COMMON PATHOGENIC MECHANISM IN DEVELOPMENT PROGRESSION OF LIVER INJURY CAUSED BY NON-ALCOHOLIC OR ALCOHOLIC STEATOHEPATITIS

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(Received September 7, 2007; Accepted September 10, 2007)

ABSTRACT — This review showed the common pathogenic mechanism in the development of non-alcoholic or alcoholic steatohepatitis. In particular, we describe the role of innate immune system and oxidative stress caused by gut-derived endotoxin. Gut-derived endotoxin plays an important role in alcoholic liver injury. It was reported that acute ethanol administration reduced activation of Kupffer cells. It is therefore possible that alcohol-induced hepatocellular damage occurs as a result of bacterial or endotoxin translocation under a reduction of the reticuloendothelial system (RES) function in alcoholic liver disease (ALD). On the other hand, recently, attention has been directed toward the effect of ethanol ingestion on Kupffer cell function, which is stimulated by gut-derived endotoxin via mechanisms dependent on increased gut permeability and the possible relationship between Kupffer cells and alcohol-induced liver injury. It is generally accepted that activation of the innate immune system and increased release of proinflammatory cytokines and other mediators plays an important role in the development of ALD. It was shown that Kupffer cells activation by endotoxin via Toll-like receptor (TLR-4) is involved in alcohol-induced liver injury and that ethanol-induced oxidative stress is important in the regulation of transcription factor NF-κB activation and that cytokine production by Kupffer cells. TNF-α and free radicals are produced in early alcohol-induced liver injury. In support of this finding, the pathology caused by alcohol was blocked nearly completely in TNF-α receptor 1. Many pathways have been suggested to contribute to the ability of ethanol to induce a state of oxidative stress. One central pathway appears to be the induction of the CYP2E1 form of cytochrome P450 enzymes by ethanol. Initial efforts to clarify the mechanisms that promote the progression from steatosis to steatohepatitis somewhat artificially divides disease mechanisms into “first and second” hit. The best candidates for these second hits were considered to be oxidative stress (CYP2E1 induction) and associated lipid peroxidation and cytokines, principally, TNF-α. Some of the most definitive data on the importance of the innate immune system or oxidative stress in the pathogenesis of liver disease come from studies of alcoholic and non-alcoholic steatohepatitis in animals.

KEY WORDS: Alcoholic steatohepatitis, Non-alcoholic steatohepatitis, Gut-derived endotoxin, Innate immune system, Oxidative stress, CYP2E1

INTRODUCTION

Alcoholic liver disease (ALD) progresses through several stages of tissue damage and liver dysfunction. Alcoholic hepatitis and other forms of ALD are major complications of chronic excessive ethanol intake. Therefore, long-term excessive consumption of alcohol can result in a spectrum of liver abnormalities, ranging from simple fatty liver (steatosis) or fatty liver accompanied by inflammation (steatohepatitis) to scar tissue formation (fibrosis), the destruction of the normal liver structure (cirrhosis), and even liver cancer. However, the mechanisms underlying alcohol-induced hepatotoxicity are complex and remain to be poorly understood.

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understood. One of the early stages, alcohol-induced steatohepatitis, is characterized by the accumulation of fat molecules in the liver tissue, accompanied by the migration into the liver of cells associated with inflammation processes. More recent experimental and clinical data suggest that a stimulation of the innate immune system plays a key role in the development and progression of ALD (Nagy, 2003; Bode and Bode, 2005). Interestingly, numerous anecdotal reports point to an association between episodes of gastrointestinal bleeding or sepsis and an acute, clinical decompensation of ALD, suggesting that endotoxia potentiates the progression of the disease. Gut-derived, endotoxin-mediated hepatocellular damage has been postulated to play a crucial role in the pathogenesis of alcohol-induced liver injury in rodents.

Spillover-endotoxia from the gastrointestinal tract is an important factor in the relationship between endotoxin and hepatotoxicity. Endotoxins, one of the components of the outer wall of gram-negative bacteria, were initially believed to be detoxified in the reticuloendothelial system (RES), particularly in the liver’s Kupffer cells. It has been known that acute ethanol administration reduced activation of Kupffer cells. On the other hand, activation of Kupffer cells by gut-derived endotoxin plays a pivotal role in alcoholic liver injury. Macrophages stimulated by microorganisms or their toxins induce a variety of biologically active mediators known as cytokines, and tumor necrosis factor (TNF-α) is recognized as an important mediator in the development of endotoxia. This mediator is responsible, at least in part, for a number of pathophysiological responses in the liver, including the acute phase response, hyperlipidemia, free oxygen radical formation, fibrogenesis and cholestasis (Fiers, 1991; Camussi et al., 1991).

In recent years, a significant role for proinflammatory cytokines such as TNF-α in the onset of liver disease has been indicated both by clinical observations of an enhanced circulating level of TNF-α and other cytokines in patients with ALD, and by results of studies with animal models of alcohol-induced liver damage (Bode and Bode, 2005; McClain et al., 2002; Tilg and Diehl, 2000). In fact, there is good evidence that ethanol treatment changes the response of Kupffer cells and monocytes to endotoxin, resulting in an enhanced production of TNF-α and other cytokines (McClain et al., 1999). On the other hand, Immune host-bacteria interactions have also begun to be better characterized. It was shown that innate immune cells recognize conserved molecular patterns associated with pathogen through pattern recognized with receptors, among which the family toll-like receptors (TLRs) occupies an important place (Akira et al., 2001). Endotoxins induced production of TNF-α by Kupffer cells via TLR-4 and contribute to liver injury (Uesugi et al., 2001).

A recognized mechanism of ethanol action is its ability to enhance oxidative stress. Ethanol is metabolized to acetaldehyde through alcohol dehydrogenase (ADH) in cytosol, through cytochrome P450 2E1 (CYP2E1) in the microsomes, and through catalase in the peroxisomes (Lieber, 1997). In addition, ADH-mediated ethanol metabolism generates the reduced form of nicotinamide adenine dinucleotide (NADH), which promotes steatosis by stimulating the synthesis of fatty acid and opposing their oxidation. All of the rodent models of ALD are characterized by significantly increased expression of the major alcohol-metabolizing CYP2E1 and it has been suggested that oxidative metabolism of ethanol by CYP2E1 contributes significantly to the development of ALD through generation of free radical reactive oxygen species (ROS) (Lieber, 1997, 2004; Cederbaum, 2003; Wu and Cederbaum, 2005). The most common cause for CYP2E1 induced is early alcoholic liver injury, such as alcoholic steatosis and alcoholic steatohepatitis. CYP2E1, however, is also induced in non-alcoholic steatohepatitis (NASH), which can be understood on the basis of the physiologic role of CYP2E1 (Lieber, 1997, 2004).

Understanding the biochemical and toxicological properties of CYP2E1 is important for many reasons, even besides the role in contributing to alcohol-induced liver injury since CYP2E1 is induced under a variety of pathophysiological conditions, such as fasting, diabetes, obesity, and high-fat diet. Non-alcoholic fatty liver disease (NAFLD) is a condition with a wide spectrum of liver damage including steatosis, which may have the best prognosis in spite of having the potential to progress to steatohepatitis, with or without fibrosis and cirrhosis (Angulo, 2002). On the other hand, NASH is an important type of liver disease because it is an intermediary stage of liver pathology that develops as fatty liver disease progresses to cirrhosis and hepatocellular carcinoma. The pathological picture resembles that of alcohol-induced liver injury, but it occurs in patients who do not abuse alcohol. In humans, NASH is associated with the metabolic syndrome, i.e., obesity, diabetes, dyslipidemia and insulin resistance.

As mentioned previously, the importance of
cytokines as effector molecules in liver damage has been particularly well demonstrated in patients and animals with alcoholic or non-alcoholic liver diseases ranging from steatosis to cirrhosis. In combination with data from clinical studies, these findings indicate that TNF-α mediates not only the early stages of fatty liver disease but also the transition to more advanced stages of liver damage. Although there have been several excellent reviews of innate immune system in ALD or NASH (Diehl, 2005; Cortez-Pinto et al., 2006; McClain et al., 2002; Wheeler, 2003; Bode and Bode, 2005), in this review we in contrast, put forward the concept of a role for gut-derived bacterial toxins, innate immune system and oxidative stress in common pathogenic mechanism in ALD or NASH.

Role of gut-derived endotoxin in ALD
1. Gut-derived endotoxin and Kupffer cells depression in ALD

The endotoxin, lipopolysaccharide, elicits various responses in the host, involving hemodynamic, cardiovascular, immunologic and metabolic mechanisms. Most administered endotoxin is located in cells of the RES in animals, particularly in Kupffer cells and splenic macrophages. The ability of Kupffer cells to eliminate and detoxify various exogenous and endogenous substances (e.g., endotoxin) is an important physiological regulatory function. Several observations support the hypothesis that Kupffer cells are involved in hepatic injury caused by ethanol. Chronic alcohol abuse impairs the phagocytic activity of the hepatic RES and depresses the clearance of gut-derived endotoxin by Kupffer cells (Wheeler, 2003; Bode and Bode, 2005). Several authors have suggested that gut bacteria play a crucial role in the development of ALD. Not only inactivation of RES function, which reduces clearances of endotoxin, but also an increase in absorption of endotoxin from the intestine may be involved in mechanisms of ethanol-induced endotoxemia. Consumption of large doses of alcoholic beverages increases the risk to develop hemorrhagic erosions in the stomach both in experimental animals and in humans.

Several mechanisms may underlie the significant increase in endotoxin levels in the bloodstream following chronic alcohol use. In addition, increased absorption of endotoxin from the intestine plays a role in ALD (Adach et al., 1994). Alcohol ingestion disrupts gastrointestinal barrier function and subsequently induces the diffusion of luminal bacterial products into the portal blood. The damaging effect of alcohol on the mucosa of the upper gastrointestinal tract is associated with an enhanced permeability of the gut mucosa. On the other hand, direct evidence of increased translocation of endotoxin across the gut mucosa caused by ethanol was obtained in experiments using rat (Mathurin et al., 2000). They have been demonstrated in a pronounced increase in translocation of endotoxin from the gut lumen into the portal blood caused by alcohol. Acute ethanol ingestion, especially at high concentrations, facilitates the absorption of endotoxin from rat small intestine via an increase in intestinal permeability, which may play an important role in endotoxemia observed in alcoholic liver injury (Tamai et al., 2000). When ethanol-fed rats received endotoxin by constant infusion into a peripheral vein, ALD was not potentiated despite markedly increased plasma endotoxin levels, suggesting the development of tolerance to endotoxin in these animals (Jarvelainen et al., 1999).

Bacterial translocation from the gastrointestinal tract, i.e., spillover endotoxemia, is important in the relationship between endotoxin and hepatotoxicity, since endotoxin clearance may be due to rapid uptake in the RES, especially by Kupffer cells in the liver. We have previously reported (Sakaguchi et al., 1982) that the blockade of RES by lead acetate (an RES depressor agent) markedly enhances endotoxin sensitivity on superoxide generation. The early works by Rush et al. (1988) or Deith et al. (1989) reported the increased permeability of the gut under shock conditions, with the spreading of endotoxin or bacteria into the circulating blood and translocation into other organs, and they emphasized the role of the gut barrier failure. Bacterial translocation induced with hemorrhagic shock is an etiologic factor in the pathogenesis of multiple organ failure. In a recent study, Mori et al. (2005) suggested that the lipid peroxidation of intestinal neutrophils is involved in bacterial translocation during hemorrhagic shock and that a free radical scavenger, edaravone, is potentially useful in diminishing bacterial translocation after hemorrhagic shock. The results of these and other experimental studies strongly suggest that increased concentrations of endotoxin in the portal blood are essential for initiation and progression of ALD (Bode and Bode, 2005). It is therefore possible that alcohol-induced hepatocellular damage occurs as a result of bacterial or endotoxin translocation under a reduction of RES function in ALD.

2. Activation of Kupffer cells by gut-derived endotoxin in ALD

As noted above, gut-derived endotoxin plays an
important role in alcoholic liver injury. Attention has been directed towards the effect of ethanol ingestion on Kupffer cells function, which is stimulated by gut-derived endotoxin via mechanisms dependent on increased gut permeability and the possible relationship between Kupffer cells and alcohol-induced liver injury. As reviewed by Wheeler (2003), when activated, Kupffer cells produce signaling molecules (i.e., cytokines) that promote inflammatory reactions as well as molecules called ROS, which can damage liver cells (see Fig. 1). Actually previous works have shown that activation of Kupffer cells are involved in alcohol-induced liver injury, that plasma endotoxin levels increased in rats on the Tsukamoto-French protocol,

![Diagram](https://via.placeholder.com/150)

**Fig. 1.** Role of Kupffer cells and gut-derived endotoxin in alcoholic liver injury. It was shown that acute ethanol administration reduced activation of Kupffer cells. On the other hand, activation of Kupffer cells by gut-derived endotoxin plays a pivotal role in alcoholic liver injury. Following chronic alcohol ingestion, endotoxin released from certain intestinal bacteria moves from gut into the bloodstream and into liver. Endotoxin, also called lipopolysaccharide (LPS), in association with soluble LPS-binding protein (LBP), interacts with a receptor complex consisting of the mCD14 (membrane-associated CD14) and Toll-like receptor-4 (TLR-4). This interaction causes the production of the regulatory nuclear factor kappa B (NF-κB), which in turn leads to the generation of significant amount of cytotoxic factors, namely free radicals (O$_2^-$ etc) and various cytokines, most prominently TNF-α. TNF-α has been shown to be an essential factor in the injury to primary liver cells (i.e., hepatocytes) associated with alcoholic liver disease (ALD).
Common pathogenic mechanism of non-alcoholic or alcoholic steatohepatitis.

and that levels correlated well with pathology (Nanji et al., 1993; Thurman, 1998). Activation of Kupffer cells results from exposure to endotoxin, which derives from the cell wall material of Gram-negative bacteria in the gut. An elevated circulating endotoxin level has been detected in heavy drinkers, as well as in experimental animals after chronic ethanol administration. Following the ingestion of ethanol, significant alterations occur in RES function which results in increased host susceptibility to infection. Kupffer cells comprise the largest resident macrophage population within the RES and this tissue-fixed macrophage population is a key component of the inflammatory response and elaborates many mediators that initiate, perpetuate and modulate this response. The macrophages, when stimulated with endotoxin, release numerous cytokines. The increased release of pro-inflammatory mediators (e.g., TNF-α, interleukin (IL)-1, IL-6, reacting oxygen species) and infiltration of other inflammatory cells (e.g., neutrophils) finally cause liver damage. Among the various cytokines, the proinflammatory cytokine TNF-α has emerged as a key factor in various aspects of liver disease. Activation of Kupffer cells is a prominent event in the initiation of ALD. During the past decade increasing evidence has accumulated that TNF-α is important in the development of ALD (Bode and Bode, 2005). Patients with alcoholic hepatitis (AH) have elevated serum TNF-α levels, and the elevated serum TNF-α levels have been correlated with poor prognosis in AH. This assumption was supported by the finding that antibodies against TNF-α attenuate alcohol-induced liver injury in rats. Direct evidence for a central role of TNF-α in the pathogenesis of ALD stems from experiments using TNF-α receptor 1 (TNF-α-R1) and TNF-α receptor 2 (TNF-α-R2) knockout mice (Yin et al., 1999). Thurman's laboratory suggested that long-term ethanol feeding caused liver injury in wild-type and TNF-α-R2 knockout mice but not in TNF-α-R1 knockout mice, providing solid evidence in support of the hypothesis that TNF-α plays an important role in the development of early alcohol-induced liver injury via the TNF-α-R1 pathway (Yin et al., 1999). On the other hand, the increased serum TNF-α concentration in alcoholics reported by Stahneke et al. (1991) is consistent with the idea that Kupffer cells of patients with ALD are activated, because TNF-α is produced exclusively by the monocyte-macrophage lineage, which is mostly made up of Kupffer cells.

The hypothesis suggested that alcoholic liver injury may contribute to the extent of the TNF-α release triggered by gut-derived endotoxin in ALD. It was concluded that these results from some experiments strongly support the hypothesis that increased TNF-α release from activated Kupffer cells plays an important role in the development of ALD.

3. Kupffer cells activity by gut-derived endotoxin via CD14 or Toll-like receptor (TLR)-4 in ALD

Activation of macrophages, including Kupffer cells, by low concentration of endotoxin (pg/ml to ng/ml range) depends on the expression of CD14 receptor. This receptor exists in two forms, membrane-associated CD14 (mCD14) and soluble CD14 (sCD14). CD14 recognizes extremely low dose of endotoxin (LPS) in the presence of LPS binding protein (LBP), an acute-phase reactant produced from hepatocytes. CD14 alone, however, is unable to transduce intracellular signaling, since CD14 is only tethered to cytoplasmic membrane by a glycosyl phosphatidylinositol (GPI) anchor. Therefore, researchers have postulated that endotoxin also must interact with another receptor which transmits a signal into and within the Kupffer cells. Indeed, investigators recently identified several molecules called TLRs that serve as coreceptors for endotoxin, with different TLR proteins interacting with endotoxin from different bacteria (Beutler and Rietschel, 2003). To date, at least ten TLRs (TLR1-10) have been discovered; among these, TLR-2 and TLR-4 have been shown to act as transmembrane receptors for endotoxin, which coordinately function with CD14 in terms of the cellular response to endotoxin. In fact, a defective murine TLR-4 is responsible for the endotoxin-hyposensitivity in two mouse strains C3H/HeJ and C57BL10ScCr (Poltorak et al., 1998). Endotoxin has long been known to bind to an LBP in the plasma, with LBP also being a hepatic acute-phase reactant (McClain et al., 2002; Bode and Bode, 2005). Furthermore, it has been reported that TLR-4 is essential for the activation of Kupffer cells by endotoxin (Su et al., 2000). Thus far, endotoxin toxicity and TNF-α production seem to be closely related to TLR-4 receptor density, and mutants-lacking functional TLR-4 are resistant to endotoxin toxicity and lethality, and do not produce TNF-α in response to endotoxin (Beutler, 2000). The receptor most relevant to endotoxin-induced ALD is TLR-4.

Animal models of ALD demonstrate increased LBP and CD14 (Su et al., 1998), and patients with alcoholic hepatitis have increased serum LBP levels. Thurman and colleagues have shown that elevated endotoxin after given ethanol triggers more activation...
of Kupffer cells via enhanced CD14 expression in female rats (Kono et al., 2000). It is noteworthy that nuclear factor kappa B (NF-κB) is activated in this process, leading to increases in TNF-α mRNA expression in the liver and more severe liver injury in females. Furthermore, increases of LBP and CD14 mRNAs occur after only 24 to 48 hr of ethanol feeding to rat, supporting the suggestion that these binding proteins may play a role in the liver damage instead of merely being a consequence of liver injury (Lukkari et al., 1999). On the other hand, LBP plays an important role in early alcohol-induced liver injury by enhancing endotoxin-induced signal transduction, most likely in Kupffer cells (Uesugi et al., 2002). In addition, Uesugi et al. (2001) showed that C3H/HeJ strains that carry a mutation in the TOLL/interleukin 1 receptor domain of the TLR-4 gene develop less severe early alcohol-induced liver injury than wild-type mice. In their animal model, the expression of functional TLR-4 is essential for alcohol-induced expression of TNF-α in the liver, which is responsible for hepatocyte injury via TNF-α receptor 1 (p55). Other studies using animals in which the genes for CD14 were removed or which lacked LBP demonstrated that signaling processes mediated by endotoxin receptor were critical to the development of liver disease associated with chronic alcohol administration (Yin et al., 2001a).

These reports have shown that mice lacking CD14 and mice lacking functional TLR-4 are protected against early alcohol-induced hepatotoxicity. It is, therefore, of interest that activation of Kupffer cells by gut-derived endotoxin may occur via CD14 or TLR-4 pathways in ALD (see Fig.1, Fig.2).

**Role of TNF-α in alcohol-induced steatohepatitis**

Cytokines are pleiotropic regulatory peptides that can be produced by virtually every nucleated cell in the body, including most types of liver cells. Among the various cytokines, the proinflammatory cytokine TNF-α in the pathogenesis of liver disease comes from stud-

![Fig. 2](image-url). Gut-derived endotoxin interactions with monocytes and the Toll-signaling pathway in acute ethanol administration. LPS, lipopolysaccharide; LBP, LPS binding protein; TLR4, Toll-like receptor-4; NF-κB, nuclear factor kappa B; ALD, alcoholic liver disease.
ies of alcoholic and nonalcoholic steatohepatitis in animals. In combination with data from clinical studies, these findings indicate that TNF-α mediates not only the early stage of fatty liver disease but also the transition to more advanced stage of liver damage (Tiilg and Diehl, 2000). On the other hand, close temporal associations among Kupffer cells activation, increased transcription of the genes for TNF-α and related cytokines, hepatic inflammation, and liver-cell death have been reported in rodents with alcohol-induced steatohepatitis. It is of interest that pathology caused by alcohol was blocked nearly completely in TNF-α receptor 1 (Yin et al., 1999). In this animal model, it was also shown that free radicals act as redox signals for TNF-α production and not directly damage cells in early alcohol-induced hepatic injury (Yin et al., 2001b).

Like chronic ethanol feeding, TNF-α cytotoxicity is also associated with alterations of mitochondrial function. The mitochondria of TNF-α-exposed cells overproduce ROS derived from the respiratory chain. The mitochondria themselves then become the targets of ROS, thus setting up a cycle of injury. In addition to ROS production, TNF-α prompts the opening of the mitochondrial permeability transition (MPT). The MPT is the regulatable opening of a large, nonspecific pore across the outer and inner mitochondrial membrane. Interestingly, ethanol may also increase the susceptibility to MPT induction by TNF-α at the mitochondrial level, possibly through an increase in ROS production caused by respiratory chain dysfunction and/or CYP2E1 (Pastorino and Hoek, 2000).

1. TNF-α and oxidative stress in ALD: synergism between endogenously produced endotoxin and TNF-α

As mentioned above, in recent years most authors agree that an activation of the innate immune system is a key event in initiating and progression of ALD beyond the stage of fatty liver (Bode and Bode, 2005; Tiilg and Diehl, 2000; Thurman, 1998). The research findings from McClain’s laboratory have shown that activation of monocytes and macrophages with subsequent proinflammatory cytokine production plays an important role in certain metabolic complications of ALD and is a component of the liver injury of ALD (McClain et al., 2002). Important elements of the innate immune system are phagocytes such as macrophages and neutrophils, and macrophage-derived soluble mediators. The translocation of bacterial products from the intestinal lumen to the mesenteric circulation and its lymphatics induces regional and systemic production of TNF-α, and other proinflammatory cytokines. Increased circulating levels of cytokines have been postulated to cause many of the metabolic and nutritional abnormalities observed in ALD, especially in alcoholic hepatitis, and in more decompensated liver disease (McClain et al., 1999).

The importance of ROS in the development of ALD is well documented. As mentioned earlier, one consequence of Kupffer cells activation is the production of ROS, particularly superoxide, which in large amounts can lead to oxidative stress. Results of several studies suggest that the oxidative stress associated with chronic alcohol consumption is largely attributable to endotoxin-induced activation of Kupffer cells (Kono et al., 2000; Yin et al., 2001a, 2001b). This hypothesis expanded the current assumption that alcohol-associated oxidative stress results primarily from the degradation of alcohol in the liver by a monooxygenase system called CYP 2E1. Oxidative stress promotes inflammation, which is aggravated by an increase of the proinflammatory cytokine TNF-α in the Kupffer cells. TNF-α administered intravenously with nanogram quantities of the endotoxin has been reported to cause lethal shock, and it appears that TNF-α and endogenously produced endotoxin act synergistically in activating the complement system, which plays an important role in mediating tissue injury and lethality (Hsueh et al., 1990; Leist et al., 1997; Sakaguchi and Fusuraya, 2006). In addition, we have reported that the oxidative stress caused by TNF-α occurs as an enhancing effect of endotoxin or by bacterial translocation from the intestinal gut under reduction of RES function in various disease states, and that the effect of TNF-α may cause a marked increase of toxicity of oxidative stress by endotoxin (Sakaguchi and Fusuraya, 2006; Sakaguchi et al., 1996). In support of our findings, previous studies (Sakaguchi et al., 2000) have indicated that endogenously produced endotoxin may contribute to the extent of TNF-α-hypersensitivity caused by D-galactosamine. Several experiments using a variety of antioxidants and inhibitors of ROS-producing enzymes have explored the relationship of superoxide generation and oxidative stress to alcohol-induced liver injury (Kono et al., 2001; McKim et al., 2002). Moreover, in vitro and in vivo antioxidant supplementation has been shown to inhibit monocyte NF-κB activation and proinflammatory cytokine production. Thus, inadequate levels of antioxidants may play a role in monocyte activation, and antioxidant therapy may represent a target for therapeutic intervention in ALD (Hill et al., 2000). Interestingly, in most studies using antioxi-
The alcohol-induced production of TNF-α was also reduced, suggesting that oxidative stress promotes TNF-α production. In addition, it was shown that Kupffer cells activation by endotoxin via TLR-4 is involved in alcohol-induced liver injury and alcohol-induced oxidant stress is important in the regulation of transcription factor, NF-κB, activation and cytokine production by Kupffer cells (MaClain et al., 2002). This supports the suggestion that oxidative stress-sensitive transcription factor such as NF-κB may be potential sites of intervention in the treatment of inflammatory diseases, including AH (Hill et al., 2000). Moreover, study findings from McClain’s laboratory have shown that intravenous administration of the glutathione prodrug procystein decreases cytokine production in stable alcoholic cirrhosis (Pena et al., 1999). Recently, Yamashina et al. (2005) has shown that IL-1 receptor-associated kinase (IRAK), one of the signaling molecules of TLR-4, regulates tolerance and sensitization to endotoxin and acute alcohol increases in IRAK expression through a mechanism dependent upon oxidant production. Further, NADPH oxidase plays a pivotal role in the increase in IRAK expression due to ethanol via activation of NF-κB signaling. These data indicate that acute ethanol can cause sensitization to endotoxin through mechanisms dependent upon oxidative stress. It is therefore possible that TNF-α production is occurring as a result of bacterial or endotoxin translocation from the intestinal gut under conditions of reduced RES function in ALD. It is generally accepted that activation of pro-inflammatory cytokines and other mediators play an important role in the development of ALD.

Oxidative stress and CYP2E1 in ALD

The modifications induced by the apolar side residues of membrane phosphoglycerides by active oxygen generation are thought to bring about structural alterations in the membrane. Biomembranes and subcellular organelles are therefore the major sites of lipid peroxide damage. These peroxidative metabolic pathways are linked by a cytoplasmic glutathione (GSH) shuttle system, namely NAD(P)H oxidase and GSH peroxidase (GSH-Px). Furthermore, superoxide dismutase (SOD) may play an important role in protecting cells or tissues against the toxic effects of these superoxide radicals.

Interestingly, chronic ethanol consumption increases the content of CYP2E1 in the liver. Progress in the understanding of the pathogenesis of alcoholic liver disease was achieved when it was discovered that alcohol affects the liver through not only nutritional disturbances but also its direct toxicity because of its predominant metabolism in the liver associated with oxidation-reduction (redox) changes and oxidative stress. Redox changes are mediated by ADH, and oxidative stress is generated mainly by the activity of the microsomal ethanol oxidizing system (MEOS) and its key enzyme CYP2E1, which releases free radicals (Lieber, 2004). Despite the discovery of CYP2E1 and its prevailing role in microsomal ethanol oxidation, the term MEOS was maintained because cytochromes P450 other than CYP2E1 (such as CYP1A2 and CYP3A4) (Salmela et al., 1998) may also contribute to ethanol metabolism in liver microsomes. Thus, the term MEOS characterizes total microsomal ethanol oxidation, not only catalyzed by CYP2E1. Ethanol can enhance ROS formation through induction of CYP2E1 in the liver and in the brain. Further oxidation of acetaldehyde to acetate is accompanied by the generation of ROS.

Wu and Cederbaum (2003) described that the linkage between CYP2E1-dependent oxidative stress, stellate cell activation, mitochondrial injury and GSH homeostasis contributes to the toxic action of ethanol on the liver (see Fig.3). CYP2E1 has a very high NADPH oxidase activity and extensively produces $O_2^-$ and $H_2O_2$ as well as hydroxethyl radicals that are likely to be responsible for oxidative stress and lipid peroxidation by ethanol induction. It is of interest that some of the more stable CYP2E1-derived reactive species are diffusible and are postulated to exit the hepatocyte and interact with other liver cells such as hepatic stellate cells to initiate a fibrotic response. This free radical formation in biological systems in the presence of ethanol has been detected by spin trapping techniques (Reinke, 2002). Deterioration of liver mitochondrial function after chronic ethanol treatment has been well documented. For example, the acetaldehyde produced by the oxidation of ethanol has toxic effects, inhibiting the repair of alkylated nucleoproteins, decreasing the activity of key enzymes, and markedly reducing oxygen use in mitochondria damaged by long-term ethanol consumption. Ethanol can also induce lesions between complex I and complex III of the mitochondrial electron transport chain, and enhance superoxide anion production (Bailey et al., 1999). It is noteworthy that the ethanol-induced oxidative damage on the mitochondrial proteins could not only diminish mitochondrial function, but also promote ROS because of loss of protein activity in the mitochondrial electron respiratory chain. In addition,
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Ethanol treatment induces changes in mitochondrial membrane structure that have been reported to account for defective mitochondrial uptake of GSH and a decline in its defense capacity against oxidative stress (Femandez-Cheea et al., 1997). It is therefore indicated that ethanol-induced oxidative stress is the result of the combined impairment of antioxidant defense and the production of reactive oxygen species by the mito-

\[ \text{Ethanol} \rightarrow \text{CYP2E1} \]

\[ \text{O}_2 \rightarrow \text{NADPH oxidase} \]

Superoxide generation

SOD

Hydrogen peroxide

Hepatic stellate cells

Fibrotic response

Fe\(^{2+}\) Fenton reaction

Hydroxyl radical

Oxidant injury

GSH ↓

Lipid peroxide formation

Plasma membrane damage

Mitochondrion injury

\( \{ \text{ATP ↓} \}

\{ \text{MMP ↓} \}

\]

Cell injury

Fig. 3. CYP2E1-dependent oxidant stress and toxicity in alcohol liver injury. Ethanol elevates CYP2E1 protein and activity by stabilizing the enzyme against proteasome-mediated degradation. CYP2E1, a loosely coupled enzyme, generates reactive oxygen species during its catalytic cycle. In the presence of iron, which is increased after ethanol treatment, more powerful oxidants including hydroxyl radical. Lipid peroxide formations are postulated to be key mediators of the CYP2E1 toxicity and mitochondrial injury. MMP; mitochondrial membrane potential.
chondrial electron transport chain, the alcohol-induced CYP2E1 and activated phagocytes (Albano, 2006). Indirectly, chronic ethanol may augment oxidative stress by decreasing antioxidant defenses such as reducing GSH-Px and GSH homeostasis. Supplementation of antioxidants has been shown to prevent ethanol-induced injury in liver, brain, heart and skeletal muscle (Mansouri et al., 2001). The end-products of the peroxidation of polyunsaturated fatty acids, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), are used as markers to assess ROS-induced lipid peroxidation. Several other studies have suggested that ethanol may cause tissue damage through lipid peroxidation (Ohhira et al., 1998). Besides being specific markers of lipid peroxidation, both MDA and HNE have been implicated as mediators of diverse biologic effects (Parola et al., 1999) and may play roles in the pathogenesis of alcoholic myopathy and liver disease. Oxidation of ethanol through ADH pathway produces acetaldehyde, which is converted to acetate. Both reactions reduce nicotinamide adenine dinucleotide (NAD) to its reduced form NADH. In this animal model, it was also shown that, in addition to metabolic abnormalities caused by ADH activity, a new pathway of ethanol metabolism, namely MEOS, plays a key role in the progression of the disease. It is likely that several mechanisms contribute to alcohol-induced liver injury, the linkage between CYP2E1-dependent oxidative stress, mitochondrial injury, stellate cell activation, and GSH homeostasis may contribute to the toxic action of ethanol on the liver.

Enhanced concentration of both hepatic CYP2E1 protein and mRNA was found in actively drinking patients. It is suggested that the most common cause for CYP2E1 induction is early alcoholic liver injury, such as alcoholic steatosis and alcoholic steatohepatitis (Lieber, 1997, 2004). In addition, as reviewed by Lieber (2004), liver disease in the alcoholic is due not only to malnutrition but also to ethanol’s hepatotoxicity linked to its metabolism by means of the ADH and CYP2E1 pathways and the resulting production of toxic acetaldehyde. Ethanol, its metabolites arising during its metabolic degradation as well as novel compounds formed via ethanol-induced oxidative stress, especially during the action of the ethanol inducible microsomal CYP2E1, may apart from direct damage to biological structures affect signal transduction pathways thus modulating and potentiating damage (Zima and Kalousova, 2005). As noted above, induction of CYP2E1 by ethanol may play a role in the mechanisms by which ethanol generates a state of oxidative stress and in mechanism responsible for alcoholic liver injury, although this is currently controversial (Lieber, 1997, 2004; Cederbaum, 2003; Wu and Cederbaum, 2005). To try to understand basic effects and actions of CYP2E1, cell lines that constitutively express human CYP2E1 were developed. HepG2 cells are a human hepatoblastoma cell line which maintains several liver functions but does not express significant amounts of CYP2E1. HepG2 cells lines, which overexpress CYP2E1, were established either by retroviral infection methods (MV2E1-9 cells, or E9 cells) or by plasmid transfection methods (E47 cells). These cell lines have been used to study CYP2E1-catalyzed toxicity of alcohol-induced liver injury. It seems that HepG2 cell lines overexpressing CYP2E1 may be a valuable model to characterize the biochemical and toxicological properties of CYP2E1.

Pathogenesis of NAFLD and NASH

The difficulties in differentiating between NASH and alcoholic steatohepatitis at the histological level, the recognition that specific underlying disorders are associated with NAFLD and NASH, the acceptance that the criteria used for excluding use of alcohol are not strictly defined and the possible concurrence of steatohepatitis with other forms of chronic liver disease, have led hepatopathologists and clinicians alike to reconsideration of the term “non-alcoholic steatohepatitis” (Cortez-Pinto et al., 2006). The term steatohepatitis followed by the underlying clinical condition if provided (i.e. diabetes, obesity, hyperlipidemia, etc.) may be more appropriate to use in histopathological diagnosis. Recently, there have been several excellent reviews (Angulo, 2002; Diehl, 2005; Cortez-Pinto et al., 2006) of pathogenesis in NAFLD and NASH. NAFLD is an increasingly recognized liver disease is an progress to end-stage liver disease. Frequently associated with obesity, non-insulin-dependent diabetes mellitus, and hyperlipidemia, NAFLD is related to insulin resistance and oxidative stress as critical pathogenic factor. Similarly NASH is intimately related to the insulin resistance syndrome, a constellation of disorders that result from abnormal production of hormones and cytokines that regulate inflammatory responses. The pathological picture resembles that of alcohol-induced liver injury, but it occurs in patients who do not abuse alcohol. NAFLD is becoming the preferred term, and it refers to a wide spectrum of liver damage, ranging from steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. Steatohepatitis (NASH) represent only a stage within the spectrum of NAFLD. NAFLD should be differenti-
Common pathogenic mechanism of non-alcoholic or alcoholic steatohepatitis.

1. Progression from NAFLD to NASH

Understanding the mechanisms that lead to the progression from steatosis to advanced disease is clearly important to inform the rational design of treatment strategies directed at those who have developed progressive disease. Initial efforts to clarify the mechanisms that promote the progression from simple hepatic steatosis to steatohepatitis somewhat artificially divides disease mechanisms into “first and second hit” (Day and James, 1998). This model considered the development of steatosis to be the “first hit” increasing the sensitivity of liver to the putative “second hit” leading to hepatocyte injury, inflammation and fibrosis. The best candidates for these “second hits” were considered to be oxidative stress, associated CYP2E1 induction and cytokines, principally, TNF-α (see Fig.4). Rather, initial and subsequent mechanisms blend into each other. For example, the “second hit” that drives progression to steatohepatitis may be ongoing in individuals with steatosis, but these individuals are capable of compensating for the stress, thereby limiting disease progression (Diehl, 2005).

2. Oxidative stress (CYP2E1 induction) as common mechanism of alcoholic or non-alcoholic steatohepatitis progression

In any event, the induction of CYP2E1 has been shown to play a key role in the pathogenesis of alcoholic liver, including alcoholic steatohepatitis, because of the oxidative stress it generates (Lieber, 1997). On the other hand, NASH caused steatosis, liver cell injury, inflammation and variable necrosis. NASH is associated with obesity, type 2 diabetes, and hyperlipidemia, conditions in which CYP2E1 is induced. As noted above, although the pathogenesis of NAFLD and NASH has not yet been elucidated, a popular mechanism is the two “hit” theory (Day and James, 1998), with the ‘first hit’ being the accumulation, by several causes, of fatty acids in the liver. The ‘second hit’ is the peroxidation of these fatty acids because of the oxidative stress production by different factors such as CYP2E1 induction. Most of the available data from animal models and patients with NAFLD suggest that mitochondria are the most important intracellular source of ROS in NASH (Pessayre and Fromenty, 2005). Indeed, the oxidative stress caused by CYP2E1 induction and mitochondrial injury results in lipid per-

First hit

Accumulation of fat

Insulin resistance

Normal liver

Diabetes

Obesity

Hyperlipidemia

Second hit

Gut-derived endotoxin

(TNF-α etc production)

Oxidative stress

(CYP2E1 etc induction)

NAFLD (steatosis)

NASH

Fig. 4. Progression of NASH by “two hit theory”. This model considered the development of simple hepatic steatosis to be the “first hit” increasing the sensitivity of liver to the putative “second hit” leading to hepatocyte injury. NAFLD, non-alcoholic fatty disease; NASH, non-alcoholic steatohepatitis; CYP2E1, cytochrome P450 2E1.
oxidation and membrane damage. In several excellent reviews findings from Lieber (1997, 2004), the damage caused by oxidative stress in both alcoholic and non-alcoholic steatohepatitis induces mitochondrial injury, which, in turn, exacerbates the oxidative stress. That mitochondrial damage is a key component of alcoholic liver injury is well established with mitochondrial structural defects, whereas NAFLD is not. This mitochondrial dysfunction contributes to the oxidative stress in NASH. In vitro studies conducted in CYP2E1-overexpressing hepatocyte cell lines indicate potential links between CYP2E1-dependent oxidative stress, GSH homeostasis, and mitochondrial damage leading to cell death. It is therefore of interest that hepatic CYP2E1 is consistently upregulated in both clinical and experimental NASH and that evidence of both oxidative stress and mitochondrial injury can be found (Lieber, 2004; Pessayre and Fromenty, 2005). CYP2E1 is invariably elevated in the liver of patients with NASH because fatty acids (which increase in obesity) and ketones (which increase in diabetes) are also substrates for CYP2E1; their excess up-regulates CYP2E1. CYP2E1 leaks oxygen radicals as part of its operation, and when they exceed the cellular defense systems they result in oxidative stress with its pathologic consequences. This is true when excess alcohol has to be metabolized, as in alcoholic steatohepatitis, or when CYP2E1 is confronted by an excess of ketones and fatty acids associated with diabetes, obesity, or both, resulting in NASH (Lieber, 2004). It is likely that CYP2E1 overexpression occurs in animals and humans with ALD and NASH. Interestingly, TNF-α has also been implicated as a critical mediator of liver in ALD and NASH. The known role of oxidative stress in TNF-α cytotoxicity leads to the hypothesis that CYP2E1-induced oxidative stress may act to sensitize hepatocytes to death from TNF-α. It is therefore of interest that the ability of CYP2E1 overexpression to sensitize hepatocytes to TNF-α death receptor-mediated cytotoxicity may be a mechanism of liver cell injury and death in liver diseases such as alcoholic and nonalcoholic steatohepatitis in which CYP2E1 overexpression occurs (Liu et al., 2002).

**CONCLUSION**

Fig. 5 shows a hypothetical scheme for common pathogenic mechanism in non-alcoholic or alcoholic

![Fig. 5. Hypothetical schematic of common pathogenic mechanism in the liver injury caused by non-alcoholic or alcoholic steatohepatitis: role of gut-derived endotoxin, innate immune system and oxidative stress. NAFLD, non-alcoholic fatty disease; NASH, non-alcoholic steatohepatitis; RES, reticuloendothelial system; ROS, reactive oxygen species; CYP2E1, cytochrome P450 2E1; * gastrointestinal tract.](image-url)
Common pathogenic mechanism of non-alcoholic or alcoholic steatohepatitis. It is known that chronic ethanol ingestion increases hepatotoxicity and produces fatty liver, hepatitis and cirrhosis. Gut-derived endotoxin has been implicated in the pathogenesis and progression of ALD. Not only inactivation of RES function, which reduces clearance of endotoxin, but also an increase in absorption of endotoxin from the intestine may be involved in mechanisms of ethanol-induced endotoxemia. On the other hand, activation of Kupffer cells by gut-derived endotoxin plays a pivotal role in ALD. Activated monocytes and macrophages have been postulated to play an important role in the pathogenesis of ALD. NASH is a liver disease characterized by histopathological features similar to those observed in alcoholic liver disease, in the absence of significant alcohol consumption. Although the pathogenesis of NAFLD and NASH has not yet been fully elucidated, a popular mechanism is the "two hit theory". This model considered the development of steatosis to be the "first hit" increasing the sensitivity of liver to the putative "second hit" leading to hepatocyte injury, inflammation and oxidative stress. These common pathogenic mechanisms may partly explain the development of progressive liver damage by non-alcoholic or alcoholic steatohepatitis. In our present review, we would like to emphasize an important role of gut-derived bacterial toxins, innate immune system and oxidative stress in development of steatohepatitis.

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