Tight junctions (TJ) form a physical barrier to the diffusion of allergens, toxins, and pathogens through the paracellular space in intestinal epithelium (1). It is suggested that adherens junctions (AJ) localized just beneath the TJ indirectly regulate the integrity of TJ. A number of specific proteins is localized at the TJ and AJ. Occludin (1), claudins (26), and junction adhesion molecule (16) are the currently known transmembrane proteins localized at the TJ. Zonula occludens (ZO)-1, -2 and -3 are the major TJ-plaque proteins that bind to the intracellular domain of occludin (8, 9, 27). The interaction between occludin and ZO-1 plays a crucial role in maintaining the structure of TJ and epithelial barrier function (8). AJ are composed of the transmembrane protein E-cadherin and the plaque proteins α-, β-, and γ-catenins. The transmembrane proteins of TJ and AJ interact with the actin cytoskeleton, and these interactions are crucial for the integrity of TJ and AJ (1).

Alcoholic liver disease is associated with increased intestinal permeability and endotoxemia (22). Recent studies (2, 19) demonstrated that acetaldehyde, the metabolic product of ethanol, increases the paracellular permeability of the Caco-2 cell monolayer. Acetaldehyde-induced increase in the paracellular permeability was associated with the reorganization of TJ proteins such as occludin and ZO-1 (2). Increase in paracellular permeability and redistribution of TJ proteins by acetaldehyde required tyrosine kinase activity. Acetaldehyde-induced increase in the paracellular permeability was also associated with the inhibition of protein tyrosine phosphatase activity and increase in tyrosine phosphorylation of a wide spectrum of proteins including ZO-1 and β-catenin (2).

A significant body of evidence indicates that l-glutamine is essential for intestinal epithelial cell growth and differentiation (20). Glutamine prevents intestinal hyperpermeability and bacterial translocation and was found to be helpful in the treatment of trauma and surgery patients (7). Glutamine reduces intestinal permeability in piglet intestine (6) and total parenteral nutrition-induced permeability in rats (4, 14). Bacterial translocation and endotoxemia caused by abdominal radiation were ameliorated by l-glutamine (7, 12, 25). However, the effect of l-glutamine on alcohol or acetaldehyde-induced cell injury or epithelial permeability is not known.

In the present study, we determined the effect of L-glutamine on acetaldehyde-induced disruption of TJ and increase in paracellular permeability to LPS. This study shows that 1) l-glutamine significantly reduces acetaldehyde-induced increase in paracellular permeability in dose-dependent and time-dependent manners, 2) l-glutamine prevents acetaldehyde-induced redistribution of TJ and AJ proteins from the intercellular junctions and release of TJ and AJ proteins from the actin cytoskeleton, and 3) l-glutamine induces tyrosine phosphorylation of EGF receptor, and 4) l-glutamine-induced protection of barrier function is prevented by AG1478, a specific inhibitor of EGF receptor tyrosine kinase.

Materials and Methods

Cell Culture

Caco-2 cells purchased from American Type Cell Culture (Manassas, VA) were maintained under standard cell culture conditions at 37°C in a medium containing 10% (vol/vol) fetal bovine serum. Cells were grown on polycarbonate membranes in Transwells (6.5 or 24 mm; Costar, Cambridge, MA), and experiments were performed on 12–14 (6.5 mm) or 15–17 days (24 mm) after seeding.

Acetaldehyde Treatment

Acetaldehyde was administered by exposing cell monolayers in PBS (Dulbecco’s saline containing 1.2 mM CaCl2, 1 mM MgCl2, and 0.6% BSA) to vapor phase acetaldehyde as described previously (2, 19) to achieve acetaldehyde concentration of 100–600 μM in the

Address for reprint requests and other correspondence: R. K. Rao, Dept. of Physiology, Univ. of Tennessee, 894 Union Ave., Memphis, TN 38163 (E-mail: rkrao@physio1.utmem.edu).
buffer bathing the cell monolayer. L-Glutamine, other amino acids, and AG1478 were administered to the apical, basal, or apical and basal compartments.

**Treatment with LPS, EGTA, and Mannitol Load**

Caco-2 cell monolayers were incubated with 2 mM EGTA for 60 min or 100 μM LPS for 30 min on both the apical and basal surfaces. For mannitol load experiment, cell monolayers were incubated with 0.25 M mannitol on the apical surface for 90 min. Transepithelial electrical resistance (TER) and inulin flux were measured.

**Measurement of TER**

TER was measured as described by Hidalgo et al. (10) using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). TER was calculated as Ohms per centimeter squared by multiplying it with the surface area of the monolayer (0.33 cm² for 6.5 mm wells). The resistance of the supporting membrane in Transwells (which is usually ~30 Ω·cm²) was subtracted from all readings before calculations.

**Unidirectional Flux of Inulin and Endotoxin**

Cell monolayers in Transwells were incubated under different experimental conditions in the presence of FITC-inulin (0.5 mg/ml) or FITC-LPS (0.1 mg/ml) in the basal well. At different times after experimental conditions in the presence of FITC-inulin (0.5 mg/ml) or before calculations. (which is usually

**Association of TJ and AJ Proteins with the Actin Cytoskeleton**

Preparation of actin cytoskeleton. Cytoskeletal fractions were prepared as described previously (21). Briefly, cell monolayers were lysed in Tris buffer containing 1.0% Triton X-100, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml bestatin, 10 μg/ml pepstatin-A, 2 mM vanadate, and 1 mM PMSF. Extracts were centrifuged at 15,600 × g for 4 min at 4°C to sediment high-density actin cytoskeleton. The pellet was suspended in 200 μl of Tris buffer and sonicated to homogenize actin filaments. After withdrawal of aliquots for protein assay, cytoskeletal fractions were mixed with an equal volume of Laemmli’s sample buffer (2X concentrated) and heated at 100°C for 5 min.

Western blot analysis. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Blots were probed for occludin, ZO-1, E-cadherin, or β-catenin using horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-occludin, mouse monoclonal anti-E-cadherin, mouse monoclonal anti-β-catenin, or rabbit polyclonal anti-ZO-1 antibodies in combination with HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG antibodies. The blots were developed by using the enhanced chemiluminescence method (Amersham, Arlington Heights, IL).

**Tyrosine Phosphorylation of EGF Receptor**

After treatment with L-glutamine (2 mM) for varying times, proteins were extracted in lysis buffer D (0.3% SDS in 10 mM Tris buffer, pH 7.4, containing 1 mM vanadate and 0.33 mM PMSF). P-Tyr was immunoprecipitated as described previously (21) using biotin-conjugated anti-p-Tyr antibodies. Immune complexes were isolated by precipitation using streptavidin-agarose. Immunoprecipitates were immunoblotted for EGF receptor using anti-EGF receptor antibodies as described above.

**Statistics**

Comparison between two groups was made by Student’s t-tests for grouped data. Significance in all tests was set at 95% or greater confidence level.

**RESULTS**

**L-Glutamine Prevents Acetaldehyde-Induced Increase in Paracellular Permeability to Macromolecules Including Endotoxin**

Administration of acetaldehyde (600 μM) resulted in a time-dependent decrease in TER (Fig. 1A) and an increase in inulin permeability (Fig. 1B). L-Glutamine effectively prevented the acetaldehyde-induced changes in TER (Fig. 1, A and C) and inulin permeability (Fig. 1, B and D). This effect of L-glutamine on TER (Fig. 2A) and inulin flux (Fig. 2B) was concentration dependent. L-Glutamine also significantly reduced the acetaldehyde-induced increase in permeability to LPS (Fig. 2C). Preincubation of cells with L-glutamine and its removal before acetaldehyde treatment also significantly reduced acetaldehyde-induced changes in TER and inulin flux (data not shown). The glutaminase inhibitor, 6-diazo-5-oxo-L-norleucine (DON) failed to prevent the L-glutamine-mediated prevention of acetaldehyde-induced changes in TER and flux (Fig. 3). DON by itself did not alter paracellular permeability in untreated or acetaldehyde-treated cells in the absence of glutamine. n-Glutamine also failed to influence the acetaldehyde-induced decrease in TER (Fig. 3A) and increase in inulin permeability (Fig. 3B).
Fig. 1. Effect of L-glutamine on acetaldehyde-induced paracellular permeability. A and B: Caco-2 cell monolayers were incubated for 4 h without (○, ●) or with (□, ■) acetaldehyde in the absence (○, □) or presence (●, ■) of 2 mM L-glutamine. Transepithelial electrical resistance (TER; A) and inulin flux (B) were measured at varying times. Values are means ± SE (n = 6). TER (C) and inulin flux (D) were measured in cell monolayers treated with varying concentrations (Conc.) of acetaldehyde in the absence (○) or presence (●) of 2 mM L-glutamine for 5 h. Values are means ± SE (n = 6). *Values significantly different (P < 0.05) from the corresponding values for the acetaldehyde group.

flux (Fig. 3B). D-Glutamine or glutaminase inhibitor by themselves did not influence TER or inulin flux in control or acetaldehyde-treated cell monolayers. L-arginine, L-lysine, and L-alanine were not effective in preventing acetaldehyde-induced changes in TER or inulin flux, whereas L-asparagine produced a mild protective effect (Fig. 4, A and B). Different amino acids by themselves produced no significant effect on TER or inulin flux in control cell monolayers (Fig. 4, C and D). L-Glutamine was effective in protecting the barrier function when it was administered to either the apical or the basal surface of the epithelium (Fig. 5). L-Glutamine failed to pre-

Fig. 2. Dose-response relationship of glutamine-mediated prevention of acetaldehyde-induced increase in paracellular permeability. Caco-2 cell monolayers were incubated for 4 h with acetaldehyde in the presence of varying concentrations of L-glutamine (0–2 mM), TER (A), FITC-inulin flux (B), and FITC-LPS flux (C) were measured. Values are means ± SE (n = 6). *Values significantly different (P < 0.05) from the corresponding values for the acetaldehyde group.

Fig. 3. Effect of L-glutamine and glutaminase inhibitor on acetaldehyde-induced permeability. Caco-2 cell monolayers were incubated for 4 h without or with acetaldehyde (600 μM) and L-glutamine or D-glutamine (2 mM) in the absence or presence of 6-diazo-5-oxo-L-norleucine (DON). TER (A) and FITC-inulin flux (B) were measured. Values are means ± SE (n = 6). Values significantly different (P < 0.05) from the corresponding values for the acetaldehyde group and acetaldehyde + L-glutamine groups are indicated by * and #, respectively.
vent EGTA and mannitol load-induced decrease in TER (Fig. 6A) and increase in inulin flux (Fig. 6B). However, changes in TER and inulin flux induced by LPS were significantly reduced by L-glutamine.

**Fig. 5.** Polarity of L-glutamine-mediated prevention of acetaldehyde-induced increase in paracellular permeability. Caco-2 cell monolayers were incubated for 4 h with acetaldehyde (600 μM) in the absence or presence of glutamine (2 mM) administered to apical, basal, or apical and basal compartments. TER (A) and FITC-inulin flux (B) were measured. Values are means ± SE (n = 6). *Values significantly different (P < 0.05) from the corresponding value for the acetaldehyde group.

**Fig. 6.** Effect of glutamine on LPS, EGTA, and mannitol-induced permeability. Caco-2 cell monolayers were incubated without (black bars) or with (grey) L-glutamine followed by 1 mM EGTA (for 60 min) or 100 μg/ml of LPS (for 30 min) on both the apical and basal surfaces, or incubated with 0.5 M mannitol on the apical surface. TER (A) and inulin flux (B) were measured. Values are means ± SE (n = 4). *Values significantly different (P < 0.05) from values for corresponding controls.

**1-Glutamine Prevents Acetaldehyde-Induced Reorganization of TJ and AJ Proteins**

Confocal immunofluorescence microscopy showed that occludin and ZO-1 are localized at the intercellular junctions (Fig. 7).
Acetaldehyde treatment induced considerable reduction in the distribution of occludin and ZO-1 at the intercellular junctions. ZO-1 appears to be redistributed into the intracellular compartments, whereas occludin level in the intracellular compartment was not altered. Therefore, it is quite likely that occludin is released into the extracellular space or is digested by proteases as soon as it is released into the intracellular compartment. However, acetaldehyde in the presence of L-glutamine failed to induce redistribution of occludin and ZO-1. Acetaldehyde also induced redistribution of E-cadherin and β-catenin from the intercellular junctions into the intracellular compartments (Fig. 8). The presence of L-glutamine reduced the acetaldehyde-induced redistribution of E-cadherin and β-catenin (Fig. 8).

L-Glutamine Prevents Acetaldehyde-Induced Reduction of Detergent-Insoluble of TJ and AJ Proteins

Interaction of TJ and AJ proteins with the actin cytoskeleton is essential for the integrity of these junctional complexes (1, 27). Our recent study indicated that the detergent-insoluble pool of occludin, ZO-1, E-cadherin, and β-catenin is important in the regulation of paracellular barrier function (18). Occludin, ZO-1, E-cadherin, and β-catenin were detected in the detergent-insoluble fractions prepared from Caco-2 cell monolayers (Fig. 9). The amounts of occludin, ZO-1, E-cadherin, and β-catenin present in the detergent-insoluble fraction prepared from acetaldehyde-treated cells were considerably lower than those in untreated cells (Fig. 9). L-Glutamine prevented acetaldehyde-induced reduction in the detergent-insoluble E-cadherin, β-catenin, occludin, and ZO-1. Acetaldehyde-induced reduction of occludin and β-catenin in detergent-insoluble fraction was associated with a slight increase in these proteins in detergent-soluble fractions. However, there was no increase in the levels of ZO-1 and E-cadherin in the detergent-soluble fractions.

Role of EGF Receptor in the Mechanism of L-Glutamine-Mediated Prevention of Acetaldehyde-Induced Permeability

Our recent study (24) demonstrated that EGF protects the TJ and the AJ from acetaldehyde-induced disruption in Caco-2 cell monolayers.
DISCUSSION

Evidence indicates that l-glutamine protects the barrier function of the gastrointestinal mucosa and prevents bacterial translocation under many pathophysiological conditions (4, 6, 7, 12, 14, 25). In the present study, we show that l-glutamine prevents the acetaldehyde-induced increase in permeability to endotoxin by preventing the disruption of the TJ and AJ in the Caco-2 cell monolayer. Our previous studies (2, 19) showed that acetaldehyde, the metabolic product of ethanol in gastrointestinal lumen and mucosa, disrupts TJ and increases paracellular permeability. It is suggested that acetaldehyde-induced endotoxin permeability may contribute to endotoxemia in alcoholic liver disease (22). This study therefore raises the possibility that l-glutamine may help reduce the intestinal permeability to endotoxemia in alcoholic liver disease.

The normal plasma glutamine concentration is nearly 1 mM, and it is significantly reduced during sepsis, trauma, major burns, and postoperatively (3). Glutamine supplementation in burn-injury patients resulted in an increase in plasma glutamine concentration from 0.44 to 0.61 mM, significantly reduced intestinal permeability to lactulose/mannitol, and accelerated wound healing (17). Therefore, alteration of the plasma glutamine level has serious consequences for the integrity of gastrointestinal mucosa. Our present study shows that glutamine at 0.02–2 mM concentration significantly reduces the acetaldehyde-induced increase in paracellular permeability in Caco-2 cell monolayers, and therefore, this effect of glutamine has physiological importance. The present study shows that the extent of permeability to LPS and inulin is somewhat similar. Although the exact molecular weight of the LPS used in this study is not clear, the molecular weight of different LPSs varies from 10 to 20 kDa, and the molecular weight of inulin used in this study is 4 kDa. The similar rates of permeability to inulin and FITC-LPS may indicate that TJs are uniformly modified throughout the monolayer, and the junction is wide enough to allow permeability to inulin and LPS.

**Fig. 9.** Effect of acetaldehyde on association of tight junction and adherens junction proteins with the actin cytoskeleton; prevention by l-glutamine. Caco-2 cell monolayers were incubated without or with acetaldehyde for 4 h in the absence or presence of l-glutamine (2 mM). The Triton-insoluble actin cytoskeleton was prepared from these cell monolayers and immunoblotted for occludin, ZO-1, E-cadherin, and β-catenin, using specific antibodies.

**Fig. 10.** Role of epidermal growth factor receptor (EGFR) activation in l-glutamine-mediated protection of barrier function from acetaldehyde. A and B: Caco-2 cell monolayers were preincubated without or with AG1478 and incubated without or with acetaldehyde for 4 h in the absence or presence of l-glutamine (2 nM). TER (A) and inulin flux (B) were measured. Values are means ± SE (n = 6). Values that are significantly different (P < 0.05) from the acetaldehyde and acetaldehyde + l-glutamine groups are indicated by * and #, respectively. C: Caco-2 cell monolayers were incubated with l-glutamine for varying times and the proteins extracted under denaturing conditions. Phosphotyrosine (PY) was immunoprecipitated by using a biotin-conjugated anti-p-Tyr antibody. Immunoprecipitates (IP) were then immunoblotted for the EGFR receptor.
Prevention of acetaldehyde-induced decrease in TER and increase in permeability to inulin and LPS by L-glutamine indicates that L-glutamine protects the barrier function in Caco-2 cell monolayers from acetaldehyde-induced injury. The lack of protective effect by L-asparagine, L-arginine, L-lysine, and L-alanine indicates that glutamine-mediated protection of barrier function is mediated by a specific mechanism. Lack of effect of D-glutamine in protection from acetaldehyde indicates that the L-glutamine-mediated protection is stereospecific. Previous studies (20) showed that the effect of glutamine on cell growth and proliferation involves metabolism of glutamine to provide an energy source and precursors for synthesis of pyrimidines and amino acids. However, the failure of 6-diazoo-5-oxo-L-norleucine to prevent L-glutamine-mediated protection of the barrier function indicates that the metabolism of glutamine is not required for its protective effect.

The interaction between occludin and ZO-1 was shown to be crucial for the assembly of TJ at the intercellular junctions. Confocal immunofluorescence microscopy of occludin and ZO-1 indicates that glutamine prevents the acetaldehyde-induced reduction of occludin and ZO-1 at the intercellular junctions. ZO-1 appears to be redistributed into the intracellular compartments by acetaldehyde, which was prevented by L-glutamine. On the other hand, reduction in occludin at the intercellular junctions by acetaldehyde was not associated with an increase in fluorescence in the intracellular compartment. It is likely that occludin is degraded by proteolysis immediately after its release into the intracellular compartment. Results also show that L-glutamine prevents acetaldehyde-induced redistribution of E-cadherin and β-catenin, suggesting that L-glutamine protects the integrity of AJ from the acetaldehyde-mediated injury. The preservation of the integrity of TJ and AJ may contribute to L-glutamine-induced protection of barrier function from acetaldehyde.

A significant body of evidence indicates that occludin and ZO-1 of TJ and E-cadherin and β-catenin of AJ interact with the actin cytoskeleton, and this interaction of TJ and AJ proteins with the cytoskeleton is essential for the maintenance of the integrity of TJ (1). Disruption of actin cytoskeleton by cytochalasin-D disrupts TJ and AJ and increases the paracellular permeability (15). Previous studies (21) showed that actin-bound occludin, ZO-1, E-cadherin, and β-catenin are important for the integrity of TJ and AJ. Our recent study (24) showed that acetaldehyde disrupts the organization of actin cytoskeleton and induces the release of TJ and AJ proteins from the actin cytoskeleton, suggesting that disruption of the actin cytoskeletal architecture is involved in the mechanism of acetaldehyde-induced disruption of barrier function. The present study shows that L-glutamine prevents acetaldehyde-induced reduction of the detergent-insoluble occludin, ZO-1, E-cadherin, and β-catenin. Therefore, L-glutamine may protect the TJ and AJ by interfering with the acetaldehyde-induced reorganization of the actin cytoskeleton and the disruption of interaction between the actin cytoskeleton and the TJ and AJ proteins. Although there was a slight increase in occludin and β-catenin in the detergent-soluble fraction in acetaldehyde-treated cells, no increase in ZO-1 and E-cadherin in the detergent-soluble fraction was observed. Therefore, it is likely that these proteins are degraded by proteases in the intracellular compartments.

EGF, a peptide growth factor, is secreted in saliva at high concentrations (17), and it plays an important role in cell growth and differentiation (5). A significant body of evidence indicates that EGF protects the gastrointestinal mucosa from a variety of injurious factors (13). Our recent study (24) showed that EGF prevents acetaldehyde-induced disruption of the barrier function in Caco-2 cell monolayers. EGF prevents acetaldehyde-induced changes in TER and inulin permeability and redistribution of occludin, ZO-1, E-cadherin and β-catenin from the intercellular junctions. EGF also attenuated the acetaldehyde-induced reduction in the amounts of occludin, ZO-1, E-cadherin, and β-catenin bound to the actin cytoskeleton. Activation of the EGF receptor involves dimerization of the receptor and autophosphorylation of the receptor on tyrosine residues (5). The present study shows that L-glutamine-mediated protection of the barrier function of Caco-2 cell monolayer is inhibited by AG1478, a specific inhibitor of EGF receptor tyrosine kinase. This observation suggests that EGF receptor tyrosine kinase activity is required for the L-glutamine-mediated protection of TJ and AJ from acetaldehyde. Results also show that L-glutamine rapidly induces tyrosine phosphorylation of EGF receptor, indicating the transactivation of EGF receptor by L-glutamine.

The mechanism involved in this transactivation of EGF receptor is not clear, but the mechanism of L-glutamine-induced protection of TJ and AJ from acetaldehyde may involve EGF receptor activation and subsequent signaling pathways. L-Glutamine failed to prevent EGTA and mannitol load-mediated increase in permeability, whereas it partially reduced the permeability induced by LPS. Although acetaldehyde and glutamine both induce protein tyrosine phosphorylation, different proteins are likely to be phosphorylated in these two conditions. Our previous studies (20, 24) demonstrated that EGF, which induces protein tyrosine phosphorylation, prevents paracellular permeability induced by acetaldehyde and hydrogen peroxide. EGF reduced hydrogen peroxide-induced tyrosine phosphorylation of certain proteins without affecting that of others. Therefore, overlap in tyrosine phosphorylation may not be a concern, because a growing network of signaling pathways appears to be regulated by interphosphorylation on tyrosine residues. It is quite likely that the effect of glutamine is mediated by the interaction with an amino acid receptor. The dose-response data for glutamine in Fig. 2 show that glutamine at concentration as low as 20 μM shows a significant protective effect.

This study therefore shows that acetaldehyde compromises the barrier function of the Caco-2 cell monolayer by disrupting the TJ and the AJ and reducing the interaction between TJ and AJ proteins and the actin cytoskeleton. L-glutamine protects the barrier function of Caco-2 cell monolayer by preventing the acetaldehyde-induced disruption the TJ and AJ by a mechanism involving EGF receptor tyrosine kinase activity.

ACKNOWLEDGMENTS

This study was supported by the National Institutes of Health Grants DK-55532 and AA-12307 (to R. K. Rao).

REFERENCES


