Published in final edited form as:

Alcohol Clin Exp Res. 2009 July ; 33(7): 1220–1230. doi:10.1111/j.1530-0277.2009.00946.x.

Nitric oxide mediated intestinal injury is required for alcohol-induced gut leakiness and liver damage

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Abstract

Background—Alcoholic liver disease (ALD) requires endotoxemia and is commonly associated with intestinal barrier leakiness. Using monolayers of intestinal epithelial cells as an in vitro barrier model, we showed that ethanol-induced intestinal barrier disruption is mediated by iNOS (inducible nitric-oxide synthase) upregulation, NO (nitric oxide) overproduction, and oxidation/nitration of cytoskeletal proteins. We hypothesized that iNOS inhibitors (L-NAME, L-NIL) in vivo will inhibit the above cascade and liver injury in an animal model of alcoholic steatohepatitis (ASH).

Methods—Male Sprague-Dawley rats were gavaged daily with alcohol (6 g/kg/day) or dextrose for 10 weeks ± L-NAME, L-NIL or vehicle. Systemic and intestinal NO levels were measured by nitrites and nitrates in urine and tissue samples, oxidative damage to the intestinal mucosa by protein carbonyl and nitrotyrosine, intestinal permeability by urinary sugar tests, and liver injury by histological inflammation scores, liver fat, and myeloperoxidase activity.

Results—Alcohol caused tissue oxidation, gut leakiness, endotoxemia and ASH. L-NIL and L-NAME, but not the D-enantiomers, attenuated all steps in the alcohol-induced cascade including NO overproduction, oxidative tissue damage, gut leakiness, endotoxemia, hepatic inflammation and liver injury.

Conclusions—The mechanism we reported for alcohol-induced intestinal barrier disruption in vitro – NO overproduction, oxidative tissue damage, leaky gut, endotoxemia and liver injury – appears to be relevant in vivo in an animal model of alcohol-induced liver injury. That iNOS inhibitors attenuated all steps of this cascade suggests that prevention of this cascade in alcoholics will protect the liver against the injurious effects of chronic alcohol and that iNOS may be a useful target for prevention of ALD.

Keywords

intestinal hyperpermeability; inducible nitric-oxide synthase (iNOS); L-NIL; oxidative stress; endotoxemia; alcoholic liver disease

Introduction

The intestinal epithelium is a highly selective barrier that permits the absorption of nutrients from the gut lumen into the circulation, but, normally, restricts the passage of harmful and potentially toxic compounds such as products of the luminal microbiota (Clayburgh et al., 2004; Hollander, 1992; Keshavarzian et al., 1999). Disruption of intestinal barrier integrity (leaky gut) may lead to the penetration of luminal bacterial products such as endotoxin, into the mucosa and then into the systemic circulation and initiate local inflammatory processes in the intestine and even in distant organs (Clayburgh et al., 2004; Hollander, 1992; Keshavarzian et al., 1999). Indeed, disrupted intestinal barrier integrity has been implicated in a wide range of illnesses such as inflammatory bowel disease, systemic disease such as cancer, and even hepatic encephalopathy (Clayburgh et al., 2004; Hollander, 1992; Keshavarzian et al., 2001; Keshavarzian and Fields, 2003; Keshavarzian et al., 1994; Keshavarzian et al., 1999; Mathurin et al., 2000; Sawada et al., 2003; Turner et al., 1997).

Several studies, including our own, indicate that EtOH disrupts the functional and structural integrity of intestinal epithelial cells and results in hyperpermeability of intestinal cell monolayers and gut leakiness (Banan et al., 1999; Banan et al., 2000; Banan et al., 2001; Keshavarzian et al., 2001; Keshavarzian and Fields, 2000; Keshavarzian and Fields, 2003; Keshavarzian et al., 1994; Keshavarzian et al., 1999; Keshavarzian et al., 1996; Robinson et al., 1981; Tang et al., 2008).

We also found, using monolayers of Caco-2 cells as an in vitro model of gut barrier function, that oxidative stress plays an important role in EtOH-induced loss of intestinal barrier integrity (Banan et al., 2000; Banan et al., 2001; Banan et al., 2007).

One endogenous oxidant in particular, nitric Oxide (NO), appeared to be involved. At normal levels, NO is a key mediator of intestinal cell and barrier function (Alican and Kubes, 1996; Kubes, 1992; Lopez-Belmonte and Whittle, 1994; Unno et al., 1996; Unno et al., 1997a; Unno et al., 1995). When NO is present in excess, however, the result is barrier dysfunction (Colgan, 1998; Invernizzi et al., 1997; Unno et al., 1997b) including EtOH-induced barrier dysfunction (Banan et al., 1999; Banan et al., 2000). Many studies (Chow et al., 1998; Greenberg et al., 1994; Lancaster, 1992; Sisson, 1995) found that chronic EtOH raises NO levels and that EtOH-induced cytotoxicity is mediated via excess levels of NO and its metabolite, peroxynitrite (ONOO⁻). Our previous studies (Banan et al., 1999; Banan et al., 2000) showed that EtOH upregulates iNOS and increases NO and ONOO⁻ in Caco-2 cells. Because monolayers of these intestinal epithelial cells constitute a model of the gut barrier, our in vitro data suggest that the main mechanism by which NO overproduction induces intestinal barrier dysfunction is oxidation and nitration of cytoskeletal proteins (Banan et al., 1999; Banan et al., 2000). However, this mechanism, which involves excessive NO signaling, needs to be investigated in vivo.

Accordingly, we hypothesized that inhibition of iNOS activity will prevent EtOH-induced intestinal barrier dysfunction in an animal model of alcoholic steatohepatitis (ASH), and will do so by inhibiting EtOH-induced production of excess NO and the oxidative injury to the intestinal epithelium that ensues. To test this hypothesis, and to study the role of iNOS in EtOH-induced oxidative injury, gut leakiness, and liver damage in an animal model of ASH, we used a nonselective inhibitor (NG-nitro-L-arginine methyl ester, L-NAME) and a selective inhibitor (L-N⁶-(1-iminoethyl)-lysine, L-NIL) of iNOS, which have been studied in different models of intestinal inflammation (Kawachi et al., 1999; Kriegelstein et al., 2001; Obermeier et al., 1999).

Materials and Methods

Animal Subjects

Male Sprague-Dawley rats (250–300 g at intake) were obtained from Harlan (Indianapolis, IN). During experiments, each rat was given either alcohol or an isocaloric *amount of dextrose in a liquid diet intragastrically 2 times each day*. The ethanol dose was gradually increased every 2 to 3 days from 2 g/kg/day to a maximum of 6 g/kg/day by 2 weeks. Study Day 1 was defined as the first day rats received 6 g/kg/day alcohol. The dextrose dose for control rats was isocaloric to the amount of EtOH given. L-NAME (20 mg/kg) or D-NAME (20 mg/kg) or L-NIL (20 mg/kg) or D-NIL (20 mg/kg) were given by gastric gavage in two, equally divided portions to both dextrose and alcohol fed rats. D-NAME and D-NIL are the inactive enantiomers of L-NAME and L-NIL and were used as controls. Doses were chosen from either the literature (Kawachi et al., 1999; Krieglstein et al., 2001; Obermeier et al., 1999) or from our preliminary data. All agents were purchased from Sigma (St. Louis, MO). Rats received chow ad lib and were weighed daily. Intestinal permeability was measured just before sacrifice. Sacrifice was done by CO₂ inhalation, followed immediately by cardiac puncture (for blood draw) and harvesting of intestine and liver. All animal protocols and practices were reviewed and approved in advance by the Rush University Institutional Animal Care and Use Committee.

Intestinal permeability

We used an oral sugar test to assess intestinal permeability as we described (Farhadi et al., 2006; Farhadi et al., 2003; Keshavarzian et al., 2001). After an 8 h fast, rats were given, intragastrically, 2.0 ml of a solution containing 107 mg/kg lactulose, 30 mg/kg mannitol, 15 mg/kg sucralose, and 570 mg/kg sucrose. Rats were housed individually in metabolic cages and urine samples were collected over 5 h. To promote urine output, each rat was subcutaneously injected with 10 ml of lactated Ringer's solution, just prior to sugar administration. Urinary sugar levels were measured by gas chromatography as we reported (Farhadi et al., 2006; Farhadi et al., 2003; Keshavarzian et al., 2001).

Plasma Endotoxin

Blood was collected from rats at the time of sacrifice. Serum samples were then analyzed for endotoxin by a kit (Kinetic-QLC; Whittaker Bioproducts) following the protocol from the manufacturer.

Liver Injury

- a. *Liver Histology*. Formalin fixed liver tissues were stained with H&E. Blinded assessment was done by a GI pathologist (SJ). Histological slides were assessed for liver disease by grading steatosis, necrosis, inflammation & fibrosis (Keshavarzian et al., 2001). Our scoring system was based on one widely used for the scoring of liver disease (Kleiner et al., 2005). Severity of steatosis (% of liver cells containing fat) was scored 1 to 4, corresponding to the fraction of liver cells with fat. These were, respectively, <25%, 26–50%, 51–75%, and >75%. Necrosis was quantified as the number of necrotic foci/mm² and number of Councilman Bodies per high power field (HPF) and severity of necrosis was graded on a scale of 0–4. Severity of inflammation was graded on a scale of 0–4 based on the extent [number of inflammatory foci per HPF and number of HPF with inflammatory foci] and density of inflammatory foci (from a few inflammatory cells to a dense inflammatory infiltrate). Severity of fibrosis was scored on a scale of 0 to 4 using trichrome staining. However, the fibrosis score in all rats was zero and thus fibrosis stage was not included in the histology score. A total histological score (including

fatty liver grade) and necroinflammatory score (inflammation and necrosis score representing the presence and severity of steatohepatitis) was then calculated. The maximum histology score and necroinflammatory scores were 12 and 8 respectively. When assessing slides for pathology studies, at least 3 different sections were studied for each rat. Alcoholic steatohepatitis (ASH) was defined as the presence in the liver of inflammatory cell infiltration, spotty necrosis, and liver cell necrosis.

- b. *Liver Fat Content.* Total liver fat was measured gravimetrically as previously described (Folch et al., 1957) and used by us (Keshavarzian et al., 2001).

Serum alanine aminotransferase (ALT)—ALT was measured in blinded serum samples by the Rush University Medical Center clinical laboratories and the data provided as Units/dl $\pm$ S.E.

NO Levels in Intestinal Mucosa and Urine—Nitrite and nitrate in urine and tissue samples were measured (umol/ml or umol/mg) using Nitrate/Nitrite Colorimetric Assay Kits (Cayman Chemical, Ann Arbor, MI).

Quantitative Slot-Immunoblotting for oxidation (carbonylation) and nitration (nitrotyrosination)

Oxidation and nitration of mucosal proteins were assessed by measuring protein carbonyl and protein nitrotyrosine formation using a slot-blotting method we previously described (Banan et al., 2004; Keshavarzian and Fields, 2003).

Data and Statistical Analysis—Data are presented as mean $\pm$ S.E. For parametric analyses of two groups, we used Student's t-test; ANOVA was used when we compared >2 groups. Least Standard Deviation (LSD) was used for post-hoc analysis and a paired t-test was used for comparison of paired data such as data on intestinal permeability. $P < 0.05$ was regarded as significant.

Results

EtOH-induced NO₂/NO₃ overproduction in urine and colonic mucosa was prevented by iNOS inhibitors

To confirm, in an in vivo model, our in vitro data that EtOH induces iNOS activation and oxidative stress (Banan et al., 1999; Banan et al., 2000; Banan et al., 2001), we measured total NO levels in the urine from dextrose-fed and EtOH-fed rats at week 10. Total NO production in urine was increased 8.5 fold from 81 ± 11 to 690 ± 75 $\mu\text{mol/mg}$ ($p < 0.05$) in EtOH fed rats compared with dextrose fed control rats (Fig. 1a). The EtOH-induced increase in NO was inhibited in rats that were fed, in addition to EtOH, a nonselective iNOS inhibitor, L-NAME (46% decrease to 410 ± 35 , $p < 0.05$, Fig. 1a) or a selective iNOS inhibitor, L-NIL (98% decrease to 90 ± 8 , $p < 0.05$, Fig. 1a). D-NAME and D-NIL had no effect on EtOH-induced NO production.

To determine if EtOH not only affects systemic NO production, but also affects NO production in the intestinal epithelium, we measured NO production in the colonic mucosa in our rats. EtOH induced a 4.1 fold increase in NO production in colonic mucosa (from 850 ± 89 to 3548 ± 350 $\mu\text{mol/mg}$; $p < 0.05$) (EtOH-fed rats compared with dextrose-fed control rats) (Fig. 1b). The EtOH-induced increase in colonic tissue NO was inhibited by a nonselective iNOS inhibitor, L-NAME (64% decrease to 1800 ± 160 , $p < 0.05$, Fig. 1b) or a selective iNOS inhibitor, L-NIL (97% decrease to 910 ± 98 , $p < 0.05$, Fig. 1b).

iNOS inhibitors prevented EtOH-induced increases in oxidative tissue damage in intestinal tissues

Our in vitro data suggested that EtOH-induced NO overproduction was required for EtOH-induced oxidative damage in monolayers of intestinal epithelial cells (Banan et al., 1999; Banan et al., 2000; Banan et al., 2001). Because daily chronic alcohol feeding induced NO overproduction in intestinal tissues in our rats, we determined whether alcohol also causes oxidative damage in intestinal tissues and whether an iNOS inhibitor can prevent oxidative tissue damage. To measure changes in oxidative tissue damage, we used the same markers in vivo that we had in vitro – protein-nitrotyrosines and protein-carbonyls. The abundance of nitrotyrosine and carbonyl epitopes were determined by slot blot immunostaining and quantitative densitometry in tissue samples obtained after sacrifice from rat duodenum, jejunum, ileum, and colon mucosa. Chronic alcohol feeding markedly elevated tissue protein nitration (nitrotyrosine levels; Fig. 2) (e.g., 4.6 fold in colon) and protein oxidation (protein carbonyl levels; Fig. 3) (e.g., 4.3 fold in colon) in intestine of alcohol-fed rats. iNOS inhibitors significantly inhibited this effect of EtOH (Fig. 2 and Fig. 3). For example, in colon, L-NAME inhibited EtOH-induced increases in nitrotyrosines by 58% and carbonyls by 53%; for L-NIL, the corresponding inhibitions were 63% and 72%.

iNOS inhibitors prevented intestinal hyperpermeability induced by chronic alcohol consumption

To determine if EtOH-induced oxidative damage contributes to EtOH-induced disruption of the intestinal barrier and if iNOS inhibitors can prevent EtOH-induced hyperpermeability by blocking EtOH-induced increases in oxidative damage, we measured intestinal permeability in rats using an oral sugar test. Daily alcohol feeding for 10 weeks disrupted intestinal barrier integrity in rats (Fig. 4). Urinary lactulose (an index of small bowel permeability) was significantly higher (~7 fold) in alcohol-fed rats than in dextrose fed rats (controls) (Fig. 4a, $p<0.05$). Urinary sucralose (an index of whole gut [small bowel + large bowel] permeability) was also significantly increased (~5 fold) in alcohol-fed rats (Fig. 4b, $p<0.05$). iNOS inhibitors significantly inhibited alcohol-induced gut leakiness and markedly decreased urinary lactulose and sucralose in alcohol fed rats (Fig. 4, $p<0.05$). L-NAME caused 83% and 100% inhibition of the EtOH-induced increase in urinary lactulose and sucralose, respectively; for L-NIL, the corresponding inhibitions were 100% and 80%.

iNOS inhibitors prevented EtOH-induced increases in serum endotoxin

Since leaky gut can result in endotoxemia, we determined whether iNOS inhibitors also prevent EtOH-induced increases in serum endotoxin. Feeding of alcohol to rats for 10 weeks significantly increased serum endotoxin levels 7 fold, from 0.08 ± 0.02 to 0.58 ± 0.11 EU/ml (Fig. 5, $p<0.05$). L-NAME reduced this increase by 46% to 0.35 ± 0.02 EU/ml in alcohol fed rats (Fig. 5, $p<0.05$); L-NIL reduced the increase by 84% to 0.16 ± 0.02 EU/ml. Both agents thus attenuated alcohol-induced endotoxemia.

iNOS inhibitors prevented EtOH-induced increases in inflammatory damage and in fat content in liver

Because EtOH-induced endotoxemia is an important factor in development of ALD (Criado-Jimenez et al., 1995; Hunt and Goldin, 1992; McClain and Cohen, 1989; Purohit et al., 2008), we determined whether iNOS inhibitors prevent EtOH-induced liver damage. To study the effect of iNOS inhibitors on EtOH-induced steatohepatitis, we measured hepatic MPO activity, fat content and serum ALT and also assessed liver tissue histologically in rats fed EtOH with or without iNOS inhibitors.

Feeding of alcohol to rats for 10 weeks increased MPO activity about 18 fold from 2.1 ± 0.03 to 38 ± 4.11 Units/mg tissue (Fig. 6a, $p < 0.05$). iNOS inhibitors significantly decreased hepatic MPO activity in alcohol fed rats. For L-NIL, the inhibition was 92% (to 5.1 ± 0.7 Units/mg tissue); for L-NAME, inhibition was 86% (7.2 ± 0.6 ; Fig. 6a, $p < 0.05$).

Chronic EtOH consumption by rats significantly increased inflammatory injury to the liver; the inflammatory score increased from zero to 2.5 ± 0.31 (Fig. 6b). iNOS inhibitors (L-NIL; L-NAME) reduced the EtOH-induced increase in inflammatory score to zero (100% inhibition; Fig. 6b; $p < 0.05$). Histological scores were assessed for liver injury by grading steatosis, necrosis, inflammation & fibrosis. EtOH increased histology scores from zero to 11 ± 1.2 (Fig. 6c). iNOS inhibitors significantly reduced the EtOH-induced increase in histology score. L-NIL reduced it to 2.1 ± 0.3 (–81%); L-NAME reduced it to 3.1 ± 0.4 (–72%; Fig. 6c).

EtOH increased liver fat content (~2 fold), from 4.1 ± 0.5 to 9.2 ± 1.2 (Fig. 6d). iNOS inhibitors reduced fat content to 4.7 ± 0.5 (–88%) and 5.6 ± 0.6 (–71%) for L-NIL and L-NAME, respectively (Fig. 6d).

To further study the effect of iNOS inhibitors on the severity of alcoholic steatohepatitis, we measured serum ALT levels in rats fed EtOH with or without iNOS inhibitors. Chronic EtOH consumption by rats significantly increased ALT from 85 ± 20 to 152 ± 25 U/L (Fig. 7). iNOS inhibitors significantly reduced serum ALT levels to 91 ± 15 U/L and 96 ± 16 U/L for L-NIL and L-NAME, respectively (Fig. 7, $p < 0.05$).

Comparison of the effect of specific iNOS inhibition by L-NIL and total inhibition of NOS by L-NAME on intestinal barrier function and severity of steatohepatitis

The basal level of NO in the urine of dextrose fed rats was 81 ± 11 $\mu\text{mol/mg}$. Inhibition of NOS by a non-specific NOS inhibitor, L-NAME, significantly decreased total urinary NO levels in dextrose fed rats (52 ± 8 $\mu\text{mol/mg}$, $p < 0.05$ compared to vehicle treated rats); inhibition by the specific iNOS inhibitor L-NIL had no significant effect (78 ± 10 $\mu\text{mol/mg}$). This indicates that iNOS does not significantly contribute to NO production in control rats. In contrast, iNOS appears to be the key source of the EtOH-induced increase in NO levels because, in alcohol fed rats, iNOS inhibition by L-NIL significantly decreased NO levels in both urinary and colonic mucosa. Both L-NAME and L-NIL decreased NO levels in the urine and in colonic mucosa of alcohol fed rats. However, the inhibitory effect of L-NIL was significantly greater than L-NAME (98% vs 46% for urine NO; 97% vs 64% for colonic mucosa NO; $p < 0.05$, Fig. 1).

Discussion

Because alcoholic liver disease (ALD), including alcoholic steatohepatitis (ASH), has high morbidity and mortality but no satisfactory therapy (Burbige et al., 1984; Galambos, 1972; Grant et al., 1988; Maher, 2002; O'Connor and Schottenfeld, 1998), our broad long-term research objective has been to develop effective therapies to prevent or reverse liver disease in alcoholics. Since endotoxemia is an essential co-factor for the development of ALD (Criado-Jimenez et al., 1995; Hunt and Goldin, 1992; McClain and Cohen, 1989; Purohit et al., 2008), we traced the origins of endotoxemia back to leakiness of the intestinal barrier – alcoholics with liver disease have more gut leakiness than alcoholics without liver disease (Keshavarzian et al., 1999). However, it is not yet established that endotoxemia is a prerequisite for development of ASH. Moreover, the mechanism by which EtOH causes intestinal barrier hyperpermeability is still not clear.

To answer these key questions regarding mechanisms, we turned to an animal model of alcoholic steatohepatitis. Our experimental model of ASH, which involves daily administration of EtOH chronically (10 to 12 weeks) to rats by gavage, has been validated in our previous studies (Keshavarzian et al., 2001). In those studies we showed that alcoholic steatohepatitis can be prevented by protecting intestinal barrier integrity from disruption (Keshavarzian et al., 2001). Recently, to determine whether endotoxemia occurs prior to development of ASH and whether gut leakiness and endotoxemia are one of the key co-factors for development of alcoholic steatohepatitis, we studied time courses for development of gut hyperpermeability, endotoxemia, and liver injury and showed that gut leakiness and endotoxemia occurred several weeks prior to development of ASH (Keshavarzian A et al., 2009). These data show that gut leakiness and endotoxemia cannot be the consequence of ALD and support the notion that gut leakiness causes endotoxemia, which leads to alcoholic steatohepatitis and serious ALD.

To determine the mechanism of alcohol-induced disruption of intestinal barrier integrity, we first turned to a well-established in vitro model of intestinal barrier function – monolayers of intestinal (Caco-2) cells. We demonstrated that alcohol-induced overproduction of NO and the attendant oxidative injury to key proteins, were necessary for dysregulation of the monolayer barrier and barrier hyperpermeability (Banan et al., 2000; Banan et al., 2001; Banan et al., 2007; Forsyth et al., 2007). In the current study, we extended our studies of this mechanism of the effects of EtOH on the intestinal tract to the in vivo situation. Using our animal model of ASH, we confirmed the mechanism suggested by our in vitro findings. We showed that chronic daily alcohol feeding of rats for 10 weeks causes overproduction of nitric oxide (NO) that in turn results in (i) oxidative tissue damage to the intestinal mucosa and (ii) intestinal hyperpermeability. EtOH-induced leaky gut was associated with endotoxemia and hepatic inflammation and liver cell injuries in our animal model of alcoholic steatohepatitis. More importantly, iNOS inhibitors reduced these effects of EtOH and prevent the downstream cascade of injurious events in the intestine and the liver that would have otherwise occurred (Fig. 8). We thus established the relevance of the in vitro mechanism we observed in monolayers of intestinal cells to the in vivo situation. Our findings could be clinically useful because inhibiting iNOS activation may become a novel and effective therapeutic strategy for preventing and/or treating ALD. This, of course, would require a study showing that iNOS inhibitors in ALD patients prevent EtOH-induced oxidative tissue damage to the intestines, prevent the hyperpermeability, and prevent the subsequent endotoxemia-mediated inflammation and liver injury.

Our studies indicate that EtOH-induced oxidative stress results in gut leakiness by inducing nitration and oxidation of intestinal mucosa. However, the mechanisms by which NO induces intestinal epithelial barrier dysfunction are not clearly understood. NO-mediated oxidation of cellular proteins is due to its metabolite, peroxynitrite, which is a product of the reaction of NO with superoxide radicals (Banan et al., 2001; Kolios et al., 2004). Peroxynitrite oxidizes and damages proteins by reacting with amino acid residues such as cysteine (Banan et al., 2001; Kolios et al., 2004). For example, nitration of phenolic amino acid residues produces nitrotyrosine, a stable foot-print of peroxynitrite reactions and thus an index of peroxynitrite formation (Banan et al., 2001; Kolios et al., 2004). Our previous in vitro studies demonstrated that EtOH increases NO and ONOO⁻ formation, and that this overproduction of ONOO⁻ causes nitration and carbonylation of cytoskeletal proteins; this damage then disrupts intestinal integrity (Banan et al., 2000; Banan et al., 2001; Banan et al., 2007).

Our current findings, which demonstrate the importance of NO in alcohol-induced gut leakiness and steatohepatitis, are supported by these prior reports. Most (Chow et al., 1998; Greenberg et al., 1994; Lancaster, 1992; Sisson, 1995) but not all (Neiman and Benthin,

1997; Persson and Gustafsson, 1992) reports indicate that chronic EtOH raises NO levels in multiple organs. Indeed, many have suggested that EtOH-induced cytotoxicity is mediated via upregulation of NO and its metabolite, peroxynitrite (ONOO⁻). For example, NO appears to be a key mediator of EtOH's cytotoxic effect on the CNS (Lancaster, 1992). Increased NO levels in primary cultures of bovine bronchial epithelial cells are responsible for the harmful effects of EtOH (Sisson, 1995). iNOS upregulation in adrenal glands (Greenberg et al., 1994) may cause EtOH-induced blunting of the ACTH response to sepsis. EtOH damage to gastric mucosa appears to be mediated via increased iNOS/NO (Chow et al., 1998). Finally, EtOH-induced damage to the liver may be NO-mediated (Nanji et al., 1995). Indeed, our own published in vitro data indicate that EtOH upregulates iNOS and increases NO and ONOO⁻ in Caco-2 cells (Banan et al., 2000; Banan et al., 2001; Banan et al., 2007).

The next question is how chronic alcohol feeding increases NO levels. NO is synthesized from L-arginine by nitric oxide synthases (NOS). Three isoforms of nitric oxide synthases have been identified: neuronal (nNOS), endothelial (eNOS), and inducible NOS (iNOS) (Kolios et al., 2004). nNOS and eNOS are referred to as constitutive NOS (cNOS) (Kolios et al., 2004). NO production by cNOS modulates several aspects of intestinal physiology and is considered to be required for maintaining epithelial cell barrier integrity (Collins, 1996; Takahashi, 2003; Vallance et al., 2004). In contrast, NO produced by iNOS is believed to occur under pathological conditions such as inflammation and is believed to be harmful to the integrity of the intestinal barrier. Several studies have shown that iNOS is induced by bacterial products, microbes and certain cytokines (Kolios et al., 2004) resulting in production of high levels of NO. For example, several studies have demonstrated upregulation of NOS activity in the inflamed mucosa of patients with ulcerative colitis and in animal models of colitis (Vallance et al., 2004). We also showed that alcohol upregulates iNOS in Caco-2 cell monolayers, resulting in increased levels of NO and ONOO⁻ formation (Banan et al., 2000; Banan et al., 2001; Banan et al., 2007).

Our data in the present study support the conclusion of these prior reports that iNOS is not a major source of NO under normal, physiological conditions, but is a primary source of increased tissue NO under pathological conditions such as alcohol-induced organ dysfunction. We showed that L-NAME, but not the specific iNOS inhibitor L-NIL, significantly decreases urinary NO levels in dextrose fed control rats. This finding supports the notion that cNOS and not iNOS is the major source of NO under normal, physiological conditions. In contrast, we showed that L-NIL is more potent in preventing alcohol-induced increases in NO levels in the urine and in colonic tissue, supporting the notion that iNOS is the major source of elevated NO levels under pathological conditions.

Our studies show that inhibition of iNOS not only decreased the severity of EtOH-induced hepatic inflammation and injury, but also, it markedly attenuated (2 fold) the increase in liver fat content caused by EtOH and reduced EtOH-induced steatosis by 71 to 88%. The mechanism by which iNOS inhibitors attenuate alcoholic steatosis is unclear. iNOS inhibition can potentially reduce steatosis indirectly by inhibiting the endotoxin-cytokine cascade and/or directly by affecting hepatic lipid homeostasis. Although, our current study can not differentiate between these two mechanisms, our time course study, using the same model of alcoholic steatohepatitis as used here, support the direct effects of iNOS inhibition on hepatic lipid metabolism (Keshavarzian A et al., 2009). We demonstrated that fatty liver occurs early, within the first 2 weeks of daily alcohol gavage and at least 2 weeks before significant endotoxemia occurs. Our data support the conclusion of prior studies that steatosis is primarily due to the direct effects of alcohol on hepatic lipid metabolism and is not dependent on endotoxin (Hall, 1994). Indeed, it is well established that chronic alcohol exposure induces hepatic enzymes involved in lipid metabolism and the alcohol-induced

increase in NO levels can inhibit these enzymes including ALDH2, APT synthase and other mitochondrial proteins and enzymes involved in mitochondrial beta-oxidation of fatty acids and steatosis (Deng and Deitrich, 2007). Our finding of a marked reduction in alcoholic steatosis by iNOS inhibition was previously shown for another model of alcoholic steatohepatitis by McKim et al (McKim et al., 2003) who reported that alcohol-induced fatty accumulation is significantly attenuated in iNOS knockout mice. Using a specific iNOS inhibitor (1400W) in wild type mice, they also found similar protective effects against alcohol-induced steatosis and liver damage (McKim et al., 2003). Endotoxin mediated changes in hepatic lipid metabolism and fat accumulation can clearly exaggerate the direct effects of alcohol and worsen alcoholic steatosis. Thus, our findings that iNOS inhibitors reduce steatosis can be due to their direct hepatic effects and their ability to decrease endotoxin levels and endotoxin-mediated hepatic inflammatory cascades.

Indeed, the importance of activation of the Kupffer cells induced by gut-derived endotoxin in the development of alcoholic liver disease is now well established (Nagata et al., 2007). It is generally accepted that endotoxin can activate Kupffer cells via Toll-like receptors (TLR-4) resulting in upregulation of the transcription factor NF κ -B and increased release of proinflammatory cytokines and other mediators like TNF- α , IL-6, IFN, and NO that are known to play key roles in the development of ALD (Baraona et al., 2002; Lieber, 2004; Nagata et al., 2007). Endotoxin-induced TNF- α production and iNOS activation in Kupffer cells may worsen hepatic oxidative stress caused by direct effects of EtOH such as EtOH induction of CYP1 and associated lipid peroxidation (Lieber, 2004; Nagata et al., 2007; Yuan et al., 2006). The synergy between the effect of EtOH and endotoxin on the liver not only can initiate liver injury, but also, can create a vicious circle that sustains a chronic necroinflammatory process and hastens the onset of liver failure. Thus, in addition to its well established role in hepatic inflammation and liver cell injury, the gut-derived endotoxin-cytokine cascade may also contribute directly to EtOH-induced steatosis. Our observed beneficial effects of iNOS inhibition, therefore, could be due to attenuation of both endotoxin-cytokine cascades and prevention of NO-mediated disruption of hepatic lipid homeostasis.

Several studies have demonstrated the importance of NO in alcoholic liver disease. However, there are conflicting results for the consequence of NOS inhibition on the severity of liver disease (Nanji et al., 1995; Nanji et al., 2001; Uzun et al., 2005). For example, Nanji, et al reported that L-NAME worsened EtOH-induced liver injury (Nanji et al., 1995). In contrast, Uzun et al found that L-NAME decreased the severity of ethanol-induced liver damage by decreasing oxidative stress and increasing antioxidant status (Uzun et al., 2005). Our results are consistent with Uzun's study that L-NAME prevents EtOH-induced NO overproduction and decreases oxidative stress and liver injury. Furthermore, we showed that inhibition of iNOS is more effective in alcohol-induced injury, suggesting that iNOS is the primary source of NO overproduction and oxidative tissue damage caused by alcohol.

Several other studies have confirmed the importance of NO homeostasis in liver injury. It has been shown by several groups that while iNOS activation is associated with hepatic necro-inflammation and liver injury (Yuan et al., 2006), eNOS activation can protect liver against injurious agents. For example, eNOS had a protective effect on the liver injury induced by carbon tetrachloride (Leung et al., 2008; Tipoe et al., 2008), but iNOS exacerbated the liver injury (Aram et al., 2008). Also, chronic ethanol and LPS significantly inhibited eNOS activity, leading to extensive steatohepatitis with extensive focal necrosis (Karaa et al., 2005; Yuan et al., 2006). Our study confirmed the importance of NO upregulation in the development of alcoholic steatohepatitis and, for the first time, also shows that the beneficial effects of NOS inhibition is due, at least in part, to prevention of alcohol-induced gut leakiness and endotoxemia.

The importance of iNOS in alcohol-induced gut leakiness in man is not surprising. Other studies using iNOS inhibitors have also shown that NO plays an important role in the regulation of the functions of the human intestinal epithelium (Kawachi et al., 1999; Krieglstein et al., 2001; Obermeier et al., 1999). Increased NO production has been described in intestinal inflammation associated with hyperpermeability (Lefer and Lefer, 1999; McCafferty et al., 1999; Vallance et al., 2004). Our studies show that inhibition of iNOS reduces EtOH-induced gut leakiness. The iNOS inhibitors may directly inhibit iNOS activation in intestinal epithelial cells. iNOS expression in intestinal epithelial cells was demonstrated by immunohistochemistry in the epithelial cells of colonic mucosa of patients with active ulcerative colitis (UC) and infectious colitis (Kolios et al., 1998). Using in situ hybridization and immunohistochemistry, other studies have demonstrated that iNOS expression is localized to the surface epithelium and crypts in mucosa from UC patients (Godkin et al., 1996; Singer et al., 1996). These studies strongly indicate that colonic epithelial cells are the major source of NO production and iNOS activity in the mucosa of patients with UC (Godkin et al., 1996; Kolios et al., 1998; Singer et al., 1996). Therefore, we suspect that iNOS inhibition of EtOH-induced NO overproduction is mainly occurring in intestinal epithelial cells. This is consistent with a recent study which showed that epithelial iNOS makes a larger contribution to intestinal inflammation induced by Dextran sodium sulfate (DSS), although both blood cell-derived and epithelium-derived iNOS contribute to the mechanism (Krieglstein et al., 2007). Our previous in vitro experiments using monolayers of intestinal epithelial cells without other types of immune cells demonstrate the importance of iNOS in intestinal epithelial cell in alcohol-induced intestinal leakiness.

However, our findings and these data do not exclude the possibility that iNOS in non-epithelial cells is also involved in alcohol-induced gut leakiness. Indeed, iNOS is expressed in a variety of cells including epithelial cells, neutrophils, macrophages, neurons, glia, and endothelial cells (Krieglstein et al., 2007). Some studies suggest that iNOS from bone marrow-derived cells plays a critical role in regulating colonic inflammation (Beck et al., 2007). A limitation of using iNOS inhibitors in vivo is that it is difficult to determine which cell or tissue source of iNOS is mediating the effects of EtOH. To overcome this problem, tissue specific iNOS knock out mice may be useful (Beck et al., 2007; Krieglstein et al., 2007). Indeed, some studies using a tissue-specific iNOS knock out animal model of colitis demonstrated the relative contribution of bone marrow-derived or epithelial-derived iNOS in the regulation of colonic inflammation (Beck et al., 2007; Krieglstein et al., 2007). Further studies in animal and human are now required to determine the cellular source of the upregulated iNOS in the intestinal mucosa, to determine how iNOS upregulation disrupts intestinal barrier integrity, and to identify the optimal target for future therapeutic interventions.

The mechanism by which EtOH induces iNOS upregulation in intestinal epithelial cells has not been fully explored. Our monolayer studies showed that alcohol upregulates iNOS in Caco-2 cells through NF κ B signaling (Banan et al., 2007). Yuan et al also showed that upregulation of iNOS in the liver is associated with activation of NF κ B, TNF- α expression and liver injury (Yuan et al., 2006). Further studies in both in vitro and in vivo models and in patients with ALD are now required to identify the signaling pathways involved in alcohol-induced upregulation of iNOS and disruption of barrier function in intestinal epithelial cells.

In summary, our findings demonstrate that iNOS inhibitors reduce EtOH-induced steatosis and liver cell injury by preventing oxidative stress-induced intestinal hyperpermeability and the consequent endotoxemia – at least in our animal model of alcoholic steatohepatitis. These results indicate that strategies designed to target iNOS could lead to therapeutic

agents for the treatment and prevention of ALD and of other diseases associated with NO overproduction and intestinal hyperpermeability.

Acknowledgments

This study was supported by NIH grant AA13745 (AK).

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Fig. 1. iNOS inhibitors prevented EtOH-induced NO overproduction in urine and colon mucosa. Total NO ($\text{NO}_2 + \text{NO}_3$) in urine and colonic mucosa from dextrose fed (control) and alcohol fed (10 weeks) rats was assayed after sacrifice (see Methods). Data are shown as mean for total NO ($\mu\text{mol/mg}$) $\pm$ SE for $n=6$ for each group. The difference between groups was analyzed using ANOVA, *: $p<0.05$ compared to dextrose fed rats (controls), #: $p<0.05$ compared to alcohol fed rats, &: $p<0.05$ compared to alcohol + L-NAME group.

Fig. 2. iNOS inhibitors prevented EtOH-induced increases in nitration (protein-nitrotyrosination) in intestinal tissues. Levels of nitrotyrosine epitopes, a marker of tissue nitration, were determined by slot blotting and quantitative densitometry in tissue (mucosal) samples obtained after sacrifice from duodenum (a), jejunum (b), ileum (c), and colon (d). Data are shown as the mean $\pm$ SE for each group (n=6/group). The difference between groups was analyzed using ANOVA, *: p<0.05 compared to dextrose-fed rats (controls), #: p<0.05 compared to alcohol fed rats (10 weeks). A representative slot blot image for colonic mucosa is shown in Fig. 2d.

Fig. 3.

iNOS inhibitors prevented EtOH-induced increases in oxidation (protein-carbonyls) in intestinal tissues. Levels of the carbonyl epitope, an oxidative stress marker, were determined by slot blots and quantitative densitometry in tissue (mucosal) samples obtained after sacrifice from duodenum (a), jejunum (b), ileum (c), and colon (d). Data are shown as the mean $\pm$ SE for each group (n=6). The difference between groups was analyzed using ANOVA, *: $p < 0.05$ compared to dextrose-fed rats (controls), #: $p < 0.05$ compared to alcohol-fed rats (10 weeks). A representative slot blot image for colonic mucosa is shown in Fig. 3d.

Fig. 4. iNOS inhibitors prevented EtOH-induced increases in intestinal permeability. Intestinal permeability was assayed by measuring urinary lactulose and sucralose after an oral dose of these sugars. Urinary lactulose (a marker of small intestine permeability and urinary sucralose (a marker of whole gut permeability) increased in alcohol fed rats. iNOS inhibitors (L-NIL or L-NAME) significantly prevented EtOH-induced hyperpermeability. Data are means of the fraction of the oral dose recovered $\pm$ S.E. for $N = 6$ rats for each group. The difference between groups was analyzed by ANOVA, *: $p < 0.05$ compared to dextrose-fed rats (controls), #: $p < 0.05$ compared to alcohol fed rats (10 weeks).

Fig. 5.

iNOS inhibitors prevented EtOH-induced increases in serum endotoxin. Serum endotoxin was determined for control and alcohol-fed rats at 10 weeks after alcohol feeding as described in Methods. Serum endotoxin levels were significantly higher in alcohol-fed rats. iNOS inhibitors (L-NIL or L-NAME) prevented EtOH-induced increases in endotoxin levels. Data are expressed as mean endotoxin Units (EU) per ml serum $\pm$ S.E. for N=6 rats for each group. The difference between groups was analyzed by ANOVA, *: $p < 0.05$ compared to the control group, #: $p < 0.05$ compared to alcohol-fed rats (10 weeks), &: $p < 0.05$ compared to alcohol + L-NAME group.

Fig. 6. iNOS inhibitors prevented EtOH-induced steatohepatitis. EtOH-induced inflammatory reactions were determined by measuring hepatic myeloperoxidase (MPO) activity (a) and by calculating histological inflammatory scores (b). Total histological score represents a combined inflammatory score (i.e. hepatitis) and score for the severity of fat accumulation in the liver (i.e. steatosis) and thus it represents the severity of steatohepatitis (c). EtOH-induced fatty liver was determined by measuring hepatic fat content (d). Feeding of alcohol to rats for 10 weeks significantly increased MPO activity, inflammation score, histology score, and fat content in liver tissue. iNOS inhibitors (L-NIL or L-NAME) significantly decreased all indices of alcoholic steatohepatitis. Data are expressed as mean $\pm$ S.E. for N=6 rats for each group. The difference between groups was analyzed using ANOVA, *: $p < 0.05$ compared to dextrose-fed rats (controls), #: $p < 0.05$ compared to alcohol-fed rats (10 weeks).

Fig. 7. iNOS inhibitors prevented EtOH-induced increases in alanine aminotransferase (ALT). Chronic EtOH consumption by rats significantly increased serum ALT levels, an index of liver cell injury. iNOS inhibitors significantly inhibited EtOH-induced increases in ALT: back to 91±15 U/dl by L-NIL and 96±16 U/dl by L-NAME. Data are expressed as mean ± S.E. for N=6 rats for each group. Differences between groups were analyzed using ANOVA, *: p<0.05 compared to dextrose-fed rats (controls), #: p<0.05 compared to alcohol-fed rats (10 weeks).

Fig. 8.

Our current model for the mechanism by which iNOS inhibitors reduce EtOH-induced steatohepatitis. They do so by preventing oxidative stress-induced increases in intestinal hyperpermeability and the endotoxemia that would otherwise result.