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Article

Egg White Protein Ovotransferrin-Derived IRW (Ile-Arg-Trp) Inhibits LPS-Induced Barrier Integrity Dysfunction and Inflammation in Caco-2 Cells

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ABSTRACT: Tripeptide IRW derived from egg ovotransferrin was initially identified to be an inhibitor of angiotensin-converting enzyme. Later, IRW has been shown to possess various bioactivities, including anti-inflammatory activity and the ability to suppress colitis development. Nevertheless, its role in protecting intestinal barrier integrity has not been reported. This study aims to investigate the effect of IRW on inhibiting intestinal barrier dysfunction and inflammation in lipopolysaccharide (LPS)-treated Caco-2 cells. Pretreatment with IRW could mitigate the LPS-induced reduction of transepithelial electronic resistance values and decrease the paracellular permeation of differentiated Caco-2 cell monolayers. Meanwhile, IRW restored the expression level and cell surface distribution of the tight junction protein occludin. Furthermore, IRW showed LPS-neutralizing activity and could significantly inhibit LPS-induced activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. In conclusion, our study demonstrated the ability of IRW to prevent LPS-induced intestinal barrier dysfunction and prohibit inflammatory responses.

KEYWORDS: *IRW, intestinal barrier integrity, Caco-2 cells, LPS, tight junction, inflammation*

INTRODUCTION

Intestinal barrier integrity plays a pivotal role in maintaining intestinal function and holistic health. Defects of intestinal barrier integrity, such as increased intestinal permeability, are associated with various diseases,[1] including inflammatory bowel disease,[2] celiac disease,[3] irritable bowel syndrome,[4] metabolic syndrome,[5] and central nervous system disorders.[6] Interventions targeting impaired intestinal barrier function[7,8] are a promising approach to preventing colitis pathogenesis[9] and controlling the onset of arthritis.[10] For example, the probiotic *Akkermansia muciniphila*, indirectly correlated with hepatic disease severity, can promote intestinal barrier integrity and ameliorate experimental alcoholic liver disease.[11] Food components such as polyphenols[12] and milk proteins[13] can improve intestinal integrity and may have clinical efficacy in disorders characterized by intestinal barrier dysfunction. In addition, there is a growing interest in investigating the effects of food-derived bioactive peptides on intestinal barrier function and their potential applications in disease management.[14,15]

Bioactive peptides are mostly composed of 2−20 amino acid residues and can exert a wide range of bioactivities once released from their parent proteins. The tripeptide IRW was initially characterized from egg white ovotransferrin as a novel angiotensin-converting enzyme inhibitory peptide.[16] Later studies demonstrated its anti-inflammatory activity.[17−19] This peptide was also shown to attenuate the tumor necrosis factor (TNF)-induced endothelial inflammatory response and oxidative stress by inhibiting the activation of the nuclear factor-κB (NF-κB) pathway.[20] It also improved insulin resistance in skeletal muscle cells by inhibiting TNF-α-induced activation of mitogen-activated protein kinase (MAPK) p38 and c-Jun N-terminal kinases (JNK) 1/2 molecules.[21] Its regulation on these two pathways was also found in murine monocytes RAW 264.7,[22] vascular smooth muscle cells,[23,24] and spontaneously hypertensive rats,[25] supporting its great potential to suppress inflammation and prevent chronic diseases. Additionally, IRW was also shown to maintain the intestinal histomorphology, improve the activities of antioxidative enzymes, modulate intestinal immune defense by decreasing the expressions of inflammatory cytokines, and increase gut microbial abundance and diversity in experimental mouse models of pathogenic infection or dextran sodium sulfate-induced colitis,[26−29] indicating its colitis-alleviating potential. However, its role in protecting intestinal barrier integrity by modulating tight junction proteins and inflammation has not been studied.

Tight junction proteins, including claudins, occludin, and zonula occludens (ZOs), are responsible for constituting effective barrier structures and supporting the selective transport of molecules across the epithelial barrier.[30] Altered expression and organization of tight junction proteins have been observed in the pathogenesis of gastrointestinal disorders.[31−33] Under normal circumstances, the transmembrane tight junction

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components like claudins and occludin interact with cytosolic scaffolding proteins like ZO-1, which directly anchor the transmembrane proteins to the actin cytoskeleton. This allows the cytoskeletal regulation of the tight junction complex and barrier integrity. Both NF-κB and MAPK signaling pathways are predominantly involved in the inflammatory and immune responses induced by the exposure of immune cells, stromal cells, and epithelial cells to the ligands of the pattern-recognition receptors.[34] Activation of the key molecules in these two pathways has been demonstrated to play an important role in increasing intestinal tight junction permeability.[35−37] Lipopolysaccharide (LPS), also known as endotoxin, is a key component of the Gram-negative bacterial cell wall that can trigger inflammatory responses and pathogenic infection through activating downstream signaling cascades, including NF-κB and MAPK pathways.[34] In this study, we investigated the anti-inflammatory and intestinal barrier-benefiting properties of IRW in LPS-treated Caco-2 cells.

MATERIALS AND METHODS

Reagents and Antibodies. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (penicillin−streptomycin, 10,000 U/mL), trypsin-EDTA solution (0.25%, phenol red), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and nonessential amino acids (NAA) were purchased from Thermo Fisher Scientific (Burlington, ON, Canada). The tracers FITC-dextran and Lucifer yellow, Triton X 100, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), glass coverslips, lipopolysaccharides from *Escherichia coli* O111:B4, the goat antirabbit CF 594 antibody, the goat antimouse CF 488A antibody, and 24-well transwell polyester membrane cell culture inserts (0.4 μm pore size, 6.5 mm diameter, 0.33 cm^2 grown surface, Costar, Corning, NY) were obtained from Sigma-Aldrich (St. Louis, MO). Rabbit monoclonal primary antibodies against claudin-3, occludin, ZO-1, phospho-NF-κB p65 (Ser 536), phospho-ERK1/2 (Thr 202/Tyr 204), p38 MAPK, JNK, GAPDH, mouse monoclonal primary antibodies against NF-κB p65, ERK1/2, phospho-p38 (Thr180/Tyr182), and phospho-JNK (Thr 183/Tyr 185) were purchased from Cell Signaling Technology (Whitby, ON, Canada). The donkey antimouse 800CW secondary antibody and the goat antirabbit IRDye 680RD secondary antibody were purchased from LI-COR Biosciences (Lincoln, NE).

Cell Culture and Treatment. Caco-2 cells (32-45 passage) were grown in DMEM with 10% FBS, 1% antibiotics, 25 mM HEPES, and 1% NEAA under a 5% CO_2 atmosphere at 37 °C. The culture medium was replaced every other day, and cells were cultured for 21 days to undergo differentiation. Differentiated Caco-2 cells were incubated with IRW (50 and 100 μM) for 24 h, followed by LPS treatment. The concentration of IRW used in the study was based on previous studies.[21,38,39] Then, parameters were detected and samples were collected for the following analyses.

Transepithelial Electrical Resistance (TEER) Value Detection and the Permeability Tracer Flux Assay. Cells seeded on permeable filters were cultured for differentiation with TEER values ≥400 Ω·cm^2. After treatment, TEER values were measured using an ohmmeter (World Precision Instruments, Sarasota, FL). Data were expressed as the percentage of the reduction of the initial values. FITC-dextran (4 and 40 kDa) and Lucifer yellow at the concentration of 100 μg/mL in PBS were added to the upper chambers of the transwell plates for 4 h, and solutions were collected from the basolateral compartments. The fluorescence density was detected using a microplate reader (SpectraMax, Molecular Devices, CA). The excitation and emission wavelengths for FITC-dextran are 490 and 520 nm, respectively, and 410 and 520 nm for Lucifer yellow.

Western Blot Analysis. Cell lysates were collected by hot Laemmli's buffer with 2% DTT and applied to SDS-PAGE. Then, the gels were transferred to nitrocellulose membranes, which were then blocked in 5% skim milk powder in PBS for 1 h on a shaker at room temperature (RT). The membranes were subsequently incubated with different antibodies at 4 °C on a low shaker overnight, followed by TPBS washing three times (5 min/time) and incubation with secondary antibodies for 1 h on a shaker at RT. The bands were detected with an LI-COR Odyssey Bioimager and analyzed by Image Studio Lite 5.2 (LI-COR Biosciences). GAPDH was used as a reference. All data are presented as the percentage of control groups.

Immunofluorescent Staining. After treatment, cells grown on coverslips were washed with ice-cold PBS, followed by fixation with formalin solution for 10 min at 4 °C and permeation with 0.1% Triton X 100 in PBS for 10 min. Then, the cells were incubated with blocking buffer (5% BSA in PBS) for 1 h on a shaker at RT and subsequently primary antibody against occludin or NF-κB p65 overnight on a slow shaker at 4 °C. After washing with TPBS (0.1% tween 20 in PBS) 3 times (5 min/time), cells were incubated with the goat antirabbit CF 594 antibody or the goat antimouse CF 488A antibody for 1 h at RT. Finally, the coverslips were mounted in 50% glycerol in ddH_2O, and images were captured by a confocal laser scanning microscope (Olympus FV3000).

Endotoxin-Neutralizing Activity (LAL Assay). The quantitative chromogenic Limulus amebocyte lysate (LAL) assay kit (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher, Canada) was used to evaluate the endotoxin-neutralizing activity of IRW according to the manufacturer's instructions. IRW at different concentrations was incubated with LPS [0.5 endotoxin units (EU)/mL] in microtubes at 37 °C for 30 min to allow binding. Then, 50 μL of standard (0.1−1.0 EU/mL) and sample mixtures were added to the prewarmed microplate, followed by incubation with an equal volume of LAL reagent containing a chromogenic substrate [butyloxycarbonyl-(Boc)-LeuGly-Arg-p-nitroanilide] from the amebocytes of the horseshoe crab Limulus polyphemus at 37 °C for 10 min. After 6-min incubation with 100 μL of the prewarmed chromogenic substrate solution, 100 μL of stop solution (25% acetic acid) was dispensed into all wells before measuring the absorbance at 405 nm by a microplate reader (SpectraMax M3, Molecular Devices, CA). Endotoxin-free water was used as a reference control, and the absorbance of the reaction mixture solely containing 0.5 EU/mL of LPS was represented as 100%. The relative absorbance of reaction mixtures with IRW was calculated as the ratio of their absorbance to that of the corresponding reaction mixtures without IRW.[40]

Statistical Analysis. Data are represented as mean ± standard error of the mean of 4−6 independent experiments and analyzed using software PRISM 6 (GraphPad Software, San Diego, CA) with one-way analysis of variance and Bonferroni post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Transepithelial Electronic Resistance (TEER) Values. The TEER values of the Caco-2 cell monolayers were significantly decreased by the LPS (20 μg/mL) challenge compared with those in the control group (Figure 1). Adding peptide alone also reduced the TEER values, which might be due to the increased transport of IRW across the cell monolayer.[41] Pretreatment with IRW (50 and 100 μM) for 24 h could effectively prevent the LPS-induced reduction of TEER values.

Permeation of FITC-Dextran and Lucifer Yellow. The paracellular passage of fluorescent tracers across Caco-2 cell monolayers was performed to evaluate the barrier permeability. Treatment with LPS for 2 h significantly increased the permeation of FITC-dextran (4 and 40 kDa) and Lucifer yellow across the cell monolayers (Figure 2). Pretreatment with IRW significantly inhibited the LPS-elevated passage of FITC-dextran (40 kDa) (Figure 2B) and Lucifer yellow (Figure 2C). Thus, IRW showed the potential to protect intestinal barrier integrity from LPS treatment. Adding peptide alone did not affect the barrier permeability.

Expression Levels of Tight Junction Proteins. The abundance of tight junction proteins was determined by

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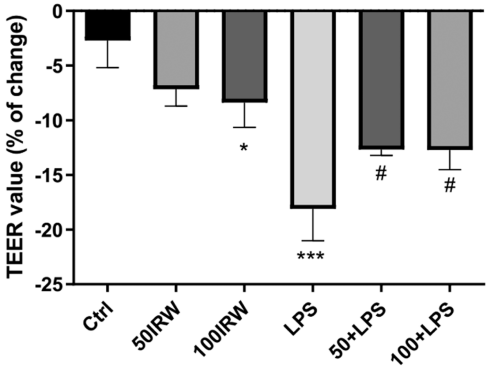

Figure 1. Effect of IRW on transepithelial electronic resistance (TEER) values. Cells on transwell plates were pretreated with IRW for 24 h, followed by LPS treatment for 2 h. TEER values were measured using a voltmeter (Millicell-ERS; Millipore, MA). Data are representative of the reduction percentage of the initial values from four independent experiments and expressed as mean ± SEM. Ctrl means the control group. 50 and 100 IRW refer to the treatments with 50 and 100 μM IRW, respectively. 50 + LPS and 100 + LPS refer to the treatments with IRW (50 and 100 μM, respectively), followed by 20 μg/mL LPS. *P < 0.05, ***P < 0.001, significantly different from the control group. #P < 0.05, significantly different from the LPS group.

Western blot to study the effect of IRW on the LPS-impaired barrier structure of Caco-2 cell monolayers. Treatment with LPS (20 μg/mL) for 1 h significantly decreased the expression of occludin (Figure 3A1) but did not change the levels of claudin-3 and ZO-1 (Figure 3A2,A3). At the concentration of 100 μg/mL for 6 h, LPS significantly decreased their expression levels (Figure 3B1−B3). Pretreatment with LPS could restore the expression level of occludin, claudin-3, and ZO-1 (Figure 3A1,B2−B3).

Distribution of Occludin. The cell surface distribution of occludin was studied by immunofluorescence staining. In control and IRW groups, occludin assembled as a clear and organized network between adjacent cells (Figure 4). Treatment with LPS decreased the fluorescence intensity and disrupted the integrity of the network; however, IRW pretreatment could prevent the LPS-induced deterioration of the network.

MAPK and NF-κB Signaling Pathways. As shown in Figure 5, LPS treatment significantly upregulated the phosphorylation of p38, ERK1/2, JNK, and NF-κB p65. The translocation of NF-κB p65 from the cytoplasm to cell nuclei was also enhanced by LPS stress (Figure 5E), while IRW pretreatment could suppress NF-κB p65 translocation. These data indicated that activation of MAPK and NF-κB signaling pathways are involved in LPS-induced inflammation and intestinal barrier

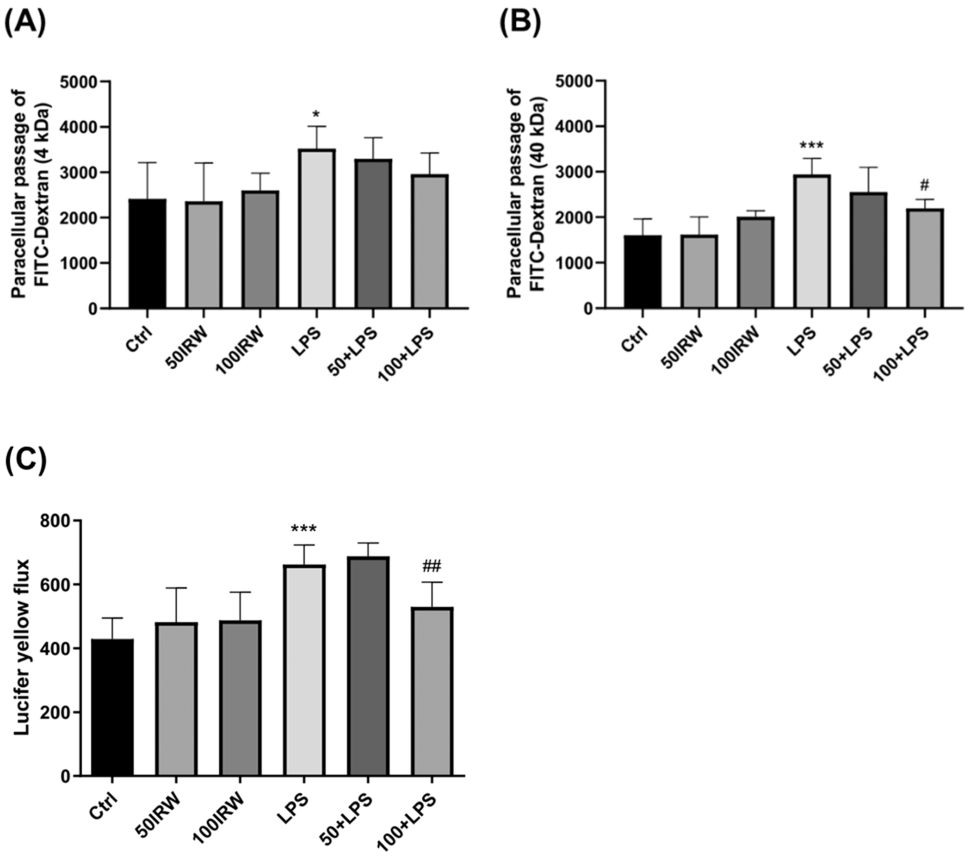

Figure 2. Effect of IRW on paracellular tracer flux across Caco-2 cell monolayers. Cells were incubated with IRW (50 and 100 μM) for 24 h and then exposed to LPS (20 μg/mL) for 2 h. FITC-dextran 4 kDa (A) and 40 kDa (B) and Lucifer yellow (C) in PBS were added to the apical chamber, and solutions were collected from the basal chamber after 4 h of incubation for fluorescence detection by a microplate reader (SpectraMax M3, Molecular Devices, CA). The excitation and emission wavelengths for FITC-dextran are 490 and 520 nm, respectively, and 410 and 520 nm for Lucifer yellow. The results are representative of four independent experiments expressed as mean ± SEM. Ctrl means the control group. 50 and 100 IRW refer to the treatments with 50 and 100 μM IRW, respectively. 50 + LPS and 100 + LPS refer to the treatments with IRW (50 and 100 μM, respectively), followed by 20 μg/mL LPS. *P < 0.05, ***P < 0.001, significantly different from the control group. #P < 0.05, ##P < 0.01, significantly different from the LPS group.

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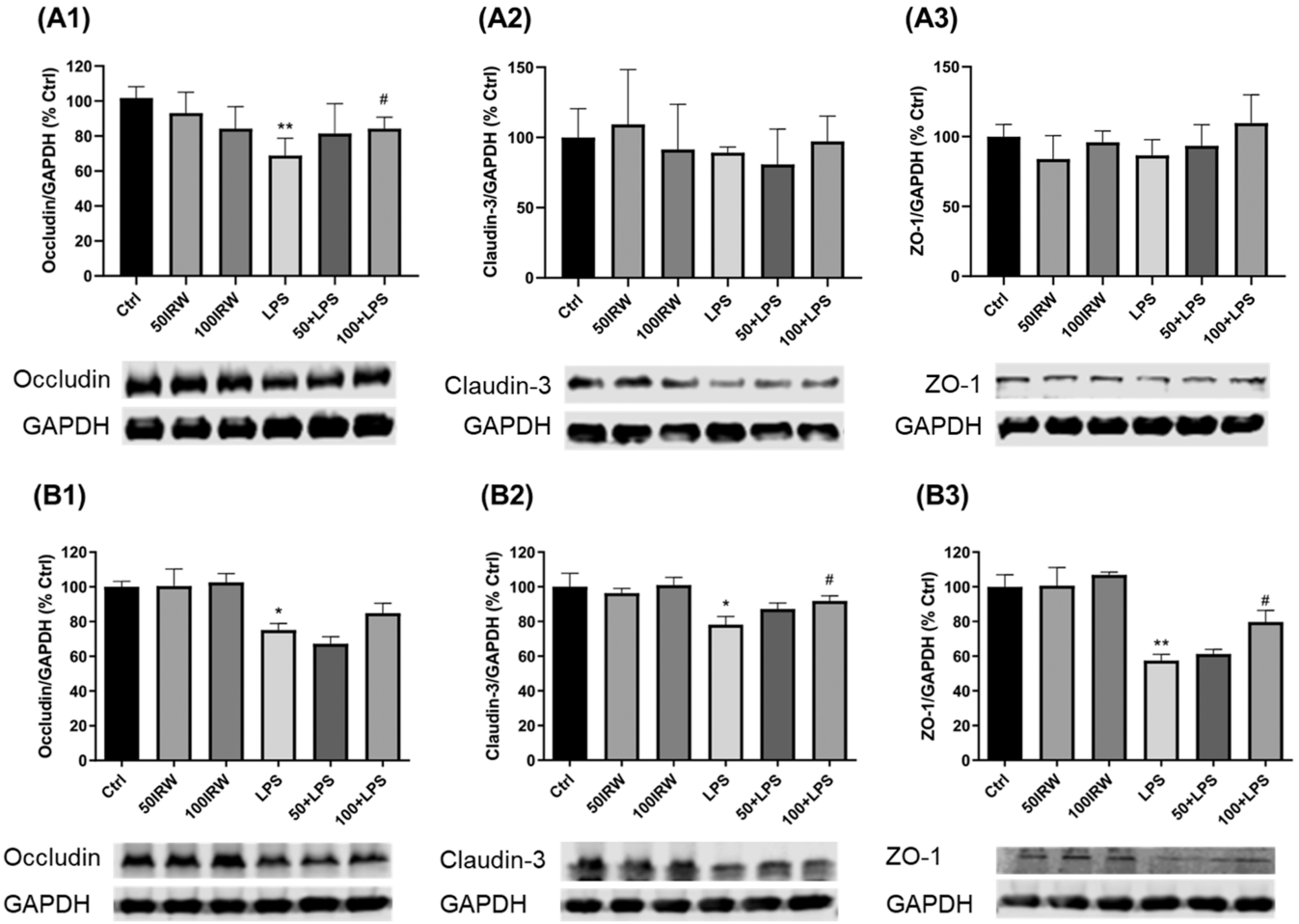

Figure 3. Effect of IRW on tight junction protein expression levels. Differentiated Caco-2 cells were treated with IRW for 24 h and then stimulated with LPS (20 μg/mL) for 1 h (A1−A3) or LPS (100 μg/mL) for 6 h (B1−B3). Cell lysates were applied for Western blot analysis of occludin (A1, B1), claudin-3 (A2, B2), and ZO-1 (A3, B3). Band density quantitation was performed by Image Studio software (LI-COR Biosciences). Data were expressed as mean ± SEM of four independent experiments. Ctrl means the control group. 50 and 100 IRW refer to the treatments with 50 and 100 μM IRW, respectively. 50 + LPS and 100 + LPS refer to the treatments with IRW (50 μM and 100 μM, respectively), followed by 20 μg/mL LPS. *$P < 0.05$, **$P < 0.01$, significantly different from the control group. #$P < 0.05$, significantly different from the LPS group.

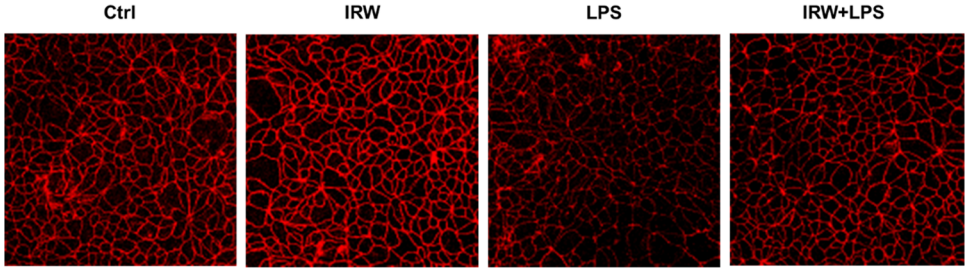

Figure 4. Effect of IRW on tight junction protein distribution on cell membrane surfaces. Cells were grown on coverslips for 21 days to allow differentiation and incubated with IRW (100 μM) for 24 h prior to LPS (20 μg/mL) treatment for 1 h. After treatment, cells were fixed with 4% paraformaldehyde, permeabilized with Triton X 100, and stained with the primary anti-occludin antibody at 4 °C on a slow shaker overnight. The antirabbit CF 594 antibody (Sigma) was used to visualize the localization of occludin by a confocal laser scanning microscope (Olympus FV3000). Images were captured under the 10× objective lens. Ctrl means the control group.

dysfunction. Pretreatment with IRW could significantly inhibit LPS-activated MAPK and NF-κB pathways.

Endotoxin-Neutralizing Activity. The tripeptide IRW at 50, 100, and 200 μM showed a 10−15% reduction of endotoxic activity, as shown in Figure 6. This indicates that IRW exhibited LPS-neutralizing activity.

DISCUSSION

The tripeptide IRW has been reported with multiple bioactivities, including anti-inflammatory, vasodilatory, and insulin-sensitizing properties.[42] In Caco-2 cells, IRW could decrease H_2O_2-induced reactive oxygen species generation and apoptosis and restore cell viability.[38] It also alleviated dextran sodium sulfate-induced colitis and pathogen infection by

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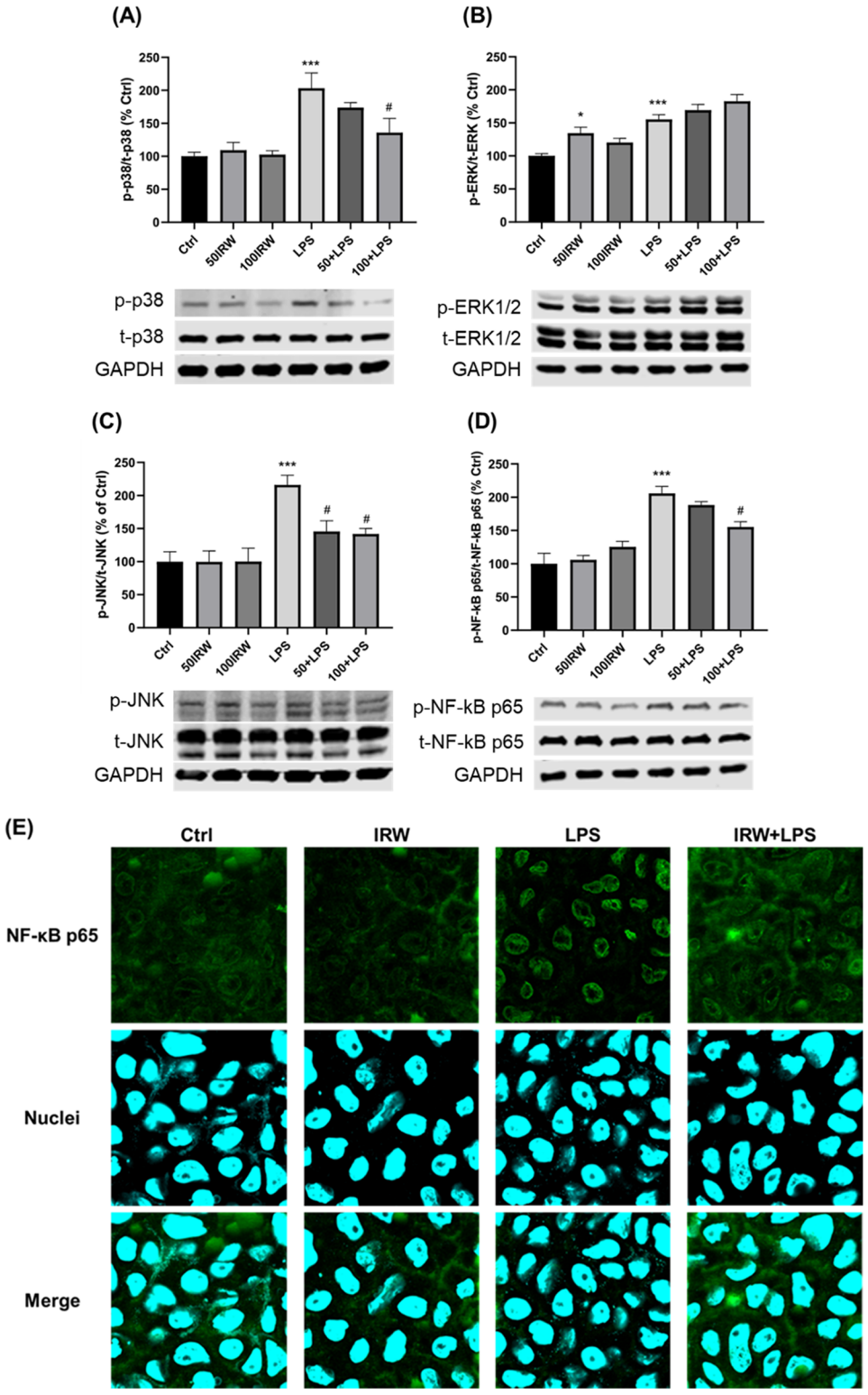

Figure 5. Effect of IRW on expression levels of molecules in MAPK (A–C) and NF-κB (D-E) signaling pathways. Caco-2 cells were treated with IRW (50 or 100 μM) for 24 h, followed by the LPS (20 μg/mL) challenge for 1 h. Whole-cell lysates were used for Western blot analysis of p-p38 (Thr180/Tyr182)/p38 (A), p-ERK1/2 (Thr 202/Tyr 204)/ERK1/2 (B), p-JNK (Thr 183/Tyr 185)/JNK (C), and p-NF-κB p65 (Ser 536)/NF-κB p65 (D). Ctrl means the control group. 50 and 100 IRW refer to the treatments with 50 and 100 μM IRW, respectively. 50 + LPS and 100 + LPS refer to the treatments with IRW (50 and 100 μM, respectively), followed by 20 μg/mL LPS. The data are expressed as mean ± SEM of 4–6 independent experiments. *$P < 0.05$, ***$P < 0.001$, significantly different from the control group. #$P < 0.05$, significantly different from the LPS group. (E) Fixed and permeabilized cells were immunostained for NF-κB p65 translocation to nuclei by the goat antimouse CF 488A antibody. Cell nuclei were stained with DAPI at RT for 10 min. Images were captured by a confocal laser scanning microscope (Olympus FV3000) under a 60× oil lens. IRW+LPS means 100 μM IRW pretreatment for 24 h, followed by the LPS (20 μg/mL) challenge for 1 h.

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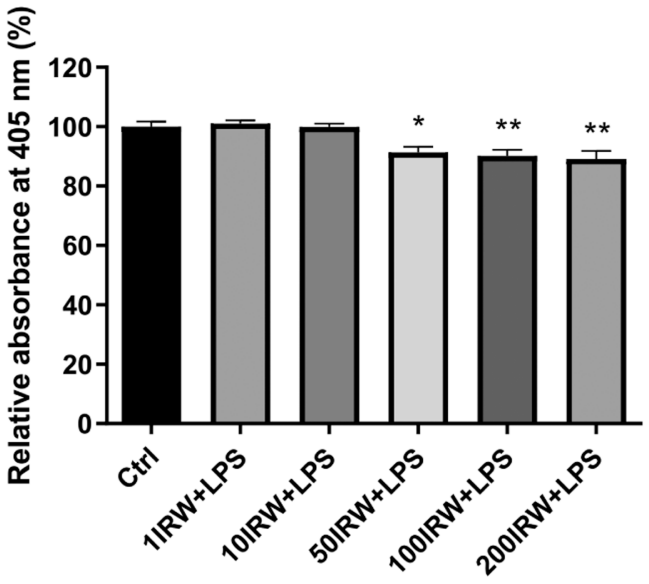

Figure 6. Endotoxin-neutralizing activity of IRW. IRW at different concentrations (1−200 μM) was incubated with LPS (0.5 EU/mL) at 37 °C for 30 min and then applied for the following procedures. After the reaction, absorbance was measured at 405 nm. A_{405} in 0.5 EU/mL of the LPS reaction mixture without IRW was referenced as 100%. The relative absorbance was expressed as the percentage of A_{405} of the reaction mixtures with the peptide to that of the reaction mixture without the peptide. The data are expressed as mean ± SEM of six independent experiments. Ctrl means the control group. *P < 0.05, **P < 0.01, significantly different from the control group.

regulating host metabolism, enhancing intestinal immune defense, mitigating oxidative stress, and modulating gut microbial diversity and abundance.[26−29] These studies suggested the ability of IRW to improve intestinal health and its potential application in treating intestinal disorders. The Caco-2 cell line from a human adenocarcinoma is widely used to investigate barrier function after achieving differentiation. Here, we used differentiated Caco-2 cells to study the effect of IRW on intestinal barrier function, aiming to illustrate the mechanisms of IRW toward improving intestinal homeostasis. The major findings of this study are that IRW can restore barrier permeability and tight junction expression and organization and mitigate LPS-induced inflammation by partially neutralizing LPS and suppressing the activation of NF-κB and MAPK signaling pathways.

Tight junction proteins are essential for constructing the barrier architecture between adjacent intestinal epithelial cells, and they function to regulate the paracellular diffusion of ions and solutes.[43] Downregulated synthesis or increased disassembly of claudin-3, occludin, and ZO-1 was closely correlated with impaired intestinal barrier properties and intestinal inflammation in gastrointestinal disorders.[44,45] In the present study, we found that LPS (20 μg/mL for 1 h) significantly reduced the expression level and organization of occludin but did not change the expression of claudin-3 and ZO-1, while at higher concentration (100 μg/mL) and longer treatment (6 h), LPS significantly decreased the expression levels of occludin, claudins-3, and ZO-1 (Figure 3). This is consistent with our previous study that LPS exposure at a relatively shorter period of time might not exert a dramatic influence on their abundance and assembly.[46] In this study, IRW could mitigate the breakdown of tight junction proteins and maintain the functional property of the Caco-2 cell monolayer. Similarly, casein-derived peptide NPWDQ could upregulate the expression of occludin and enforce the tight junction barrier.[47,48] Bovine lactoferrin could increase the tight junction expression and strengthen intestinal barrier function in Caco-2 cells and suckling piglets.[49,50] And porcine lactoferrin-derived peptide LFP-20, a 20 amino acid antimicrobial peptide at the N terminus with a sequence of KCRQWQSKIRRTNPIFCIRR, could increase the transepithelial resistance and tight junction protein levels, modulate the intestinal inflammatory response, and maintain functional intestinal structures in LPS-stimulated porcine intestinal epithelial cells and mice.[51] Other food-derived bioactive peptides regulating intestinal tight junction permeability are summarized in our review article, with introduction of some food protein hydrolysates that need further identification and exploration of responsible peptides.[14] Bioactive peptides were considered to function as trophic factors to facilitate the intestinal barrier reconstruction and energize tight junctions via increased protein synthesis, improving intestinal permeability and symptom scores of IBD patients.[52] Thus, this might be one of the crucial mechanisms of IRW benefiting intestinal barrier function. In addition, a transport study showed that IRW was partially hydrolyzed after apical uptake in Caco-2 cells.[41] Amino acids can also energize the turnover of tight junctions. For example, arginine and tryptophan were reported to promote intestinal barrier function and tight junction expression via inhibiting NF-κB and MAPK pathways and enhancing intracellular protein turnover by activating the mammalian target of rapamycin complex 1 (mTORC1), a Ser/Thr protein kinase that mediates cell growth, protein synthesis, and proteolysis.[53−56] Besides, individual IRW treatment (100 μM) decreased the TEER values and slightly increased the paracellular flux of FITC-dextran 40 kDa, which may be induced by the increased transport of IRW through passive absorption.[41] With an advanced understanding of the architecture of tight junction proteins and their functions, it is well recognized that various stimuli can regulate their production and assembly and that they can mediate multidirectional signaling transmission from and to the matrix, cytoskeleton, and nucleus.[43] These findings provide a potential explanation for peptide IRW effectively restoring the expressions of these tight junction proteins and attenuating LPS-elevated permeability, but further research is warranted.

Stimulation of LPS is transmitted to its receptor toll-like receptor 4. Then, effectors are recruited to the cytosolic domain of the receptor, which will cause downstream cascade reactions, including the activation of NF-κB and MAPK pathways. Phosphorylated NF-κB p65 will be translocated into the nuclei to initiate the expression of proinflammatory cytokines. The MAPK signaling modules containing different elements, including the extracellular signal-regulated kinases 1/2 (ERK1/2), p38, and c-Jun N-terminal kinases (JNK) 1/2, function with NF-κB in response to stresses and inflammatory stimuli.[34] Activation of these pathways also increases the expression and activity of myosin light chain kinase, a crucial molecule that regulates the interaction of tight junctions with the cytoskeleton, thus disrupting intestinal barrier integrity.[57,58] This study showed that IRW could prevent the LPS-induced phosphorylation of NF-κB p65 and inhibit its translocation into the nuclei. Also, IRW inhibited the activation of JNK1/2 and MAPK p38. These data proved the anti-inflammatory ability of IRW and potentiated its further investigation in benefiting intestinal barrier function. ERK1/2 molecules play a vital role in mediating cell survival, proliferation, mitigation, cytoskeleton

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remodeling, and transcriptional processes following the extracellular stimulation of cells.[59] Interestingly, IRW at 50 μM was shown to increase the phosphorylation of ERK1/2 (Figure 5B), indicating its ability to modulate cellular biological processes; however, a dose-dependent response did not exist, which may be due to a temporal difference for the characteristic phosphorylation time of a protein kinase, indicating that IRW at different dosages may upregulate the phosphorylation of ERK1/2 at different time points.[60] In this study, IRW could mitigate the breakdown of tight junction proteins and maintain the functional property of the Caco-2 cell monolayer. Taken together, further research is needed to understand the detailed molecular mechanisms of IRW modulating proinflammatory pathways and the tight junction assembly.

To understand why IRW could reverse the LPS-induced impairment, the LAL assay was conducted to test its endotoxin-neutralizing ability. At 50, 100, and 200 μM, IRW showed a reduction of LPS toxicity. With an increasing number of research unraveling the structure properties of peptides with anti-inflammatory bioactivity, it is assumed that the hydrophobicity of the peptides and hydrophobic amino acid residues clustered at the N-terminal-like IRW may contribute to their activity.[61] Also, the Arg residue in the peptides may enable their binding to LPS and subsequently block or alleviate inflammatory responses.[61] Besides, the molecular weight and length of the peptides are also crucial for their bioactivities. Some longer peptides derived from rice and soybean were reported with a significant effect on LPS detoxification.[40,62] These data indicated the importance of structural features in the anti-inflammatory activity of IRW. However, further studies are required to elucidate the role of amino acid residues and their electrochemical properties in IRW's anti-inflammatory activity.

In conclusion, the present study demonstrated that IRW could maintain tight junction permeability and barrier integrity and prevent inflammatory response by neutralizing LPS and inhibiting LPS-activated NF-κB and MAPK pathways in Caco-2 cells. These data explain the underlying mechanisms by which IRW improves intestinal barrier function. Further studies are necessary to elaborate on the molecular basis of IRW enhancing the turnover and trafficking of intestinal tight junction proteins and investigate the correlation between the IRW structure and activity.

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Notes

The authors declare no competing financial interest.

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