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Alcoholic-induced hepatic steatosis – role of ceramide and protein phosphatase 2A

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Hepatic steatosis, once considered benign, is now being recognized as a condition leading to steatohepatitis, fibrosis, and ultimately cirrhosis ^{1,2}. The mechanisms underlying alcohol-induced hepatic steatosis are complex, involving the disturbance of several signaling pathways. We have gained a better understanding of the role of the innate immune system in the liver and its effects on lipid metabolism and uncovered a number of circulating factors that can influence the response of the liver to ethanol. The detailed reviews of alcohol and hepatic metabolism have been extensively described elsewhere ¹. In this report, we will focus on the potential role of ceramide on AMP-activated protein kinase, as a mediator of alcohol-induced hepatic steatosis.

1. Role of ethanol on AMP-activated protein kinase (AMPK)

AMPK is a master regulator of metabolism that senses cellular stresses. Once activated, it increases fatty acid oxidation and inhibits its synthesis ^{3–5}. Inhibition of AMPK, on the other hand, blocks fatty acid oxidation and promotes fatty acid synthesis ⁵. It regulates lipid synthesis both by direct effects on sterol regulatory element-binding protein (SREBP)-1c and through phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. Inhibition of ACC by AMPK leads to decreased levels of malonyl-CoA, an inhibitor of carnitine palmitoyltransferase I (CPT I), the rate-limiting enzyme for fatty acid oxidation. This allows increased rates of transport of acyl-CoAs into the mitochondrion of oxidation. Taken together, activation of AMPK leads to decreased fatty acid synthesis and increased fatty acid oxidation ⁵.

In ethanol-fed rodents, AMPK activity was decreased ⁵. This led to decreased phosphorylation and increased activity of ACC, increased levels of malonyl-CoA, and decreased activity of CPT I ⁵. Treatment with metformin, an activator of AMPK, returned the phosphorylation levels of ACC toward those of controls ⁵. Decreased AMPK activity seen with chronic ethanol feeding also allows the mature and transcriptionally active form of SREBP-1c, a key transcription factor for lipid synthesis, to accumulate in the liver ⁶. Ethanol's effects on AMPK appear to play a role in both the decreased fatty acid oxidation and the increased fatty acid synthesis seen in alcoholic liver disease.

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Traditionally, an increased intracellular level of AMP was considered the main activator of AMPK⁷, acting through several mechanisms. First, AMP itself causes direct activation of AMPK^{7,8}. Second, AMP also activates the upstream kinase, LKB1 leading to phosphorylation of the α -subunit on Thr-172 on AMPK protein^{3,9}. Phosphorylation of this residue is essential for activity. Third, the binding of AMP to AMPK renders it a better substrate for the upstream kinase, LKB1 and a worse substrate for protein phosphatase¹⁰. AMPK also responds to oxidative stress and reactive oxygen species (ROS). Exposure of endothelial cells to peroxynitrite increased phosphorylation of AMPK, without changing the cellular AMP/ATP ratio¹¹. Xie et al. suggested that this was mediated by protein kinase C ζ , which phosphorylated LKB1 at Ser 428¹². More recently, this group¹³ suggested that phosphorylation of LKB1 on Ser 428 promoted its export from the nucleus resulting in activation of AMPK. AMPK was activated by hydrogen peroxide in NIH 3T3 cells, and the effect was reversed by the hydroxyl radical scavenger dimethylsulfoxide¹⁴. The mechanism of activation of AMPK by oxidative stress is poorly understood and there may be several pathways involved. Transient increases in AMP levels were shown in some experimental models following hydrogen peroxide exposure¹⁵. The tyrosine kinase inhibitor, genistein, was demonstrated to further stimulate hydrogen peroxide-induced AMPK activity without altering the AMP levels¹⁴. The potential mechanism for how ethanol inhibits AMPK was recently reported by our group. We found that ethanol inhibited AMPK through the inhibition of the upstream kinases for AMPK, PKC- ζ and LKB1. Ethanol (50 mM for 24 hours) significantly reduced the level of p-PKC- ζ and p-LKB1 by ~40% and ~60%, respectively, in H4IIEC3 cells compared with controls⁴.

2. Protein phosphatases in control of AMPK activity

Dephosphorylation of AMPK at Thr172 is likely due to protein phosphatase 2C (PP2C α),¹⁶. Recombinant PP2C catalytic subunit dephosphorylated rat liver AMPK in a fashion blocked by the presence of AMP¹⁰. This was felt to be due to binding of AMP to the AMPK γ subunit, rather than a direct effect on the phosphatases. Okadaic acid is a potent inhibitor of protein phosphatases, inhibiting PP2A completely at 1–2 nM, and PP1A at 10–15 nM¹⁷. PP2C is insensitive to this toxin. In freshly isolated rat hepatocytes, the dephosphorylation of AMPK after treatment with fructose (which generates AMP) was insensitive to okadaic acid¹⁸. These data suggest that PP2C is directly responsible for dephosphorylation of Thr172. However, PP2A may also play a role in controlling the level of pAMPK. Okadaic acid increased pAMPK in rat hepatocytes¹⁹ and in MDCK cells treated with vasopressin²⁰. Palmitate inactivated AMPK in endothelial cells via increased PP2A activity²¹. High glucose reduced pAMPK in β cells by way of activation of PP2A²².

PP2A belongs to a family of trimeric serine/threonine phosphatases controlling many cellular functions and signaling pathways, including apoptosis, insulin signaling, and the Wnt/ β -catenin pathways²³. The C subunit is catalytic; the A subunit is the scaffolding subunit; B subunits influence the target protein specificity and cellular location of the complex. There are 2 isoforms of both subunits A and C, and a dozen B subunits divided into B, B', and B'' families²⁴. Experiments using the yeast two-hybrid technique showed that the A subunit of PP2A interacted with the AMPK α subunit and regulated the interaction between α and γ subunits²⁵. This group showed that the PP2A A and AMPK α subunits could be co-immunoprecipitated; we also reported that the PP2A-C subunit co-immunoprecipitated with AMPK α ⁴. With this information in mind, we identified another potential mechanism for the inhibitory effect of ethanol on AMPK. We found that ethanol increased PP2A activity by 30% in hepatoma cells treated with ethanol (50 mM for 24 hours)⁴. We examined the effect of ethanol on AMPK in the presence of the PP2A inhibitor okadaic acid and found that okadaic acid significantly attenuated the inhibitory effect of ethanol on AMPK phosphorylation. We further tested this hypothesis by testing the effect of

using PP2A siRNA on the ability of ethanol to inhibit AMPK. . When the hepatoma cells were transfected with siRNA, the inhibitory effect of ethanol on AMPK and ACC phosphorylation was attenuated ⁴. Our results implied that ethanol-induced AMPK inhibition is also due to the activation of PP2A.

3. Role of ceramide in activation of PP2A

PP2A is involved in the regulation of many cellular functions and signaling pathways ^{24, 26}. PP2A can be activated by ceramide ²⁷, which was reported to bind to the B subunit; hence a form of PP2A was identified as a “ceramide-activated protein phosphatase” ²⁸. Recently, another mechanism for ceramide activation of PP2A was suggested involving the inhibitor of protein phosphatase 2A, I₂PP_{2A}. This protein has the ability to bind directly with ceramide, which decreases its association with PP2A, leading to increasing PP2A activity *in vitro* ²⁹.

Ethanol has been reported to be involved in ceramide metabolism. It can activate both acid and neutral sphingomyelinase, and thereby increase cellular ceramide *in vivo* ³⁰. Acid sphingomyelinase (ASMase) knockout mice fed ethanol are resistant to hepatic steatosis ³¹. A recent study showed that ethanol treatment of isolated hepatocytes reduced the levels of sphingomyelin and sphingosine and increased ceramide content ³². In *in vitro* experiments using H4IIEC3 cells, a reduction in AMPK phosphorylation and its downstream target, phospho-ACC, were observed when the cells were treated with exogenous C₂-ceramide (at 15 μ M) for between 4 and 12 hrs ³³. When the cells were treated with fumonisins B1, which inhibits intracellular ceramide synthesis, we found that the levels of cellular ceramide were reduced by 58%, when compared to controls, and this coincided with an increase in AMPK and ACC phosphorylation. These experiments confirmed that altered levels of ceramide can influence AMPK phosphorylation. Ethanol treatment of hepatoma cells significantly increased cellular ceramide content by ~ 20%, and increased PP2A activity by 18%–23% ^{4, 33}. To further understand the mechanism by which ethanol increased intracellular ceramide levels, several experiments were conducted with inhibitors of the key enzymes of ceramide metabolism. Ceramide can be synthesized by the hydrolysis of sphingomyelin by sphingomyelinases (SMases), of which the acid and neutral isoforms are of major relevance in the cells. Ceramide can also be synthesized *in vivo* in the endoplasmic reticulum through the condensation of serine and palmitoyl-CoA, catalyzed by serine palmitoyl transferase (SPT). It can be salvaged from sphingosine by (dihydro)ceramide synthase ³⁴. To differentiate which pathways might be involved in the action of ethanol, we used the following inhibitors to test their ability to block the effect of ethanol on AMPK activation: myriocin (an inhibitor of serine-palmitoyl transferase), GW4869 (an inhibitor of neutral sphingomyelinase, NSMase), fumonisins B1, and imipramine (an inhibitor of acid sphingomyelinase, ASMase). We found that the myriocin and GW4869 did not interfere with the ability of ethanol to inhibit AMPK phosphorylation ³³. However, the effect of ethanol on AMPK phosphorylation was reversed by imipramine and fumonisins B1, even though there were no significant changes in the levels of mRNA of acid SMase, neutral SMase, or SPT in ethanol-treated H4IIEC3 cells. In mice fed with ethanol for 4 weeks, we found a 28% and 36% increase in hepatic C-16 ceramide and C-18 ceramide, respectively ³³. The level of acidic SMase mRNA, but not neutral SMase and SPT, was increased by 1.7-fold in ethanol-fed liver compared to controls. Taken together, we concluded that ethanol increased ceramide, thus increasing PP2A activity, by activating acid SMase *in vivo*.

4. Other possible effects of ethanol on ceramide generation, and roles of ceramide in the development of alcoholic steatosis

Additional effects of ethanol on the liver *in situ* could affect ceramide metabolism. Dysregulated cytokine signaling, particularly of those released from the Kupffer cells such as TNF- α , occurs with chronic ethanol use. TNF α antibody prevented ethanol-induced liver injury^{1, 35}, and TNFR1 knockout mice were protected against ethanol-induced liver injury³⁶. Kupffer cells from ethanol-fed animals are sensitized to LPS, responding with increased TNF α production. TNF α is a well-known stimulus for the generation of ceramide via activation of acid SMase³⁷. Acid SMase knockout animals were resistant to the steatotic and apoptotic effects of TNF α on the liver³⁴. Another mechanism by which ethanol could raise ceramide levels is through induction of stearoyl-CoA desaturase-1 (Scd-1)³⁸. In Scd-1 knock-out mice, ceramide levels and SPT mRNA and activity are reduced in oxidative-type muscles. This was associated with increased rates of fatty acid oxidation, reduced fatty acyl-CoA levels, and activation of AMPK³⁸. Ethanol feeding induces Scd-1 in mouse liver^{39, 40} through its effect on SREBP-1 and ceramide. Ethanol metabolism results in oxidative stress generated at the mitochondrion and in the ER. Reactive oxygen species are reported to both stimulate ER stress, and to increase ceramide generation³⁴. Glutathione (GSH) has been reported to inhibit neutral SMase 2 in liver, and thus the ethanol-induced depletion of GSH could lead to increased activity of this enzyme despite there being no change in the mRNA for the enzyme (46). The schematic diagram on ethanol effect on ceramide is shown in Figure 1.

Ceramide signaling has been linked to other pathways aside from those directly related to lipid metabolism that are implicated with alcohol-induced liver injury. Ceramide can activate Jun N-terminal kinase (JNK) and protein kinase C ζ , each of which participates in a network of signaling cascades. Increased levels of ceramide observed in ethanol-treated cells and mouse liver have been shown to increase hepatocyte apoptosis and mitochondrial dysfunction^{31, 34, 41}. Ethanol feeding alters hepatic methionine metabolism. Methionine is normally converted to *S*-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT). SAM is then converted to *S*-adenosylhomocysteine (SAH) by donating a methyl group to an accepting molecule, and SAH is then converted to homocysteine. Homocysteine is converted back to methionine by methionine synthase (MS) using *N*-5-methyl-THF (5-methyl-tetrahydrofolate), or by betaine-homocysteine methyltransferase (BHMT), using betaine as a methyl donor. Interestingly, ceramide was found to mediate the down-regulation of methionine adenosyltransferase-1A, which has been shown to play a key role in alcohol-induced liver injury⁴¹ as well as changes in DNA methylation status.

Our review provides the insight into the possible role of ceramide and ASmase as the key elements in ethanol-induced hepatic steatosis. Work from our laboratory has shown that ethanol leads to 'metabolic remodeling' of the liver resulting in hepatic steatosis. This process involves the inhibition of AMP-activated protein kinase (AMPK) leading to decrease in fatty acid oxidation and increase in fatty acid synthesis. Activation of protein phosphatase 2A (PP2A) through the generation of ceramide by *activation of acidic sphingomyelinase (ASmase)* is likely the key mechanism in the inhibitory effect of ethanol on AMPK phosphorylation. Given that ceramide is involved in several pathways of ethanol-induced liver injury, future studies to explore pharmacological therapy that blocks ceramide synthesis or increases its rate of degradation on alcoholic liver diseases are warranted.

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Figure 1.

We propose that the inhibitory effects of ethanol on AMPK phosphorylation are secondary to increased intracellular ceramide concentrations (through acid SMase and ceramide synthase) leading to increase in PP2A activity, thus inhibiting AMPK phosphorylation. Additional effects of ethanol on the liver *in situ* could affect ceramide metabolism. Ethanol stimulates Kupffer cells to release cytokines such as TNF α , a well-known stimulus for the generation of ceramide via activation of acid SMase. Ethanol also causes oxidative and ER stress, both of which were shown to stimulate ceramide generation.