

4.2 Materials and methods

4.2.1 Chemicals

DSS (molecular weight 36,000–50,000) was purchased from MP Biomedicals (Santa Ana, CA, USA). Rabbit anti-claudin -7 and goat Alexa Flour 488-conjugated anti-rabbit Ig-G were purchased from Life Technologies, Ltd (Carlsbad, CA, USA). Rabbit anti-phospho (p)-NF-κB, p65/Ser 536, stress activated-phospo kinase/c-Jun N-terminal protein kinase (p-SAPK/JNK), Thr183/Tyr185), p-44/42 MAPK/ ERK 1/2, spleen tyrosine kinase (p-Syk, Tyr 525/526), and p-p38 MAPK (Thr 180/Tyr 182) were purchased from Cell Signaling Technology (Danvers, MA, USA). Goat fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and FITC-conjugated-anti-mouse Ly-6G (Gr-1) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All other chemicals were obtained as described previously (Chapter 3).

4.2.2 Animals

All study protocols were preapproved by the Animal Use Committee of the Hiroshima University and all mice were maintained in accordance with the Hiroshima University guidelines for the care and use of laboratory animals (approval number: C15-10-4). A total of 21 7-week-old male BALB/c mice (Charles River, Tokyo, Japan) were used in this study. They were acclimatized in our animal laboratory under standard conditions (temperature 22 ± 2 °C, humidity 40%–60%, lighting regimen of 12D/12L) and were given an AIN-93G-based standard diet and distilled water *ad libitum* for 1 week before the start of the experiment.

4.2.3 Experimental design of the animal study

To investigate the effects of resveratrol on the regulation of the intestinal barrier and inflammation, DSS-induced colitic mice were used. Mice were randomized into three groups: control, DSS, and DSS + resveratrol (Res) (n = 7 per group). The DSS + Res group was provided with the AIN-93G based diet containing 0.1% (w/w) resveratrol in the standard diet for 14 days before and during DSS treatment. The control and DSS groups were provided with the standard diet throughout the study. The DSS and DSS + Res groups received 2% (w/v) DSS in the drinking water for 8 days to induce colitis, whereas the control mice received distilled water only. The body weight of the mice and their clinical scores for colitis were evaluated every day using a previously described qualitative disease activity index (DAI) (Chasaing, 2014). Briefly, the DAI was calculated as the sum of diarrhea, bloody stool and body weight loss (Table 4.1).

Table. 4.1. Clinical scoring system

Score	Diarrhea stool	Bloody stool	Weight loss (% of initial)
0	Normal	Normal color	<1
1	Mildly soft	Brown color	1–5
2	Very soft	Reddish color	6–10
3	Watery	Bloody stool	11–20
4	More watery	More bloody	> 21

At the end of the experiment, blood was collected from the abdominal vein under isoflurane anesthesia for measuring the lipopolysaccharide binding protein (LBP) and mice were killed by exsanguination. The colon was dissected and its length was measured. Plasma LBP was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biometec, Greisfswald, Germany). The colon tissues were subjected to hematoxylin and eosin (HE)

staining in standard fashion, immunoblot, immunofluorescence, and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses as described below.

4.2.4 Immunoblot analysis

Protein expression of ZO-1, ZO-2, occludin, JAM-A, claudin-2, claudin-3, claudin-4, and claudin-7 in mouse colonic tissues of mice were determined by immunoblot analysis as described previously (Chapter 3).

4.2.5 qRT-PCR analysis

The mRNA expression of TNF-α, chemokine motif ligand 2 (CXCL-2), IL-6, IL-17A, IL-1β, and monocyte chemotactic protein (MCP)-1 in mouse colonic tissues were determined by qRT-PCR as described previously (Azuma et al, 2013; Kaikiri et al., 2017). Total RNA from mouse colonic tissues was isolated using NucleoSpin® RNA (Takara Bio Inc., Kusatsu, Japan) and reverse-transcribed using a ReverTra Ace® qPCR RT kit (TOYOBO) according to the manufacturers' instructions. Data were analyzed by the ΔΔCt method and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression level as an internal control. Primer sequences used for PCR are listed in Table 4.2.

Table 4.2. Primer sequences for qRT-PCR

Target gene	Forward	Reverse
Mouse IL-6	5′- CTGATGCTGGTGACAACCAC - 3′	5′- TCCACGATTTCCCAGAGAAC - 3′
Mouse IL-17A	5′- TGGATTCAGAGGCAGATTCA - 3′	5′- CAGTTTGGGACCCCTTTACA - 3′
Mouse CXCL-2	5′- AGTGAACTGCGCTGTCAATG - 3′	5′- ACTTTTTGACCGCCCTTGAG - 3′
Mouse IL-1β	5′- TGGCAACTGTTCCTGAACTCA - 3′	5′- CAAAGGTTTGGAAGCAGCCC - 3′
Mouse MCP-1	5′- GGAATGGGTCCAGACATACATTA - 3′	5′- TAGCTTCAGATTTACGGGTCAAC - 3′
Mouse GAPDH	5′- TCAAGAAGGTGGTGAAGCAG - 3′	5′- AAGGTGGAAGAGTGGGAGTTG - 3′

4.2.6 Immunofluorescence analysis and neutrophil staining in mouse colon tissues

Immunostaining in mouse colonic tissues for TJ proteins ZO-1, claudin-3, and claudin-7, and for Ly-6G, a neutrophil marker, was performed as described previously (Azuma et al., 2013). Briefly, mouse colons were embedded in an optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) and frozen sections were prepared on glass slides (8 μm thickness). Colon sections were fixed with 4% (w/v) paraformaldehyde for 10 min and permeabilized with 0.2% Triton X for 5 min. The sections were blocked with 5% normal goat serum in 4% skim milk and incubated with corresponding antibodies at 4 °C for 16 h, followed by incubation for 1 h with goat AlexaFluor 488-conjugated anti-rabbit IgG. For neutrophil staining, the colon sections were incubated with the FITC-conjugated anti-mouse Ly-6G at 4 °C for 16 h. The specimens were preserved in a mounting medium, and the fluorescence was visualized with the use of an LCM710 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

4.2.7 Calculation and statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed Statistical analysis was conducted as described previously (Chapter 3).

4.3 Results

4.3.1 Dietary resveratrol suppresses colitis and colon barrier defects in DSS-administered mice

DSS administration resulted in dramatic body weight loss and watery and bloody diarrhea, but supplemental resveratrol ameliorated these symptoms. Body weight in the DSS group was lower than that in the control and the DSS+Res groups at and after day 3 ($p < 0.05$), and no difference in body weight was observed between the control and the DSS+Res groups during DSS administration (Fig. 4.1.A). DSS administration increased the DAI scores at and after day 4 ($p < 0.05$), but supplemental resveratrol effectively suppressed the scores (Fig. 4.1.B). Colon length shortening—another indicator of colonic inflammation and damage (Rose et al., 2012), induced by DSS administration—was restored by supplemental feeding with resveratrol (Fig. 4.1.C).

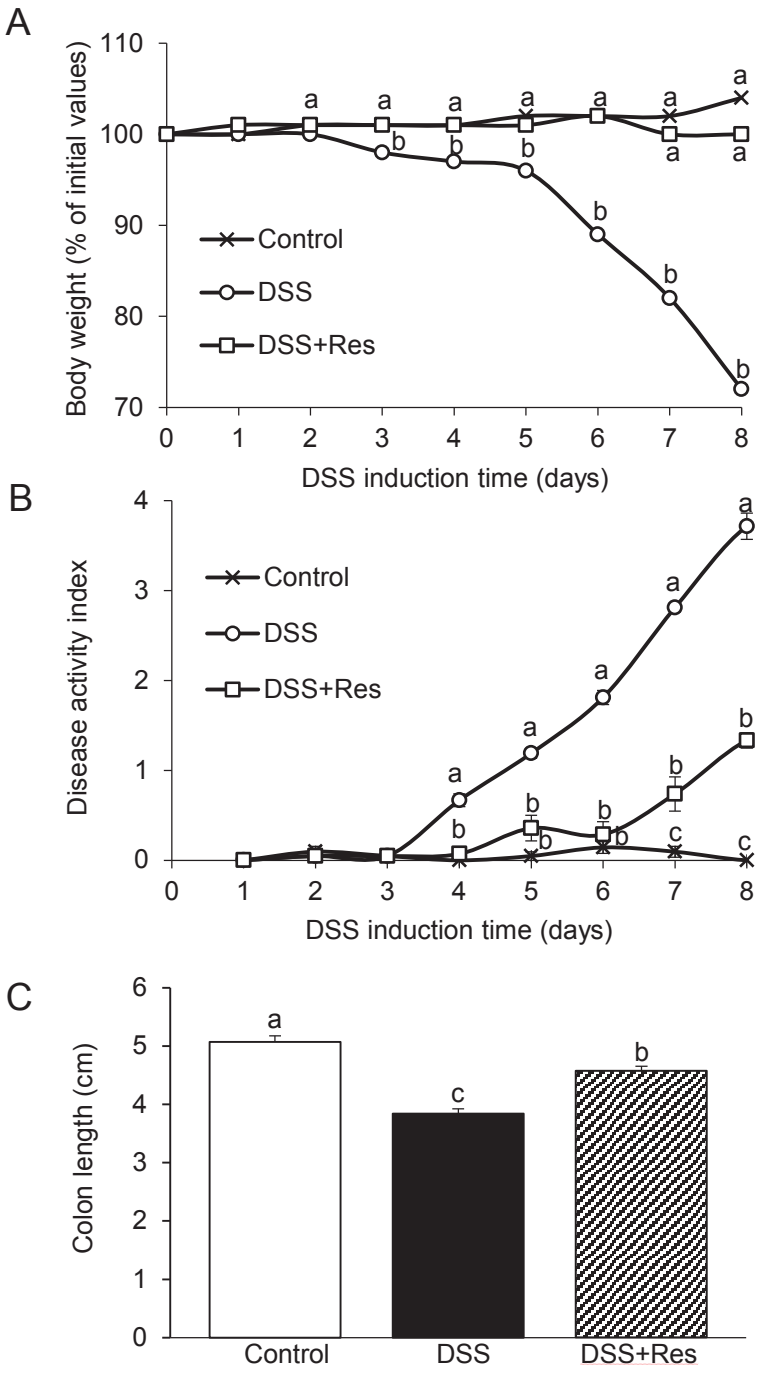


Figure 4.1. Resveratrol supplementation ameliorated symptoms in the dextran sulfate sodium (DSS)-induced colitic mouse model. Resveratrol supplementation ameliorated symptoms in the dextran sulfate sodium (DSS)-induced colitic mouse model. A, Percentage of initial body weight. B, disease activity index (DAI) score; score 0 represents no disease symptoms, and score 4 represents the most severe symptoms. (C) Colon length at necropsy. Data presented as the mean ± standard error measurement (SEM), n = 7. Means without a common letter differ significantly, $p < 0.05$. Res, resveratrol.

Histological evaluation of colitis was carried out by the HE method in colon tissues. DSS administration induced pathological features of colitis, such as loss of crypts, ulceration of the mucosa, edema, and infiltration of inflammatory cells in the mucosa. However, supplemental resveratrol reduced these alterations and the control and DSS+Res groups showed a normal structure of the colon (Fig. 4.2)

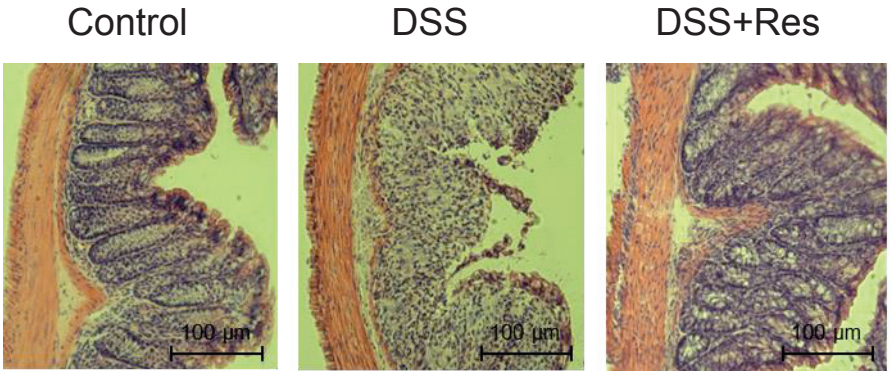


Figure 4.2. Dietary resveratrol supplementation ameliorated DSS-induced histological alteration in colitic mice. Sectioned colon tissues of mice were stained with hematoxylin and eosin. The images are representative of 7 mice.

The plasma concentration of LBP—an indicator of intestinal barrier impairment—in the DSS group was higher than that in the control group, but this imbalance was reversed with supplemental resveratrol (Fig 4.3).

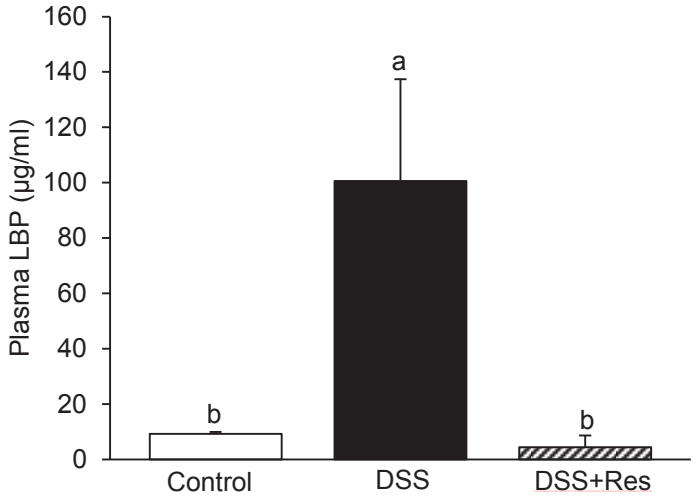


Figure 4.3. Dietary resveratrol supplementation mitigated plasma lipopolysaccharide binding protein (LBP) increase in the DSS-induced colitic mice. Data presented as the mean ± SEM, n = 7. Means without a common letter differ significantly, $p < 0.05$.

4.3.2 Dietary resveratrol restores colonic TJ structure in DSS-administered mice

Intercellular TJ structure has an important role in physical barrier function and regulates paracellular permeability in colons. Mice administrated with DSS showed decreased expression of TJ proteins ZO-2, occludin, JAM-A, claudin-3, claudin-4, and claudin-7 in the colon compared to that of normal mice. Supplemental resveratrol partially restored or resolved these decreases in the TJ proteins induced by DSS administration, although the restoration of JAM-A was not significant (Fig. 4.4). Immunofluorescence analysis showed the expression of

TJ proteins ZO-1, claudin-3, and claudin-7 in the colonic epithelial cells of normal mice with different localization patterns. ZO-1 was predominantly located at the apical portion of the lateral membranes, whereas claudin-3 and -7 were expressed at the lateral and basolateral membranes. DSS administration severely impaired the localization and expression of these TJ proteins, whereas resveratrol supplementation clearly attenuated these impairments induced by DSS administration (Fig. 4.5).

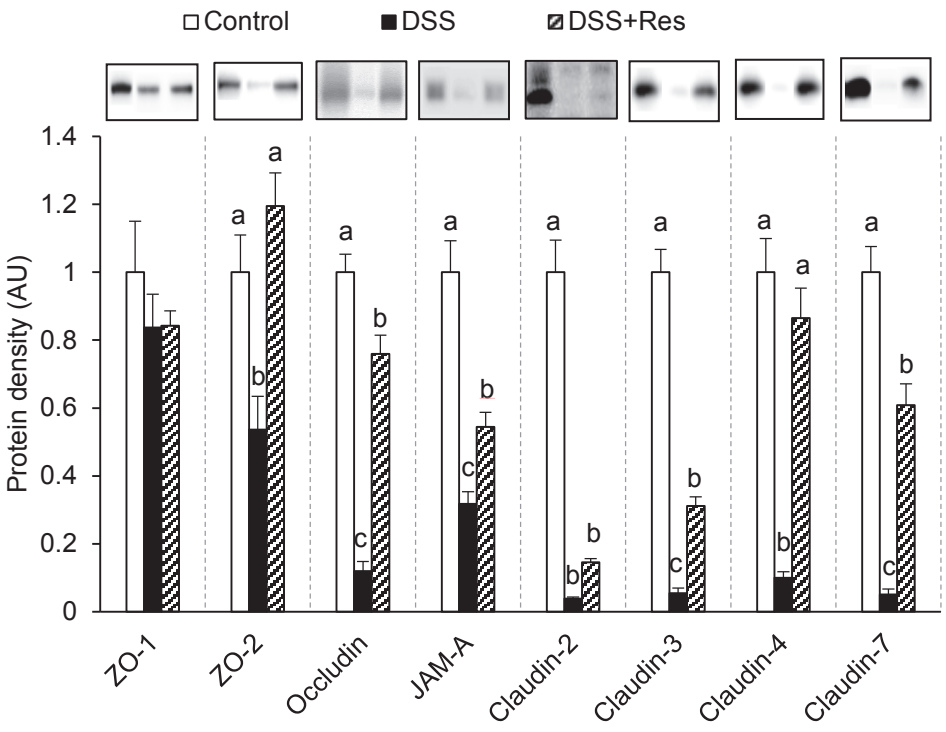


Figure 4.4. Dietary resveratrol supplementation ameliorated tight junction (TJ) proteins zonula occludens (ZO)-1, ZO-2, occludin, junctional adhesion molecule (JAM)-A, claudin-3, claudin-4, and claudin-7 decrease in DSS-induced colitic mice. Protein density values were normalized to the values from the control group. Data presented as the mean ± SEM, n = 7. Means without a common letter differ significantly, $p < 0.05$. AU, arbitrary unit.

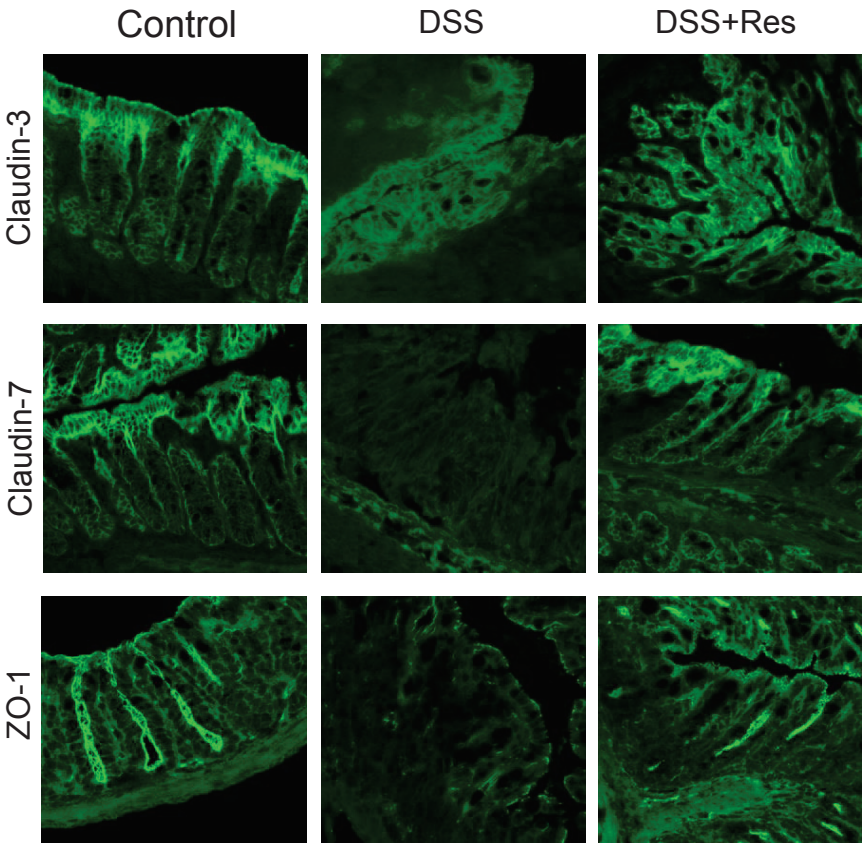


Figure 4.5. Immunolocalization of TJ proteins claudin-3, claudin-7, and ZO-1 in the colons of mice administered DSS by water or in combination with feeding with or without 0.1% resveratrol (w/w) in the standard diet. Sectioned colon tissues were fixed and stained for claudin-3, claudin-7, and ZO-1 using an immunofluorescence method. Images were collected by confocal microscopy. The images are representative of 7 mice.

4.3.3 Dietary resveratrol suppresses colonic pro-inflammatory cytokine expression in DSS-administered mice

The increased expression of inflammatory cytokines and chemokines in the colon plays an important role in IBD pathogenesis. Gene expression of inflammatory molecules—including TNF-α, IL-6, IL-17A, CXCL-2, IL-1β, and MCP-1—was markedly increased by DSS, although the increase in expression of TNF-α and IL-17A was not significant (Fig. 4.6). Especially, the CXCL-2 (a mouse homologue of IL-8) expression in the DSS group was nearly 700-fold

higher than that in the control group. The up-regulations of these inflammatory markers—apart from TNF-α were—mitigated by resveratrol supplementation, although the suppression of IL-1β and IL-17 were not statistically significant. Apparently, TNF-α expression was not influenced by resveratrol.

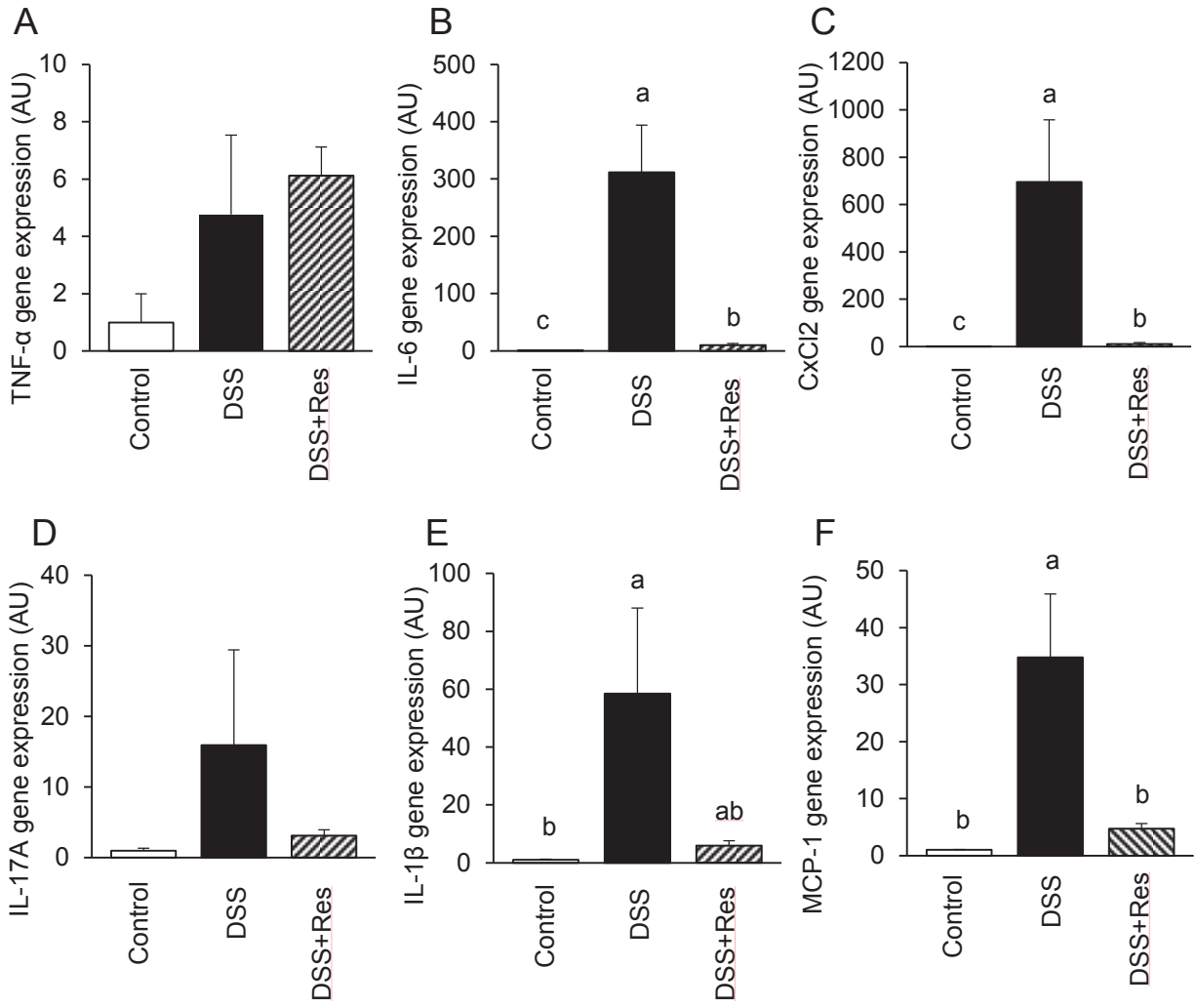


Figure 4.6. Dietary resveratrol supplementation ameliorated pro-inflammatory biomarkers TNF-α (A), IL-6 (B), CXCL-2 (C), IL-17A (D), IL-1β (E), and MCP-1 (F) gene expression in DSS-induced colitic mice. Gene expression levels were normalized to the values of the control group. Data are presented as the mean ± SEM, n = 7. Means without a common letter differ significantly, $p < 0.05$. TNF, tumor necrosis factor; IL, interleukin; CXCL, chemokine motif ligand; MCP, monocyte chemoattractant protein.

4.3.4 Resveratrol suppresses neutrophil infiltration into colonic tissues of DSS-administered mice

Recruitment of neutrophils from blood into inflamed tissues is a hallmark of intestinal inflammation. To determine the neutrophil infiltration into inflamed colonic tissues, immunohistochemical analysis using anti-Ly6G, a marker of peripheral neutrophils, was performed. The Ly6G-positive cells were observed in the submucosa and lamina propria of control tissues, but their number was quite limited (Fig. 4.7.A and 4.7.B). The DSS-administered mice showed a higher population of Ly6G-positive cells in the submucosa and lamina propria of colonic tissues compared to that of the normal mice, indicating an increased neutrophil infiltration, whereas the increase was effectively mitigated by resveratrol supplementation.

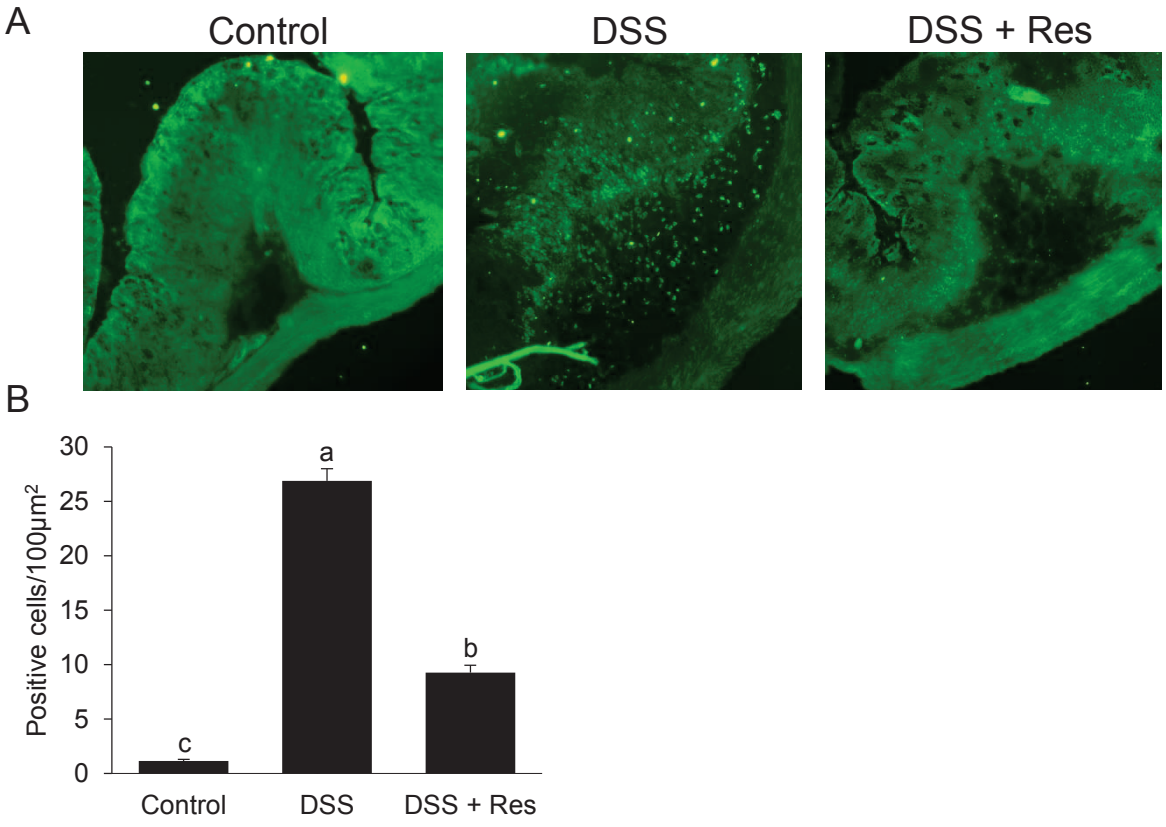


Figure 4.7. Dietary resveratrol supplementation mitigated neutrophil infiltration in DSS-induced colitic mouse colons. Sectioned colon tissues were fixed and stained with anti-mouse Ly-6G (Gr-1) FITC. The images are representative of 7 mice (A). Data presented as the mean ± SEM. Means without a common letter differ significantly, $p < 0.05$ (B).

4.4 Discussion and conclusion

Previous studies have demonstrated that dietary resveratrol reduces the colonic damage in a murine model of colitis, and some molecular mechanisms—such as the regulation of redox status and helper T cell differentiation—have been proposed (Yao et al., 2015). A clinical study has also shown that supplementation of resveratrol in patients with IBDs reduces DAI and improves the quality of life. However, the precise underlying mechanisms of the resveratrol-mediated beneficial effects for intestinal homeostasis remain unclear. The present study shows the capability of resveratrol to prevent barrier defects and inflammation through the suppression of neutrophil infiltration in colitic mice.

In the inflamed tissues of patients with IBDs, the alteration and impairment of some TJ proteins have been observed, and can therefore be understood as measurable features for the progression of IBDs. Because of the essential roles of the intestinal TJ barrier for intestinal homeostasis, much attention has been attracted to TJ regulation by dietary components. Our study provides evidence that resveratrol protects the TJ barrier integrity in the mouse colon against DSS administration. Although the IBD pathogenesis is complicated, monocytes and macrophages seem to be initially triggered to produce TNF-α in response to the bacteria-derived endotoxin (Parameswaran and Patial, 2010) and the moderate immune activation by TNF-α induces the subclinical hyperpermeability of intestines. Subsequent permeation of luminal endotoxins and antigens results in the robust and chronic inflammatory response, including abnormal production of cytokines (Bruggen et al., 1999; Lissner et al., 2015).

In regard to human consumption, both the effectiveness and the safety of resveratrol should be carefully considered. The human equivalent dose (HED) can be estimated using no observed adverse effect levels (NOAEL) (Contrera et al., 2004). In our study we fed mice with 0.1% resveratrol in the diet. This is equivalent to 120 mg/kg body weight of animal NOAEL value, since a mouse weighing 25 g feeds on a diet of approximately 3 g per day. Based on the equation HED (mg/kg body weight) = animal NOAEL (mg/kg body weight) x [body weight of animal (kg)/body weight of human (kg)]$^{(1-0.67)}$, the HED calculated in our study is 513 mg for a 60 kg human and the calculation is close to an acceptable daily intake (ADI) of resveratrol in food of 450 mg/day, defined in the previous studies. In addition, resveratrol is known to be a substance of low oral toxicity and the NOAEL for humans has been defined at 750 mg/kg body weight·day (Williams et al, 2009). Accordingly, the resveratrol-mediated reduction of intestinal inflammation observed in our study could be translated into a similar effect in humans. Further studies, however, are required to establish the safety and effectiveness of resveratrol for human consumption.

In conclusion, this study suggests that supplemental resveratrol prevents barrier defects and inflammation in DSS-induced colitic mice through the prevention of neutrophil infiltration into inflamed tissues.

CHAPTER 5

RESVERATROL RESTORES TNF-α-INDUCED-IL-8 PRODUCTION IN INTESTINAL CACO-2 CELL MONOLAYERS

5.1 Introduction

In the chapter 4, this study has performed the ability of resveratrol in preventing barrier defects and inflammation in DSS-induced colitic mice through the prevention of neutrophil infiltration into inflamed tissues. It is known that IL-8, secreted by intestinal epithelial cells, plays a critical role in the chemoattraction of neutrophils. Secretion of IL-8 in healthy tissues is scarce, but its production is rapidly and considerably induced in response to a wide range of pro-inflammatory stimuli, including tumor necrosis factor-α (TNF-α). Indeed, the mucosal IL-8 level has been reported to be elevated in patients with IBD (Mahida et al., 1992). In addition, it is known that TJ barrier integrity is negatively regulated by pro-inflammatory mediators such as TNF-α (Piechota-Polanczyk and Fichna, 2014). Based on this evidence, the suppression of IL-8 production and the protection of the TJ structure could be effective approaches to preventing intestinal barrier impairment-related disease. This chapter was aimed to examine the effect of resveratrol in the molecular mechanisms for the resveratrol-mediated suppression of neutrophil infiltration using human intestinal Caco-2 cell monolayers.

5.2 Materials and methods

5.2.1 Chemicals

All chemicals were obtained as described previously (Chapter 3 and 4).

5.2.2 Cell culture

Human intestinal Caco-2 cells (HTB-37; American Type Culture Collection, Manassas, VA, USA) were propagated and maintained under standard cell culture conditions as described previously (Chapter 3).

5.2.3 Treatment of intestinal Caco-2 cells

Caco-2 cell monolayers were incubated with or without TNF-α (30 ng/mL). Resveratrol (100 μmol/L) was added to the cell culture medium 1 h before stimulation with TNF-α. Culture medium was collected 24 h after TNF-α administration to determine the IL-8 concentrations by ELISA as described previously (Oyama et al., 2017). Caco-2 cell extracts were collected at 24 h for qRT-PCR analysis and at 0.5 h for immunoblot analysis as described below.

5.2.4 Immunoblot analysis

Protein expression p-NF-κB p65, p-SAPK/JNK, p-ERK 1/2, p-Syk and p-p38 MAPK were determined by immunoblot as described previously (Chapter 3).

5.2.5 qRT-PCR analysis

The mRNA expression of IL-8 in Caco-2 cells was determined and analyzed by qRT-PCR as described previously (Chapter 4). Total RNA from Caco-2 cells was isolated using NucleoSpin® RNA or RNA Iso Plus (Takara Bio Inc., Kusatsu, Japan) and reverse-transcribed using a ReverTra Ace® qPCR RT

kit (TOYOBO) according to the manufacturers' instructions. Primer sequences used for PCR are listed in Table 5.1.

Table 5.1. Primer sequences for qRT-PCR

Target gene	Forward	Reverse
Human IL-8	5′-ATGACTTCCAAGCTGGCCGTGGCT- 3′	5′- TCTCAGCCCTCTTCAAAAACTTCTC- 3′
Human Claudin-2	5′- CTCCCTGGCCTGCATTATCTC - 3′	5′- ACCTGCTACCGCCACTCTGT - 3′
Human GAPDH	5′- CAACGGATTTGGTCGTATTGGG - 3′	5′- AAGGGGTCATTGATGGCAAC - 3′

5.2.6 Calculation and statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed Statistical analysis was conducted as described previously (Chapter 3).

5.3 Results

Resveratrol restores TNF-α-induced-IL-8 production in intestinal Caco-2 cell monolayers

Human intestinal Caco-2 cells were used to examine the effect of resveratrol on the TNF-α-induced inflammatory signaling and IL-8 expression. Protein and mRNA expression levels of IL-8 in Caco-2 cells were potently increased by TNF-α stimulation (Fig. 5.1.A and 5.1.B). Resveratrol treatment suppressed these increases in a dose-dependent manner, indicating that resveratrol showed anti-inflammatory properties against TNF-α. Stimulation of Caco-2 cells with TNF-α increased the phosphorylation of NF-κB p65, SAPK/JNK, ERK 1/2, Syk, and p38 MAPK, whereas resveratrol treatment suppressed these increases (Fig. 5.2).

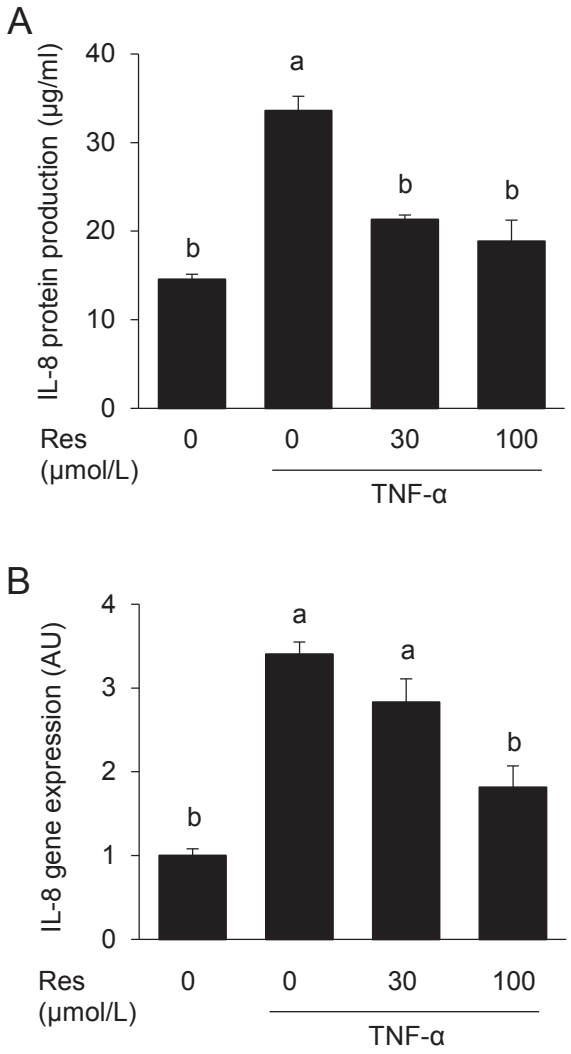


Figure 5.1. Resveratrol suppressed TNF-α-induced IL-8 protein (A) and gene (B) expression in Caco-2 cell monolayers. Protein expression levels were analyzed using the ELISA method and were normalized to the values of control group. Data are presented as the mean ± SEM, n = 6. Means without a common letter differ significantly, $p < 0.05$.

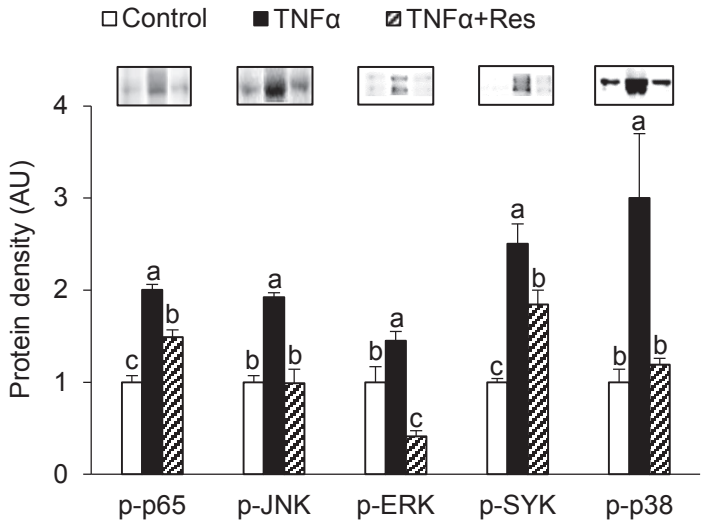


Figure 5.2. Resveratrol blocked the TNF-α-induced cellular signaling in Caco-2 cells. Phosphorylation of p-NF-κB p65, p-SAPK/JNK, p-ERK 1/2, p-Syk and p-p38 MAPK were determined by immunoblot analysis. Data are presented as the mean ± SEM, n = 6. Means without a common letter differ significantly, $p < 0.05$. JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; SYK, spleen tyrosine kinase; NF-κB, nuclear factor kappa B; MAPK, mitogen-activated protein kinase.

5.4 Discussion and conclusion

In line with the *in vivo* study in chapter 4, resveratrol was shown to suppress TNF-α-induced inflammatory signaling and IL-8 production in human intestinal Caco-2 cells. Our findings, therefore, shed new light on the beneficial role of resveratrol for intestinal homeostasis.

Suppression of CXCL-2 (a mouse homolog of IL-8) expression seems to be one of the central events in the resveratrol-mediated reduction of colonic inflammation in mice. IL-8 secreted by epithelial cells recruits neutrophils into the inflamed colonic tissues, where the neutrophils promote and develop the mucosal inflammation through the intercellular interaction with other immune cells such as macrophages and T cells (Mitsuyama, et al., 1994). Clinical studies demonstrate that various immune cells, including neutrophils, accumulate in the inflamed tissues of patients with ulcerative colitis. Immunofluorescence analysis using anti-Ly6G confirmed that resveratrol mitigated the neutrophil infiltration induced by DSS administration (Chapter 4). It should be noted that resveratrol did not influence the DSS-induced TNF-α expression (Chapter 4), which is known to stimulate IL-8 (and CXCL-2) expression in the epithelial cells. Although the precise mechanisms for the resveratrol-mediated suppression of CXCL-2 and neutrophil infiltration are still unclear, this evidence led us to hypothesize that the inhibition of TNF-α-induced inflammatory signaling and CXCL-2 expression in the epithelial cells was a proximal event in the resveratrol-mediated effect. This hypothesis is supported by the observation that resveratrol suppressed IL-8 expression in the Caco-2 cells stimulated by TNF-α.

Resveratrol suppressed the phosphorylation (that is, the activation) of all signaling molecules examined in Caco-2 cells stimulated with TNF-α—such as NF-κB p65, SAPK/JNK, ERK 1/2, Syk, and p38 MAPK. Activation of these signaling pathways is known to enhance the transcription of IL-8. These findings suggest that resveratrol influences the upstream cellular molecules to the activation of these signaling pathways. Upon binding of TNF-α to TNF-receptors (TNFRs), TNFR-associated factors (TRAFs), which are multifunctional adaptor proteins, recruit additional proteins to form multiple signaling complexes capable of promoting cellular response. Resveratrol may interfere with the binding of TRAF to the intracellular domain of TNF-R, resulting in the suppression of IL-8 expression. It has been reported that TRAF plays a crucial role in the immune responses and contributes to chronic inflammation and infection (Xie, 2013), this factor thus potentially becomes a platform for the development of therapeutic intervention in IBD progression.

In conclusion, this study suggests that supplemental resveratrol prevents barrier defects and inflammation through the inhibition of IL-8 production in inflamed tissue, in turn resulting in the prevention of neutrophil infiltration into inflamed tissues. This study demonstrated that resveratrol suppresses TNF-α-induced-inflammatory signaling and IL-8 production in Caco-2 cell monolayers. Our study, therefore, elucidates the novel molecular mechanisms underlying the beneficial role of resveratrol for maintaining intestinal homeostasis.

CHAPTER 6

RESVERATROL AMELIORATES H_2O_2-INDUCED INTESTINAL BARRIER DISRUPTION IN CACO-2 CELL MONOLAYERS

6.1 Introduction

In Chapter 3, we has described about the promotion of intestinal barrier function by resveratrol. This chapter describes the effect of resveratrol in impaired intestinal barrier model by oxidative stress agent. The intestinal barrier is always threatened by different stimuli such as pathogens, inflammatory cytokines, and ROS, which are closely associated with the development of intestinal inflammation. Major sources of inflammatory cytokines and ROS are activated immune cells such as macrophages, neutrophils, dendritic cells, T cells, and mast cells. Mucosal infiltration of polymorphonuclear neutrophils is common in IBD. The neutrophils predominantly generate H_2O_2, which leads to chronic inflammation and tissue injury. Sheth et al. (2009) demonstrated that H_2O_2-induced increase in intestinal hyperpermeability is associated with tyrosine (Tyr) phosphorylation of TJ proteins such as occludin. This chapter was aimed to investigate the protective effect of resveratrol in H_2O_2-induced impaired barrier model in human intestinal Caco-2 cell monolayers.

6.2 Materials and methods

6.2.1 Chemicals

Mouse anti-phosphoTyr (PY-20) was purchased from Southern Biotech (Birmingham, USA). Protein A-Sepharose was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). All other chemicals were obtained as described previously (Chapter 3).

6.2.2 Cell culture

Human intestinal Caco-2 cells (HTB-37; American Type Culture Collection, Manassas, VA, USA) were propagated and maintained under standard cell culture conditions as described previously (Chapter 3).

6.2.3 Treatment of Caco-2 cells with resveratrol and H_2O_2

Caco-2 cell monolayers were incubated with or without resveratrol (50, 100, and 200 μmol/L) at their apical sides. H_2O_2 (50 μmol/L) was added 30 min after the treatment with resveratrol at the basolateral side of the cell monolayers. TER was measured before and at 1, 2, and 3 h after the treatment with H_2O_2. Whole Caco-2 cell extracts were collected to analyze protein expression and Tyr phosphorylation of occludin at 3 h as described below. The cell monolayers were then fixed for immunostaining.

6.2.4 Measurement of TER across Caco-2 cell monolayers

Intestinal TJ barrier integrity was evaluated by measuring TER as described previously (Chapter 3).

6.2.5 Preparation of detergent-insoluble fractions and whole-cell extracts

Detergent-insoluble fractions, which correspond to actin-cytoskeleton-associated protein content, and whole-cell extracts were prepared as described previously (Chapter 3).

6.2.6 Immunoblot analysis

The protein expression levels of occluding and PY-20 in Caco-2 cells were determined by immunoblot analysis as described previously (Chapter 3).

6.2.7 Immunoprecipitation and occludin phosphorylation

Tyr phosphorylation of occludin in Caco-2 cells was demonstrated by immunoprecipitation of phosphoTyr followed by immunoblot analysis for occludin (Elias et al., 2009). Briefly, Caco-2 cell monolayers lysed with lysis buffer D (0.3% (w/v) SDS and 10 mmol/L Tris solution containing protease inhibitors, pH 7.4) were heated at 95 °C for 10 min and homogenized using an ultrasonic homogenizer (TAITEC, Saitama-ken, Japan). The protein extracts were mixed with the same volume of immunoprecipitation (IP) buffer (20 mmol/L Tris, 300 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid-2Na, 2 mmol/L EGTA-4Na, and 1% Nonidet P-40) and incubated with anti-occludin antibody at 4 °C for 16 h. The immune complexes were isolated by precipitation using Protein A-Sepharose at 4 °C for 1 h. The sepharose beads were washed with IP buffer. The immune complex was extracted with Laemmli sample buffer and subjected to immunoblot analysis of occludin as described above.

6.2.8 Immunostaining method

Caco-2 cells were stained as described previously (Chapter 3).

6.2.9 Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed Statistical analysis was conducted as described previously (Chapter 3).

6.3 Results

6.3.1 TER

In order to evaluate the protective effect of resveratrol against oxidative-stress-induced TJ barrier disruption in intestinal Caco-2 cells, monolayers of the cells were incubated with resveratrol in the presence of H_2O_2. TER across Caco-2 cell monolayers incubated with 50 μmol/L H_2O_2 was rapidly decreased within 1 h of incubation. This decrease in TER was sustained for 3 h, which indicated oxidative-stress-induced cell damage (barrier disruption). Resveratrol effectively suppressed reduction in TER in a dose-dependent manner. Moreover, TER at and after 1 h following treatment with 200 μmol/L resveratrol plus H_2O_2 was higher than that after treatment with H_2O_2 alone (Fig. 6.1).

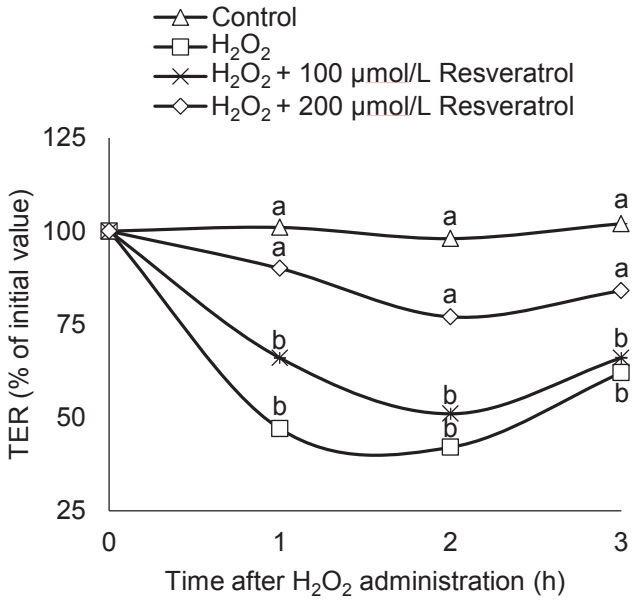


Figure 6.1. Resveratrol restores tight junction (TJ) integrity following H_2O_2-induced oxidative stress damage in Caco-2 cell monolayers. Transepithelial electrical resistance (TER) across cell monolayers incubated with or without 50-200 µmol/L resveratrol and 50 µmol/L H_2O_2 was measured. All the values are expressed as mean ± standard error of measurement (n = 3). Means without a common letter differ significantly from each other ($P < 0.05$).

6.3.2 Occludin protein expression

Occludin protein expression in Caco-2 cell monolayers was reduced by H_2O_2, which led to reduced TJ integrity; however, 200 µmol/L resveratrol reduced the decrease (Fig. 6.2.A). Results of the confocal fluorescence microscopy confirmed that H_2O_2 downregulated occludin protein expression in the Caco-2 cell monolayers, whereas resveratrol restored it (Fig. 6.2.B).

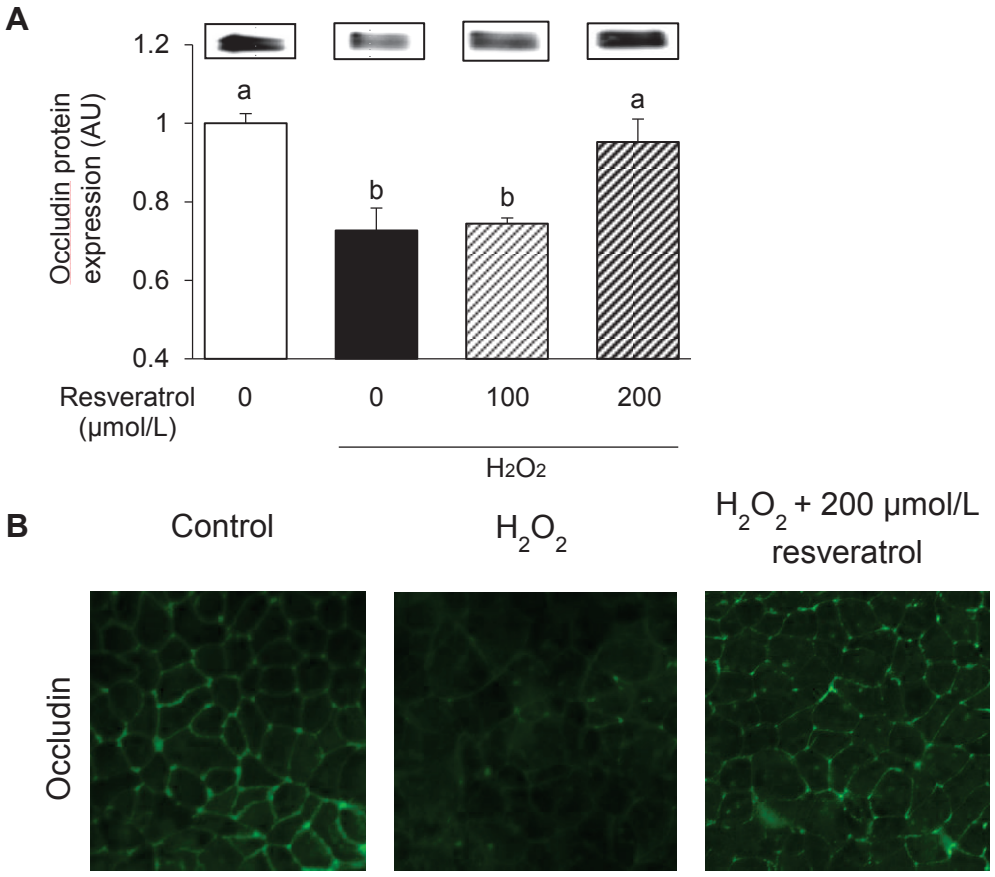


Figure 6.2. Resveratrol induces occludin expression in Caco-2 cell monolayers following H_2O_2-induced oxidative stress damage. A, Immunoblot analysis of occludin protein expression in the cell monolayers incubated with or without 100-200 µmol/L resveratrol and 50 µmol/L H_2O_2. Values are expressed as mean ± standard error of measurement (n = 3). Means without a common letter differ significantly from each other ($P < 0.05$). B, Immunofluorescence analysis of occludin expression in cell monolayers incubated with or without 200 µmol/L resveratrol and 50 µmol/L H_2O_2. Images were collected using a confocal microscope. Each image is representative of three monolayers.

6.3.3 Occludin phosphorylation

H_2O_2-induced impairment of TJ barrier is associated with Tyr phosphorylation of occludin (Elias et al., 2009). In the present study, treatment with H_2O_2 resulted in increased Tyr phosphorylation of occludin in the Caco-2 cells. In contrast, resveratrol suppressed occludin phosphorylation (Fig. 6.3).

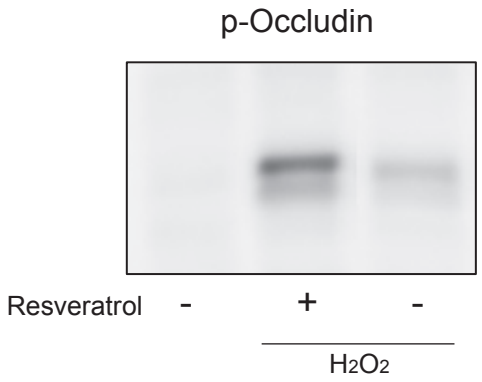


Figure 6.3. Resveratrol mitigates Tyr phosphorylation of occludin induced by H_2O_2 in Caco-2 cell monolayers. Protein extracts from the cell monolayers incubated with or without 200 μmol/L resveratrol and 50 μmol/L H_2O_2 were immunoprecipitated to obtain phospho-Tyr, followed by immunoblot analysis for occludin. Each image is representative of the results from three independent experiments.

6.4 Discussion and conclusion

A growing body of evidence suggests that barrier dysfunction is associated with robust production of ROS (Nakamura et al., 1992; Sommer et al., 2014). Furthermore, H_2O_2 and other ROS are produced in large quantities during intestinal inflammation. Inflamed epithelial cells and activated immune cells, mainly macrophages and neutrophils, generate ROS through the activities of several enzymes such as peroxidases, myeloperoxidase, and xanthine oxidase, which is considered a risk factor for IBD. The present results show that resveratrol effectively ameliorates intestinal barrier disruption in Caco-2 cell model H_2O_2-induced barrier impairment. Resveratrol clearly prevented H_2O_2-mediated disruption by restoring occludin protein expression. The protective effect of resveratrol against damages caused by H_2O_2 may help in preventing or reducing the progression of different intestinal diseases associated with inflammation.

The molecular mechanisms underlying resveratrol-mediated protection against the effects of H_2O_2 seem to differ from the improvement of TJ barrier integrity that was observed in the unstimulated Caco-2 cells. This is because resveratrol suppressed intracellular events induced by H_2O_2, such as phosphorylation occludin. It has been demonstrated that H_2O_2-induced disruption of the TJ is associated with Tyr phosphorylation at the C-terminal domain of occludin. Additionally, it plays a role in the disruption of junctional complexes between occludin and ZO proteins and the actin cytoskeleton (Kale et al., 2003; Rao, 2009; Seth et al., 2007). The present results demonstrate that resveratrol restored H_2O_2-induced intestinal barrier integrity by upregulating occludin expression through mitigation of Tyr phosphorylation. H_2O_2 alters various protein

kinases and the activities of phosphatases, some of which are involved in the Tyr phosphorylation of occludin. A previous *in vitro* study revealed that Tyr residues (Tyr-398 and Tyr-402) in occludin are phosphorylated by c-Src. Additionally, the role of the Tyr residues in regulating the interaction between occludin and ZO-1 and TJ assembly was determined (Elias et al., 2009). It is reported that H_2O_2 inhibits protein Tyr phosphatases such as PTP1B, which results in increased Tyr phosphorylation of occludin (Atkinson and Rao, 2001). Although the precise underlying mechanisms are unclear, resveratrol may reduce H_2O_2-mediated occludin phosphorylation and barrier defect by affecting the activities of these kinases or phosphatases.

In conclusion, this study shows that that resveratrol inhibits inflammatory or harmful intracellular signaling to protect intestinal barrier against H_2O_2-induced barrier disruption.

CHAPTER 7

RESVERATROL AMELIORATES INTERLEUKIN-6-INDUCED INTESTINAL BARRIER DISRUPTION IN CACO-2 CELL MONOLAYERS

7.1 Introduction

In Chapter 3 and 6, we have showed the resveratrol promotive effect in intestinal TJ barrier regulation and its protective effect in the intestinal barrier in oxidative cell damage. Besides of ROS, inflammatory cytokines become major factor which are closely associated with the development of intestinal inflammation. Major sources of inflammatory cytokines are activated immune cells such as macrophages, neutrophils, dendritic cells, T cells, and mast cells. Mucosal infiltration of polymorphonuclear neutrophils is common in IBD. IL-6 is a pleiotropic cytokine that acts as a pro-inflammatory and anti-inflammatory cytokine (Alonzi et al., 1998). In pathological states, excessive IL-6 production and activation of IL-6 signaling pathways play key roles in acute and chronic inflammation. Previous studies have demonstrated that IL-6 induces the expression of the pore-forming claudin isoform claudin-2 in intestinal epithelial cells, which then results in hyperpermeability (Nakamura et al., 1992; Suzuki et al., 2011). An abnormally high expression of claudin-2 is observed when biopsies are performed in patients with UC and CD. The above-mentioned findings indicate that protection of the intestinal barrier from insults by may be an effective approach in preventing intestinal inflammation. This chapter was aimed to

investigate the protective effect of resveratrol in IL-6-induced impaired barrier model in Caco-2 cell monolayers.

7.2 Materials and methods

7.2.1 Chemicals

Rabbit anti-claudin-2, anti-p-44/42 MAPK/ ERK 1/2 and goat Alexa Flour 488-conjugated anti-rabbit immunoglobulin (Ig)G were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HRP-conjugated anti-rabbit and anti-mouse IgG was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were obtained as described previously in Chapter 3.

7.2.2 Cell culture

Human intestinal Caco-2 cells (HTB-37; American Type Culture Collection, Manassas, VA, USA) were propagated and maintained under standard cell culture conditions as described previously (Chapter 3).

7.2.3 Treatment of Caco-2 cells with resveratrol and IL-6

Caco-2 cell monolayers were incubated with or without resveratrol (100 and 200 μmol/L) for 1 h, after which IL-6 (10 ng/mL) was added to the culture. TER was measured before and at 1, 3, 6, 12, and 24 h after the treatment with IL-6. Caco-2 cell extracts were collected to analyze claudin-2 protein expression at 24 h and ERK phosphorylation at 15 and 30 min after the treatment with IL-6. The cell monolayers were then fixed for immunostaining

7.2.4 Measurement of TER across Caco-2 cell monolayers

Intestinal TJ barrier integrity was evaluated by measuring TER as described previously (Chapter 3).

7.2.5 Preparation of detergent-insoluble fractions and whole-cell extracts

Detergent-insoluble fractions, which correspond to actin-cytoskeleton-associated protein content, and whole-cell extracts were prepared as described previously (Chapter 3).

7.2.6 Immunoblot analysis

The protein expression levels of claudin-2 and p-ERK in Caco-2 cells were determined by immunoblot analysis as described previously (Chapter 3).

7.2.7 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

The mRNA expression of claudin-2 in Caco-2 cells was determined by qRT-PCR as described previously (Azuma, et al. 2013; Kaikiri et al., 2017). Total RNA was isolated using RNA Iso Plus (Takara Bio Inc., Kusatsu, Japan) and were analyzed as described previously (Chapter 4). Primer sequences used for PCR are listed in Table 5.1.

7.2.8 Immunostaining method

Caco-2 cells were stained as described previously (Chapter 3).

7.2.9 Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed Statistical analysis was conducted as described previously (Chapter 3).

7.3 Results

7.3.1 TER

TER decreased at and after 12 h of incubation with 10 ng/mL of IL-6; however, resveratrol effectively restored TER in a dose-dependent manner (Fig. 7.1).

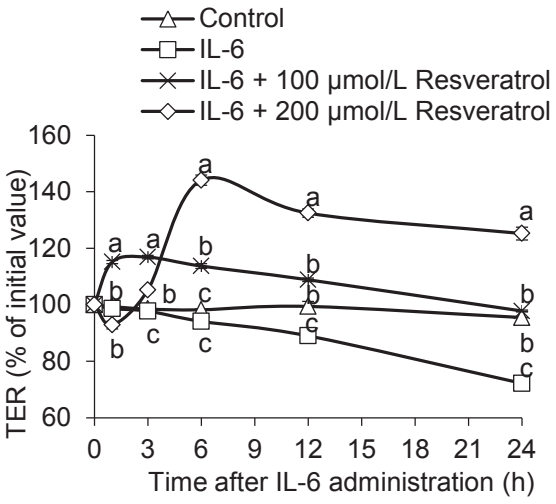


Figure 7.1. Resveratrol restores tight junction integrity in interleukin (IL)-6-induced hyperpermeability in Caco-2 cell monolayers. Transepithelial electrical resistance (TER) across cell monolayers incubated with or without 100-200 µmol/L resveratrol and 10 ng/mL IL-6 was measured. All the values are expressed as mean ± standard error of measurement (n = 3). Means without a common letter differ significantly from each other ($P < 0.05$).

7.3.2 Protein and gene expression of claudin-2

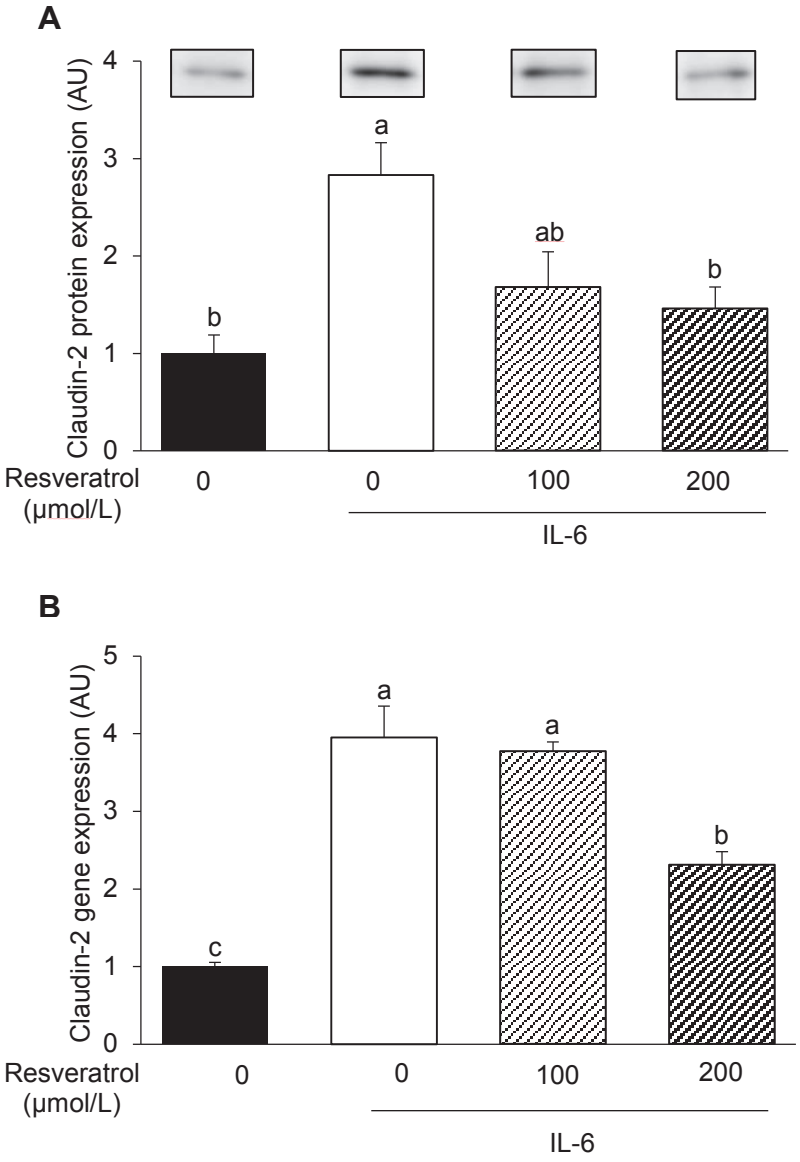


Figure 7.2. Resveratrol downregulates claudin-2 expression in interleukin (IL)-6-induced hyperpermeability in Caco-2 cell monolayers. A, Immunoblot analysis of claudin-2 protein expression in cell monolayers incubated with or without 100-200 µmol/L resveratrol and 10 ng/mL IL-6. B, Quantitative reversed transcriptase polymerase chain reaction analysis of claudin-2 gene expression in the cell monolayers incubated with or without 100-200 µmol/L resveratrol and 10 ng/mL IL-6. Values are expressed as mean ± standard error of measurement (n = 3). Means without a common letter differ significantly from each other ($P < 0.05$).

IL-6 increased claudin-2 protein expression in the Caco-2 cells; however, this increase was suppressed by resveratrol in a dose-dependent manner (Fig. 7.2.A). Furthermore, results of the qRT-PCR analysis showed that IL-6 increased claudin-2 gene expression in the Caco-2 cells, whereas 200 µmol/L resveratrol decreased this increase (Fig. 7.2.B). Additionally, the microscopy analysis revealed that resveratrol reduced the protein expression of claudin-2 that had been increased by IL-6 (Fig. 7.3).

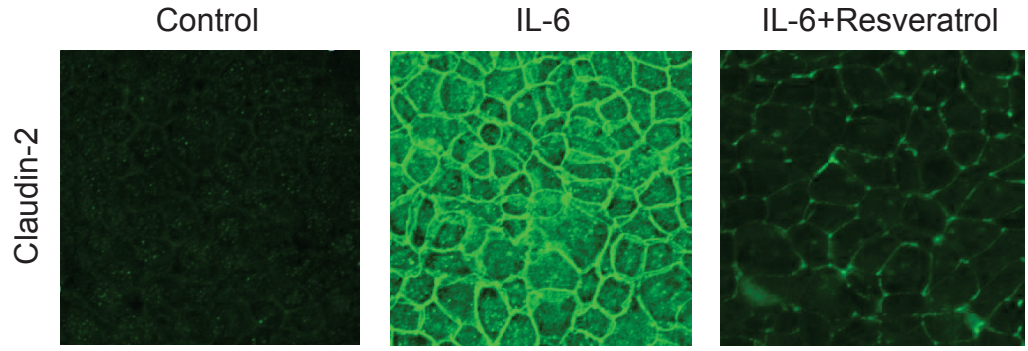


Figure 7.3. Immunofluorescence analysis of claudin-2 expression in cell monolayers incubated with or without 200 µmol/L resveratrol and 10 ng/mL IL-6. Images were collected using a confocal microscope. Each image is representative of three monolayers.

7.3.3 Phosphorylation of ERK

It has been reported that the MEK/ERK pathway has a primary role in mediating IL-6-induced claudin-2 expression (Suzuki et al., 2011). The immunoblot analysis showed that IL-6 increased ERK expression in the Caco-2 cell monolayers; however, this increase was decreased by resveratrol (Fig. 7.4).

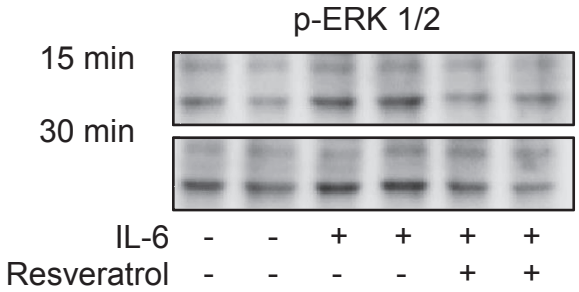


Figure 7.4. Resveratrol suppresses interleukin (IL)-6-induced phosphorylation of extracellular signal-regulated kinase (ERK) in Caco-2 cell monolayers. Immunoblot analysis was performed to assess p-ERK 1/2 expression in cell monolayers incubated with or without 100-200 μmol/L resveratrol and 10 ng/mL IL-6 for 15 and 30 min. Each image is representative of the results from three independent experiments.

7.4 Discussion and conclusion

A growing body of evidence suggests that barrier dysfunction is associated with robust production of pro-inflammatory cytokines such as tumor necrosis factor-α, interferon-γ, IL-6, IL-1β, and IL-13. IL-6 is produced in substantially higher amounts in the serum and tissues of patients with active IBD; therefore, increased IL-6 levels are correlated to IBD severity (Nakamura et al., 1992; Sommer et al., 2014). Our previous study showed that IL-6 induces intestinal hyperpermeability by increasing claudin-2 expression (Suzuki et al., 2011). Moreover, it has been reported that claudin-2 is highly expressed in the inflamed tissues of patients with different intestinal diseases such as ulcerative colitis, Crohn's disease, and celiac disease (Prasad et al., 2005). Although the precise role of claudin-2 expression in the pathogenesis of these diseases is unclear, increased claudin-2 expression seems to contribute to breaks in TJ strands and induction of diarrhea. Therefore, suppression of increased claudin-2 expression by food factor could become a new approach to mitigate the disease progression. This study showed that resveratrol suppressed claudin-2 overexpression in the inflamed Caco-2 cells, however, this increase did not occur in the DSS-treated mice (Fig. 4.4). It is suggested that DSS administration disrupts the colon structure and TJ barrier.

The molecular mechanisms underlying resveratrol-mediated protection against the effects of IL-6 seem to differ from the improvement of TJ barrier integrity that was observed in the unstimulated Caco-2 cells. This is because resveratrol suppressed intracellular events induced by IL-6, such as phosphorylation of ERK. We have previously shown that IL-6 activates the

MEK/ERK pathway through gp130/IL-6Rα interaction, which in turn enhances Cdx2 expression and results in increased claudin-2 expression (Suzuki et al., 2011). Immunoblot analysis reveals that resveratrol reduces IL-6-induced phosphorylation of ERK. Our results corroborate previous findings that resveratrol induces apoptosis of colonic cancer SW480 cells by inhibiting the MEK/ERK pathway. Accordingly, resveratrol reduces IL-6-induced claudin-2 expression and hyperpermeability by targeting MEK/ERK signaling.

The results of the present study show that resveratrol has the potential to inhibit inflammatory or harmful intracellular signaling to protect against IL-6-induced barrier disruption. Resveratrol-mediated regulation of intestinal barrier integrity may be associated with prevention or alleviation of inflammatory diseases.

CHAPTER 8

OVERALL CONCLUSIONS

The purpose of this study was to survey and select polyphenols having potentials to promote intestinal TJ barrier integrity, to examine alleviative effects of selected polyphenol on intestinal barrier defects and inflammation in both *in vitro* and *in vivo* studies, and to unveil molecular mechanisms underlying the polyphenol-mediated protection in intestinal TJ barrier.

The result of this study shows that among three polyphenols tested including resveratrol, EGCG and delphinidin, only resveratrol showed to increase the intestinal integrity in Caco-2 cell monolayers.

In the *in vitro* study, this study shows that resveratrol has the potential to protect the TJ barrier and promote TJ barrier integrity in intestinal Caco-2 cells. In the absence of harmful substances, resveratrol-mediated promotion of TJ barrier integrity occurs through increased expression and assembly of TJ proteins in intestinal epithelial cells. However, resveratrol inhibits inflammatory or harmful intracellular signaling to protect against IL-6- and H_2O_2-induced barrier disruption. Resveratrol-mediated regulation of intestinal barrier integrity may be associated with prevention or alleviation of inflammatory diseases.

In the *in vivo* study, this study suggests that supplemental resveratrol prevents barrier defects and inflammation in DSS-induced colitic mice through the inhibition of IL-8 production in inflamed tissue, in turn resulting in the prevention of neutrophil infiltration into inflamed tissues. In addition, the study using Caco-2 cell monolayers demonstrated that resveratrol suppresses TNF-α-

induced-inflammatory signaling and IL-8 production. This study, therefore, elucidates the novel molecular mechanisms underlying the beneficial role of resveratrol for maintaining intestinal homeostasis.

Although the research has reached its aims, there were some unavoidable limitations. First, the DSS-induced colitis model used in this study is an acute model; because IBDs are chronic inflammatory diseases, the result interpretation to the implementation in disease therapeutic seems not enough. Second, this study suggested potential benefits of resveratrol derivatives and other stillbenes in the IBDs therapeutic approach. However, due to the limited time, the research could not cover these issues. Therefore, for the future studies, it is necessary to examine the protective effect of resveratrol in the chronic colitis model, and it is promising to investigate the protective effects of resveratrol derivatives such as piceatannol or naturally resveratrol analog such as pterostilbene in IBDs.

REFERENCE

Abouaf-tabet, E.R., Kolkhorst, F.W., Hong, M.Y., 2014. Effects of resveratrol on inflammatory bowel disease : A Review. Journal of Nutritional Health & Food Science 2(3), 1–6.

Aichinger, G., Puntscher, H., Beisl, J., Kütt, M.L., Warth, B., Marko, D., 2018. Delphinidin protects colon carcinoma cells against the genotoxic effects of the mycotoxin altertoxin II. Toxicology Letters 284, 136–142.

Al-Sadi, R., Khatib, K., Guo, S., Ye, D., Youssef, M., Ma, T., 2011. Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. AJP: Gastrointestinal and Liver Physiology 300(6), G1054–G1064.

Alfadda, A.A., Sallam, R.M., 2012. Reactive oxygen species in health and disease. Journal of Biomedicine & Biotechnology 2012, 936486.

Alonzi, T., Fattori, E., Lazzaro, D., Costa, P., Probert, L., Kollias, G., De Benedetti, F., Poli, V., Ciliberto, G., 1998. Interleukin 6 is required for the development of collagen-induced arthritis. The Journal of Experimental Medicine 187(4), 461–8.

Amasheh, S., 2002. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. Journal of Cell Science 115(24), 4969–4976.

Atkinson, K.J., Rao, R.K., 2001. Role of protein tyrosine phosphorylation in acetaldehyde-induced disruption of epithelial tight junctions. American journal of physiology. Gastrointestinal and Liver Physiology 280(6), G1280–G1288.

Atreya, R., Neurath, M.F., 2014. IBD pathogenesis in 2014: Molecular pathways

controlling barrier function in IBD. Nature Reviews Gastroenterology & Hepatology 12(2), 67–68.

Azuma, T., Shigeshiro, M., Kodama, M., Tanabe, S., Suzuki, T., 2013. Supplemental naringenin prevents intestinal barrier defects and inflammation in colitic mice. Journal of Nutrition 143, 827–834.

Balda, M.S., 2000. The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. The EMBO Journal 19(9), 2024–2033.

Basuroy, S., Seth, A., Elias, B., Naren, A.P., Rao, R., 2006. MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. The Biochemical Journal 393(Pt 1), 69–77.

Bazzoni, G., 2003. The JAM family of junctional adhesion molecules. Current Opinion in Cell Biology 15(5), 525-530.

Beaurepaire, C., Smyth, D., McKay, D.M., 2009. Interferon-γ regulation of intestinal epithelial permeability. Journal of Interferon & Cytokine Research 29(3), 133–144.

Broer, S., 2008. Amino acid transport across mammallian intestinal and renal epithelia. Physiological Reviews 88(1), 249–286.

Bruggen TVD, Nijenhuis S, Verhoef EVR, A.B., 1999. Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the Raf-1/MEK1-MEK2/ERK1-ERK2 pathway. Infection and Immunity 67(8), 3824–3829.

Cabré, E., Domènech, E., 2012. Impact of environmental and dietary factors on

the course of inflammatory bowel disease. World Journal of Gastroenterology 18(29), 3814–3822.

Chamcheu, J.C., Pal, H.C., Siddiqui, I.A., Adhami, V.M., Ayehunie, S., Boylan, B.T., Noubissi, F.K., Khan, N., Syed, D.N., Elmets, C.A., Wood, G.S., Afaq, F., Mukhtar, H., 2015. Prodifferentiation, anti-inflammatory and antiproliferative effects of delphinidin, a dietary anthocyanidin, in a full-thickness three-dimensional reconstituted human skin model of psoriasis. Skin Pharmacology and Physiology 28(4), 177–188.

Cheon, J.H., 2013. Genetics of inflammatory bowel diseases: A comparison between western and eastern perspectives. Journal of Gastroenterology and Hepatology (Australia) 28(2), 220–226.

Contrera, J.F., Matthews, E.J., Kruhlak, N.L., Benz, R.D., 2004. Estimating the safe starting dose in phase I clinical trials and no observed effect level based on QSAR modeling of the human maximum recommended daily dose. Regulatory Toxicology and Pharmacology 40(3), 185–206.

Crandall, J.P., Oram, V., Trandafirescu, G., Reid, M., Kishore, P., Hawkins, M., Cohen, H.W., Barzilai, N., 2012. Pilot study of resveratrol in older adults with impaired glucose tolerance. The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences 67(12), 1307–12.

Cui, X., Jin, Y., Hofseth, A.B., Pena, E., Habiger, J., Chumanevich, A., Poudyal, D., Nagarkatti, M., Nagarkatti, P.S., Singh, U.P., Hofseth, L.J., 2010. Resveratrol suppresses colitis and colon cancer associated with colitis. Cancer Prevention Research 3(4), 549–559.

Denker, B.M., Sabath, E., 2011. The biology of epithelial cell tight junctions in

the kidney. Journal of the American Society of Nephrology: JASN 22, 622–625.

Elias, B.C., Suzuki, T., Seth, A., Giorgianni, F., Kale, G., Shan, L., Turner, J.R., Naren, A., Desiderio, D.M., Rao, R., 2009. Phosphorylation of Tyr-398 and Tyr-402 in occludin prevents Its interaction with ZO-1 and destabilizes Its assembly at the tight junctions. Journal of Biological Chemistry 284(3), 1559–1569.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K., Tsukita, S., 1998. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. Journal of Cell Biology 141(7), 1539–1550.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S., Tsukita, S., 1993. Occludin: A novel integral membrane protein localizing at tight junctions. Journal of Cell Biology 123(6 II), 1777–1788.

Ganesan, K., Xu, B., 2017. A critical review on polyphenols and health benefits of black soybeans. Nutrients 9(5), 455.

Ghanim, H., Sia, C.L., Korzeniewski, K., Lohano, T., Abuaysheh, S., Marumganti, A., Chaudhuri, A., Dandona, P., 2011. A resveratrol and polyphenol preparation suppresses oxidative and inflammatory stress response to a high-fat, high-carbohydrate meal. Journal of Clinical Endocrinology and Metabolism 96(5), 1409–1414.

Gonzalezmariscal, L., Tapia, R., Chamorro, D., 2008. Crosstalk of tight junction components with signaling pathways. Biochimica et Biophysica Acta - Biomembranes 1778(3), 729–756.

Gonzalezmariscal, L., 2003. Tight junction proteins. Progress in Biophysics and Molecular Biology 81(1), 1–44.

Higdon, J. V., Frei, B., 2003. Tea Catechins and polyphenols: health effects, metabolism, and antioxidant functions. Critical Reviews in Food Science and Nutrition 43(1), 89-143.

Holmes, J.L., Van Itallie, C.M., Rasmussen, J.E., Anderson, J.M., 2006. Claudin profiling in the mouse during postnatal intestinal development and along the gastrointestinal tract reveals complex expression patterns. Gene Expression Patterns 6(6), 581–588.

Jaeschke, H., 2011. Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. Journal of Gastroenterology and Hepatology, 26, 173–179.

Johns Hopkins Medicine. 2003. Crohn's disease : introduction. Retrieved https://www.hopkinsmedicine.org/gastroenterology_hepatology, from Johns Hopkins Medicine Organization.

Kaikiri, H., Miyamoto, J., Kawakami, T., Park, S.B., Kitamura, N., Kishino, S., Yonejima, Y., Hisa, K., Watanabe, J., Ogita, T., Ogawa, J., Tanabe, S., Suzuki, T., 2017. Supplemental feeding of a gut microbial metabolite of linoleic acid, 10-hydroxy-cis-12-octadecenoic acid, alleviates spontaneous atopic dermatitis and modulates intestinal microbiota in NC/nga mice. International Journal of Food Sciences and Nutrition 68(8), 941–951.

Kale, G., Naren, A.P., Sheth, P., Rao, R.K., 2003. Tyrosine phosphorylation of occludin attenuates its interactions with ZO-1, ZO-2, and ZO-3. Biochemical and Biophysical Research Communications 302(2), 324–329.

Kang, Y., Xue, Y., Du, M., Zhu, M.-J., 2017. Preventive effects of Goji berry on dextran-sulfate-sodium-induced colitis in mice. The Journal of Nutritional Biochemistry 40, 70–76.

Khan, N., Mukhtar, H., 2007. Tea polyphenols for health promotion. Life Sciences 81(7), 519–533.

König, J., Wells, J., Cani, P.D., García-Ródenas, C.L., MacDonald, T., Mercenier, A., Whyte, J., Troost, F., Brummer, R.J., 2016. Human intestinal barrier function in health and disease. Clinical and Translational Gastroenterology 7(10), e196.

Kopylov, U., Ben-Horin, S., Seidman, E., 2014. Therapeutic drug monitoring in inflammatory bowel disease. Annals of gastroenterology : quarterly publication of the Hellenic Society of Gastroenterology 27(4), 304–312.

Kühn, R., Löhler, J., Rennick, D., Rajewsky, K., Müller, W., 1993. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75(2), 263–274.

Kunzelmann, K., Mall, M., 2002. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. Physiological Reviews 82(1), 245–289.

Lambert, J.D., Yang, C.S., 2003. Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis. pp. 201–208.

Landy, J., Ronde, E., English, N., Clark, S.K., Hart, A.L., Knight, S.C., Ciclitira, P.J., Al-Hassi, H.O., 2016. Tight junctions in inflammatory bowel diseases and inflammatory bowel disease associated colorectal cancer. World Journal of Gastroenterology 22(11), 3117-3126.

Laukoetter, M.G., Nava, P., Lee, W.Y., Severson, E.A., Capaldo, C.T., Babbin, B.A., Williams, I.R., Koval, M., Peatman, E., Campbell, J.A., Dermody, T.S., Nusrat, A., Parkos, C.A., 2007. JAM-A regulates permeability and inflammation in the intestine in vivo. The Journal of Experimental Medicine 204(13), 3067–3076.

Lee, H.S., Chauhan, S.K., Okanobo, A., Nallasamy, N., Dana, R., 2011. Therapeutic efficacy of topical epigallocatechin gallate (EGCG) in murine dry eye. Cornea 30(12), 1465–72.

Lissner, D., Schumann, M., Batra, A., Kredel, L.-I., Kühl, A.A., Erben, U., May, C., Schulzke, J.-D., Siegmund, B., 2015. Monocyte and M1 macrophage-induced barrier defect contributes to chronic intestinal inflammation in IBD. Inflammatory Bowel Diseases 21(6), 1297-1305.

Lopez-Bayghen, E., Jaramillo, B.E., Huerta, M., Betanzos, A., Gonzalez-Mariscal, L., 2007. TJ proteins that make round trips to the nucleus. Tight Junctions, 76–100.

Magyar, K., Halmosi, R., Palfi, A., Feher, G., Czopf, L., Fulop, A., Battyany, I., Sumegi, B., Toth, K., Szabados, E., 2012. Cardioprotection by resveratrol: a human clinical trial in patients with stable coronary artery disease. Clinical Hemorheology and Microcirculation 50(3), 179–187.

Mahida, Y.R., Ceska, M., Effenberger, F., Kurlak, L., Lindley, I., Hawkey, C.J., 1992. Enhanced synthesis of neutrophil-activating peptide-1/interleukin-8 in active ulcerative colitis. Clinical science 82(3), 273–5.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. American Journal of

Clinical Nutrition 79(5). 727-747.

Matricon, J., Barnich, N., Ardid, D., 2010. Immunopathogenesis of inflammatory bowel disease. Self/Nonself Immune Recognition and Signaling 1(4), 299–309.

Meiers, S., Kemény, M., Weyand, U., Gastpar, R., von Angerer, E., Marko, D., 2001. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. Journal of Agricultural and Food Chemistry 49(2), 958–962.

Molodecky, N.A., Soon, I.S., Rabi, D.M., Ghali, W.A., Ferris, M., Chernoff, G., Benchimol, E.I., Panaccione, R., Ghosh, S., Barkema, H.W., Kaplan, G.G., 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology 142(1), 46–54.

Murakami, Y., Tanabe, S., Suzuki, T., 2016. High-fat diet-induced intestinal hyperpermeability is associated with increased bile acids in the large intestine of mice. Journal of Food Science 81(1), H216–H222.

Nakamura, M., Saito, H., Kasanuki, J., Tamura, Y., Yoshida, S., 1992. Cytokine production in patients with inflammatory bowel disease. Gut 33(7), 933–937.

Ng, S.C., Tang, W., Ching, J.Y., Wong, M., Chow, C.M., Hui, A.J., Wong, T.C., Leung, V.K., Tsang, S.W., Yu, H.H., Li, M.F., Ng, K.K., Kamm, M.A., Studd, C., Bell, S., Leong, R., De Silva, H.J., Kasturiratne, A., Mufeena, M.N.F., Ling, K.L., Ooi, C.J., Tan, P.S., Ong, D., Goh, K.L., Hilmi, I., Pisespongsa, P., Manatsathit, S., Rerknimitr, R., Aniwan, S., Wang, Y.F.,

Ouyang, Q., Zeng, Z., Zhu, Z., Chen, M.H., Hu, P.J., Wu, K., Wang, X., Simadibrata, M., Abdullah, M., Wu, J.C., Sung, J.J.Y., Chan, F.K.L., 2013. Incidence and phenotype of inflammatory bowel disease based on results from the Asia-Pacific Crohn's and colitis epidemiology study. Gastroenterology 145(1), 158–165.

Niewiadomski, O., Studd, C., Wilson, J., Williams, J., Hair, C., Knight, R., Prewett, E., Dabkowski, P., Alexander, S., Allen, B., Dowling, D., Connell, W., Desmond, P., Bell, S., 2016. Influence of food and lifestyle on the risk of developing inflammatory bowel disease. Internal Medicine Journal 46(6), 669–676.

Noda, S., Tanabe, S., Suzuki, T., 2014. Quercetin increases claudin-4 expression through multiple transcription factors in intestinal Caco-2 cells. Journal of Functional Foods 10, 112–116.

Noda, S., Tanabe, S., Suzuki, T., 2012. Differential effects of flavonoids on barrier integrity in human intestinal Caco-2 cells. Journal of Agricultural and Food Chemistry 60(18), 4628–4633.

Ogita, T., Tanii, Y., Morita, H., Suzuki, T., Tanabe, S., 2011. Suppression of Th17 response by *Streptococcus thermophilus* ST28 through induction of IFN-γ. International Journal of Molecular Medicine 28(5), 817–822.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nuñez, G., Cho, J.H., 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn ' s disease. Nature 411(6837), 603–606.

Ordás, I., Eckmann, L., Talamini, M., Baumgart, D.C., Sandborn, W.J., 2012. Ulcerative colitis. Lancet 380(9853), 1606–19.

Oyama, M., Van Hung, T., Yoda, K., He, F., Suzuki, T., 2017. A novel whey tetrapeptide IPAV reduces interleukin-8 production induced by TNF-α in human intestinal Caco-2 cells. Journal of Functional Foods 35, 376–383.

Pandey, K.B., Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity 2(5), 270-278.

Parameswaran, N., Patial, S., 2010. Tumor necrosis factor-α signaling in macrophages. Critical Reviews in Eukaryotic Gene Expression 20(2), 87–103.

Piechota-Polanczyk, A., Fichna, J., 2014. Review article: The role of oxidative stress in pathogenesis and treatment of inflammatory bowel diseases. Naunyn-Schmiedeberg's Archives of Pharmacology 387(7), 605-620.

Ponder, A., Long, M.D., 2013. A clinical review of recent findings in the epidemiology of inflammatory bowel disease. Clinical Epidemiology 5(1), 237-247.

Rao, R., 2009. Occludin phosphorylation in regulation of epithelial tight junctions. Annals of the New York Academy of Sciences 1165, 62–68.

Rose, W.A., Sakamoto, K., Leifer, C.A., 2012. TLR9 is important for protection against intestinal damage and for intestinal repair. Scientific Reports 2, 574.

Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y., Tsukita, S., 1997. Possible involvement of phosphorylation of occludin in tight junction

formation. Journal of Cell Biology 137(6), 1393–1401.

Sánchez-Fidalgo, S., Cárdeno, A., Villegas, I., Talero, E., de la Lastra, C.A., 2010. Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. European Journal of Pharmacology 633(1–3), 78–84.

Seth, A., Sheth, P., Elias, B.C., Rao, R., 2007. Protein phosphatases 2A and 1 interact with occludin and negatively regulate the assembly of tight junctions in the CACO-2 cell monolayer. Journal of Biological Chemistry 282(15), 11487–11498.

Sheth, P., Samak, G., Shull, J.A., Seth, A., Rao, R., 2009. Protein phosphatase 2A plays a role in hydrogen peroxide-induced disruption of tight junctions in Caco-2 cell monolayers. Biochemical Journal 421(1), 59–70.

Singh, U.P., Singh, N.P., Singh, B., Hofseth, L.J., Price, R.L., Nagarkatti, M., Nagarkatti, P.S., 2010. Resveratrol (trans-3,5,4'-trihydroxystilbene) induces silent mating type information regulation-1 and down-regulates nuclear transcription factor-kappaB activation to abrogate dextran sulfate sodium-induced colitis. The Journal of pharmacology and experimental therapeutics 332(3), 829–39.

Sommer, J., Engelowski, E., Baran, P., Garbers, C., Floss, D.M., Scheller, J., 2014. Interleukin-6, but not the interleukin-6 receptor plays a role in recovery from dextran sodium sulfate-induced colitis. International Journal of Molecular Medicine 34(3), 651–660.

Statovci, D., Aguilera, M., MacSharry, J., Melgar, S., 2017. The impact of western diet and nutrients on the microbiota and immune response at

mucosal interfaces. Frontiers in Immunology 8, Article 838.

Stervbo, U., Vang, O., Bonnesen, C., 2007. A review of the content of the putative chemopreventive phytoalexin resveratrol in red wine. Food Chemistry 101(2), 449–457.

Suzuki, T., 2013. Regulation of intestinal epithelial permeability by tight junctions. Cellular and Molecular Life Sciences 70(4), 631-659.

Suzuki, T., Hara, H., 2010. Dietary fat and bile juice, but not obesity, are responsible for the increase in small intestinal permeability induced through the suppression of tight junction protein expression in LETO and OLETF rats. Nutrition and Metabolism (Lond) 7, 19.

Suzuki, T., Yoshinaga, N., Tanabe, S., 2011. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. Journal of Biological Chemistry 286(36), 31263–31271.

Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S., LoeningBaucke, V., Ortner, M., Weber, J., Hoffmann, U., Schreiber, S., Dietel, M., Lochs, H., 2002. Mucosal flora in inflammatory bowel disease. Gastroenterology 122(1), 44–54.

Szekeres, T., Saiko, P., Fritzer-Szekeres, M., Djavan, B., Jäger, W., 2011. Chemopreventive effects of resveratrol and resveratrol derivatives. Annals of the New York Academy of Sciences 1215(1), 89–95.

Szigethy, E., McLafferty, L., Goyal, A., 2010. Inflammatory bowel disease. Child and Adolescent Psychiatric Clinics of North America 19(2), 301–318.

Timmers, S., Auwerx, J., Schrauwen, P., 2012. The journey of resveratrol from yeast to human. Aging 4(3), 146–158.

Tipoe, G.L., Leung, T.-M., Hung, M.-W., Fung, M.-L., 2007. Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection. Cardiovascular & Hematological Disorders: Drug Targets 7(2), 135–144.

Tomé-Carneiro, J., Gonzálvez, M., Larrosa, M., Yáñez-Gascón, M.J., García-Almagro, F.J., Ruiz-Ros, J.A., Tomás-Barberán, F.A., García-Conesa, M.T., Espín, J.C., 2013. Grape resveratrol increases serum adiponectin and downregulates inflammatory genes in peripheral blood mononuclear cells: A triple-blind, placebo-controlled, one-year clinical trial in patients with stable coronary artery disease. Cardiovascular Drugs and Therapy 27(1), 37–48.

Tontini, G.E., Vecchi, M., Pastorelli, L., Neurath, M.F., Neumann, H., 2015. Differential diagnosis in inflammatory bowel disease colitis: state of the art and future perspectives. World Journal of Gastroenterology 21(1), 21–46.

Turner, J.R., 2009. Intestinal mucosal barrier function in health and disease. Nature reviews. Immunology 9(11), 799–809.

Turner, J.R., 2006. Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. American Journal of Pathology 169(6), 1901–1909.

Tsao, Rong., 2010. Chemistry and biochemistry of dietary polyphenol: a review. Nutrients 2, 1231-1246.

Ueno, F., Nakayama, Y., Hagiwara, E., Kurimoto, S., Hibi, T., 2017. Impact of inflammatory bowel disease on Japanese patients' quality of life: results of

a patient questionnaire survey. Journal of Gastroenterology 52(5), 555–567.

Umeda, K., Matsui, T., Nakayama, M., Furuse, K., Sasaki, H., Furuse, M., Tsukita, S., 2004. Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. The Journal of biological chemistry 279(43), 44785–44794.

Van Itallie, C.M., Anderson, J.M., 2006. Claudins and epithelial paracellular transport. Annual Review of Physiology 68(1), 403–429.

Van Staa, T.P., Card, T., Logan, R.F., Leufkens, H.G., 2005. 5-Aminosalicylate use and colorectal cancer risk in inflammatory bowel disease: a large epidemiological study. Gut 54(11), 1573–1578.

Wang, C., Zhu, L., Ju, K., Liu, J., Li, K., 2017. Anti-inflammatory effect of delphinidin on intramedullary spinal pressure in a spinal cord injury rat model. Experimental and Therapeutic Medicine 5583–5588.

Williams, L.D., Burdock, G.A., Edwards, J.A., Beck, M., Bausch, J., 2009. Safety studies conducted on high-purity trans-resveratrol in experimental animals. Food and Chemical Toxicology 47(9), 2170–2182.

Wong, V., Gumbiner, B.M., 1997. Synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. Journal of Cell Biology 136(2), 399–409.

Yang, S.K., Loftus, E. V., Sandborn, W.J., 2001. Epidemiology of inflammatory bowel disease in Asia. Inflammatory Bowel Diseases 7(3), 260-270.

Yao, J., Wang, J.Y., Liu, L., Li, Y.X., Xun, A.Y., Zeng, W. Sen, Jia, C.H., Wei, X.X., Feng, J.L., Zhao, L., Wang, L.S., 2010. Anti-oxidant effects of

resveratrol on mice with DSS-induced ulcerative colitis. Archives of Medical Research 41(4), 288–294.

Yao, J., Wei, C., Wang, J.Y., Zhang, R., Li, Y.X., Wang, L.S., 2015. Effect of resveratrol on Treg/Th17 signaling and ulcerative colitis treatment in mice. World Journal of Gastroenterology 21(21), 6572–6581.

Yen, D., Cheung, J., Scheerens, H., Poulet, F., McClanahan, T., Mckenzie, B., Kleinschek, M.A., Owyang, A., Mattson, J., Blumenschein, W., Murphy, E., Sathe, M., Cua, D.J., Kastelein, R.A., Rennick, D., 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. Journal of Clinical Investigation 116(5), 1310–1316.

ACKNOWLEGDEMENT

My sincere thanks to all people who have helped and inspired me to be completed my Ph.D degree in Food Science. This thesis would not have been possible without those supports from many people.

First of all, my deepest and foremost gratitude is given to my supervisor, Professor Takuya Suzuki, for the continuous support of my Ph.D study and related research, for his patience, motivation and immense knowledge. His guidance helped me in all the time of research and writing of this thesis and related publications. I could not have imagined having a better advisor and mentor for my Ph.D study.

I would like to express my appreciation to the members of committee, Professor Manabu Asakawa and Professor Tadashi Shimamoto for the comprehensive and critical judgement of this thesis.

I would like to deeply acknowledge the financial support that I mainly received from the Indonesian government throughout my Ph.D study. I received a grant from Indonesia Endowment Fund for Education (LPDP), Ministry of Finance for Ph.D program in 2015-2019 and another one from the Japan Society for The Promotion of Science in Grant-in-Aid (KAKENHI) B: 16K07737 that provided financial support for the scientific research.

My warmest thanks to the Graduate School of Biosphere Science staff members, especially Ms. Himiko Koi who helped me to contribute official documents and further information are necessary in my Ph.D education.

Many thanks also go to all my lab mates in the Food Chemistry Laboratory for their friendly help and cooperation. I thank to all my Indonesian

friends who study in Hiroshima University, without them my Ph.D life would have been so lifeless.

My deepest gratitude goes to my husband, my parents, my son and my family for their unconditional love and support throughout my life.

Last but not least, I would like to thank Hiroshima University for giving me a good memories and special experiences in oversea education.

YUNIKA MAYANGSARI

2019