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Alcohol metabolites and lipopolysaccharide: Roles in the development and/or progression of alcoholic liver disease

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tion and fibrosis, and play a role in the development and/or progression of ALD.

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Abstract

The onset of alcoholic liver disease (ALD) is initiated by different cell types in the liver and a number of different factors including: products derived from ethanol-induced inflammation, ethanol metabolites, and the indirect reactions from those metabolites. Ethanol oxidation results in the production of metabolites that have been shown to bind and form protein adducts, and to increase inflammatory, fibrotic and cirrhotic responses. Lipopolysaccharide (LPS) has many deleterious effects and plays a significant role in a number of disease processes by increasing inflammatory cytokine release. In ALD, LPS is thought to be derived from a breakdown in the intestinal wall enabling LPS from resident gut bacterial cell walls to leak into the blood stream. The ability of adducts and LPS to independently stimulate the various cells of the liver provides for a two-hit mechanism by which various biological responses are induced and result in liver injury. Therefore, the purpose of this article is to evaluate the effects of a two-hit combination of ethanol metabolites and LPS on the cells of the liver to increase inflamma-

INTRODUCTION

It has become increasingly clear that alcohol alone is not solely responsible for the initiation and/or progression of alcoholic liver disease (ALD). While alcohol consumption does increase fatty liver, lipid peroxidation, and reactive oxygen species (ROS), these insults are typically not enough to induce the onset of more severe forms of liver disease^[1]. The "two-hit" proposal of ALD is of interest because inflammation caused by the metabolites of ethanol [ROS, aldehyde modified proteins or lipopolysaccharide (LPS)] increase the levels of cytokines/chemokines resulting in a deleterious positive feedback loop that propagates liver inflammation, infiltration of inflammatory cells^[2,3], and fibrosis. To support this, aldehyde modified proteins^[4,5] and endotoxin (LPS)^[6,7] have been detected in the serum and/or livers of patients with ALD. These substances have been shown to increase the release of TNF- α , interleukin-1 β , and prostaglandin by Kupffer cells, sinusoidal endothelial cells and stellate cells. This release in turn promotes an influx of

inflammatory cells leading to an increase in cellular damage promoting the development of necrosis and eventually liver failure^[8-11]. The purpose of this review was to more closely examine how a “two-hit” model of ethanol metabolism and LPS interaction affects resident liver cells in the progression and/or development of ALD. Many excellent reviews^[12-14] exist concerning inflammatory cell activation and recruitment to the damaged liver during the development of ALD and therefore will not be discussed here^[15-17].

ANIMAL MODELS OF ETHANOL AND LPS-INDUCED LIVER INJURY

Current animal models of ALD have provided many valuable findings^[18]. However, in general, these models do not produce the type of end-stage liver failure observed in patients with ALD. To counter this, models have been developed that combine ethanol treatment strategies with an additional injury cofactor. One way of developing more overt injury (e.g. steatohepatitis and fibrosis) in order to mimic the human disease is to combine ethanol and LPS treatment. LPS is a component from the cell walls of bacteria found in the gut as normal flora. Typically, when gram-negative bacteria break down, LPS is released and removed by endothelial cells lining the blood vessels or Kupffer cells (KCs) in the liver. If the normal activity of the gut epithelium is disrupted, as has been shown to happen with acute or chronic ethanol ingestion, the LPS released from degrading bacteria can cross into the bloodstream^[6,7,19]. Even though the exact mechanism of this is unknown, it is suggested that with chronic ethanol consumption, ethanol can damage the cells lining the interior of the intestine and increase the amount of LPS entering the blood stream. In addition, ethanol impairs KCs and prevents them from clearing LPS from the bloodstream^[11,20]. When LPS enters into the bloodstream and moves to the liver, it activates KCs by interacting with the CD14 and Toll-like receptor 4 (TLR-4) molecules on the surface of the cell. This interaction causes a cascade which results in production of ROS and release of inflammatory cytokines (i.e. TNF- α , IL-1 β , IL-6, IL-10), which in turn activates signaling cascades and causes injury to the primary liver cells, the hepatocytes^[8,21].

There have been a number of different approaches utilized to investigate the effects of ethanol consumption and LPS on ALD. The first hit from ethanol metabolism results in the production of ethanol metabolites (i.e. acetaldehyde and acetate). These metabolites increase redox state, steatosis, production of ROS, and lipid peroxidation. This in turn, increases other reactive aldehydes like malondialdehyde and 4-hydroxynonenal. These aldehydes can react with or adduct proteins to alter normal liver functions, induce cell death, and/or liver inflammation. This makes the liver more susceptible to a second hit, probably by LPS. The second hit perpetuates liver injury and fibrosis as a result of LPS-induced oxidative stress, cytokine release, and subsequent infiltration of immune cells^[2].

Two-hit animal models

In a two-hit model of ALD described by Koteish *et al*^[22], C57BL-6 mice were maintained on diets containing 4 mL/L ethanol for five weeks. Pair-fed control mice were fed an identical volume of ethanol-free diet. At the end of the five weeks, the mice received one 10 μ g injection of LPS and sacrificed at 0.5, 1.5 and 6 h post-injection. Histological results showed that the livers from control pair-fed mice had features of apoptosis, but no inflammatory cell infiltrates or hemorrhage. In contrast, hepatocytes in the ethanol-fed mice showed fat accumulation, inflammatory cell infiltration, and hemorrhage.

Procaspase-3, procaspase-8, Jun N-terminal kinase (JNK), TNF- α and TNFR-1 are mediators of LPS-induced hepatotoxicity. Apoptosis in this model was evaluated by determining the levels of procaspase-3 and procaspase-8. Immunoblot analysis to examine procaspase-3 levels showed that the ethanol-fed mice exhibited a decreased content of procaspase-3 relative to the pair-fed mice before and after LPS injections. Evaluation of procaspase-8 showed that it was elevated in the ethanol-fed mice compared to the pair-fed mice.

Examination of JNK activity demonstrated an eight-fold increase 1.5 h after LPS exposure alone. In contrast, LPS had no effect on JNK activity in ethanol-fed mice. TNF- α can interact with TNFR-1 and initiate a signaling cascade that activates procaspase-3 and inducing apoptosis. The expressions of TNF- α and TNFR-1 were found to be steady between the ethanol-fed and control pair-fed mice. Cytokines (IL-10, IL-15 and IL-6) that inhibit TNF- α activity have been shown to prevent liver damage from LPS. These cytokines were up-regulated in both groups but to a greater extent in the ethanol-fed mice.

Thus, the Koteish mouse animal model provides an excellent way to set up a long-term study showing the interaction of ethanol and LPS. This model is beneficial as there is no surgery to perform and only one injection of LPS is administered at the end of the study.

In the von Montfort *et al*^[23] model, four groups of C57BL-6J mice were given different treatments with epinephrine and ethanol. In the first group, 2 mg/kg per day of epinephrine was administered *via* an osmotic pump implanted in the dorsal area for five days. In previous studies this epinephrine dose had been shown to effectively mimic the effects of ethanol. The second group (controls) underwent sham surgery to mimic the implantation of the osmotic pump. The third group was injected with ethanol (6 g/kg per day) for three days. The last group was given a maltose/dextrin injection that was comparable calorifically to the ethanol injection. An injection of 10 mg/kg LPS was administered after the 5-d period and 24 h after the last ethanol dose, the mice were sacrificed at one, eight and 24 h later.

Histological results showed normal hepatocytes in the control animals and in the animals with the epinephrine pump/no LPS injection. Mice injected with LPS alone showed mild inflammation, whereas the mice receiving the epinephrine pump/LPS showed enhanced

liver damage. Importantly, the mice that were given ethanol/LPS showed dramatic liver damage. Plasma AST levels showed that the mice infused with epinephrine alone had no significant increase in plasma AST levels as compared with control mice. Animals treated with LPS only showed a progressive increase in AST with values approximately two-fold higher than the controls. Exposure of animals to epinephrine and ethanol with LPS exposure showed a significant increase in AST levels after LPS exposure by a factor of approximately four and six, respectively. The expression of pro-inflammatory genes, TNF- α , IL-6, and PAI-1 (plasminogen activator inhibitor 1) were analyzed by RT-PCR. In this part of the study, LPS alone stimulated all three genes as early as one hour after injection. Epinephrine pretreatment alone did not affect the expression of these genes, but paired with LPS showed an increased expression of all three genes. The induction of PAI-1 caused by LPS was significantly greater (two-fold) in the presence of epinephrine. The peak expression of TNF- α and IL-6, one hour after LPS exposure was not enhanced by epinephrine pre-exposure. However, these levels were significantly elevated when compared with the LPS alone group at the 8 h time point. Twenty-four hours after LPS injection, mRNA levels of all three returned to basal levels with no difference between the LPS and epinephrine/LPS groups.

Inflammation by LPS is not only enhanced by increased production of pro-inflammatory cytokines, but by impairing the expression of anti-inflammatory genes. Key anti-inflammatory genes such as IL-10, SOCS-1 and SOCS-3 were not significantly changed following epinephrine infusion alone, or in the sham-treated mice.

Curcumin (CMN) has been shown to exhibit anti-inflammatory, anti-bacterial, anti-oxidant properties, and reducing oxidative stress caused by ethanol. This animal model effectively shows how CMN pretreatment protects from LPS-induced liver damage. Kaur *et al.*^[24] used CMN in a model where six groups of male Wistar rats were administered different treatments in conjunction with CMN. In these studies CMN was administered at different doses (5, 30, 60 mg/kg) in the presence or absence of LPS. Rats were sacrificed six hours after an LPS injection on the last day.

Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), and alkaline phosphatase (AP) were analyzed in all rats which showed a marked rise in serum levels, following LPS injection causing increased liver damage. LPS also caused a rise in serum bilirubin and a decrease in serum total protein compared to control rats. Treatment with CMN significantly decreased the elevated levels of AST, ALT, AP, and bilirubin in response to LPS challenge. Serum nitrite levels and lipid peroxidation were decreased, whereas hepatic GSH, reduced glutathione, and superoxide dismutase (SOD), were increased. Additionally, TNF- α and IL-6 cytokines increased in LPS-administered rats compared to normal rats. CMN administration for seven days ef-

fectively blocked the rise of TNF- α and IL-6 cytokines in the LPS-challenged rats. Histology results did not reveal any morphological alterations in the control group but did show marked morphological disruption in LPS-administered rats. Treatment with CMN attenuated these morphological changes.

In the Gustot *et al.*^[25] animal model, C57BL-6J mice were fed a liquid ethanol diet adapted from the Lieber-DeCarli diet *ad libitum* for 10 d followed by an injection intraperitoneally with one of the following: 60 or 120 μ g lipoteichoic acid (LTA), 60 or 120 μ g peptidoglycan (PGN), 60 or 120 μ g polyinosine-polycytidylic acid (polyIC), 30 or 60 μ g LPS, 60 μ g flagellin or 60 or 120 μ g 7-allyl-8-oxoguanosine (loxoribine). Control mice were pair-fed with a control diet and injected with the same volume of saline solution after 10 d. Mice were sacrificed eight hours after injection.

The ethanol diet led to an increased liver weight compared to the control mice. Histology showed nearly 75% of hepatocytes in ethanol-fed mice exhibited steatosis. Compared to the control-fed mice, the ethanol-fed mice showed significant increase in serum (ALT) levels, and TNF- α mRNA expression. After 10 d, the expression of Toll-like receptors (TLR) 1, 2, 4, 6, 7, 8 and 9 was measured and found to have significant increases over control-fed mice. However, TLR3 and TLR5 were not statistically different in the two groups. To look at the role that endotoxin plays in liver damage in ethanol-fed mice, antibiotics were administered before the experiment began. Cultures from the ethanol-fed mice treated with antibiotics showed a clear decrease in gram-negative bacteria from the mice not treated with antibiotics prior to the start of the experiment. Addition of antibiotics reduced the severity of fatty liver, as shown by decreases in liver weight, serum ALT levels, and steatosis. An increase of lipid peroxidation products (e.g. malondialdehyde, 4-hydroxyaldenals) and a decrease of antioxidant levels (e.g. glutathione) demonstrated hepatic oxidative stress in the livers of ethanol-fed mice.

This animal model is effective because it provides a way to study ethanol consumption and LPS in a way that shows the importance of LPS in the liver for damage to occur. It is important to note that clinically, antibiotics have demonstrated some success in the treatment of ALD patients^[26-28].

The studies above used different approaches, but have shown very similar results when comparing the short-term and long-term effects of ethanol. Overall the short-term affects show that ethanol provides a protective affect from LPS by altering the expression of endotoxin receptors and intracellular signaling molecules^[20]. In contrast, the long-term effect shows an increased sensitivity to LPS and ethanol resulting in liver damage. Clearly, while these models have helped in gaining new insights into the mechanisms of ALD, the development of new and innovative animal models are needed to better elucidate the development and/or progression of ALD.

EFFECTS OF ETHANOL, METABOLITES, AND LPS *IN VITRO*

Liver sinusoidal endothelial cells (SECs)

SECs are flattened, highly fenestrated, and lack a basement membrane. This leads to their common characterization as “sieve-like”. Scanning electron microscopy has shown healthy fenestrae are approximately 150 nm in diameter and make up 6%-8% of the sinusoidal surface. These cells are active filters, selectively moving liquids and solutes from the portal blood into the Space of Disse where they are exposed to parenchymal hepatocytes and lipid storage cells. SECs are also highly endocytotic using scavenger receptors (SRs) to clear the blood of many molecular waste products. They have much higher permeability than other capillaries, and there is constant bidirectional exchange between hepatic parenchyma and blood *via* SECs. The size of the molecules that can pass through SECs is only constrained by fenestral diameter^[29]. These cells are the first line of defense against harmful molecules that could potentially damage the liver. There are many immunological functions of SECs. They have been shown to efficiently remove small (< 200 nm) molecules from the blood using innate immune mechanisms such as scavenger and mannose receptors^[29,30]. They have also been shown to express MHC class II, and co-stimulatory molecules (CD40, CD80, and CD86). They also express intercellular adhesion molecules including CD54 and CD106, which attract immune cells during inflammation. This suggests that they are involved in antigen processing and presentation as well as leukocyte recruitment^[30].

Capillarization of the SECs occurs when they form basement membranes that have been shown to precede fibrosis^[31]. Also, hepatic stellate cells (HSCs), when activated to their collagen producing form, induce fibrosis^[32]. It has been shown *in vitro* that quiescent rat HSCs maintain their inactivity and active HSCs revert to quiescence when grown in co-culture with healthy, nitric oxide (NO) producing SECs in the presence of vascular endothelial growth factor (VEGF). However, this effect is not observed when HSCs are co-cultured with capillarized SECs or those not producing NO^[33].

There is also evidence that malondialdehyde and acetaldehyde-two ethanol metabolites-form an adduct (MAA) which modifies proteins such that they increase the expression of fibrotic molecules by SECs. One such molecule is fibronectin. Fibronectin is expressed following insult to the liver and is known to activate HSCs and induce fibrosis. After stimulation with MAA-modified bovine serum albumin (MAA-Alb), isolated rat SECs show significant increases over negative controls in expression of soluble fibronectin, cellular fibronectin, and of the EIIIA fibronectin variant (the form most closely associated with activation of HSCs)^[34]. Incubation of MAA-Alb with isolated rat SECs for four hours has been shown to elicit increases in pro-inflammatory cytokines/chemokines: an eight-fold increase in TNF- α ; a two-fold increase in MCP-1; and a four-fold increase in MIP-2^[8]. This increase in fibrotic molecules and pro-

inflammatory cytokines provides a potential mechanism by which damage to the liver is mediated by metabolites of ethanol adducted proteins.

Due to their participation in the clearance of LPS from the blood, SECs have mechanisms for the control of inflammatory, leukocyte-mediated response to LPS, while maintaining the population of SRs necessary for toxin and waste clearance. Upon initial contact with LPS, cultures of mouse hepatic SECs release and activate IL-6. This occurs without regard to the presence of TNF- α and requires functional TLR4. SEC responsiveness is decreased following repeated stimulation by LPS. This occurs *via* reduced nuclear translocation of transcriptionally active nuclear factor $\kappa\beta$ (NF $\kappa\beta$). Importantly, repeated exposure to LPS diminishes SEC scavenger function but does not eliminate it. Initial exposure to LPS results in an increased expression of adhesion molecules on leukocytes including CD54, CD106, MCP-1, and IFN-inducible protein 10. None of the above are up-regulated in cultured SECs with previous LPS exposure, and in the case of CD54, this was shown to occur *via* reduced gene expression^[35].

Exposure to LPS *in vivo* has been shown to increase both the quantity of small (20 nm) latex beads and the maximum size (from 100 nm to 500 nm) of latex beads that can be ingested by mouse SECs. Increases were not seen in the rate of ingestion of 100 nm beads, which were the most aggressively ingested regardless of LPS stimulation. LPS was also shown to increase the uptake of both BSA (soluble protein) and dextran (soluble carbohydrate). These increases are attributed to LPS-induced actin remodeling by protein kinase C (PKC) and phosphoinositide 3-kinase (PI3-K) as indicated by increased expression of *src*-suppressed C kinase substrate (SseCKS) on the endothelial cells^[36].

Two-hit model effects on SECs

Lining the portal veins of the liver, the SECs are the first line of defense against LPS derived from the gut^[30]. Most of the attention in LPS clearance has been attributed to the KCs. However, it has been shown that SECs contribute to the regulation of LPS in the liver^[30,35,36]. This regulation of LPS under normal physiological conditions is kept in check by both KC and SECs, which keep the amount of inflammation to a minimum. If LPS levels are increased due to other factors (i.e. alcohol or aldehyde modified proteins), then the potential for disruption of normal homeostasis exists. The fact that alcohol increases both gut permeability (increasing the LPS)^[7] and aldehyde modified liver proteins^[4], provides a possible two-hit mechanism by which the liver could become damaged.

Chronic exposure to ethanol metabolites in the form of MAA-adducted albumin has been shown to alter the SEC response to LPS. In hepatic SECs isolated from rats, LPS-induced secretions of TNF- α , MCP-1, and MIP-2 all show at least two-fold increases in the presence of MAA modified albumin, but no increase in the presence of unmodified albumin. The TNF- α

response is decreased by chronic ethanol consumption, but MCP-1 and MIP-2 responses are not^[8]. The MAA-adduct has also been shown to bind to, and be degraded by, SECs *via* SRs on their surface^[37]. It has also been shown that SECs have CD14 and TLR receptors, which are involved in the uptake of LPS^[38,39]. The potential exists for both aldehyde modified proteins and LPS to bind their receptors simultaneously, increasing the normal release of pro-inflammatory factors that promote inflammation of immune cells to the liver.

While chronic ethanol consumption and LPS stimulation independently increase apoptosis of SECs (as measured by caspase-3 activity) in pre-ALD rat livers, the combination elicits no additional increases. However, data generated from this study did demonstrate that LPS treatment of animals increased the amount of malondialdehyde (MDA) in the hepatocytes. The increase in MDA provides additional substrate for the potential formation of MAA adducts. Therefore an increase in AA and MDA from alcohol metabolism and an increase in MDA from inflammation-induced cell damage could lead to increased MAA-adducted self cellular material and the subsequent initiation of an autoimmune disease^[40].

KCs

KCs are the resident macrophages found in the liver. It is believed that KCs play an important role in the development of ALD^[41]. When stimulated with LPS they become activated and release pro-inflammatory and fibrotic cytokines, along with ROS, which can contribute to liver injury^[11]. Interestingly, it has been shown that circulating LPS concentrations are increased in the blood of alcoholics, and in rats fed alcohol intragastrically, due to the effects ethanol on increasing the permeability on the intestinal mucosa^[42,43]. The circulating LPS derived from intestinal bacteria in turn activate KCs, which initiate their pro-fibrotic and pro-inflammatory effects. KCs can also be activated by interactions with proteins modified by reactive aldehydes associated with ethanol metabolism increasing oxidative stress due to [acetaldehyde (AA) and malondialdehyde (MDA)]. These modified proteins have been associated with ALD^[44-47]. LPS may further sensitize KC interactions with aldehyde-modified proteins^[8] and may actually be involved in their formation^[48].

Chronic ethanol does more than provide the KCs with LPS; it also directly affects their sensitivity to LPS. This section will examine the role that LPS activation of KCs plays in the development of ALD and whether ethanol directly affects KC responses to LPS. The role(s) of aldehyde modified proteins in KC activation and how LPS may affect KC sensitivity to stimulation by these modified proteins will also be examined.

Alcohol causes both tolerance and sensitization to KC activation by LPS^[49-51]. Studies have shown that acute ethanol administration inactivates KCs probably due to ethanol's effects on calcium channels and their requirement for TNF- α release^[52]. KCs isolated from rats two hours after ethanol treatment indeed lacked increased intracellular calcium normally observed when KCs are

treated with LPS. However, when KCs were isolated 24 h after the rats were treated with ethanol, the cells displayed their normal TNF- α production and histological changes upon LPS stimulation. These cells also displayed higher levels of the LPS binding receptor, CD14, which may explain their increased sensitivity to LPS. Treating the rats with antibiotics, which sterilizes the gut removing portal LPS, lowered CD14 expression on KCs isolated 24 h after the administration of ethanol, suggesting that gut derived LPS was the cause of the increased CD14 expression and resultant sensitivity to LPS^[53]. From these studies it can be concluded that a single dose of ethanol can either sensitize KCs or induce tolerance to LPS based on timing.

Two-hit model effects on KCs

As discussed above, chronic ethanol exposure seems to increase the sensitivity of KCs to LPS stimulation. One of the major cytokines released by KCs exposed to LPS is TNF- α , which plays a role in the development of ALD^[41]. Use of anti TNF- α antibody has been shown to protect against ALD in certain animal models^[54]. The role of TNF- α in ALD was also confirmed in studies using TNF- α receptor 1 knockout mouse. In these mice, the pathological changes associated with ethanol treatment were greatly diminished^[55]. KCs may become extra sensitive to LPS and as a result increase their production of TNF- α when chronically exposed to ethanol. This increases CD14 expression and changes the signaling cascade molecules involved in LPS stimulation induced by ROS. These events change transcription factor binding to DNA and increase stability of mRNA involved in TNF- α production.

KCs stimulated by LPS also have effects on HSCs. Hepatic fibrosis is characterized by an over deposition of extracellular matrix components. HSCs are involved with the production of extracellular matrix (ECM) in the liver and during fibrogenesis they undergo a process of activation and proliferation leading to excess collagen synthesis^[56]. LPS activated KCs have been shown to be capable of activating HSC *in vitro*, and levels of ECM production have been directly correlated to increased HSC proliferation^[57].

The *in vitro* effects that LPS-stimulated KCs have on HSCs may be similar to what is seen during *in vivo* fibrogenesis. Higher levels of LPS in the blood of chronic alcoholics may serve as the catalyst for KC activation and therefore may promote HSC activation and fibrogenesis. The production of TGF- β 1 by LPS-stimulated KCs is one of the most significant steps in the activation of HSCs^[57].

Studies have indicated that the detection of acetaldehyde (AA), malondialdehyde (MDA), and AA MDA hybrid (MAA) modified proteins adducts, correlate with increased liver enzymes and liver damage^[45,47,58]. Experiments have also demonstrated that administering gadolinium chloride with ethanol results in the decreased accumulation of MDA and especially AA protein adducts in the livers of rats chronically fed alcohol indicating a role for KCs in AA protein adduct formation^[48].

LPS stimulation of KCs and their ability to release

pro-inflammatory cytokines might also promote immune system recruitment and surveillance, which might help to promote an immune response to these modified proteins. Circulating antibodies to aldehyde-derived epitopes have been identified^[59]. KCs might be involved in the actual presentation of these molecules to the immune cells and be stimulated by these adducted proteins to release pro-inflammatory cytokines. SRs found on their surfaces can bind MAA modified proteins and subsequent binding of these proteins leads to increased levels of TNF- α . When KCs are stimulated with low levels of LPS in addition to MAA modified albumin (MAA-Alb), TNF- α secretion increases six to eight fold. The levels of LPS used for co-stimulation were so low that LPS alone did not result in any TNF- α secretion^[8].

In summary, KCs are a key component in the development and/or progression of ALD. LPS is a potent activator of KCs, causing them to release cytokines such as TNF- α and TGF- β 1, which have been indicated in the development of ALD. Acute administration of ethanol can lead to tolerance or sensitivity to LPS in isolated KCs, while chronic ethanol exposure usually induces a state of ethanol sensitivity marked by increased cytokine production. Cytokines produced by KCs stimulated by LPS can lead to proliferation and activation of HSCs resulting in ECM production. KCs might be involved in the formation of aldehyde protein adducts and these adducts might promote a pro-inflammatory response in KCs, especially in the presence of LPS.

Stellate cells (HSCs)

HSCs undergo activation and proliferation when under the influence of acute and chronic liver injury events. Liver fibrosis and cirrhosis occur in the chronic stages of injury and represent activation of HSCs and secretion of matrix from these cells. Activation of HSCs also occurs in liver injury in acute stages where this damage is known to be able to resolve on its own^[60]. Chronic liver injury events influenced by alcohol and LPS are of interest as they are associated with a constant hepatic insult that leads to life threatening complications with liver transplantation as the only viable option^[60]. As HSCs are involved in the pathway of the wound healing of the liver, their association with alcohol and LPS is an important relationship to understand.

Metabolism of ethanol by the liver is an extremely oxidative event resulting in the development of acetaldehyde (AA). AA is further metabolized into acetate *via* the mitochondrial enzyme acetaldehyde dehydrogenase (ALDH)^[61]. This is a slow reaction that allows for AA buildup over time when alcohol is consumed, and while ADH activity is not greatly influenced, CYP2E1 and other microsomal enzymes are greatly stimulated^[61]. This buildup of AA has also been shown to form stable aldehyde adducts on proteins, which stimulate collagen synthesis, activate protein kinase C, and promote the release of chemokines MCP-1 and MIP-2^[62-64]. Activation of HSCs increases the release of collagen and matrix proteins, which begin the fibrogenic response of wound healing^[32,61].

TGF β 1 is the major profibrotic cytokine in the nor-

mal wound healing process. TGF β 1 messenger expression in HSCs is elevated by ethanol and acetaldehyde, whereby collagen type I gene expression is up-regulated^[32]. Indeed, mouse α 2 (I) collagen promoter was shown to have greater activation in transient transfection experiments when TGF β 1 and acetaldehyde were introduced in tandem, rather than alone, suggesting that TGF β 1 could play a direct part in collagen I gene activation^[65].

Two-hit model effects on stellate cells

In rodent models, using ethanol to induce substantial fibrotic liver injury is problematic regardless of the concentration or length of treatment. Therefore two-Hit models have been used in rodents with the hope to better emulate the fibrotic injury found in human disease states, using some secondary factor with ethanol. Degradation of ethanol increases oxidative stress within the hepatic system, generating free radicals, leading to further hepatic events such as endotoxemia. These events increase gut permeability allowing for more LPS release making LPS clearance more difficult^[10,11,19].

Karaa *et al*^[66] looked for therapeutic agents that slow or possibly prevent ALD progression. S-adenosyl-L-methionine (SAME) has previously been shown to be a precursor in glutathione (GSH) synthesis in the transsulfuration pathway, which is an important hepatic antioxidant. The fact that chronic ethanol consumption greatly limits hepatic SAME storage, GSH synthesis, and increases gut permeability, allows LPS to act as a secondary agent in the two-hit model.

Karaa *et al*^[66] examined how SAME acted as an anti-fibrotic agent by looking at liver fibrosis, HSC activation, and collagen deposition. This model used Lieber DiCarli liquid diet containing ethanol (or a calorie matched diet) concurrently with twice weekly LPS injections during an eight week period. Ethanol alone led to steatosis, some immune cell infiltration (mainly neutrophils), HSC activation, and increased hepatic collagen production. When LPS was introduced with ethanol, hepatic infiltration of neutrophils was increased as well as increased activation of HSCs and preferential pericellular collagen deposition. When SAME was introduced with LPS and chronic ethanol administration, the antioxidative properties of SAME were apparent. GSH stores depleted by ethanol were replenished and hepatic oxidative stress was reduced.

Quiroz *et al*^[67] also looked at the effect of LPS on rat HSC (CFSC-2G) in relation to ethanol and AA. GSH, oxidized GSH (GSSG), IL-6, and collagen secretion were measured. The authors found that lipid peroxidation levels were increased in all experimental conditions versus controls (controls being HSCs with LPS, ethanol, or AA alone). MDA response to ethanol and acetaldehyde exposure did not show a significant change with LPS pretreatment. Experimental cells showed a 2.5 fold increase in GSSG content with LPS and ethanol, and a 5.5 fold increase with LPS and AA, with control values of GSH being much lower. Collagen content was also greatly enhanced with pretreatment of LPS, 120%

greater with ethanol and 209% greater with AA. TGF- β secretion was similar to that of the controls, but IL-6 was greatly up-regulated^[3].

Pretreatment of LPS resulted in an increase of intracellular GSSG, leading to the formation of mixed disulfides with protein thiols, thus lowering the ability to fight oxidative stress induced hepatic injury^[67]. The authors theorized that with the aforementioned change in GSH and GSSG levels, HSCs pretreated with LPS might also generate additional ROS. They also speculated that IL-6 not only promotes hepatocyte proliferation, but enhances collagen production by these activated HSCs. Therefore, "LPS pretreatment of HSC adds to the damage produced by ethanol and acetaldehyde by diminishing GSH content and increasing GSSG content, collagen, and IL-6 secretion"^[67].

Precision cut liver slices (PCLS)

Precision cut liver slices (PCLS) may provide an alternative to other *in vitro* model systems using isolated liver cells to study the combined effects of ethanol and LPS. PCLS are representative of the whole liver and have recently been developed as an *in vitro* model of ethanol induced liver injury^[68]. PCLS exhibit significant ethanol-induced damage in as little as 24 h. This model uses PCLS originating from Wistar rats, cultured in the presence or absence of 25 mmol/L ethanol in a roller system under 95% O₂^[68]. Over a 96 h time period this model efficiently metabolizes ethanol, produces AA, develops a reduced redox state and fatty liver, and exhibits impaired albumin secretion. All of these phenomena are characteristics of early liver injury. Interestingly, in the presence of 4-methylpyrazole (4-MP), an inhibitor of ethanol metabolism, all of the ethanol-mediated effects are ameliorated or significantly reduced, indicating that the metabolites of ethanol are responsible. In addition, recent studies have shown that 25 mmol/L ethanol induces sustained production of IL-6, depletion of GSH, increased lipid peroxidation, and induction of fibrogenesis (increased expression of smooth muscle actin and deposition of collagen in sinusoidal areas). All these phenomena are inhibited by 4-MP, implicating ethanol metabolites. The production of IL-6, GSH depletion, and lipid peroxidation all precede the induction of fibrogenesis, suggesting that inflammation and production of reactive oxygen species are responsible.

PCLS have also been used to examine the effects of LPS on the liver^[69,70]. LPS induced expression of TNF- α , IL-1 β , IL-6 and IL-10 within 24 h of incubation. Consistent with other studies indicating TNF- α induces expression of other cytokines, TNF- α expression was maximal by five hours, whereas expression of the other cytokines was maximal by 16-24 h^[69]. The authors attributed this to activation of KCs, but activation of SECs cannot be ruled out, as they also respond to LPS. Additionally, production of nitric oxide (NO) gradually increased after LPS treatment, starting at five hours after treatment, and continuing throughout the 24 h treatment period^[70]. This increase was paralleled by an increase in inducible nitric oxide synthase (iNOS) expression in the hepatocytes. Inhibition of

TNF- α and IL-1 β attenuated iNOS expression, indicating a paracrine effect by the cytokines induced by LPS treatment. Production of NO *in vivo* has been shown to have protective effects in inflammation and endotoxemia-induced hepatic injury^[71]. Thus, these results might indicate a compensatory response by the PCLS to the inflammatory response induced by LPS treatment.

One previous study examined the combined effects of LPS and ethanol on PCLS cytotoxicity^[72]. This study incubated PCLS in the absence or presence of 0.5%-8% ethanol and/or 0.1-100 μ g/mL LPS for up to 12 h. Ethanol at 1% or more exhibited a time- and dose-dependent hepatotoxicity by itself, whereas LPS had no appreciable effect. However, when combined, 2% ethanol and increasing concentrations of LPS exhibited additive effects on hepatotoxicity with 100 μ g/mL LPS inducing the most injury (70% viable) compared to 2% ethanol alone (90% viable) at 12 h. While these concentrations of both ethanol and LPS are extremely high, they do provide an upper limit for future studies to examine the combined effects of ethanol and LPS. In fact, preliminary data by this laboratory suggests that more physiological levels of ethanol (25 mmol/L) and LPS (10 ng) exhibit additive effects on inflammatory processes and induction of oxidative stress in PCLS.

CONCLUSION

Studies into the pathogenesis of ALD have demonstrated that ethanol, LPS, and the metabolites of ethanol all may have a significant role in the development and/or progression of this disease. However, the data also strongly suggests that while each of these components are necessary, alone they are not sufficient to induce the pathogenesis of ALD. Indeed, it is becoming more apparent that combinations of one or more of these components must occur to induce ALD, which would suggest that at the very least a "two-hit" model is involved in the pathogenesis of ALD. Figure 1 shows a proposed hypothesis of the "two-hit" model of ALD.

Support for this concept can be found in some very simple observations. Acute (down-regulation) versus chronic (no effect) ethanol exposure decreases the response of KCs and SECs to LPS, which is thought to be a major co-factor in ALD. It has also been shown that on some cell types, multiple receptors (Scavenger Receptor A; SRA-1) are present and may bind LPS and degrade this ligand before it binds to TLR4. When the expression of these SRs is altered (ethanol inhibits degradation), LPS is now free to bind to CD14/TRL4 and initiate pro-inflammatory responses. It has also been shown that multiple ligands, ROS, aldehyde-modified proteins, and hyaluronan are involved in the development and/or progression of ALD. Therefore, depending upon which ligands were to bind to their appropriate receptors, then the response would be totally different than if only one of these ligands were to bind. Couple this with the fact that each cell type in the liver expresses different receptors, and it is easy to imagine that the interactions

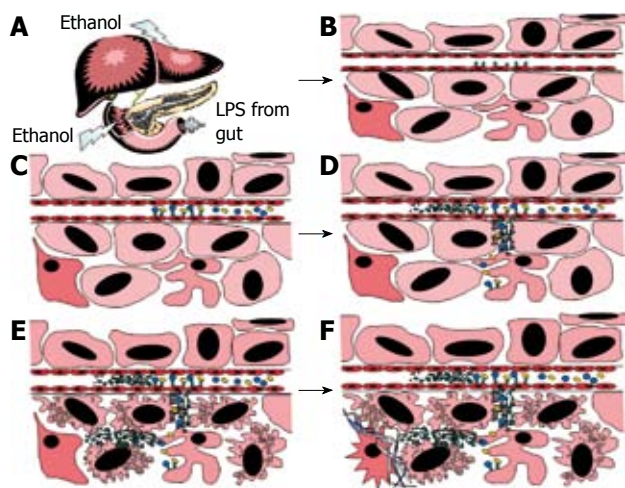


Figure 1 A proposed hypothesis of the two-hit model for the onset and/or progression of alcohol liver disease. A: The prolonged consumption of ethanol has been associated with an increase in gut permeability. Lipopolysaccharide (LPS) may leak out of the gut and into the blood stream, finding its way back to the liver. B: Meanwhile, the breakdown of alcohol and fats in the liver could modify cellular proteins with malondialdehyde and/or acetaldehyde and result in increased levels of these modified proteins. Receptors on endothelial cells (SECs) specific for LPS and aldehyde modified proteins might also be up-regulated. C: The various scavenger receptors bind LPS and/or aldehyde modified proteins circulating in the blood stream. D: Binding of these molecules causes an increased release of pro-inflammatory cytokines, which are dumped into the blood stream and into the liver through the Space of Disse. The release into the liver causes activation of kupffer cells, binding of LPS and modified proteins, and their release of more pro-inflammatory cytokines. E: The cytokine release from SECs and/or kupffer cells signals immune cells infiltrate into the liver and result in damage to the hepatocytes. F: Damage to the hepatocytes increases the amount of TGF- β and other cytokines, causing the stellate cells and SECs to secrete pro-fibrogenic factors. These factors help to rebuild and remodel the liver parenchyma initiating the wound healing responses. Following repair of the liver, there is scarring and some irreparable damage, which can get better if the insults (LPS and modified proteins) are taken away. However, if alcohol consumption remains there becomes a point whenentence, preferably as two shorter sentences, to clarify this?

between the different cell types might change depending upon the receptors expressed. As discussed in this review, it is apparent that these interactions do occur and the development and/or progression of ALD is a complex interaction that may be investigated utilizing a “two hit” or “multiple hit” model.

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