Mechanism of TNF-α modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression

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Ma, Thomas Y., Michel A. Boivin, Dongmei Ye, Ali Pedram, and Hamid M. Said. Mechanism of TNF-α modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. Am J Physiol Gastrointest Liver Physiol 288: G422–G430, 2005; doi:10.1152/ajpgi.00412.2004.—TNF-α plays a central role in the intestinal inflammation of various inflammatory disorders including Crohn’s disease (CD). TNF-α-induced increase in intestinal epithelial tight junction (TJ) permeability has been proposed as one of the proinflammatory mechanisms contributing to the intestinal inflammation. The intracellular mechanisms involved in the TNF-α-induced increase in intestinal TJ permeability remain unclear. The purpose of this study was to investigate the possibility that the TNF-α-induced increase in intestinal epithelial TJ permeability was regulated by myosin light-chain kinase (MLCK) protein expression, using an in vitro intestinal epithelial model system consisting of the filter-grown Caco-2 intestinal epithelial monolayers. TNF-α (10 ng/ml) produced a time-dependent increase in Caco-2 MLCK expression. The TNF-α increase in MLCK protein expression paralleled the increase in Caco-2 TJ permeability, and the inhibition of the TNF-α-induced MLCK expression (by cycloheximide) prevented the increase in Caco-2 TJ permeability, suggesting that MLCK expression may be required for the increase in Caco-2 TJ permeability. The TNF-α increase in MLCK protein expression was preceded by an increase in MLCK mRNA expression but not an alteration in MLCK protein degradation. Actinomycin-D prevented the TNF-α increase in MLCK mRNA expression and the subsequent increase in MLCK protein expression and Caco-2 TJ permeability, suggesting that the increase in MLCK mRNA transcription led to the increase in MLCK expression. The TNF-α increase in MLCK protein expression was also associated with an increase in Caco-2 MLCK activity. The cycloheximide inhibition of MLCK protein expression prevented the TNF-α increase in MLCK activity and Caco-2 TJ permeability. Moreover, inhibitors of MLCK, Mg2+-myosin ATPase, and metabolic energy prevented the TNF-α increase in Caco-2 TJ permeability, suggesting that the increase in MLCK activity was required for the TNF-α-induced opening of the Caco-2 TJ barrier. In conclusion, our results indicate for the first time that 1) the TNF-α increase in Caco-2 TJ permeability was mediated by an increase in MLCK protein expression, 2) the increase in MLCK protein expression was regulated by an increase in MLCK mRNA transcription, and 3) the increase in Caco-2 TJ permeability required MLCK protein expression-dependent increase in MLCK activity.

intestinal permeability; tight junctions; intestinal barrier

TUMOR NECROSIS FACTOR-α (TNF-α) has been shown to play an important role in the intestinal inflammation of Crohn’s disease (CD) and other inflammatory conditions (28, 32, 34, 39, 41). It is well established that TNF-α levels are significantly elevated in intestinal tissue, serum, and stool of patients with CD (3, 28, 41). In the stools collected from patients with CD, the excreted TNF-α concentration ranged from 420 to 4,322 pg/g stool compared with 40–84 pg/g stool in controls (3). The central importance of TNF-α in the intestinal inflammation of CD has been demonstrated by the clinical studies showing the efficacy of anti-TNF-α antibody infusion therapy in the treatment of severe active CD (32, 34, 39, 41). The recently completed ACCENT I clinical trial also showed that the chronic maintenance therapy of anti-TNF-α antibody was effective in the prevention of CD exacerbation (8). Consistent with these studies, thalidomide (an inhibitor of TNF-α expression) has also been shown to be effective in the treatment of CD (4). Thus the clinical importance of TNF-α in the inflammatory process of CD has been well validated. The proposed proinflammatory actions of TNF-α include production and stimulation of proinflammatory cytokines including IL-1,-6,-8,-12, and granulocyte macrophage colony-stimulating factor, increased expression of endothelial adhesion molecules, activation of acute-phase response, and stimulation of metalloproteinases and collagen synthesis (34, 41). In addition to its immune modulating effects, TNF-α also caused a disruption of the intestinal epithelial tight junction (TJ) barrier (23, 26, 33, 35). A number of investigators have shown that TNF-α produces an increase in intestinal TJ permeability manifested by an increase in paracellular permeability and a drop in transepithelial resistance (TER) (23, 26, 33, 35). Thus it had been proposed that the TNF-α disturbance of intestinal TJ barrier contributes to the intestinal inflammation in CD by allowing increased intestinal permeation of toxic luminal antigens (23, 34).

It is well established that the patients with CD have a defective intestinal epithelial TJ barrier function manifested by an increase in intestinal permeability to the permeability markers (11, 12, 15, 18, 27). The defective intestinal TJ barrier has been proposed as a primary etiologic factor of CD (11, 15, 18, 27). It had been proposed by several groups that the preexisting defect in the intestinal TJ barrier in CD patients allows increased intestinal penetration of toxic luminal antigens, culminating in intestinal inflammation (11, 18, 27). Because TNF-α levels are markedly increased in CD patients, it is likely that
the abnormal increase in intestinal TJ permeability present in these patients are, in part, related to the TNF-α effect on the intestinal TJ barrier (23). Consistent with such possibility, Svenaert et al. (37) found that the CD patients treated with anti-TNF-α antibody had a significant decrease in intestinal permeability after therapy. Additionally, it had been shown that the therapeutic maneuvers that decrease the luminal antigenic load such as the use of total parenteral nutrition therapy, antibiotic treatment, and the use of elemental diet are effective in the treatment of active CD (2, 17, 18, 29, 46). Conversely, external factors that cause disturbance of intestinal TJ barrier such as NSAID usage, ethanol binges, and acute gastroenteritis induce exacerbation of CD (5, 16, 24). The clinical relevance of abnormal increase in intestinal TJ permeability in CD was further evidenced by the clinical studies that show that the level of increase in intestinal permeability in CD patients predicts poor response to drug therapy and early relapse after therapy (43).

Intracellular mechanisms involved in the TNF-α-induced increase in intestinal epithelial TJ permeability remain unclear. The purpose of this study was to elucidate some of the intracellular mechanisms involved in the TNF-α modulation of the intestinal epithelial TJ barrier function, using an in vitro intestinal epithelial model system consisting of the filter-grown Caco-2 intestinal epithelial monolayers (22, 23, 31, 40). Understanding the mechanisms involved in the TNF-α modulation of intestinal TJ permeability will be important in gaining further insight into the pathophysiology of intestinal TJ barrier defect in CD and other inflammatory conditions (11, 18, 37). In this study, we tested the hypothesis that the TNF-α increase in intestinal TJ permeability was regulated by an increase in myosin light-chain (MLC) kinase (MLCK) protein expression. Our hypothesis was generated based on our previous studies showing the importance of MLCK activity in the regulation of the pharmacological agent-induced increase in intestinal epithelial TJ permeability (19, 24, 25). In this study, we provide new experimental data showing that the TNF-α-induced increase in intestinal TJ permeability was regulated by an increase in MLCK protein expression.

MATERIALS AND METHODS

DMEM, trypsin, and FBS were purchased from Life Technologies (Gaithersburg, MD). Glutamine, penicillin, streptomycin, and PBS were purchased from Irvine Scientific (Santa Ana, CA). TNF-α was purchased from Sigma (St. Louis, MO). Transwell permeable filters were purchased from Corning (Corning, NY). [3H]mannitol and [14C]inulin were obtained from New England Nuclear Research Products (Wilmington, DE). All other chemicals were of a reagent grade.

Cell cultures. Caco-2 cells (passage 19) were purchased from American Type Culture Collection (Rockville, MD), and the stock cultures were grown in a culture medium composed of DMEM with 4.5 mg/ml glucose, 50 U/ml penicillin, 50 U/ml streptomycin, 4 mM glutamine, and 10% FBS (10, 31). Culture medium was changed every 1–2 days. The cells were subcultured by partial digestion with 0.25% trypsin and 0.9 mM ethylenediamine-tetraacetic acid in Ca2+/Mg2+-free PBS. For growth on filters, Caco-2 cells were plated on Transwell microporous filters and monitored regularly by measuring TER. For experimental purposes, only Caco-2 cells between passages 20 and 26 were used.

Determination of Caco-2 intestinal monolayer resistance and paracellular permeability. The epithelial electrical resistance of the filter-grown Caco-2 intestinal monolayers was measured by using an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) as previously described (20, 21). For resistance measurements, both apical and basolateral sides of the epithelia were bathed in appropriate buffer solution. Electrical resistance was measured until similar values were recorded on three consecutive measurements.

The effect of TNF-α on Caco-2 monolayer paracellular permeability was determined by using the paracellular marker mannitol (20, 24, 25). Unless specified otherwise, DMEM (pH 7.4) was used as the incubation solution during the experiments. Buffered solution (100 μl) was added to the apical compartment, and 600 μl were added to the basolateral compartment to ensure equal hydrostatic pressure as recommended by the manufacturer. Known concentrations of permeability marker (10 μM) and its radioactive tracer were added to the apical solution. Low concentrations of permeability markers were used to ensure that negligible osmotic or concentration gradient was introduced. All flux studies were carried out at 37°C. All of the experiments were repeated three to six times in triplicates to ensure reproducibility.

Assessment of MLCK protein expression by immunoprecipitation and Western blot analysis. The MLCK protein expression was determined by Western blot analysis as previously described (19, 24, 25). The filter-grown Caco-2 monolayers were serum deprived overnight to synchronize the cell cycle and were treated with appropriate experimental reagents for varying experimental periods. At the end of the experimental period, Caco-2 monolayers were washed twice with cold HBSS, and cells were lysed by using 500 μl lysis buffer (lysine buffer in mM: 50 Tris-HCl, pH 7.5, 100 NaCl, 50 NaF, 5 EDTA, 40 β-glycerophosphate, 40 mM P-nitrophenyl phosphate, plus 200 μM vanadate, 100 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 0.5% Nonidet P-40). Monolayers were scraped, and the cell lysates were placed in microfuge tubes (tube A) (7, 9). Anti-MLCK antibody (5 μl/200 μl) in lysis buffer was added to the microfuge tubes containing the protein A Sepharose beads. MLCK antibody and protein A Sepharose bead mixture were incubated end-over-end for 2 h at 4°C to form the antibody-bead complex (tube B). Cleared lysate (100 μl; tube A) was then added to tube B containing the antibody-bead complex and incubated end-over-end for 2 h at 4°C. Subsequently, the tubes containing the immunoprecipitates were microfuged, and the supernatants were aspirated. Pellets were washed twice with lysis buffer. Gel-loading buffer (50 μl) was then added to the pellets. The tubes were boiled for 5 min at 95°C, after which MLCK proteins were separated on a 7% SDS-PAGE gel. The gel was transblotted against anti-MLCK antibody using the Amersham Western blot analysis kit.

Caco-2 MLCK activity determination. The Caco-2 MLCK activity was determined by measuring the in vitro kinase activity of the immunoprecipitated MLCK as previously described (24, 25). Caco-2 MLCK was immunoprecipitated as described above. Immunoprecipitates were washed sequentially with lysis buffer and solution of 10 mM HEPES and 10 mM magnesium acetate at 4°C. Immunoprecipitated MLCK was then used in an in vitro kinase reaction in microfuge tubes. The MLCK activity was determined by measuring the rate of MLCK phosphorylation by the immunoprecipitated MLCK. In brief, 20 μl purified chicken gizzard MLCK protein (2 mg/ml) and 20 μl ATP, 30 mM MgCl2, 30 mM magnesium acetate, and 30 mM HEPES were added and mixed with the immunoprecipitated MLCK for a 10-min reaction period at 30°C. The MLCK catalyzed phosphorylation reaction was terminated by the addition of 20 μl stop buffer solution (1 ml 2 M Tris buffer, pH 6.8, 2 ml 20% SDS, 4 ml glycerol, 3 ml water, 308 mg dithiothretiol, trace of bromphenol blue). Subsequently, the reaction mixture was boiled for 3 min, microfuged for 10 s, and then the supernatant (40–50 μl) was separated on 10% SDS-PAGE. The gel was fixed in 40% MeOH/10% acetic acid overnight, stained with Coomassie blue solu-
tion, dried, then autoradiographed, and the MLCK band at 19.5 kDa was identified. Experiments were repeated three to five times to ensure reproducibility.

**RNA isolation and RT.** A 5 × 10⁵ Caco-2 cells/filter were seeded into six-well Transwell permeable inserts and grown until ~90% confluency. Filter-grown Caco-2 cells were then treated with appropriate experimental reagents for desired time periods. At the end of the experimental period, cells were washed with ice-cold PBS. Total Caco-2 RNA was isolated by using Qiagen RNeasy Kit according to the manufacturer’s protocol. Total RNA concentration was determined by absorbance at 260/280 nm. RT was carried out by using the GeneAmp Gold RNA PCR core kit (Applied Biosystems, Foster City, CA). From each sample 2 μg of total RNA were reverse-transcribed into cDNA in a 40-μl reaction containing 1 × RT-PCR buffer, 2.5 mM MgCl₂, 250 μM of each dNTP, 20 units of Rnase inhibitor, 10 mM DTT, 1.25 μM random hexamer, and 30 units of multiscribe RT. RT reactions were performed in a thermocycler (PTC-100, MJ Research, Waltham, MA) at 25°C for 10 min, 42°C for 30 min, and 95°C for 5 min.

**Quantitation of gene expression using real-time PCR.** Real-time PCRs were carried out by using ABI prism 7900 Sequence Detection System and TaqMan Universal PCR Master Mix kit (Applied Biosystems, Branchburg, NJ) as previously described (38, 44). Each real-time PCR reaction contained 10 μl of RT reaction mix, 25 μl of 2× TaqMan Universal PCR Master Mix, 0.2 μM of probe, and 0.6 μM of primers. Primer and probe design for the real-time PCR was made with Primer Express version 2 from Applied Biosystems. The primers used in this study are as follows: MLCK specific primer pairs consisted of 5′-AACGAGATCAACATCTAGAACC-3′ (forward), 5′-CAGTGTGCCTGCCTGGAAG-3′ (reverse); probe specific for MLCK consisted of FAM 5′-AGCCAGTTGACCTGAC-3′ TAMRA; the internal control GAPDH-specific primer pairs consisted of 5′-ACACCCATGGCAAATTC-3′ (forward), 5′-TGGGATTCTCCATGATGCG-3′ (reverse); probe specific for GAPDH consisted of JOE 5′-TGGAGGACGCTCAAGGCT-GAAGACG-3′ TAMRA.] All runs were performed according to the default PCR protocol (50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min). For each sample, real-time PCR reactions were performed in triplicate and the average threshold cycle (Ct) was calculated. Standard curve was generated to convert the Ct to copy numbers. Expression of MLCK mRNA was normalized with GAPDH mRNA expression. The average copy number of MLCK mRNA expression in control samples was set to 1.0. The relative expression of MLCK mRNA in treated samples was determined as a fold increase compared with control samples (38, 44).

**[35S]methionine pulse-chase experiments.** Filter-grown Caco-2 cells were pulse labeled with [35S]methionine as previously described (42). Caco-2 monolayers were incubated in methionine-free DMEM onine for 2 hours at 37°C. The radioactive media was removed, and cells were pulse labeled with [35S]methionine as previously described and MLCK protein degradation assessed by counting the radioactivity of [35S]methionine in the immunoprecipitated sample (20 μl) and also by separation of MLCK protein on a 7% SDS-PAGE followed by autoradiography as described above. The experiments were repeated four times to ensure reproducibility.

**Statistical analysis.** Results are expressed as means ± SE. Statistical significance of differences between mean values was assessed with Student’s t-tests for unpaired data. All reported significance levels represent two-tailed P values. A P value of ≤ 0.05 was used to indicate statistical significance. All experiments were repeated a minimum of three times to ensure reproducibility.

**RESULTS**

**TNF-α effect on Caco-2 MLCK protein expression and TJ permeability.** In the following studies, we examined the possibility that the TNF-α-induced increase in Caco-2 TJ permeability was mediated by an increase in MLCK protein expression. The time-course effect of TNF-α on Caco-2 MLCK protein expression was determined by Western blot analysis (Fig. 1). By ~6 to 12 h of TNF-α treatment, there was a significant increase in MLCK protein expression; and by 48 h of treatment, the relative MLCK protein levels were ~300–400% of control levels (Fig. 1A) as determined by the densitometry measurement. The TNF-α increase in protein expression was specific to MLCK protein, as TNF-α did not increase the expression of other cellular proteins such as β-actin and caused a decrease in ZO-1 protein expression (23). The time course of the TNF-α-induced increase in MLCK protein expression correlated with the time course of the TNF-α-induced drop in Caco-2 TER and increase in permeability to paracellular marker mannitol (Fig. 1, B and C), suggesting that the TNF-α-induced increase in Caco-2 TJ permeability was related to the increase in MLCK protein expression. In a previous report (23), we showed that the TNF-α-induced drop in Caco-2 TER correlated directly with the increase in paracellular permeability. To confirm the importance of MLCK protein expression, the effect of protein synthesis inhibitor cycloheximide on TNF-α-induced increase in Caco-2 MLCK protein expression and Caco-2 TJ permeability was determined (Fig. 2). Cycloheximide (10 ng/ml) at a dose that inhibited the TNF-α increase in Caco-2 MLCK protein expression (Fig. 2A) prevented the TNF-α-induced drop in Caco-2 epithelial resistance (Fig. 2B), suggesting that the TNF-α-induced increase in MLCK protein expression was required for the TNF-α-induced drop in Caco-2 TER.

**TNF-α-induced increase in MLCK protein expression is associated with an increase in MLCK activity.** In the following studies, the effect of TNF-α-induced increase in MLCK protein expression on Caco-2 MLCK activity was determined. The TNF-α effect on Caco-2 MLCK activity was assessed by a direct in vitro kinase activity measurement as previously described (19, 25). After TNF-α treatment (10 ng/ml) for increasing time periods, Caco-2 MLCK was isolated by immunoprecipitation, and the kinase activity of the immunoprecipitated MLCK was determined by measuring the in vitro phosphorylation of ML. The TNF-α treatment of filter-grown Caco-2 monolayers resulted in a time-dependent increase in Caco-2 MLCK activity (Fig. 3A). The time course of TNF-α increase in MLCK activity mirrored the TNF-α increase in MLCK protein expression (Fig. 1A), suggesting a correlation between the increase in Caco-2 MLCK protein level and the increase in MLCK activity. To confirm the role of MLCK protein expression in the TNF-α increase in Caco-2 MLCK activity, the effect of cycloheximide on TNF-α increase in MLCK activity was also examined. The cycloheximide treatment (10 ng/ml) at a dose that inhibits MLCK protein synthesis (Fig. 2A) resulted in an inhibition of the TNF-α-induced increase in Caco-2 MLCK activity (Fig. 3B), suggesting that the increase in MLCK activity was due to the increase in MLCK protein synthesis.

To determine whether the TNF-α increase in Caco-2 MLCK activity was required for the TNF-α increase in Caco-2 TJ permeability, the effect of MLCK inhibitors ML-7 and ML-9 on TNF-α increase in MLCK activity and drop in Caco-2 epithelial resistance was examined (19, 25, 40). The MLCK inhibitors ML-7 (1 μM) and ML-9 (10 μM) (at doses shown to specifically inhibit MLCK activity) significantly inhibited the TNF-α increase in Caco-2 MLCK activity (Fig. 4A) and prevented the TNF-α-induced drop in Caco-2 epithelial resis-
TNF-α INCREASE IN INTESTINAL TIGHT JUNCTION PERMEABILITY

A

MLCK

β-actin

Time (hrs) 0 0.5 1 6 12 24 48

B

Epithelial Resistance (ohm cm²)

24 48

Time (h)

C

Mannitol flux (nmol/cm²)

0 24 48

Time (h)

Fig. 1. Time-course effect of TNF-α on Caco-2 MLCK protein expression, transepithelial resistance (TER), and paracellular permeability. Filter-grown Caco-2 monolayers were treated with TNF-α (10 ng/ml) for increasing time periods (0–48 h). TNF-α MLCK protein expression was determined by Western blot analysis as described in MATERIALS AND METHODS. The effect of TNF-α (10 ng/ml) on Caco-2 TER and mucosal-to-serosal flux of paracellular marker mannitol (10 μmol/ml) were measured sequentially over the 48-h experimental period as described in MATERIALS AND METHODS. A: time course effect of TNF-α on Caco-2 MLCK protein expression (β-actin was used as an internal control for protein loading). B: time course effect of TNF-α on Caco-2 TER (means ± SE, n = 4). C: time course effect of TNF-α on mucosal-to-serosal mannitol flux (means ± SE, n = 4).

Fig. 2. Effect of protein synthesis inhibitor cycloheximide on TNF-α-induced increase in MLCK protein expression (A) and drop in Caco-2 TER (B). Filter-grown Caco-2 monolayers were treated with TNF-α (10 ng/ml) for a 48-h experimental period. Cycloheximide (5 μM) significantly prevented the TNF-α (10 ng/ml)-induced increase in MLCK protein expression (A) and drop in Caco-2 TER (n = 4) (B). *P < 0.001 compared with the controls; **P < 0.001 compared with the TNF-α-treated monolayers.

Fig. 3. Effect of TNF-α on Caco-2 MLCK activity. TNF-α-induced increase in MLCK activity of filter-grown Caco-2 monolayers was determined by in vitro kinase activity measurements as described in MATERIALS AND METHODS. After appropriate experimental treatments, Caco-2 MLCK was isolated by immunoprecipitation with anti-MLCK antibody. Subsequently, MLCK activity of the immunoprecipitated MLCK was assessed by measuring the in vitro phosphorylation of MLC (P-MLC) over a 10-min reaction period. A: time course of TNF-α (10 ng/ml) effect on MLCK activity as assessed by the level of MLC phosphorylation by the immunoprecipitated MLCK. B: cycloheximide (5 μM) effect on TNF-α-induced increase in MLCK activity after 48-h experimental period. TNF-α treatment resulted in a time-dependent increase in Caco-2 MLCK activity and cycloheximide prevented the TNF-α-induced increase in Caco-2 MLCK activity.

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following studies the effect of Mg$^{2+}$-myosin ATPase inhibitor 2,3-butanone monoxide (BDM), metabolic inhibitors 2,4-dinitrophenol (DNP), and sodium azide on TNF-α-induced drop in Caco-2 epithelial resistance was examined (19, 25). DNP (2.5 mM) treatment resulted in a significant inhibition of the TNF-α-induced drop in Caco-2 epithelial resistance (Fig. 5A). The metabolic inhibitors DNP (30 μM) and sodium azide (0.2 mM) also prevented the TNF-α-induced drop in Caco-2 epithelial resistance (Figs. 5B). These findings further substantiated the role of MLCK pathways in the TNF-α-induced increase in Caco-2 TJ permeability.

Mechanism of TNF-α-induced increase in MLCK protein expression. In the following studies, the mechanism of TNF-α-induced increase in MLCK protein expression was examined. The TNF-α effect on MLCK mRNA transcription and MLCK protein degradation was determined by real-time PCR and [35S]methionine pulse-chase studies. The TNF-α treatment of filter-grown Caco-2 monolayers resulted in a significant increase in MLCK mRNA expression (Fig. 6A), suggesting that the increase in Caco-2 MLCK protein expression was related to the increase in MLCK transcription. The maximal increase in MLCK mRNA was seen between 2 and 4 h after TNF-α treatment. To verify the role of MLCK transcription in the TNF-α modulation of MLCK protein expression, the effect of RNA polymerase inhibitor actinomycin-D on MLCK protein expression was determined. As shown in Fig. 6B, actinomycin-D (10 ng/ml) inhibited the TNF-α increase in Caco-2 MLCK mRNA expression, indicating that actinomycin-D at the dose used (10 ng/ml) inhibited MLCK mRNA expression. Actinomycin-D (10 ng/ml) also prevented the TNF-α increase in MLCK protein expression (Fig. 7A), suggesting that the TNF-α-induced increase in MLCK protein expression was due to the increase in MLCK mRNA transcription. In separate experiments, the effect of actinomycin-D on TNF-α modulation of Caco-2 epithelial resistance was also determined (Fig. 7B). Actinomycin-D (10 ng/ml) significantly inhibited the TNF-α-induced drop in Caco-2 epithelial resistance (Fig. 7B), indicating the requirement of MLCK transcription in the modulation of the TNF-α increase in Caco-2 TJ permeability.

In subsequent studies, the TNF-α effect on MLCK protein degradation was determined by [35S]methionine pulse-chase studies. The filter-grown Caco-2 monolayers were treated with TNF-α (10 ng/ml) for increasing time periods (0, 6, 12, 24, 36, 48 h), and the rate of MLCK protein degradation was assessed. MLCK proteins were labeled with [35S]methionine, and TNF-α effect on MLCK protein degradation was assessed by immunoprecipitation of MLCK followed by quantitation of 35S-labeled MLCK present in the immunoprecipitated MLCK or by the gel electrophoresis. There was a progressive degradation of MLCK protein in the untreated Caco-2 cells (controls) during the 48-h experimental period (Fig. 8). TNF-α (10 ng/ml) treatment did not have significant effect on the rate of MLCK protein degradation as assessed by the pulse-chase experiments (Fig. 8).

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**Fig. 4.** Effect of MLCK inhibitors ML-7 and -9 on TNF-α-induced increase in Caco-2 MLCK activity (A) and drop in Caco-2 TER (B). Filter-grown Caco-2 monolayers were treated with TNF-α (10 ng/ml) for a 48-h experimental period. ML-7 (1 μM) and ML-9 (10 μM) significantly prevented the TNF-α (10 ng/ml)-induced increase in MLCK activity (A) and drop in Caco-2 TER (n = 4) (B). *P < 0.001 compared with the controls; **P < 0.001 compared with the TNF-α-treated monolayers.

**Fig. 5.** Effect of Mg$^{2+}$-myosin ATPase and metabolic inhibitors on TNF-α-induced drop in Caco-2 TER. Filter-grown Caco-2 monolayers were treated with TNF-α (10 ng/ml) and selected inhibitors for a 48-h experimental period. A: effect of Mg$^{2+}$-myosin ATPase inhibitor 2,3-butanone monoxide (BDM) (2.5 mM) on TNF-α (10 ng/ml)-induced drop in Caco-2 TER after the 48-h treatment period. B: effect of metabolic inhibitors 2,4-dinitrophenol (DNP) (30 μM) and sodium azide (0.2 mM) on TNF-α (10 ng/ml)-induced drop in Caco-2 TER after the 48-h treatment period. *P < 0.001 compared with the controls; **P < 0.001 compared with the TNF-α-treated monolayers.
DISCUSSION

The intracellular mechanisms involved in the TNF-α-induced increase in intestinal epithelial TJ permeability remain unclear. Previous studies from our laboratory (23) demonstrated a central role for the NF-κB in the TNF-α modulation of the intestinal epithelial TJ barrier function. In the present study, we investigated the possibility that the TNF-α-induced increase in intestinal epithelial TJ permeability was regulated by modulation of MLCK protein expression. In previous studies of acute pharmacological modulation of intestinal TJ permeability in Caco-2 intestinal epithelial cells, we showed that MLCK activation was required for the cytochalasin B and D, low extracellular Ca²⁺, or ethanol-induced increase in intestinal epithelial TJ permeability (19, 24, 25). Similarly, other investigators (9, 40, 45) have also shown that MLCK activation and the subsequent contraction of perijunctional actin/myosin filaments were necessary for the bacterial toxin, pathogenic bacteria, and Na⁺-glucose cotransport-induced increase in intestinal epithelial TJ permeability. In each of these TJ modulating systems, there was a rapid activation of MLCK followed by an increase in intestinal TJ permeability (19, 24, 25). We have also found that the acute increase in MLCK activity by the pharmacological agents was due to an increase in the specific activity of the preexisting MLCK mediated by an intracellular signaling process and not increased expression of the MLCK protein (19, 24, 25).

In the present study, we examined a novel hypothesis that the TNF-α increase in Caco-2 intestinal epithelial TJ permeability was regulated by an increase in MLCK protein expression. This hypothesis was formulated on the basis of our previous observations that the pharmacological agent-induced increase in Caco-2 intestinal TJ permeability occurred within minutes after the pharmacological treatment (19, 24, 25), whereas the TNF-α effect was delayed until ~24 h after the treatment (23), suggesting that the TNF-α effect on Caco-2 TJ permeability was not strictly due to an acute intracellular signaling process but involved a more complex sequence of events, perhaps requiring a new protein synthesis. Thus based on our studies showing the importance of MLCK activation in the modulation of intestinal epithelial TJ barrier function, we advanced the hypothesis that the TNF-α modulation of Caco-2 intestinal TJ barrier was regulated by an increase in MLCK protein expression. Our findings indicated that TNF-α at a clinically relevant dose (10 ng/ml) induces a sequential increase in Caco-2 MLCK protein expression (Fig. 1) (23). The time course of TNF-α increase in MLCK protein expression...
paralleled the increase in Caco-2 TJ permeability, and the inhibition of the TNF-α-induced increase in MLCK protein expression prevented the TNF-α increase in Caco-2 TJ permeability (Fig. 2). Together, these findings indicated that the TNF-α increase in Caco-2 TJ permeability was mediated, in part, by an increase in Caco-2 MLCK protein expression.

Subsequently, we assessed the mechanisms involved in the TNF-α-induced increase in Caco-2 MLCK protein expression. We considered the possibility that the TNF-α increase in MLCK protein expression may have resulted from an increase in MLCK mRNA transcription, a decrease in the rate of MLCK protein degradation, or combination of the two. We found that TNF-α produced a rapid increase in MLCK mRNA expression (Fig. 6A), and the inhibition of TNF-α increase in MLCK mRNA expression prevented the TNF-α increase in MLCK protein expression and the subsequent increase in Caco-2 TJ permeability. Moreover, TNF-α did not affect the rate of Caco-2 MLCK protein breakdown. In combination, these studies indicated that the TNF-α increase in MLCK protein expression and the subsequent increase in Caco-2 TJ permeability were mediated by an increase in MLCK transcription and not a decrease in MLCK protein breakdown.

Our data also indicated that the TNF-α increase in Caco-2 MLCK protein expression was associated with an increase in Caco-2 MLCK activity. The regulatory action of MLCK protein expression on MLCK activity was confirmed by the cycloheximide treatment studies (Fig. 3B), which indicated that the inhibition of the TNF-α increase in MLCK expression prevented the increase in Caco-2 MLCK activity. These findings suggested that the TNF-α increase in MLCK activity was a result of an increase in MLCK protein expression and not due to an increase in the specific activity of the preexisting MLCK protein. Thus the mechanism of TNF-α-induced increase in MLCK activity was different from the pharmacological agent (including cytochalasins, ethanol, and low extracellular Ca2+-induced increase in MLCK activity, which resulted from an increase in the specific activity of the preexisting MLCK protein (19, 24, 25). Our results also confirmed that the TNF-α-induced increase in MLCK activity was required for the increase in Caco-2 TJ permeability. It had been previously demonstrated in muscle cells that MLCK activation leads to the enzymatic phosphorylation of MLCK. The MLCK phosphorylation then leads to the activation of Mg2+-myosin ATPase, which provides the metabolic energy required for the mechanical contraction of actin and myosin filaments (1, 13, 14).

Other intracellular mechanisms that may be involved in the TNF-α regulation of intestinal TJ permeability include NF-κB activation and the induction of apoptosis (25). Studies from our laboratory (23) suggested that the TNF-α-induced increase in Caco-2 TJ permeability was mediated, in part, by activation of the NF-κB pathways. Our data indicated that the TNF-α increase in Caco-2 TJ permeability was accompanied by an increase in NF-κB activation, and inhibition of NF-κB activation prevented the TNF-α increase in Caco-2 TJ permeability (23). Thus NF-κB activation was required for the TNF-α-induced opening of the Caco-2 TJ barrier. In 1996, Peralta Soler et al. (29a) reported the possibility that the TNF-α increase in LLC-PK1 renal epithelial TJ permeability may be related to an increase in apoptosis. They found that the TNF-α increase in LLC-PK1 TJ permeability was associated with a small but significant increase in apoptosis (an increase in apoptosis from a baseline of 0.66 to 1.79% after TNF-α treatment) and suggested that the increase in apoptosis may be involved in the increase in LLC-PK1 epithelial TJ permeability. In a subsequent study (26), the same group reported that the TNF-α increase in Caco-2 TJ permeability was not associated with an increase in apoptosis in the Caco-2 BBE cells, and concluded that the TNF-α increase in Caco-2 TJ permeability was not due to apoptosis. Consistent with this report, we also found that the TNF-α increase in Caco-2 TJ permeability was not associated with an increase in apoptosis, and also concluded that apoptosis was not the mechanism involved in the TNF-α increase in Caco-2 TJ permeability (23). Schmitz et al. (35) also found no correlation between cytokine-induced apoptosis and increase in TJ permeability in HT-29/B6 intestinal epithelial cells, and concluded that the cytokine increase in intestinal TJ permeability was not due to apoptosis. In contrast, Gitter et al. (7) using single cell conductance experiments in Caco-2.
HT-29 cells suggested the possibility that single cells undergoing apoptosis may account for the alteration in epithelial TJ permeability. In their study (7), they reported that 1–2% of the cells underwent apoptosis after TNF-α treatment. In our previous report in Caco-2 cells, we showed that TNF-α caused a diffuse alteration in TJ protein localization in the majority of the cells in the visual field, and concluded that the TNF-α-induced increase in Caco-2 TJ permeability was due to a diffuse change in the TJ protein complex (23).

There have been varying conclusions regarding the role of MLCK in the cytokine-induced alteration of intestinal epithelial TJ permeability. Ferrier et al. (6) found that repeated stress (mixed restraint and acoustic stress) produced an “overexpression” of colonic IFN-γ and an increase in colonic permeability in mice. The stress did not cause an increase in colonic permeability in the IFN-γ deficient mice, suggesting that the IFN-γ upregulation was required for the increase in colonic permeability (6). The repeated stress did not affect the MLCK protein level but caused an increase in the level of phosphorylated MLC; ML-7 prevented the stress-induced increase in colonic permeability and bacterial translocation. Thus Ferrier et al. (6) concluded that the stress-induced increase in intestinal permeability was mediated by an IFN-γ-induced MLCK activation without a change in the MLCK protein level. Consistent with these studies, Zolotarevsky et al. (47) also reported that the IFN-γ (100 U/ml) and TNF-α (100 ng/ml) combination-induced drop in TER in Caco-2 BBE monolayers was associated with an increase in phosphorylated MLC. They found that the membrane-permeant peptide inhibitor of MLCK prevented the IFN-γ/TNF-α combination-induced drop in TER and increase in MLC phosphorylation, suggesting that MLC phosphorylation was required for the IFN-γ/TNF-α-induced increase in Caco-2 TJ permeability (47). Petrace et al. (30) also found TNF-α (20 ng/ml) to cause an increase in endothelial TJ permeability in pulmonary artery endothelial cells. The TNF-α increase in endothelial permeability was associated with an increase in MLC phosphorylation and apoptosis. They found that the treatment of the endothelial cells with MLCK and Rho kinase inhibitors prevented the TNF-α increase in MLC phosphorylation and endothelial apoptosis, but not the increase in endothelial permeability. Thus these investigators concluded that in the pulmonary endothelial cells MLC phosphorylation was responsible for the TNF-α mediated apoptosis but not the increase in endothelial permeability.

Based on our present data combined with our published report (23) showing the central role of NF-κB in the TNF-α modulation of Caco-2 TJ barrier, we propose a novel hypothesis that the TNF-α increase in intestinal TJ permeability requires NF-κB-mediated activation of MLCK promoter. We propose that the TNF-α-induced NF-κB activation results in: 1) cytoplasmic-to-nuclear translocation of NF-κB, 2) binding of the activated NF-κB to the MLCK promoter region and upregulation of MLCK promoter activity, 3) increase in MLCK mRNA transcription and MLCK protein expression, 4) increase in MLCK activity with activation of MLCK pathway, 5) MLCK activated contraction of perijunctional actin-myosin filaments, and 6) alteration of TJ protein localization and expression and functional opening of the intestinal TJ barrier. To validate this hypothesis, we have recently initiated studies to examine the molecular interaction between NF-κB and the MLCK promoter.

In conclusion, our data indicated for the first time that the TNF-α-induced increase in Caco-2 TJ permeability required upregulation of MLCK protein expression. The TNF-α increase in Caco-2 MLCK protein expression was mediated by an increase in MLCK mRNA transcription. The TNF-α-induced increase in MLCK protein expression produced a stepwise increase in MLCK activity, activation of MLCK pathway, and functional opening of the Caco-2 TJ barrier. Thus our findings provide an important new insight into the mechanism of the TNF-α modulation of the intestinal epithelial TJ barrier.

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