TNF-α-induced increase in intestinal epithelial tight junction permeability requires NF-κB activation


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INTACT INTESTINAL EPITHELIAL barrier is essential in preventing intestinal penetration of luminal antigens (21, 26, 30). The epithelial structures that form the intestinal epithelial barrier are the apical plasma membrane and the intercellular tight junctions (TJs) of the enterocytes. The bilipid composition of the enterocyte membrane provides a diffusion barrier against transcellular permeation of large, water-soluble molecules. The intercellular TJs provide the paracellular barrier function by forming an extracellular seal across the intercellular spaces between adjacent cells. The TJs act as a physical and functional barrier against the paracellular penetration of noxious substances present in the lumen (including bacteria, bacterial toxins, bacterial byproducts, digestive enzymes, and degraded food products) (1, 21, 26, 30). The disruption of the intestinal TJ barrier results in a “leaky” TJ barrier and leads to an increase in intestinal permeability. The leaky intestinal TJs allow paracellular permeation of luminal antigens that promote intestinal inflammation (1, 21, 23, 26).

It is well established that patients with Crohn’s disease (CD) have a defective intestinal TJ barrier, manifested by an increase in intestinal permeability (21–24, 26, 33, 54). The defective intestinal TJ barrier has been proposed as a primary etiologic factor of CD (21, 24, 26, 33). Numerous studies have shown CD patients to have an abnormal increase in intestinal permeability to paracellular markers including lactulose, PEG-400, 51Cr-EDTA, and cellobiose (22–24, 26, 33, 54). Additionally, it has been demonstrated that 10–30% of healthy first-degree relatives of CD patients (an at-risk population for CD) also have an increase in intestinal permeability (23, 24, 33, 60).

Recent studies have shown TNF-α to play a central role in the intestinal inflammation of CD (38, 44, 46, 52, 66). The TNF-α levels are markedly elevated in patients with CD including in serum, stool, and intestinal tissue (6, 37, 56). The increased levels of TNF-α lead to increased production and activation of other proinflammatory cytokines and factors that promote intestinal inflammation (38, 44, 56). The central importance of TNF-α in intestinal inflammation in CD has been well validated by clinical studies that show anti-TNF-α antibody to be an effective agent in the treatment of active CD (21, 46, 52, 56). Consistent with the clinical studies, anti-TNF-α antibody has also been shown to be effective in the
treatment of animal models of intestinal inflammation (38, 41, 59).

In addition to activating the endogenous inflammatory cascade, a potential proinflammatory action of TNF-α in CD includes the alteration of intestinal epithelial TJ barrier. TNF-α has been shown to produce an increase in epithelial TJ permeability in various cell types, including in intestinal epithelial cells (17, 35, 36, 45). Thus elevated TNF-α levels could be an important factor contributing to the intestinal TJ permeability defect in CD patients. The intracellular mechanisms involved in TNF-α modulation of intestinal TJ barrier remain unclear. Understanding the intracellular mechanisms involved could be important in devising future therapeutic strategies to induce retightening of the leaky TJ barrier. The purpose of this study was to determine some of the intracellular mechanisms involved in TNF-α modulation of intestinal epithelial TJ barrier by using an in vitro intestinal epithelial model system consisting of filter-grown Caco-2 monolayers (27, 29). Because many of the proinflammatory actions of TNF-α are mediated by NF-κB pathways (9, 39, 43), we tested the hypothesis that the TNF-α increase in intestinal TJ permeability was regulated by NF-κB activation. We also examined the involvement of apoptosis in mediating the TNF-α-induced increase in Caco-2 TJ permeability. Our results indicate for the first time that the TNF-α-induced increase in Caco-2 TJ permeability is regulated by NF-κB activation. Additionally, our results also indicate that apoptosis is not the mechanism involved in the TNF-α-induced increase in Caco-2 TJ permeability.

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, curcumin, and triptolide were purchased from Sigma (St. Louis, MO). Transwell permeable filters were purchased from Corning (Corning, NY). Anti-sonal occludens (ZO-1) antibody, anti-NF-κB p65 antibody, and FITC-streptavidin were obtained from Zymed Laboratories (San Francisco, CA), and fluorescein-conjugated rabbit anti-rat antibodies were obtained from Boehringer Mannheim (Indianapolis, IN). [14C]mannitol and [14C]insulin were obtained from NEN Research Products (Wilmington, DE). All other chemicals were of reagent grade.

Cell cultures. Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD). 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 mmol/l glutamine, and 10% FBS (27, 42). Culture medium was changed every 2 days. The cells were subcultured by partial digestion with 0.25% trypsin and 0.9 mmol/EDTA in Ca2+-free and Mg2+-free PBS. For growth on filters, high-density Caco-2 cells (1 x 106 cells) were plated on Transwell filters and monitored regularly by visualization with an inverted microscope and by epithelial resistance measurements.

Determination of epithelial monolayer resistance and paracellular permeability. The electrical resistance of the filter-grown Caco-2 intestinal monolayers was measured by using an epithelial volthometer (World Precision Instruments, Sarasota, FL) as previously reported (27, 29). For resistance measurements, both apical and basolateral sides of the epithelium were bathed with buffer solution. Electrical resistance was measured until similar values were recorded on three consecutive measurements. The resistances of monolayers are reported after subtraction of the resistance value of the filters alone. The effect of TNF-α on Caco-2 monolayer paracellular permeability was determined by using an established paracellular marker mannotol or inulin (25, 28). For determination of mucosal-to-serosal flux rates of the paracellular probes mannotol and inulin, Caco-2-plated filters having epithelial resistance of 400–550 Ω·cm² were used. The filter-grown Caco-2 monolayers reached epithelial resistance of 400–550 Ω·cm² by 3 wk postplating (27, 42). 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 was added to the basolateral compartment to ensure equal hydrostatic pressure as recommended by the manufacturer. Known concentrations of permeability marker (10 μmol/l) 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. Test reagent was added to both the apical and the basolateral compartments as indicated. All flux studies were carried out at 37°C. All of the experiments were repeated three to five times to ensure reproducibility.

Fluorescein localization of ZO-1 proteins. Cellular localization of the TJ protein ZO-1 was assessed by an immunofluorescent antibody-labeling technique as previously described (27, 29). Caco-2 monolayers grown on Transwell filters were fixed with 2.0% formaldehyde and permeabilized in acetone at −20°C for 5 min. The Caco-2 monolayers were then labeled with rabbit anti-ZO-1 antibody; this was followed by incubation with 1:30 dilution of Triton-X-100 containing secondary anti-rabbit IgG biotinylated antibody (Zymed Laboratories) and incubation with 1:20 diluted Tris-buffered saline solution containing FITC-labeled streptavidin (Zymed Laboratories).

Assessment of ZO-1 protein expression by immunoprecipitation and Western blot analysis. Filter-grown Caco-2 monolayers were serum deprived overnight. Caco-2 monolayers were then treated with appropriate experimental reagents for the 48-h treatment period. At the end of the experimental period, Caco-2 monolayers were washed twice with cold HBSS, and cells were lysed with 500 μl lysis buffer (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM b-glycerophosphate, 200 μM vanadate, 100 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 40 mM paraaminophenyl phosphate, 1 μg/ml aprotinin, and 0.5% NP-40) and scraped, and the cell lysates were then labeled with rabbit anti-ZO-1 antibody; this was followed by incubation with 1:30 dilution of Tris-buffered saline solution containing the antibody-bead complex. The ZO-1 antibody-bead complex was washed twice with lysis buffer. One hundred microliters of each cleared lysate was then added to the pellet; antibody-bead complex and incubated end-over-end for 2 h at 4°C to form the antibody-bead complex. The ZO-1 antibody-bead complex was microfuged for 5 min at 7,000 g to yield a clear lysate. ZO-1 antibody was diluted (1:50), and 200 μl was added to the Microfuge tubes containing the protein A-Sepharose beads. ZO-1 antibody and protein A-Sepharose bead mixture were incubated end-over-end for 2 h at 4°C to form the antibody-bead complex. The ZO-1 antibody-bead complex was washed twice with lysis buffer. One hundred microliters of each cleared lysate was then added to the pellet; antibody-bead complex and incubated end-over-end for 2 h at 4°C. Subsequently, the tubes were microfuged and the supernatants were aspirated. Pellets were washed twice with lysis buffer. Next, 50 μl of gel loading buffer was added to the pellets. The tubes were boiled for 5 min at 95°C, after which ZO-1 proteins were separated on a 7% SDS-PAGE gel. The gel was transblotted against anti-ZO-1 antibody by using the Amersham Western blot kit.

Assessment of apoptosis and cell death. The effect of TNF-α on Caco-2 apoptosis was assessed by immunostaining of poly(ADP-ribose) (PAR) and by annexin V-FITC labeling (7, 10, 11, 13, 29, 53). For PAR staining studies, filter-grown Caco-2 monolayers were exposed to TNF-α (10 ng/ml) for increasing time periods (0, 24, and 48 h). Subsequently, Caco-2 monolayers were labeled with mouse anti-PAR antibody (Alexis Biochemicals, Carlsbad, CA) followed by labeling with Cy3-conjugated donkey anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA). The Caco-2 apoptosis was then quantitated by assessing the relative intensity of PAR staining by using the Nikon-PCM 2000 confocal imaging system and image analysis software. Filter-grown Caco-2 monolayers were treated with TNF-α (10 ng/ml) for varying time periods (0, 6, 24, and 48 h). Subsequently, Caco-2 cells were trypsinized and assessed for...
apoptosis by using the annexin V-FITC apoptosis detection kit II from BD Sciences Pharmingen (San Diego, CA). Annexin V-FITC was used to stain for the apoptotic cells, and propidium iodide (PI) was used to stain the necrotic cells (10, 11, 53). Apoptosis was measured by flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA). Fluorescence of all dyes was excited with the 488-nm line of an argon ion laser. Fluorescence emission was detected in the FL-1 channel (530 ± 15 nm) for cells labeled with annexin V-FITC and in the FL-2 channel (585 ± 21 nm) for cells labeled with PI; subsequently, a FL-1/FL-2 dot plot was generated. For each experimental sample, a total of 20,000 cells were counted for annexin V-FITC and PI stain. Camptothecin was used as a positive control to induce apoptosis in Caco-2 cells. Each apoptosis experiment was carried out in triplicate and repeated three times to ensure reproducibility.

In vitro determination of nuclear NF-κB binding to the DNA binding site. The binding of the nuclear NF-κB to a DNA binding site on oligonucleotide probe was assessed by EMSA as previously described (2, 16, 20). Oligonucleotide containing the consensus NF-κB binding sequence (5'-AGTTGAGGGGACTTTCAGGC-3') was labeled with [32P]ATP and T4 polynucleotide kinase as described (2, 16, 20). The filter-grown Caco-2 monolayers were treated with TNF-α for 30 min, and the nuclear extracts were harvested as previously described (20). Briefly, cells were washed with PBS and resuspended in a low-salt buffer and allowed to swell. The cells were lysed with 15 passes through a 25-gauge needle, and cell nuclei were separated by centrifugation. The cell nuclei were then placed in a high-salt buffer to extract the nuclear proteins. Equal amounts of nuclear proteins were incubated with the [32P]-labeled oligonucleotide NF-κB probe for 20 min and then run on a 4% polyacrylamide gel at 100 V. Gels were scanned and quantitated using the Storm phosphorimaging system. Binding specificity was then assessed by the addition of specific unlabeled oligonucleotide NF-κB probe (competition assay) as described (20).

Statistical analysis. Results are expressed as means ± SEM. Statistical significance of differences between mean values was assessed by Student’s t-tests for unpaired data. All reported significance levels represent two-tailed P values. A P value < 0.05 was used to indicate statistical significance. All experiments were repeated a minimum of 3 times to ensure reproducibility.

RESULTS

Effect of TNF-α on Caco-2 intestinal epithelial TJ permeability. TNF-α treatment of filter-grown Caco-2 monolayers (0–100 ng/ml) produced a concentration-dependent drop in Caco-2 transepithelial resistance (TER) during the 48-h experimental period (Fig. 1). TNF-α was added to both apical and basolateral compartments. The maximum drop in Caco-2 TER occurred at a TNF-α concentration of 10 ng/ml, and increasing TNF-α concentration above 10 ng/ml did not produce a further drop in Caco-2 TER. The time-course effect of TNF-α (10 ng/ml) on Caco-2 TER over the 96-h treatment period is shown in Fig. 2A. There was a progressive time-dependent drop in Caco-2 TER over the first 48 h of treatment. The TNF-α exposure beyond 48 h produced only a small additional drop in TER. Most of the drop in TER occurred between 24 and 48 h. The TNF-α effect on Caco-2 paracellular permeability was also examined by using the paracellular markers mannitol and inulin. TNF-α (10 ng/ml) produced a progressive time-dependent increase in transepithelial permeability (TER) to mannitol and inulin (Fig. 2, B and D). The TNF-α exposure beyond the 48-h time period did not produce further increase in paracellular flux rates of inulin or mannitol. The plot of the effect of TNF-α on Caco-2 TER vs. TEP revealed a linear relationship (r = 0.98) between TNF-α decrease in Caco-2 TER and increase in TEP (Fig. 2, C and E), indicating that the TNF-α-induced drop in TER directly correlates with an increase in paracellular permeability. Subsequently, the membrane specificity of the effect of TNF-α on Caco-2 TJ permeability was determined. TNF-α (10 ng/ml) was selectively added to the basolateral, apical, or combined apical and basolateral membrane compartments. The apical treatment of TNF-α did not have significant effect on Caco-2 TER (Fig. 3). In contrast, the addition of TNF-α to the basolateral compartment produced a significant drop in Caco-2 TER. The extent of the TER drop was similar whether TNF-α was added to the basolateral compartment alone or to both basolateral and apical compartments together. These findings indicated that TNF-α action at the basolateral membrane compartment was required and sufficient for the TNF-α-induced opening of the Caco-2 TJ barrier. Unless stated otherwise, in all subsequent studies, TNF-α was added to the basolateral compartment only.

TNF-α-induced increase in Caco-2 TJ permeability requires NF-κB activation. In the following studies, we examined the possible involvement of NF-κB pathways in TNF-α-induced increase in Caco-2 TJ permeability. The effect of TNF-α on Caco-2 NF-κB activation was determined by cytoplasmic-to-nuclear translocation of NF-κB subunit p65 using immunofluorescent antibody labeling of the p65 subunit (19). As shown in Fig. 4, in the control monolayers, NF-κB p65 subunits were present mostly in the cytoplasm, with minimal localization in the nucleus. TNF-α (10 ng/ml) produced a rapid translocation of NF-κB p65 into the nucleus, and by 30 min, most of the NF-κB p65 had translocated into the nucleus (Fig. 4B). The nuclear translocation was accompanied by a corresponding decrease in NF-κB p65 in the cytoplasm (Fig. 4B). Thus TNF-α-induced increase in Caco-2 TJ permeability correlated with a rapid activation of NF-κB in the Caco-2 cells. Consistent with the above epithelial resistance studies, apical treatment of TNF-α (10 ng/ml) did not have any significant effect on NF-κB p65 nuclear translocation (data not shown). To determine whether NF-κB activation was a prerequisite for the TNF-α-induced increase in Caco-2 TJ permeability, the...
The effect of NF-\(\kappa\)B inhibition on Caco-2 TJ permeability was examined. The TNF-\(\alpha\)-induced activation of NF-\(\kappa\)B pathways was prevented by NF-\(\kappa\)B inhibitors curcumin and triptolide. Curcumin is an upstream inhibitor of NF-\(\kappa\)B and prevents the activation of inhibitory \(\kappa\)B (IkB) kinase (IKK), an enzyme required for the activation of NF-\(\kappa\)B (49). Triptolide inhibits NF-\(\kappa\)B activity further downstream by interfering with the NF-\(\kappa\)B-mediated transcription process (42, 62). Curcumin pretreatment (5 \(\mu\)M) inhibited the TNF-\(\alpha\) (10 ng/ml)-induced activation and nuclear translocation of NF-\(\kappa\)B p65 in Caco-2 cells (Fig. 4C). Curcumin (5 \(\mu\)M) also prevented the TNF-\(\alpha\)-induced drop in Caco-2 TER (Fig. 5A), indicating that NF-\(\kappa\)B activation was required for the TNF-\(\alpha\)-induced increase in Caco-2 TJ permeability. Triptolide (1 \(\mu\)M) pretreatment also prevented the TNF-\(\alpha\)-induced drop in Caco-2 TER, further confirming the requirement of activation of NF-\(\kappa\)B pathways in this process (Fig. 5B).

The above studies indicated that the TNF-\(\alpha\)-induced increase in Caco-2 TJ permeability was dependent on activation of NF-\(\kappa\)B pathways. In the following studies, we examined the functional relevance of cytoplasmic-to-nuclear translocation of

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Fig. 2. Time-course effect of TNF-\(\alpha\) on Caco-2 epithelial resistance and paracellular permeability. The effect of TNF-\(\alpha\) (10 ng/ml) on Caco-2 epithelial resistance and mucosal-to-serosal flux of paracellular markers mannitol (10 \(\mu\)mol/ml) and inulin (10 \(\mu\)mol/ml) were measured sequentially over the 96-h experimental period. A: time-course effect of TNF-\(\alpha\) on Caco-2 epithelial resistance (\(n = 4\)). B: time-course effect of TNF-\(\alpha\) on mannitol flux (\(n = 4\)). C: graph of epithelial resistance vs. mannitol flux (\(r = 0.98\)). D: time-course effect of TNF-\(\alpha\) on inulin flux (\(n = 4\)). E: graph of epithelial resistance vs. inulin flux (\(r = 0.98\)).

Fig. 3. Membrane specificity of the effect of TNF-\(\alpha\) on Caco-2 epithelial resistance. TNF-\(\alpha\) (10 ng/ml) was added to either apical (AP), basolateral (BL), or combined apical and basolateral compartments for the 48-h experimental period. Addition of TNF-\(\alpha\) to the basolateral or combined basolateral and apical compartments produced a significant drop in Caco-2 epithelial resistance (\(n = 4\)). *\(P < 0.01\).

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NF-κB by assessing NF-κB binding to the DNA by EMSA as previously described (2, 16, 20). In the following studies, Caco-2 nuclear proteins were extracted after TNF-α treatment and allowed to bind to the DNA binding site on a 32P-labeled oligonucleotide DNA probe having the consensus NF-κB binding motif (2, 16, 22). NF-κB binding to the 32P-labeled oligonucleotide DNA probe was then assessed by EMSA as previously described (20). TNF-α treatment of filter-grown Caco-2 cells resulted in a significant increase in NF-κB binding to the 32P-labeled oligonucleotide DNA probe (Fig. 6). To confirm the specificity of NF-κB binding to the 32P-labeled oligonucleotide DNA probe, excess (100-fold higher concentration) "cold" or nonradioactive labeled oligonucleotide DNA probe was added to the reaction mixture (20). The addition of excess (100:1 ratio) cold oligonucleotide DNA probe almost completely inhibited NF-κB binding to the 32P-labeled oligonucleotide DNA probe, indicating a competitive inhibition process (Fig. 6). These findings confirmed that TNF-α activation and nuclear translocation of NF-κB leads to an increase in NF-κB binding to the DNA binding site.

Effect of TNF-α on Caco-2 apoptosis. TNF-α is known to cause apoptosis in various cell types. Thus the possibility exists that the effect of TNF-α on Caco-2 TJ permeability may be due to apoptosis. Several groups have examined this possibility but have reached opposite conclusions (18, 32, 47, 51). To determine the possible role of apoptosis in the TNF-α-induced increase in Caco-2 TJ permeability, the effect of TNF-α on apoptosis was examined. The effect of TNF-α on Caco-2 apoptosis was determined by immunostaining of PAR and by labeling apoptotic cells with annexin V-FITC. During apoptosis, PAR production markedly increases due to activation of PAR polymerase (7, 13). The increase in PAR production is a positive indication of apoptosis (7, 13). TNF-α (10 ng/ml) treatment did not cause a significant increase in PAR production in filter-grown Caco-2 monolayers as assessed by PAR staining (Table 1). In separate experiments, the effect of TNF-α on Caco-2 apoptosis was also assessed by labeling of apoptotic cells with annexin V-FITC. In early stages of apoptosis, phosphatidylserine located on the inner (cytoplasmic) leaflet of the plasma membrane in healthy cells translocates to the outer leaflet (or external surface) of the membrane. The translocation of phosphatidylserine to the external surface of the plasma membrane allows annexin V-FITC to bind directly to cells undergoing apoptosis (10, 11, 53). Thus cells undergoing apoptosis stain positive for annexin V-FITC. The mean basal rates of apoptosis in control Caco-2 cells as determined by flow cytometry detection of annexin V-FITC-labeled cells ranged between 0.5 and 3% (n = 9). TNF-α treatment for increasing time periods (0, 6, 24, and 48 h) did not have a significant effect on Caco-2 cell apoptosis (P < 0.05; Table 2). These findings in combination indicate that TNF-α does not induce apoptosis in Caco-2 cells.

TNF-α downregulates ZO-1 protein expression and alters junctional localization of ZO-1 proteins via a NF-κB-depen-
Caco-2 monolayers, ZO-1 proteins were localized at the apical cellular junctions and appeared as continuous belt-like structures encircling the cells at the cellular borders (Fig. 9). TNF-α (10 ng/ml) caused a progressive disturbance in the continuity of ZO-1 localization at the cellular borders. By 48 h, ZO-1 localization at the cellular junctions were characterized by zig-zagging appearance and discrete punctate or gaplike appearance at points of multiple cellular contact. Curcumin (5 μM) prevented the TNF-α-induced disturbance of ZO-1 localization at cellular junctions (Fig. 9). Similarly, triptolide also prevented the TNF-α-induced disturbance of ZO-1 localization (Fig. 9). These findings indicated that the TNF-α-induced increase in Caco-2 TJ permeability correlates with NF-κB-dependent downregulation of ZO-1 protein expression and alteration in ZO-1 localization.

**DISCUSSION**

The intestinal epithelial TJs provide the paracellular barrier function against epithelial permeation of toxic luminal antigens. The breach in the TJ barrier allows intestinal permeation of luminal antigens and substances that promote intestinal inflammation (21, 26, 30). The defect in intestinal TJ barrier in patients with CD is well documented and has been proposed to be an etiologic factor of CD (21, 23, 26). Since TNF-α levels are markedly elevated in CD, TNF-α-induced alteration of intestinal TJ barrier could be an important contributing factor in the intestinal permeability defect of CD. However, the intracellular mechanisms involved in the TNF-α-induced increase in intestinal TJ permeability remains unknown. Understanding the mechanisms that lead to the abnormal increase in

**Table 1. Effect of TNF-α on Caco-2 cell apoptosis as assessed by immunolabeling of poly(ADP-ribose)**

<table>
<thead>
<tr>
<th>Relative Intensity of PAR labeling</th>
<th>P value vs. Control</th>
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<tbody>
<tr>
<td>Control (14 h)</td>
<td>373.9±28.2</td>
</tr>
<tr>
<td>TNF-α (24 h)</td>
<td>345.2±24.2</td>
</tr>
<tr>
<td>TNF-α (48 h)</td>
<td>337.3±16.2</td>
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Values are means ± SE. The effect of TNF-α on Caco-2 apoptosis was assessed by immunolabeling of poly(ADP-ribose) as described in MATERIALS and METHODS. The filter-grown Caco-2 monolayers were exposed to TNF-α (10 ng/ml) for 24 and 48 h. Caco-2 monolayers were labeled with anti-PAR antibody, and apoptosis was determined by the intensity of PAR labeling in high power (×40 objective lens) fields (n = 10).

**Table 2. Effect of TNF-α on Caco-2 cell apoptosis as assessed by annexin V-FITC labeling**

<table>
<thead>
<tr>
<th>% Cells Undergoing Apoptosis</th>
<th>P value vs. Control</th>
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</thead>
<tbody>
<tr>
<td>Control (6 h)</td>
<td>1.49±0.41</td>
</tr>
<tr>
<td>TNF-α (6 h)</td>
<td>1.55±0.63</td>
</tr>
<tr>
<td>Control (24 h)</td>
<td>2.65±1.44</td>
</tr>
<tr>
<td>TNF-α (24 h)</td>
<td>2.78±0.82</td>
</tr>
<tr>
<td>Control (48 h)</td>
<td>2.21±0.86</td>
</tr>
<tr>
<td>TNF-α (48 h)</td>
<td>1.58±0.81</td>
</tr>
<tr>
<td>Camptothecin (48 h)</td>
<td>54.22±3.41</td>
</tr>
</tbody>
</table>

Values are means ± SE. The effect of TNF-α on Caco-2 apoptosis was assessed by annexin-V-FITC labeling as described in MATERIALS and METHODS. The filter-grown Caco-2 monolayers were exposed to TNF-α (10 ng/ml) for increasing (6, 24, and 48 h) time periods. Subsequently, Caco-2 cells were trypsinized and labeled with annexin-V-FITC (apoptosis) or propidium iodide (necrosis). Caco-2 apoptosis (positive stain for annexin-V-FITC) was detected by using a FACScan flow cytometer. Total of 20,000 cells were counted for each experimental point (N = 3). Camptothecin was used as a positive control for apoptosis. The experiment was repeated 3 times to ensure reproducibility.

**Fig. 6.** EMSA of NF-κB binding to the 32P-labeled oligonucleotide DNA probe containing the DNA binding site. Filter-grown Caco-2 monolayers were treated with either buffer solution or buffer solution containing TNF-α (10 ng/ml) for 30 min. Subsequently, Caco-2 nuclear proteins were extracted and binding to 32P-labeled oligonucleotide probe containing the consensus NF-κB binding site (5’-AGTTGAGGGGACTTTCCAGGC-3’) was assayed by EMSA as described in MATERIALS and METHODS. Binding of nuclear proteins from control monolayers (lane 1) and from TNF-α-treated monolayers (lane 2) to the 32P-labeled oligonucleotide probe is shown. Inhibition of NF-κB binding to the 32P-labeled oligonucleotide probe by addition of excess unlabeled oligonucleotide probe (100:1 ratio of unlabeled vs. 32P-labeled oligonucleotide probe) to the nuclear extracts obtained from control monolayers (lane 3) and TNF-α-treated monolayers (lane 4) is also shown. TNF-α caused a marked increase in NF-κB binding to the 32P-labeled oligonucleotide probe, and the addition of 100-fold excess unlabeled oligonucleotides inhibited NF-κB binding to the 32P-labeled oligonucleotide probe. The experiments were run in duplicate and repeated 6 times to ensure reproducibility.

**Fig. 7.** Time-course effect of TNF-α on Caco-2 cell apoptosis as assessed by annexin V-FITC labeling.
intestinal TJ permeability will be important in devising therapeutic approaches to retighten the leaky TJ barrier in CD and other intestinal permeability disorders. Our results demonstrate for the first time that TNF-α-induced increase in intestinal epithelial TJ permeability is mediated by NF-κB activation.

NF-κB is a transcription factor that plays a key role in immunity and inflammation. It is an essential factor that controls gene expression of various proinflammatory cytokines, chemokines, cell adhesion molecules, and acute-phase proteins (4, 9, 40). NF-κB also plays a crucial role in the maturation of the immune system, including development of B lymphocytes, T lymphocytes, thymocytes, dendritic cells, and macrophages (3, 48). NF-κB in resting or unstimulated cells is localized mostly in the cytoplasm in an inactive state bound to the inhibitory protein IκB (9). On stimulation by an external stimulus, upstream protein kinase IKK is activated, which catalyzes phosphorylation of IκB and leads to ubiquination and subsequent degradation of IκB by proteasomes (12). The degradation of IκB leads to activation and nuclear translocation of NF-κB. In the nucleus, NF-κB binds to the DNA binding sites having the NF-κB motif GGGRNNYCC, where R is purine and Y is pyrimidine (9). The binding of NF-κB to the DNA promoter region leads to either up- or downregulation of the target gene transcription and translation (9). Thus NF-κB acts as a genetic switch controlling the target gene transcription.

In this study, we show that TNF-α-induced increase in Caco-2 TJ permeability was dependent on NF-κB activation. TNF-α caused a rapid activation of NF-κB with cytoplasmic-to-nuclear translocation of NF-κB in Caco-2 cells. The nuclear translocation of NF-κB occurred within minutes of TNF-α treatment. However, there was a delay of ~24 h between TNF-α activation of NF-κB and the functional increase in TJ permeability. The delay in the time course between NF-κB nuclear translocation and the functional opening in TJ permeability suggested that NF-κB-dependent transcription may be required for the TNF-α modulation of the TJ barrier. Consistent with the requirement of transcription in this process, actinomycin D (a transcription inhibitor) prevented the TNF-α-induced increase in Caco-2 TJ permeability (data not shown). The studies with NF-κB inhibitors confirmed the requirement of NF-κB activation in the TJ opening process. Curcumin (which inhibits NF-κB activation by preventing IKK activation and subsequent degradation of IκB) prevented the TNF-α activation and nuclear translocation of NF-κB and the increase in TJ permeability, indicating the requirement of NF-κB activation in this process. Similarly, triptolide [which inhibits the NF-κB-induced transcription process (42, 62)] also inhibited the TNF-α-induced increase in Caco-2 TJ permeability. TNF-α activation and nuclear translocation of Caco-2 NF-κB were associated with an increase in NF-κB binding to the DNA binding site on the oligonucleotide probe, confirming that TNF-α activation of NF-κB leads to an increase in NF-κB binding to the DNA.
Our data also indicated that TNF-α-induced increase in Caco-2 TJ permeability was associated with downregulation of ZO-1 protein expression. The TNF-α-induced decrease in ZO-1 protein expression was accompanied by an alteration in ZO-1 junctional localization. The TNF-α modulation of ZO-1 protein expression and junctional localization were prevented by NF-κB inhibitors, also indicating the requirement of NF-κB activation in this process. These findings show for the first time that NF-κB has a modulatory action on ZO-1 protein expression and localization. These findings raise the possibility that the genes regulating the TJ proteins may be targeted by NF-κB. Since ZO-1 proteins are an integral component of other junctional complexes, including adherens junctions and gap junctions, TNF-α modulation of ZO-1 proteins may also affect these junctions. Although the precise molecular and intracellular mechanisms involved in TNF-α regulation of ZO-1 protein expression remain to be defined, there are several possibilities. One possible scenario may be that, following TNF-α activation, NF-κB binds to the DNA binding site on or near the ZO-1 promoter region, leading to the downregulation of ZO-1 protein expression. A second possibility is that the activated NF-κB induces transcription and translation of certain target genes, which subsequently lead to the inhibition of ZO-1 protein expression. Another possibility is that NF-κB activation leads to an accelerated degradation of ZO-1 proteins, perhaps by activation of proteasomes. In astrocytes, Wachtel et al. (57) found that TNF-α also downregulates the transmembrane TJ protein occludin in astrocytes. However, TNF-α did not affect occludin expression in cells that form the TJ barrier, including the brain epithelial and endothelial cells (57). TNF-α also did not affect ZO-1 proteins in the astrocytes (57).

Since TNF-α is also known to induce apoptosis in various cell types (8, 55), the possibility that the TNF-α-induced increase in epithelial TJ permeability could be due to cell damage or cell death was also considered. In the present study, TNF-α did not induce apoptosis in Caco-2 cells, indicating that apoptosis was not the mechanism responsible for the TNF-α-induced increase in Caco-2 TJ permeability. Consistent with our present data, Marano et al. (32) also found lack of correlation between TNF-α-induced increase in TJ permeability and apoptosis in Caco-2 cells. In their study, they found TNF-α to cause a small but significant decrease in apoptosis in Caco-2 cells. Our findings are in line with other studies that show NF-κB activation to have an inhibitory effect on apoptosis (5, 55). A number of investigators have shown NF-κB activation to prevent apoptosis and NF-κB inhibition to induce cell death (34, 58). In this regard, an important rationale for the use of NF-κB inhibitors in cancer therapy is to induce apoptosis in cancer cells (58).

There have been several conflicting reports regarding the role of apoptosis in the modulation of epithelial TJ barrier in other cell types. Soler et al. (51), using LLC-PK1 renal epithelial cells, reported that the TNF-α-induced drop in epithelial resistance was associated with an increase in apoptosis (0.64% for control vs. 1.79% for TNF-α-treated) and suggested that the TNF-α-induced increase in apoptosis may contribute to the breakdown of the TJ barrier. The same group later reported lack of correlation between TNF-α-induced increase in TJ permeability and apoptosis in Caco-2 cells and concluded that the effect of TNF-α on epithelial TJ permeability was not related to apoptosis (32). Gitter et al. (18) also found TNF-α to cause an increase in apoptosis in HT-29/B6 cells and suggested that the increase in epithelial TJ permeability may be related to single cells undergoing apoptosis. However, Schmitz et al. (47) using the same cell line (HT-29/B6) found no correlation between apoptosis and increase in epithelial TJ permeability. They reported that although IFN-γ produced markedly higher levels of apoptosis than TNF-α, IFN-γ had only a small effect on TJ permeability and concluded that the cytokine-induced increase in TJ permeability was not related to apoptosis (47). Thus it remains unclear whether apoptosis truly contributes to the observed increase in TJ permeability in these cell types.

The explanation for the varying apoptotic response to TNF-α in different cell types is unclear. On the basis of our present data, we suggest that perhaps apoptotic response to TNF-α may be dependent on TNF-α activation of “antiapoptotic factor” NF-κB, such that, in cell types like Caco-2 cells where TNF-α causes rapid activation of NF-κB, TNF-α-induced apoptosis may be inhibited. On the other hand, in cell types that do not respond by NF-κB activation, apoptosis may be the predominant response to TNF-α. In support of such a mechanism, Manna et al. (31) showed that, in Jurkat T cells, inhibition of TNF-α activation of NF-κB produces a potentiation of TNF-α-induced apoptosis and cell death.

In a previous report in LLC-PK1 kidney cells, NF-κB inhibition (with either curcumin or transfection with a dominant-negative mutant gene of IκBα) did not prevent the TNF-α-induced increase in epithelial permeability (50). In fact, NF-κB inhibition was associated with an enhancement of TNF-α-induced increase in epithelial permeability and prevented the recovery of epithelial barrier function in LLC-PK1 monolayers. The varying response of LLC-PK1 cells to NF-κB inhibition could be explained by the differences in the two epithelial systems in how they respond to TNF-α stimulation. Unlike the Caco-2 cells, the LLC-PK1 respond by having a rapid increase in TJ permeability within 1–2 h of TNF-α treatment, followed by a rapid decrease in TJ permeability to baseline levels by 6 h of TNF-α treatment (50). As shown in the present study, the effect of TNF-α on Caco-2 TJ permeability required ~24 h for the initial changes, and the TJ barrier defect persisted and did not recover in the presence of TNF-α (Fig. 2). The TNF-α-induced increase in LLC-PK1 TJ permeability was accompanied by an increase in apoptosis, whereas no such increase was seen in the Caco-2 cells (Tables 1 and 2). Additionally, the alteration in ZO-1 protein localization was limited to the single cell undergoing apoptosis in LLC-PK1 cells (50), whereas in Caco-2 cells alteration in ZO-1 was diffuse and affected a majority of the cells (Fig. 9). Thus the effects of TNF-α on TJ permeability in these two epithelial cell types are quite different and likely to involve different mechanisms.

In conclusion, our results demonstrate for the first time that NF-κB pathways play a central role in TNF-α-induced increase in intestinal epithelial TJ permeability. Our data indicate that TNF-α activation of NF-κB pathways leads to cytoplasmic-to-nuclear translocation of NF-κB, increased NF-κB binding to the DNA binding site, downregulation of ZO-1 protein expression, disturbance in junctional localization of ZO-1 protein, and functional opening of the TJ barrier. Moreover, our results indicate that the effect of TNF-α on Caco-2 TJ permeability was not due to an increase in apoptosis. Since TNF-α is a key proinflammatory cytokine in intestinal inflammation in CD, TNF-α-induced increase in intestinal TJ permeability repre-
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REFERENCES


