Ethanol modulation of intestinal epithelial tight junction barrier

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Ma, Thomas Y., Don Nguyen, Vuong Bui, Hanh Nguyen, and Neil Hoa. Ethanol modulation of intestinal epithelial tight junction barrier. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G965–G974, 1999.—Previous studies have shown that high concentrations of ethanol (≥40%) cause functional damage of the gastrointestinal epithelial barrier by direct cytotoxic effect on the epithelial cells. The effects of lower noncytotoxic doses of ethanol on epithelial barrier function are unknown. A major function of gastrointestinal epithelial cells is to provide a barrier against the hostile substances in the gastrointestinal lumen. The apicodlaterally located tight junctions (TJ)s form a paracellular seal between the lateral membranes of adjacent cells and act as a paracellular barrier. In this study, we investigated the effects of lower doses of ethanol on intestinal epithelial TJ barrier function using filter-grown Caco-2 intestinal epithelial monolayers. The Caco-2 TJ barrier function was assessed by measuring epithelial resistance or paracellular permeability of the filter-grown monolayers. Ethanol (0, 1, 2.5, 5, 7.5, and 10%) produced a dose-related drop in Caco-2 epithelial resistance and increase in paracellular permeability. Ethanol also produced a progressive disruption of TJ protein (ZO-1) with separation of ZO-1 proteins from the cellular junctions and formation of large gaps between the adjacent cells. Ethanol, at the doses used (≥10%), did not cause cytotoxicity (lactate dehydrogenase release) to the Caco-2 cells. Ethanol produced a disassembly and displacement of perijunctional actin and myosin filaments from the perijunctional areas. On ethanol removal, actin and myosin filaments rapidly reassembled at the cellular borders. Ethanol stimulated the Caco-2 myosin light chain kinase (MLCK) activity but did not affect the MLCK protein levels. Specific MLCK inhibitor ML-7 inhibited both ethanol increases in MLCK activity and TJ permeability without affecting the MLCK protein levels. Consistent with these findings, metabolic inhibitors sodium azide and 2,4-dinitrophenol significantly prevented ethanol-induced increase in Caco-2 TJ permeability, whereas cycloheximide or actinomycin D had no effect. The results of this study indicate that ethanol at low noncytotoxic doses causes a functional and structural opening of the Caco-2 intestinal epithelial TJ barrier by activating MLCK.

paracellular permeability; myosin light chain kinase; actin

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the Caco-2 intestinal epithelial TJ barrier. Additionally, some of the intracellular processes involved in the ethanol opening of the intestinal epithelial TJ barrier is elucidated.

MATERIALS AND METHODS

DMEM, trypsin, and fetal bovine serum were purchased from Life Technologies (Gaithersburg, MD). Glutamine, penicillin, streptomycin, and PBS solution were purchased from Irvine Scientific (Santa Ana, CA). Colchicine, cytochalasin B, and cycloheximide were purchased from Sigma Chemical (Indianapolis, IN). Millicell-HA 0.4-µm permeable filters (12 mm) were purchased from Millipore (Bedford, MA). Anti-(Indianapolis, IN). Millicell-HA filters and monitored regularly by measuring cellular permeability.

Determination of epithelial monolayer resistance and paracellular permeability. The electrical resistance of the filter-grown intestinal monolayers was measured with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) as previously reported (18, 27). For resistance measurements, both apical and basolateral sides of the epithelium were bathed with the same buffer solution. Electrical resistance was measured until similar values were recorded on three consecutive measurements. The resistances of monolayers in this study are reported after subtraction of the resistance value of the filters alone. The effect of ethanol on Caco-2 monolayer paracellular permeability was examined with the established paracellular marker mannitol (23, 24). For determination of mucosal-to-serosal flux rates of the paracellular probe mannitol, only Caco-2-plated filters having epithelial resistance of 400–450 Ω·cm² were used. The filter-grown Caco-2 monolayers reached epithelial resistance of 400–450 Ω·cm² by 3–4 wk after plating (10, 20). Unless specified otherwise, Krebs-PBS (pH 7.4) was used as the incubation solution during the experiments. Buffer solution (300 µl) was added to the apical compartment, and 450 µl were added to the basolateral compartment to ensure equal hydrostatic pressure as recommended by the manufacturer. Known concentrations of mannitol (10 µmol/l) and its radioactive tracer ([14C]mannitol) were added to the apical solution. Low concentrations of mannitol were used to ensure that negligible osmotic or concentration gradient was introduced. Test reagent was added to both the apical and basolateral compartments as indicated. All flux studies were carried out at 37°C.

All of the experiments were repeated four to six times to ensure reproducibility.

Fluorescent labeling of cytoskeletal elements and TJ proteins. Distribution of actin microfilaments was assessed using fluorescent labeling techniques as previously described (20). Monolayers grown on coverslips were fixed in 3.75% formaldehyde solution in PBS for 20 min at room temperature and were permeabilized in acetone at −20°C for 5 min and washed with 1 M PBS solution. Then, 10 U of fluorescein-labeled phalloidin (Molecular Probes, Eugene, OR) dissolved in 200 µl of PBS was placed on the coverslips for 40 min. After PBS rinse, coverslips were mounted on a slide with the cell side down in a 1:1 solution of PBS and glycerol.

The Caco-2 myosin filaments were labeled with anti-myosin antibody. After fixation with 2.0% formaldehyde and permeabilization in acetone as previously described, Caco-2 monolayers were labeled with 1:10 diluted anti-myosin antibody (Amersham, Arlington Heights, IL) in PBS. This was followed by incubation with 1:10 fluorescein-conjugated rabbit anti-mouse antibody (Amersham) in PBS. Coverslips were mounted in 60% glycerol-PBS, 0.4% n-propyl gallate.

The tight junctional protein ZO-1 was labeled with anti-ZO-1 antibody (26, 27). Epithelial monolayers grown on coverslips were fixed with 2.0% formaldehyde and permeabilized in acetone as described previously. The Caco-2 monolayers were labeled with anti-ZO-1 antibody diluted 1:20 with Tris-buffered saline solution; this was followed by incubation with 1:30 diluted Tris-buffered saline solution containing secondary anti-rabbit IgG biotinylated antibody (Zymed Laboratories) and incubation with 1:20 diluted Tris-buffered saline solution containing FITC-streptavidin (Zymed Laboratories). Coverslips were mounted in 60% glycerol-Tris-buffered saline solution, 0.4% n-propyl gallate, and viewed on a Nikon epifluorescence microscope. All of the fluorescent labeling experiments were repeated three to five times to ensure reproducibility.

In vitro myosin light chain kinase activity determination. Caco-2 myosin light chain kinase (MLCK) activity was determined by direct in vitro kinetic measurement of MLCK activity of the immunoprecipitated MLCK. For MLCK activity studies, Caco-2 monolayers were serum deprived overnight. After appropriate experimental treatment, cells were immediately rinsed with ice-cold Hanks’ balanced salt solution. Cells were then lysed using 0.8 ml lysis buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 µM pepstatin, 1 µg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 2 µg/ml aprotinin, 40 mM para-nitrophenol phosphate-di-cyclohexylammonium salt) and scraped, and lysates were placed in Microfuge tubes (tube A) and microcentrifuged 5 min to yield a clear lysate.

Anti-MLCK antibody (5 µl/200 µl lysis buffer) was added to a separate Microfuge tube (tube B) containing the protein A beads and incubated end-over-end for 1 h at 4°C. Then 100 µl of each cleared lysate (tube A) were added to the microvial (tube B) containing the pelleted protein A bead-MLCK antibody complex and incubated end-over-end for 2 h at 4°C. The microvial containing the immune mixture was microfuged, and the supernatant was aspirated and washed sequentially with lysis buffer and solution of 10 mM HEPES and 10 mM magnesium acetate at 4°C.

Protein A bead-MLCK antibody immunoprecipitated MLCK (immune mixture) was then used in an in vitro kinase reaction in microfuge tubes to determine the phosphorylation of MLC by the isolated MLCK. For this, 20 µl myosin light chain protein (2 mg/ml), 20 µl of 3 times hot mix [150 µM ATP, 10 µl [32P]ATP (5 µCi/reaction), 30 mM magnesium acetate, 30 mM HEPES] were added, mixed with the immune mixture,
and incubated for 30 min at 30°C. The MLCK-catalyzed phosphorylation reaction was terminated by 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 µg dithiothreitol, and trace of bromphenol blue). Subsequently, the reaction mixture was boiled for 3 min and microcentrifuged for 10 s, and then the supernatant (40–50 µl) was separated on 10% SDS-PAGE. The gel was fixed in 40% MeOH and 10% acetic acid overnight and stained with Coomassie blue solution, dried, and then autoradiographed.

RESULTS

Ethanol modulation of Caco-2 TJ barrier. The effect of ethanol on Caco-2 intestinal epithelial TJ barrier function or TJ permeability was determined by measuring Caco-2 epithelial resistance and/or epithelial permeability to paracellular marker mannitol (23, 24). Addition of varying doses (0, 1, 3, 5, 7.5, 10%) of ethanol produced a dose-related drop in Caco-2 epithelial resistance over the 60-min treatment period (Fig. 1). As shown in Fig. 2A, ethanol (7.5%) produced a progressive decrease in Caco-2 epithelial resistance over the 60-min treatment period. Ethanol also caused a progressive increase in Caco-2 epithelial permeability to paracellular marker mannitol over the 60-min period (Fig. 2B). Previous studies (20, 24) have demonstrated an inverse relationship between intestinal epithelial resistance and paracellular permeability. To verify such a relationship, we compared ethanol-induced alteration in Caco-2 epithelial resistance and paracellular permeability (Fig. 2C). There was a linear relationship ($r = 0.97$) between ethanol-induced decrease in Caco-2 epithelial resistance and increase in epithelial permeability to mannitol, confirming an inverse relationship between Caco-2 epithelial paracellular permeability and epithelial resistance. [As in other studies (8, 20, 23, 25, 39), increase in paracellular permeability or drop in epithelial resistance was used as an indication of increase in TJ permeability.]

Next, we examined whether ethanol alteration of Caco-2 TJ barrier function was reversible. After a 60-min treatment, ethanol was removed and Caco-2 epithelial monolayers were incubated in Krebs buffer solution for 3 h. Similar to above, ethanol treatment resulted in an increase in Caco-2 paracellular permeability and a decrease in epithelial resistance. After ethanol removal, Caco-2 epithelial resistance (Fig. 3A) and paracellular permeability (Fig. 3B) rapidly returned to the baseline levels, indicating retightening of the TJ barrier. These findings suggested that ethanol-induced increase in TJ permeability resulted from a reversible functional change in the TJ barrier rather than from a permanent cell injury or cell death. The possible cytotoxic effect of ethanol on Caco-2 monolayers was also determined by measuring lactate dehydrogenase (LDH) release using the LDH assay kit from Sigma Chemical. Ethanol, at the doses used ($\leq$10%), did not result in a significant increase in LDH release by the Caco-2 cells (Fig. 4). There was a significant increase in LDH release at ethanol concentrations $>12%$. To examine the possible “late LDH release” by the ethanol-treated cells, LDH release was also measured for an additional 24 h after ethanol treatment. There was no significant increase in late LDH release by the ethanol ($\leq$10%)-treated cells compared with the control or untreated cells (data not shown).

Ethanol modulation of Caco-2 ZO-1 tight junctional proteins. In the following studies, the modulatory effect of ethanol on Caco-2 TJ was examined by immunofluorescent labeling of tight junctional proteins ZO-1. In the control monolayers, ZO-1 proteins were present in the cellular periphery and appeared as a continuous band localized at the intercellular borders (Fig. 5A). Ethanol treatment produced a progressive disruption of ZO-1 proteins and displacement of ZO-1 proteins away from the cellular borders with formation of large paracellular openings between the adjacent cells (Fig. 5B). On ethanol removal, ZO-1 proteins rapidly reassembled at the apical cellular borders with reclosure of the paracellular gaps (Fig. 5D). These findings visually correlated with ethanol-induced functional “opening” (increased in TJ permeability) and subsequent reclosure of the TJ barrier, confirming a structural-functional relationship.

Ethanol perturbation of Caco-2 actin microfilaments. Previous studies have suggested a central role for perijunctional actin and myosin filaments in modulation of intestinal epithelial TJs (20, 22, 23). In the following studies, the possible involvement of actin microfilaments in ethanol modulation of Caco-2 TJ barrier was examined. In the mature Caco-2 monolayers, F-actin filaments were present as brightly staining structures localized mainly at the perijunctional area.

Fig. 1. Effect of varying concentrations of ethanol on Caco-2 epithelial resistance. Filter-grown Caco-2 monolayers were incubated with varying concentrations of ethanol for 60 min. A: time course of ethanol effect on Caco-2 epithelial resistance. B: dose effect of ethanol on Caco-2 epithelial resistance after 60 min of treatment. Data represent means ± SE of epithelial resistance; n = 4.
(Fig. 6A) as visualized by immunofluorescent labeling. Ethanol treatment resulted in breakage of the F-actin filaments with clumping and displacement of the perijunctional actin filaments from the cellular borders (Fig. 6B). This was associated with a pulling apart of the adjacent cells and formation of large paracellular gaps. On removal of ethanol (Fig. 6D), actin filaments rapidly reassembled at the perijunctional location correlating with the functional changes in Caco-2 TJ barrier. (It should be noted that ZO-1 and F-actin labeling studies were also performed on the Caco-2 cells grown on permeable inserts with similar results, suggesting that ethanol-induced morphological changes in these structures were similar between the two systems.)
Ethanol modulation of Caco-2 myosin filaments and MLCK.

In small intestinal epithelial cells, a peripheral band of F-actin and myosin filaments encircle the cells near the apical junctions. As shown in Figs. 6 and 7, perijunctional belt of actin and myosin filaments also encircle the Caco-2 cells at the apical borders (en face views). In the following experiments, the effect of ethanol on perijunctional myosin filaments was determined. In the Caco-2 intestinal monolayers, myosin filaments were present near the apical junctions forming a continuous peripheral belt encircling the cells (Fig. 7A). Ethanol treatment produced a disassembly and displacement of perijunctional myosin filaments internally with visible separation of myosin filaments from the adjacent cells (Fig. 7B). On removal of ethanol, myosin filaments rapidly reassembled at the cellular borders (Fig. 7D), correlating with the functional changes in Caco-2 TJ barrier.

The MLCK plays an integral role in activating contraction of actin and myosin filaments (13, 14). In the following studies, the possible role of MLCK in ethanol modulation of Caco-2 TJ barrier was examined. The modulatory effect of ethanol on Caco-2 MLCK activity was determined by direct in vitro kinetic measurements of MLC phosphorylation by the immunoprecipitated Caco-2 MLCK. The Caco-2 MLCK immunoprecipitated from ethanol-treated cells greatly increased MLC phosphorylation compared with the untreated cells (control), indicating ethanol activation of Caco-2 MLCK. In contrast, ethanol did not affect Caco-2 MLCK protein level (Fig. 8). Specific MLCK inhibitor 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) inhibited the ethanol-induced increase in MLCK activity without affecting the MLCK protein levels. ML-7 also inhibited the ethanol-induced drop in Caco-2 epithelial resistance (Fig. 9), suggesting that MLCK activation was necessary for the increase in Caco-2 TJ permeability.

Role of metabolic energy and protein synthesis on ethanol modulation of Caco-2 permeability and perijunctional structures. In smooth muscle cells, the contraction of actin-myosin filaments requires metabolic energy generated by MLCK-induced activation of myosin-Mg$^{2+}$-ATPase (26). In the following studies, the possible roles of protein synthesis, transcription, and metabolic energy in ethanol-induced increase in Caco-2 TJ permeability were examined. Metabolic inhibitors sodium

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**Fig. 4.** Cytotoxicity of ethanol as measured by lactate dehydrogenase (LDH) release. Filter-grown Caco-2 monolayers were treated with various concentrations of ethanol for 60 min; subsequently, incubation solution was collected and LDH release was measured. Values are means ± SE; n = 4. Total LDH release (determined by treatment with 1 times Triton X-100) was 1,438 U/l. Experiments were repeated in quadruplicate to ensure reproducibility. *P < 0.001.

**Fig. 5.** Effect of ethanol on immunofluorescent localization of Caco-2 ZO-1 proteins (en face views). Caco-2 ZO-1 proteins were labeled with immunofluorescent antibody labeling technique as described in MATERIALS AND METHODS. A: untreated or control monolayer. B: Caco-2 cells treated with ethanol for 60 min. C: Caco-2 cells pretreated with sodium azide (30 mM) and then treated with ethanol for 60 min. D: Caco-2 cells treated with ethanol for 60 min, followed by removal of ethanol and incubation in cell culture medium for 2 h (original magnification ×200).
azide (Fig. 10A) and 2,4-dinitrophenol (Fig. 10B) prevented the ethanol-induced drop in Caco-2 epithelial resistance. The pretreatment of Caco-2 monolayers with cycloheximide (70 µM) and actinomycin D (1 µg/ml) at the doses previously shown to inhibit Caco-2 protein synthesis and transcription did not affect the ethanol-induced drop in epithelial resistance (Fig. 10, C and D). These findings suggest that the ethanol-induced increase in Caco-2 TJ permeability was dependent on metabolic energy but not new protein synthesis.

To further examine the structural-functional relationship, the effect of sodium azide, cycloheximide, and actinomycin D on ethanol modulation of perijunctional structures was determined. Consistent with the above findings, cycloheximide and actinomycin D did not have a significant effect on ethanol modulation of actin or myosin filaments (data not shown). In contrast, sodium
azide and 2,4-dinitrophenol almost completely inhibited ethanol-disruption of actin and myosin filaments (Figs. 6C and 7C). Cycloheximide and actinomycin D also did not have a significant effect on ethanol modulation of ZO-1 proteins, whereas sodium azide and 2,4-dinitrophenol prevented ethanol disruption of ZO-1 proteins and formation of paracellular openings (Fig. 5C). These findings confirm the requirement of metabolic energy in the ethanol modulation of actin and myosin filaments and the ZO-1 tight junctional proteins.

**DISCUSSION**

An important function of gastrointestinal epithelia is to provide a barrier against the mucosal penetration of toxic substances and antigens such as H⁺, bile acids, proteolytic enzymes (e.g., pepsin, trypsin, and chymotrypsin), bacteria and bacterial by-products, and food additives present in the gastric and the intestinal lumen. The gastrointestinal epithelial TJs act as a structural barrier against the paracellular permeation of luminal compounds (1, 21). The disruption of the TJ barrier allows increased epithelial penetration of normally excluded luminal substances that may promote mucosal injury.

The results of the present study indicate that ethanol at low noncytotoxic doses produces a functional and morphological opening of the Caco-2 intestinal epithelial TJ barrier. The ethanol disruption of the Caco-2 tight junctional proteins (ZO-1) and the increase in the TJ permeability were accompanied by structural disturbance and displacement of perijunctional actin and myosin filaments. The functional retightening or reclosure of the Caco-2 TJ barrier following ethanol removal paralleled the structural reassembly of perijunctional actin and myosin filaments and the TJ proteins, as well as the morphological reclosure of the paracellular gaps, demonstrating a structural-functional relationship. Additionally, our data suggest that ethanol increase in Caco-2 TJ permeability was mediated by the activation of Caco-2 MLCK, so that the ethanol increase in TJ permeability correlated with an increase in MLCK activity and inhibition of MLCK activity with prevention of ethanol-induced increase in TJ permeability. Consistent with these findings, inhibition of metabolic energy (which is required for MLCK activation of actin-myosin contraction) prevented both ethanol increases in TJ permeability and the alteration of the perijunctional structures.

Ethanol produces many morphological and functional disturbances of the gastrointestinal epithelium (2, 12, 15, 36, 38, 40). In previous studies, it was demonstrated that high doses of ethanol (=40%) caused
a direct cytotoxic injury of the epithelial cells at the gastrointestinal surface with a resultant rupture and exfoliation of the epithelial layers and formation of large open wounds in the epithelial surface (2, 15, 36, 38, 39). The effect of lower noncytotoxic doses of ethanol on gastrointestinal epithelial barrier is not well understood. The small intestinal luminal levels of ethanol routinely reach concentrations of 2–10% following moderate consumption of ethanol (50 ml ethanol in 20% solution) (3, 28). It had been demonstrated that the peak serum levels of ethanol were 100–150 times higher than the peak small intestinal levels following moderate ethanol consumption, suggesting that the peak serum ethanol levels of 100 mg/dl correspond to a small intestinal level of 10–15% (28). This is the first study to demonstrate that ethanol at lower clinically achievable doses produces a functionally reversible opening of the intestinal epithelial TJ barrier. The low doses of ethanol (<10%) used in this study did not cause permanent cell damage or cell death. Thus ethanol-induced increase in TJ permeability was due to a reversible change in the TJ barrier and not from cell death or formation of large open wounds in the epithelial surface as seen with the higher doses of ethanol (2, 15, 36, 38, 39).

The intestinal epithelial TJs are the apical-most structures, which encircle the cells at the lateral borders in a beltlike manner. The TJs make homotypic contact across the intercellular space between the adjacent cell (1). The lateral contacts, which may be visualized by electron microscopy and freeze-fracture analysis, act as structural barrier against the paracellular permeation of luminal substances (1, 21, 24). An apicolateral ring or belt of actin and myosin filaments also encircles the intestinal epithelial cells near the TJs (22). The proximity of the “perijunctional actomyosin ring” to the apical TJs suggested a possible interdependent relationship (22). Indeed, a correlation between disturbance of perijunctional actin and myosin filaments and an increase in TJ permeability have been previously demonstrated (20, 22, 23). The treatment of intestinal epithelial cells with actin-depolymerizing agents (cytochalasins) caused a condensation and disruption of perijunctional actin microfilaments and structural and functional opening of the intercellular TJ complexes (20, 23). Some studies have also shown that an increase in intestinal epithelial TJ permeability is associated with an increase in MLC phosphorylation (7, 8, 25, 29, 32). Based on these findings, it was suggested that MLC phosphorylation might be an important step in the propagation of a series of intracellular processes resulting in the opening of the TJ barrier (29, 39). It had been hypothesized that MLC phosphorylation induces contraction of perijunctional actin and myosin filaments, which in turn produces tension on the apical
Recent studies suggest that altered intestinal epithelial TJ permeability may be an important etiologic factor in a number of diseases, including Crohn’s disease, nonsteroidal anti-inflammatory drug-associated enteritis, and diarrhea syndromes caused by Clostridia difficile, Vibrio cholerae, and enteropathogenic Escherichia coli (4, 5, 8, 11, 16, 32). It had been proposed in these diseases that the increase in intestinal epithelial TJ permeability allows the paracellular penetration of the toxic luminal substances, culminating in intestinal inflammation and mucosal injury (4, 11, 16, 32). The ethanol consumption in these clinical conditions may accentuate the increase in TJ permeability, allowing greater mucosal penetration of the luminal substances and further exacerbating intestinal inflammation. Consistent with this possibility, in our preliminary studies, combined treatment of ethanol and indomethacin had an agonistic effect on ethanol-induced increase in Caco-2 TJ permeability (data not shown), suggesting that the combination of these two drugs was more damaging to the TJ barrier than either drug individually.

In summary, the results of this study indicate that ethanol at low nontoxic doses causes an opening of the Caco-2 TJ barrier. The ethanol opening of Caco-2 TJ barrier appeared to be mediated by activation of MLCK and subsequent modulation of perijunctional actin and myosin filaments. These findings demonstrate a mechanism by which low doses of ethanol may alter intestinal epithelial TJ barrier and possibly promote mucosal inflammation.

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