World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i11.1321

World J Gastroenterol 2010 March 21; 16(11): 1321-1329
ISSN 1007-9327 (print)
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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Alcoholic liver disease and the gut-liver axis

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Author contributions: Szabo G and Bala S worked together on the concept and outline of the article and the specific chapters were written by one of the authors in equal distribution.
Supported by NIAAA Grants No. AA017729 and No. AA011576
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Received: January 5, 2010 Revised: February 10, 2010
Accepted: February 17, 2010
Published online: March 21, 2010

Abstract

Alcoholic liver disease (ALD) is one of the leading causes of liver diseases and liver-related death worldwide. Of the many factors that contribute to the pathogenesis of ALD, gut-derived lipopolysaccharide (LPS) plays a central role in induction of steatosis, inflammation, and fibrosis in the liver. In this review, we discuss the mechanisms by which alcohol contributes to increased gut permeability, the activation of Kupffer cells, and the inflammatory cascade by LPS. The role of the Toll-like receptor 4 (TLR4) complex in LPS recognition and the importance of the TLR4-induced signaling pathways are evaluated in ALD.

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Key words: Kupffer cell; Gut permeability; microRNA; Tumor necrosis factor-α; Endotoxin

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Szabo G, Bala S. Alcoholic liver disease and the gut-liver axis. *World J Gastroenterol* 2010; 16(11): 1321-1329 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i11/1321.htm
DOI: http://dx.doi.org/10.3748/wjg.v16.i11.1321

INTRODUCTION

The clinical spectrum of alcoholic liver disease (ALD) includes alcoholic fatty liver, alcoholic steatohepatitis, alcoholic cirrhosis (Laennec's cirrhosis), and increased risk of hepatocellular carcinoma[1,2]. The pathomechanism of ALD involves complex interactions between the direct effects of alcohol and its toxic metabolites on various cell types in the liver, induction of reactive oxygen species (ROS), upregulation of the inflammatory cascade, and other cell-specific effects in the liver[3,4]. Lipopolysaccharide (LPS), also known as endotoxin, has been identified as a major factor in the pathogenesis of ALD. Indeed, LPS can lead to liver steatosis, as it induces inflammation and contributes to cirrhosis, which are all features of ALD[5,6]. These effects of LPS are manifested in the various cell types in the liver and the source of LPS appears to be the gut in ALD, resulting from alcohol-induced disturbance of gut permeability. At the cellular and molecular level, LPS is recognized by the Toll-like receptor 4 (TLR4) complex and induces specific intracellular activation pathways. This review will focus on the role of LPS in ALD and will summarize the current state of art on alcohol-related changes in the gut-liver axis.

GUT-LIVER AXIS

The gut is a habitat for billions of microorganisms and the gut mucosal epithelium serves as a barrier between microbiota and gut lumen[7]. LPS (endotoxins) derived from Gram-negative bacteria in the intestinal microflora normally penetrate the mucosa only in trace amounts,

enter the portal circulation, and become cleared in the liver to maintain the control of immune homeostasis. Resident macrophages (Kupffer cells) and hepatocytes both contribute to this process through different LPS recognition systems[3,8-10]. There is a positive correlation between liver dysfunction and the occurrence of bacterial translocation[11-14], and the clearance of LPS from the circulation is decreased in states of hepatic dysfunction, such as cirrhosis[15]. Studies in animals suggest that the liver quickly removes about 40%-50% of an intravenous dose of LPS from the bloodstream[16,17]. Hepatic uptake and detoxification is important for preventing systemic reactions to blood-borne LPS. It has been proposed that LPS initially is taken up by Kupffer cells and then by hepatocytes[18]. LPS is removed *via* several mechanisms, including molecules that bind LPS and prevent it from activating TLR4, enzymes that degrade the lipid A moiety to decrease its activity, inactivation of LPS following uptake into the liver and spleen, and cellular adaptations that modify target cell responses[19]. Another mechanism for LPS neutralization is by serum lipoproteins, HDL, LDL, VLDL, and chylomicrons, apolipoproteins apoE and apoA-I LPS[20-22]. All of these mechanisms can chaperone endotoxin to hepatocytes, Kupffer cells, or sinusoidal endothelial cells, resulting in clearance of LPS without significant inflammatory cell activation.

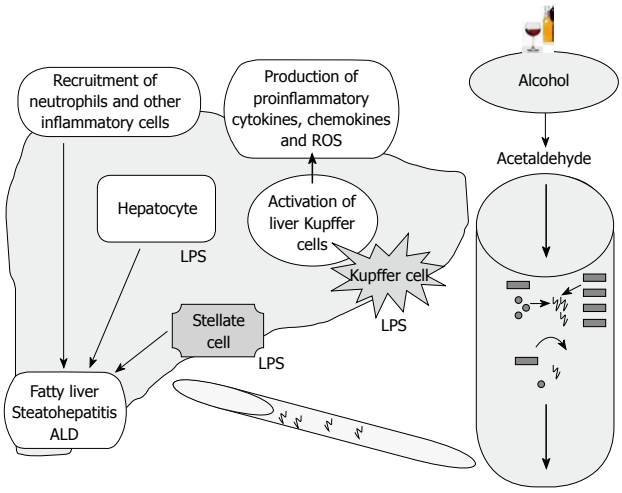


Figure 1 Mechanisms of alcohol induced liver damage. Alcohol consumption alone, or with its metabolites, disrupts the gut integrity by various mechanisms, including increased reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), alteration of microRNAs, proliferation of Gram-negative bacteria, and changes in bacterial species. These factors alone, or in combination, mediate increased gut permeability and subsequent bacterial or microbial translocation into intestinal lumen and thus an increase in lipopolysaccharide (LPS) in the portal circulation. The excess of LPS in the liver affects immune, parenchymal, and non-immune cells and in response there is release of various inflammatory cytokines, and recruitment of neutrophils and other inflammatory cells. Persistence of the above mentioned factors are hallmark of alcoholic liver disease (ALD).

ALD AND ENDOTOXIN

The role of LPS in alcoholic liver injury has been shown in several studies[12,23,24]. The importance of gut-derived endotoxin in ALD was suggested by experiments where treating the animals either with antibiotics or with lactobacilli to remove or reduce the gut microflora provided protection from the features of ALD[12,25,26]. In mice and rats, circulating endotoxin levels were increased after chronic alcohol feeding[27,28] and plasma endotoxin levels were also increased in patients with ALD compared to normal subjects[29]. The persistence of endotoxin not only activates the liver immune cells but also affects the function of liver parenchymal cells.

The progression of ALD is a complex phenomenon, as it not only results from the direct effects of alcohol and its metabolites, but other factors also play an important role in its pathogenesis, such as leaky gut, which results in endotoxemia[30]. Both chronic ethanol- mediated microbial proliferation[31,32] and acetaldehyde-mediated opening of intestinal tight junctions (TJs)[33] enhance the passage or release of endotoxins into the intestinal lumen, which are later transported to liver. However, when excess amounts of endotoxin are not cleared efficiently by the liver and accumulate in blood circulation, innate immune cells, including Kupffer cells, are activated, leading to the release of various pro-inflammatory cytokines, chemokines, and other factors[34,35].

Kupffer cell activation has been identified as one of the key elements in the pathogenesis of alcoholic steatohepatitis. Studies in mice and rats demonstrated that inactivation of Kupffer cells with gadolinium chloride or clodronate injection can almost fully ameliorate alcohol-induced liver disease[36,37]. These observations led to the currently accepted model of ALD, where Kupffer cell activation by gut-derived endotoxin, induction of chemokines such as MCP-1, and upregulation of the inflammatory cascade represent a central component of the pathomechanisms of ALD (Figure 1).

MECHANISMS OF GUT BARRIER DISRUPTION BY ALCOHOL

The mechanisms underlying the disruption of the intestinal barrier by alcohol appear to be at multiple levels, including disruption of the gut barrier and changes in microbial flora.

Disruption of gut integrity by alcohol and its metabolites

Tight junctions are scaffolds of various transmembrane proteins (e.g. claudins, occludin, JAMs, and tricellulin) and a complex network of adaptors proteins that cross-link junctional membrane proteins (i.e. ZO-1/2/3, PATJ, PAR-3, and PAR-6) to the actin cytoskeleton as well as to different intracellular signaling components. Both alcohol and its metabolites affect the integrity of TJs.

Several studies in the literature suggest the role of acetaldehyde (one of the metabolites of alcohol) in increasing intestinal permeability[30,38,39]. Acetaldehyde causes the redistribution of tight junction proteins (occludin and ZO-1) and adherens junction (E-cadherin and

β-catenin) proteins from the intercellular junctions[40,41]. Furthermore, acetaldehyde increases the tyrosine phosphorylation of ZO-1, E-cadherin, and β-catenin, without affecting tyrosine kinase activity[40]. Acetaldehyde also disrupts the interactions between E-cadherin, β-catenin, and PTP1B, which are the vital components of adherens junctions and epithelial cell-cell adhesion[42]. These studies indicate the central role of acetaldehyde in disruption of gut integrity, however, not much is known about the effects of other metabolic products of alcohol on gut permeability.

Increased expression of inducible nitric oxide synthase (iNOS) is another factor by which alcohol disrupts the intestinal barrier function. Increase in iNOS, NO, and superoxide correlates with an increase in nitration and oxidation of tubulin, causing increased levels of disassembled tubulin that subsequently damage the microtubule cytoskeleton and result in disruption of barrier function in alcohol treated CaCo2 cells[43,44]. NF-κB is involved in oxidation-induced upregulation of iNOS as well in nitration and oxidation of cytoskeleton[45]. Interestingly epidermal growth factor has a protective role in intestinal barrier function *via* downregulation of iNOS activity, which results in the stabilization of cytoskeleton[46-48].

Not only chronic alcohol intake results in the disruption of intestinal barrier, but acute alcohol consumption also damages intestinal mucosal membrane, as reported in a rat model[49]. In a mouse model, a single dose of acute ethanol (6 g/kg) causes injury to the mucosal lining of the small intestine[50].

Exploitation of mircoRNAs (miRs) by alcohol to target the tight/adherent junction proteins

Another mechanism by which alcohol increases intestinal permeability is by indirectly affecting tight junction proteins through miRs. In particular, a recent study showed the involvement of miRs in gut barrier disruption in alcohol treated cells. miR-212 targets the ZO-1 protein negatively, thus increasing intestinal permeability[51]. Consistent with this *in vitro* observation, higher levels of miR-212 and lower amounts of ZO-1 protein were found in colon biopsy tissues from patients with ALD[51]. However, more work needs to be done to explore the role of miRs in regulating tight and adherent junction proteins in ALD.

Change or increase in gut microflora by alcohol

Chronic alcohol abuse not only causes gut leakiness, but also affects the composition of colonic mucosa-associated bacterial microbiota in alcohol-fed rats[52]; however, the latter finding needs to be validated in human subjects. While there is evidence of bacterial overgrowth (Gram negative) in the gut of alcoholics[53], little is known about how alcohol consumption is related to increased intestinal bacterial growth. Interestingly, we do not know whether alcohol consumption affects Gram-positive bacteria, which are the source of peptidoglycan. Nevertheless, increased peptidoglycan levels were found in mice after prolonged administration of alcohol in their drinking water. Interestingly, this mode of alcohol administration does not result in ALD[54].

ENDOTOXIN RECEPTORS AND SIGNALING PATHWAYS

LPS is a major component of the outer membrane of Gram-negative bacteria and it comprises three distinct parts: a carbohydrate "O-antigen", the oligosaccharide core region, and a lipid portion "lipid A". Only the lipid A moiety is toxic and is responsible for the activation of the innate immune response in mammals[55]. LPS and other bacterial cell wall constituents are released during bacterial multiplication or when bacteria die or lyse[56]. As soon the immune system recognizes the presence of microorganisms (bacteremia) or LPS in the blood stream (endotoxemia), various proinflammatory cytokines, chemokines, ROS, and other mediators are released to activate monocytes, macrophages, and to recruit lymphocytes. The liver plays an important role in the body's defense mechanism against bacteria and bacterial products.

LPS is recognized by various receptors in the cells. CR3 (CD11b/CD18) was the first described LPS receptor[57] in human macrophages. Later on, cluster of differentiation 14 (CD14) and LPS binding protein (LBP) were recognized as receptors for LPS[58]. Recently, myeloid differentiation factor-2 (MD-2) was found as another LPS binding molecule (direct binding)[59]. However, CD14 and MD-2 lack a transmembrane domain and, therefore, a second receptor is required to activate the signaling cascade, which was recently described as TLR4 (indirect binding)[60,61].

TLR4

Toll receptors were first discovered in Drosophila[62] and later on their human homologs were identified[63]. TLR4 recognizes LPS with the cooperation of its co-receptors, CD14 or MD-2[64,65]. LPS recognition by TLR4 results in recruitment of the adaptor molecules MyD88 and TRIF, which each activate separate downstream signaling cascades (Figure 2). Formation of the TLR4-MyD88 complex activates the IRAK kinases, which turn on the IKK complex to activate NF-κB, which results in increased production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β[64]. Activation of TRIF pathway results in TBK/IKKe phosphorylation and activation of the interferon regulatory factor-3 (IRF3), which leads to induction of Type- I interferons (IFNs)[66]. Activation of both of the pro-inflammatory and Type- I IFN pathways by TLR4-LPS is unique, and evaluation of these specific pathways has recently received attention in ALD.

The TLR4-LPS signaling pathway plays a critical role in alcohol-induced liver injury. Both chronic and acute (or binge) alcohol use affect the various components of TLR4 signaling[67-71]. The effect of alcohol use on TLR4

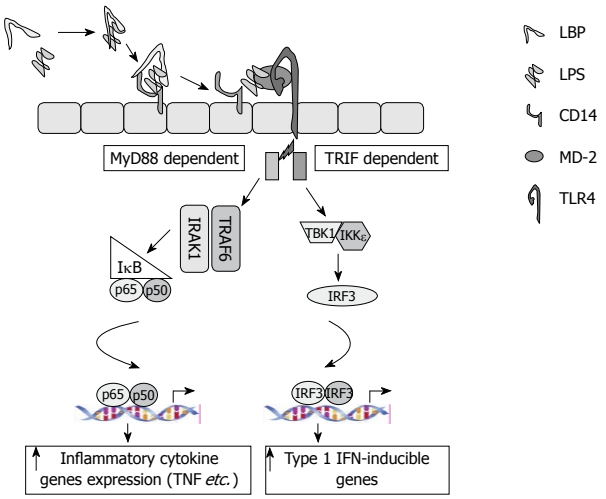


Figure 2 Role of the Toll-like receptor 4 (TLR4)-LPS signaling cascade in alcohol induced liver injury. LPS binds to LPS binding protein (LBP), transfers to cluster of differentiation 14 (CD14) and then to the TLR4 myeloid differentiation factor-2 (MD-2) complex. This signal is passed either through MyD88-dependent or TRIF-dependent intracellular pathways, which activate various transcription factors and induce various pro-inflammatory cytokine and Type Ⅰ interferon genes.

signaling was recently reviewed in detail[3]. There is increased expression of TLR4 and its co-receptors, as well as other TLRs, in ALD in mice. Early studies in TLR4 mutant mice demonstrated protection from early ALD[72] and more recent reports using TLR4 deficient mice validated the important role of TLR4 in the pathogenesis of ALD[73]. We also investigated the specific role of the MyD88 adapter in ALD and found that MyD88-deficient mice were not protected from alcoholic steatosis and inflammation. Consistent with the hypothesis that MyD88-independent, TLR4-mediated, pathways are involved in ALD, we found protection from ALD in TLR4-deficient, as well as in IRF3-deficient, mice[73]. The role of IRF3 in ALD was also indicated by another study[74].

MD-2

MD-2 is a type Ⅱ acute phase protein and is expressed on the surface of myeloid and endothelial lineage cells[75,76]. Although it lacks a transmembrane domain, it attaches to the cell surface through its interaction with TLR4[77,78]. MD-2 also presents in a soluble form (sMD-2) and is secreted by various cells[79,80]. Increased sMD-2 activity is found in plasma of sepsis patients[81,82]. It is postulated that at high concentrations, sMD-2 might inhibit endotoxin induced cell activation in a similar way to LBP and soluble CD14[78,83]. IL-1β regulates the production of MD-2 in hepatocytes and myeloid cells[83]. Chronic alcohol feeding results in an upregulation of MD-2 in the liver[73].

CD14

CD14 is expressed in various cell types, including monocytes, macrophages, B cells, liver parenchymal cells, and some fibroblast cells[84,85]. It is absent in early myeloid progenitor cells; however, with maturation, its expression increases. Human CD14 transgenic mice are hypersensitive to LPS[86], whereas CD14 knockout mice are resistant to endotoxin shock[87], indicating its crucial role in LPS signaling. CD14 is also present in soluble forms, as sCD14 α and sCD14 β, and is secreted by macrophages[88] and liver parenchyma cells[89].

Alcohol consumption affects CD14 expression and plays an important role in LPS induced immune activation in alcoholics. Increased expression of CD14 is found in Kupffer cells or whole livers of chronic ethanol-fed animals[25,90,91]. A correlation between CD14 expression and the severity of ALD has been reported in humans and it has been suggested that CD14 is one of risk factor in ethanol-induced pathology[92,93]. Interestingly, acute alcohol treatment also induces CD14 expression in whole liver cells[94] and CD14-deficient mice were protected from alcohol-induced liver steatosis[92].

LBP

LBP is an acute phase protein and is induced by LPS, IL-6, and IL-1β[95,96]. Although liver is a major source of LBP production, other organs, such as lungs, kidneys, and heart, also produce LBP[97]. This protein is present in normal serum; however, its levels become elevated during acute phase responses[98,99]. LBP catalyzes the transfer of LPS to CD14, and thus enhances the LPS-induced activation of monocytes, macrophages, and other immune cells[100]. Anti-LBP antibodies, together with LPS, protected the mice from death[101]. Neutralization of LBP protects the host from LPS-induced toxicity, suggesting its critical role in innate immunity[102].

In addition to its pro-inflammatory role, it also acts as an antiinflammatory, where it transfers LPS (Gram negative) or LTA (Gram positive) to HDL and other lipoproteins, and also aids the neutralization of LPS[103]. The antiinflammatory role of this protein is well described in various reports[99,104-107]. It is postulated that low concentrations of LBP enhance the LPS-induced activation of mononuclear cells, whereas the acute-phase rise in LBP concentrations inhibit LPS-induced immune cell activation[108].

Not much is known about the role of LBP in alcoholics, except one report where its role is described in early alcohol-induced liver injury where it enhances the production of cytokines, such as TNF-α. Ethanol fed LBP KO mice showed reduced TNF-α expression and reduced liver damage[109]. There was no change in endotoxin levels of both wild-type and LBP knockout mice; however, decreased steatosis in LBP knockout ethanol-fed mice was observed[109]. A potential antiinflammatory role of the above mentioned LBPs in the pathogenesis of ALD is yet to be explored.

In summary, it appears that LBPs and receptors modulate the LPS response bifunctionally, either by neutralizing or enhancing its response.

EFFECTS OF LPS ON THE LIVER

Activation of inflammatory cells in ALD

There is ample evidence for increased inflammatory

cascade activation in ALD[3]. Alcoholic steatohepatitis is characterized by infiltration of various inflammatory cells into the liver, including neutrophils, leukocytes, monocytes, and macrophages and this occurs as a result of chemokine activation (e.g. IL-8, MCP-1, and MIPs)[110-112]. In humans with alcoholic steatohepatitis, serum TNF-α, IL-6, and IL-8 levels are increased and there is also evidence for activation of circulating monocytes based on increased TNF-α production and increased NF-κB activation[113-115]. Serum levels and liver expression of these LPS-inducible pro-inflammatory cytokines are also increased in animal models of ALD[73]. Isolated Kupffer cells from mice and rats show increased production of TNF-α after chronic alcohol feeding[116] and this has been linked to increased TNF-α mRNA stability, as well as to upregulation of Erk, MAPK, and Egr-1 kinases[117]. While LPS has been proposed to play a major role in Kupffer cell and macrophage activation in ALD, *in vitro* studies in human monocytes/macrophages suggest that chronic alcohol exposure itself can promote a pro-inflammatory phenotype and amplify LPS-induced pro-inflammatory responses[71]. Our laboratory showed that increased LPS responsiveness after chronic alcohol exposure in monocytes is due to reduced expression of IRAK-M, which is a negative regulator of TLR4 activation[71]. Thus, chronic alcohol exposure alone not only results in pro-inflammatory activation of macrophages, but also sensitizes cells to LPS-induced pro-inflammatory signals[71].

Effects of endotoxin on the liver parenchymal and other non-immune cells in ALD

TLR4, the LPS receptor, is expressed in all cell types in the liver; thus, gut-derived endotoxin can modulate the function of all liver cells in ALD[3]. In hepatocytes, LPS can promote apoptosis, particularly in combination with other hepatotoxins[118,119]. TLR4 expression in hepatic stellate cells (HSC) has been shown to mediate inflammatory signaling by LPS and manifests in activation of Jnk kinase and NF-κB[120]. Oxidative stress induced by alcohol and its metabolites has also been shown to sensitize HSC to LPS-induce activation and subsequent induction of hepatic fibrosis[121,122]. Thus, LPS affects hepatocytes as well as HSC, both directly and *via* inflammatory cell activation.

ROLE OF GUT-LIVER AXIS IN OTHER LIVER DISEASES

The balance of gut microbial flora, intestinal permeability, hepatocyte function, and Kupffer cell activation appears to be critical in the maintenance of normal homeostasis (Figure 1). Indeed, increasing evidence suggests an importance for a gut-liver connection in different liver diseases where gut-derived LPS delivered to the liver through the portal circulation might play a role. For example, increased intestinal permeability was detected in patients with intrahepatic cholestasis of pregnancy[123], and in hepatitis C virus (HCV)-induced liver injury in human immunodeficiency virus infected individuals[124]. An increase in serum endotoxin levels was associated with pro-inflammatory activation of circulating monocytes in chronic HCV infection, even in the absence of cirrhosis[125]. These observations underscore the importance of the gut-liver axis in the pathogenesis of ALD, as well as in other types of liver injuries.

CONCLUSION

The gut-liver axis, particularly gut-derived endotoxin, seems to play a crucial role in the pathogenesis of liver diseases caused by various insults, including alcohol. However, the mechanisms and source of endotoxin in liver diseases are not fully understood. The importance of alcohol-induced alterations in the gut and the role of the liver in elimination of gut-derived pathogen-derived compounds require further investigation. Furthermore, interactions between immune, non-immune, and parenchymal cells, which take place *in vivo*, contribute and determine the progression of ALD. Understanding the role of TLR signaling and the cell-specific effects of gut-derived microbial products will provide new insights, not only into the pathomechanisms of ALD, but might also reveal new targets for therapeutic interventions.

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