THE PROTECTIVE MECHANISMS OF QUERCETIN ON THE LEAKINESS OF INTESTINAL EPITHELIAL CELLS INDUCED BY IL-1β THROUGH MLCK DEPENDENT SIGNALING PATHWAYS

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THE PROTECTIVE MECHANISMS OF QUERCETIN ON THE LEAKINESS OF INTESTINAL EPITHELIAL CELLS INDUCED BY IL-1B THROUGH MLCK DEPENDENT SIGNALING PATHWAYS

BY

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Title	THE PROTECTIVE MECHANISMS OF QUERCETIN ON THE LEAKINESS OF INTESTINAL EPITHELIAL CELLS INDUCED BY IL-1β THROUGH MLCK DEPENDENT SIGNALING PATHWAYS
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Interleukin-1beta (IL-1β) is an important mediator in gastrointestinal inflammation and increased intestinal tight junction permeability via nuclear factor-kappa B (NF-**K**B)/Extracellular signal-regulated kinases (ERK)1/2/myosin light chain kinase (MLCK) pathway. Flavonol quercetin has potent anti-inflammation and intestinal barrier-promoting effects. This study investigates the effect of quercetin on intestinal barrier function, its protective effect and mechanism of quercetin on IL-1β induced tight junction (TJ) barrier dysfunction via NF-**K**B/ERK1/2/MLCK pathway in human colonic epithelial cancer cell line. The Caco-2 cells were treated with quercetin 1, 10 and 100 μM in the presence or absence of IL-1β 10 ng/ml for 24 and 48 h. Intestinal barrier function was assessed by Transepithelial Electrical Resistance (TER) and paracellular permeability assay of fluorescein isothiocyanate-labeled dextran (4 kDa, FD-4). The expression of NF-**K**B, i**K**B, ERK1/2, MLCK and phospho-MLC (p-MLC) proteins and *NF-**K**B* and *MLCK* gene were analyzed by western blot and real-time PCR. The NF-**K**B translocation was determined by immunofluorescence staining. All concentrations of quercetin, with or without IL-1β, were not toxic to Caco-2 cells, as determined by MTT assay. Quercetin 1 and 10 μM had no significant effect on TER and FD-4 flux, while quercetin 100 μM decreased TER at 24 h and increased FD-4 flux at 48 h. Cell exposure to IL-1β for 24-48 h significantly increased FD-4 flux and inhibited by quercetin pretreatment at 1 and 10 μM for 48 h. All of the target protein expressions were unaffected by quercetin alone. In contrast, IL-1β increased NF-**K**B, ERK1/2, MLCK and p-MLC and decreased i**K**B protein expression during 12-24 h. The IL-β induced increase in NF-**K**B, ERK1//2 MLCK and p-MLC proteins was prevented by quercetin 1 and 10 μM. The inhibitory effect of IL-1β on i**K**B protein was also prevented by quercetin 10 and 100 μM. However, the expression of *NF-**K**B* and *MLCK* gene in response to quercetin and IL-1β were not to parallel with protein expressions. The preventive effect of quercetin on IL-1β was correlated with the quercetin effect on decreased FD-4 flux induced by IL-1β. In addition, quercetin 1 μM was found to inhibit nuclear translocation of NF-**K**B induced by IL-1β. All of the results suggested that quercetin, especially at low concentrations maintains proper intestinal barrier function under normal condition and prevents an increased paracellular permeability of intestinal epithelium induced by IL-1β via inhibition of NF-**K**B/ERK1/2/MLCK pathway. This provides the potential role of quercetin for therapeutic application in alleviation and protection of intestinal barrier leakiness associated with inflammation.

Keyword : Caco-2 cells Intestinal inflammation Intestinal barrier Myosin light chain kinase Quercetin

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CHAPTER 1
INTRODUCTION

Gastrointestinal inflammation is one of the most common inflammatory processes in humans. Inflammation is the complex biological response resulting from harming gastrointestinal tissues by various stimuli especially toxins and pathogens. In the acute phase, inflammatory cells are recruited to the sites of infection to eliminate the stimuli and induce tissue healing. However, persistence of inflammatory stimuli or failure of healing process can lead to chronic inflammation [(1)]. The important cause of chronic inflammation is leaky gut which is mainly resulted from impairment of intestinal barrier due to damage of epithelial cell lining or leakiness of intestinal tight junction. This intestinal barrier impairment induces an increased intestinal permeability, allowing the movement of toxins and pathogens from the lumen into the bloodstream. Various substances and conditions have been reported to induce leaky gut such as dietary nutrients, infection, toxin, stress, and drugs [(2)]. Many diseases may be arisen or be worse due to leaky gut including inflammatory bowel disease (IBD), gastric ulcer, infectious diarrhea, celiac disease, esophagus and colorectal cancer, inflammatory skin condition, allergy, respiratory infection, arthritis, and diabetes [(3)]. One of the most common disease of chronic gastrointestinal inflammation is IBD which can induce the systemic infection and sepsis [(4)]. From the clinical studies, IBD patients have high levels of pro-inflammatory cytokines such as TNF-α and IL-1β in the intestinal tissues and blood circulation, which are associated with increased intestinal permeability [(5)]. The treatments of IBD usually use anti-inflammatory or anti-microbial drugs which have disadvantages such as drug resistance and immune imbalance [(6)]. To avoid these problems, the promotion of intestinal barrier by naturally occurring medicine may be the key approach to prevent the penetration of luminal pathogens from leaky gut, and consequent inflammation and systemic diseases.

The intestinal epithelium is composed of a single layer of epithelial cells lining the luminal surface of small and large intestines. The intestinal epithelial cells play an

important role in the secretion of hormones and digestive enzymes, absorption of nutrients, electrolytes and water, and protection of the host from pathogens and toxic molecules in the lumen. The absorptive process across the intestinal epithelium can occur through two routes, transcellular and paracellular pathways. The transcellular pathway is associated with the transport of sugars, amino acid, electrolytes, and short chain fatty acid predominately regulated by selective membrane transporters [(7)]. The paracellular transport through the space between epithelial cells is the main route of passive diffusion of water, solutes, and macromolecules, and is regulated by intercellular junctional complexes known as tight junction. The ability of intestinal epithelial cells to prevent the harmful intraluminal antigens penetrating the body makes the intestinal epithelium the most important physical barrier which is a part of innate host defense.

The intestinal barrier is an important host defense as epithelial cells contact luminal environments which consist of tremendous antigens, commensal bacteria, pathogens, and toxins. The intestinal barrier can be divided into three functions including physical barrier, chemical barrier, and immune barrier. Firstly, the physical barriers are composed of mucins secreted by goblet cells and a single layer of epithelial cells with tight junction (TJ). Tight junction is composed of a network of strands of tight junction proteins, claudins and occludins. These proteins of adjacent epithelial cells hold cells together to form protective and functional barriers to limit the passage of luminal antigens, toxins, and macromolecules into the blood circulation. Secondly, the chemical barrier involves secretions to counter pathogens and toxins such as antimicrobial peptide and pro-inflammatory cytokines. Lastly, the immune barrier comprises neutrophil, lymphocyte, and macrophage at submucosal sites. It is the last barrier to prevent the infection from luminal antigen by phagocytosis, neutralizing antigen, secreting antibodies, and pro-inflammatory cytokines [(8)].

As previously mentioned, intestinal tight junction is the most important physical barrier which allows water, ions, and organic molecules, but not pathogens, to pass through paracellular pathway. The permeability of paracellular pathway reflects the tight

junction integrity which is commonly detected by transepithelial electrical resistance (TER) and paracellular permeation of macromolecule [9]. The impairment or leakiness of intestinal tight junction is correlated with a decreased TER and an increased paracellular permeability to macromolecules. Accumulating evidence have been demonstrated that paracellular permeability is regulated by assembly of tight junction proteins and activation of myosin light chain kinase (MLCK) pathway which induces tight junction opening [7, 10, 11]. Main compositions of tight junction proteins i.e., claudin, occludin and junctional adhesion molecule (JAMs) form the complex protein and link with zonula occluden (ZO)-1, 2 which attaches to actin filament. The increasing of tight junction proteins which are categorized as barrier builder, i.e., occludin, claudin-1, claudin-4, JAMs increases TER and decreases paracellular permeability [10]. On the other hand, the increasing of pore forming proteins such as claudin-2 decreases TER and increases paracellular permeability. Furthermore, MLCK which is the enzyme to phosphorylate myosin light chain (MLC) is the key target to control tight junction permeability. The activation of MLCK pathway induces tight junction opening by activating actomyosin contraction, leading to decreasing TER and increasing paracellular permeability [2].

IL-1β is the pro-inflammatory cytokines which can be secreted from epithelial cells and immune cells. IL-1β is an important mediator for inflammation, proliferation, differentiation, and apoptosis. High levels of IL-1β in the intestinal tissues and blood circulation have been found in IBD patients. Moreover, human intestinal cancer Caco-2 cells challenged by IL-1β showed a decrease in TER and an increase in paracellular permeability of inulin flux due to impairment of tight junction [2]. Several signaling pathways including NF-κB and kinases, such as mitogen-activated protein kinase (MAPK) and MLCK, have been reported to mediate the IL-1β effect on intestinal inflammation by disrupting the tight junction permeability [11, 12]. IL-1β binding to its receptor induces NF-κB translocation into nucleus to activate MLCK gene transcription. The increased levels of MLCK gene and then MLCK protein turns to convert myosin light chain (MLC) to the phosphorylated form of myosin light chain (p-MLC). The active p-MLC interacts with actin to induce the contraction of actomyosin and open the tight

junction leading to increased paracellular permeability [13]. Experiment in Caco-2 cells shows that the pathological dose of IL-1β (10 ng/ml) decreases TER and increases the paracellular permeability during 0-72 h. These results are correlated with IL-1β induced MLCK mRNA and protein expression resulting in increased MLCK activity and tight junction opening [2]. Moreover, IL-1β induces intestinal permeability by activating the p38 MAPK signaling pathway in Caco-2 cells [12]. Similarly, ERK1/2, which is the important signaling molecule of IL-1β in MAPK pathway can induce MLCK gene transcription. IL-1β 10 ng/ml also decreases TER and increases paracellular permeability during 0-72 h in Caco-2 cells via ERK1/2 signaling pathway. ERK1/2 activates transcription factor Elk-1 to bind with MLCK promotor leading to increased MLCK protein synthesis and opening tight junction [11]. From the above mentioned, IL-1β impairs tight junction barrier via NF-κB, ERK1/2 and MLCK signaling pathways. Therefore, an inhibition of NF-κB and ERK1/2 associated with MLCK pathway is the target mechanism to prevent or alleviate the effect of IL-1β-on inducing tight junction permeability of intestinal epithelial barrier.

Flavonoids are polyphenolic compounds that are secondary metabolites from various plants including vegetables, fruits, bark, grain, root, flower, stem, wine, and tea [14]. The flavonoids consist of six major groups: isoflavone, anthocyanidins, flavones, flavonols, flavan-3-ols and flavonones. Among these flavonoid groups, flavonols get much attention regarding their biological activities on promoting intestinal barrier. Quercetin is one of the most flavonol in human diets, fruits, and vegetables such as apples and onions. Quercetin has many biological effects such as anti-inflammation, anti-oxidation, anti-cancer, anti-allergy, promotion of immunity and intestinal barrier. Regarding the promotive effect on intestinal barrier, treatment of quercetin 100 μM for 48 h increases TER and decreases paracellular permeability of lucifer yellow (LY) flux in Caco-2 cells [15]. These results are correlated with the quercetin effect on increasing the expression and assembly of tight junction proteins claudin-4, occludin, ZO-2, and claudin-1, leading to barrier integrity enhancement via PKCδ inhibition [16]. Quercetin

also induces occludin phosphorylation leading to tight junction assembly [17]. The anti-inflammatory effect of quercetin is evidenced by decreasing pro-inflammatory cytokines, TNF-α, COX-6, IL-6 in Caco-2 cells [18]. Moreover, quercetin exerts potent anti-oxidative effect by inducing glutathione (GSH) synthesis and enhancing the Cu/Zn SOD, catalase (CAT), Mn SOD and GSH peroxidase which are the anti-oxidative enzymes in the hippocampal CA1 pyramidal neurons [19]. As mentioned above, quercetin has benefits to promote intestinal barrier via increasing tight junction protein function and expression; however, the mechanism of quercetin action on MLCK pathway is still unclear. Additionally, the protective effect on the leakiness of intestinal epithelial cells induced by IL-β through MLCK dependent signaling pathways remain unknown. The present study aimed to investigate the effect and underlying mechanisms of quercetin on intestinal barrier function via NF-κB and ERK1/2 associated with MLCK pathway in Caco-2 cells challenged by IL-1β. The predicted results of this study may provide the potential benefit of quercetin as the candidate therapeutic compound used for promoting intestinal barrier and preventing inflammation-associated leaky gut.

RESEARCH HYPOTHESES

1. Quercetin may change TER and paracellular permeability in Caco-2 cells
2. Quercetin prevents the changes in TER and paracellular permeability related with tight junction impairment in Caco-2 cells challenged with IL-1β
3. Quercetin inhibits the activation of MLCK pathway via NF-κB and ERK1/2 signaling molecules in Caco-2 cells challenged with IL-1β

OBJECTIVE OF THIS STUDY

1. To examine the effect of quercetin on tight junction permeability in Caco-2 cells
2. To investigate the protective effect and underlying mechanism of quercetin on IL-1β induced intestinal barrier dysfunction through NF-κB/ERK1/2/MLCK pathway in Caco-2 cells

CHAPTER 2
REVIEW LITERATURE

INTESTINAL EPITHELIAL CELL AND FUNCTION

Intestinal epithelial cells (IECs) are a single layer of polarized cells lining the luminal surface of small and large intestine. They joined together with tight junctions between adjacent cells to be the continuously impermeable layer. IECs are replaced every 4-5 days via renewal and migration. The crypts at the base of intestinal glands produce the new IECs, while the aged IECs will be apoptosis and shed into the lumen [20]. Basically, intestinal epithelia are composed of many cell types, including enterocytes, goblet cells, Paneth cells, microfold cells (M cells), neuroendocrine cells, exocrine cells and endocrine cells [20]. First, the enterocytes are the main population of intestinal epithelium. The structure of enterocytes comprises the simple columnar cells which plays role in absorption of water, ions, peptide, sugar and lipid and secretion of immunoglobulin [20]. The enterocytes express the protein and receptor for M cells at the membrane. The binding of specific antigen to its membrane receptor stimulates the production of proinflammatory cytokines. Moreover, they function as physical barrier to separate the environment between the intestinal lumen and the body. This function helps prevent the luminal pathogen invading into the body [20]. Second, goblet cells have the number almost 10% of IECs which secrete mucus for lubricating food and protecting the intestinal wall from enzyme [21]. Third, Paneth cells, only found in small intestines, synthesize, and secrete proteins and antimicrobial peptides. Moreover, Paneth cells can interact with antigen via MyD88-dependent pathway of toll-like receptor leading to expression of anti-microbial factors [22]. Forth, M cells of small intestine are located in gut-associated lymphoid tissue (GALT) of the Peyer's patches. M cells endocytose the luminal antigen to lamina propria to interact with immune dendritic cells and B lymphocyte [23]. Fifth, neuroendocrine cells can secrete peptides and hormones into the circulatory system in response to neural stimuli [24]. Besides, neuroendocrine cells are chemoreceptor which interacts with digestive substance to activate protective response

[25]. Sixth, exocrine cells at the mucosal layer of small intestine secrete mucus, sucrase, lactase, peptidase, lipase, and enteropeptidase [24]. At last, endocrine cells function to secrete secretin and cholecystokinin from chyme activation [26].

From all above, IECs have the many functions related with defensive mechanism, immunity, environment including communication with neighbor cells [27]. Besides, IECs are the barrier to protect antigen, toxin and pathogen invading from the lumen into the blood circulation [7]. Moreover, they can be selectively absorb electrolytes, nutrients and other substances [7]. At last, IECs can express toll-like receptor and nucleotide oligomerization domain proteins which recognize the specific pathogen leading to induction of immune response [28]. Thus, IECs are not only a physical barrier but also antigen recognition to stimulate the immune system.

EPITHELIAL PERMEABILITY

The intestinal epithelia are polarized with apical surfaces facing the lumen and basolateral surfaces closed to the underlying tissues which regulate the vectorial transportation of nutrients and ions [29]. The intestinal transport has two permeability routes, transcellular and paracellular permeability [9]. Transcellular permeability regulates transportation of sugars, amino acid, electrolytes, short chain fatty acid by transporters [7]. This transport allows the solutes to pass the epithelial membrane by active process. Meanwhile, lipophilic and small hydrophilic substances can pass through the lipid bilayer of membrane by passive process. Active transport is mediated by transporters, endocytosis, exocytosis, and transcytosis for ions, specific antigen, and amino acids. Large molecules such as bacterial products or proteins are captured by endocytosis and transcytosis by vector via cytoplasm and present antigen leading to immune response. On the other hand, virus, bacteria and particles entry host by endocytosis and phagocytosis via binding with the receptors at the cell membrane [30, 31].

Paracellular permeability allows 85% of the passive transepithelial flow passing between space of epithelial cells. This route allows the medium-sized hydrophilic molecule of <10 kDa passing through a tight junction. However, this tight junction barrier

is impermeable to the macromolecule of proteins and luminal antigen [32]. Paracellular route is the main route of water, solutes and macromolecules permeability [33]. The size and charge of substances are selected by tight junction proteins which allows the positive charge molecules and ions pass easily [32].

The paracellular permeability is influenced by the complex structure in terms of assembly or disassembly of tight junction proteins or the number of tight junction proteins. Meanwhile, MLCK pathway can regulate paracellular permeability by phosphorylating cytoskeleton resulting in tight junction opening in short term [34]. Tight junction is the most important physical barrier to prevent pathogens invading into the circulatory system. The parameters of paracellular permeability are TER and macromolecule permeability [35]. TER is mainly referred to paracellular integrity and paracellular permeability related with ion and solute across the tight junction. Meanwhile, macromolecule passing is the specific parameter to indicate paracellular permeability [35]. There are many stimuli to affect tight junction and cytoskeleton leading to paracellular permeability change. The main signaling pathway of tight junction permeability is MLCK pathway which phosphorylate MLC by MLCK resulting in peri-junctional contraction and opening tight junction. This pathway is found in physiological condition of nutrient uptake such as glucose in which Na^+-glucose cotransporter (SGLT-1) is activated by MLCK [32]. The impairment of tight junction causes the decreased TER and increased paracellular permeability, called leaky epithelia. The leaky epithelia promote the pathogen and toxin permeability leading to intestinal inflammation and systemic infection [36]. In the early response of infection, immune cells and intestinal epithelium respond by secreting many chemical substances including pro-inflammatory cytokines, TNF-α and IL-1β. The high levels of pro-inflammatory cytokines can impair tight junction barrier function and increased paracellular permeability [37].

STRUCTURE AND FUNCTION OF TIGHT JUNCTION BARRIER

The epithelial cells can be assembled by apical junctional complex which contains tight junctions, adherens junction, and desmosomes. Adherens junction and

desmosome build the strength between epithelium but do not have the effect on paracellular permeability. The apical junctional complex (AJC) comprises tight junctions and adherens junction which are located at the apical side. The epithelial barrier and paracellular transport are controlled by these structures. The adherens junction does not even directly control the paracellular permeability, it controls structural stability and integrity. The predominant structural tight junction proteins that regulates paracellular permeability and integrity are claudin and occludin [38]. Both tight junctions and adherens junctions bind with peri-junctional actinmyosin ring located around an epithelial cell. The binding between AJC and actin cytoskeleton establish the epithelial barrier ability [13].

The tight junctions have the important role to control the permeability of water, ion, macromolecules [39]. The ions, water and solutes can pass through the pore of tight junction. Tight junction is composed of transmembrane proteins which are located at the apical side of epithelial cells [40]. The main transmembrane proteins comprise occludin, claudin and JAM including scaffold proteins such as ZO 1-3.

Occludin is the first protein located at the uppermost of tight junction complex. It contains two extracellular loops, intracellular turn, and N- and C-terminal cytoplasmic domain [41]. Its extracellular loop is composed of tyrosine, glycine and charged amino acids. Likewise, C-terminal domain is rich in serine and tyrosine that can be phosphorylated by protein kinase [42]. Moreover, C-terminal domain binds with ZO-1 which links with the cytoskeleton of actin [41] **(Fig. 1)**. Occludin has the role of being the barrier to select the macromolecule. The abnormality of occludin expression and function leading to decreasing the ability of macromolecule barrier is indicated in inflammatory diseases such as ulcerative colitis, collagenous colitis, Crohn's disease, and pleural inflammation. High level of cytokines production in inflammatory diseases, such as TNF-α and IFN-γ, or some pathogens decrease the occludin expression [43].

Claudins are the second transmembrane proteins which have 24 members in humans and mice. Claudins consist of short cytoplasmic N-terminal, two extracellular loops and C-terminal cytoplasmic domain [9]. Moreover, claudins attach with the core

proteins, PDZ domain ZO-1, ZO-2 and ZO-3 [44]. Claudins 1, 3, 4, 5, 8, 9, 11 and 14 are the barrier builder proteins which promote intestinal barrier function and decrease paracellular permeability. Claudins 2, 7, 12 and 15 are the pore forming proteins which form pore at the tight junctions resulting in an increased paracellular permeability [9].

The third tight junction protein is JAMs which are glycosylated transmembrane proteins. The JAMs family has two subgroups that consist of two extracellular Ig-like domains, membrane spanning domain, and C-terminal cytoplasmic domain. JAM-A, JAM-B and JAM-C in the first subgroup consist of class II PDZ domain-binding at C-terminal binding with ZO-1 and PAR-3 [45]. JAMs select nutrients and solute passing the epithelium. JAMs do not have the direct function to be a barrier but also mediate proliferation and migration. However, loss of JAM-A expression increases the paracellular permeability [46]. Meanwhile, ZO proteins link with transmembrane proteins and interact with actin cytoskeleton and cytoskeleton-associated proteins [40]. Epithelia are classified as tight and leaky epithelia by the ability of permeable to water and substances. Normally, tight epithelia are found in distal convoluted tubule, collecting duct and bile duct. However, some organs have the leaky epithelia such as proximal tubule which has the transmembrane proteins less than tight epithelia [47].

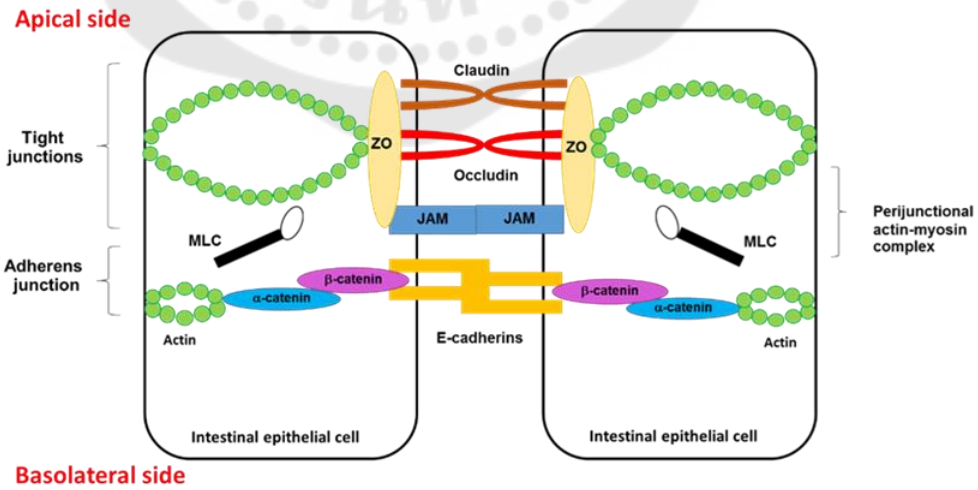


Figure 1 The intracellular structure of tight junctions of intestinal epithelial cells Adapted from Chiba et al.[48]

Normally, healthy tight junctions prevent the translocation. However, the impairment of tight junction such as changing some transmembrane proteins induces translocation of pathogen. Tight junction barrier function can be altered by many conditions which are ischemia/reperfusion, shock, pancreatitis, metabolic syndrome, diabetes, chronic kidney disease, hepatic disease, burn, sepsis, stress, etc [49]. The initial effect of tight junction impairment is translocation of LPS into circulatory system leading to endotoxemia, local inflammation and apoptosis of enterocyte [50]. The impairment of tight junction decreases TER and increases paracellular permeability allowing the pathogen passing to interact with immune cells under the epithelium. This evidence induces the intestinal inflammation and systemic infection [39]. The claudin 1, 4, occludin and JAM-A are internalized in T84 cells with challenged by IFN-γ and increasing paracellular permeability [49]. Pro-inflammatory cytokines such as TNF-α and anti-inflammatory cytokines such as IL-4, IL-6 and IL-13 increased paracellular permeability in association with the increased claudin-2 expression [42].

The studies in intestinal epithelial cell lines like Caco-2, T84 or HT-29 revealed the impairment of AJC increasing paracellular permeability. The inflammatory bowel disease has the high level of pro-inflammatory cytokines i.e., TNF-α, IL-1β and IFNγ. This evidence suggests pro-inflammatory cytokine affects AJC functions. The phosphorylation of myosin light chain (MLC) induces peri-junctional contraction. The serine-threonine protein kinases which phosphorylate MLC are myosin light chain kinase (MLCK) and Rho-associated protein kinase (ROCK) [13]. The inhibitions of MLCK and RhoA decrease the epithelial barrier impairment induced by TNF-α and IFNγ [51]. Interestingly, the inhibition of MLCK can decrease paracellular permeability from tight junction opening [13, 43]. Moreover, other intracellular signaling molecules including tyrosine kinase are associated with AJC function mediated by MLCK [10, 38].

THE REGULATION OF TIGHT JUNCTIONS

The assembly and disassembly of tight junction depend on physiological stimuli such as nutrients and pathological stimuli such as pathogen and toxin. The tight junction proteins and actomyosin ring are regulated by many signaling molecules including PKC, MAPK, MLCK and Rho family of small GTPase [10]. The phosphorylation of tight junction proteins affects intestinal barrier function. For example, phosphorylation of claudin can increase or decrease paracellular permeability depending on type of claudin [52].

The expression of PKC isoforms affects membrane integrity of intestinal epithelial cell monolayer. PKC has been related with the Toll-like receptor 2 (TLR2) pathway which has the roles in immune modulation and microbial recognition [53]. TLR2 stimulation induces PKCα and PKCδ leading to an increased TER and ZO-1 redistribution. Meanwhile, MAPK can be stimulated by growth factor and stress [54]. For example, epithelial growth factor inhibits tight junction impairment induced by hydrogen peroxide via MAPK pathway [55]. Moreover, ERK is one of the signaling molecules in the MAPK pathway which affects C-terminal of occludin leading to the prevention of tight junction disruption from hydrogen peroxide [55].

Actomyosin contraction is regulated by MLCK which phosphorylates myosin II regulatory light chain (MLC), resulting in tight junction opening and increased paracellular permeability. The stimulation of Na^+-glucose cotransport induced phosphorylation of MLC in Caco-2 cells [56]. The inhibition of MLCK decreases tight junction permeability. MLCK can be activated by many stimuli such as bacteria and pro-inflammatory cytokines. MLCK phosphorylation is associated with the disassembly of tight junction. The activation of MLCK reduces TER and redistribution of occludin and ZO-1 [10].

The Rho family of small GTPase, Rac, Rho kinase (ROCK), RhoA and Cdc42 regulates tight junction structure and actomyosin ring. The activation of ROCK phosphorylates MLC and induces perijunctional contraction [57]. The inactivation of Rho can redistribute occludin and ZO-1 from cell membrane and reorganize actin filament leading to a decreased TER and an increased paracellular permeability [58]. Then the

activation of Rho/ROCK signaling pathway affects the tight junction disassembly via MLCK pathway [59].

PATHOGENESIS AND MECHANISMS OF TIGHT JUNCTION IMPAIRMENT

Pathogens and toxin can invade into the blood circulation through intestinal epithelium leading to damaged intestinal barrier [60, 61]. The intestinal barrier has the function to control the substances and nutrients passing including prevention of the pathogens and toxins invading. Many gastrointestinal diseases such as inflammatory bowel disease (IBD) have the intestinal barrier impairment from pro-inflammatory cytokines of bacteria [62]. Normally, immune cells and epithelium can recognize the pathogen-associated molecular pattern (PAMPs). In mammals, the cells express numerous pattern-recognition receptors (PRRs) especially toll-like receptors (TLRs) that recognize PAMP and transduce signaling pathway to induce immune response [63].

The lipopolysaccharide (LPS) is the endotoxin which is located at the outer membrane of enteric bacteria, especially gram-negative bacteria [63]. Lipid binding protein (LBP) is the soluble protein of LPS which binds to the surface protein CD14 at the outer membrane [64]. CD14 induces LPS binding with toll-like receptor complex TLR4/MD-2 receptor complex to activate signal transduction [65]. The TLR-4 signaling is characterized into two pathways, which are dependent or independent on MyD88 to stimulate pro-inflammatory cytokines and interferon. These two groups of cytokines cause inflammation or neutralize viruses, respectively [15]. The MyD88-dependent pathway induces many transcription factors such as interleukin-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6) and nuclear factor-kappa B (NF-κB), activator protein1 (AP-1), interferon regulatory factor 5 (IRF5). Moreover, this pathway also induces transcription of pro-inflammatory cytokine genes, including tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12). Meanwhile, the MyD88-independent pathway activates TRIF signals to induce transcription factor TRAF3 and RIP1 to stimulate IRF3, NF-kB and AP-1 leading to interferon type I expression [66] **(Fig. 2)**. From the previous studies, LPS with physiological and clinical concentrations (0-10 ng/ ml) can increase tight junction

permeability without cell death [67]. However, LPS can induce TNF-α and IL-1β expression leading to diarrhea, inflammation, and septicemia [68].

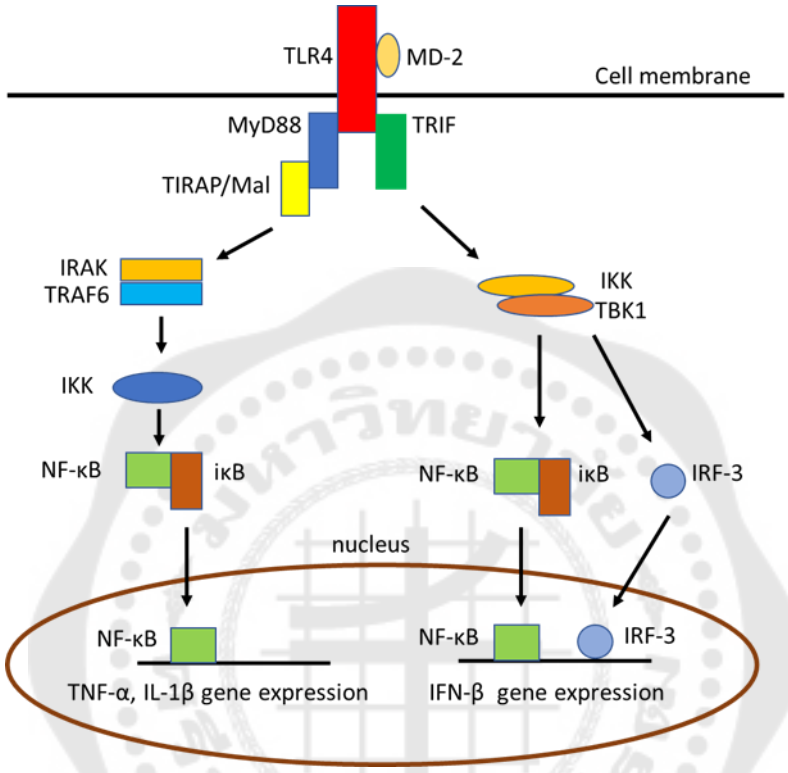


Figure 2 The TLR signaling pathway is characterized into two pathways, which are dependent or independent of MyD88. The MyD88-dependent signaling pathway produces TNF-α and IL-1β via NF-κB, while the MyD88-dependent signaling pathway stimulates interferon production via NF-κB and IRF-3.
Adapted from Elsamanoudy et al.[69]

One of the most common diseases of chronic gastrointestinal inflammation is inflammatory bowel disease (IBD) which can induce the systemic infection and sepsis [4]. Nowadays, the number of patients with inflammatory bowel disease (IBD) is increasing globally in many countries. In industrialized countries of western world, the prevalence of IBD population increases from 0.5 to 0.75% in 2010-2020 and maybe rise until 1% in 2030 [70]. Even the causes of IBD are unknown, but the main cause of IBD is

the leaky gut which increases intestinal permeability by disrupting intestinal barrier. Leaky gut allows pathogen and toxin invading into the circulatory system leading to malabsorption, malnutrition, chronic inflammation, and systemic infection [5]. There are many causes of leaky gut which impair mucosal barrier function such as ischemia/reperfusion, shock, pancreatitis, diabetes, chronic kidney disease, metabolic syndrome, hepatic disease, sepsis, burn, psychogenic stress including pro-inflammatory cytokines [49]. IBD associated with leaky gut can be characterized into two types i.e., Crohn's disease and ulcerative colitis. The lesion of Crohn's disease can diffuse from mouth to anus, but the lesion of ulcerative colitis is limited to the colon. Both diseases induce abdominal pain and diarrhea. Rectal bleeding can be found in ulcerative colitis more than Crohn's disease. Moreover, Crohn's disease can occur weight loss and perianal disease [71]. From the previous studies, IBD patients have high level of TNF-α and IL-1β in intestinal tissues and blood circulation which are associated with increased intestinal permeability [5].

TNF-α and IL-1β have many functions i.e., intestinal immunity, inflammation, infection, cell differentiation, apoptosis, and tissue remodeling. From the preliminary study, IL-1β 10 ng/ml significantly decreased TER and increased paracellular permeability of FD-4 at 24 and 48 h in Caco-2 cells. However, TNF-α had the trend to increase paracellular permeability but not significant. Then, IL-1β is chosen for induction of the intestinal barrier impairment in this study. High level of IL-1β is found in the intestinal fluid and blood circulation during inflammation period. The increased IL-1β level (1-10 ng/ml) decreases the transepithelial electrical resistance and increases the paracellular permeability in 0-72 h in Caco-2 [2]. The IL-1β mechanism involves the induction MLCK mRNA and protein expression leading to increased MLCK activity and opening tight junction [2] **(Fig. 3)**. The IL-1β binding with interleukin-1 receptor type I (IL-1RI) and TLR binding associated MyD88 signaling pathway can stimulate the release of NF-κB from IκBα. The activated NF-κB can induce MKK enzyme to activate ERK, JNK, and p38 MAP kinase pathways to induce the target gene transcription induced by

IL-18 in neutrophil [72]. IL-1β induces intestinal permeability by the activation of the p38 MAPK signaling pathway in Caco-2 cells [12]. Moreover, IL-1β induces migration of mesenchymal cells related with MLCK activation via PKC signaling [73].

Thus, IL-1β is the important pro-inflammatory cytokine induced by LPS. IL-1β stimulates tyrosine kinase and MLCK pathways to form peri-junctional actin-myosin contraction and elevate paracellular permeability. Interestingly, tyrosine kinase inhibitor inhibits the phosphorylation of MLC to p-MLC in MLCK pathway to prevent the opening of tight junction and decrease the paracellular permeability [10, 74]. Moreover, IL-1β induces intestinal permeability via MAPK associated with MLCK pathway. Then it is possible that protein kinase inhibitors can inhibit MAPK-associated MLCK pathway leading to decreased paracellular permeability.

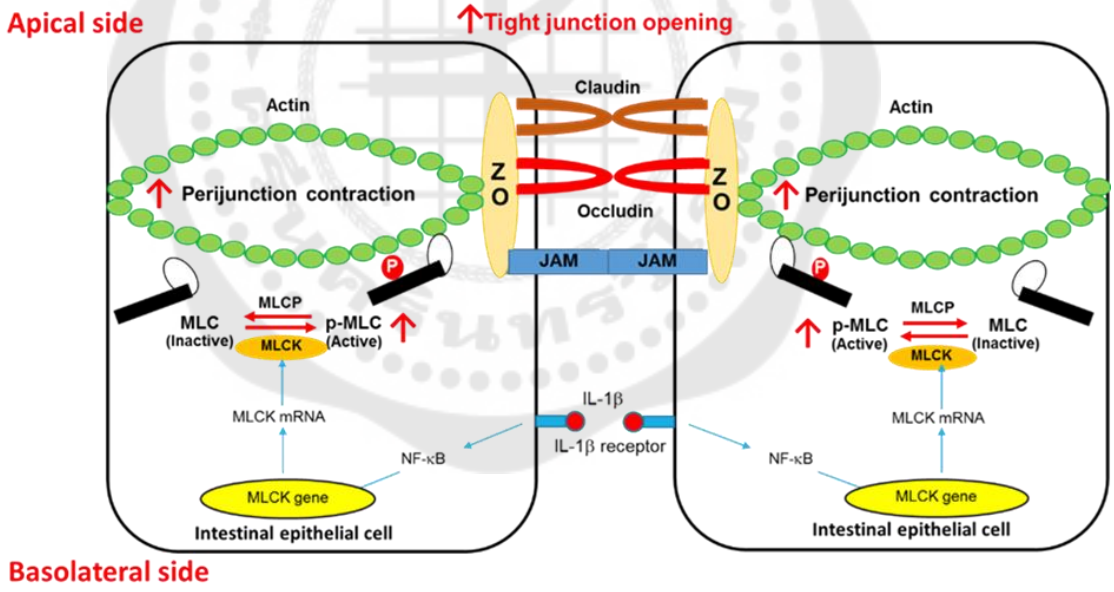


Figure 3 Cellular mechanism of IL-1β involves stimulation of NF-κB associated with MLCK pathway and leads to the opening of tight junctions in intestinal epithelial cells Adapted from Al-Sadi et al and Ma et al.[2, 75]

Interestingly, ERK1/2 is one of the signaling molecules in MAPK pathway that stimulates MLCK gene transcription. From the previous studies, IL-1β 10 ng/ml

decreased TER and increased paracellular permeability in 0-72 h in Caco-2 cells correlated with increased MLCK mRNA and protein expression. ERK1/2 blocker PD 98058 and siERK1/2 inhibited MLCK mRNA expression and decreased inulin flux induced by IL-1β. Moreover, Elk-1 (transcription factor of ERK1/2) activity was increased by IL-1β challenge. siElk-1 blocked the increased MLCK mRNA, MLCK protein, TER and decreased inulin flux induced by IL-1β. These results suggested IL-1β activation of MLCK via ERK1/2 signaling pathway [11]. In conclusion, IL-1β attaches with IL-1β receptor to phosphorylate ERK1/2 to P-ERK1/2 which is the active form. P-ERK1/2 phosphorylates Elk-1 to P-Elk-1 which is the transcription factor. P-Elk-1 translocates into nucleus to attach MLCK promotor leading to activating MLCK gene and protein expression. MLCK will phosphorylate MLC to p-MLC resulting in opening tight junction and increased paracellular permeability [11] **(Fig. 4)**. From all above, the inhibition of NF-κB/ERK1/2/MLCK signaling pathways are the important keys to decrease paracellular permeability from IL-1β and prevent leaky gut.

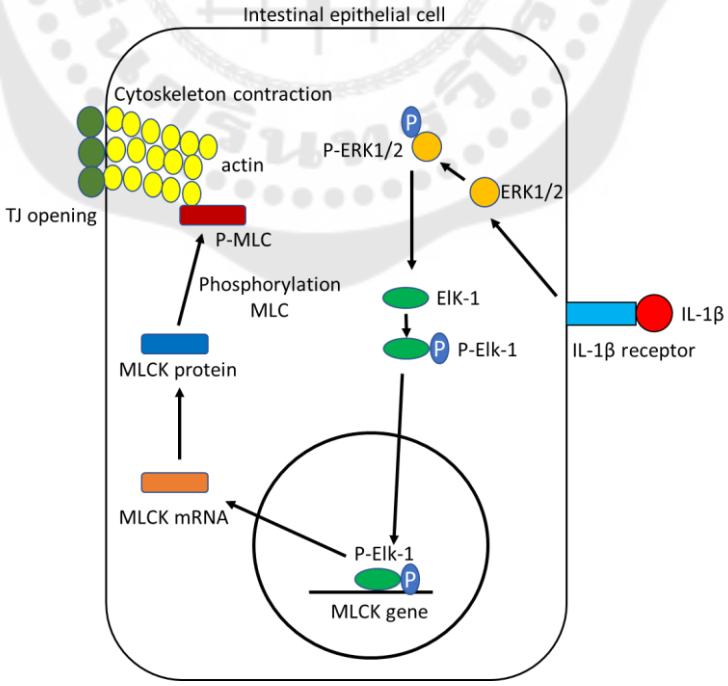


Figure 4 Cellular mechanism of IL-1β in ERK1/2 associated with MLCK pathway Adapted from Al-Sadi et al.[11]

QUERCETIN

Polyphenolic compounds, flavonoids are the secondary metabolites of plants that are characterized into six groups i.e., anthocyanin, flavonol, flavanone, flavanol, flavone, isoflavone [76]. Flavonols have been shown to promote tight junction permeability induced by bacterial challenge in the mucosal tissue and epithelial cell culture [77-79]. The mechanisms improving the epithelial barrier and integrity is not only the expression of tight junction proteins, adherens junction or scaffold proteins, but also inhibition of protein kinase A and tyrosine kinase activity [16, 78, 79].

Quercetin is one of the most abundant flavanols found in many fruits and vegetables such as apples, berries, grapes, tea, tomato, onion, etc. Quercetin has many biological properties especially anti-carcinogenic, anti-inflammatory, anti-viral, decreased platelet aggregation, decreased capillary permeability, and reduced lipid peroxidation.

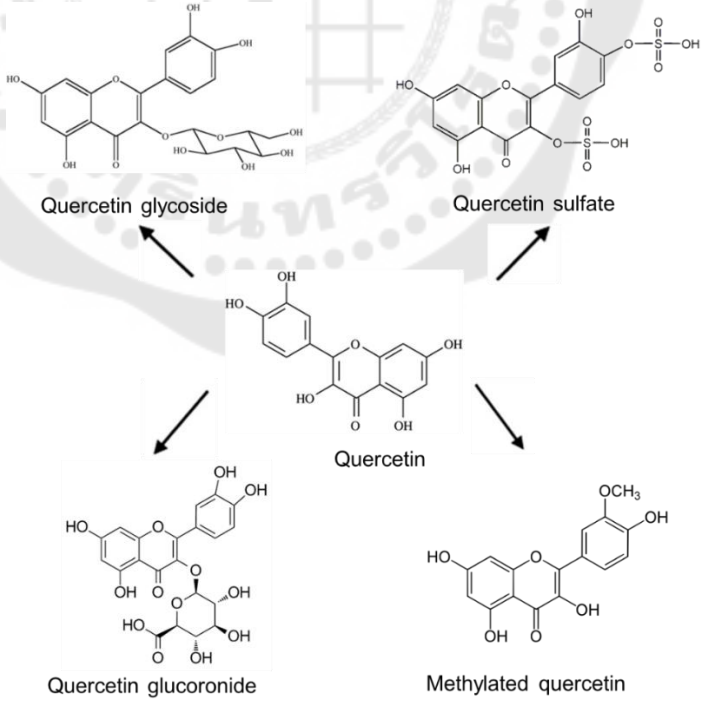


Figure 5 Chemical structures of quercetin and its conjugated forms
Adapted from Li et al.[80]

The chemical structure of quercetin is 3, 3′, 4′, 5, 7-pentahydroxyflavanone which contains OH group binding at position 3, 3′, 4′, 5, 7 **(Fig. 5)**. Quercetin can be formed in other structures, for example quercetin glycoside, quercetin sulfate, quercetin glucuronide, and methylated quercetin **(Fig. 5)**. Quercetin is yellow crystal, insoluble in water but soluble in alcohol and lipids except quercetin glycoside with increased water solubility [80].

The bioavailability of quercetin varies depending on its chemical structure. Quercetin glycosides are hydrolyzed by beta-glycosidase to aglycone and absorbed in the small intestine. Meanwhile, quercetin sulfate and quercetin glucuronic acid are easily absorbed more than quercetin [81]. Therefore, the absorption of quercetin is different depending on many factors including glycosylation, food, co-dietary food such as fat and fiber, types of sugar and sugar conjugation sites. Normally, quercetin and its derivatives are constant in gastric acid but are absorbed in the upper small intestine [82, 83]. After absorption, quercetin is metabolized in many organs such as small intestine, large intestine, liver, and kidney. In small intestine and liver, quercetin is metabolized by biotransformation of enzymes to sulfo-substituted, methylated and glucuronidated forms [84, 85]. Moreover, quercetin and derivatives are formed into phenolic acid by enteric bacteria and enzymes in intestinal epithelial cells of small intestine and colon. The bacteria break the backbone of quercetin and form into smaller phenolics for absorption [86]. The concentration of quercetin metabolites is found in plasma more than liver after diet consumption. The hepatic metabolites of quercetin are methylated 90-95%. Meanwhile, quercetin glycoside is the major form in blood [87]. Moreover, quercetin is also conjugated with albumin in plasma leading to a decrease in bioavailability in cells [88]. Quercetins accumulate in mitochondria in the organs associated with metabolism and excretion. The major organ of excretion is the kidney. Quercetin is excreted in urine as well as eliminated in feces and exhaled in air in extensively metabolized condition. The terminal half-life of quercetin is 3.5 h [89]. From all above, quercetin is absorbed by upper small intestine after that is methylated, glucuronidated and sulfo-substituted by biotranformation enzymes in small intestine and liver, and mainly excreted by kidney.

Quercetin exerts many actions including anti-inflammation, antioxidant, and promotion of barrier function. The inflammatory actions have been shown in many studies. Quercetin 100 μM has been found to inhibit PARP-1, preserve NAD-1 and energy production in pulmonary epithelial cells (A549) [90]. In N9 microglial cells, quercetin 100 μM inhibits TNF-α and IL-1α leading to decreased apoptosis of neuron cells [91]. Moreover, quercetin 3 μM inhibits cyclooxygenase and lipoxygenase activities in guinea pig epithelial cells [92]. Quercetin 15-30 μM have also inhibited arsenic-induced COX-2 expression by blocking PI3K activation in rat liver epithelial (RLE) cells and inhibited Src- and Syk-mediated PI3K-(p85) tyrosine phosphorylation via TLR4/MyD88/PI3K pathways in macrophage-like cell line (RAW 264.7 cells) [93, 94]. Quercetin also inhibits TNF-α-induced inflammation by preventing the activation of ERK, c-Jun, JNK, NF-κB, signaling molecules of inflammatory gene and protein expression. In addition, quercetin can indirectly prevent inflammation by activating PPARγ activity which antagonizes NF-κB and AP-1 transcription factors for inflammatory gene [80].

Quercetin has the potent antioxidant effects as evidenced by stimulation of glutathione (GSH) synthesis and enhancement of the Cu/Zn SOD, catalase (CAT), Mn SOD and GSH peroxidase which are the antioxidant enzymes in the hippocampal CA1 pyramidal neurons [19]. In fact, there are many environmental factors that can induce reactive oxygen species (ROS). Meanwhile, the intracellular ROS is generated from mitochondrial electron transport. Quercetin can activate antioxidant enzyme and non-enzyme antioxidant mechanisms. It can regulate NRF-2, MAPK, and AMPK caused by ROS via stimulating antioxidant mechanism such as CAT, SOD, GSH peroxidase and GSH synthesis leading to oxidative balance [19].

Quercetin has the function to control tight junction permeability and epithelial barrier. Quercetin decreased lucifer yellow (LY) flux by dose-dependent manner in rat intestine [77]. Treatment with quercetin 100 μM for 48 h dose-dependently decreased LY flux and increased TER in Caco-2 cells [16]. The TER started to increase at 0.5 h and significantly increased after 6 h of administration of 100 quercetin μM while decreased until 24 h followed by increasing again between 24 and 48 h. However, the claudin-4

protein expression markedly increased after 12 h of quercetin treatment [15]. Quercetin enhanced claudin-4 promotor activity and claudin-4 mRNA expression in Caco-2 cells [77]. In addition, tight junction associated with cytoskeleton such as claudin-1, occludin and ZO-2 increased in first 6 h after quercetin treatment, but cytoskeleton associated with claudin-4 still increased after 12 h [15]. These results suggested the enhancing of claudin-1, occludin and ZO-2 assembly was mediated by quercetin leading to TER increasing in the early phase, and the promotion of claudin-4 expression increased TER in the late phase.

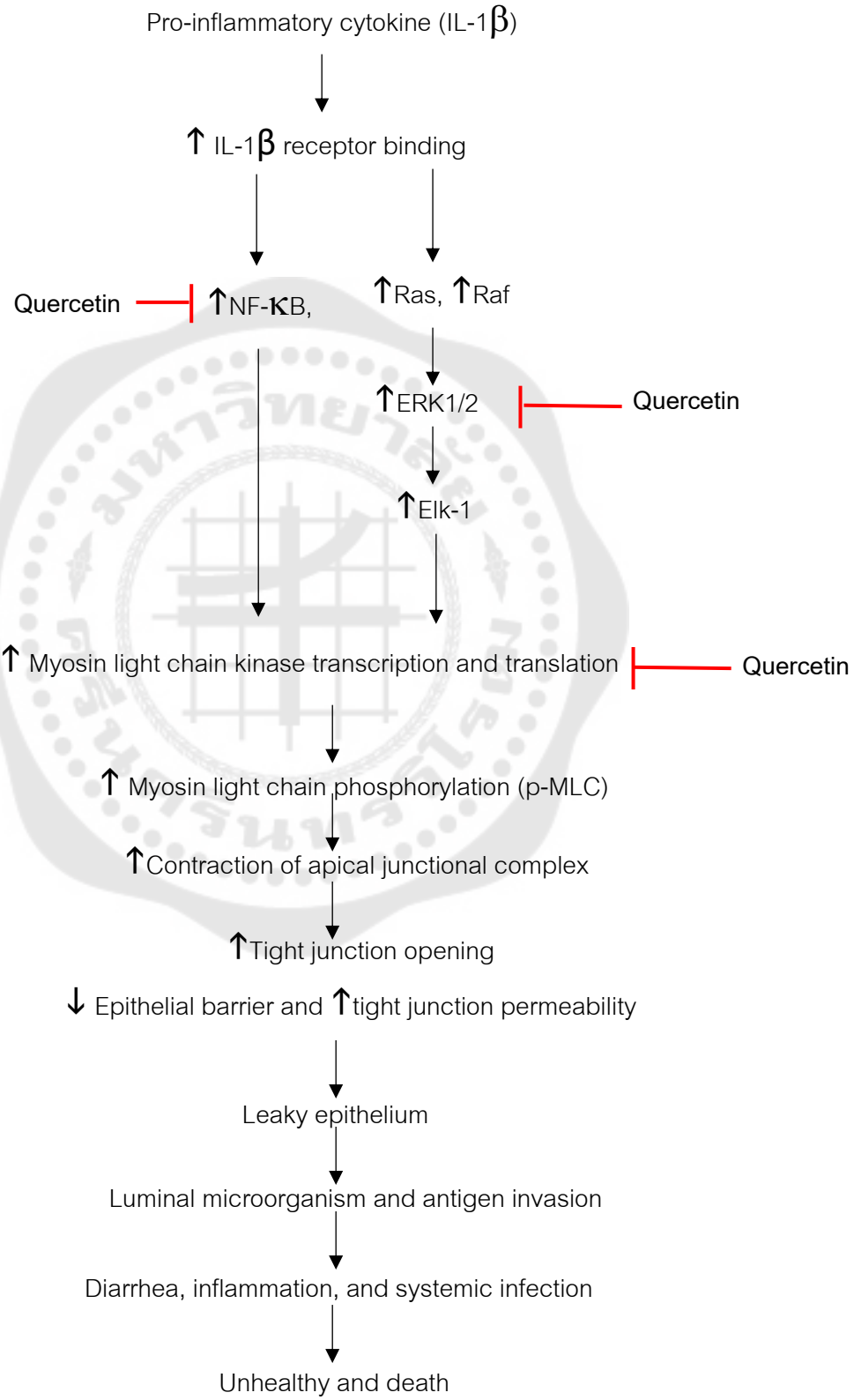
The important signaling molecule of flavonoid such as protein kinase has been demonstrated to relate with antioxidant property. The selective protein kinase Cδ (PKCδ) inhibitor promoted tight junction barrier in a similar manner with quercetin, for example, increasing cytoskeleton in association with claudin-1, occludin, ZO-2 and total claudin-4 expression [15]. The PKCδ activity was decreased after 1 h of quercetin treatment until 60% from initial activity at 24 h in Caco-2 cells [15]. These results show that quercetin stimulates tight junction barrier via an inhibition of PKCδ. Moreover, quercetin induces tight junction protein assembly via phosphorylation of occludin in Caco-2 cells. The occludin phosphorylation is increased within 1 h of quercetin treatment. Many protein kinases such as PKCξ [95], PKCη [16], casein kinase I/II [96] and protein phosphatase PP1 and PP2A [95, 97] have the ability of occludin phosphorylation and dephosphorylation. Then, quercetin affects kinase and phosphatase activities via PKCδ inhibition leading to inducing occludin phosphorylation and tight junction assembly. Even though protein kinases tend to relate with intestinal tight junction barrier, the effect of quercetin effect on the modulation of NF-κB/ERK1/2/MLCK signaling pathways induced by IL-1β is still unclear.

CACO-2 CELLS

Caco-2 cells are human colorectal adenocarcinoma which are the monolayer of adherent epithelial cells. It has an ability to differentiate and form polarization in the

specific condition [98]. Caco-2 cells differentiate morphology and change function from colonic-like cells to jejunal-like cells at day 14-21 in culture. Cell morphology is polarized with brush border and tight junction at the apical side. Moreover, Caco-2 cells can express many enzymes, microvilli, transporters such as peptidases, esterases, disaccharidase and P-glycoprotein which are characteristics of small intestine. Amino acids, sugar, vitamins, and hormones are absorbed by active transport. Caco-2 cells express Na^+/H^+ exchange, H^+/K^+ ATPase, Na^+/K^+ ATPase, $Na^+/K^+/Cl^-$ cotransport and apical Cl^- channels needed for transport process. Many receptors are represented such as vitamin B12, vitamin D3, EGFR and sugar transporters. Several cytokines including TNF-α, TGF-β1, IL-6, IL-8, IL-15, thymic lymphopoietin (TSLP) can be secreted [99]. Caco-2 cells are the in vitro model widely used for drug absorption. The absorption model can be performed by seeding cells into membrane filter and allowed to form the complete monolayer which acts as physical barrier for selective passing of ion and macromolecules [100]. In addition, TER measurement and paracellular permeability test can be employed to observe the macromolecule transportation from apical to basolateral side [101]. The TER is measured in ohm resistance for detecting the transport of substances of transcellular and paracellular routes. The decreased TER not only indicates the tight junction impairment, allowing macromolecule passing but also increases transcellular ion transport [79]. The TER of colonic-like and jejunal-like cells are between 150-400 $\Omega.cm^2$ and 600-800 $\Omega.cm^2$ respectively [100]. However, it can be changed by many factors such as cell passage, number of cells, media, and temperature [102]. The leaky epithelia can be investigated by paracellular permeability to macromolecules. The macromolecule conjugated with fluorescein isothiocyanate-dextran (FITC-dextran, MW = 4 kDa; FD-4) has been used for detection of paracellular flux. The increased FD-4 permeability indicates tight junction impairment and leaky gut [103].

CONCEPTUAL FRAMEWORK



CHAPTER 3

MATERIALS AND METHODS

MATERIAL AND CHEMICAL REAGENTS

Table 1 List of materials and chemical reagent

Materials and chemical reagents	Manufacturers
Dulbecco modified eagle's medium (DMEM)	Gibco, USA
Fetal bovine serum	Gibco, USA
Non-essential amino acid	Gibco, USA
Antibiotic-antimyotic	Gibco, USA
Phosphate buffer saline (PBS)	Gibco, USA
0.25% trypsin/EDTA	Gibco, USA
DMSO	Chemical express, Italy
IL-1β	Genscript, USA
Quercetin (glycoside form)	Sigma, USA
MTT	Sigma, USA
FITC-Dextran (FD) 4 kDa	Sigma, USA
Trizol™	Thermofisher, USA
Bicinchoninic acid assay (BCA assay)	Thermofisher, USA
Ammonium persulfate (APS)	Biorad, USA
β-mercaptoethanol	Biorad, USA
30% Acrylamide/bisacrylamide	Biorad, USA
Tetramethylethylenediamine (TEMED)	Biorad, USA
SDS	Biorad, USA
Loading dye	Biorad, USA
Bovine serum albumin	Chemical express, Italy
Anti-NF-κB antibody	Santa cruz Biotechnology, USA
Anti-iκB, MLCK, p-MLC, ERK1/2 antibody	Abcam, USA

Anti-β-actin antibody	Santa cruz Biotechnology, USA
Horseradish peroxidase (HRP)-conjugated secondary antibody	Abcam, USA
NF-κB, MLCK GAPDH primer	Iscience tech, Thailand
ECL	Santa cruz Biotechnology, USA
Polyvinyldene difluoride membrane	Millipore, USA
Short plate	Biorad, USA
Thick blot paper	Biorad, USA
Precision Plus protein standard dual color	Biorad, USA
iScript™ Select cDNA synthesis kit	Biorad, USA
ToptaqTM master Mix Kit	Biorad, USA
qPCRBIO SyGreen Mix Lo-ROX	PCR Biosystems, UK
Ethanol	Merck, USA
Chloroform	Sigma, USA
Agarose	Sigma, USA
Paraformaldehyde	Sigma, USA
Triton-X100	Union carbide chemicals and plastic, USA
FITC-conjugated secondary antibody	Abcam, USA
DAPI	Abcam, USA
Vectashield	Vector laboratories, USA
100-mm cell culture dish	Corning-Costar, USA
Microporous membrane 12 mm diameter	Corning-Costar, USA
Microporous membrane 24 mm diameter	Corning-Costar, USA
12-well plate	Corning-Costar, USA
6-well plate	Corning-Costar, USA
60-mm culture dish	Corning-Costar, USA
0.2 μm filter	Corning-Costar, USA
Microscopic slide and cover slip	Sail brand, China

EXPERIMENTAL PROCEDURES

Cell culture

Human colonic adenocarcinoma Caco-2 cell line (passage 17) was obtained from The American Type Culture Collection (Manassas, USA). Caco-2 cells were grown in a 100-mm cell culture dish. The culture medium consists of Dulbecco's Modified Eagle Medium (DMEM) with supplements of 20% fetal bovine serum, 1% non-essential amino acid, and 1% antibiotic-antimyotic at 37°C in a humidified incubator with 5% CO_2 in air. After 80% confluence, the cells were dissociated with 0.25% trypsin/EDTA in Hank's balanced solution (HBSS) pH 7.4. After that appropriate cell density was seeded on Transwell microporous filters (12-mm and 24-mm polycarbonate membrane, 0.4 μm pore size) (Corning-Costar, USA) for permeability test and immunohistochemistry, 60-mm culture dish for western blot analysis, or 6-well plate for mRNA expression. The culture medium was changed every 2 days.

Drug treatment

After culture of Caco-2 for 7 days in culture dishes or membrane filters, the experiments were performed by dividing cells into five groups, i.e., control group without any treatment, control DMSO, quercetin, IL-1β, and quercetin + IL-1β groups. Quercetin at the concentrations of 1, 10, 100 μM and IL-1β 10 ng/ml were chosen for all experiments except immunofluorescence experiment which cells were challenged with only quercetin 1 μM. In the previous study, quercetin at 1, 10, 100 μM have been shown to increase intestinal barrier by decreasing paracellular permeability at 24 and 48 h via claudin-1, claudin-4, occludin and ZO-2 [15]. Meanwhile, IL-1β at 10 ng/ml is the clinical plasma concentration which induces the leaky gut by increasing paracellular permeability at 24 and 48 h via MLCK pathway [2]. In the present experiment, Caco-2 cells were incubated with quercetin at 1, 10, 100 μM in culture media for 1, 12, 24, 48 h. Since quercetin was dissolved in 0.01% v/v DMSO, the equivalent volume of DMSO

added to the treated group was used as vehicle control. In the groups treated with quercetin and IL-1β, cells were pre-incubated with three different concentrations of quercetin or DMSO for 24 and then the culture media were the same concentration of quercetin in the presence of IL-1β 10 ng/ml for 1, 6, 12, 24 and 48 h depending on the experiments.

Cytotoxicity test

Cytotoxicity effect of all drugs used in the experiment was first determined using the MTT assay. Caco-2 cells, seeded in a 48-well plate at a density of $3X10^4$ cells/well, were allowed to grow for 7 days. Cells were then treated with drugs as previously stated for 24 and 48 h. Untreated cells were used as negative control and DMSO-treated cells as vehicle control. After removing the test medium, 125 µl/well of MTT 5 mg/ml diluted in culture medium was added and incubated for 3 h at 37°C in a 5% CO_2 incubator. After removing excess MTT solution, cells were washed with PBS 200 µl/well, and formazan crystal was lysed with DMSO 100 µl/well at 37°C in a 5% CO_2 incubator. The formazan solution was collected to 96-well plate for measuring optical density (OD) at the specific wavelength 570 mm and non-specific wavelength 620 nm as a background using the Microplate reader spectrophotometer (Epoch, BioTek, VM, USA). The OD unit of DMSO, quercetin with or without IL-1β at 570 nm minus with 620 nm (OD 570-620) was calculated for cytotoxicity analysis or cell viability. Moreover, percentage of viability was calculated and reported using following equation:

$$\% \text{ Viability} = \frac{(OD570\text{-}620) \text{ of treatment at 24 or 48 h}}{(OD570\text{-}620) \text{ of DMSO at 24 h}} \times 100$$

Measurement of transepithelial electrical resistance (TER)

Caco-2 at 10^5 cells/well were plated in 12-mmTranswell microporous filters and maintained for 7 days for complete cell monolayer. Transepithelial electrical resistance

(TER) measurements were taken every two days during the culture to assess the tightness and integrity of the epithelial barrier. Cells formed complete monolayer at day 7 with a TER value of 150-400 $\Omega.cm^2$. After that, cells were treated with drugs as previously stated for 24 and 48 h. The TER was detected using volt-ohmmeter (Millipore, USA) which connected to chopstick silver/silver chloride (Ag/AgCl) electrodes in which one tip of electrode was dipped in the apical and another tip in the basolateral solution of filter (**Fig. 6**). After each measurement, the electrodes were cleaned with 70% ethanol before the next measurement. The resistance measurement of each filter was done in duplicate at three different locations. The average resistance values were used to calculate TER by multiplying the resistance value with the area of the filter insert (R*A= $\Omega.cm^2$). To obtain the absolute TER value, the resistance values of the filters without cells were subtracted from that of the filters with cells. High TER value reflects membrane integrity and increased tight junction barrier, whereas low TER value indicates loss of membrane integrity or impairment of tight junction barrier. The percentage of TER change from the initial TER (before drug added) was calculated and reported using following equation:

$$\% \text{ Change of TER} = \frac{(\text{TER after treatment} - \text{TER before treatment})}{\text{TER before treatment}} \times 100$$

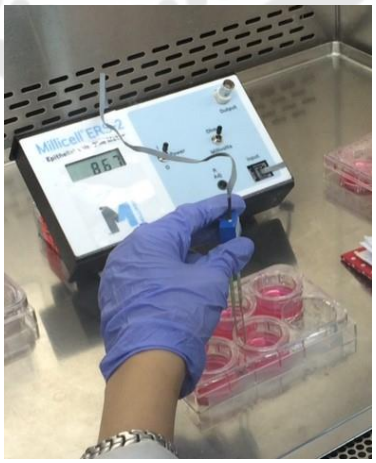


Figure 6 Measuring transepithelial electrical resistance across Caco-2 cell monolayer by volt-ohmmeter

Measurement of macromolecule permeability

At the end of TER measurement following drug treatment for 24 and 48 h, the macromolecule permeability of Caco-2 was determined by flux of paracellular marker FD-4 (FITC-Dextran; MW=4 kDa) across the monolayer. Normally, tight junction does not allow the macromolecule passing unless the tight junction is impaired as in leaky gut. FD-4 at 1 mg/ml was dissolved in Hanks' Balanced Salt Solution (HBSS) and purified by 0.2 µm filter before the permeability experiment. After removing the culture medium and washing with PBS, cell monolayers were incubated in the apical membrane with FD-4 in HBSS and the basolateral side with HBSS alone. After incubation at 37°C for 4 h, 100 µl of the samples from the apical and basolateral sides was collected and transferred to a 96-well plate for the fluorescent microplate reader (BioTek, USA) to measure the fluorescence intensity at 485/530 nm. The FD-4 concentration of each sample was calculated from the FD-4 standard curve. The FD-4 flux ($h^{-1}.mm^{-2}$) was calculated and reported using the following equation.

$$\text{FD-4 flux} = dq/dt \times 1/(A \times C)$$

q was the concentration of FD-4 at the basolateral sides (µg/ml) at the end of incubation period. t was the duration of FD-4 incubation period. A was the surface area of monolayer (1.2 mm^2), and C (µg/ml) was the concentration of fluorescence at the apical side at the end of incubation period.

RNA isolation and cDNA synthesis by PCR

The protective effect of quercetin on NF-κB and MLCK gene expression was determined by conventional PCR and qPCR. Caco-2 cells at $5x10^5$ cells were cultured in 6-well plate and maintained for 7 days followed by drug treatment as previously stated. After removing treatment media, the cells were washed and added Trizol 200 µl/well for cell lysis. After collecting sample into Eppendorf, chloroform 40 µl/sample was added for phase separation to collect RNA and incubated at 4°C for 10 min, followed by centrifugation at 12,000 rpm for 15 min to separate phase of RNA, DNA, and protein.

The RNA was collected, precipitated with 100 µl of isopropanol at 4°C for 10 min, and then centrifuged at 12,000 rpm for 15 min. RNA was centrifuged at 7500 rpm for 10 min at 4 °C after being washed twice with 200 µl of 75% ethanol. After removing ethanol, RNA was dried for 30 min before eluting RNA with nuclease free water 20 µl for preserving RNA. The RNA sample was heated in block heater at 65°C for 5 min. A nanodrop spectrophotometer (Thermo Scientific, USA) operating at 260/280 nm was used to measure the concentration of RNA. For the synthesis of cDNA, RNA with an OD260/OD280 ratio of 1.8 to 2.0 was employed.

RNA was transformed into cDNA by Thermocycler (Biorad, USA) using the iScript Select cDNA synthesis kit. The reagent volume per reaction of iScript™ Select cDNA synthesis was composed of nuclease free water, 5x iScript select reaction mix (dNTPs, magnesium chloride, stabilizer, Oligo (dT) primer and random hexamer primer) 4 µl, iScript reverse transcriptase (RNase H^+ Moloney murine leukemia virus (MMLV) reverse transcriptase and RNase inhibitor protein) 1 µl. The volume of RNA 3 µg was added into iScript reagents and adjusted to 20 µl by nuclease-free water. All the components were mixed in PCR tube and synthesized cDNA by Thermocycle. The concentration of cDNA was measured by spectrophotometer at wavelength 260/280 nm. The ratio OD260/OD280 of cDNA 1.8 was used for PCR.

Toptaq™ master Mix Kit (Biorad, USA) and specific primers were used for increasing PCR products by Thermocycler. The components of master mix were 2xToptaq master mix (Toptaq PCR buffer, dNPT, Toptaq DNA polymerase) 12.5 µl, 10xCoralLoad concentrate (gel tracking reagent and gel tracking dye) 2.5 µl, forward primer 10 µM 1 µl, reverse primer 10 µM 1 µl, nuclease free water. The volume of cDNA 3 µg was added into master mix and adjusted to 25 µl by nuclease-free water. All the components were mixed in PCR tube and PCR products increased by Thermocycle. Denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min made up the three steps of a PCR cycle. The PCR products were run to 35 cycles and stopped at 65°C.

The agarose gel electrophoresis was prepared for detecting PCR product. Gel electrophoresis consists of power supply, supporting media and buffer system. Agarose gel (1.5% w/v) was dissolved in 1xTAE (Tris/Acetic acid/EDTA buffer) and heat at 100-200°C until transparent color. The fluorescence color Gel red was added in agarose solution and moved the solution to tray with comb. After the hard agarose, the gel was moved to chamber. 1xTAE was added after removing comb from gel. The loading dye 1 µl was mixed with PCR sample on paraffin. The mixture of sample was added into well. The PCR sample was run at 80 volts, 3 amps, 300 watts for 85 min. The samples in agarose gel were detected for specific bands by Gel documentation (Sygene Gene Genius Bio Imaging System).

Real-time polymerase chain reaction (RT-PCR)

After confirming specific band, the quantitative mRNA expression of NF-κB and MLCK was assessed by real-time PCR or qPCR using 2X qPCRBIO SyGreen Mix Lo-ROX. The volume per reaction of master mix was 2x qPCRBIO SyGreen Mix 10 µl, up-stream primer 10 µM 0.8 µl, down-stream primer 10 µM 0.8 µl, template DNA 1 µg and nuclease free water up to 20 µl total reaction volume. Real-time PCR was performed on the mixture using a thermocycler in 40 cycles of initial denaturation at 95°C for 2 min, denaturation at 95°C for 5 sec, annealing/extension at 65°C for 30 sec, and repeat. The amplification product was confirmed by gel electrophoresis and melting curve. GAPDH was used as the endogenous control for analyzing threshold cycle (Ct). The relative fold of target gene expression in treatment groups was compared with control group by using a modification of $2^{-\Delta\Delta Ct}$ equation. The sequence and reference of NF-κB and MLCK primer were designed from Iscience tech as shown in **Table 2 and 3**.

Table 2 List of primers in qPCR experiment

Gene	Primer sequence (5'→3')	Accession number	Product size (bp)
MLCK	5′- GGGGACTTTCAGCCTTGTGA-3′ (forward) 5′- GACCAAGCTGCTTCGCAAAA-3′ (reverse)	U48959.2	140
NF-κB	5′- TTGGGGACTTCTCTCCCACA-3′ (forward) 5′- TGCACCTCTTCCTTGTCTTCC-3′ (reverse)	X61498.1	190
GAPDH	5′- TGCACCACCAACTGCTTAGC -3′ (forward) 5′- GGCATGGACTGTGGTCATGAG -3′ (reverse)	NM_02046	87

Table 3 Reference of primers in qPCR experiment

Gene	Reference
MLCK	https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?ORGANISM=9606&INPUT_SEQUENCE=U48959.2&LINK_LOC=nuccore
NF-κB	https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?ORGANISM=9606&INPUT_SEQUENCE=X61498.1&LINK_LOC=nuccore
GAPDH	Design from Iscience tech, Thailand

Western blot analyses

The expression levels of the proteins NF-κB, iκB, ERK1/2, MLCK, and p-MLC were evaluated by Western blot. Caco-2 at $2x10^5$ cells were grown in 60-mm culture dish and maintained for 7 days followed by drug treatment as previously stated. After treatment, Caco-2 cells were washed and added with lysis buffer (Tris 46.5 mM, NP-40 1%, sodium dechelate 6.02 mM, NaCl 150 mM, PMSF 1 mM, EGTA 1 mM, NaF 1 mM and aprotinin 0.01 mg/ml). Cell lysate was collected in eppendorf and incubated for 30 min on ice. To collect the supernatant, the cells were centrifuged at 12,000 rpm and 4°C for 15 min. The concentration of protein was measured by Biscinchoninic acid (BCA) assay in 96-well plate. BCA was made by combining reagent B:A at a ratio of 1:50 which

was incubated in dark room for 30 min. The standard bovine serum albumin (BSA) was prepared at 125, 250, 500, 750, 1000, 1500, 2000 µg/ml. The protein sample was diluted eight folds by nanopure water. The standard and samples of 5 µl were mixed with BCA reagent 100 µl in 96-well plate and incubated at 37°C for 30 min. The absorbance of proteins was measured by spectrophotometer at OD 570/620 nm for concentration calculation compared with standard curve of BSA. The volume of protein 30 µg/ml was calculated for using in the next step.

Protein separation was performed by 10%SDS-PAGE. The 10% resolving (nanopure water, resolving buffer, 10% SDS, 10% APS, 30% acrylamide and TEMED) and 4% stacking gel (nanopure water, stacking buffer, 10% SDS, 10% APS 30% acrylamide, and TEMED) were prepared for setting gel. Following gel setting, 2.5 µl of the precision plus marker and a 1:1 mixture of protein sample and loading dye that were pre-heated at 65°C for 5 min were loaded into the gel. The gel with samples was run at 200 volts, 30 A for 45 min. Using Transfer blot turbo, the isolated protein sample was transferred to ImmobilonTM transfer polyvinylidene difluoride membrane (Millipore, USA). The membrane was washed by 1xtris/tween one time followed by 3% BSA/0.05% Tris-tween for blocking non-specific proteins for 1 h. After washing with 1xtris-tween 6 times for 30 min, the membrane was incubated with primary antibodies, anti-NF-κB, iκB, ERK1/2, MLCK, and p-MLC **(Table 4)**, overnight. After 1 h of HRP-secondary antibody incubation **(Table 4)**, the membrane was washed 6 times with 1xtris-tween for 30 min. The membrane was incubated for 5 min with ECL substrate. The immunoreactive band was detected by gel documentation. The internal control protein β-actin was compared with the target protein band. The band density was measured by densitometer by adobe photoshop and Image J.

Table 4 List of antibodies used in western blot experiment

Antibodies	Manufacturers
Mouse-anti-NF-κB IgG	Santa cruz Biotechnology, USA
Rabbit-anti-iκB IgG	Abcam, USA
Rabbit-anti-ERK1/2 IgG	Abcam, USA
Rabbit-anti-MLCK IgG	Abcam, USA
Rabbit-anti-p-MLC IgG	Abcam, USA
Mouse-anti-β-actin IgG	Santa cruz Biotechnology, USA
Donkey-anti-rabbit IgG-HRP	Abcam,USA
Goat-anti-mouse IgG-HRP	Santa cruz Biotechnology, USA

Immunofluorescence

To investigate the impact of quercetin on NF-κB translocation, immunofluorescence staining was performed. Caco-2 at $5x10^5$ cells/well were plated in 24-mm Transwell microporous filters and maintained until 7 days prior to drug treatment as previously stated. The membrane was rinsed with PBS and fixed with 4% paraformaldehyde after receiving drug treatment for 1 h. The membrane was then cut into size of 0.2-0.3 cm/well and put into 48-well plate followed by PBS washing. The 0.1% Triton-X/PBS 300 µl/well was added to permeabilize membrane for 10 min. After being cleaned with PBS, the membrane was incubated for 30 min at room temperature in 300 µl/well of 1% BSA/PBST (0.1% Tween-20/PBS) to inhibit non-specific antibody. Membrane washed with PBST before being incubated with mouse anti-NF-κB primary antibody in 1%BSA/PBST 100 µl/well for an overnight period. The membrane was cleaned with PBST again before being incubated for 1 h at room temperature with 100 µl/well of goat anti-mouse IgG-FITC secondary antibody. After washing membrane by PBST, DAPI 1 µM was added in wells for staining nucleus 100 µl/well for 30 min at 4°C in the dark chamber followed by PBST washing. The target membrane and negative controls were mounted on the slide by vectashield. All membranes were closed by

cover slips until dry for 30 min. The membrane was detected for immunoimaging picture by Zen confocal microscope software (Carl Zeiss Microscope LLC, USA). The picture was shown in the green and dark bule colors of positive results of FITC and DAPI respectively. The NF-κB translocation was detected from co-localized color between green FITC and blue DAPI and reported as the immunofluorescence pictures.

Statistical analyses

Data was presented as means ± SEM, and n was number of independent experiments using cell passage number 30 to 40 of Caco-2 cells. Quercetin or IL-1β with DMSO group was compared with DMSO vehicle control, and quercetin with IL-1β was compared with IL-1β group. Multiple comparisons were evaluated using two-way repeated measure analysis of variance (ANOVA) followed by a Dunnett's post hoc test for comparing treatments at 12, 24, 48 h in MTT, permeability and western blot experiments. One-way ANOVA was used for evaluating the qPCR results at 12 h followed by Dunnett's post hoc test. The GraphPad prism software (version 8.0.1) was used for all statistical calculations. A p value < 0.05 was accepted statistically different.

EXPERIMENTAL PROTOCOL

Part 1 Evaluation of cytotoxicity of quercetin and IL-1β in Caco-2 cells

Cytotoxic effect to Caco-2 cells of DMSO, quercetin (1, 10, 100 μM), IL-1β (10 ng/ml) and quercetin+IL-1β were evaluated using MTT assay. A 48-well plate was seeded with $3X10^4$ cells per well and cultivated for 7 days. The cells were exposed to DMSO or quercetin in culture media at concentrations of 1, 10, or 100 μM for 24 or 48 h. Cells in the quercetin and IL-1β treatment groups were pre-treated for 24 h with varying concentrations of quercetin or DMSO, and then incubated in the same culture conditions with IL-1β 10 ng/ml for 24 and 48 h. At the end of treatment period, the colorimetric MTT assay was performed to assess cellular metabolic activity, and the formazan crystal

concentration was measured for optical density at 570 nm and 620 nm by spectrophotometer. The high OD unit of 570 minus 620 nm indicates cell viability or low cytotoxicity (Fig. 7).

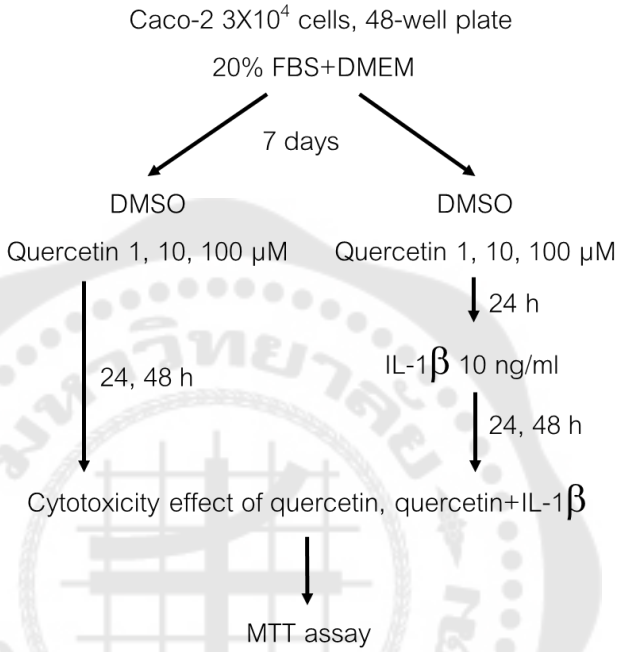


Figure 7 Protocol of determining the cytotoxic effect of quercetin and IL-1β in Caco-2 cells using MTT assay

Part 2 Evaluation of effects of quercetin on membrane permeability in Caco-2 cells induced by IL-1β

Effect of quercetin on transepithelial electrical resistance in cells induced by IL-1β

TER is overall resistance of transcellular and paracellular pathway which reflects the transcellular and paracellular permeability. As the paracellular permeability mainly regulates the membrane resistance, TER is used to indicate the integrity or strength of tight junction. To assess the protective impact of quercetin on the TER of Caco-2 cells induced by IL-1β, cells were plated in 12-mm Transwell microporous filters at a density

of 10^5 cells/well and maintained for 7 days for confluent monolayer. The TER at day 7 was 150-400 $\Omega.cm^2$. Cells were exposed with DMSO and quercetin 1, 10, 100 μM for 24 and 48 h. In the quercetin with IL-1β treatment, cells were pre-treated for 24 h with quercetin or DMSO, then incubated in the same culture conditions with IL-1β 10 ng/ml for another 24 or 48 h. The TER was measured before quercetin or IL-1β treatment (0 h), 24 and 48 h after treatment by volt-ohmmeter in duplicate at three different locations. The % change of TER from initial was calculated in each treatment **(Fig. 8)**.

Effect of quercetin on paracellular permeability in cells induced by IL-1β

Cell permeability to macromolecules reflects tight junction permeability. The increased macromolecule permeability indicates tight junction impairment. The protective effect of quercetin on macromolecule permeability of Caco-2 cells induced by IL-1β was measured by a flux of paracellular marker FD-4. Cells were treated in the same manner as TER experiment. The permeability assay was performed at the end of TER measurement at 24 and 48 h. After adding FD-4 to the apical side of the cell monolayer and incubating for 4 h, the solutions from the apical and basolateral sides were collected for fluorescence intensity measurement using a fluorescent microplate reader. The data was calculated to apparent permeability coefficient **(Fig. 8)**.

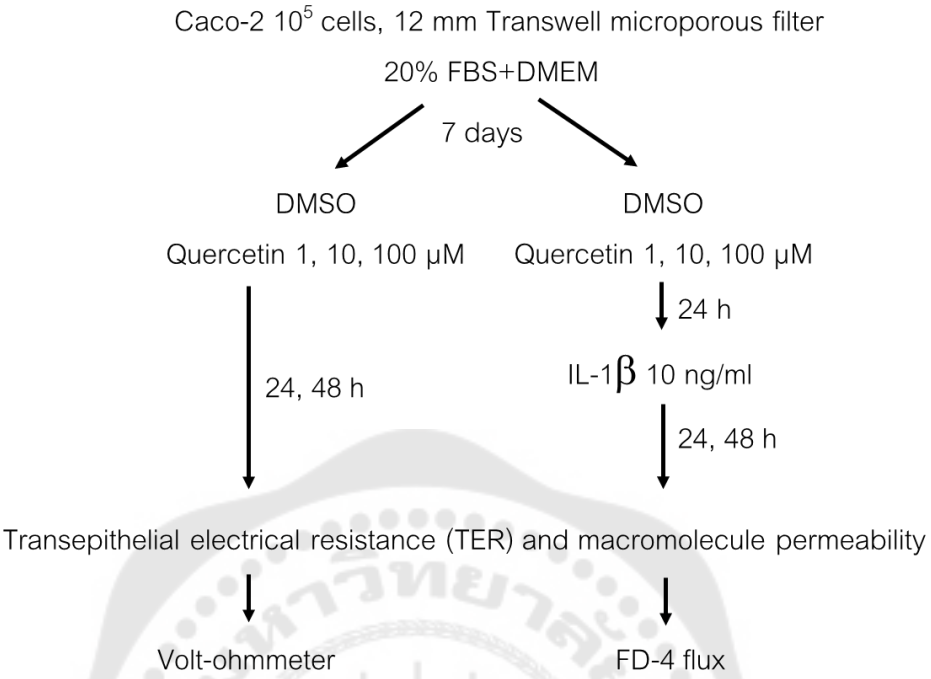


Figure 8 Protocol of evaluating the effect of quercetin and IL-1β on tight junction permeability in Caco-2

Part 3 Evaluation of cellular mechanisms of quercetin in Caco-2 cells induced by IL-1β

Effect of quercetin on NF-κB, iκB, ERK1/2, MLCK and p-MLC protein expression in cells induced by IL-1β

The target proteins were the signaling molecules NF-κB, iκB, ERK1/2, MLCK, and p-MLC, which have been identified to mediate the IL-1β action on intestinal barrier dysfunction. All of the target proteins were determined by western blot analysis to investigate the mechanism of quercetin on the intestinal barrier induced by IL-1β. Caco-2 at 2X10^5 cells/well were plated in 60 mm plate and maintained for 7 days. Cells were challenged with DMSO or quercetin at 1, 10, 100 μM in culture media for 12 and 24 h. In quercetin and IL-1β treatment groups, cells were pre-treated with quercetin or DMSO for 24 h, and then the same culture media in the presence of IL-1β 10 ng/ml were

further incubated for another 12 and 24 h. After that, the total proteins from each treatment were isolated, separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and determined for the target proteins using specific primary antibodies and their secondary antibodies. The immunoreactive bands on the membrane were detected by gel documentation. The target protein band and the internal β-actin control were compared. The band density was measured by densitometer by adobe photoshop and Image J (Fig. 9).

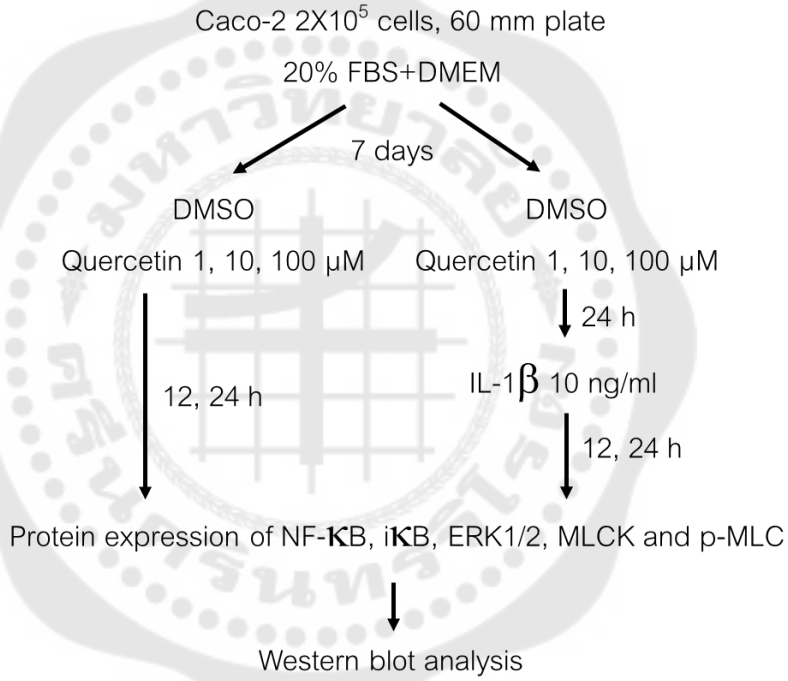


Figure 9 Protocol of evaluating the effect of quercetin and IL-1β on NF-κB, iκB, ERK1/2, MLCK and p-MLC protein expression in Caco-2 using western blot analysis

Effect of quercetin on NF-κB and MLCK gene expression in cells induced by IL-1β

In addition to protein expression, the *NF-κB* and *MLCK* genes were identified by RT-PCR for quercetin's protective mechanism on the intestinal barrier induced by IL-1β. Similar to previous protocol, cells at 5X10^5 cells/well in 6-well plate were maintained for 7

days. The cells were treated with DMSO or quercetin at 1, 10, 100 μM in culture media for 12 h. In quercetin and IL-1β treatment groups, cells were pre-treated with quercetin or DMSO for 24 h, and then the same culture media in the presence of IL-1β 10 ng/ml were further incubated for 6 h. Using real-time PCR, total RNA was extracted, and specific mRNA expression was evaluated. The amplification product was confirmed by gel electrophoresis and melting curve. GAPDH was used as endogenous control for analyzing threshold cycle (Ct). The relative fold of target genes in each treatment group was compared with control group and calculated using a modification of $2^{-\Delta\Delta Ct}$ equation (Fig. 10).

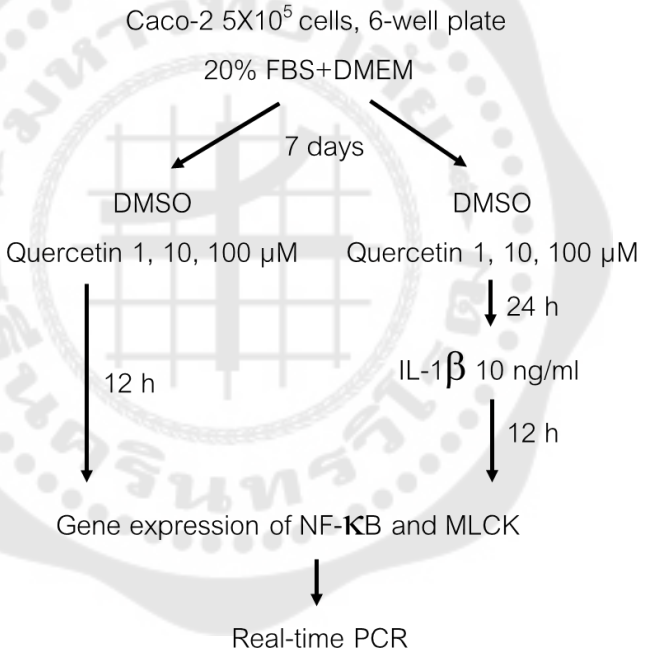


Figure 10 Protocol of evaluating the effect of quercetin and IL-1β on NF-κB and MLCK gene expression in Caco-2 cells by real-time PCR

Effect of quercetin on NF-κB translocation in cells induced by IL-1β

The translocation of NF-κB from cytoplasm into nucleus was also performed to confirm the transcriptional activation of specific gene i.e., MLCK that induces intestinal barrier impairment. To investigate the protective effect of quercetin on NF-κB

translocation in Caco-2 cells induced by IL-1β, Caco-2 at 5X10^5 cells/well seeded in 24 mm Transwells were treated with DMSO, quercetin 1 μM, IL-1β with or without quercetin for 1 h. The membrane was then stained with DAPI after treated with the primary NF-κB antibody and its secondary antibody-FITC. The immunoimaging picture as visualized using confocal microscope showed the positive fields of FITC in green and DAPI in dark blue. The NF-κB translocation was detected from co-localized color of green FITC and blue DAPI as shown in immunofluorescence pictures (Fig. 11).

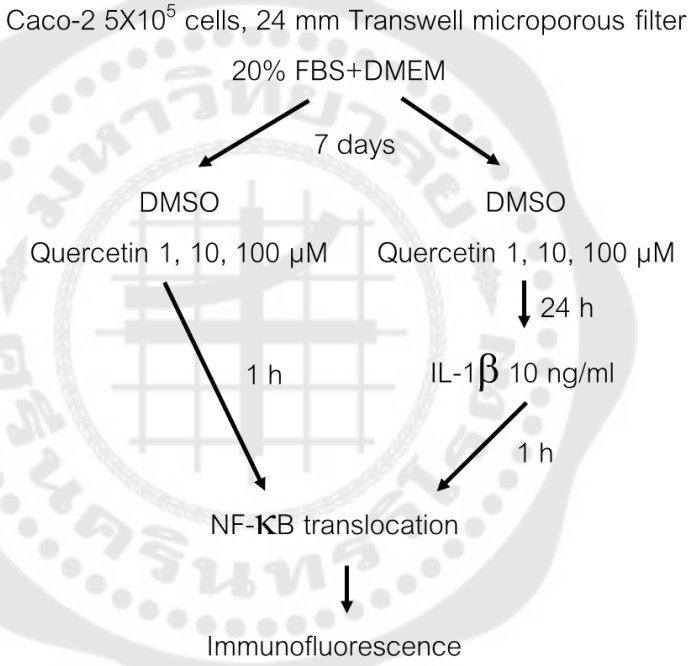


Figure 11 Protocol of determining the effect of quercetin and IL-1β on NF-κB translocation in Caco-2 cells using immunofluorescence staining

CHAPTER 4

RESULTS

Cytotoxicity of quercetin and IL-1β on Caco-2 cells

We first evaluated whether all drugs or vehicle DMSO used in the present study were toxic to Caco-2 cells using MTT assay. Cells cultivated for 7 days that had colonic epithelial characteristics were incubated for 24 and 48 h with quercetin or IL-1β in the presence or absence of quercetin. The true valves of OD 570-620 and calculated % viability was represented in **Fig. 12 and 13**, respectively.

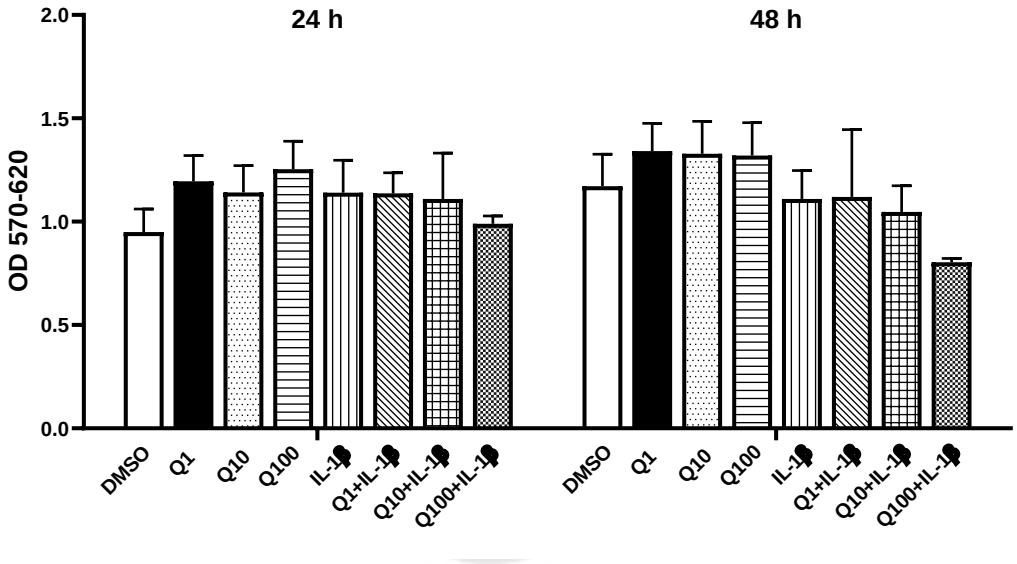


Figure 12 Cytotoxic effect of quercetin, IL-1β and quercetin with IL-1β expressed as OD (570-620) unit in Caco-2 cells. Cells incubated with DMSO, quercetin (1, 10, 100 μM) or IL-1β (10 ng/ml) in the presence or absence of quercetin for 24 and 48 h were determined for cell viability using MTT assay. Each value represents the mean ± SEM from 7 independent experiments in duplicate. No statistical difference between treatment groups and DMSO or IL-1β group as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

The results showed that treatment with quercetin 1, 10 or 100 μM for 24 and 48 h did not affect the OD570-620 as compared with the corresponding DMSO control (*p>0.05*; **Fig. 12**). Similarly, the OD570-620 was unaffected by IL-1β (10 ng/ml) alone or IL-1β in the presence of three concentrations of quercetin after 24 and 48 h (*p>0.05*; **Fig. 12**). It is noted that treatment with quercetin 100 μM with IL-1β for 48 h tended to decrease the OD570-620 of Caco-2 cells (*p>0.05*; **Fig. 12**), but no significant difference from DMSO and IL-1β groups.

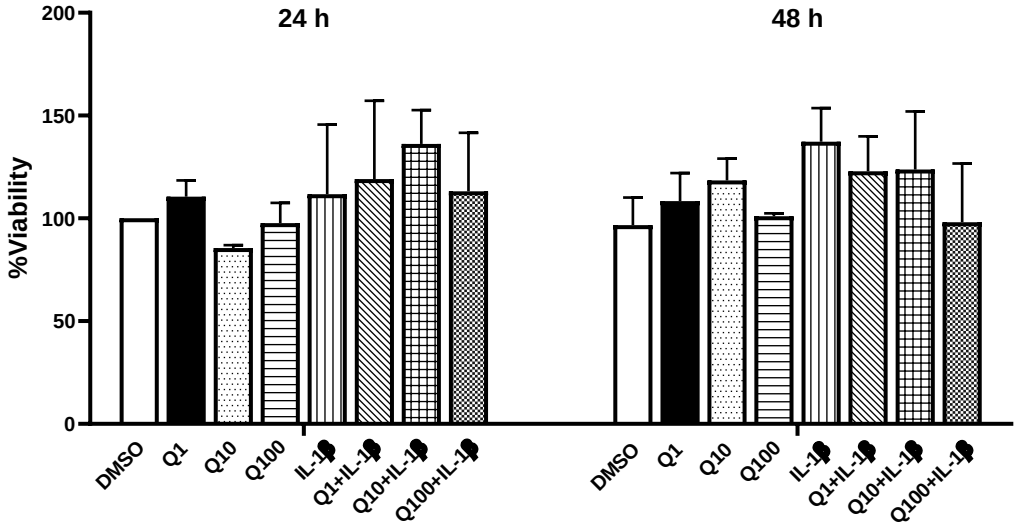


Figure 13 Percent of viability to quercetin, IL-1β and quercetin with IL-1β of Caco-2 cells. Cells incubated with DMSO, quercetin (1, 10, 100 μM) or IL-1β (10 ng/ml) in the presence or absence of quercetin for 24 and 48 h were determined for cell viability using MTT assay. Each value represents the mean ± SEM from 7 independent experiments in duplicate. No statistical difference between treatment groups and DMSO or IL-1β group as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

Similarly, quercetin 1, 10, 100 μM did not change the % viability compared with DMSO at 24 and 48 h (*p>0.05*; **Fig. 13**). The IL-1β with or without quercetin did not affect % viability at the same time point compared with IL-1β alone or DMSO, respectively (*p>0.05*; **Fig. 13**). These results suggest that all concentrations of quercetin with or without IL-1β used were suitable for the present study.

Effects of quercetin on the epithelial barrier function of Caco-2 cells challenged by IL-1β

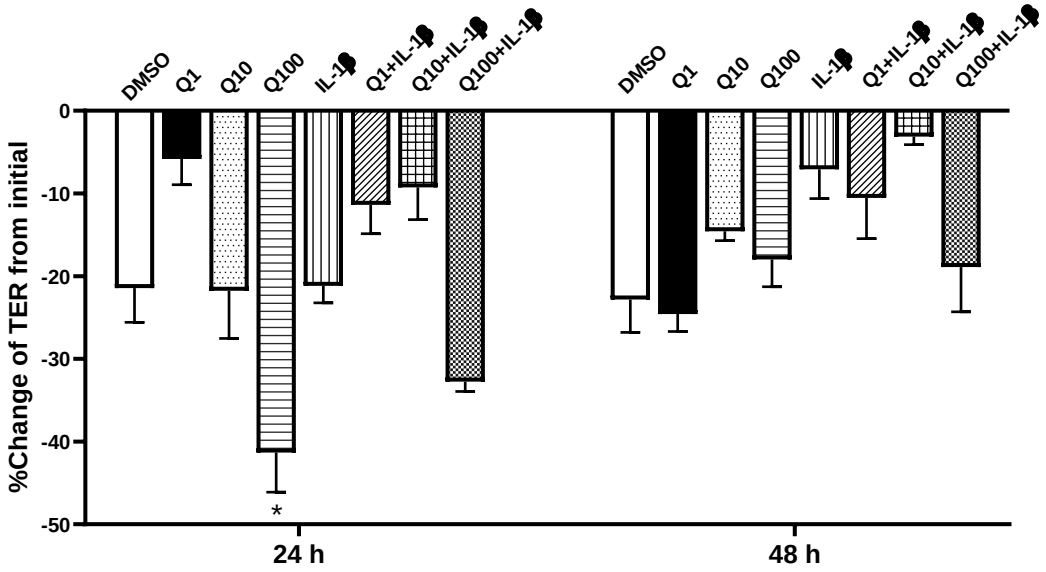


Figure 14 Effects of quercetin, IL-1β and quercetin with IL-1β on the percent changes of TER from initial. Caco-2 cells cultured in microporous filter were incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 24-48 h. Data showed the mean ± SEM from 7 independent experiments in triplicate. **p<0.05* compared with DMSO as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

To examine the barrier function of intestinal epithelial cells, Caco-2 cells treated with quercetin with or without IL-1β for 24 and 48 h were assessed for transepithelial

electrical resistance (TER) and paracellular permeability to FITC-conjugated 4-kD dextran (FD4). To further investigate the alteration in paracellular permeability to specific FD-4 macromolecules in Caco-2 cell monolayer, the results in **Fig. 15** showed that the permeability of Caco-2 cells to FD-4 (FD-4 flux) increased depending on time (*p<0.01*). Treatment with quercetin 100 μM increased the FD-4 flux which was significantly observed at 48 h compared with DMSO control. This increased FD-4 flux was correlated with the decreased TER in the previous experiment **(Fig. 14)**. However, quercetin 1 or 10 μM treatment did not significantly alter the FD-4 flux in Caco-2 monolayer at any time point compared with DMSO (*p>0.05*; **Fig. 15**).

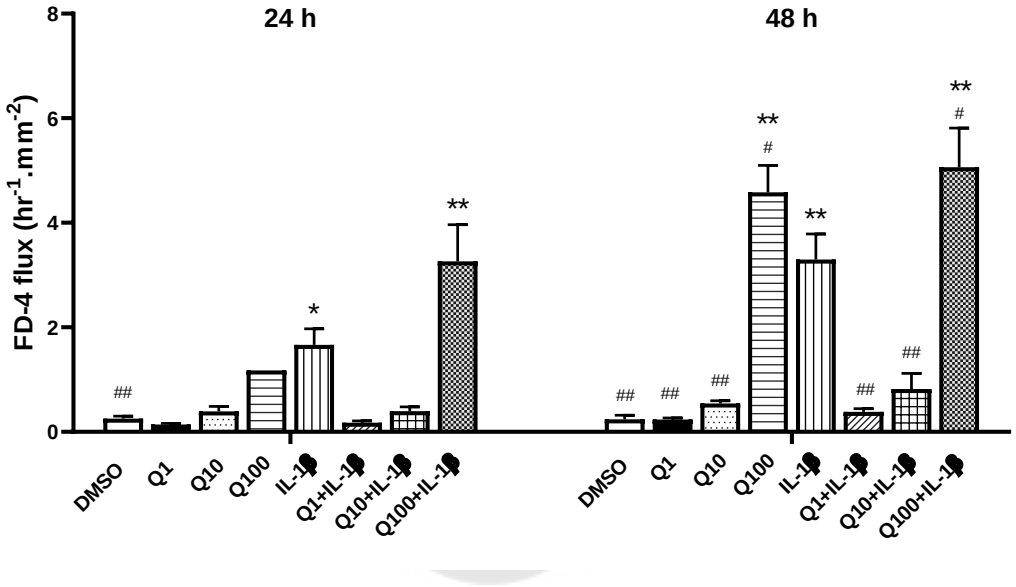


Figure 15 Effects of quercetin, IL-1β and quercetin with IL-1β on the FD-4 flux. Caco-2 cells cultured in microporous filter were incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 24-48 h. Data showed the mean ± SEM from 7 independent experiments in triplicate. **p<0.05* or ***p<0.01* compared with DMSO. $^{\#}p<0.05$ or $^{\#\#}p<0.01$ compared with IL-1β as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

In the present study, although cells challenged with IL-1β (10 ng/ml) had no change in the TER, the FD-4 flux were significantly increased after IL-1β treatment at both 24 and 48 h (*p<0.05 and 0.01*, respectively; **Fig. 15**). When IL-1β was co-incubated with either quercetin 1 or 10 μM, the FD-4 flux significantly decreased at 48 h (*p< 0.01*; **Fig. 15**). However, in the presence of quercetin 100 μM, IL-1β significantly increased the FD-4 flux compared with IL-1β alone. (*p< 0.01*; **Fig. 15**). Interestingly, the FD-4 flux in response to quercetin 1 and 10 μM with IL-1β was not different from that of quercetin alone. From these results, IL-1β induced increase in paracellular permeability was prevented by pretreatment with quercetin 1 and 10 μM for 48 h.

Effects of quercetin on the expression of nuclear factor kappa-B (NF-κB) protein in Caco-2 cells challenged by IL-1β

The stimulation of NF-κB by IL-1β is the important mechanism to induce MLCK gene expression. To determine the mechanism of quercetin in the prevention of IL-1β induced increase in the paracellular permeability to macromolecules, the expression of NF-κB protein was determined by western blot analysis. The 7days-Caco-2 cells were incubated with varies doses of quercetin or IL-1β in the presence or absence of quercetin for 12 and 24 h. The mouse-anti-NF-κB (p65) antibody was used to detect a protein band with a molecular weight of 65 KDa. The results showed that treatment with quercetin at the concentrations of 1, 10, 100 μM for 12 and 24 h did not change NF-κB protein expression as compared with corresponding DMSO control (*p>0.05*; **Fig. 16**). Meanwhile, the effect of IL-1β alone significantly increased NF-κB protein expression compared with DMSO at both 12 and 24 h (*p<0.01*; **Fig. 16**). However, pretreatment with three concentrations of quercetin significantly decreased the effect of IL-1β on increased NF-κB protein expression to a level that was not different from DMSO or quercetin alone at 12 and 24 h (*p<0.01*; **Fig. 16**). However, quercetin 10, 100 μM with

IL-1β was found to significantly increase NF-κB protein expression compared with DMSO at 24 h (*p<0.01*; Fig. 16). From these results, pretreatment with quercetin 1, 10 and 100 μM was found to prevent the effect of IL-1β on increased NF-κB protein expression at 12 and 24 h.

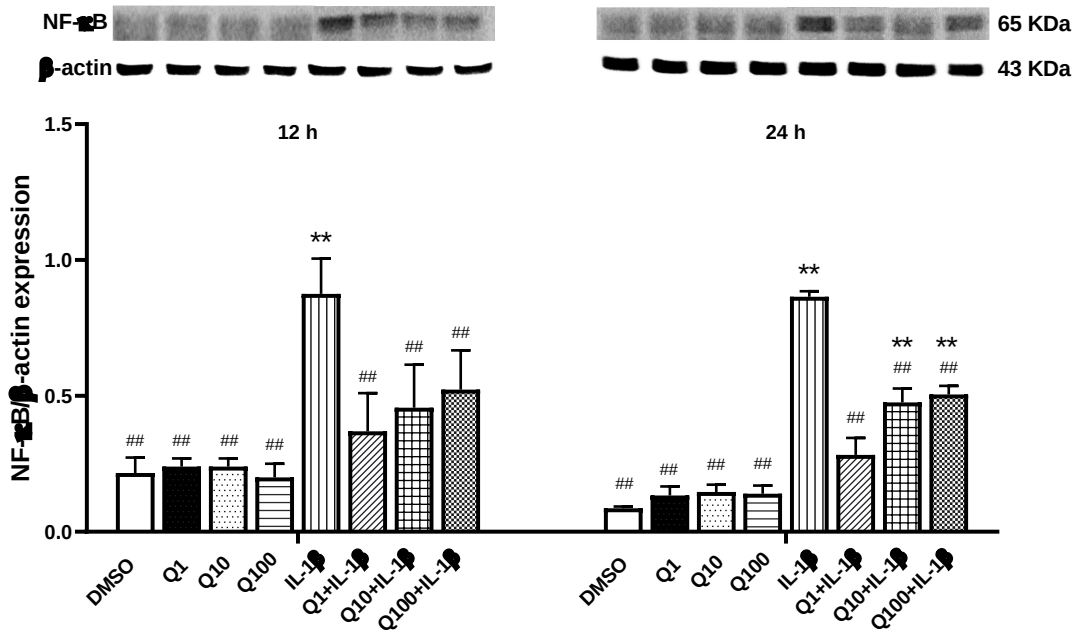


Figure 16 Effects of quercetin, IL-1β and quercetin with IL-1β on the NF-κB protein expression. Caco-2 cells incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 12-24 h were extracted for total protein. Representative NF-κB protein bands were analyzed by Western blot analysis compared to β-actin. Data showed the mean ± SEM from 7 independent experiments by densitometry analysis. ***p<0.01* compared with DMSO. $^{\#\#}p<0.01$ compared with IL-1β as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

Effects of quercetin on the expression of nuclear factor kappa-B inhibitor (iΚB) protein in Caco-2 cells challenged by IL-1β

Normally, iΚB acts as an inhibitor by binding to NF-ΚB and thus blocking NF-ΚB translocation into nucleus. To further determine the mechanism of quercetin in the prevention of IL-1β induced increase in the permeability to macromolecules, the expression of iΚB protein was determined by semi-quantitative Western blot analysis at 12-24 h.

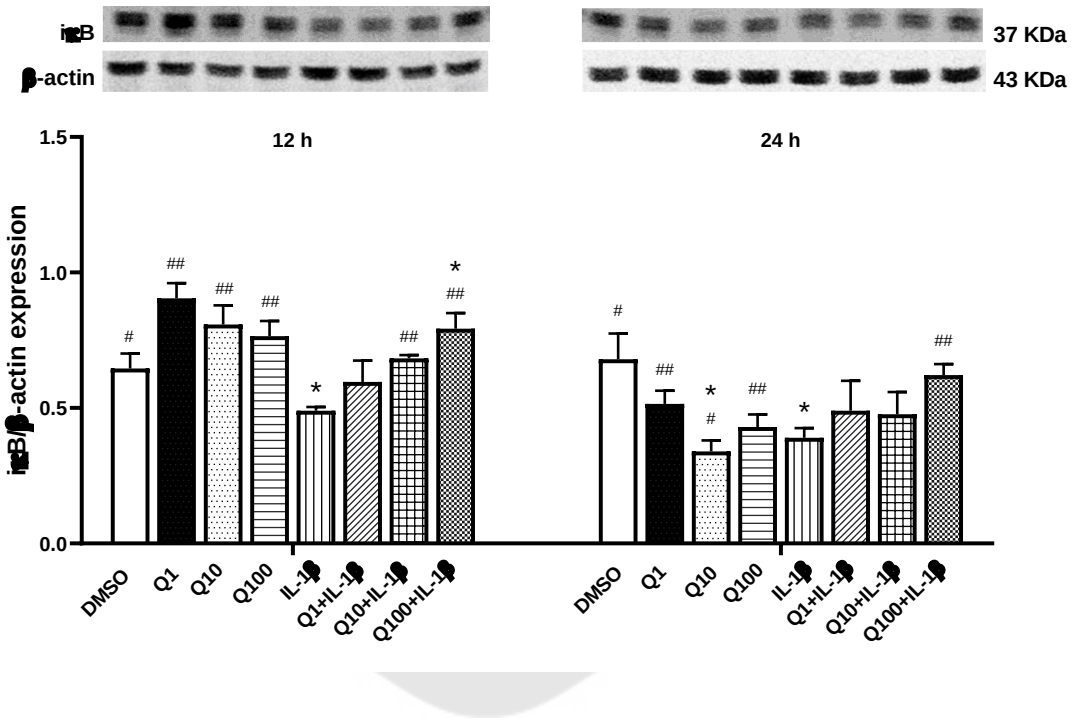


Figure 17 Effects of quercetin, IL-1β and quercetin with IL-1β on the iΚB protein expression. Caco-2 cells incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 12-24 h were extracted for total protein. Representative iΚB protein bands were analyzed by Western blot analysis compared to β-actin. Data showed the mean ± SEM from 7 independent experiments by densitometry analysis. *$*p<0.05$* compared with DMSO. $^{\#}p<0.05$ or $^{\#\#}p<0.01$ compared with IL-1β as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

Cells were treated with drugs using the same protocol for protein expression as previously mentioned. The rabbit-anti-iΚB antibody was used to detect a protein band with a molecular weight of 37 KDa. Treatment with quercetin alone for 12 and 24 h had no significant effect on iΚB protein expression except quercetin 10 μM for 24 h significantly decreased iΚB compared with DMSO control ($p<0.05$; Fig. 17). IL-1β alone significantly decreased iΚB protein expression at both 12 and 24 h compared with DMSO ($p<0.01$; Fig. 17). However, co-incubation of cells with IL-1β and quercetin 10 and 100 μM for 12 h or quercetin 100 μM for 24 h significantly increased iΚB as compared with IL-1β ($p<0.01$; Fig. 17). From these results, pretreatment with quercetin 10 and 100 μM was found to prevent the effect of IL-1β on decreased iΚB protein expression at 12 and 24 h.

Effects of quercetin on the expression of extracellular signal-regulated protein kinase 1/2 (ERK1/2) protein in Caco-2 cells challenged by IL-1β

The stimulation of MLCK protein and gene expression is involved in ERK1/2 signaling molecule. The activation of ERK1/2 by IL-1β induces the phosphorylation of ERK1/2 to Elk-1 which acts as transcription factor of MLCK gene. To determine the mechanism of quercetin in the prevention of IL-1β increased the macromolecule permeability, the expression of ERK1/2 protein was determined by semi-quantitative Western blot analysis at 12-24 h. Following the drug treatment protocol, rabbit-anti-ERK1/2 antibody detected a protein band with a molecular weight of 44 KDa. Treatment with quercetin 1, 10, 100 μM at 24 and 48 h did not change ERK1/2 protein expression as compared with DMSO ($p>0.05$; Fig. 18). After IL-1β treatment, the expression of ERK1/2 protein was significantly increased compared with DMSO at both 24 and 48 h ($p<0.05$ and $p<0.01$; Fig. 18). Pretreatment with quercetin 100 μM significantly decreased ERK1/2 protein expression induced by IL-1β to a level that did not change

from DMSO at 12 h (*p<0.05*; **Fig. 18**). Similarly, quercetin 1, 10 μM significantly decreased the effect of IL-1β on ERK1/2 protein expression at 24 h (*p<0.01*; **Fig. 18**), and this significant level after IL-1β treatment was not different from DMSO and quercetin alone.

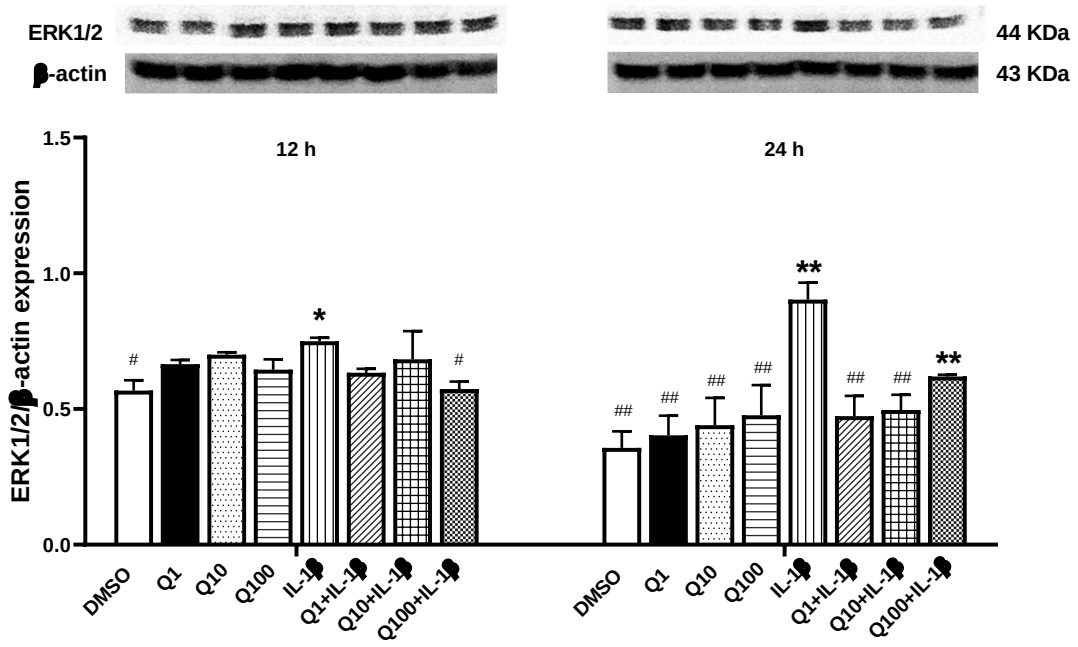


Figure 18 Effects of quercetin, IL-1β and quercetin with IL-1β on the ERK1/2 protein expression. Caco-2 cells incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 12-24 h were extracted for total protein. Representative ERK1/2 protein bands were analyzed by Western blot analysis compared to β-actin. Data showed the mean ± SEM from 7 independent experiments by densitometry analysis. **p<0.05* or ***p<0.01* compared with DMSO. $^{\#}p<0.05$ or $^{\#\#}p<0.01$ compared with IL-1β as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

It is noted that quercetin 100 μM tended to decrease the effect of IL-1β on ERK1/2 protein expression at 24 h, but not significantly different (*p>0.05*; **Fig. 18**). From

these results, pretreatment with quercetin 1, 10 and 100 μM was found to prevent the effect of IL-1β on increasing iκB protein expression at 12 and 24 h.

Effects of quercetin on the expression of myosin light chain kinase (MLCK) protein in Caco-2 cells challenged by IL-1β

The MLCK is the important enzyme which phosphorylates myosin light chain (MLC) to p-MLC resulting in increased paracellular permeability via tight junction opening. Thus, an inhibition of MLCK protein expression is the key target to treat the leaky gut associated with tight junction impairment. We further determine the preventive mechanism of quercetin on IL-1β increased the macromolecules permeability through the expression of MLCK protein. Following the same protocol of drug treatment, western blot analysis using the rabbit anti-MLCK antibody detected a protein band with a molecular weight of 50 KDa. The results showed no significant difference in MLCK protein expression in response to all quercetin treatment for 12 and 24 h when compared to DMSO ($p>0.05$; **Fig. 19**). Cell exposure to IL-1β (10 ng/ml) alone revealed significant increase in MLCK protein following treatment for 24 h, but not 12 h when compared with DMSO ($p<0.05$; **Fig. 19**). Pretreatment with quercetin at 1 and 10 μM significantly decreased MLCK protein expression induced by IL-1β to a level that was not different from DMSO at 24 h ($p<0.01, <0.05$; **Fig. 19**). In contrast, co-incubation with quercetin 100 μM with IL-1β turned to significantly increase MLCK protein compared with IL-1β at 12 h ($p<0.05$; **Fig. 19**). The significant levels of quercetin 1, 10 μM with IL-1β did not differ from quercetin alone. Based on these results, pretreatment with quercetin 1 and 10 μM could prevent the effect of IL-1β on increasing MLCK protein expression which was only evidenced at 24 h treatment period.

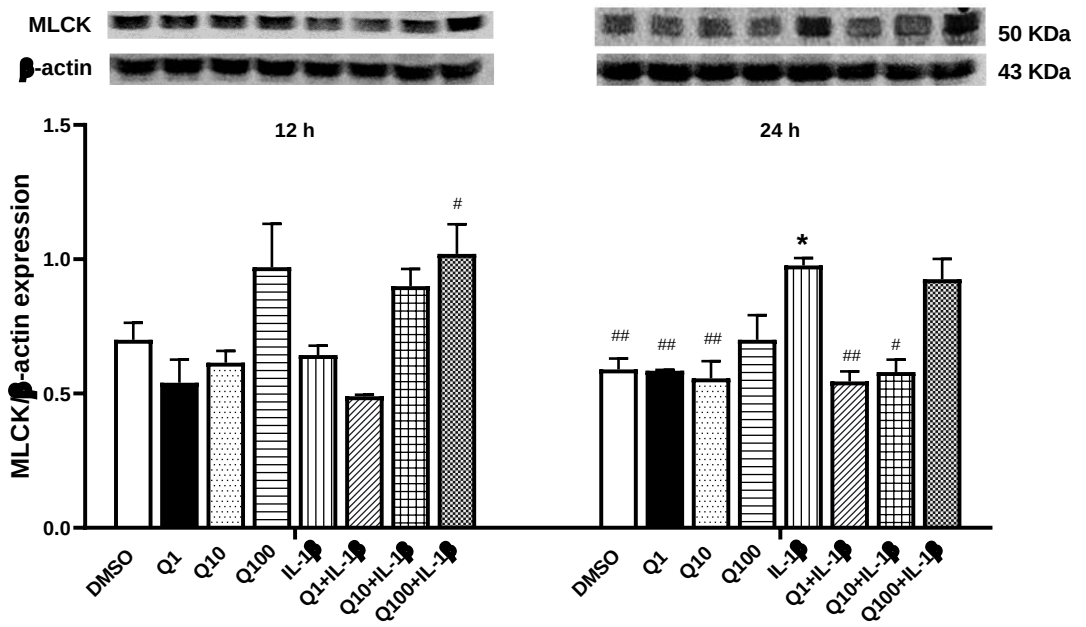


Figure 19 Effects of quercetin, IL-1β and quercetin with IL-1β on the MLCK protein expression. Caco-2 cells incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 12-24 h were extracted for total protein. Representative MLCK protein bands were analyzed by Western blot analysis compared to β-actin. Data showed the mean ± SEM from 7 independent experiments by densitometry analysis. *$*p<0.05$* compared with DMSO. $^{\#}p<0.05$ or $^{\#\#}p<0.01$ compared with IL-1β as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

Effects of quercetin on the expression of phosphorylated myosin light chain kinase (p-MLC) protein in Caco-2 cells challenged by IL-1β

p-MLC is the most important target of MLCK pathway. Activation of MLCK by IL-1β induces the phosphorylation of MLC to p-MLC leading actomyosin contraction and a consequent tight junction opening. Thus, the p-MLC inhibition is the greatest effective target to treat the increased tight junction permeability. To determine the mechanism of

quercetin in the prevention of IL-1β increased the macromolecules permeability, the expression of p-MLC protein was determined by semi-quantitative Western blot analysis at 12-24 h. Following the same protocol of drug treatment, the result using the rabbit-anti-p-MLC antibody detected a protein band with a molecular weight of 37 KDa.

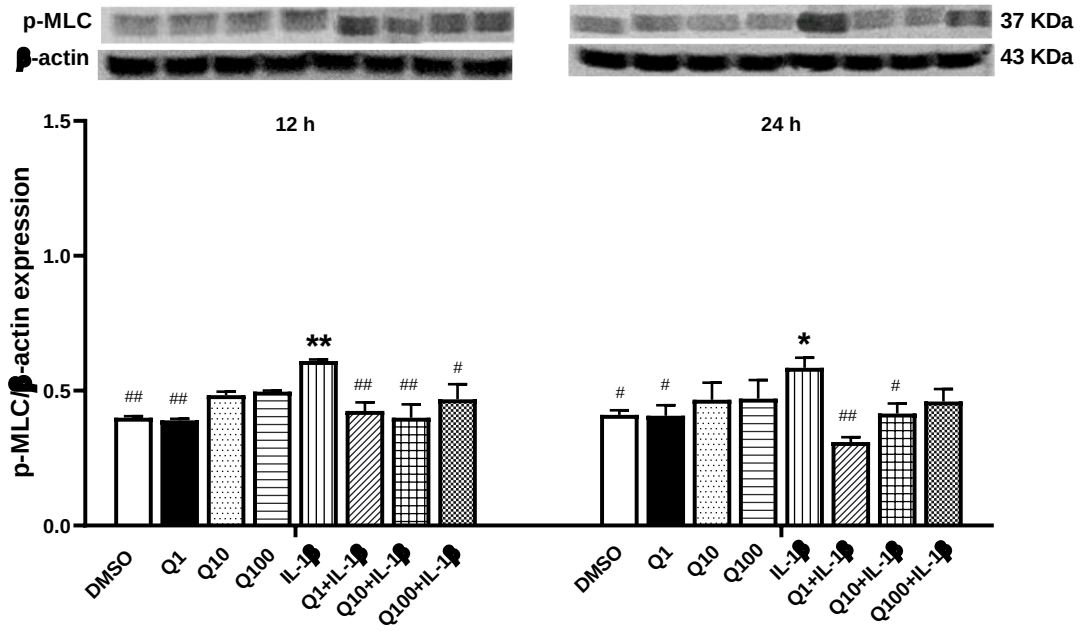


Figure 20 Effects of quercetin, IL-1β and quercetin with IL-1β on the p-MLC protein expression. Caco-2 cells incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 12-24 h were extracted for total protein. Representative p-MLC protein bands were analyzed by Western blot analysis compared to β-actin. Data showed the mean ± SEM from 7 independent experiments by densitometry analysis. *$p<0.05$ or **$p<0.01$ compared with DMSO. $^{\#}p<0.05$ or $^{\#\#}p<0.01$ compared with IL-1β as analyzed by two-way repeated measure ANOVA and Dunnett's post-hoc test.

Incubation with all quercetin concentrations for 12 and 24 h did not change p-MLC protein compared to DMSO (*p>0.05*; **Fig. 20**). Cell exposure to IL-1β (10 ng/ml) alone, for both 12 h and 24 h, significantly increased p-MLC protein when compared with DMSO (*p<0.01, p<0.05*; **Fig. 20**). Pretreatment with all quercetin concentrations with IL-1β significantly decreased p-MLC protein expression level that was not different from DMSO at both 12 h and 24 h (*p<0.05*; **Fig. 20**), except co-treatment with quercetin 100 μM and IL-1β for 24 h. Therefore, pretreatment with quercetin 1 and 10 μM could prevent the IL-1β increasing p-MLC protein expression which was evidenced at both 12 h and 24 h treatment periods.

Effects of quercetin on the expression of *nuclear factor kappa-B (NF-κB)* gene in Caco-2 cells challenged by IL-1β

In addition to NF-κB protein expression, the expression of *NF-κB* mRNA in response to quercetin with or without IL-1β in Caco-2 cells was determined by real-time PCR. The RNA extracted from Caco-2 cells treated with DMSO, quercetin 1-10 μM or IL-1β in the presence and absence of quercetin for 12 h to determine *NF-κB* mRNA using specific primers. The relative fold change of *NF-κB* mRNA normalized to *GAPDH* and then compared to control DMSO using $2^{-\Delta\Delta Ct}$ calculation was shown in **Fig. 21**. From the results, quercetin 1 and 10 μM, but not 100 μM, significantly up-regulated *NF-κB* mRNA compared with DMSO (*p<0.05; p<0.01*; **Fig 21**). Cells exposed to IL-1β did not significantly increase *NF-κB* mRNA. When cells were co-incubated with quercetin and IL-1β, a twofold increase in *NF-κB* mRNA was observed in groups with quercetin pretreatment at 1 μM (*p<0.01*; **Fig. 21**) whereas quercetin pretreatment at 10 and 100 μM did not change *NF-κB* mRNA expression (*p>0.05*; **Fig. 21**) compared to IL-1β. From this result, quercetin 1 μM with or without IL-1β and quercetin 10 μM induced *NF-κB* mRNA expression. Meanwhile, IL-1β did not affect *NF-κB* mRNA in this experiment.

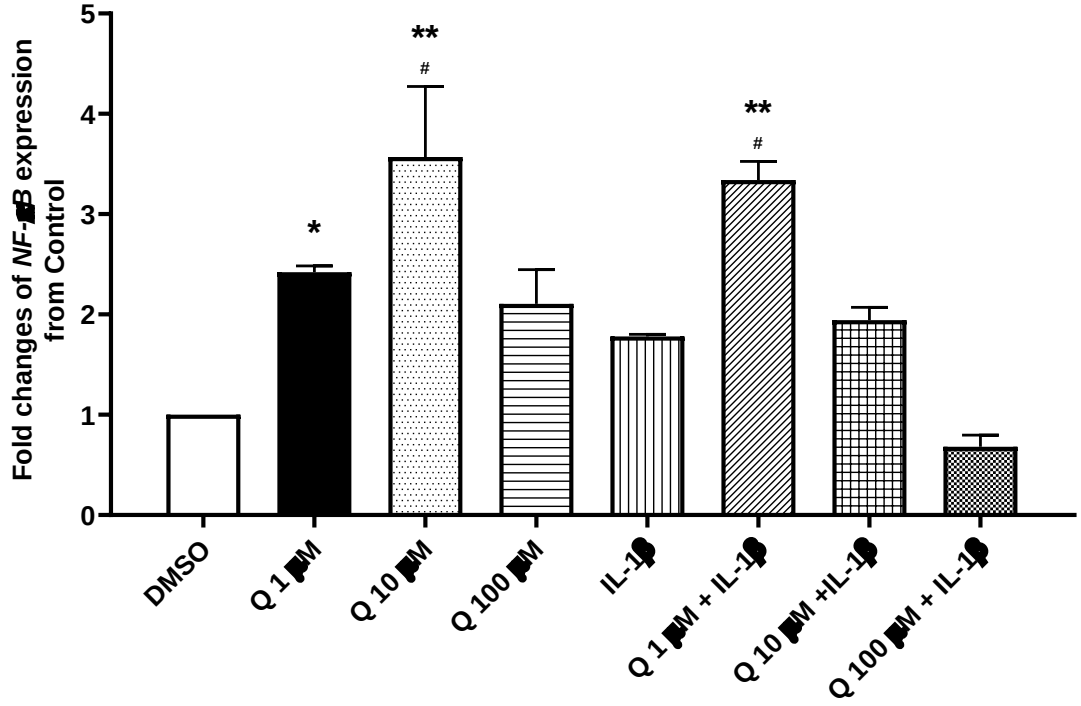


Figure 21 Effects of quercetin, IL-1β and quercetin with IL-1β on the NF-κB gene expression. Caco-2 cells incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 12 h. The total RNA was extracted to determine NF-κB mRNA expression using reverse transcription and real-time PCR. Fold change of NF-κB expression normalized to GAPDH was compared to DMSO using $2^{-\Delta\Delta Ct}$ calculation. Data showed the mean ± SEM from 7 independent experiments. *$p<0.05$* or **$p<0.01$* compared with DMSO. $^{\#}p<0.05$ compared with IL-1β as analyzed by one-way ANOVA and Dunnett's post-hoc test.

Effects of quercetin on the expression of *myosin light chain kinase (MLCK)* gene in Caco-2 cells challenged by IL-1β

The expression of *MLCK* mRNA expression in response to drug treatments was additionally investigated in the same manner as NF-κB. The results in **Fig. 22** showed that all quercetin with or without IL-1β treatment for 12 h significantly decreased NF-κB

mRNA from control DMSO (*p<0.01*; Fig. 22). Moreover, cells exposed to IL-1β alone or in the presence of quercetin at any concentrations had no significant effect on *MLCK* mRNA (*p>0.05*; Fig. 22).

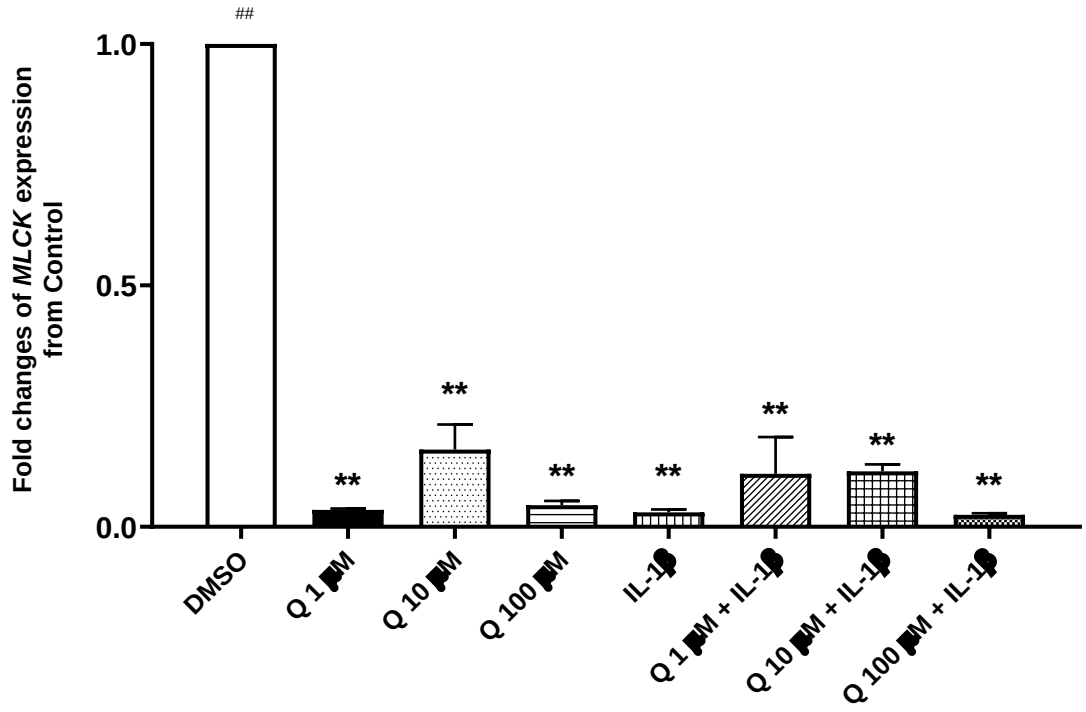


Figure 22 Effects of quercetin, IL-1β and quercetin with IL-1β on the MLCK gene expression. Caco-2 cells incubated with DMSO, quercetin (1, 10, 100 μM), or IL-1β (10 ng/ml) in the presence or absence of quercetin for 12 h. The total RNA was extracted to determine MLCK mRNA expression using reverse transcription and real-time PCR. Fold change of MLCK expression normalized to GAPDH was compared to DMSO control using $2^{-\Delta\Delta Ct}$ calculation. Data showed the mean ± SEM from 7 independent experiments. ***p<0.01* compared with DMSO. $^{\#\#}p<0.01$ compared with IL-1β as analyzed by one-way ANOVA and Dunnett's post-hoc test.

Effects of quercetin on the nuclear factor kappa-B (NF-κB) translocation of Caco-2 cells challenged by IL-1β

Apart from the expression of mRNA and protein, the preventive effect of quercetin on NF-κB translocation induced by IL-1β was determined using immunofluorescence staining. In this experiment, Caco-2 cells was incubated with DMSO, quercetin 1 μM or IL-1β (10 ng/ml) or quercetin 1 μM+ IL-1β for 1 h. Quercetin at concentration of 1 μM was chosen for testing as it was previously shown to markedly inhibit the IL-1β induced increase in NF-κB protein expression. The incubation time at 1 h was chosen because the translocation of NF-κB into nucleus should occur before gene expression at 6-12 h. Immunofluorescence using mouse anti-NF-κB antibody followed by rabbit anti-mouse-FITC antibody detected NF-κB protein which was represented by green color while DAPI localized nucleus represented by blue color.

The result presented in **Fig. 23** showed that cells in the control group and quercetin group expressed green color of NF-κB and blue color of nucleus in separate indicating NF-κB was not translocated into nucleus. Treatment with IL-1β induced the appearance of green-stained within nucleus and co-localized with blue color. The co-localization indicated NF-κB translocation into nucleus. When cells were treated with IL-1β in the presence of quercetin, the NF-κB translocation was reduced compared with IL-1β alone and was not different from control **(Fig. 23)**.

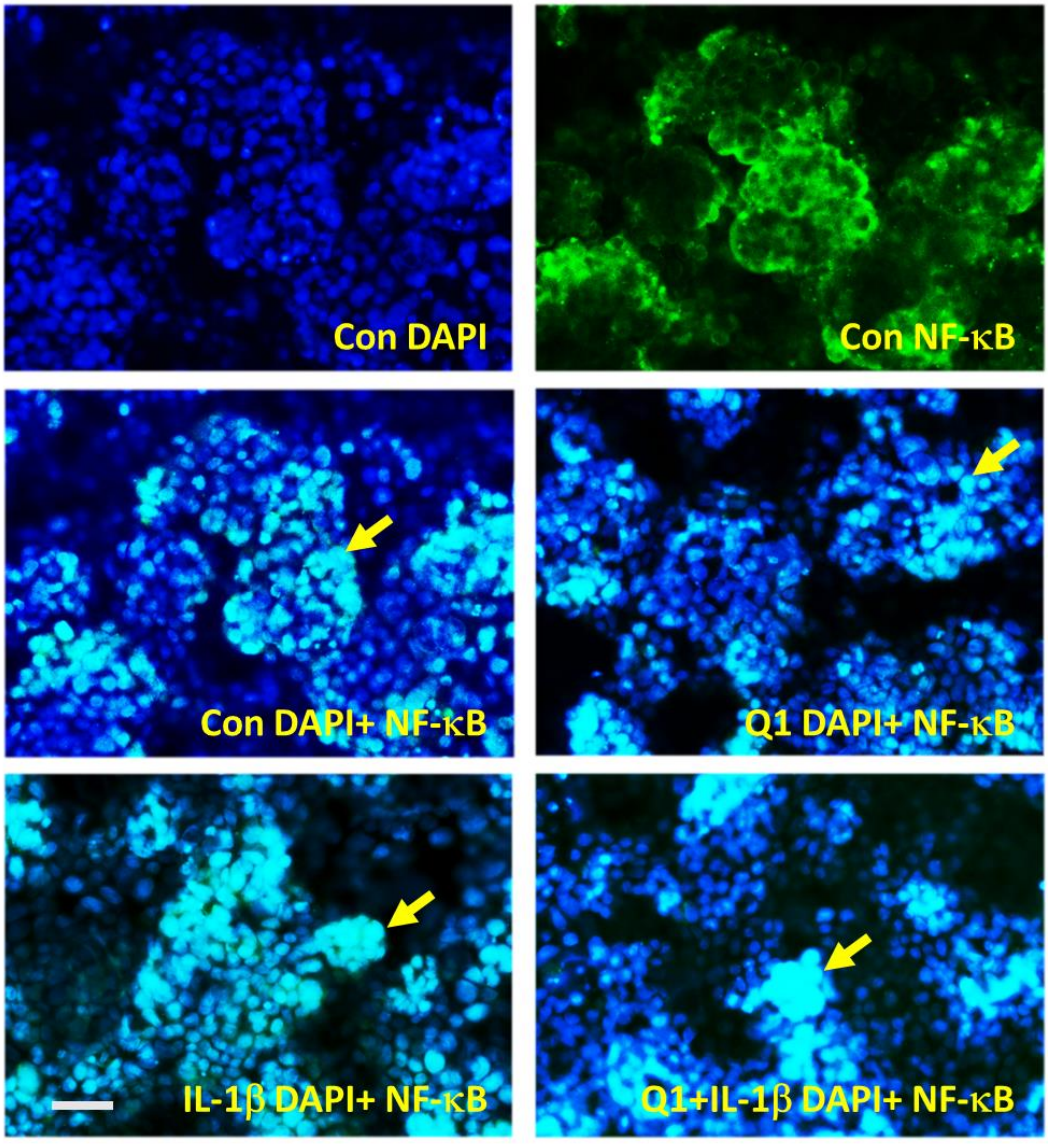


Figure 23 Immunofluorescence staining of NF-ΚB translocation in Caco-2 cell monolayer. Cells were incubated with DMSO (Con), quercetin 1 μM (Q1) for 24 h and challenged with IL-1β for 1 h. The immunoreactive results were scanned by confocal microscope with magnification X200. Cell nuclei were labeled with DAPI in blue at excitation/emission at 358/461 nm and NF-ΚB in green at excitation/emission at 493/519 nm. (n=7 experiments) Scale bar: 200 μm. Arrow: co-localization.

CHAPTER 5
SUMMARY DISCUSSION AND SUGGESTION.

The intestinal epithelial barrier especially tight junction (TJ) is an important physical barrier which protects pathogens and toxin invading into the blood circulation [5]. Impairment of intestinal TJ barrier due to cell damage or inflammation leads to increased intestinal permeability via a leak pathway or called leaky gut. Pro-inflammatory cytokine IL-1β which is produced at high level (10-100 μM) during inflammation, has been shown to disrupt intestinal TJ barrier. The IL-1β-induced increase in intestinal TJ permeability is mediated by NF-κB/ERK1/2/MLCK pathway leading to opening the TJ [104]. As quercetin exerts potent antioxidant, anti-inflammation, and promotive effect on epithelial barrier function, we therefore investigated the effect of quercetin on intestinal barrier function and the protective effect and mechanism of quercetin on IL-1β induced TJ barrier dysfunction via NF-κB/ERK1/2/MLCK pathway in human intestinal epithelial Caco-2 cell line. The present study showed that quercetin (1, 10, 100 μM), IL-1β or each combination was not toxic to Caco-2 cells. Low concentration of quercetin (1, 10 μM) had no effect on the intestinal epithelial barrier function as detected by TER and paracellular permeability to FD-4. However, these quercetin concentrations significantly inhibited an increase in paracellular permeability induced by IL-1β. An analysis of western blot revealed that cell exposed to IL-1β for 12-24 h increased expression of NF-κB, ERK1/2, MLCK and p-MLC protein and decreased iκB protein. Alterations in these signaling molecules were well correlated with increased paracellular permeability, suggesting that IL-1β induced TJ dysfunction was mediated through an activation of NF-κB/ERK1/

2/MLCK pathway. Moreover, the NF-κB pathway activated by IL-1β was confirmed by nuclear translocation of NF-κB. Importantly, the effect of IL-1β on activation of NF-κB/ERK1/2/MLCK pathway and NF-κB translocation leading to intestinal TJ impairment was prevented by pretreatment with quercetin especially at 1 and 10 μM.

All concentrations of quercetin (1-100 μM) used in the present study had no toxicity to Caco-2 cells when treated up to 48 h as shown by no significant change in in both OD570-620 and % cell viability to quercetin. Our finding contrasts to other studies that demonstrate the anticancer effect of quercetin by decreasing cell viability in several types of cancer cells such as HepG2, Hep3B, Hct116 and MDA-MB-231 using the same range of concentration [12]. Although the cytotoxicity of quercetin has not been investigated in Caco-2 cells, the increased TER in response to quercetin treatment indicates membrane integrity of living cells and no toxic effect of quercetin on Caco-2 cells [16]. The different cell viability by quercetin is likely due to different types of cancer cells. Like quercetin, IL-1β (10 ng/ml) was not toxic to Caco-2 cells. This result indicates that the effect of IL-1β on increased TJ permeability is not a result of cell death which is similar to a previous study indicating that IL-1β 10 ng/ml increased paracellular permeability without cell apoptosis and necrosis [2]. However, co-treatment of cells with quercetin 100 μM with IL-1β for 48 h tended to decrease cell viability expressed as OD570-620 unit. This decreased viability was probably correlated with a marked impairment of intestinal barrier function by decreased TER at 24 h and increased paracellular permeability to FD4 at 48 h, suggesting a substantial reduction of cell viability even though not significantly different from control. However, this result indicates all concentrations of quercetin are not toxic to Caco-2 cells.

The integrity of TJ is crucial for intestinal epithelial barrier function which is widely assessed by transepithelial electrical resistance (TER) and paracellular permeability to marker macromolecule like FD-4. The TER value reflects ion permeability

of paracellular pathway and, to a lesser extent, transcellular pathway. Basically, the paracellular permeation of small ions and solutes represents the pore pathway that can be assessed by measurement of TER. On the other hand, the paracellular permeation of large solutes or a specific macromolecule through the leak pathway indicates increased permeability of TJ which normally restricts ions and substances in a selective manner [35]. Under normal conditions, the TJ permeability can be activated, especially post-ingestion to absorb nutrients and water. Afterward, the permeability will decrease to limit pathogen and macromolecule passing after absorption [105]. In the present study, quercetin at 1 and 10 μM did not change TER and FD-4 flux, indicating its effect on maintenance of intestinal barrier function. Indeed, an appropriate TER is required for normal intestinal function since very high integrity of monolayer may limit the transport of ions and nutrients across cell monolayer. In contrast, low TER indicates not only ion permeability but also tight junction barrier impairment as in leaky gut syndrome. From the present results, incubation of quercetin 100 μM for 24 h was found to decrease TER with no change in FD-4 flux suggesting an increase in ion permeability through both transcellular and paracellular pathway. From the previous studies, quercetin 100 μM stimulated Cl^- and HCO_3^- secretion in small and large intestine and basolateral K^+ channels in distal colon of rat [106, 107] as well as stimulated Cl^- secretion in T84 cells [108]. The evidence of quercetin effect on ion transport across intestinal cells and tissues at least explains the decreased TER by quercetin.

In the current study, IL-1β increased paracellular permeability of-FD-4 flux at 24 and 48 h without a significant change in TER. This result contrasts to the previous study demonstrating an increase in paracellular permeability associated with a decrease in TER in Caco-2 cells following treatment with IL-1β for 24-72 h [104]. The different TER responses are likely due to different intestinal cell types and conditioned media used for studying. In Al-Sadi's study, 21-day Caco-2 cells were used while our study used 7-day cells. The 14-21 days of cultured Caco-2 shows a characteristic of jejunal like-cells

whereas 7-day culture cells are colonic-like cells [109]. In addition, 10% FBS containing media were used in that study instead of 20% FBS in media being used to maintain cell growth in the present study. Therefore, these different factors may be at least the cause of the different TER response. As the paracellular permeability to macromolecule is more reliable than the TER in determination of tight junction leakiness, our results suggest that the impairment of TJ barrier function was induced by IL-1β.

Interestingly, pretreatment with quercetin 1 and 10 μM for 24 h was found to inhibit an increase paracellular permeability induced by IL-1β. This is the new finding due to lack of investigations regarding the effect of quercetin and IL-1β on paracellular permeability in intestinal or other epithelial cells. Most previous studies consider the effect of quercetin on other parameters associated with inflammation such as pro-inflammatory cytokine secretion or adhesion molecules of white blood cells [110]. Conversely, pretreatment with quercetin 100 μM with IL-1β for 24 and 48 h significantly increased paracellular permeability more than IL-1β alone. The additive effect of quercetin and IL-1β on tight junction impairment could be because of the IL-1β effect on decreasing cell viability at high quercetin concentration.

To investigate the signaling mechanism of quercetin on inhibition of IL-1β-increased in paracellular TJ permeability through signaling molecules involving MLCK signaling pathway, the expression of NF-κB, iκB, ERK1/2, MLCK and p-MLC protein and gene expression including NF-κB translocation were determined. Western blot results revealed that most of quercetin tested did not alter NF-κB, iκB, ERK1/2, MLCK and p-MLC protein expression. These findings were not surprising since quercetin did not affect TER and permeability test. Meanwhile, IL-1β-induced macromolecule permeability was correlated with increased NF-κB, ERK1/2, MLCK, p-MLC and decreased iκB protein expression that occur at the earlier time. Additionally, the role of NF-κB in mediating an increase TJ permeability activated by IL-1β is confirmed by

increasing NF-κB translocation into nucleus. Several studies have demonstrated signaling pathways that mediate the effect of IL-1β on increasing TJ permeability [111, 112]. In gastrointestinal tract, IL-1β induced by pathogen-associated molecular pattern (PAMP) especially lipopolysaccharide stimulates degradation of iκB leading to NF-κB translocation and activation of MLCK gene in human intestinal epithelial cells [111]. Moreover, IL-1β induces MAPK pathway by mitogen-activated protein kinase kinase-1 (MEKK-1) and iκB kinase (IKK) activation leading to translocation of NF-κB and increase permeability in Caco-2 cells and mouse intestinal epithelial cells [112, 113]. Thus, IL-1β increased in TJ permeability is mediated by MEKK-1-induced IKK to phosphorylate and degrade iκB, resulting in translocation of NF-κB. Other previous studies have shown that IL-1β induced increases in MLCK gene activity and protein expression is mediated by NF-κB activation, involving the MEKK-1 and IKK activation in human intestinal epithelial cells [2, 114]. IL-1β also increases ERK1/2 protein expression and Elk-1 translocation which attaches with MLCK promotor region, leading to increased MLCK gene and TJ permeability [115]. From these evidence, IL-1β induces TJ permeability via MLCK pathway by NF-κB activation and ERK1/2 transcription factors.

Our findings that co-incubation of quercetin and IL-1β significantly decreased NF-κB, ERK1/2, MLCK, p-MLC and increased iκB protein appeared to depend on concentrations of quercetin and incubation time. Significant changes of these protein expressions induced by IL-1β were observed following co-incubation with quercetin 1 or 10 μM for 24 h, except a decreased IκB expression was observed with quercetin 100 μM. In overall, the effects of quercetin on inhibiting IL-1β induced changes in NF-κB, iκB, ERK1/2, MLCK and p-MLC protein expression were associated with decreased TJ permeability. Quercetin 1 μM also decreased NF-κB translocation induced by IL-1β. The present results are supported by several previous studies.

Quercetin has been demonstrated to prevent NF-κB activation and Nrf2 translocation induced by indomethacin in Caco-2 cells [114]. Quercetin also reduces oxidative stress and inflammation via inhibition of NOX2/ROS/NF-κB in lung epithelial cells [116]. Quercetin also inhibits NF-κB activation of pro-inflammatory gene promotor induced by TNF-α in murine intestinal epithelial cells [117]. Moreover, quercetin inhibits IL-1β-induced MCP-1, IL-6, IL-8, ICAM-1 and sICAM-1 by inhibiting NF-κB and MAPK including ERK1/2, p38 and JNK signaling molecules in human retinal pigment epithelial cells ARPE-19 [118]. At last, quercetin inhibits MAPK pathway induced by LPS via inhibition of JNK, ERK, and p38 MAPK phosphorylation in chicken intestine [119]. However, no previous studies have been investigated the effect of quercetin to NF-κB/ERK1/2/MLCK pathway on Caco-2 cells induced by IL-1β. This study demonstrates that quercetin can decrease paracellular permeability induced by IL-1β via inhibition of NF-κB/ERK1/2/MLCK pathway on Caco-2 cells. In further study, the signaling molecules such as MEKK-1, IKK, p38, Elk-1 and JNK associated with MLCK pathway should be investigated in intestinal epithelial cells induced by IL-1β.

To investigate whether quercetin prevented the IL-1β-increased in NF-κB and MLCK proteins was due to its modulation of gene expression, the real-time PCR was additionally performed. The results showed that quercetin increased *NF-κB* gene and decreased *MLCK* gene expression. Moreover, IL-1β did not change *NF-κB* gene expression but decreased *MLCK* gene expression. These *NF-κB* and *MLCK* gene expressions were not related to NF-κB and MLCK protein expression as well as barrier function. Although no previous studies have investigated the direct effect of quercetin on *NF-κB* and *MLCK* gene expression in intestinal epithelial cells, IL-1β-decreased *MLCK* gene conflicts with the previous study in which IL-1β induced *MLCK* gene expression in Caco-2 cells [104]. Normally, gene expression can happen any time between 12-24 h. There are a variety of factors that affect gene expression such as

post-transcription, translation (2-7 h), protein degradation (48 h) and low rate of gene transcription compared to protein translation [120]. Therefore, the gene expression is unnecessary to relate with protein expression, probably depending on duration in each process. Like quercetin alone, quercetin with IL-1β increased *NF-κB* and decreased *MLCK* gene expression. It is noted that the significance changes between quercetin and quercetin with IL-1β were not different, and IL-1β did not induce *NF-κB* and *MLCK* gene. Therefore, the effect of quercetin with IL-1β on *NF-κB* and *MLCK* gene may result from quercetin alone. These results suggested the quercetin alone inhibits *MLCK,* but not *NF-κB* gene expression. Normally, gene expression happens at 6-12 h. However, the time of real-time PCR in this study investigates the effect of quercetin and IL-1β on gene expression at 12 h which happens before protein expression at 12 and 24 h. Therefore, *NF-κB* and *MLCK* gene should be performed during 6-<12 h to confirm the alteration of gene expression.

Quercetin is one of the most abundant flavanols which has many biological properties including anti-carcinogenic, anti-inflammatory, and intestinal barrier-promoting effects [84, 85]. Due to its alleviation of the pro-inflammatory cytokine effect on intestinal epithelial cells, quercetin receives much attention as natural medicine for treatment of gastrointestinal inflammation. Dietary quercetin usually presents in quercetin glycoside form which conjugated with sugar especially glucose. The glucose conjugated with quercetin is absorbed into the cells by glucose transporter and passed into the blood circulation. In the our study, we used the active form quercetin glycoside which have been shown to affect intestinal epithelial cells [121]. Quercetin at the concentrations of 1, 10, 100 μM are chosen for our experiments based on previous studies of Egert et al., 2008 [122] and Suzuki and Hara, 2009 [15] showing that it dose-dependently decreases the paracellular permeability of lucifer yellow (LY) flux in Caco-2 cells [15]. Normally, taking quercetin 400-600 mg of coated tablet 1-3 times daily [123] has

the various biological actions depending on its concentration in the blood circulation (0->200 μM) i.e., low range (0-10 μM) resulting in chemopreventive effect, mid ranges (10-200 μM) resulting in mixed effects, and high concentration (>200 μM) resulting in anti-oxidant or direct therapeutic properties [124] in human body. The concentration of quercetin in human body is comparable to the concentration in cell culture; therefore, quercetin 1, 10, 100 μM is suitable for determining the effect of quercetin in paracellular permeability to macromolecule in Caco-2 cells. We found the protective effect of quercetin 1 and 10 μM on decreased paracellular permeability in Caco-2 cells induced by IL-1β is correlated to the anti-inflammatory effect of quercetin. However, quercetin 100 μM either in the presence or in the absence of IL-1β increased paracellular permeability to macromolecule. This effect of quercetin 100 μM is different from the other previous studies which decreased paracellular permeability [77]. This difference may be due to, as previously stated, the difference population of Caco-2 cell model. The previous studies used the 21-day Caco-2 cells which were small intestine-liked cells [77]. Meanwhile, this study used the 7-day Caco-2 cells representing colonic-like cells which have the barrier integrity less than the 21-days cells [109]. Therefore, the 7-day cells cannot tolerate quercetin at high concentration and IL-1β leading to increased permeability. However, the 7-day colonic-like cells were chosen to be the model because the pathogens in the gastrointestinal tract mostly locate in the colon which is likely to induce IL-1β secretion more than the small intestine.

Taken altogether, our results demonstrate that quercetin maintains intestinal barrier function in normal condition and prevents an increased paracellular permeability of intestinal epithelial cells induced by IL-1β via inhibition MLCK pathway associated with NF-κB, iκB and ERK1/2 signaling molecules. The underlying mechanism of quercetin on inhibition of IL-1β-induced the intestinal barrier impairment is shown in **Fig. 24**. The binding of IL-1β to IL-1β receptors activate the phosphorylation of iκB and

release NF-ΚB to cytoplasm. The free NF-ΚB translocate into nucleus leading to stimulation of MLCK gene and protein expression. The MLCK phosphorylates MLC to p-MLC which induces peri-junctional contraction and tight junction opening. The increased tight junction permeability permits the macromolecule, pathogen and toxin invading into the blood circulation. Treatment of quercetin inhibits the effect of IL-1β on NF-ΚB/ERK1/2/MLCK pathway by blocking NF-ΚB activation and iΚB degradation leading to inhibition of the NF-ΚB translocation. The decreased NF-ΚB translocation decreases *MLCK* gene and protein expression resulting in reduction of p-MLC. The reduction of p-MLC alleviates peri-junction contraction, TJ opening and macromolecule, pathogen or toxin passing. Moreover, quercetin also blocks IL-1β-induced ERK1/2 leading to an inhibition of MLCK pathway in a similar manner to NF-ΚB cascade.

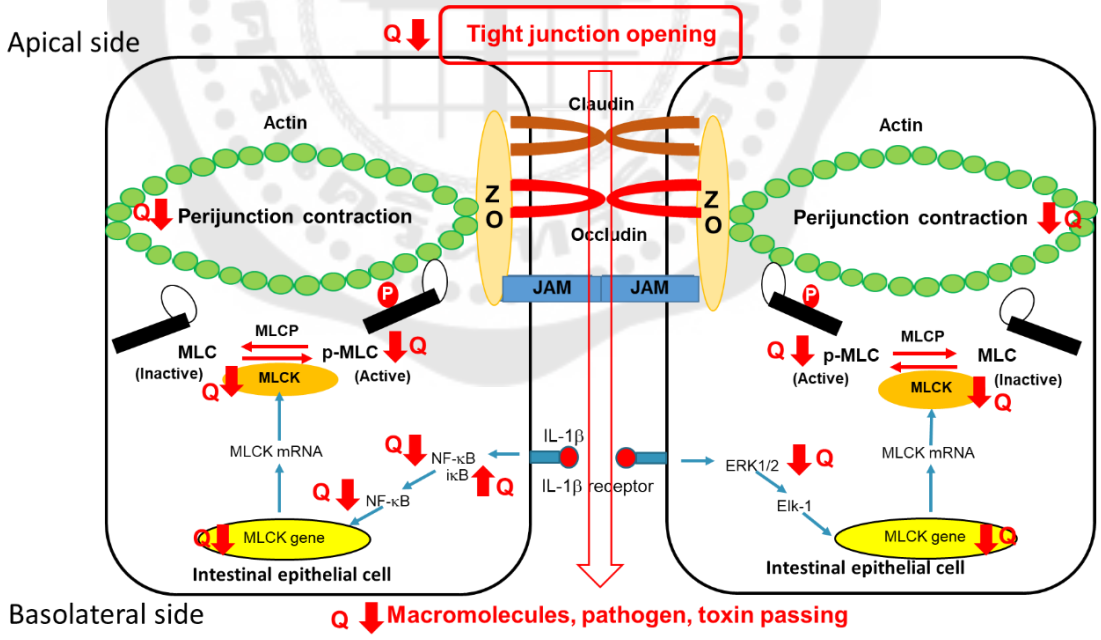


Figure 24 Model summarizing NF-ΚB/ERK1/2/MLCK pathway of quercetin on the prevention of IL-1β-induced increase in tight junction permeability in intestinal epithelia

In conclusion, quercetin especially at low concentrations maintains proper intestinal barrier function under normal condition and prevents an increased paracellular permeability of intestinal epithelium induced by IL-1β via inhibition of NF-κB/ERK1/2/MLCK pathway. This study provides the potential role of quercetin for therapeutic application in alleviation and protection of intestinal barrier leakiness associated with inflammation.

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