Protective Role of Oligomycin Against Intestinal Epithelial Barrier Dysfunction Caused by IFN- γ and TNF- α

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Key Words

Interferon-gamma • Tumor necrosis factor-alpha • Oligomycin • Intestinal barrier dysfunction • Permeability • Myosin light chain kinase • Tight junction

Abstract

Although the precise mechanisms involved in intestinal barrier dysfunction induced by proinflammatory cytokines are incompletely understood, pharmacological restoration of barrier function is very important to the management of intestinal disease. This study was aimed to investigate the protective role of HIF-1 α inhibitor oligomycin against intestinal epithelial barrier dysfunction induced by proinflammatory cytokines IFN- γ and TNF- α , and the underlying mechanisms. To induce barrier dysfunction, Caco-2 monolayers were treated with IFN- γ and TNF- α simultaneously. The cytokines-treated Caco-2 monolayers in the absence and in the presence of oligomycin were used for physiological, morphological, and biochemical analyses. The results showed that at the concentration of blocking HIF-1 α activation, oligomycin significantly ameliorated TER reduction and paracellular permeability increase in Caco-2 monolayers

challenged with IFN- γ and TNF- α . Oligomycin also largely attenuated the IFN- γ and TNF- α -related relocalization of tight junction proteins ZO-1 and occludin. Western blot analysis revealed that oligomycin abolished the increases of both MLC phosphorylation and MLCK protein expression induced by IFN- γ and TNF- α challenge. Quantitative RT-PCR analysis showed that oligomycin inhibited the IFN- γ and TNF- α -induced up-regulation of MLCK mRNA. It is concluded that oligomycin is able to attenuate intestinal epithelial barrier dysfunction induced by proinflammatory cytokines IFN- γ and TNF- α . The mechanism by which oligomycin protects intestinal barrier function may, at least in part, be attributed to block the up-regulated MLCK transcription and protein expression induced by IFN- γ and TNF- α .

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Introduction

It is well known that the luminal surface of the gastrointestinal tract is lined with a highly polarized and continuously renewing epithelium, so as to form epithelial

barrier. Intact intestinal epithelial barrier is crucial for the maintenance of mechanical defence to luminal bacteria or other antigens. Intercellular tight junctions, mainly composed of cytoplasmic proteins, including zona occludens (ZO) proteins, and two distinct transmembrane proteins, occludin and claudin, are the key structures responsible for intestinal epithelial barrier integrity [1-3]. Disruption of intestinal epithelial barrier is compromised in a variety of intestinal disease, including inflammatory bowel disease [4, 5]. The compromised intestinal barrier dysfunction may be either causative or consequential. Previous studies have clearly established that proinflammatory cytokines including IFN- γ and TNF- α , either alone or together, play very important roles in disrupting intestinal epithelial barrier function [6-12].

Despite the molecular mechanism involved in intestinal barrier dysfunction caused by proinflammatory cytokines is still unclear, it has been well demonstrated that the proinflammatory cytokines disrupt intestinal barrier function largely by inducing myosin light chain kinase (MLCK), which leads to increased myosin light chain (MLC) phosphorylation and in turn destabilizes tight junctions [8-10, 13-15]. Thus, the activation of MLCK-dependent MLC phosphorylation can be viewed as a common pathway involved in intestinal barrier dysfunction induced by proinflammatory cytokines [1, 16].

Hypoxia-inducible factor-1 alpha (HIF-1 α) has emerged as a central determinant in the pathophysiological response to cellular hypoxia [17, 18]. HIF-1 α is also a critical regulator in immunity, inflammation and infection [19-21]. Previous studies from several groups have shown that HIF-1 α activation is deleterious to intestinal barrier function associated with hypoxia, ischemia/reperfusion, and inflammation [22-25]. It has also been reported that HIF-1 α is involved in barrier dysfunction and disorganization of tight junction proteins of endothelial cells exposed to hypoxia or high glucose [26, 27]. We have previously demonstrated the involvement of HIF-1 α in MLCK-dependent endothelial barrier dysfunction induced by hypoxia [28]. Based on these, we hypothesize that pharmacological HIF-1 α inhibition may be protective against proinflammatory cytokines-induced intestinal barrier dysfunction.

It has been shown that oligomycin inhibits HIF-1 α expression both *in vitro* and *in vivo*. For example, oligomycin prevents HIF-1 α accumulation caused by hypoxia in Mum2B and U87 cells [29]. Oligomycin also inhibits HIF-1 α in vascular smooth muscle cells of rats suffering from hemorrhagic shock [30]. Thus, the aim of this study was to determine whether oligomycin is

protective against intestinal barrier dysfunction caused by proinflammatory cytokines and, if so, whether MLCK-dependent MLC phosphorylation is involved in the action of oligomycin on intestinal barrier function. Our data provide the direct evidence that oligomycin attenuates cytokines-induced intestinal barrier dysfunction by inhibiting MLCK-dependent MLC phosphorylation *in vitro*.

Materials and Methods

Caco-2 monolayer preparation

Caco-2 cells purchased from ATCC (Manassas, VA) were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 4.0 mM L-glutamine (Invitrogen), 1% non-essential amino acid (Invitrogen), 100 U/ml penicillin, 100 U/ml streptomycin and 15% fetal bovine serum (Invitrogen). The cells were partial digested with 0.25% trypsin and 0.53 mM EDTA in Ca²⁺- and Mg²⁺-free Hank's balanced saline solution (HBSS). Caco-2 cells were planted at a density of 0.5x10⁵/cm² and grown as confluent monolayers on collagen-precoated permeable polycarbonate membrane Transwell supports with 0.4 μ m pores (Corning, NY), and used for experiments after confluence.

Monolayer treatment

For hypoxia treatment experiments, monolayers were exposed to 1% O₂ for 6 hours as we described previously [28] without or with oligomycin treatment. For cytokine treatment experiments, recombinant human IFN- γ (10 ng/ml) and TNF- α (10 ng/ml) (R&D Systems, Minneapolis, MN), without or with oligomycin (40 μ g/mL, ALEXIS, San Diego, USA), were simultaneously added to the basal chamber without manipulating the apical DMEM media. Control Caco-2 monolayers were only incubated with DMEM without additional treatment. The monolayers were incubated for 24 or 48 hours.

Measurement of transepithelial electrical resistance

Transepithelial electrical resistance (TER) values of monolayers grown on 0.33-cm² Transwell supports were determined with a Millicell-ERS voltohmmeter (Millipore, Bedford, MA) as we described previously [9]. To facilitate comparisons between conditions, TER was normalized to initial value, and expressed as percentage of initial resistance values.

Measurement of paracellular permeability

Fluorescein isothiocyanate (FITC)-labeled dextran (Sigma, St. Louis, MO) across Caco-2 monolayers was used to represent the apical-to-basal paracellular permeability of intestinal epithelial barrier as we described previously [31]. Briefly, after 48 hours treatment, monolayers were washed with HBSS and transferred to 500 μ l HBSS. Media in apical chamber were gently aspirated and replaced with 100 μ l of 1mg/ml FITC-dextran dissolved in HBSS. Then, monolayers were incubated at 37°C for 2 hours. 100 μ l sample was taken from basal chamber for fluorescence determination using a fluorescent plate reader

(Varioskan Flash, Thermo Electron Corporation, Vantaa, Finland) with excitation wavelength of 480nm and emission wavelength of 520nm. The paracellular flux was calculated from a standard curve and expressed as pmole.

Immunofluorescent staining of HIF-1 α

At the end of hypoxia experiments, HIF-1 α staining was performed by immunofluorescent assay as we described previously [28].

Western blot analysis of HIF-1 α , MLCK, and phosphorylated MLC

Caco-2 monolayers grown on 5-cm² Transwell supports were washed twice with pre-cooling phosphate-buffered saline (PBS), then cells were lysed with SDS-PAGE sample buffer containing 50mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 5% β -mercaptoethanol, 10% glycerine. After briefly sonicated by a sonicator (Tomy Seiko, Tokyo, Japan), cell debris were separated by centrifugation at 15,000 g for 10min at 4°C. The supernatant protein concentrations were tested with RC DC kit (Bio-Rad, Hercules, CA). The cellular proteins were separated on 10% SDS-PAGE gel, and then transferred to PVDF membrane (Millipore). After blocking with 5% non-fat milk for 1 hour, membranes were respectively incubated with specific antibodies directed to HIF-1 α (1:1000, Upstate, NY), phosphorylated MLC (1:1000, Cell Signaling, MA), MLC (1:1000, Sigma), MLCK (1:1000, Sigma), and β -actin (1:5000, Sigma) overnight at 4°C. After washing four times with TBS-T, membranes were incubated with peroxidase-conjugated secondary antibodies (1:5000, Southern Biotech, Birmingham, AL) for 1 hour. After extensive washing with TBS-T, the blots were visualized with enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK) and imaged using a ChemiDoc XRS system (Bio-Rad). Densitometry of immunoblot data was performed using Quantity One (Bio-Rad).

Immunofluorescent staining of tight junction proteins ZO-1 and occludin

At the end of experiments, Caco-2 monolayers grown on 0.33-cm² Transwell supports were fixed with 1% paraformaldehyde for 30 minutes, incubated with 50mmol/L NH₄Cl for 15 min, then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. After incubation with mouse anti-ZO-1 or rabbit anti-occludin antibodies (1:200, Invitrogen) diluted in PBS containing 5% bovine serum albumin (BSA) at 4 °C overnight, monolayers were washed three times with PBS containing 2.5% BSA, and incubated with Texas red-conjugated goat anti-mouse or rabbit secondary antibodies (1:50, EMD Chemicals Gibbstown, NJ) for 1 hour at room temperature. After mounted with Slowfade (Molecular Probes, Eugene, OR), monolayers were imaged using a TCS SP5 laser scanning fluorescence microscopy (Leica, Germany).

Quantitative RT-PCR analysis of MLCK

Cell lysates in TRIzol (Invitrogen) were sonicated using a sonicator (Tomy Seiko, Tokyo, Japan), and total RNA was extracted with chloroform, precipitated with isopropyl alcohol, and resuspended in diethyl pyrocarbonate (DEPC)-treated

water. cDNA was generated from total RNA using First-Strand cDNA Synthesis Kit (OriGene Technologies, Rockville, MD). MLCK mRNA expression was determined by qSTAR SYBR Master Mix-Low Rox Kit (OriGene Technologies) using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers were 5'-GAG GTG CTT CAG AAT GAG GAC G-3' and 5'-GCATCAGTGACACCTGGCAACT-3' for detection of human MLCK, and 5'-GTC TCC TCT GAC TTC AACAGC G-3' and 5'-ACC ACC CTG TTG CTG TAG CCAA-3' for detection of human GAPDH. GAPDH was used as a reference.

Statistical analysis

Results are presented as means $\pm$ SEM. The statistical significance of differences between two groups was determined by the Student's *t* test. For multi-group data analysis, an ANOVA analysis was used. Correlation between TER and HIF-1 α expression was assessed using the Pearson correlation analysis. SPSS statistical software (version 13.0) was used for all statistical analysis. A *p* value of <0.05 was considered as the minimum level of significance in all cases. All reported significance levels represent two-tailed *p* values.

Results

Oligomycin blocks IFN- γ and TNF- α -induced HIF-1 α accumulation

Oligomycin has been reported to be capable of inhibiting HIF-1 α expression both *in vitro* and *in vivo* [29, 30]. In contradiction to this, an *in vitro* study has demonstrated that oligomycin has no effect on HIF-1 α accumulation caused by hypoxia [32]. To investigate the effect of oligomycin on HIF-1 α accumulation in intestinal epithelial cells, we first detected HIF-1 α protein expression in hypoxic intestinal epithelial cells. As shown in Fig. 1A, as compared with control, hypoxia treatment for 6 hours caused a dramatic increase of HIF-1 α protein expression. In contrast, oligomycin significantly inhibited the hypoxia-induced increase of HIF-1 α protein expression in a dose-dependent manner. In addition, as illustrated in Fig. 1B, oligomycin at 40 μ g/ml almost completely abrogated the nuclear accumulation of HIF-1 α in intestinal epithelial cells exposed to hypoxia for 6 hours. Thus, the dose of 40 μ g/ml was selected for further studies.

Based on the results presented above, we further investigated the effect of oligomycin on HIF-1 α accumulation in Caco-2 monolayers treated with simultaneous IFN- γ and TNF- α . As shown in Fig. 1C, HIF-1 α protein expression in Caco-2 monolayers treated with simultaneous IFN- γ and TNF- α was significantly higher than that in control monolayers. However,

Fig. 1. Oligomycin blocks IFN- γ and TNF- α -induced HIF-1 α accumulation. **A.** Cells were cultured under hypoxia (1% O₂) without or with oligomycin for 6 hours. Hypoxia induced a significant increase of HIF-1 α protein expression, whereas oligomycin inhibited the hypoxia-caused increase of HIF-1 α expression at the concentration indicated. *, p<0.05, compared with control. #, p<0.05, compared with hypoxia without oligomycin. Data are representative of five similar experiments. **B.** Cells were treated as noted in Panel A, and HIF-1 α was stained by immunofluorescence. Hypoxia caused a dramatically HIF-1 α accumulation within the nucleus. Data are representative of three independent experiments performed in duplicate. Scale bar =10 μ m. **C.** Caco-2 monolayers were incubated with cytokines (IFN- γ 10 ng/ml, TNF- α 10 ng/ml) in the absence or presence of 40 μ g/ml oligomycin added to the basal chamber for 24 hours. Treatment of simultaneous IFN- γ and TNF- α significantly increased HIF-1 α protein expression, whereas oligomycin abolished IFN- γ and TNF- α -induced increase of HIF-1 α expression. *, p<0.05, compared with control without both cytokines and oligomycin. #, p<0.05, compared with cytokines without oligomycin. Data are representative of five similar experiments.

oligomycin blocked the increased HIF-1 α protein expression induced by IFN- γ and TNF- α .

Oligomycin alleviates IFN- γ and TNF- α -induced intestinal barrier dysfunction in vitro

Previous studies have shown that preincubation with IFN- γ for 24 or 19 hours followed by TNF- α treatment could disrupt barrier function in both Caco-2 and T84 intestinal epithelial cell monolayers [9-11, 33]. In present study, to assess the effect of oligomycin on intestinal barrier function, we developed an *in vitro* model in which Caco-2 monolayers were treated with simultaneous IFN- γ and TNF- α for 48 hours, and TER, a very sensitive measure of barrier integrity, was employed as an indicator of barrier function. Our preliminary experiments proved that oligomycin alone had no obvious effects on both TER and paracellular permeability in Caco-2 monolayers (data not shown). As shown in Fig. 2A, TER levels of control Caco-2 monolayers were maintained over the time-course of the experiments. However, basolateral treatment of Caco-2 monolayers with simultaneous IFN- γ and TNF- α caused a progressively time-dependent decrease of TER with a maximal action at 48 hours. By contrast, the TER drop induced by IFN- γ and TNF- α was significantly dampened at 24, 36 and 48 hours after oligomycin

Fig. 2. Oligomycin alleviates IFN- γ and TNF- α -induced intestinal barrier dysfunction. **A.** Caco-2 monolayers were treated as described in Panel C of Figure 1 for the hours indicated. Oligomycin significantly inhibited TER drop induced by simultaneous IFN- γ and TNF- α treatment. *, $p < 0.05$, compared with control at the same time point. #, $p < 0.05$, compared with cytokines IFN- γ and TNF- α at the same time point. $n = 7$. **B.** Caco-2 monolayers were treated as noted in Panel C of Figure 1 for the hours indicated. Treatment with simultaneous IFN- γ and TNF- α caused a markedly increase of HIF-1 α protein expression, whereas oligomycin inhibited IFN- γ and TNF- α -induced increase of HIF-1 α expression. Data are representative of five similar experiments. **C.** Caco-2 monolayers were treated as noted in Panel C of Figure 1. The IFN- γ and TNF- α -caused increase of paracellular permeability to FITC-dextran was significantly attenuated by oligomycin. *, $p < 0.05$, compared with control in the absence of both cytokines and oligomycin. #, $p < 0.05$, compared with cytokines in the absence of oligomycin. $n = 5$.

treatment applied with both cytokines. Consistent with the changes of TER, after treatment with simultaneous IFN- γ and TNF- α , HIF-1 α expression increased progressively in a time-dependent manner over the 48 hours period (Fig. 2B). By contrast, the IFN- γ and TNF- α -induced increase of HIF-1 α expression was significantly inhibited by co-administration of oligomycin, but still higher than that of control (Fig. 2B). In addition, there was a significantly negative correlation between TER and HIF-1 α expression ($r = -0.814$, $p = 0.001$).

Next, paracellular flux of FITC-labeled dextran across Caco-2 monolayers, a measure of paracellular permeability, was also tested. As illustrated in Fig. 2C, when compared with control, paracellular flux was significantly increased after treatment with simultaneous IFN- γ and TNF- α for 48 hours. In contrast to this, the increased paracellular flux caused by IFN- γ and TNF- α was significantly diminished by oligomycin treatment for 48 hours, albeit it was still higher than that of control monolayers without cytokine treatment. These results were consistent with the TER data mentioned above. Taken together, these results indicate that oligomycin has beneficial effect on cytokine-induced barrier dysfunction in Caco-2 monolayers.

Oligomycin preserves tight junction morphology against the actions of IFN- γ and TNF- α

We [9, 10] and others [11, 33] have previously demonstrated that IFN- γ and TNF- α synergistically lead to morphological disruption of tight junction in both Caco-2 and T84 intestinal epithelial cells. Thus, we considered the possibility that the protective effect of oligomycin against simultaneous IFN- γ and TNF- α -induced barrier dysfunction might be associated with the maintenance of tight junction morphology. With the use of immunofluorescent antibody labeling assay, we detected the morphological distribution of tight junction proteins ZO-1 and occludin.

As shown in Fig. 3, in control Caco-2 monolayers, the tight junction protein ZO-1, which is a member of the membrane-associated guanylate kinase homologs and a linker between the transmembrane protein occludin and the actin cytoskeleton [34], was localized to the tight junction, along the edge of the cells. Similarly, another tight junction protein occludin, one transmembrane protein of tight junction that may be integral to tight junction structure, was also detected at the tight junction, along the regular profile of cells. By contrast, after the treatment with simultaneous IFN- γ and TNF- α for 48 hours, ZO-1 distribution was transformed from regularly smooth arcs

Fig. 3. Oligomycin preserves tight junction morphology against the actions of IFN- γ and TNF- α . Caco-2 monolayers were treated as described in Panel C of Figure 1 for 48 hours. Tight junction proteins ZO-1 and occludin were stained by immunofluorescence. Oligomycin alleviated the IFN- γ and TNF- α -caused morphological redistributions of tight junction proteins ZO-1 and occludin. Data are representative of five independent experiments. Scale bar= 10 μ m.

into irregularly undulating profiles and discontinuous punctae along the tight junction. Similar to ZO-1, occludin distribution was also transformed into irregular arrangement, with partially transfer from the tight junction into cytoplasmic vesicles. However, oligomycin markedly prevented the morphological disturbances of ZO-1 and occludin in Caco-2 monolayers treated with simultaneous IFN- γ and TNF- α for 48 hours. These results suggest that oligomycin could preserve tight junction morphology against the actions of IFN- γ and TNF- α in Caco-2 monolayers.

Fig. 4. Oligomycin prevents the increases of MLC phosphorylation and MLCK expression caused by IFN- γ and TNF- α . A. Caco-2 monolayers were treated as described in Panel C of Figure 1. Oligomycin prevented the IFN- γ and TNF- α -induced increase of phosphorylated MLC expression. *, $p<0.05$, compared with control without both cytokines and oligomycin. #, $p<0.05$, compared with cytokines without oligomycin. Data are representative of five similar experiments. B. Oligomycin treatment blocked the up-regulated MLCK protein expression caused by IFN- γ and TNF- α . *, $p<0.05$, compared with control without both cytokines and oligomycin. #, $p<0.05$, compared with cytokines without oligomycin. Data are representative of five similar experiments. C. Oligomycin treatment inhibited the IFN- γ and TNF- α -induced up-regulation of MLCK mRNA. *, $p<0.05$, compared with control without both cytokines and oligomycin. #, $p<0.05$, compared with cytokines without oligomycin. Data are representative of three similar experiments.

Oligomycin prevents the increases of MLC phosphorylation and MLCK expression caused by IFN- γ and TNF- α

MLCK activation and subsequent MLC phosphorylation play central roles in the pathophysiological regulation of intestinal epithelial tight junction barrier [8-10, 13-15, 35]. Our previous studies have shown that increases of MLC phosphorylation and MLCK protein expression are involved in barrier function disruption induced by TNF- α treatment of IFN- γ -primed or TNF receptor 2-transfected Caco-2 monolayers [9, 10]. Thus, to better understand the mechanism involved in the protective action of oligomycin against intestinal barrier dysfunction induced by simultaneous IFN- γ and TNF- α treatment, we assessed the effect of oligomycin on both MLC phosphorylation and MLCK expression.

Treatment of Caco-2 monolayers with simultaneous IFN- γ and TNF- α caused a significantly increase of phosphorylated MLC, rather than total MLC, as compared with control (Fig. 4A). The increased expression of phosphorylated MLC was efficiently prevented by oligomycin treatment (Fig. 4A). Similar to the change profile of MLC phosphorylation, MLCK protein expression in Caco-2 monolayers treated with simultaneous IFN- γ and TNF- α was also significantly higher than that in control monolayers (Fig. 4B). On the contrary, oligomycin treatment almost completely blocked the IFN- γ and TNF- α -induced increase of MLCK protein expression (Fig. 4B). Consistent with these, MLCK mRNA was increased by IFN- γ and TNF- α treatment, whereas oligomycin inhibited the IFN- γ and TNF- α -induced up-regulation of MLCK mRNA (Fig. 4C). Collectively, these results imply that oligomycin is able to prevent the IFN- γ and TNF- α -induced up-regulation of MLCK expression and subsequent MLC phosphorylation in intestinal epithelia *in vitro*.

Discussion

The principle novel finding of this study is that oligomycin, at the concentration of blocking HIF-1 α accumulation induced by proinflammatory cytokines IFN- γ and TNF- α , is able to attenuate intestinal epithelial barrier dysfunction caused by IFN- γ and TNF- α co-stimulation, and to prevent the IFN- γ and TNF- α -induced morphological disruption of tight junction in intestinal epithelia. The current findings, for the first

time, also demonstrate that dampening the up-regulation of MLCK expression and subsequent MLC phosphorylation, at least in part, might be the mechanism by which oligomycin attenuates intestinal barrier dysfunction induced by simultaneous IFN- γ and TNF- α .

A number of intestinal diseases mediated by inflammation, infection, ischemia, and immune, such as ulcerative colitis, Crohn's disease, and irritable bowel syndrome, involve intestinal epithelial barrier dysfunction, which is characterized by the increased paracellular permeability as well as the changes of tight junction protein expression and organization [36, 37]. Although the precise molecular mechanism is still unclear, proinflammatory cytokines have been reported to be the most important mediators leading to intestinal epithelial barrier disruption. Previous published data have well demonstrated that IFN- γ , TNF- α , IL-1 β , IL-6, IL-13 or TNF superfamily member LIGHT alone is able to induce intestinal barrier defects [6-8, 15, 38-40]. Moreover, sequential IFN- γ and TNF- α treatment can also synergize to cause intestinal barrier disruption. We [9, 10] and others [11, 33] have demonstrated that TNF- α treatment of Caco-2 or T84 intestinal epithelial cell monolayers primed with IFN- γ for 24 or 19 hours leads to barrier function impairment. In present study, we show that IFN- γ and TNF- α , when administered simultaneously, produce a significant reduction of barrier function as measured by the decrease of TER and increase of paracellular permeability, which is accompanied by the localization changes of tight junction proteins ZO-1 and occludin.

A novel finding of this study is that at the concentration of blocking HIF-1 α activation, oligomycin, a specific inhibitor of HIF-1 α , alleviates the IFN- γ and TNF- α -induced barrier function disruption and relocation of tight junction proteins ZO-1 and occludin in intestinal epithelia, implying that HIF-1 α might be involved in the intestinal barrier dysfunction induced by IFN- γ and TNF- α . HIF-1 α , the inducible subunit of HIF-1, heterodimerizes HIF-1 β , which is the constitutive subunit of HIF-1 and also known as aryl hydrocarbon receptor nuclear translocator (ARNT), forming the functional nuclear transcription factor HIF-1 [17, 18]. Upon activation, HIF-1 induces the transcription of numerous genes involved not only in cellular adaption to hypoxia but also in regulation of intestinal epithelial barrier function [22, 23, 41, 42]. Previously *in vivo* or *in vitro* studies have shown that HIF-1 α activation is deleterious to intestinal or endothelial barrier integrity

[22–25]. For example, partial HIF-1 α deficiency alleviates gut injury induced by ischemia/reperfusion, and HIF-1 α activation contributes to the loss of gut barrier function [24, 25]. Mice conditionally lacking intestinal epithelial *hif1 α* are resistant to hypoxia-induced increase of intestinal epithelial permeability [22]. HIF-1 α has also been reported to augment experimental murine colitis [23]. A recent *in vitro* study has revealed that up-regulating HIF-1 activity by cobalt chloride increases the paracellular permeability of the endothelial cells exposed to normal glucose, whereas downregulating HIF-1 activity by HIF-1 α inhibitors and HIF-1 α specific siRNA ameliorates the increased paracellular permeability and the alterations of distribution pattern of occludin and ZO-1 induced by high glucose [27].

Another novel finding of this study is that oligomycin prevents the increases of MLC phosphorylation and MLCK expression in intestinal epithelia caused by simultaneous IFN- γ and TNF- α . Previous *in vitro* or *in vivo* studies from several groups have proven that MLCK-dependent MLC phosphorylation signaling pathway is critical to intestinal epithelial barrier dysfunction induced by proinflammatory cytokines [8–10, 13–15]. It has been reported that MLCK activation alone is sufficient to increase tight junction permeability, both *in vitro* and *in vivo* [43, 44]. We have previously demonstrated that MLC phosphorylation mediated by up-regulated MLCK protein expression is necessary to barrier function disruption of Caco-2 monolayers treated with sequential IFN- γ and TNF- α [9, 10], and that the barrier function is restored upon pharmacological MLCK inhibition [9]. Here, we reveal that oligomycin not only attenuates barrier function disruption and relocalization of tight junction proteins, but also blocks the increased MLC phosphorylation and MLCK protein expression in Caco-2 monolayers treated with simultaneous IFN- γ and TNF- α . Thus, it is suggested that inhibition of MLCK-mediated MLC phosphorylation might be the molecular mechanism involved in the protective effect of oligomycin on the intestinal barrier dysfunction induced by IFN- γ and TNF- α . It should be noted that oligomycin just makes the intestinal barrier dysfunction attenuated, but not restored, albeit it blocks HIF-1 α activation as well as up-regulation of MLC phosphorylation and MLCK protein expression in intestinal epithelia challenged with IFN- γ and TNF- α . Thus, other mediators and signaling pathways should be considered in the pathogenesis of intestinal barrier dysfunction

induced by proinflammatory cytokines. For example, some previous studies have shown that matrix metalloproteinase-9 (MMP-9), adenosine monophosphate-activated protein kinase (AMPK), GDP-GTP exchange factor H1 (GEF-H1), phosphatidylinositol 3'-kinase (PI3K), extracellular signal regulated kinases 1/2 (ERK1/2), and Src kinase Fyn are involved in the barrier dysfunction induced by proinflammatory cytokines [45–51].

The molecular mechanism by which oligomycin blocks the IFN- γ and TNF- α -induced up-regulation of MLCK protein expression is currently unknown. However, in this study we show that oligomycin inhibits the up-regulation of MLCK mRNA induced by IFN- γ and TNF- α , suggesting that oligomycin inhibits transcriptional activation of MLCK. We have previously demonstrated that up-regulating HIF-1 α by dimethylxaloyl glycine, a specific HIF-1 α inducer by inhibiting prolyl-4-hydroxylase, increases MLCK protein expression, whereas downregulating HIF-1 α by HIF-1 α specific siRNA ameliorates the hypoxia-induced increase of MLCK protein expression in endothelial cells [28]. It has been reported that NF- κ B contributes to the transcriptional up-regulation of MLCK in intestinal epithelial cells challenged with cytokines [14, 52, 53], and that HIF-1 α activates NF- κ B, both *in vitro* and *in vivo* [54, 55]. Here, we show that oligomycin abolishes HIF-1 α accumulation triggered by both hypoxia and simultaneous IFN- γ and TNF- α . Thus, it is speculated that inhibiting HIF-1 α activation, at least in part, might be the molecular mechanism by which oligomycin blocks MLCK up-regulation caused by IFN- γ and TNF- α .

In conclusion, our current data provide an original protective role for oligomycin against intestinal epithelial barrier disruption induced by IFN- γ and TNF- α *in vitro*. This work clearly identifies oligomycin as a putative therapeutic agent for intestinal barrier dysfunction associated with proinflammatory cytokines, although the need for further studies to be addressed.

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