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MOLECULAR ASPECTS OF ALCOHOL METABOLISM: Transcription Factors Involved in Early Ethanol-Induced Liver Injury

Laura E. Nagy
Department of Nutrition, Case Western Reserve University, Cleveland, Ohio 44106-4906; email: len2@po.cwru.edu

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■ **Abstract** Alcohol metabolism takes place primarily in the liver. Initial exposures to ethanol have a major impact on the hepatic redox state and intermediary metabolism as a consequence of ethanol metabolism via alcohol dehydrogenase. However, upon continued exposure to ethanol, the progression of liver injury involves ethanol metabolism via CYP2E1 and consequent oxidant stress, as well as potential direct effects of ethanol on membrane proteins that are independent of ethanol metabolism. Multiple organ systems contribute to liver injury, including the innate immune system and adipose tissue. In response to ethanol exposure, specific signal transduction pathways, including NFκB and the mitogen-activated protein kinase family members ERK1/2, JNK, and p38, are activated. These complex responses to ethanol exposure translate into activation of nuclear transcription factors and altered gene expression within the liver, leading to the development of steatosis and inflammation in the early stages of alcohol-induced liver injury.

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INTRODUCTION

Alcohol metabolism takes place primarily in the liver. The consequences of ethanol on hepatic function can be dependent and/or independent of the metabolism of ethanol by the hepatocyte. Initially, changes in the nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide (NAD/NADH) ratios in the hepatocyte, a consequence of metabolism via alcohol dehydrogenase, have a major impact on intermediary metabolism in the liver. However, as the organism adapts to continued exposure to ethanol, the functional consequences of ethanol exposure involve more than just changes in the redox state of the hepatocyte (70). These responses involve multiple organ systems interacting with the hepatocytes, including possible effect on the intestinal barrier function, as well as the innate immune system (81) and adipose tissue. Additional pathways of ethanol metabolism via the cytochrome P450 system are induced, likely resulting in oxidant stress during ethanol exposure (70). Finally, continued chronic exposure to ethanol may impair liver function via pathways that are independent of ethanol metabolism per se, perhaps via direct interaction of the alcohol with membrane proteins involved in signal transduction pathways, as observed in neural cells (95). Each of these complex mechanisms of ethanol metabolism translates into changes in gene expression within the liver, leading to the progression of alcohol-induced liver injury.

Liver injury is a complex process involving both parenchymal and nonparenchymal cells resident in the liver, as well as the recruitment of other cell types to the liver in response to damage and inflammation (36). The progression of the alcohol-induced liver injury follows a pattern characteristic to all types of liver fibrosis, regardless of the causative agent. This progression is marked by the appearance of fatty liver, hepatocyte necrosis, inflammation, regenerating nodules, fibrosis, and cirrhosis (74). Fibrosis is thought to be initiated in response to hepatocellular damage, with inflammatory processes contributing to the progression of the disease (36). Interestingly, many of the events involved in the development of fibrosis are typical of other tissue responses to injury, such as wound healing in the skin and soft tissues (98). Ethanol metabolism may disorder this highly regulated "wound healing response," resulting in continued hepatocellular damage, inflammation, and fibrosis. Several recent reviews have covered the basic aspects of the enzymology of alcohol metabolism in the liver (see 68–70). The focus of this review is on understanding the consequences of alcohol metabolism in the liver on changes

in key signal transduction pathways leading to the activation of nuclear transcription factors that regulate the changes in hepatic gene expression that are ultimately responsible for the development of alcoholic liver injury. The specific focus of this review will be on the transcription factors regulating fatty acid homeostasis during the development of steatosis and the factors involved in the activation of the inflammatory response in Kupffer cells, the resident macrophage in the liver.

ENZYMATIC PATHWAYS OF ETHANOL METABOLISM

Ethanol is primarily metabolized in the liver by three enzymatic pathways. Recent reviews have extensively covered these pathways in regard to their enzymatic characteristics, as well as molecular and genetic regulation (see 2, 70, 100), so only a brief overview is provided here. Alcohol dehydrogenase (ADH, EC1.1.1.1) is responsible for the bulk of ethanol oxidation. ADH is predominantly expressed in the liver, but other tissues, including gastric mucosa, express ADH and contribute to overall oxidation of ethanol (70). Allelic variation in the ADH and aldehyde dehydrogenase (ALDH) genes are thought to contribute to differential rates of ethanol elimination in human populations, as well as be potential contributors to variations in the susceptibility to alcohol dependence and/or organ damage in response to prolonged alcohol consumption (2, 38). The expression of ADH in the liver is also highly regulated at the transcriptional level (70). Differential regulation of ADH transcription may contribute to diurnal variations in the rate of ethanol metabolism, as well as differences in rates of ethanol elimination between individuals (7, 70).

The second major pathway for ethanol elimination is the microsomal ethanol oxidizing system (MEOS) catalyzed by cytochrome P4502E1 (CYP2E1). Activity of the CYP2E1 pathway is induced in chronic alcoholics; induction of this pathway is thought to contribute to the metabolic tolerance to ethanol observed in alcoholics (70). Because a number of xenobiotics are also substrates for CYP2E1, increased expression of CYP2E1 can have a major impact on the production of highly toxic metabolites in the liver of alcoholics (70). Importantly, metabolism of ethanol via the CYP2E1 pathway also results in the production of several species of reactive oxygen that are thought to contribute to ethanol-induced liver injury (70). As with ADH, CYP2E1-dependent ethanol oxidation takes place primarily in the hepatocyte (70); however, additional sites of CYP2E1 expression include Kupffer cells (18). At least 10 polymorphisms have been reported in the human CYP2E1 gene (38). In contrast to the polymorphisms in the ADH gene, no association between the CYP2E1 polymorphisms and susceptibility to alcoholism or alcohol-induced organ damage has yet been reported (38).

The final pathway for ethanol metabolism is a nonoxidative pathway catalyzed by fatty acid ethyl ester (FAEE) synthase, leading to the formation of fatty acid ethyl esters (9). FAEE concentrations are highest in organs susceptible to the toxic effects of ethanol, including pancreas and liver (9). FAEEs accumulate in the plasma membrane, as well as in membranes of organelles including mitochondria

and lysosomes (9), potentially having a negative impact on the activity of these organelles. Accumulation of FAEE is currently being developed as a marker of long-term ethanol consumption. Accumulation of FAEE may have very specific mechanisms of actions, as suggested by a recent report that FAEE can activate the mitogen-activated protein kinases, externally regulated kinases (ERK), and c-Jun N-terminal kinase (JNK) in hepatic stellate cells (65).

METABOLIC CONSEQUENCES OF ETHANOL METABOLISM IN THE LIVER

The metabolic consequences of ethanol metabolism vary depending on the pathway utilized for metabolism. Although the pathways for ethanol metabolism never work in isolation, the initial response to ethanol, metabolized primarily via alcohol dehydrogenase, would be expected to have a different impact on the liver than chronic exposure to ethanol when the CYP2E1 system is induced.

The major metabolic consequence to an initial exposure to ethanol is a shift in the redox state favoring the accumulation of NADH in the hepatocyte. This shift has important consequences on fuel utilization in the liver, favoring the synthesis and accumulation of fatty acids, preventing gluconeogenesis, and inhibiting the TCA cycle. These metabolic derangements have been extensively and recently reviewed (70). Interestingly, this initial shift in redox status is not sustained over longer periods of ethanol consumption. As the animal adapts to continued exposure to ethanol, the hepatic redox states tends to normalize (104) as other pathways of ethanol metabolism make more substantial contributions to ethanol disposal.

In addition to the shift in NAD/NADH ratios, the production of acetaldehyde is an important metabolic consequence of ethanol metabolism via the ADH pathway (28, 70). Acetaldehyde is a highly reactive molecule that can form stable adducts on proteins. Acetaldehyde-modified proteins have been identified in human liver after alcohol consumption (87). In addition to stable acetaldehyde adducts, other aldehydes generated during ethanol metabolism, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), have been observed in the liver after ethanol consumption (93). Aldehyde adducts can be detected early in the progression of liver injury, prior to extensive inflammation and necrosis (105). MDA adducts and 4-HNE adducts appear to be localized primarily with microvesicular lipid deposits during these early phases of liver injury (105). Different reactive aldehydes (malondialdehyde and acetaldehyde) have also been found to target the same polypeptide, resulting in the formation of hybrid adducts, called MAA adducts (126). Although their mechanism of toxicity is largely unknown, both individual and hybrid adducts can elicit proinflammatory and profibrogenic responses that are likely to contribute to the progression of liver injury (126). In addition to understanding the potentially toxic effects of aldehyde adducts, another challenge is to identify specific molecular targets that are vulnerable to aldehyde adduct formation.

Acetaldehyde is rapidly metabolized in the liver, generating acetate and AMP. AMP can be metabolized in the hepatocyte to adenosine, which in turn can interact

with adenosine receptors on the surface of the hepatocyte and/or nonparenchymal cells in the hepatic sinusoid (19, 91). Acetate produced during ethanol metabolism can have a significant impact locally on the liver, as well as more distal effects on other organs, including the central nervous system (47). Similarly, adenosine produced during ethanol metabolism has a long-term effect on cAMP production in the hepatocyte (80, 83).

Reactive oxygen species (ROS) are highly reactive intermediates generated during ethanol oxidation via CYP2E1 that contribute to ethanol-induced liver injury (40, 49). In addition, ROS may be derived from activated Kupffer cells in the liver during the inflammatory phase of liver disease (116). Although ROS can have beneficial effects in the liver, acting as effectors in the innate immune response (14), they also cause oxidative damage to macromolecules in the liver. It is widely accepted that oxidative damage to macromolecules plays a critical role in the progression of alcoholic liver disease (ALD) (40, 49), but ROS may also have an impact on hepatic metabolism via changes in the regulation of key signal transduction processes, including the transcription factors NFκB and AP-1, as well as on members of the mitogen-activated protein kinase family, ERK1/2 and JNK (81). The potential impact of these specific effects of ROS on signal transduction cascades on liver function is discussed below.

MECHANISMS OF ETHANOL ACTION INDEPENDENT OF ENZYMATIC METABOLIC PATHWAYS

Because the liver is the primary site of ethanol metabolism, most investigations have focused on the role of ethanol metabolism per se in mediating the effects of ethanol in the liver. However, it is important to remember that ethanol may have direct effects on cellular function, independent of metabolism. Recent studies indicate that ethanol can directly interact with membrane proteins, including ion channels, kinases, and adhesion molecules (95, 114, 136). Studies have documented molecular "cut-offs" indicating that ethanol may be interacting with specific low-affinity binding sites on different polypeptides (30, 95, 114, 136). In addition to specific interactions with proteins, it is also hypothesized that ethanol may interact directly with lipid microdomains in membranes and influence the activity of membrane proteins (95). However, these direct effects of ethanol on membrane fluidity are generally observed at much higher concentrations of ethanol than is observed in animals or humans consuming ethanol ad libitum.

PATHOLOGICAL MECHANISMS OF ETHANOL ACTION: PROGRESSION OF LIVER INJURY

The progression of alcoholic liver injury is characterized by the initial appearance of fatty liver, followed by inflammation, necrosis and apoptosis, followed by fibrosis and, then, in some individuals, by cirrhosis (122). It is generally thought

that the early stages of alcoholic liver injury are reversible, so a focus of current research is to understand the molecular mechanisms for the development of fatty liver and inflammation. Indeed, in most models, therapeutic or molecular strategies to prevent the steatosis also prevent the resulting inflammatory responses, as well as fibrosis (121, 122). This initiating role of fatty liver in the disease process has led investigators to describe the accumulation of excess fat in the liver as "lipotoxic," but the mechanisms by which excess lipid accumulation in the liver sensitizes the hepatocyte to further damage are not understood (58). Of particular interest is to understand how steatosis sensitizes the liver to inflammation. Critical interactions between lipid accumulation in the hepatocyte and activation of macrophages in the liver are likely involved in accelerating/exacerbating the early response of the liver to ethanol, leading to liver injury during long-term exposure to ethanol.

STEATOSIS

What causes the accumulation of lipid in the liver during ethanol consumption? In the initial stages of ethanol exposure, ethanol metabolism via alcohol dehydrogenase results in a shift in the redox state of the hepatocyte, favoring the accumulation of fatty acids, rather than oxidation of fatty acids as fuels. This aspect of ethanol metabolism and the early stages of fat accumulation have been extensively studied (see reviews 26, 58). However, as the organism adapts to the continued presence of ethanol, the redox state of the hepatocyte normalizes (104). Thus, it is likely that alternative mechanisms are involved in the subsequent accretion of triglyceride in the liver during chronic ethanol exposure. Here I discuss recent data that suggest further accumulation of lipid involves changes in the expression of genes that govern lipid homeostasis in the liver. Increases in the expression of genes regulating fatty acid synthesis, coupled with suppression of genes for fatty acid oxidation and transport from the liver, have been reported in a number of animal models of chronic ethanol exposure.

Transcriptional Regulation of Genes Governing Lipid Homeostasis

The regulation of fatty acid synthesis and oxidation is complex, involving multiple enzyme systems within different compartments of the hepatocyte. However, the expression of many of the genes involved in fatty acid metabolism is coordinated and responds to stimulation by specific transcription factors (115). Although there is a large body of literature characterizing the effects of chronic ethanol on transcription factors involved in coordinating the inflammatory response (see below), recent evidence has suggested that chronic ethanol exposure also affects the activity of several transcription factors that coordinate expression of genes involved in fatty acid metabolism, including sterol regulatory element binding protein (SREBP), which primarily coordinates genes involved in the regulation of fatty acid

synthesis, and peroxisomal proliferating factor α (PPARα), which coordinates expression of genes involved in fatty acid oxidation.

Sterol Regulatory Element Binding Proteins

Sterol response element binding proteins (SREBPs) are a family of membrane-bound transcription factors. SREBPs are synthesized as 125 kD precursors embedded in the endoplasmic reticulum. Activation by proteolytic cleavage allows for the accumulation of active SREBP in the nucleus (41). SREBP1a and 1c are important in the regulation of genes required for hepatic triglyceride synthesis, including acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase 1, malic enzyme, and ATP citrate lyase. SREBP2 is involved in the regulation of genes required for cholesterol synthesis, including HMG-CoA synthase, HMG-CoA reductase, and low-density lipoprotein receptor (41, 42). SREBP1c and SREBP2 are expressed in the liver, while SREBP1a is expressed only at very low levels in the liver of adult mice, rats, and humans (41). Information on the metabolic role of this family of transcription factors is primarily derived from a series of experiments in genetically manipulated mice that overexpress truncated versions of the SREBPs that lack their transmembrane domain and thus act as dominant-positive forms of the transcription factors (109, 110, 113). Liver-specific overexpression of either SREBP1a or SREBP1c results in fatty liver with 22-fold and 4-fold increases in hepatic triglyceride content, respectively (41). The enhanced accumulation of triglyceride with overexpression of SREBP1a is consistent with its greater transcriptional potency for activation of target genes (110).

Increased expression of SREBP1c has been observed in several models of diabetes, including genetic models such as the ob/ob mouse (111) and transgenic mice overexpressing SREBP1c or SREBP1a in adipose tissue (43, 111). Interestingly, overexpression of SREBP1c in adipose tissue impairs adipocyte differentiation, leading to insulin resistance and a massive accumulation of triglyceride in the liver (111). In contrast, overexpression of SREBP1a in adipose induces fatty acid biosynthetic enzyme expression in adipose, leading to increased secretion of fatty acids and a modest accumulation of triglyceride in the liver (43). High-fat diets induce SREBP1 expression in ICAM-1 $-/-$, a transgenic mouse line that is very sensitive to high-fat-diet-induced fatty liver, but not in wild-type mice (35). In contrast, polyunsaturated fatty acid diets reduce SREBP1 expression in the liver, primarily via enhanced turnover of SREBP1c messenger ribonucleic acid (mRNA) turnover (22).

In hepatoma cell lines exposed to ethanol in culture, as well as in mice allowed to consume an ethanol-containing diet for four weeks, mature/active SREBP1 levels are increased (143). This increase was associated with increased expression of genes involved in the regulation of hepatic lipogenesis, as well as the accumulation of triglyceride in the liver (143). Increased expression of SREBP1 mRNA and protein in mouse liver is also observed in response to intragastric exposure to ethanol for six weeks (53).

The mechanisms for chronic ethanol-induced expression of SREBP-1, as well as the increase in activation, are not understood. SREBP-1 expression in the liver is induced by insulin and suppressed by glucagon/cAMP (41). Chronic ethanol exposure results in insulin resistance (90, 138) and impairs some insulin-mediated signals in the liver (106). However, insulin can still induce SREBP-1c expression in several models of insulin resistance, including the ob/ob mouse (111), and in streptozotocin-induced diabetes (112). Therefore, the potential role for insulin in chronic ethanol-induced SREBP1 expression needs to be further investigated. In contrast, glucagon-stimulated cAMP production is increased in cultured hepatocytes exposed to chronic ethanol (82), as well as in the liver of rats fed ethanol for four weeks (45), making it unlikely that a suppression of glucagon-stimulated cAMP production contributes to SREBP1 induction during chronic ethanol exposure.

Chronic ethanol-induced liver injury is exacerbated by fish oils (86). However, because PUFAs suppress SREBP-1 expression (22), it is unlikely that increased SREBP-1 expression is involved in the ability of fish oils to exacerbate chronic ethanol-induced liver injury (86).

The localization of SREBP to the endoplasmic reticulum (ER) suggests that its activation may be regulated during ER stress. Hyperhomocysteinemia leads to an ER stress response, as well as activation of SREBPs in hepatocytes (134). Because chronic ethanol exposure is associated with hyperhomocysteinemia, Ji & Kaplowitz (53) hypothesize that induction of SREBP1 mRNA during intragastric ethanol exposure may be related to hyperhomocysteine-induced ER stress. Activation of SREBP-1 by ethanol in cultured hepatoma cells requires ethanol metabolism, which suggests a role for acetaldehyde in the response to ethanol (143). Further, the transcriptional activity of SREBP-1 and SREBP-2 can be enhanced by ERK1/2-dependent phosphorylation (60, 103). Ethanol exposure has complex effects on ERK1/2 activation (21, 64) that may impact on SREBP-1 expression. Further studies designed to assess the potential role of ER stress, acetaldehyde, and altered signaling in mediating the effects of ethanol on SREBP1 activity need to be carried out.

Peroxisome Proliferator-Activated Receptor α

The peroxisome proliferator-activated receptor (PPAR) family of transcription factors is a member of the nuclear hormone receptor superfamily (for recent reviews, see 101, 115). In the liver, PPARα is an important coordinator of the expression of genes involved in fatty acid oxidation in mitochondria, peroxisomes, and microsomes (63, 101). Upon activation by ligand, PPARα forms a heterodimer with retinoid X receptor α (RXRα) to increase transcription of target genes (101). Although wild-type animals respond to a fast by increasing the expression of PPARα-dependent genes regulating fatty acid oxidation, PPARα-null mice develop a severe hepatic steatosis when subjected to fasting (67). Fasting increases the release of fatty acids from adipose stores for oxidation in the liver. The

development of steatosis in PPARα-null mice with fasting demonstrates the critical role that PPARα plays in sustaining lipid homeostasis in response to increased availability of fatty acids to the liver (67).

Based on this essential regulatory role for PPARα in up-regulating fatty acid oxidation in response to increased availability of fatty acids to the liver (79), investigators have asked whether this homeostatic response is disrupted during chronic ethanol exposure. French and colleagues (132) first reported that chronic ethanol feeding to rats decreases PPARα mRNA. Further, exposure of hepatoma cells or primary rat hepatocytes to ethanol during culture decreases the PPARα DNA binding activity and transcriptional activation of PPARα-dependent genes (32). Impaired PPARα DNA binding activity was dependent on ethanol metabolism because it is prevented by the addition of 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. The response was exacerbated by the addition of cyanamide, an inhibitor of acetaldehyde metabolism, and could be mimicked by incubation of in vitro transcribed PPARα with acetaldehyde (32). Chronic ethanol feeding to mice for four weeks also decreased hepatic PPARα DNA binding activity (29). Decreased PPARα binding was associated with decreased expression of PPARα-dependent genes, with the exception of liver fatty acid–binding protein, which was induced fivefold with ethanol feeding (29). Treatment of these chronic ethanol-fed mice with WY14,643, a PPARα agonist, restored PPARα DNA binding activity and, importantly, prevented the development of chronic ethanol-induced steatosis (29).

These recent data suggest that chronic ethanol feeding disrupts the normal homeostatic response of the liver to increased fatty acid availability; fatty acid oxidation pathways are not induced, despite elevation of plasma free fatty acids (29), and excess lipid is stored in the liver as triglyceride. The mechanism for this failure of PPARα activation is not clear. Decreased expression of PPARα mRNA (132) and PPARα and RXRα protein (29) likely contribute to decreased activity. However, because WY14,643 restored DNA binding activity without restoring PPARα quantity, additional mechanisms of chronic ethanol action are likely. Further studies are required to ascertain whether chronic ethanol impacts on the ability of ligand to activate PPARα due to acetaldehyde-dependent modifications to the PPARα protein, as suggested by Fischer and colleagues (29, 32). A mechanism of action involving acetaldehyde would likely be specific to ethanol-induced steatosis. Interestingly, steatohepatitis induced by feeding a methylcholine-deficient diet to mice, a model of nonalcoholic steatohepatitis, could also be prevented by treatment with WY14,643 (46). As in the chronic ethanol-fed mouse, most PPARα-dependent genes were not induced with the methylcholine-deficient diet; supplementation of the PPARα agonist increased expression of genes involved in fatty acid oxidation and prevented liver injury (46). These data suggest that activation of PPARα is suppressed in steatosis independent of a specific etiology. This suggests a more generalized mechanism for reduced activation of PPARα, one that is not mediated by acetaldehyde per se. Possible alternative mechanisms of ethanol action include decreased availability of low-affinity, endogenous lipid PPARα ligands and/or

impaired PPARα signaling via interactions with other nuclear receptors. For example, activation of liver X receptor, a nuclear receptor activated by SREBP-1c, can reduce the formation of the PPARα-RXRα heterodimer and thus impair transcriptional activation (44).

PPARγ, another member of the PPAR family, is primarily expressed in adipose tissue, where it regulates adipogenesis and modulates insulin resistance (24). Our understanding of the functional significance of PPARγ has recently expanded beyond its role in adipocytes. PPARγ is expressed in macrophages and has been implicated in innate immune responses (23). Of particular interest is an emerging role of PPARγ in the development of hepatic steatosis (34, 144). PPARγ expression is increased in the liver during steatosis in response to high-fat diets, obesity, and chronic ethanol exposure (13, 77, 99, 130). Expression is observed in both Kupffer cells, the resident macrophage in the liver, and in the parenchymal cells (13). However, it is not clear whether PPARγ expression is a cause or consequence of steatosis. Treatment of rats with pioglitizone, a PPARγ agonist, during chronic ethanol exposure prevented the development of steatosis and inflammation (27). This protective effect of PPARγ activation was associated, at least in part, with decreased activation of Kupffer cells in the liver (27) (see below); however, it is not clear whether activation of PPARγ with pioglitizone also modulated the expression of genes involved in fatty acid oxidation to prevent the development of ethanol-induced steatosis.

INFLAMMATION/ALCOHOLIC STEATOHEPATITIS

Role of Kupffer Cells

Chronic ethanol consumption is characterized by an increase in the expression of a number of inflammatory mediators, including cytokines, reactive oxygen and nitrogen species, and chemokines (121, 122). One of the primary mechanisms for increased production of inflammatory mediators in response to ethanol exposure is due to activation of Kupffer cells in the liver (121). Ablation of Kupffer cells prevents the development of fatty liver and inflammation in rats chronically exposed to ethanol via intragastric feeding (1, 51). Thurman (121) developed a working model for the progression of ALD, proposing that increased activation of Kupffer cells during chronic ethanol consumption results in increased production of inflammatory mediators, in particular TNFα and reactive oxygen species, leading to the progression of fatty liver, inflammation, and fibrosis (121).

Increased production of TNFα by Kupffer cells is thought to be of particular importance in the pathogenesis of ALD. TNFα is one of the principal mediators of the inflammatory response in mammals, transducing differential signals that regulate cellular activation and proliferation, cytotoxicity, and apoptosis (10, 48). The role of TNFα in the development of ethanol-induced liver injury has been

well characterized in animal models (121, 122). Although it is clear that other inflammatory mediators are involved in the progression of alcohol-induced liver injury, many studies have focused on the regulation of TNFα expression as a model to study the effects of chronic ethanol on the inflammatory response in the liver. As with the effects of chronic ethanol exposure in the regulation of lipid homeostasis, changes in the activation of key transcription factors in response to chronic ethanol coordinate the inflammatory response in the liver. Understanding the mechanisms by which chronic ethanol exposure disrupts the activity of these key transcription factors is a central theme of much of the current research aimed at preventing the development of ALD.

Kupffer Cell Activation

Kupffer cells, the resident macrophages in the liver, are critical to the onset of ethanol-induced liver injury, at least in part because of their function as the first site of exposure to gut-derived lipopolysaccharide/endotoxin (LPS). Alcohol consumption is associated with impaired barrier function of the intestinal mucosa in patients with various stages of alcoholic liver injury, as well as rodents exposed to short-term or long-term ethanol (94, 118). LPS concentration is increased in the blood of alcoholics (11, 31) and rats exposed to ethanol via gastric infusion (85). Moreover, LPS clearance from the blood is decreased in patients with various degrees of ALD (128), as well as in the presence of an acute alcohol dose in rats chronically fed alcohol in their diet (57). In rats exposed to ethanol via intragastric infusion, antibiotic treatment decreases TNFα expression and ethanol-induced liver injury (121), suggesting that increased TNFα after ethanol exposure is due, at least in part, to increased exposure to LPS. However, while antibiotic treatment trials of patients with ALD demonstrate a reduction in endotoxemia, improvement in liver function is not observed in many individuals (12).

In addition to the increased exposure of Kupffer cells to LPS in response to ethanol exposure, chronic ethanol also sensitizes Kupffer cell responses to LPS-mediated activation (4, 18, 55, 56). Chronic ethanol exposure increases the expression of CD14, part of the LPS receptor complex on the surface of the Kupffer cells (52, 55, 119), as well as increases the ability of LPS to stimulate key CD14 signaling intermediates including members of the mitogen-activated protein kinase family, ERK1/2 and p38 (55, 56, 108). The effect of chronic ethanol on LPS-mediated signal transduction in Kupffer cells has been recently reviewed (81).

Additional mechanisms of Kupffer cell activation likely occur during chronic ethanol exposure and the development of ALD. It is widely accepted that ROS play a critical role in the development of alcoholic liver injury (40, 90). Although the targets of ROS during ethanol exposure have not been completely elucidated, ROS-mediated mechanisms are likely to impact the signal transduction pathways involved in Kupffer cell activation. The most well studied example is the activation of NFκB by ROS (see below); however, recent analyses have questioned the role of oxidative stress in the activation of NFκB (15). ROS may also act indirectly on

signaling pathways; for example, oxidant production by HepG2 cells overexpressing CYP2E1 has been implicated in the activation of the ubiquitin-proteosomal pathway for protein degradation (92), a pathway essential to the complex regulation of NFκB activation (25). Additional mechanisms of Kupffer cell activation during chronic ethanol exposure may involve activation of auto-immunity in response to oxidative damage to hepatic proteins and phospholipids (3, 131). Although oxidized macromolecules, such as oxidized low-density lipoproteins, are known to activate the innate immune response via scavenger receptors (50), it is not known whether oxidized macromolecules observed after chronic ethanol exposure (3) can also activate the innate immune system in the liver.

NFκB

Activation of NFκB and the stimulation of NFκB-dependent gene transcription coordinate the production of the inflammatory response to bacterial endotoxin (88). Activation of NFκB is also required for the activation of the innate immune system in response to necrotic cells (66). In monocytes from patients with alcoholic hepatitis, NFκB is activated compared to monocytes from controls (76, 120). Activation of DNA binding activity of NFκB in the liver has also been reported in experimental models of ALD. In rats chronically exposed to ethanol via gastric infusion, NFκB DNA binding activity is increased in the liver (84, 125). This response is thought to occur primarily in Kupffer cells (84) and precedes the appearance of pathological liver injury (54). The greatest increases in NFκB activation are observed in animals fed ethanol within a diet high in fat and/or containing fish oils (84, 124). The functional significance of this activation in the pathophysiology of alcoholic liver injury has been demonstrated by inhibiting NFκB activity in the liver via transduction with the IκB super-repressor (127). Suppression of NFκB activation with the IκB super-repressor prevents the appearance of ethanol-induced liver injury (127).

Thus, while data from several laboratories have convincingly demonstrated an increase in NFκB activation during chronic exposure to ethanol in rodent liver, as well as increases in monocytes isolated from human alcoholics, the mechanisms for this activation are not clear. Several lines of evidence suggest that in vivo activation of NFκB after chronic ethanol exposure is not due to a direct effect of LPS on Kupffer cells. For example, LPS increases hepatic NFκB DNA binding activity in control mice; however, after chronic ethanol feeding, LPS did not activate hepatic NFκB DNA binding activity (59). A similar loss of LPS-stimulated NFκB activity was reported in Kupffer cells isolated from rats allowed free access to ethanol-containing diets for four weeks and then cultured overnight. In this model, LPS treatment increased NFκB activity in Kupffer cells from control rats, but not in Kupffer cells from ethanol-fed rats (55). Similarly, although LPS increases NFκB DNA binding activity in RAW 264.7 macrophages, this response is suppressed after chronic exposure to ethanol in culture (108). However, in a similar study of isolated Kupffer cells, chronic ethanol feeding increased LPS-stimulated NFκB binding to the TNFα promoter (18). Fatty acids can modulate LPS-stimulated

NFκB activation in macrophages, with saturated fatty acids stimulating and unsaturated fatty acids inhibiting NFκB activation via the toll-like receptor 4 receptor (61). However, the interaction of ethanol with fatty acid regulation of toll-like receptor 4 signaling to NFκB has not been investigated.

Many studies have described a potential role of ROS in activation of NFκB during ethanol exposure (for examples see 39, 84). However, it is likely that the activation of NFκB in response to ethanol involves complex mechanisms, possibly involving ROS, acetaldehyde, and/or activation by inflammatory cytokines. Increased accumulation of nonheme iron in Kupffer cells during chronic ethanol feeding may also contribute to activation of NFκB (140). Finally, it is important to consider that chronic ethanol exposure may activate NFκB via differential mechanisms within each cell type in the liver. For example, acetaldehyde may be an important contributor in hepatocytes, where presumably the local concentration of acetaldehyde may be higher as a result of ethanol metabolism, while LPS- and/or ROS-dependent mechanisms may be more important in Kupffer cells. Data from cell culture experiments support this hypothesis. For example, in HepG2 cells overexpressing cytochrome P450 2E1, ethanol exposure results in CYP2E1-dependent oxidative stress. However, activation of NFκB was independent of oxidative stress, but instead required acetaldehyde (102). In contrast, treatment of Kupffer cells with dilinoleoylphosphatidylcholine (DPC), which protects against liver injury and acts as an antioxidant, prevents the increase in NFκB activation observed in Kupffer cells isolated from rats chronically fed ethanol (18).

Early Growth Response Factor 1

Early growth response factor 1 [Egr-1, also known as nerve growth factor induced-A (NGFI-A), Krox-24, ZIF268, ETR103, and TIS8] is a zinc finger transcription factor discovered for its role in regulation of cell growth and proliferation (33). Synthesis of Egr-1, an immediate early gene, is rapidly and transiently induced in response to a variety of stimuli, including cytokines and growth factors, as well as environmental stress, including ischemic stress and tissue damage (16). Egr-1 has been characterized as a transcription factor that coordinates cellular responses to environmental stress, mediating increased expression of a number of target genes, including TGFβ, platelet-derived growth factor (PDGF), chemokines, and adhesion molecules (142). These gene products are required for progression of tissue repair involving the acute inflammatory response and the release of cytokines and growth factors, neutrophil migration, collagen synthesis, and extracellular matrix remodeling (16). Studies aimed at improving tissue repair in response to injury, such as dermal wound healing or during angiogenesis, suggest that increased Egr-1 expression/activity may enhance tissue repair, while lowering expression may provide a therapy for chronic inflammatory diseases, such as rheumatoid arthritis (16).

Transcription of Egr-1 is the primary mediator for increased Egr-1 DNA binding activity, although reports of posttranscriptional and posttranslational regulation

have suggested a more complex regulatory process (33). The promoter sequence of the Egr-1 gene contains five serum response elements, as well as two AP-1 sites (37). LPS activation of Egr-1 requires three of the serum response element sites (37). LPS-stimulated Egr-1 expression is dependent on the activation of ERK1/2 in Kupffer cells and macrophages (37, 55, 108). Kupffer cells isolated from rats fed an ethanol-containing diet for four weeks exhibit increased LPS-stimulated ERK1/2 activation compared to cells from pair-fed controls (55). Activation of ERK1/2 was required for maximal increases in TNFα and interleukin-1β mRNA and was associated with increased binding of Egr-1 to the TNFα promoter after ethanol feeding (55). Further, in RAW 264.7 macrophages cultured with ethanol for 48 h, LPS stimulation of Egr-1 transcription and mRNA accumulation were increased above controls (108). Importantly, overexpression of a dominant negative form of Egr-1 prevented chronic ethanol-induced increases in TNFα expression (108).

The mechanism for increased ERK1/2 activation and Egr-1 production are not known, but may involve ROS. ROS can contribute to the activation of both ERK1/2 and Egr-1 in other model systems (20, 139). In addition to ROS activation of mitogen-activated protein kinase family members, recent data also indicate that H_2O_2 inhibits protein tyrosine phosphatases (62), which could lead to delayed inactivation of kinases. A recent report from by Cao and colleagues (18) finds that treatment of Kupffer cells with DPC, which protects against liver injury and acts as an antioxidant, prevents enhanced LPS-stimulated TNFα production after chronic ethanol feeding. Moreover, DPC also prevents the increase in LPS-stimulated ERK1/2 activation observed in Kupffer cells isolated from rats chronically fed ethanol (18). Taken together, these data suggest that increased activation of the ERK1/2–Egr-1 pathway may be an important contributor to the increased sensitivity of ethanol-fed animals to LPS and may contribute to the progression of ethanol-induced liver damage. However, in vivo studies testing this hypothesis have not yet been reported.

Activator Protein 1

Activator protein 1 (AP-1) is a transcription factor formed as a c-Jun homodimer or heterodimer with c-fos (5). Activation of the mitogen-activated protein kinases, JNK and ERK1/2, in response to a number of extracellular signals increases expression and activity of c-fos and c-Jun. Ethanol activates AP-1 in HepG2 cells (102), and isolated pancreatic stellate cells (75) and chronic ethanol feeding increases AP-1 expression in the liver (133). The mechanisms for this activation are not clearly defined, but may be related to alcohol metabolism and/or the activation of mitogen-activated protein kinases, JNK and ERK1/2, in response to ethanol feeding (59, 102). Interestingly, LPS-stimulated activation of AP-1 binding to DNA was not increased in isolated Kupffer cells by chronic ethanol feeding (55). Activation of AP-1 by chronic ethanol is likely to be important in mediating the inflammatory phase of ethanol-induced liver injury because AP-1 regulates the transcription of genes involved in the inflammatory response, including TNFα

(123) and CD14 (135). Further ethanol-induced activation of AP-1 is also implicated in the development of fibrosis during ethanol exposure because AP-1 also regulates transcription of matrix metalloproteases and collagen type I (6).

Peroxisomal Proliferating Factor γ

PPARγ is expressed in macrophages and has been implicated in innate immune responses (23). As discussed above, PPARγ expression is increased during the development of hepatic steatosis in response to high-fat diets, obesity, and chronic ethanol exposure (13, 77, 99, 130). Expression is observed in Kupffer cells as well as in parenchymal cells in the liver (13). Treatment of rats with pioglitizone, a PPARγ agonist, during chronic ethanol exposure decreased activation of Kupffer cells in the liver and prevented the development of steatosis and inflammation (27). Pioglitizone therapy normalized CD14 expression and LPS-stimulated TNFα production (27). This normalization occurred despite a continued disruption of intestinal permeability during chronic ethanol exposure (27), suggesting that the activation of PPARγ modulated the sensitivity of the Kupffer cell to LPS, rather than LPS exposure per se.

PPARα and PPARγ influence the inflammatory response via transrepression of a number of activated transcription factors including NFκB, AP-1, and signal transducers and activators of transcriptions (23). PPARγ agonists also suppress the induction of Egr-1 during ischemia (89), although the regulation of Egr-1 by PPARγ ligands may vary with cell type and, in some cases, may be independent of PPARγ itself (8). The potential for PPARγ agonists as therapeutic agents during ethanol-induced liver injury make investigations into their mechanisms of action during ethanol exposure an important area of future research.

RELATIONSHIP BETWEEN STEATOSIS AND INFLAMMATION

Why is fatty liver more sensitive to inflammatory mediators? Some investigators have suggested that accumulated fat in the liver provides increased substrate for oxidation and aldehyde-adduct formation (126). Indeed, the recent localization of 4-HNE and MDA adducts to microvesicular lipid depots after chronic ethanol exposure (105) suggest that these lipid deposits are indeed sensitive to oxidative stress developing during ethanol exposure. Lipoperoxides, as well as aldehyde adducts on proteins, can activate the immune system (78, 126). Although oxidized macromolecules, such as oxidized low-density lipoproteins, are known to activate the innate immune response via scavenger receptors (50), it is not known whether oxidized macromolecules observed after chronic ethanol exposure (3) can also activate the innate immune system in the liver.

Is there a relationship between inflammatory cytokines and the development of steatosis? An important response of the organism to inflammation is a shift

in energy utilization, mediated in part by the development of peripheral insulin resistance. Cytokines, including interleukin-1β (73), interleukin-6 (96), and tumor necrosis factor α (TNFα) (43, 129) are known to cause insulin resistance. Increased expression of TNFα by adipose tissue from obese humans or rodents is implicated in the development of the insulin resistance associated with obesity (107). Rats chronically exposed to ethanol develop insulin resistance and glucose intolerance (137); however, the mechanisms for the development of insulin resistance during ethanol exposure are not understood.

Although the mechanisms for ethanol-induced insulin resistance are not clear, there is a strong association between insulin resistance and liver injury. Nonalcoholic steatohepatitis and fatty liver are associated with obesity, insulin resistance, and type 2 diabetes (17), suggesting that there may also be an important relationship between inflammatory cytokines, insulin resistance, and fatty liver during the progression of ethanol-induced liver injury. Drugs, such as metformin, which restore insulin sensitivity, also prevent the development of obesity-associated fatty liver (71).

Interestingly, the effects of ethanol on adipose tissue may be of particular importance in the development of steatosis. Adipose tissue is particularly sensitive to ethanol-induced insulin resistance (90, 97). This may be due in part to a locally increased production of TNFα in adipose tissue (72). Production of leptin, another hormone secreted by adipose tissue, is also increased during chronic ethanol exposure (72) and may contribute to the development of steatosis. Abnormal leptin production, either due to a genetic deficiency or increased expression, has been associated with steatosis (58). The adipose-derived hormone adiponectin (also termed ACRP30 and GBP28) is also involved in the development of steatosis (141). Adiponectin, secreted exclusively by adipose tissue, can antagonize the action of TNFα and regulates hepatic glucose and lipid metabolism (117). Treatment of ethanol-fed mice with adiponectin prevented the development of liver injury (141), implicating ethanol-induced changes in adipose tissue metabolism in sustaining the progression of steatosis.

These recent data illustrate the complexity of the development of ethanol-induced liver injury. As reviewed above, not only are multiple cell types in the liver involved in injury, there is likely input from other organ systems on the progression of liver injury. These systems targeted by ethanol include a compromised intestinal barrier function, as well as changes in the production of a number of endocrine signals generated by adipose tissue. The effect of ethanol on the key cell types involved in early liver injury, including hepatocytes, Kupffer cells, and adipocytes, involves the disruption of specific signal transduction pathways regulating transcription factor activation and gene expression. The impact of these changes in gene expression then culminates in the progression of fatty liver and inflammation. Recent advances in our understanding of the mechanisms of ethanol action of signal transduction pathways and activation of transcription factors should help in the design of therapeutic strategies to prevent and/or reverse the progression of these early phases of alcoholic liver injury.

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