Galangin and Kaempferol Alleviate the Indomethacin-Caused Cytotoxicity and Barrier Loss in Rat Intestinal Epithelial (IEC-6) Cells Via Mediating JNK/Src Activation

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ABSTRACT: Nonsteroidal anti-inflammatory drugs (NSAIDs) like indomethacin and others are widely used in clinics, but they have the potential to cause severe gastrointestinal damage including intestinal barrier dysfunction. Thus, two flavonols galangin and kaempferol with or without heat treatment (100 °C, 30 min) were assessed for their effect on indomethacin-damaged rat intestine epithelial (IEC-6) cells. In total, the cell exposure of 300 μmol/L indomethacin for 24 h caused cell toxicity efficiently, resulting in decreased cell viability, enhanced lactate dehydrogenase (LDH) release or reactive oxygen species (ROS) production, and obvious barrier loss. Meanwhile, pretreatment of the cells with these flavonols for 24 and 48 h before the indomethacin exposure could alleviate cytotoxicity and especially barrier loss, resulting in increased cell viability and transepithelial resistance, decreased LDH release, ROS production, and paracellular permeability, together with the promoted expression of three tight junction proteins zonula occluden-1, occludin, and claudin-1. Moreover, the intracellular Ca2+ concentration and expression levels of p-JNK and p-Src arisen from the indomethacin damage were also reduced by the flavonols, suggesting an inhibited calcium-mediated JNK/Src activation. Consistently, galangin showed higher activity than kaempferol to the cells, while the heated flavonols were less efficient than the unheated counterparts. It is thus highlighted that the two flavonols could alleviate indomethacin cytotoxicity and combat against the indomethacin-induced barrier loss in IEC-6 cells, but heat treatment of the flavonols would weaken the two beneficial functions.

■ INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most used clinical drugs in the present time. However, as highly effective drugs for the treatment of pain and inflammation, NSAIDs have a variety of side effects like gastrointestinal bleeding, cardiovascular side effects, and nephrotoxicity.1 Thus, the prevalence of inappropriate NSAID use is worrying.2,3 In the past, several scholars had conducted a retrospective study on 3050 patients with chronic pain and found that about 97% of chronic pain subjects took NSAIDs continuously for more than 21 days.4 More importantly, it was also observed that about 70% of patients, who took therapeutic levels of NSAIDs for more than 6 months, had increased intestinal mucosal permeability and enhanced blood loss,5 while the sublethal level of a NSAID, indomethacin, could lead to chronic inflammation of distal jejunum and proximal ileum.6 It was also reported that the elderly patients had a higher risk of nephrotoxicity of NSAID.6 In addition, NSAIDs are regarded to damage the intestinal barrier function.7 Thus, the FAD of the United States had officially notified a potential risk between the NSAID intake and the undesired digestive tract bleeding.8

The intestinal epithelium is a layer of polarized continuous columnar cells covering the surface of the intestine, which separates the intestinal cavity from the internal environment (mainly acts as a physical barrier) and regulates the absorption of dietary nutrients, water, and electrolytes.9 The molecules travel from the intestinal lumen to the lamina propria in two different pathways: (1) the paracellular pathway that allows small molecules to spread through the tight junctions (TJs) between the adjacent intestinal epithelial cells and (2) the transcellular pathway that allows larger particles to pass through the epithelial cells through phagocytosis or exhalation.10 Intestinal epithelial cells are the key components of the epithelial lining, while their most important task is to maintain the integrity of the intestinal physical barrier. Intestinal epithelial cells are closely bound together by apical junction complexes,11 which are composed of TJs, adhesion junctions,
and desmosomes. These junction complexes limit the uptake of antigens from both microorganisms and food sources and prevent the passage of cellular components. TJs are located at the top of the epithelium, with a function to close the intercellular space and regulate intestinal permeability. TJs have many construction elements like occludin, claudins, and junctional adhesion molecules (JAMs). It is known that TJ transmembrane proteins, claudins, occludin, and JAMs, are connected to actomyosin fibers of the cytoskeleton through members of the zonula occludent (ZO) family. This connection to the actomyosin ring around the junction is critical for the dynamic regulation of the permeability of the paracellular space. Overall, TJs are far from static but have a flexible structure that can easily adapt to both condition change and challenging stimuli. In view of the important role of TJ proteins in the barrier function of intestinal epithelial cells, it is necessary for us to investigate the effect of natural substances from the daily diets on TJ proteins as well as the barrier function of intestinal epithelial cells.

Evidence currently available in both animal models and in vitro systems suggests that dietary intervention can strengthen the intestinal barrier to prevent the development of intestinal diseases. Food-originated natural compounds and their metabolites have immunomodulatory and other physiological effects, mainly through direct interaction with immune and epithelial cells as well as an indirect alteration in the composition and function of intestinal microorganisms to regulate cell and barrier functions. Food components like dietary fibers, proteins, fats, and others have been revealed to affect the intestinal barrier function. In recent years, scientists have also paid attention to healthy diets including the so-called “superfood” with higher polyphenol contents. In chemicals, flavonols belong to the well-known flavonoid family and are the most common polyphenols in plant foods. In general, plant foods are subjected to thermal treatments (such as cooking, boiling, and sterilization) before their intake. Previous studies indicated that thermal treatment might affect polyphenol properties like antioxidation and anticancer effects greatly. Moreover, polyphenols showed an ability to enhance the barrier function of rat intestinal epithelial (IEC-6) cells or combat against the alcohol-induced TJ dysfunction for a Caco-2 monolayer. However, it is still unknown that whether two natural flavonols galangin (3,5,7-trihydroxy-2-phenyl-4H-chromen-4-one) and kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one), which are found in plant foods but have different chemical structures (none or one hydroxyl group in the B-ring) (Figure 1), might have an ability to combat against the potential cytotoxicity and barrier dysfunction of IEC-6 cells once exposed to a NSAID, indomethacin. More importantly, whether heat treatment of the two flavonols would have a positive or negative effect on the activity of the two flavonols to the cells also deserves our investigation.

In this study, galangin and kaempferol with or without heat treatment were used to pretreat IEC-6 cells and then assessed for their in vitro activity against the indomethacin-induced cytotoxicity and barrier loss, using cell viability, lactate dehydrogenase (LDH) release, reactive oxygen species (ROS) production, transepithelial resistance (TEER), paracellular permeability, and TJ protein expression as evaluation indices. Moreover, a previous study had found that the increase in the intracellular Ca²⁺ concentration ([Ca²⁺]) could mediate the increase of p-JNK and p-Src, resulting in the damage of TJs. Thereby, a possible pathway revealing how the two flavonols combated against the indomethacin-induced barrier loss was briefly clarified, via detecting [Ca²⁺], as well as the protein expression levels of JNK and Src. This study aimed to clarify a potential biological interaction between natural flavonols and the intestine by revealing the potential benefits of flavonols to the intestine health as well as a positive or negative effect of the heat treatment on these flavonol benefits.

## RESULTS

### Indomethacin Cytotoxicity and the Cytoprotective Effect of the Flavonols

The measured viability of the IEC-6 cells exposed to different concentrations of indomethacin is shown in Figure 2, which demonstrated the potential cytotoxic effect of indomethacin on the cells. The cells without indomethacin exposure were served with a viability value of 100%, while those with indomethacin exposure had viability values less than 100% (p < 0.05). More importantly, when indomethacin concentrations in the cells were enhanced from 200 to 500 μmol/L, viability values concentration-dependently decreased to about 41–85%. The results thus suggested that indomethacin indeed had an obvious cytotoxic effect on the cells while a higher indomethacin concentration (e.g. 500 μmol/L) caused greater cytotoxicity. When the indomethacin concentration was used at 300 μmol/L, the viability value of the damaged cell decreased to nearly 70%. Thus, this concentration was used to damage cells for 24 h in the later experiments.

![Figure 1. Chemical structures of two natural flavonols galangin and kaempferol.](image)

![Figure 2. Indomethacin cytotoxicity to IEC-6 cells with a treatment time of 24 h. Different lowercase letters above the columns indicate that one-way ANOVA of the mean values is different significantly (p < 0.05).](image)
It was previously confirmed that galangin and kaempferol at 2.5−20 μmol/L could promote cell viability in IEC-6 cells. It was thus interesting to explore whether the flavonols at these concentrations also could resist the cell injury caused by indomethacin. When the cells were pretreated with one of the heated or unheated flavonol compounds for 24 h before conducting the indomethacin damage, viability values of the cells were measured to be 62−86% (Figure 3A). If the cells were pretreated with one of the heated or unheated flavonol compounds for 48 h before the indomethacin damage, viability values reached 63−87% (Figure 3B). Flavonol concentration of 20 μmol/L mostly resulted in a toxic effect on the cells by decreasing viability values to 62−69% (less than 70%). At the same time, the flavonols at other concentrations always showed a protective effect on the cells. More importantly, the flavonol concentration of 5 μmol/L led to the higher viability values (ranging from 77 to 87%) for the treated cells. The results indicated that these flavonols (especially using flavonol concentration of 5 μmol/L) could antagonize the indomethacin-induced cytotoxicity. Meanwhile, intracellular LDH may pass through the damaged cell membrane; thus, the LDH level in the culture supernatant reflects the integrity of the cell membrane directly. In this study, the measured results for LDH release consistently confirmed the protective effect of the flavonols against the indomethacin-induced cytotoxicity (Figure 3C,D) because the cells pretreated with one of the heated or unheated flavonol compounds had partly reduced LDH release (i.e. attenuated cell damage). Without the indomethacin damage, the control cells had the lowest LDH release (a designed value of 100%). At the same time, the cells treated by indomethacin only showed the highest LDH release (148.1%). When the cells were pretreated by these flavonols for 24 h, after the indomethacin damage, they showed reduced LDH release ranging from 119.3 to 144.5% (Figure 3C). If the cells were pretreated by the flavonols for a longer time of 48 h, after the indomethacin damage, they were measured with much-reduced LDH release ranging from 115.2 to 143.5% (Figure 3D). In total, the flavonols at a concentration of 5 μmol/L resulted in the lowest LDH release. In addition, the results given in Figure 3 also indicated two important facts. That is, galangin was more powerful than kaempferol to alleviate indomethacin-induced cell damage, while the heated flavonols were less efficient than the unheated counterparts to increase cell viability or reduce LDH release. It is, thus, concluded that heat treatment of the two flavonols reduced their benefits to alleviate the indomethacin-induced cell damage, although the heated ones also had an obvious protective effect on the cells.

**Cellular ROS Production in Response to Flavonol Pretreatment.** The increase in the ROS level adversely affects homeostasis and function of the cells and subsequently leads to oxidative stress. Thus, the disorder of the cellular redox balance is a risk factor for all kinds of pathological development. In this study, an indomethacin exposure of the IEC-6 cells for 24 h caused obvious oxidative stress because ROS production was enhanced by near onefold (Figure 4). After pretreatment of the cells with these flavonols at 5 μmol/L for 24 h, ROS production in the indomethacin-damaged IEC-6...
cells was significantly inhibited (p < 0.05) because the measured ROS values decreased from 197.4% (model cells) to 145.5–171.3% (flavonol-treated cells) (Figure 4A). It was thus proved that these flavonoids had the ability to protect the cells from the indomethacin-induced oxidative stress. Data comparison also showed that galangin was more active than kaempferol, resulting in a much decrease in ROS production (145.5 versus 158.3%); meanwhile, the heated flavonoids also consistently exerted weaker inhibition on ROS production than their unheated counterparts (162.6–171.3 versus 145.5–158.3%). In addition, a cell treatment of 48 h with the flavonoids yielded a similar conclusion (Figure 4B). Overall, the results given in Figure 4 proved that these flavonoids in the cells had an obvious protective effect against the indomethacin-induced cytotoxicity by inhibiting ROS production; however, the conducted heat treatment reduced the capacity of the flavonoids to combat against the indomethacin-induced oxidative stress.

Physical Barrier Function of IEC-6 Cells in Response to Flavonol Pretreatment. In general, both TEER and the labeled molecules across the epithelial channel can be used to describe the physical integrity of the assessed cell monolayer. To be more specific, TEER reflects the ionic conductance of the paracellular pathway, while the flux of a nonelectrolyte tracer (e.g., FD-4 diffusion) describes the water flow around the cells as well as the pore diameter of the TJ.31 The results listed in Table 1 demonstrated the negative effect of indomethacin exposure as well as the positive effect of these flavonols on the physical barrier function of IEC-6 cells. The control cells were set with TEER and FD-4 values of 100%. Clearly, the cells treated with indomethacin only (i.e., the model cells) had the lowest TEER (70.0–70.3%) but highest FD-4 values (144.1–146.0%), indicating an injured physical barrier function. Meanwhile, the flavonol-treated cells after indomethacin damage showed increased TEER (77.1–89.7%) but decreased FD-4 values (110.7–136.4%) than the model cells, demonstrating these cells possessed an improved physical barrier function. In detail, longer pretreatment time of the cells with these flavonoids caused higher TEER but lower FD-4 values, while galangin was more effective than kaempferol to promote TEER and reduce FD-4 values. However, the heated flavonoids always showed lower potential than the unheated ones to improve the physical barrier function of the cells. It is thus concluded that these flavonoids had the potential to enhance the barrier function of the indomethacin-injured cells, but heat treatment of the two flavonoids also led to a reduction in flavonol capacity.

| Production of Three TJ-Associated Proteins in Response to Flavonol Pretreatment | Three TJ-associated proteins (ZO-1, occludin, and claudin-1) in the cells were also detected in both mRNA and protein expression levels (Figure 5). Regarding the control cells without any indomethacin and flavonol treatments, the model cells treated with 300 μmol/L indomethacin only had lower relative mRNA expression in ZO-1, occludin, and claudin-1 (0.54–0.58-fold) (Figure 5A). However, if the cells were pretreated with these flavonoids for 24 h, the relative mRNA expressions of ZO-1, occludin, and claudin-1 were enhanced to 0.64–0.82-, 0.65–0.83-, and 0.62–0.75-fold, respectively. The flavonoids, thus, had the ability to promote the mRNA expression of the three TJ proteins. At the same time, the model cells also had less relative protein expression for ZO-1, occludin, and claudin-1 (0.29–0.37-fold), but cell pretreatment with these flavonoids resulted in an enhanced protein expression because relative ZO-1, occludin, and claudin-1 expressions were promoted to 0.47–0.71-, 0.50–0.79-, and 0.46–0.67-fold (Figure 5B,C), respectively. These results, together with the assaying results of the TEER value and FD-4 diffusion, consistently indicated that indomethacin caused barrier dysfunction in the cells by decreasing the production of the three TJ proteins, which led to a decreased TEER value and increased FD-4 diffusion. Meanwhile, the flavonoids could protect indomethacin-induced barrier loss by enhancing the production of the three TJ proteins, which then resulted in a higher TEER value but lower FD-4 diffusion. In general, galangin was more effective than kaempferol to enhance both mRNA and protein expression for the three TJ proteins; however, heat treatment of the two flavonoids also led to a decrease in flavonol capacity.

Table 1. Detected TEER and FD-4 Diffusion in IEC-6 Cells Pretreated with the Flavonols (5 μmol/L) Followed by Indomethacin Damage of 24 h\(^a,b\)

| index | cell treatment time (h) | model | galangin | heated galangin | kaempferol | heated kaempferol |
|-------|------------------------|-------|----------|----------------|------------|------------------|
| TEER  | 24                     | 70.0 ± 1.1\(^c\) | 86.1 ± 1.1\(^c\) | 83.0 ± 0.8\(^b\) | 82.3 ± 0.8\(^b\) | 77.1 ± 0.9\(^b\) |
|       | 48                     | 70.3 ± 0.9\(^d\) | 89.7 ± 1.0\(^d\) | 86.2 ± 1.2\(^b\) | 83.4 ± 1.1\(^b\) | 78.3 ± 1.1\(^d\) |
| FD-4  | 24                     | 144.1 ± 2.1\(^a\) | 112.0 ± 2.9\(^d\) | 123.3 ± 1.6\(^a\) | 125.4 ± 2.0\(^b\) | 136.4 ± 2.7\(^b\) |
|       | 48                     | 146.0 ± 1.8\(^a\) | 110.7 ± 2.0\(^d\) | 120.6 ± 2.8\(^b\) | 123.4 ± 2.6\(^b\) | 136.2 ± 2.0\(^b\) |

\(^a\)The cells without any indomethacin and flavonol treatments (i.e., control cells) were regarded with respective TEER and FD-4 values of 100%.
\(^b\)Different lowercase letters as the superscripts after the data in the same row indicate that one-way ANOVA of the mean values differs significantly (p < 0.05).
to decreased flavonol efficacy to promote the expression of these TJ proteins.

**Calcium-Mediated JNK and Src Activation in Response to Flavonol Pretreatment.** A previous study revealed that the calcium-mediated JNK/Src pathway might be involved in the TJs of the damaged cells. Thus, both 

$$[\text{Ca}^{2+}]_i$$, and protein expression of the critical JNK/Src in the treated cells were measured. The model cells treated with 300 μmol/L indomethacin only had much higher $$[\text{Ca}^{2+}]_i$$ (178.0% of the control) (Figure 6), compared with the control cells without any indomethacin and flavonol treatments. When pretreating the cells with these flavonols for 24 h, $$[\text{Ca}^{2+}]_i$$ values ranged from 127.7% (galangin) to 174.7% (heated kaempferol). Galangin was more effective than kaempferol to reduce $$[\text{Ca}^{2+}]_i$$, but the heated flavonols were weaker than the unheated counterparts to perform this activity. Meanwhile, the expression levels of these verified proteins (Figure 7A) in the cells showed an obvious response to the conducted indomethacin and flavonol treatments. Compared with the control cells without any indomethacin and flavonol treatments, the cells exposed to indomethacin (alone or with these flavonols) had a higher p-JNK/p-Src expression (Figure 7A). It was thus evident that indomethacin exposure led to increased ratios of p-JNK/JNK and p-Src/Src (0.65 and 0.68) in the model cells (Figure 7B), demonstrating a calcium-mediated JNK/Src activation. That is, the indomethacin-damaged cells had enhanced $$[\text{Ca}^{2+}]_i$$, which triggered JNK activation; after then, Src was activated by a JNK-dependent mechanism, which finally induced TJ destruction in the cells. However, a cell pretreatment with these flavonols yielded an antagonistic effect because this pretreatment led to decreased ratios of p-JNK/JNK (0.28–0.56) and p-Src/Src (0.31–0.61). These flavonols were thus regarded to have an ability to inhibit the calcium-mediated JNK/Src activation (or to antagonize the indomethacin-induced TJ destruction) and subsequently to
In conclusion, these flavonoids were more effective than the heated counterparts to perform this inhibitory effect. Activation, while the unheated galangin (GA) and kaempferol (KA) of 5 μmol/L for 24 h, followed by an indomethacin (IND, 300 μmol/L) exposure of 24 h. *p < 0.05, compared with the model group.

Figure 7. Western-blotting assay (A) and expression levels (B) of p-JNK/JNK and p-Scr/Scr in the IEC-6 cells treated with the heated and unheated galangin (GA) and kaempferol (KA) of 5 μmol/L for 24 h, followed by an indomethacin (IND, 300 μmol/L) exposure of 24 h. *p < 0.05, compared with the model group.

Figure 8. Schematic diagram describing a pathway for galangin and kaempferol to combat the indomethacin-induced barrier loss in IEC-6 cells.

Exert a protective effect on the barrier function of the cells (Figure 8). Consistently, galangin always was more powerful than kaempferol to inhibit the calcium-mediated JNK/Src activation, while the unheated flavonoids had higher potential than the heated counterparts to perform this inhibitory effect.

In conclusion, these flavonoids via inhibition of the calcium-mediated JNK/Src activation exerted a beneficial effect on IEC-6 cells to combat against the indomethacin-induced barrier loss, which thus reveals a health benefit of natural flavonoids in the digestive system.

**DISCUSSION**

It is a well-known fact that polyphenols including galangin and kaempferol can affect the physiological function of various cells; for example, three previous studies found that galangin could inhibit the activation of microglia in rats with Parkinson's disease induced by lipopolysaccharides and thus might protect nerve cells.32,33 While kaempferol could partly attenuate the barrier dysfunction of Caco-2 cells caused by inflammation.34 Thus, an in vitro study to reveal the effect of galangin and kaempferol to combat against the barrier loss of the indomethacin-damaged IEC-6 cells deserves our consideration because the normal barrier function of intestinal epithelial cells is vital to the body health. Indomethacin is a common nonsteroidal drug and has been verified to injure the cells by decreasing cell viability and increasing LDH release.35 This study again demonstrated that indomethacin at a concentration of 300 μmol/L could induce sufficient

Cytotoxicity on IEC-6 cells. The damaged cells thereby were measured with lower viability together with an enhanced LDH release due to the indomethacin-caused damage on the cell membrane. However, cell pretreatment with these assessed flavonoids yielded an increase in cell viability, together with a reduction in LDH release. This fact means that the flavonoids had the ability to alleviate indomethacin cytotoxicity to IEC-6 cells. In addition, it was evident that indomethacin had an ability to increase ROS production,36 while the increased ROS production would negatively impact cell homeostasis and function.37 Although ROS are the natural byproducts of normal cells, enhanced ROS production in the normal cells reflects undesired oxidative stress.38 This study found that both galangin and kaempferol could reduce ROS production in the injured IEC-6 cells, suggesting their ability to combat against indomethacin-induced oxidative stress. In total, this study highlighted a fact, that is, both galangin and kaempferol could exert a protective effect on IEC-6 cells against the indomethacin damage via decreasing LDH release and alleviating oxidative stress.

In general, both TEER and paracellular permeability are two classical indicators describing physical barrier integrity of the cells. Paracellular permeability is involved in the transport of water flow between epithelial cells and is regulated strictly by the intercellular complexes that are located at the apical-lateral membrane junctions along the lateral membrane.39 TJ, the most adhesive junction complex in mammalian epithelial cells, perform a function to form a selective and semipermeable paracellular barrier and can promote the passage of some ions and solutes through the intercellular space but at the same time prevent the translocation of antigens, microorganisms, and their toxins in an intestinal cavity. In general, occludin is mainly expressed in the TJs of both epithelial and endothelial cells, while occludin can interact with ZO-1 connected to the actin cytoskeleton, and thus plays a critical role in the regulation of cell paracellular permeability.39,40 Claudins are also important for the TJ interaction and formation of ion-selective channels41 because they can interact with ZO-1 that anchors claudins to the actin cytoskeleton. Scaffolding proteins then interact with signal molecules, associate TJ complex with the actin cytoskeleton, and regulate the epithelial barrier function.42 To be more specific, the expression levels of these TJ proteins occludin, claudin-1, and ZO-1 thus reflect cellular permeability directly.43 The present results also proved that a cell pretreatment of the flavonoids could combat against the...
indomethacin-caused barrier dysfunction in IEC-6 cells, as the three TJ proteins in the flavonol-treated cells had higher expression levels, compared with the model cells exposed to indomethacin only.

Regulation of the barrier function of cells involves various pathways; for example, the Ca^{2+}/Ask1/MK7/JNK2/CSRc signal cascades have been revealed to mediate the dextran sodium sulfate (DSS)-induced TJ disruption and barrier damage.\(^{28}\) It is also known that a calcium signal plays a role in the assembly and destruction of TJs.\(^{15}\) A previous study has confirmed that DSS in a JNK-dependent mechanism activated Src, resulting in barrier dysfunction.\(^{28}\) Another previous study also found that Src activation led to the destruction of epithelial TJs and barrier loss.\(^{44}\) It is thus reasonable for this study that indomethacin caused barrier loss by calcium-mediated JNK/Src activation because indomethacin showed an ability to enhance [Ca^{2+}], and the ratios of p-JNK/JNK and p-Src/Src. Thereby, the calcium-mediated JNK/Src pathway was involved in the barrier loss of the indomethacin-damaged IEC-6 cells. Pretreatment of the cells with the flavonoids led to decreased [Ca^{2+}]. Meanwhile, [Ca^{2+}], is critical to JNK activation because it had been found that the DSS-induced JNK activation was partially reduced by [Ca^{2+}], depletion.\(^{28}\) Pretreatment of the cells with these flavonoids led to reduced ratios of p-JNK/JNK and p-Src/Src, indicating that the flavonoids were capable of antagonizing the calcium-mediated JNK/Src activation. Overall, it was proposed that the flavonoids could alleviate the barrier loss of the cells via inhibiting the calcium-mediated JNK/Src activation. In addition, the JNK/Src pathway also had gained attention in the other studies,\(^{45,46}\) in which this pathway was suggested to mediate the mechanical stress-induced TJ loss in response to metabolic stress.

Heat treatment can affect the bioactivity of several food components; for example, the bacteriocidal capacity of whey was negatively correlated with the conducted thermal intensity.\(^{47,48}\) The previous results also showed that heat treatment might reduce polyphenol contents or their activity to scavenge free radicals in several foodstuffs.\(^{49,50}\) In this study, the heated flavonoids showed a weaker potential than the unheated counterparts to combat against the indomethacin-induced barrier loss in IEC-6 cells, which might be one result of flavonol degradation during heat treatment. It was evident that when flavonoids were heated at 100 °C for 30 min, their UV absorption showed that the value decreases.\(^{51}\) Moreover, heat treatment of quercetin led to the generation of these degraded compounds like 2,4,6-trihydroxyxymandelate and 2,4,6-trihydroxyphenylglyoxylate.\(^{52}\) It was thus reasonable that the performed heat treatment of galangin and kaempferol resulted in the formation of some unidentified substances and thus led to lower activity to alleviate indomethacin cytotoxicity and to combat against barrier loss. In addition, a previous study of our group also found that heat treatment of quercetin and myricetin reduced their barrier-promoting efficiencies in normal IEC-6 cells.\(^{26}\) In addition, why galangin and kaempferol in this study possessed different effects on the damaged cells might arise from their structural differences. Chemically, galangin and kaempferol are different in the B-ring (no −OH group versus one −OH group); subsequently, they might have different bioactivities in the cells. It had been proposed that the flavonoids with less nonmodification in the B-ring would have the greatest interactivity with the cell membrane.\(^{51}\) Galangin with no −OH group in the B-ring thus has lower polarity and could interact with the cell membrane much more efficiently than kaempferol with one −OH group in the B-ring. Thereby, galangin showed higher activity in the cells and then gave a higher barrier-protective effect once the cells were exposed to indomethacin.

**CONCLUSIONS**

In IEC-6 cells, the assessed NSAID, indomethacin, at 300 μmol/L caused obvious cytotoxicity and barrier loss. Pretreating the cells with two natural flavonols galangin and kaempferol (especially at a concentration of 5 μmol/L) could alleviate indomethacin toxicity, resulting in improved cell viability together with decreased LDH release or ROS production. Moreover, galangin and kaempferol also could combat against barrier dysfunction to increase TEER but reduce paracellular permeability, through enhancing relative mRNA and protein expressions of three TJ proteins, ZO-1, occludin, and claudin-1, and decreasing [Ca^{2+}], and the ratios of p-JNK/JNK and p-Src/Src. Thus, galangin and kaempferol were proposed to alleviate the indomethacin-caused barrier loss of IEC-6 cells via attenuating the calcium-mediated JNK/Src activation. Galangin with no −OH group in the B-ring showed higher activity than kaempferol with one −OH group in the B-ring, while heat treatment of the two flavonols might reduce their efficacy in the cells. This study thus highlights two potential benefits of natural flavonols to the intestine, that is, they could alleviate the indomethacin-caused toxic effect and combat against barrier loss. However, heat treatment of the flavonols reduces these benefits. Thus, the present study puts forward a reasonable suggestion for the processing of these flavonol-rich plant foods to employ a suitable heat treatment.

**MATERIALS AND METHODS**

**Materials and Chemicals.** Both galangin and kaempferol (purity values larger than 98%) were purchased from Shanghai Yousi Biotechnology Co., Ltd. (Shanghai, China). The Dulbecco’s modified Eagle’s medium (DMEM), dimethyl sulfoxide (DMSO), and 4 kDa fluorescein isothiocyanate (FITC)-dextran (FD-4) were obtained from Sigma-Aldrich Co., (St Louis, MO), while bovine serum (FBS) was bought from Wisent Inc. (Montreal, Quebec, Canada). The trypsin−EDTA and phosphate-buffered saline (PBS) were obtained from Beyotime Institute of Biotechnology (Shanghai, China) and Solarbio Science and Technology Co., Ltd. (Beijing, China), respectively. The Hanks’ balanced salt solution (HBSS), Fura-2/AM, ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), the ROS assay kit, and TritonX-100 were all obtained from Beyotime Institute of Biotechnology (Shanghai, China) and Solarbio Science and Technology Co., Ltd. (Beijing, China), respectively. The Hanks’ balanced salt solution (HBSS), Fura-2/AM, ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), the ROS assay kit, and TritonX-100 were all obtained from Beyotime Institute of Biotechnology (Shanghai, China). The cell counting kit-8 (CCK-8) and lactate dehydrogenase (LDH) assay kit (A0202-2) were bought from Dojin Molecular Technologies, Inc. (Kyushu, Japan) and Nanjing Jiancheng Biological Engineering Research Institute Co., Ltd. (Nanjing, Jiangsu, China), respectively. Other chemicals used in this study were analytical reagents. In cell experiments, ultrapure water generated with a Milli-Q Plus system (Millipore Corp., New York, NY) was used.

The primary antibodies (GAPDH ab181602, occludin ab216327, and claudin-1 ab15098) were all provided by Abcam plc. (Cambridge, U.K.), while Src and phospho-Src family (Tyr 416), c-Jun N-terminal kinase (JNK), and phospho-JNK (Thr 183/Tyr 185) were brought from Cell Signaling Technology, Inc. (Danvers, MA). The ZO-1 AF5145...
was purchased from Affinity Biosciences (Cincinnati, OH), while the goat antirabbit secondary antibody was obtained from Bioss Biotechnology Co., Ltd. (Beijing, China).

**Sample Preparation and Cell Culture.** In brief, each of galangin and kaempferol was dissolved in DMSO separately to reach a concentration of 40 mmol/L, and then divided into two parts. One part was diluted to 2.5–20 μmol/L directly with a medium before being applied on the cells, while the other part was heated in a water bath operated at 100 °C for 30 min, cooled, and then diluted to 2.5–20 μmol/L with the medium before being applied on the cells.

IEC-6 cells used in this study were bought from the American Type Culture Collection (Rockville, MD), which requires cell culture using the DMEM containing 10% fetal bovine serum, 1% sodium pyruvate, 0.1 units/mL bovine insulin, and 100 μg/mL penicillin/streptomycin. The cells were placed in a carbon dioxide incubator (Type HF 90, Healbovine serum, 1% sodium pyruvate, 0.1 units/mL bovine insulin, and 100 μg/mL penicillin/streptomycin. The cells were placed in a carbon dioxide incubator (Type HF 90, Heal Force, Hongkong, China) at 37 °C with a fixed CO₂ concentration of 5%.

**Assays of Cell Viability and Indomethacin Cytotoxicity.** A CCK-8 kit was used to detect the cytotoxic effect of indomethacin on IEC-6 cells. The cells inoculated in the 96-well plates (3 × 10³ cells/well) were cultured in a normal medium for 24 h, followed by a culture in a serum-free medium for another 12 h. After cell washing with PBS (10 mmol/L, pH 7.0), indomethacin (final concentrations of 200, 300, 400, or 500 μmol/L) in a normal medium was added to injure the cells for 24 h. The cells treated with the normal medium only were set as a negative control with a 100% viability value. The cells were washed with PBS twice and then determined for viability values using the kit. In brief, 100 μL of the medium containing 10 μL of a CCK-8 solution was added to each well. The cells were cultured for 1.5 h and measured for absorbance using a microplate reader (Bio-Rad Laboratories, Hercules, CA) at 450 nm. Cell viability was expressed as a percentage value of the control.

The flavonols (unheated or heated, final concentrations of 2.5, 5, 10, or 20 μmol/L) were used separately to treat the cells for 24 and 48 h, which were then washed with PBS and damaged with 300 μmol/L indomethacin for another 24 h. The cells treated with the normal medium only were used as a control with a 100% viability value, while those treated with indomethacin in a normal medium were set as a model. The cells were washed twice with PBS and measured for their viability values using the CCK-8 method as mentioned above. Cell viability also was expressed as the percentage value of the control.

**Measurements of LDH Release and Intracellular ROS.** As mentioned above, the cells inoculated in 96-well plates (3 × 10³ cells/well) were cultured in a normal medium for 24 h, treated with flavonols (2.5, 5, 10, or 20 μmol/L) for 24 and 48 h, washed with PBS, and then damaged by 300 μmol/L indomethacin in the normal medium for 24 h. LDH release in the resultant cell supernatants was measured using an LDH assay kit and the procedure provided by the kit producer. The cells treated by the normal medium only were used as a control with a fixed LDH release value of 100%. The results were expressed as the percentages of the control.

The cells inoculated on the 6-well plate were treated with unheated and heated flavonols (5 μmol/L) for 24 h, washed with PBS, and then damaged with 300 μmol/L indomethacin in a normal medium for 24 h. The ROS was detected according to the kit instructions. In brief, 1 mL 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe (final concentration of 10 μmol/L) in serum-free medium was added to each well, while the cells were incubated at 37 °C for 30 min. The cells with the medium only were used as a control. After the cells were washed with PBS three times, they were resuspended in a serum-free medium and detected with a fluorescent microplate reader with excitation/emission wavelengths of 500/525 nm. The results were expressed as the percentage of intracellular fluorescence in the treated cells compared with the control.

**Assays of Transepithelial Electrical Resistance and Paracellular Permeability.** The cells were inoculated into the transwell inserts (Corning, Kennebunk, ME) that have a 0.4 μm membrane pore size and a 1.12 cm² growth area. A total of 0.5 mL of a cell suspension (5 × 10³ cells/mL) and 1.5 mL of a normal medium were added to the apical and basolateral compartments, respectively. When the cells adhered to the insert walls, the culture medium was changed every other day until the TEER values reached 50 Ω cm². After adding a serum-free medium, the cells were cultured for 12 h, treated with flavonoids (5 μmol/L) for 24 and 48 h, and finally treated with a normal medium containing 300 μmol/L indomethacin for 24 h. TEER values were determined using a Millicell-ERS2 Volt-Ohm Meter (Millipore, Bedford, MA). The TEER value of the cells treated with the normal medium only (i.e., control cells) was regarded as 100%.

The cells were inoculated into the transwell inserts, cultured until the TEER reached 50 Ω cm², treated with flavonoids (5 μmol/L) for 24 and 48 h, and finally damaged with 300 μmol/L indomethacin in a normal medium for 24 h. The fluorescence density of the basolateral aliquot was detected using a fluorescent microplate reader (Infinite M200 pro, TECAN, Männedorf, Switzerland) after the FD-4 of 0.5 mg/L was added to the apical compartment for 24 h. The used excitation/emission wavelengths were 490/520 nm. The value of paracellular permeability was expressed as the percentage of the FD-4 fluorescence flux. The cells treated with the normal medium only (control cells) were used as a control with an FD-4 value of 100%.

**[Ca²⁺]**, Measurement. [Ca²⁺], was evaluated as previously described. The cells inoculated in 6-well plates (1 × 10⁶ cells/well) were treated successively by a serum-free medium for 12 h, flavonoids (5 μmol/L) for 24 h, PBS washing, 300 μmol/L indomethacin in a normal medium for 24 h, and then 1 mL of a Fura-2/AM working solution (0.5 μmol/L) for 0.5 h. They were then washed with HBSS (1 mol/L, pH 7.3), adjusted to 2 × 10⁶ cells/mL, and detected using a fluorescent microplate reader at excitation wavelengths of 340–380 nm and an emission wavelength of 510 nm. The values of R₉ (F340/F380) and R₉ (F340/F380) were detected by adding 0.1% Triton X-100 (v/v) for 0.5 h and adding EGTA (5 mmol, pH 8.5) for 0.5 h, respectively. The cells treated with a fresh medium were set as a control with a designed [Ca²⁺] value of 100%.

**RT-qPCR Assay.** The cells inoculated in a Petri dish (21 cm²) were treated with flavonoids (5 μmol/L) for 24 h, washed with PBS, and then exposed to 300 μmol/L indomethacin in a normal medium for another 24 h. This RT-qPCR assay was divided into three brief steps: (1) extraction of total RNA using the RNAPrep pure cell/bacterial kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China), (2) reverse transcription of the RNA into complementary DNA using the PrimeScript TMRT Reagent Kit (Takara Bio Ltd., Kusatsu,
Western-Blotting Assay. This assay was done as previously described. In detail, the cells inoculated in 21 cm² Petri dishes were treated with flavonols (5 μmol/L) for 24 h followed by PBS washing and an exposure of 300 μmol/L indomethacin in a normal medium for 24 h. The cells were then digested with trypsin, while PBS (0.01 mol/L, pH 7.2−7.3) at 4°C was used to wash and cool the cells. Afterward, the cells were centrifuged (300g) at 4°C for 5 min. The supernatant was discarded, while cells were collected into centrifuge tubes of 1 mL. The lysate (300 μL) containing PMSF (0.1 mol/L) was added to treat the cells on ice for 0.5 h. After centrifugation (12 000 g) at 4°C for 5 min, the separated supernatants were determined for protein contents using the BCA kit (Beyotime Institute of Biotechnology, Shanghai, China), aiming to dilute them with PBS to reach a protein content of 1 mg/mL. Appropriate gels were used to separate proteins, while 50 μg of denatured proteins was added to each well. The gels were transferred to the poly(vinylidene fluoride) (PVDF) membranes after finishing the electrophoresis. The PVDF was blocked with skimmed milk in PBS of 5% including 0.1% Tween-20 (PBST) for 2 h at 20°C. The primary antibody (1:1000 dilution) was added overnight at 4°C. The second antibody (1:1500 dilution) was added, incubated at 20°C for 2 h, and then washed with PBST three times. Using a chemiluminescent HRP substrate (P90719, Millipore, Bedford, MA), a chemiluminescence imaging system (Bio-Rad Laboratories, Hercules, CA), and Image Lab software (Bio-Rad Laboratories, Hercules, CA), quantitative analysis of the proteins was performed. GAPDH was used as an endogenous standard to normalize band density.

Statistical Analyses. The data collected from at least three independent assays were reported as means or means ± standard deviations. One-way analysis of variance (ANOVA) was applied to determine the significance between the groups (p < 0.05) using the Social Science Statistical Program 16.0 software package (SPSS Inc., Chicago, IL) and the Duncan multiple comparison test.

Table 2. The Designed Primer Sequences Used In The Real-time PCR Assays

| gene    | species | primer (5’-3’)                  |
|---------|---------|---------------------------------|
| ZO-1    | rat     | FORWARD CCACCTGCGACGTATCCACAAGC  |
|         |         | REVERSE GGCATGGACACTCCCTGCTCTG   |
| occludin| rat     | FORWARD CTTCTTACAGGCGGGATGA      |
|         |         | REVERSE AGCATGTTGCGAAGCTGATC     |
| claudin-1| rat    | FORWARD GGGCAATGACACTCCCTGCTCTG  |
|         |         | REVERSE GTCCTTACGATGGTGAGATC     |
| GAPDH   | rat     | FORWARD CCCTCTGGAAAGCTGTGG       |
|         |         | REVERSE CACGACCTGCGATCGGTG       |

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