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EGF Receptor Plays a Role in the Mechanism of Glutamine-Mediated Prevention of Alcohol-Induced Gut Barrier Dysfunction and Liver Injury

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Abstract

Recent study indicated that glutamine prevents alcoholic tissue injury in mouse gut and liver. Here we investigated the potential role of Epidermal Growth Factor Receptor (EGFR) in glutamine-mediated prevention of ethanol-induced colonic barrier dysfunction, endotoxemia and liver damage. Wildtype and EGFR*^{Tg} transgenic (expressing dominant negative EGFR) mice were fed 1-6% ethanol in Lieber-DeCarli diet. Gut permeability was measured by vascular-to-luminal flux of FITC-inulin, and junctional integrity assessed by confocal microscopy. Liver injury was evaluated by plasma transaminases, histopathology and triglyceride analyses. Glutamine effect on acetaldehyde-induced tight junction disruption was investigated in Caco-2 cell monolayers. Doxycycline-induced expression of EGFR* blocked glutamine-mediated prevention of ethanol-induced disruption of colonic epithelial tight junction, mucosal permeability and endotoxemia. Ethanol activated cofilin and disrupted actin cytoskeleton, which was blocked by glutamine in an EGFR-dependent mechanism. Ethanol downregulated antioxidant gene expression and upregulated cytokine and chemokine gene expression, which were blocked by glutamine in wildtype mice in the presence or absence of doxycycline, but not in EGFR*^{Tg} mice in the presence of doxycycline. Histopathology, plasma transaminases, triglyceride and expression of chemokine and antioxidant genes indicated ethanol-induced liver damage, which were blocked by glutamine in an EGFR-dependent mechanism. Src kinase activity and extracellular ligand binding domain of EGFR are required for glutamine-mediated protection of barrier function in Caco-2 cell monolayers. Glutamine released metalloproteinases into the medium, and metalloproteinase inhibitors blocked glutamine-mediated protection of barrier function. Results demonstrate that EGFR plays an important role in glutamine-mediated prevention of alcoholic gut permeability, endotoxemia and liver damage.

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Keywords

Glutamine; colon; barrier function; alcoholic liver disease; epidermal growth factor; tight junction; fatty liver

Introduction

Disruption of apical junction complexes (AJC), epithelial barrier dysfunction and elevated endotoxin absorption in the intestine play crucial role in the pathogenesis of alcohol-related diseases, including the alcoholic liver disease (ALD) [1]. AJC consists of multiprotein junctional complexes, including the tight junction (TJ), the adherens junction (AJ) and the actomyosin belt. AJC prevents the diffusion of allergens, pathogens and toxins such as lipopolysaccharide (LPS) from the gut lumen into the mucosa, and in turn to the systemic circulation [1-3]. TJ is composed of specific proteins such as occludin, claudins, ZO-1, ZO-2 and ZO-3, while AJ is formed by the assembly of E-cadherin, α -catenin, β -catenin, and γ -catenin [4-7]. These proteins interact with the actomyosin belt and stabilize TJ and AJ [8, 9].

Ethanol (EtOH) feeding disrupts TJ and AJ in mice and rats *in vivo* [1]. Acetaldehyde, the metabolic product of EtOH, disrupts TJ and AJ in Caco-2 cell monolayers by protein phosphorylation-dependent mechanisms [10]. EtOH synergizes the acetaldehyde-induced disruption of AJC by mechanisms involving synergistic increase in intracellular calcium and production of reactive oxygen species (ROS) [11]. Disruption of TJ leads to absorption of LPS into systemic circulation. LPS acts as an important player in alcoholic inflammation in the liver and therefore a major contributing factor in the development of ALD. LPS activates Kupffer cells and hepatocytes by Toll-like receptor-4 (TLR-4)-dependent mechanism to produce inflammatory cytokines and ROS, that are known to be involved in the pathogenesis of ALD. Although it is well established that EtOH feeding disrupts intestinal epithelial TJ and cause barrier dysfunction, the effect of EtOH on antioxidant gene expression and inflammatory response in the colon remains to be determined.

Dietary factors such as protein deprivation leads to imbalance in gut microbiota and antibacterial host defense. Administration of amino acids or glucose is associated with restoration of gut microenvironment [12]. Glutamine (Gln) plays a crucial role in the maintenance of mucosal permeability and acts as a source of fuel for cells, particularly intestinal epithelial cells [13]. Evidence indicates that Gln supplementation improves gut barrier function and reduces bacterial translocation [14]. Gln deficiency promotes tumor necrosis factor- α (TNF- α)-induced bacterial translocation, and Gln supplementation inhibits bacterial translocation in Caco-2 cell monolayers [15]. Evidence suggests that Gln supplementation improves intestinal mucosal barrier function and tight junction integrity in patients with Crohn's disease [16]. In addition, Gln feeding preserves and recovers intestinal mucosal integrity following irradiation [17-19]. Gln attenuates acetaldehyde-induced TJ disruption in Caco-2 cell monolayers [20], and Gln supplementation in mice attenuates EtOH-induced disruption of apical junctional complexes in colonic epithelium and ameliorates gut barrier dysfunction and fatty liver [21]. It is likely that Gln prevents acetaldehyde-induced TJ disruption by transactivation of epidermal growth factor receptor

(EGFR) and activation of protein kinase C and MAP kinase [22, 23]. However, the molecular mechanisms involved in Gln-mediated intestinal mucosal integrity *in vivo* remains unclear. It is essential to decipher the molecular events involved in the alcohol-induced gut and liver damage, and the beneficial effect of Gln in ALD in order to gain knowledge in formulating targeted treatment strategy for ALD and other alcohol-related diseases.

Our previous study showed that Gln blocks EtOH-induced protein thiol oxidation in mouse colon [21], and studies from other laboratory indicated that Gln upregulates Nrf2 expression and elevates cellular glutathione levels [24]. We hypothesize that EGFR activation mediates the antioxidant activity of Gln potentially by transactivation of EGFR. Intestinal epithelial cells, including Caco-2 cells, express membrane-bound heparin-binding EGF (HB-EGF) and the EGFR is activated by the matrix metalloproteinase (MMP)-mediated release of HB-EGF [25, 26]. This raises the question whether Gln facilitate the release of MMP into extracellular space, which in turn releases HB-EGF or other membrane-bound EGFR ligands leading to activation of EGFR. Additionally, our previous study showed that EtOH disrupts actin cytoskeleton and Gln blocks this effect of EtOH [21]. The mechanisms by which EtOH disrupts actin cytoskeleton and Gln prevents EtOH effect are unclear. Cofilin is an actin binding protein that severs actin filaments into short fragments, thus facilitates disruption of actin cytoskeleton [27]. Previous study has indicated that activation of EGFR leads to inhibition of cofilin activity [28]. This raises the question whether cofilin activity in the colonic mucosa is regulated by EtOH and Gln.

In the present study, we investigated the role of EGFR in Gln-mediated prevention of EtOH-induced disruption of epithelial junctions, mucosal barrier dysfunction, endotoxemia and liver injury in mice *in vivo*. We also evaluated the effects of EtOH feeding on expression of antioxidant, cytokine and chemokine genes and cofilin activation in mouse colon *in vivo*, and determined the potential role of metalloproteinase activity and ligand binding domain of EGFR in Gln effects in Caco-2 cell monolayers *in vitro*.

Materials and Methods

Chemicals

Maltose dextrin was purchased from Bioserv (Flemington, NJ). Lieber DeCarli EtOH diet (Dyet no. 710260) was purchased from Dyets Inc. (Bethlehem, PA). Hoechst 33342 dye and BODIPY FL-N-(2-aminoethyl) maleimide were purchased from Life Technologies (Grand Island, NY). Doxycycline, EtOH, N-ethylmaleimide and tris(2-carboxyethyl)phosphine, FITC-Inulin, GM6001 (N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide), PP2 (4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) and 1,10-Phenanthroline were from Sigma-Aldrich (St. Louis, MO). Triglyceride assay kit was purchased from Pointe Scientific Inc., (Canton, MI). L-Gln was purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from either Sigma Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Tustin, CA).

Antibodies

Anti-ZO-1 and anti-occludin antibodies were purchased from Invitrogen (Carlsbad, CA). Anti-E-cadherin and anti- β -catenin antibodies were purchased from BD Biosciences (Billerica, MA). Anti-cofilin and anti-phospho-cofilin antibodies were purchased from cell signaling (Danvers, MA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG, Anti-EGFR and anti-actin antibodies were obtained from Sigma-Aldrich. AlexaFluor-488-conjugated phalloidin, AlexaFluor-488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR).

Animals and diets

B6;SJL-Tg(tetO-Egfr*)2-9Jek/J (EGFR**Tg*) and B6.Cg-Gt(ROSA)26Sortm1(rtTA*M2)Jae/J (rtTA) mice were purchased from Jackson Laboratories (Sacramento, CA) and cross bred to generate rtTA-B6;SJL-Tg(tetO-Egfr*)2-9Jek/J (rtTA-EGFR**Tg*). These mice (male and female) were fed doxycycline in a liquid diet to induce EGFR* gene, which was confirmed by PCR. The rtTA-EGFR**Tg* and corresponding rtTA mice were used for the study. The rtTA-EGFR**Tg* mice express a dominant negative, cytoplasmic tyrosine kinase domain-deleted mutant form of epidermal growth factor receptor (EGFR-tr) regulated by the tetracycline operator (tetO; also called tetracycline-responsive element (TRE, TetRE) or tet-operator) and cytomegalovirus minimal promoter. EGFR-tr expression disrupts subsequent activation of signal transduction pathways that are involved in regulating cell proliferation, differentiation, and survival. These TetRE-EGFR-tr mice were bred to generate bi-transgenic mutant mice with conditional (inducible/reversible) expression of a dominant negative EGF receptor.

All animal experiments were performed according to the protocols approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. Animals were housed in an institutional animal care facility with 12 h light and dark cycles. All mice had free access to regular laboratory chow and water until the start of experiments. During the experiments, they were fed a liquid diet as described below.

EtOH Feeding

Adult (8 to 10 weeks old) mice, rtTA, and rtTA-EGFR**Tg*, were fed Lieber-DeCarli liquid diet that contained EtOH with or without Gln (8.4 g/L) and with or without doxycycline (1 mg/ml) for 4 weeks. Animals were gradually adapted to an EtOH-containing diet (0% for 2 days, 1% for 2 days, and 2% EtOH for 2 days followed by 4% EtOH for 1 week, 5% for 1 week and 6% for 1 week). Corresponding control groups received isocaloric maltose dextrin in place of EtOH. Diet intake was recorded daily and body weights recorded twice a week. A schematic of timeline of feeding EtOH, Gln and doxycycline is provided (Fig. 1A). In all experiments, animals were maintained in pairs to facilitate body temperature maintenance. At the end of the experiment, the gut permeability was measured as described below. Blood samples were collected by cardiac puncture (into heparinized tubes) and centrifuged at 3,000 $\times$ g for 10 minutes at 4°C to prepare plasma samples. Pieces (approximately 0.5 cm long) of distal colon were fixed in buffered formalin or cryofixed in OCT, and remainder of the tissues was stored in RNAlater for RNA isolation.

Gut Permeability *in vivo*

At the end of each set of experiments, mice were intravenously injected with FITC-inulin (100 mg/ml solution; 2 µl/g body weight) via tail vein using a restrainer. One hour after injection, blood samples were collected by cardiac puncture under isoflurane anesthesia to prepare plasma. Mice were euthanized by cervical dislocation under isoflurane anesthesia. Luminal contents of ileum or colon were flushed with 0.9% saline. Fluorescence in plasma and luminal flushing samples was measured using a fluorescence plate reader. Fluorescence values in the luminal flushing were normalized to fluorescence values in corresponding plasma samples and calculated as percent of inulin load. This *in vivo* method to assess intestinal mucosal permeability measures vascular-to-lumen flux of inulin, which involves no physical manipulation of the intestinal segment.

Immunofluorescence microscopy

Cryosections (10 µm thickness) were fixed in acetone:methanol (1:1) at -20°C for 2 minutes and rehydrated in phosphate buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, and 1.8 mM potassium dihydrogen phosphate). Sections were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes and blocked in 4% nonfat milk in Triton-Tris buffer (150 mM sodium chloride containing 10% Tween-20 and 20 mM Tris, pH 7.4). It was then incubated for 1 hour with the primary antibodies (mouse monoclonal anti-occludin and rabbit polyclonal anti-ZO-1 antibodies or mouse monoclonal E-cadherin and rabbit polyclonal anti-β-catenin antibodies or mouse monoclonal phospho-cofilin antibody), followed by incubation for 1 hour with secondary antibodies (AlexaFluor- 488-conjugated anti-mouse IgG and cy3-conjugated anti-rabbit IgG antibodies) and 10 min incubation with Hoechst 33342 dye. Actin cytoskeleton was stained with AlexaFluor-488 conjugated phalloidin. The fluorescence was examined using a Zeiss 710 confocal microscope (Carl Zeiss Microscopy, Jena, Germany), and images from x-y sections (1 µm) were collected using LSM 5 Pascal software (Carl Zeiss Microscopy). Images were stacked by using the software Image J (NIH, Bethesda, MD), and processed by Adobe Photoshop (Adobe Systems Inc., San Jose, CA). All images for tissue samples from different group were collected and processed under identical conditions.

Quantitative RT-PCR

Total RNA (1.5 µg) was used for generation of cDNAs using the ThermoScript RT-PCR system for first strand synthesis (Invitrogen). Quantitative PCR (qPCR) reactions were performed using cDNA mix (cDNA corresponding to 35 ng RNA) with 300 nmoles of primers in a final volume of 25 µl of 2× concentrated RT2 Real-Time SYBR Green/ROX master mix (Qiagen) in an Applied Biosystems QuatStudio 6-Flex Real-Time PCR instrument (Norwalk, CT, USA). The cycle parameters were: 50 °C for 2 min, one denaturation step at 95 °C for 10 min and 40 cycles of denaturation at 95 °C for 10s followed by annealing and elongation at 60 °C. Relative gene expression of each transcript was normalized to GAPDH using the Ct method. Sequences of primers used for qPCR are provided in Table 1.

Protein thiol assay

Reduced protein thiols were evaluated by staining cryosections of colon with BODIPY FL-N-(2-aminoethyl)maleimide (Flm) and confocal microscopy at excitation and emission wavelengths of 490 and 534 nm, respectively. For oxidized protein thiols, the reduced protein thiol was first alkylated with N-ethylmaleimide followed by reduction of oxidized protein thiols with tris(2-carboxyethyl)phosphine before staining with Flm. Control staining is done after N-ethylmaleimide treatment. Fluorescence images were captured using a confocal microscope, and fluorescence in all images was quantitated by Image J software.

Plasma endotoxin assay

Plasma endotoxin concentrations were measured using Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Cat# 88282) according to vendor's instructions.

Liver Histopathology

Liver and colon tissues were fixed in 10% buffered formalin and 8 μ m-thick paraffin-embedded sections were stained with hematoxylin and eosin (H&E). Stained sections were imaged in a Nikon 80Ti microscope (Nikon Instruments, Inc., Melville, NY) using 40x objective lens and a color camera.

Plasma Transaminase Assay

Plasma alanine transaminase (ALT) (Cayman chemicals; Ann Arbor, MI) and Aspartate transaminase (AST) (BioAssay systems; Hayward, CA) activities were measured by colorimetric assay according to vendor's instructions.

Triglyceride Assay

Liver triglyceride was assayed by GPO method using the assay kit from Pointe Scientific Inc., (Canton, MI). Liver lipids were extracted by digesting the tissue with 3 M potassium hydroxide (in 65% EtOH) for 1 hour at 70°C at room temperature for 24 hours. Hepatic triglycerides were measured by enzymatic hydrolysis of triglycerides to glycerol and free fatty acids followed by colorimetric measurement (at 540 nm wavelength) of glycerol. Values for hepatic triglycerides were expressed as mg triglyceride/g liver tissue.

Cell culture and Acetaldehyde treatment

Caco-2 cells, purchased from the American Type Culture Collection (ATCC; Rockville, MD, U.S.A.), were maintained under standard cell culture conditions at 37°C in DMEM containing 10% (v/v) fetal bovine serum. Cells were grown on polycarbonate membranes in Transwells (6.5 mm; Costar, Cambridge, MA, U.S.A.), and experiments were performed on 12–14 days after seeding. Monolayers were bathed in PBS containing 1.2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose, 0.6% bovine serum albumin, and 0.1 mM cyanamide, 0.2 ml and 1.0 ml to apical and basal wells, respectively. After 1 hour equilibration, cell monolayers were exposed to acetaldehyde vapor generating 400 μ M acetaldehyde in the incubation buffer as described previously [29]. In some experiments cell monolayers were pretreated with PP2 (10 μ M; Src kinase inhibitor), GM6001 (25, 50 and 100 μ M; matrix

metalloprotease inhibitor), 1,10-Phe (30 and 100 μ M; metalloprotease inhibitor) and Anti-EGFR (20 μ g/ml) 30 min prior to Gln (2 mM) treatment.

Epithelial barrier function

Transepithelial electrical resistance (TER) was measured using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). TER was calculated as Ohms.cm² by multiplying it with the surface area of monolayer. To evaluate the paracellular permeability, cell monolayers were incubated with FITC-inulin (6 kD; 0.5 mg/ml) in the apical well and measured the unidirectional flux of FITC-inulin as described previously [29].

Protease Zymography

MMP-9 and MMP-2 protein secretion and activation by Gln were measured using gelatin zymography under non-reducing conditions [30]. Briefly, Caco-2 cell monolayers were treated with Gln for 1h and medium from the apical well were mixed with non-reducing SDS gel sample buffer and applied without boiling to a 10% polyacrylamide gel containing 0.1% SDS and 1 mg/ml gelatin. After electrophoresis, the gels were washed in 50 mmol/l Tris-HCl (pH 7.5) containing 0.15 mol/l NaCl, 5 mmol/l CaCl₂, 5 μ mol/l ZnCl₂, 0.02% NaN₃, 0.25% Triton X-100 (three changes) at room temperature, and then incubated in the same buffer without Triton X-100 (two changes) at 37°C for 20 h. Proteins were stained by Coomassie Brilliant Blue R-250 solution and photographed.

Plasma cytokine assay

Plasma cytokine levels were measured using commercially available immunoassay ELISA kits for mice (R&D System, Minneapolis, MN, USA). TNF- α (Cat# DY410) and interleukin-6 (Cat# DY406) levels were estimated according to the manufacturer's instructions. The results are expressed as picograms of cytokine per milliliter of plasma.

Statistical analyses

All data are expressed as Mean $\pm$ SEM. The differences among multiple groups were first analyzed by ANOVA (Prism 6.0). When a statistical significance was detected, Tukey's t test was used to determine the statistical significance between multiple testing groups and the corresponding control. Statistical significance was established at 95%.

Results

Gln prevents EtOH-induced colonic barrier dysfunction and endotoxemia in mice by EGFR-dependent mechanism

To determine the role of EGFR in Gln-mediated prevention of EtOH-induced colonic mucosal barrier dysfunction and endotoxin absorption we applied a chronic EtOH feeding model using Lieber-DeCarli liquid diet, with or without Gln supplementation, in rtTA control mice and rtTA-EGFR**Tg* mice that express dominant negative EGFR in a doxycycline-dependent mechanism (Fig. 1A). Therefore, EtOH + Gln groups were examined with or without doxycycline in the diet. Similar diet intakes in different groups were maintained by pair feeding. The body weights of EtOH-fed rtTA and rtTA-EGFR**Tg*

mice were not different from each other (Fig. 1B). In EtOH + Gln-fed rtTA mice, the body weights were not different in the presence and absence of doxycycline (Fig. 1C). On the other hand, in EtOH + Gln-fed rtTA-EGFR**Tg* mice, the body weights were significantly low in the presence of doxycycline compared to body weights in the absence of doxycycline. Although we do not know the precise reason for this effect, what is clear is that Dox (expression of EGFR*) reduces body weight only when the Gln is fed. Relatively, in the absence of Dox, Gln increases body weights in both WT and *Tg* mice possibly by an EGFR-dependent mechanism. The Dox-induced loss of body weight possibly targets the Gln-mediated increase in body weight.

Serosal to luminal flux of FITC-inulin in EtOH-fed rtTA mice was significantly higher than that in pair fed rtTA mice (Fig. 1D). Gln feeding blocked the effect of EtOH on inulin permeability irrespective of the presence or absence of doxycycline. In EtOH-fed rtTA-EGFR**Tg* mice, Gln blocked the EtOH-induced inulin permeability in the absence of doxycycline, but not in the presence of doxycycline. Similarly, Gln blocked EtOH-induced elevation of plasma LPS in the presence or absence of doxycycline (Fig. 1E). On the other hand, in EtOH-fed rtTA-EGFR**Tg* mice, Gln blocked EtOH-induced elevation of plasma LPS in the absence of doxycycline, but not in the presence of doxycycline.

EGFR mediates prevention of EtOH-induced colonic epithelial TJ and AJ disruption by Gln

To determine the effect of EtOH and Gln on colonic epithelial TJ integrity we examined the distribution of TJ proteins, the occludin and ZO-1, by immunofluorescence method. EtOH-induced redistribution of occludin and ZO-1 from the colonic epithelial junctions was blocked by Gln in rtTA mice in the presence or absence of doxycycline (Fig. 2A). But in rtTA-EGFR**Tg* mice, Gln blocked EtOH-induced redistribution of TJ proteins in the absence of doxycycline, but not in the presence of doxycycline (Fig. 2B). This observation was confirmed by measuring the density of ZO-1 fluorescence (Fig. 2C) at the epithelial junctions. Similarly, EtOH caused redistribution of AJ proteins (E-cadherin and β -catenin) from the colonic epithelial junctions, and Gln prevented this effect of EtOH in rtTA mice in the presence or absence of doxycycline (Fig. 3A). In rtTA-EGFR**Tg* mice, Gln blocked EtOH-induced redistribution of AJ proteins in the absence of doxycycline, but not in the presence of doxycycline (Fig. 3B). This observation was confirmed by measuring the fluorescence densities for E-Cadherin fluorescence (Fig. 3C) at the epithelial junctions.

The changes in the levels of TJ and AJ proteins were further evaluated by immunoblot analysis (Fig. 4A). Quantification of bands for occludin (Fig. 4B), ZO-1 (Fig. 4C), E-cadherin (Fig. 4D) and β -catenin (Fig. 4E) was performed by using Image J software. Data demonstrate that EtOH caused reduction in the levels of occludin and β -catenin in the colonic mucosa, and Gln prevented this effect of EtOH in rtTA mice in the presence or absence of doxycycline, but only in the absence of doxycycline in rtTA-EGFR**Tg* mice. The levels of ZO-1 and E-cadherin were not significantly altered by EtOH or Gln, except that Gln significantly reduced both ZO-1 and E-cadherin levels in rtTA-EGFR**Tg* mice in the presence of doxycycline.

Neither EtOH or Gln modulated the levels EGFR in colonic mucosa (Fig. 4F). Gln tends to elevate EGFR in rtTA-EGFR**Tg* mouse colon, but it was not statistically significant under

our experimental conditions. The levels of β -actin were unaffected by EtOH or Gln (Fig. 4G).

EtOH induces cofilin activation and disrupts actin cytoskeleton, and Gln blocks these effects of EtOH by EGFR-dependent mechanism

Integrity of actin cytoskeleton is important for assembly and maintenance of TJ and AJ. F-actin staining with AlexaFluor-488-phalloidin and immunofluorescence imaging showed that EtOH feeding caused reduced F-actin in colonic mucosa in both rtTA and rtTA-EGFR**Tg* mice (Fig. 5A). Gln supplementation blocked EtOH-induced F-actin disruption in the presence or absence of doxycycline in rtTA mice, but only in the absence of doxycycline in rtTA-EGFR**Tg* mice. Cofilin is an actin severing protein, which disrupts actin cytoskeleton when activated by dephosphorylation [31]. Cofilin is mostly present in the cell in an inactive form, in which Ser3 residue is phosphorylated. We stained colonic sections for total cofilin and phospho-cofilin (inactive) by immunofluorescence method, and the fluorescence was quantified by densitometric analysis. EtOH feeding reduced the stain for phospho-cofilin in the colonic mucosa of both rtTA and rtTA-EGFR**Tg* mice (Fig. 5B-D). Gln supplementation blocked this effect of EtOH in the presence or absence of doxycycline in rtTA mice (Fig. 5B & C), but, only in the absence of doxycycline in rtTA-EGFR**Tg* mice (Fig. 5B & D). The amounts of total cofilin were not significantly different from each other in different groups. The phospho-cofilin/cofilin ratio was significantly reduced by EtOH in rtTA mice, which was blocked by Gln in the presence or absence of doxycycline (Fig. 5E). But in rtTA-EGFR**Tg* mice, Gln blocked the EtOH-induced reduction of phospho-cofilin/cofilin ratio in the absence of doxycycline, but not in the presence of doxycycline (Fig. 5F).

EtOH feeding induces protein thiol oxidation and suppresses antioxidant gene expression in colonic mucosa, and Gln blocks these effects of EtOH by EGFR-dependent mechanism

Oxidative stress is known to disrupt TJ, AJ and actin cytoskeleton in different epithelia [32], and EtOH was shown to induce oxidative stress both in Caco-2 cells and mouse colon [11, 33]. In the present study, EtOH feeding caused a depletion of reduced protein thiols in colonic mucosa, which was associated with a corresponding increase in oxidized protein thiols (Fig. 6). Gln supplementation blocked EtOH-induced depletion of reduced-protein thiols (Fig. 6A & 6B) and elevation of oxidized-protein thiols (Fig. 6C & 6D) in colonic mucosa in the presence or absence of doxycycline in rtTA mice. In rtTA-EGFR**Tg* mice, Gln blocked EtOH-induced protein thiol oxidation in the absence of doxycycline, but not in the presence of doxycycline. Gln attenuated EtOH-induced depletion of mRNA for *SOD1* (Fig. 6E), *SOD2* (Fig. 6F), thioredoxin (*Trx1*) (Fig. 6G), glutathione peroxidase (*Gpx1*) (Fig. 6H). *Catalase* expression was unaffected by EtOH or Gln in rtTA mice (Fig. 6I). But, in rtTA-EGFR**Tg* mice, EtOH reduced catalase expression, but it was not influenced by Gln. Nuclear factor (erythroid 2)-like 2 (*Nrf2*) is the transcription factor involved in regulation of antioxidant gene expression. In the present study, *Nrf2* expression is reduced by EtOH, which was prevented by Gln in rtTA mice in the presence or absence of doxycycline (Fig. 6J). But, in rtTA-EGFR**Tg* mice, Gln blocked EtOH-induced reduction of mRNA for antioxidant and *Nrf2* genes in the absence of doxycycline, but not in the presence of doxycycline.

EtOH elevates pro-inflammatory cytokine and chemokine gene expression in colon, and Gln blocks these effects of EtOH by EGFR-dependent mechanism

The effect of chronic EtOH feeding on inflammatory responses in colon is poorly understood. The present study shows that EtOH increases *IL-6* (Fig. 7A) and decreases *IL-10* (Fig. 7B) mRNA levels by several folds in both rtTA and rtTA-EGFR**Tg* mice. Gln supplementation blocked EtOH-induced changes of mRNA for these cytokines in rtTA mice in the absence or presence of doxycycline. But in rtTA-EGFR**Tg* mice, Gln blocked EtOH-induced alteration of *IL-6* and *IL-10* gene expression in the absence of doxycycline, but not in the presence of doxycycline.

Similarly, EtOH feeding increased the levels of mRNA for *MCPI* (Fig. 7C), *CCL5* (Fig. 7D) and *CXCL1* (Fig. 7E) genes in the colon of both rtTA and rtTA-EGFR**Tg* mice. Gln blocked EtOH-induced expression of these chemokines in the presence or absence of doxycycline in rtTA mice. In rtTA-EGFR**Tg* mice, Gln blocked EtOH-induced elevation of chemokine mRNA in the absence of doxycycline, but not in the presence of doxycycline.

Gln blocks EtOH-induced liver injury by an EGFR-dependent mechanism

EtOH-induced disruption of epithelial junctions and increased mucosal permeability to LPS from the colonic lumen into the systemic circulation play a crucial role in the pathogenesis of ALD. Histopathology showed that EtOH feeding caused steatosis in both rtTA and rtTA-EGFR**Tg* mice (Fig. 8A). Gln blocked this effect of EtOH on liver in the presence or absence of doxycycline in rtTA mice, whereas Gln blocked steatosis in rtTA-EGFR**Tg* mice only in absence of doxycycline. The EtOH-induced steatosis was associated with a corresponding increase in plasma ALT (Fig. 8B) and AST (Fig. 8C) activities as well as elevation of liver triglyceride (Fig. 8D) in both rtTA and rtTA-EGFR**Tg* mice. Once again, Gln blocked these effects of EtOH in the presence or absence of doxycycline in rtTA mice, but only in the absence of doxycycline in rtTA-EGFR**Tg* mice.

MCPI (Fig. 8E) and *CXCL2* (Fig. 8F) mRNA levels in the liver were several folds higher in EtOH-fed rtTA and rtTA-EGFR**Tg* mice. Gln blocked these effects of EtOH on liver in the presence or absence of doxycycline in rtTA mice, whereas Gln blocked chemokine expression in rtTA-EGFR**Tg* mice only in the absence of doxycycline. EtOH feeding reduced the levels of mRNA for antioxidant genes, *Trx1* (Fig. 9A), *SOD1* (Fig. 9B) and *Catalase* (Fig. 9C), as well as the mRNA for *Nrf2* gene (Fig. 9D) in both rtTA and rtTA-EGFR**Tg* mice. These effects of EtOH on *Trx1*, *SOD1*, *Catalase* and *Nrf2* expression in liver was blocked by Gln in the presence or absence of doxycycline in rtTA mice, but only in the absence of doxycycline in rtTA-EGFR**Tg* mice.

Systemic inflammation was assessed by measuring the cytokine levels in plasma by ELISA. EtOH significantly elevated plasma *IL-6* (Fig. 10A) and *TNF-α* (Fig. 10B), which was effectively prevented by Gln in the presence or absence of doxycycline in rtTA mice. In rtTA-EGFR**Tg* mice, Gln prevented EtOH-induced elevation of plasma cytokines only in the absence of doxycycline, but not in the presence of doxycycline.

The mechanism of Gln-mediated prevention of acetaldehyde-induced barrier dysfunction in Caco-2 cell monolayers *in vitro*

To determine the direct effect of Gln on the intestinal epithelium and the role of EGFR in this effect of Gln we analyzed the effect of Gln on acetaldehyde-induced disruption of barrier function in Caco-2 cell monolayers. Gln treatment effectively blocked acetaldehyde-induced increase in inulin permeability (Fig. 11A). Acetaldehyde-induced permeability was blocked almost completely when Gln was administered prior to and during acetaldehyde or when present only during the acetaldehyde. Significant reduction of acetaldehyde-induced permeability was recorded even when cell monolayers were pretreated with Gln, but washed off prior to acetaldehyde treatment. Gln-mediated prevention of acetaldehyde-induced decrease in TER (Fig. 11B) and inulin permeability (Fig. 11C) was blocked by PP2, a Src kinase inhibitor, and anti-EGFR antibody targeting the extracellular, ligand binding domain. Zymographic analysis of the medium from Caco-2 cell monolayers treated with Gln showed release of two bands of proteolytic components with mobility corresponding to MMP-2 and MMP-9 (Fig. 11D). Pretreatment of cell monolayers with metalloproteinase inhibitors, GM6001 and 1,10-Phenanthroline, dose-dependently blocked Gln-mediated prevention of acetaldehyde-induced drop in TER (Fig. 11E) and inulin permeability (Fig. 11F).

Discussion

A growing body of evidence indicates that Gln supplementation may have beneficial effect in the treatment of prolonged stress, shock, trauma, radiation syndrome and intestinal atrophy during total parenteral nutrition [23, 34-37]. Experimental evidence suggests that Gln supplementation may play an important role in the prevention of alcoholic tissue injury [21, 38]. It is therefore, crucial to understand the mechanisms involved in the protective effect of Gln against alcoholic tissue injury. In the present study, we investigated the potential role of EGFR in the mechanisms involved in the Gln-mediated prevention of colonic mucosal barrier dysfunction, endotoxemia and liver damage by chronic EtOH feeding using the EGFR**Tg* mice that conditionally express a dominant negative mutant EGFR.

Vascular to luminal flux of FITC-inulin *in vivo* and plasma LPS were elevated by chronic EtOH feeding, demonstrating that EtOH feeding disrupts mucosal barrier function and allows diffusion of bacterial endotoxins from the colonic lumen into the systemic circulation in rtTA and rtTA-EGFR**Tg* mice. Gln supplementation prevents EtOH-induced gut permeability and endotoxemia in both strains of mice. Expression of dominant negative EGFR in the transgenic mice showed a significant reduction in this epithelial protective effect of Gln. Since the dominant negative EGFR is expressed by doxycycline-dependent mechanism the attenuation of Gln-mediated epithelial protection was recorded only when rtTA-EGFR**Tg* mice were pre-treated with doxycycline. In rtTA control mice, Gln was protective irrespective of the presence or absence of doxycycline. These data demonstrate, in an *in vivo* model, that Gln-mediated prevention of alcoholic gut barrier dysfunction and endotoxemia requires EGFR. Gln may indirectly activate EGFR in the colonic epithelium and activate downstream signaling to protect the epithelial junctions from EtOH. Our

previous studies indicated that EGFR activation by EGF prevents acetaldehyde-induced barrier disruption in Caco-2 cell monolayers and human colonic mucosa [29].

Gut barrier dysfunction and endotoxemia are some of the initial events involved in the development of ALD [1]. However, it is unclear whether gut permeability and endotoxemia are prerequisites for the development of alcoholic liver damage or if these are second hits that exacerbate the liver damage caused directly by EtOH and its metabolites. Either way, Gln supplementation may prove to be beneficial in reducing the symptoms of ALD. In the present study permeability in the whole colon was measured. Our previous studies showed that EtOH-mediated permeability occur predominantly in the distal colon. EtOH had no effect on mucosal permeability in the ileum. We do recognize that studies in other laboratories have shown EtOH-induced permeability in the ileum. We believe such discrepancy is mainly due to differences in the experimental conditions and the source of mice, which may carry distinct microbiome populations

The role of EGFR in Gln-mediated prevention of alcoholic gut barrier dysfunction and resulting endotoxemia is further confirmed by the lack of protective effect of Gln on disruption of colonic epithelial TJ and AJ in EGFR**Tg* mice treated with doxycycline. In *rtTA* control mice, Gln prevented the redistribution of TJ (occludin and ZO-1) and the AJ (E-cadherin and β -catenin) proteins from the epithelial junctions in the presence or absence of doxycycline treatment. Interaction between occludin and ZO-1 is essential for the assembly and maintenance of TJ and development of barrier function. Similarly, interaction between E-cadherin and β -catenin is essential for the integrity of AJ. Therefore, preservation of TJ and AJ protein organization represent the cellular mechanisms involved in the epithelial barrier protective role of Gln. Gln-mediated cell signaling may interrupt the signaling mechanisms activated by EtOH. Immunoblot analysis indicated that EtOH feeding significantly reduced the levels of occludin and β -catenin in the colonic mucosa, which was prevented by Gln in an EGFR-dependent mechanism. The effects of EtOH and Gln may be caused by modulation of either the expression or degradation of these proteins. Unlike the immunoblot analysis, quantitation of immunofluorescence densities of ZO-1 and E-cadherin were reduced by EtOH and Gln prevented it by EGFR-dependent mechanism. This suggest that the total protein may not be affected by EtOH or Gln, but its distribution at the junctions is altered. The level of EGFR was not significantly altered by EtOH or Gln. It is possible that EtOH or Gln may alter the expression of Gln transporter or the activity of Gln may exclusively involve activation of EGFR.

Assembly and maintenance of TJ and AJ requires anchoring of these protein complexes with the actin cytoskeleton [39]. Disruption of actin cytoskeleton has been shown to result in disruption of TJ and AJ [40]. Therefore, we examined the actin cytoskeleton integrity in colonic mucosa by immunofluorescence method. Images showed that EtOH disrupts actin cytoskeleton and Gln supplementation blocks this effect of EtOH. However, the protective effect of Gln was absent in EGFR**Tg* mice treated with doxycycline, indicating that EGFR is involved in this effect of Gln. The precise mechanism of EtOH-induced disruption of actin cytoskeleton is unknown. Cofilin is an actin severing protein known to play a significant role in the breakdown of actin cytoskeleton, especially in the active process of tread milling during cell migration [41]. Cofilin is normally maintained inactive by phosphorylation on

Ser3 residue by LIM kinase [42]. Cofilin is activated by dephosphorylation of cofilin. Our data show that EtOH induced activation of cofilin in colonic mucosa, and this effect of EtOH was blocked by Gln in rtTA control mice as well as in EGFR**Tg* mice in the absence of doxycycline. But, Gln failed to block cofilin activation in EGFR**Tg* mice in the presence of doxycycline, indicating the role of EGFR in Gln-mediated suppression of cofilin activation. These results suggest that EtOH-mediated cell signaling dephosphorylates cofilin and cell signaling activated by Gln interrupt this EtOH-mediated cell signaling.

The precise mechanism of Gln-mediated protection of intestinal epithelial TJ and AJ is unclear. Our previous study showed that chronic EtOH feeding induced protein thiol oxidation, and Gln supplementation blocked this effect of EtOH [21]. Our present study shows that this effect of Gln is absent in EGFR**Tg* mice treated with doxycycline, demonstrating that the antioxidant property of Gln requires EGFR. The mechanism involved in the antioxidant property of Gln is not clear. Therefore, we extended this study further to determine the influence of Gln on expression of antioxidant genes by RT-qPCR.

Results indicate that EtOH feeding down regulates the expression of *SOD1*, *SOD2*, *Trx1*, and *Gpx1* genes in the colonic mucosa of rtTA and rtTA-EGFR**Tg* mice, indicating that suppression of antioxidant genes is an important factor in the mechanism of EtOH-induced oxidative stress in mouse colon. Gln supplementation blocked EtOH-induced suppression of antioxidant gene expression in both mice. However, Gln effect on antioxidant gene expression was absent in rtTA-EGFR**Tg* mice in the presence of doxycycline. These results demonstrate that EGFR mediates the Gln effects on the regulation of antioxidant gene expression in EtOH-fed mice. The transcription factor, *Nrf2*, is known to play an important role in regulation of antioxidant gene expression [43]. Our data show that EtOH down regulates *Nrf2* gene expression and Gln blocks this effect of EtOH in rtTA mice irrespective of the presence or absence of doxycycline. But in EGFR**Tg* mice treated with doxycycline, Gln had no effect on *Nrf2* expression in colon. This observation suggests that regulation of *Nrf2* gene expression is a factor involved in EtOH and Gln-mediated regulation of antioxidant gene expression in mouse colon. Oxidative stress is previously shown to regulate the integrity of intestinal epithelial TJ and AJ [32, 44, 45]. Therefore, protein thiol oxidation caused by down regulation of antioxidant gene expression might be involved in EtOH and Gln-mediated regulation of TJ and AJ integrity in mouse colon. In addition, oxidative stress may activate cofilin and disrupt the actin cytoskeleton. It is possible that EtOH causes other types of oxidative stress, such as lipid peroxidation and DNA damage. But, our previous studies have demonstrated that protein thiol oxidation is the primary mechanism by which oxidative stress disrupts intestinal epithelial TJ and AJ.

Oxidative stress-mediated disruption of TJ and barrier dysfunction is likely to result in diffusion of luminal LPS into the intestinal mucosa. LPS in the mucosa is known to induce inflammatory responses. Therefore, we assessed the expression of inflammatory cytokine and chemokine genes in the colonic mucosa. EtOH feeding induced an increase in *IL-6* and decrease in *IL-10* mRNA levels. Gln blocked these effects in rtTA control mice in the presence or absence of doxycycline. But in EGFR**Tg* mice, this effect of Gln was absent in the presence of doxycycline, indicating that EGFR is again involved in this effect of Gln. Similarly, EtOH feeding elevated mRNA for *MCPI*, *CCL5* and *CXCL1* genes in mouse

colonic mucosa. Gln blocked the effect of EtOH on chemokine gene expression in rtTA mice, but not in EGFR**Tg* mice in the presence of doxycycline. Pro-inflammatory cytokine and chemokine gene expression is likely the result of diffusion of LPS across the epithelium into lamina propria region. But, it is unclear, at this time, whether these cytokines and chemokines play a role in further disruption of TJ and AJ under the present experimental conditions. *TNFA* [46], *IL-1* [47] and *IL-6* [48] have been previously implicated in disruption of epithelial TJ. Therefore, it is likely that these cytokines disrupt TJ and AJ, however, further studies are necessary to test this hypothesis.

Colonic barrier disruption and endotoxemia are likely to affect multiple organ systems. Liver is likely the most affected organ as it is the “organ of first pass” for absorbed LPS. Histopathologic analysis shows that EtOH induces liver steatosis in these strains of mice, and Gln supplementation blocks this effect of EtOH on liver. Similar to its effect on gut barrier function and endotoxemia, Gln prevents EtOH-induced steatosis in rtTA mice in the presence or absence of doxycycline, and in rtTA-EGFR**Tg* mice in the absence of doxycycline, but not in the presence of doxycycline, demonstrating that EGFR mediates the effect of Gln on alcoholic liver damage. The steatosis was confirmed by liver triglyceride analysis. Liver damage was assessed by ALT and AST analyses. In agreement with its effects on gut permeability and endotoxemia, Gln prevents EtOH-induced elevation of liver triglyceride and plasma ALT and AST activities in rtTA mice irrespective of doxycycline, but not in EGFR**Tg* mice in the presence of doxycycline. Inflammatory responses in the liver was confirmed by analysis of expression of inflammatory chemokines, *MCPI* and *CXCL2*. EtOH-induced elevation of *MCPI* and *CXCL2* mRNA was blocked by Gln supplementation in rtTA mice and in EGFR**Tg* mice in the absence of doxycycline.

Furthermore, EtOH feeding down regulated the expression of *Trx1*, *SOD1*, *Catalase* and *Nrf2* in the liver, which is blocked by Gln in rtTA mice irrespective of doxycycline and in EGFR**Tg* mice only in the absence of doxycycline. The parallel changes in the levels of *Nrf2* mRNA in both colon and liver suggests that changes in the expression of some of the antioxidant genes by EtOH and Gln may be mediated by NRF2. NRF2 is known to regulate the expression of *NQO1*, *GST*, *Gpx*, *Trx* and *Prdx* genes. It is however, unclear whether *SOD* and *catalase* genes are regulated by NRF2. Interestingly, the changes in the levels of catalase mRNA do not parallel the changes in *Nrf2* mRNA.

Our data demonstrates that EGFR plays an important role in Gln-mediated prevention of steatosis and inflammation in the liver. However, further studies are needed to determine whether the effect on liver was exclusively caused by EGFR-mediated effects on gut barrier and endotoxemia or a direct effect of Gln and EGFR signaling in the liver also contribute to these effects on liver. Inflammatory responses in the colon and liver and endotoxemia is also reflected in systemic inflammation with elevated IL-6 and TNF- α in the plasma of EtOH-fed mice. Systemic inflammation was also prevented by Gln in an EGFR-dependent mechanism. It is however, the source of cytokines in the plasma is unclear. In the present study, doxycycline-mediated expression of EGFR* in rtTA-EGFR**Tg* mice is global. It is likely that EGFR in the liver is also likely to be dysfunctional. Therefore, it is unclear whether the effects on the liver was caused by the dysfunctional EGFR in the liver or due to alteration of intestinal barrier disruption and LPS flux into liver.

Our previous studies have demonstrated that Gln directly influences Caco-2 cell monolayers to exert its protective effect against acetaldehyde-induced TJ and AJ disruption and barrier dysfunction. This effect of Gln on Caco-2 cell monolayer was mediated by EGFR. Our current data show that presence of Gln during the acetaldehyde exposure is suffice for protecting the epithelial barrier from acetaldehyde in Caco-2 cell monolayers. Gln-mediated prevention of barrier disruption was blocked by a Src kinase inhibitor as well as by an anti-EGFR antibody that binds to the extracellular ligand binding domain of EGFR. These data suggest that activation of EGFR by ligand binding is involved in the protective effect of Gln, suggesting that Gln may activate mechanisms to release membrane-bound EGFR ligands such as EGF and TGF α . The zymographic analysis of the medium from cells incubated with Gln showed the presence of two MMP isoforms with the mobility on gel corresponding to MMP-2 and MMP-9. Furthermore, pretreatment of cell monolayers with metalloproteinase inhibitors such as GM6001 and 1,10-phenanthroline dose-dependently blocked the barrier protective effect of Gln. These data suggest that Gln may activate cellular signaling to release MMP to the medium, which in turn releases membrane-bound EGFR ligands. The potential mechanism of EtOH-induced cofilin activation, disruption of cytoskeleton and TJ disruption, and the potential roles of MMP, EGFR and subsequent signaling in Gln-mediated prevention of EtOH effects are outlined in the schematic figure (Fig. 12). The precise mechanism involved in Gln activity is unclear at this time. It is unclear whether Gln activity is initiated at the cell membrane or in the intracellular compartment after the uptake via Gln transporter. Gln appears to release MMP into the extracellular medium, but the cellular mechanism that mediates MMP release is remains unknown. Gln metabolism has been previously shown to regulate autophagy-dependent mTOR (mammalian target of rapamycin) activation [49]. It is possible that a cross talk between mTOR activity and EGFR is involved in the mechanism of Gln-mediated protection against EtOH. However, our previous study indicated that metabolism of Gln is not necessary for its intestinal protective effect. Therefore, further studies are necessary to determine the mechanism involved in this activity of Gln.

In summary, this study demonstrates that EGFR plays an important role in Gln-mediated prevention of alcoholic gut barrier disruption, endotoxemia and liver damage in mice *in vivo*. In addition to TJ disruption, barrier dysfunction and endotoxemia, EtOH down regulates antioxidant gene expression and up regulates inflammatory cytokine and chemokine gene expression in the colon, which are blocked by Gln in an EGFR-dependent mechanism. Gln may serve as a potential preventive factor in the treatment of ALD and alcoholic injury in other organs.

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Abbreviations

AJ adherens junction

AJC	apical junctional complex
ALD	alcoholic liver disease
ALT	alanine aminotransferase
AST	aspartate aminotransferase
EGFR	epidermal growth factor receptor
EGFR*	dominant negative EGFR
EtOH	ethanol
FITC	fluorescein isothiocyanate
Gln	glutamine
Gpx	glutathione peroxidase
HRP	horseradish peroxidase
LPS	lipopolysaccharide
MMP	metalloproteinase
Nrf2	nuclear factor (erythroid 2)-like 2
ROS	reactive oxygen species
SOD	superoxide dismutase
TER	transepithelial electrical resistance
TJ	tight junction
TLR	toll-like receptor
TRE	tetracycline responsive element
Trx	thioredoxin
ZO-1	zonula occludens-1

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Figure 1: Gln prevents EtOH-induced barrier dysfunction and endotoxemia by an EGFR-dependent mechanism.

A: Schematic representation of timeline for feeding different concentrations of EtOH, doxycycline and glutamine (Gln). Adult rtTA (WT) and rtTA-EGFR**Tg* (Tg) mice were fed a liquid diet with different concentrations of EtOH (EF) or isocaloric maltodextrin (PF) and with or without Gln and with or without doxycycline (Dox). **B-E:** Body weights EtOH fed (B) and EtOH+Gln fed (C) mice were recorded. Colonic mucosal permeability *in vivo* (D) and plasma LPS levels (E) were measured. In all panels, values are mean $\pm$ sem (n = 6). In panel C, asterisks indicate the values are significantly different from corresponding values with doxycycline. In panels D and E, asterisks indicate the values that are significantly ($p < 0.05$) different from corresponding PF values, the hash tags indicate the value different from corresponding EF values, and Δ indicates the values different from corresponding EF +Gln without doxycycline values.

Figure 2: Gln prevents EtOH-induced disruption of TJ by EGFR-dependent mechanism.

Adult rtTA (A) and rtTA-EGFR*⁺Tg (B) mice were fed a liquid diet with EtOH (EF) or isocaloric maltodextrin (PF) and with or without Gln and with or without doxycycline (Dox). Cryosections of colon were stained for occludin (green), ZO-1 (red) and nucleus (blue) by immunofluorescence method. Density of ZO-1 fluorescence at the epithelial junctions was measured using Image J software (C). Values are mean $\pm$ sem (n = 6). Asterisks indicate the values that are significantly (p<0.05) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and indicates the values that are different from corresponding EF+Gln without doxycycline values.

Figure 3: Gln prevents EtOH-induced disruption of AJ by EGFR-dependent mechanism.

Adult rtTA (A) and rtTA-EGFR* Tg (B) mice were fed a liquid diet with EtOH (EF) or isocaloric maltodextrin (PF) and with or without Gln and with or without doxycycline (Dox). Cryosections of colon were stained for E-cadherin (green), β -catenin (red) and nucleus (blue) by immunofluorescence method. Density of E-cadherin fluorescence at the epithelial junctions was measured using Image J software (C). Values are mean $\pm$ sem (n = 6). Asterisks indicate the values that are significantly ($p < 0.05$) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and indicates the values that are different from corresponding EF+Gln without doxycycline values.

Figure 4: Gln prevents EtOH-induced down regulation of TJ and AJ by EGFR-dependent mechanism.

Adult rtTA (A) and rtTA-EGFR* Tg (B) mice were fed a liquid diet with EtOH (EF) or isocaloric maltodextrin (PF) and with or without Gln and with or without doxycycline (Dox). Mucosal extracts of colon were immunoblotted for TJ and AJ proteins (A). Band densities for occludin (B), ZO-1 (C), E-cadherin (D), β -catenin (E), EGFR (F) and β -actin (G) Image J software. Values are mean $\pm$ sem (n = 6). Asterisks indicate the values that are significantly (p < 0.05) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and Δ indicates the values that are different from corresponding EF+Gln without doxycycline values.

Figure 5: Expression of dominant negative EGFR attenuates Gln-mediated prevention of EtOH-induced cofilin activation and disruption of actin cytoskeleton in mouse colon.

Adult rtTA and rtTA-EGFR*⁻Tg mice were fed a liquid diet with EtOH (EF) or isocaloric maltodextrin (PF), with or without Gln and with or without doxycycline (Dox). Cryosections of distal colon were stained for F-actin (A) or co-stained for total cofilin and phospho-cofilin (B). Fluorescence densities quantified by densitometry and phosphor-cofilin to total cofilin density ratio were calculated for both rtTA (C & E) and rtTA-EGFR*⁻Tg (D & F) mice. In all panels, values are mean $\pm$ sem (n = 4 for PF and EF and 6 for Gln+EF and Dox+Gln+EF groups). Asterisks indicate the values that are significantly (p<0.05) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and # indicate the values different from corresponding values for EF+Gln without Dox.

Figure 6: Expression of dominant negative EGFR attenuates Gln-mediated prevention of EtOH-induced protein thiol oxidation and suppression of antioxidant gene expression in mouse colon. Adult rtTA and rtTA-EGFR*^{Tg} mice were fed a liquid diet with EtOH (EF) or isocaloric maltodextrin (PF), with or without Gln and with or without doxycycline (Dox). Cryosections of distal colon were stained for reduced and oxidized protein thiols (A & C) and the fluorescence densities quantified by densitometry (B & D). RNA extracted from colonic mucosa was subjected to qPCR for *SOD1* (E), *SOD2* (F), *Trx1* (G), *Gpx1* (H), *Catalase* (I), and *Nrf2* (J) genes. In all panels, values are mean $\pm$ sem (n = 4 for PF and EF and 6 for Gln +EF and Dox+Gln+EF groups). Asterisks indicate the values that are significantly (p<0.05) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and Δ indicate the values different from corresponding values for EF+Gln without Dox.

Figure 7: Expression of dominant negative EGFR attenuates Gln-mediated prevention of EtOH-induced expression of inflammatory cytokine and chemokine genes in mouse colon.

Adult rtTA and rtTA-EGFR*-Tg mice were fed a liquid diet with EtOH (EF, red bars) or isocaloric maltodextrin (PF, blue bars), with or without Gln (green bars) and with or without doxycycline (Dox). RNA extracted from colonic mucosa was subjected to qPCR for *IL-6* (A), *IL-10* (B), *MCP1* (C), *CCL5* (D) and *CXCL1* (E) genes. In all panels, values are mean $\pm$ sem (n = 4 for PF and EF and 6 for Gln+EF and Dox+Gln+EF groups). Asterisks indicate the values that are significantly ($p < 0.05$) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and Δ indicate the values different from corresponding values for EF+Gln without Dox.

Figure 8: Gln attenuates EtOH-induced liver damage by an EGFR-dependent mechanism.

Adult mice were fed a liquid diet with EtOH (EF) or isocaloric maltodextrin (PF) and with or without Gln and with or without doxycycline. Paraffin sections of formalin-fixed liver were stained with H&E (A). Plasma was assayed for ALT (B) and AST (C) activities. Liver triglyceride levels were evaluated (D). RNA extracted from liver was subjected to qPCR for *MCP1* (E) and *CXCL2* (F) genes. In all panels, values are mean $\pm$ sem (n = 4 for PF and EF & 6 for LP+EF and Dox+Gln+EF groups). Asterisks indicate the values that are significantly ($p < 0.05$) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and Δ indicate the values different from corresponding values for EF+Gln without Dox.

Figure 9: Gln attenuates EtOH-induced expression of chemokines in liver by an EGFR-dependent mechanism.

Adult rtTA and rtTA-EGFR*^{Tg} mice were fed a liquid diet with EtOH (EF) or isocaloric maltodextrin (PF) and with or without Gln and with or without doxycycline. RNA extracted from liver was subjected to qPCR for *Trx1* (A) *SOD1* (B), *Catalase* (C) and *Nrf2* (D) genes. Values are mean $\pm$ sem (n = 4 for PF and EF and 6 for LP+EF and Dox+Gln+EF groups). Asterisks indicate the values that are significantly (p<0.05) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and Δ indicate the values different from corresponding values for EF+Gln without Dox.

Figure 10: Gln attenuates EtOH-induced elevation of circulating cytokines by an EGFR-dependent mechanism.

Adult mice were fed a liquid diet with EtOH (EF) or isocaloric maltodextrin (PF) and with or without Gln and with or without doxycycline. Plasma was assayed for IL-6 (A) and TNF- α (B) by ELISA. Values are mean $\pm$ sem (n = 4 for PF and EF and 6 for LP+EF and Dox +Gln+EF groups). Asterisks indicate the values that are significantly (p<0.05) different from corresponding PF values. Hash tags indicate the values different from corresponding EF values, and Δ indicate the values different from corresponding values for EF+Gln without Dox.

Figure 11: Gln-mediated prevention of acetaldehyde-induced barrier dysfunction in Caco-2 cell monolayers involves metalloprotease activity and EGFR ligand binding domain.

A: Caco-2 cell monolayers were pre-treated with or without Gln under varying conditions: 1) 30 min prior to acetaldehyde + during acetaldehyde treatment, 2) 30 min prior to acetaldehyde, washed and replace with fresh Gln during acetaldehyde treatment, 3) present only during acetaldehyde treatment, and 4) 30 min prior to acetaldehyde and washed off prior to acetaldehyde treatment. After four hours, inulin permeability was measured. Values are mean $\pm$ sem (n = 4). Asterisks indicate the values that are significantly (p<0.05) different from control values, and hash tags indicate the values different from acetaldehyde values. **B & C:** Caco-2 cell monolayers were incubated with or without PP2 or anti-EGFR antibody 30 min prior to Gln treatment. Acetaldehyde (Acetal) was administered 30 min after Gln. TER (B) and inulin permeability (C) were measured after 4 hours. **D:** Caco-2 cell monolayers were incubated with Gln for one hour, and the medium from the apical well was assayed for proteinases by zymographic analysis. **E & F:** Caco-2 cell monolayers were incubated with or without varying concentrations of GM6001 or 1,10-phenanthroline 30 min prior to Gln treatment. Acetaldehyde (Acetal) was administered 30 min after Gln. TER (E) and inulin permeability (F) were measured after 4 hours. In all panels, the values are mean $\pm$ sem (n = 4). Asterisks indicate the values that are significantly (p<0.05) different from corresponding control values, and the hash tags indicate the Gln+Acetal values different

from corresponding Acetal values. The symbol Δ indicates the Gln+Acetal+inhibitor values are different from corresponding Acetal+Gln values.

Fig. 12: Schematic showing the potential mechanism involved in glutamine-mediated prevention of EtOH-induced gut and liver injury.

The pathway outlined by red arrows indicate the role of Ca^{2+} channels, intracellular Ca^{2+} , reactive oxygen species (ROS), disruption of actin cytoskeleton by cofilin activation and disruption of intestinal epithelial tight junctions (TJ) in EtOH-induced endotoxemia and liver damage. Pathway outlined by green arrows indicate the potential role of release of metalloproteinases (MMP) into extracellular space, release of membrane-bound growth factors, activation of epidermal growth factor receptor (EGFR) and suppression of oxidative stress and cofilin activation in glutamine (Gln)-mediated prevention of EtOH-induced TJ disruption, endotoxemia and liver damage.

Table 1:**Primers used for RT-qPCR**

<i>IL-6</i>	Forward: TAGTCCTTCCTACCCCAATTTC Reverse: TTGGTCCTTAGCCACTCCTTC
<i>CCL5/RANTES</i>	Forward: GCTGCTTTGCCTACCTCTCC Reverse: TCGAGTGACAAACACGACTGC
<i>IL10</i>	Forward: GCTCTTACTGACTGGCATGAG Reverse: CGCAGCTCTAGGAGCATGTG
<i>MCP-1/CCL2</i>	Forward: TTAAAAACCTGGATCGGAACCAA Reverse: GCATTAGCTTCAGATTTACGGGT
<i>CXCL-1</i>	Forward: CTGGGATTACCTCAAGAACATC Reverse: CAGGGTCAAGGCAAGCCTC
<i>CXCL-2</i>	Forward: CCAACCACCAGGCTACAGG Reverse: GCGTCACACTCAAGCTCTG
<i>TXN1</i>	Forward: CATGCCGACCTTCCAGTTTTA Reverse: TTTCTTGTAGCACC GGAGA
<i>SOD1</i>	Forward: AACCAGTTGTGTGTGTCAGGAC Reverse: CCACCATGTTTCTTAGAGTGAGG
<i>SOD2</i>	Forward: CAGACCTGCCTTACGACTATGG Reverse: CTCGGTGGCGTTGAGATTGTT
<i>CAT</i>	Forward: AGCGACCAGATGAAGCAGTG Reverse: TCCGCTCTCTGTCAAAGTGTG
<i>GPX1</i>	Forward: AGTCCACCGTGTATGCCTTCT Reverse: GAGACGCGACATTCTCAATGA
<i>NRF2</i>	Forward: CTCGCTGGA AAAAGAAGTG Reverse: CCGTCCAGGAGTTCAGAGG
<i>GAPDH</i>	Forward: CTGCACCACCAACTGCTTAG Reverse: GGGCCATCCACAGTCTTCT