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Paracrine Activation of Hepatic CB_1 Receptors by Stellate Cell-Derived Endocannabinoids Mediates Alcoholic Fatty Liver

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SUMMARY

Alcohol-induced fatty liver, a major cause of morbidity, has been attributed to enhanced hepatic lipogenesis and decreased fat clearance of unknown mechanism. Here we report that the steatosis induced in mice by a low-fat, liquid ethanol diet is attenuated by concurrent blockade of cannabinoid CB_1 receptors. Global or hepatocyte-specific *CB_1* knockout mice are resistant to ethanol-induced steatosis and increases in lipogenic gene expression and have increased carnitine palmitoyltransferase 1 activity, which, unlike in controls, is not reduced by ethanol treatment. Ethanol feeding increases the hepatic expression of CB_1 receptors and upregulates the endocannabinoid 2-arachidonoylglycerol (2-AG) and its biosynthetic enzyme diacylglycerol lipase β selectively in hepatic stellate cells. In control but not CB_1 receptor-deficient hepatocytes, coculture with stellate cells from ethanol-fed mice results in upregulation of CB_1 receptors and lipogenic gene expression. We conclude that paracrine activation of hepatic CB_1 receptors by stellate cell-derived 2-AG mediates ethanol-induced steatosis through increasing lipogenesis and decreasing fatty acid oxidation.

INTRODUCTION

Alcoholism is a leading cause of liver disease in Western societies. Chronic alcohol use can lead to the development of fatty liver, which can further progress into steatohepatitis and liver cirrhosis. The steatogenic action of ethanol has been attributed to enhanced hepatic lipogenesis (Lieber and Schmid, 1961; Lieber et al., 1966; Arakawa et al., 1975; You et al., 2002; Ji et al., 2006) and decreased fatty acid oxidation in the liver (You et al., 2004; Garcia-Villafranca et al., 2007). Obesity is also frequently associated with fatty liver and the subsequent development of cirrhosis, and high-fat diets in rodents induce obesity, hepatic lipogenesis, and steatosis (Osei-Hyiaman et al., 2005a; Lin et al., 2005; Savage et al., 2006; Sampath et al., 2007).

Endogenous cannabinoids (endocannabinoids) are lipid mediators that interact with cannabinoid receptors to produce effects similar to those of marijuana, the two main endocannabinoids being arachidonoyl ethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG). To date, two types of cannabinoid receptors have been identified: CB_1 receptors, which are expressed at high levels in the brain but are also present at much lower concentrations in peripheral tissues, and CB_2 receptors, which are expressed predominantly in immune and hematopoietic cells (Pacher et al., 2006). Endocannabinoids and CB_1 cannabinoid receptors have been recently identified in the mouse liver, where their expression is increased in response to a high-fat diet (Osei-Hyiaman et al., 2005a). Mice deficient in CB_1 receptors are resistant to high-fat-diet-induced obesity and steatosis (Ravinet Trillou et al., 2004; Osei-Hyiaman et al., 2005a), and in wild-type mice, both of these diet-induced effects are reversed by chronic treatment with a CB_1 receptor antagonist (Ravinet Trillou et al., 2003). The hepatic steatosis of genetically obese Zucker rats is also reversed by CB_1 antagonist treatment (Gary-Bobo et al., 2007), and in wild-type mice, CB_1 blockade attenuates the increase in hepatic lipogenesis induced by either a high-fat diet or treatment with a cannabinoid agonist (Osei-Hyiaman et al., 2005a). These findings implicated endocannabinoids acting at hepatic CB_1 receptors in diet-induced obesity and steatosis, although the possible role of CB_1 receptors at extrahepatic sites, such as the central nervous system and/or adipose tissue, could not be excluded (Osei-Hyiaman et al., 2005a).

Similar to high-fat diet, chronic ethanol exposure can increase endocannabinoid levels, at least in the brain (Basavarajappa et al., 2000). These similarities between diet- and ethanol-induced changes in hepatic fat metabolism and endocannabinoid activity, together with recent data suggesting that elevated endocannabinoid levels are associated with ectopic fat accumulation in visceral obesity (Blüher et al., 2006; Després and Lemieux, 2006; Côté et al., 2007; Matias and Di Marzo, 2007), suggest that endocannabinoids may also be involved in ethanol-induced fatty liver. We tested this hypothesis using a mouse model of alcoholic fatty liver. Through the use of wild-type mice as well as mice with either global or hepatocyte-specific genetic ablation of CB_1 receptors, we were able to provide a definitive answer as to the cellular target of endocannabinoids. Our findings also revealed that a specific endocannabinoid, 2-AG, generated in a unique

cellular source, the hepatic stellate cell, is the most likely mediator involved.

RESULTS

Ethanol Feeding Induces Fatty Liver and Activates the Hepatic Endocannabinoid System

Exposure of 8- to 10-week-old male C57BL/6N mice to a low-fat, liquid ethanol diet for 3 weeks resulted in a significant increase in the hepatic expression of the gene encoding the CB_1 receptor (Figure 1A) and in the hepatic levels of 2-AG, but not anandamide (Figure 1B). This latter change occurred selectively in hepatic stellate cells, as determined in purified hepatocytes and stellate cells isolated from the livers of ethanol-fed versus pair-fed mice (Figure 1C). In stellate cells from ethanol-fed compared to pair-fed mice, the gene expression of diacylglycerol lipase β (*DAGL*β), one of two isozymes implicated in 2-AG biosynthesis (Bisogno et al., 2003), was significantly increased, whereas the expression of *DAGL*α and monoglyceride lipase (*MGL*), the enzyme responsible for the selective degradation of 2-AG (Dinh et al., 2002), remained unchanged (Figures 1D and 1E). These findings suggest that the increased 2-AG content of stellate cells from ethanol-fed mice is related to increased biosynthesis of 2-AG. No ethanol-induced change in the expression of any of these enzymes was detected in isolated hepatocytes (data not shown). Chronic ethanol exposure also resulted in hepatocellular damage, as indicated by elevated plasma levels of alanine aminotransferase (ALT) as compared to matched controls exposed to an isocaloric control liquid diet. The ethanol-fed mice also developed fatty liver, as verified by postmortem histological and biochemical analyses (Figures 1F and 1G).

CB_1 Receptor Blockade Protects against Ethanol-Induced Steatosis

In view of the observed upregulation of CB_1 receptors and their endogenous ligand 2-AG in ethanol-fed mice, we tested whether activation of CB_1 receptors contributes to the development of ethanol-induced steatosis. Male mice were treated every other day with intraperitoneal injections of vehicle or 10 mg/kg of the CB_1 antagonist SR141716 (rimonabant) throughout their 3 week exposure to the ethanol-containing diet. Body weight gain and ethanol intake were slightly lower in the rimonabant-treated mice than in their controls, but the intake of ethanol per g of body weight was similar in the two groups, and blood ethanol concentrations were also similar (see Figure S1 available online). Despite this, rimonabant-treated mice were resistant to the steatogenic effect of ethanol: their hepatic lipid content, as verified histologically and by measuring hepatic triglyceride concentrations, was not different from that of mice on the control liquid diet (Figures 1F and 1G), although rimonabant did not prevent the rise in plasma ALT levels.

Resistance of CB_1 Receptor-Deficient Mice to Ethanol-Induced Steatosis

To further test the potential involvement of endocannabinoids, CB_1 receptor-deficient ($CB_1^{-/-}$) mice and their wild-type littermates were exposed to the ethanol diet or were pair-fed with an isocaloric control liquid diet. Similar to the rimonabant-treated wild-type mice, $CB_1^{-/-}$ mice were resistant to the steatogenic effects of ethanol and were also resistant to its hepatotoxic effects: hepatic triglyceride content and plasma ALT levels were significantly lower than in the ethanol-exposed wild-type mice and were similar to levels in wild-type mice on the control diet (Figure 2A). The absence of significant steatosis was also evident in liver sections stained with hematoxylin and eosin or oil red O (Figure 2B). These findings strongly suggest that endocannabinoids acting at CB_1 receptors mediate the effects of ethanol in the liver.

Hepatocyte-Specific Deletion of CB_1 Receptors Confers Resistance to Ethanol-Induced Steatosis

Although functional CB_1 receptors have been identified in the mouse liver (Osei-Hyiaman et al., 2005a), hepatic metabolism can be regulated by autonomic input from the brain (Pocai et al., 2005), where the level of expression of CB_1 receptors is much higher than in the liver and ethanol exposure has been shown to upregulate the endocannabinoid 2-AG (Basavarajappa et al., 2000). Accordingly, the observed hepatic effects of ethanol may be mediated indirectly by an action of ethanol on CB_1 receptors in the central nervous system.

To test whether the endocannabinoid-mediated actions of ethanol can be unequivocally attributed to a local, hepatic mechanism, we generated mice with a hepatocyte-specific deletion of the CB_1 receptor gene by crossing CB_1 floxed mice ($CB_1^{f/f}$; Marsicano et al., 2002, 2003) with transgenic mice expressing the bacterial Cre recombinase gene driven by the mouse albumin promoter. These $CB_1^{f/f;albCre}$ mice (also referred to as $LCB_1^{-/-}$ mice) lack CB_1 receptors in hepatocytes (Figure 2C; see also below) but have normal levels of CB_1 receptors in other tissues, including the brain (data not shown). $CB_1^{f/f;albCre}$ mice exposed to the ethanol diet for 3 weeks were as resistant to steatosis and hepatocellular damage as global $CB_1^{-/-}$ mice were, whereas $CB_1^{f/f}$ controls on the same diet developed steatosis and had elevated plasma ALT levels, similar to wild-type mice on the same diet (Figures 2A and 2B). Blood ethanol concentrations were similar in wild-type, $CB_1^{-/-}$, and $CB_1^{f/f;albCre}$ mice (see Figure S1), discounting the possibility that the striking resistance of the latter two strains to ethanol-induced steatosis could be due to altered ethanol metabolism. These findings indicate that the steatotic actions of ethanol are locally mediated via hepatic CB_1 receptors.

Hepatic CB_1 Receptors Mediate Ethanol Induction of Lipogenesis and Inhibition of Fatty Acid Oxidation

In agreement with previously published findings (You et al., 2002; Ji et al., 2006), ethanol feeding increased the hepatic nuclear expression of sterol regulatory element-binding protein 1c (SREBP-1c), a transcription factor with a key role in the control of lipogenic gene expression, and its target fatty acid synthase (FAS) and decreased both the hepatic expression and enzyme activity of carnitine palmitoyltransferase 1 (CPT1; Figure 3). In both $CB_1^{-/-}$ and $CB_1^{f/f;albCre}$ mice, ethanol induction of SREBP-1c and FAS expression and the parallel inhibition of CPT1 expression were blunted or absent (Figures 3A–3C). Furthermore, the enzymatic activity of CPT1 was significantly higher in both knockout strains than in their respective controls, and unlike in controls, chronic ethanol intake failed to reduce CPT1 activity (Figure 3D).

Fatty acid β-oxidation is positively regulated by the AMP-activated protein kinase (AMPK) in the liver (Muoio et al., 1999).

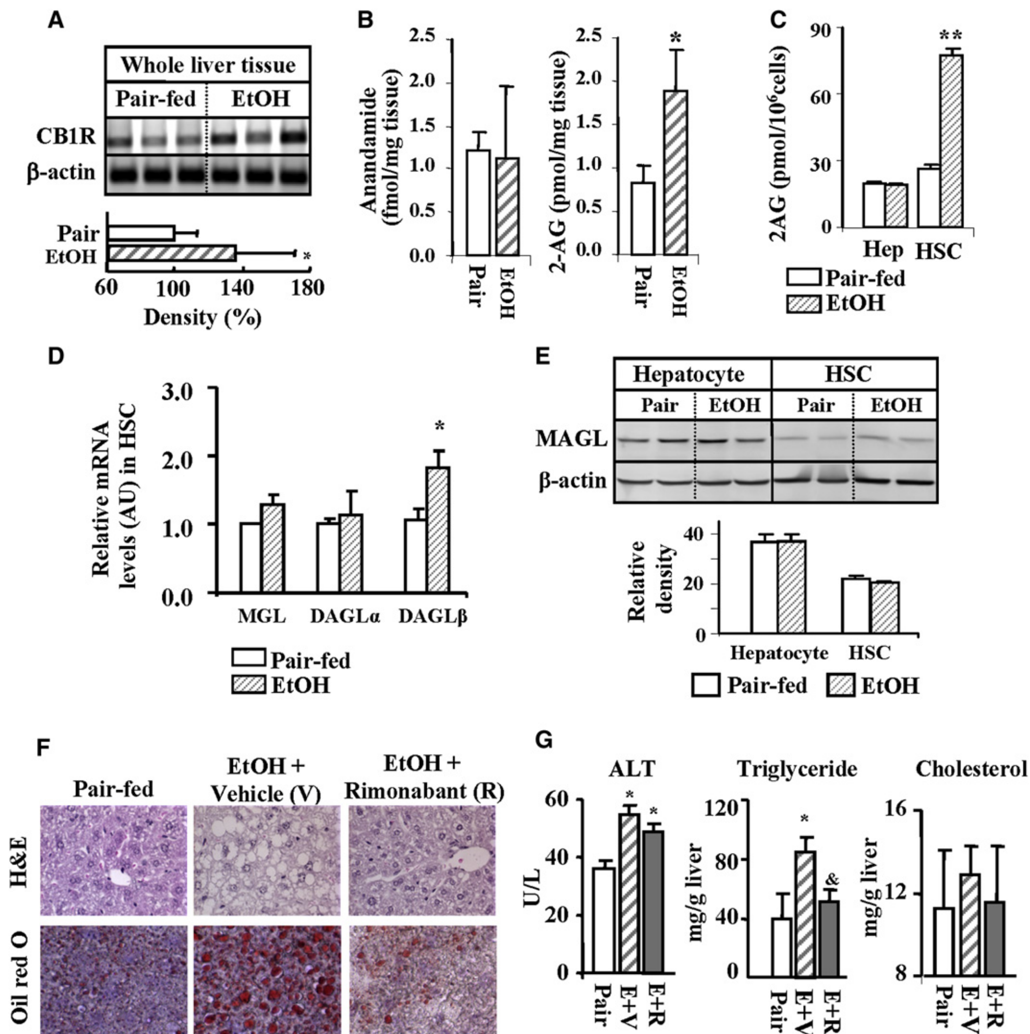

Figure 1. Alcohol-Induced Steatosis, CB_1 Receptor Expression, and Endocannabinoid Levels in the Liver

Male C57BL/6N mice were placed on a low-fat, liquid alcohol diet (EtOH) or pair-fed with an isocaloric control liquid diet for 3 weeks. Mice exposed to the EtOH diet were treated every other day with 10 mg/kg rimonabant (R) or vehicle (V).

(A) CB_1 receptor mRNA detected by RT-PCR and quantified by densitometry.

(B) Hepatic anandamide and 2-AG content in control and EtOH-treated mice as measured by liquid chromatography/mass spectrometry. *$p < 0.05$ versus pair-fed group.

(C) Anandamide and 2-AG content in isolated hepatocytes and hepatic stellate cells (HSC) in control and EtOH-fed mice. **$p < 0.01$ versus pair-fed HSC.

(D) Relative levels of *MGL*, *DAGL*α, and *DAGL*β mRNA in purified fractions of hepatic stellate cells were determined by real-time PCR. *$p < 0.05$ versus pair-fed *DAGL*β.

(E) Chronic EtOH feeding does not affect MGL protein levels in hepatocytes or stellate cells as detected by western blotting.

(F) Postmortem liver sections stained with hematoxylin and eosin (H&E) or oil red O. Note the increased deposition of lipids in the EtOH + V group as compared to the other two groups. (Colorimetric quantification of oil red O-stained sections: 132 ± 17 arbitrary units in control, 218 ± 12* in EtOH + V, 159 ± 11 in EtOH + R; *$p < 0.05$ relative to control liquid diet.)

(G) Plasma alanine aminotransferase (ALT) and hepatic triglyceride and cholesterol content. *$p < 0.05$ versus control, &$p < 0.05$ versus EtOH + V.

Columns and error bars represent means and SEM, respectively; n = 12–14 per group.

Ethanol-induced steatosis can be prevented or reversed by in vivo treatment with AMPK activators such as metformin (Yamauchi et al., 2002), adiponectin (Bergheim et al., 2006), or 5-aminoimidazole-4-carboxamide-1-β-D-furosamide (AICAR) (Tomita et al., 2005), and the phytocannabinoid Δ^9-tetrahydrocannabinol has been shown to inhibit hepatic AMPK activity (Kola et al., 2005). Unexpectedly, we found that phosphorylation of AMPK was modestly increased rather than decreased in mice fed ethanol compared to mice fed the control liquid diet, both in whole liver (Figure 4A) and in hepatocytes (Figure 4B). However, a much more robust increase in the ratio of phosphorylated AMPK (pAMPK) to AMPK was observed in the liver of ethanol-treated $CB_1^{-/-}$ and L$CB_1^{-/-}$ mice (Figure 4A, right panel). This suggests that in mice chronically exposed to ethanol, hepatic AMPK activity is tonically inhibited by endocannabinoids, and removal of this mechanism results in higher AMPK and CPT1

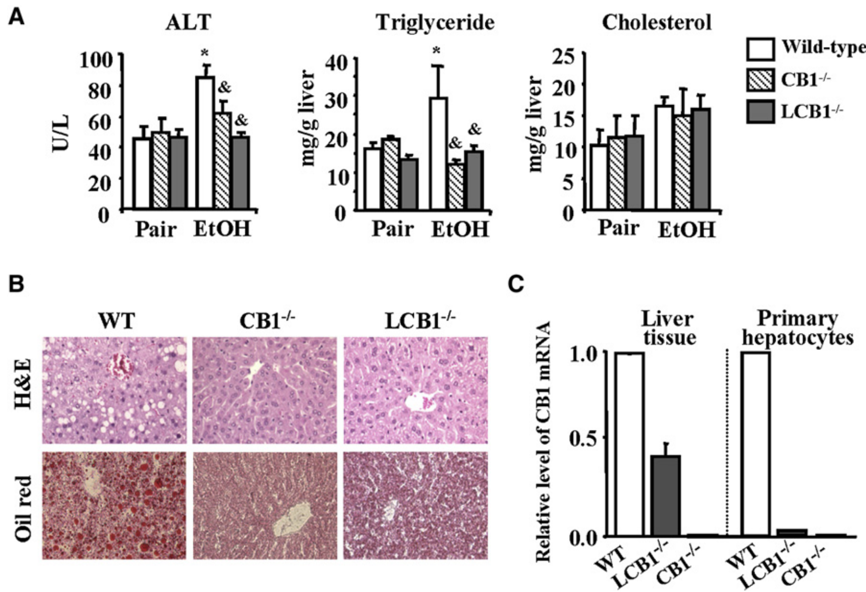

Figure 2. Mice with Global or Hepatocyte-Specific Knockout of CB_1 Receptors Are Resistant to Ethanol-Induced Steatosis

(A) The alcohol-induced increase in plasma ALT and hepatic triglyceride in wild-type mice is absent or blunted in CB_1 knockouts ($CB_1^{-/-}$, global knockout; $LCB_1^{-/-}$, hepatocyte-specific knockout). *p < 0.05 versus pair-fed mice, &p < 0.05 versus EtOH-treated wild-type mice. Columns and error bars represent means and SEM, respectively; n = 8–10 per group.

(B) H&E- or oil red O-stained liver sections from ethanol-fed mice. Note the accumulation of fat in the wild-type (WT) but not in the $CB_1^{-/-}$ or $LCB_1^{-/-}$ specimens. (Colorimetric quantification of oil red O-stained sections: 112 ± 12 arbitrary units in pair-fed wild-type, 61 ± 18** in $CB_1^{-/-}$, 60 ± 21** in $LCB_1^{-/-}$; **p < 0.01 relative to wild-type.)

(C) CB_1 receptor mRNA is absent in hepatocytes of $LCB_1^{-/-}$ mice. Relative levels of CB_1 mRNA in whole liver and purified hepatocytes were determined using real-time PCR.

activity, which likely accounts for the resistance of CB_1-deficient mice to steatosis. These findings further suggest that chronic exposure to ethanol also triggers an endocannabinoid-independent mechanism to activate AMPK, which may represent a compensatory or "repair" mechanism counteracting its steatogenic effect.

Role of Hepatic Stellate Cells in Ethanol-Induced Steatosis

Hepatic stellate cells have a key role in tissue repair. These cells are activated by fibrogenic stimuli such as CCl_4 or ethanol, and a selective increase in 2-AG levels occurs in the liver of mice chronically treated with either CCl_4 (Siegmund et al., 2007) or ethanol (Figure 1C). However, the potential role of stellate cells and their mediators in ethanol-induced steatosis has not been explored. To do this, we cocultured stellate cells freshly isolated from ethanol-fed or pair-fed mice with hepatocytes from pair-fed wild-type or $LCB_1^{-/-}$ mice and analyzed the effect of coculture on lipogenic gene expression in the hepatocytes. As illustrated in Figure 4C, the presence of stellate cells from ethanol-fed versus pair-fed mice resulted in a robust increase in the expression

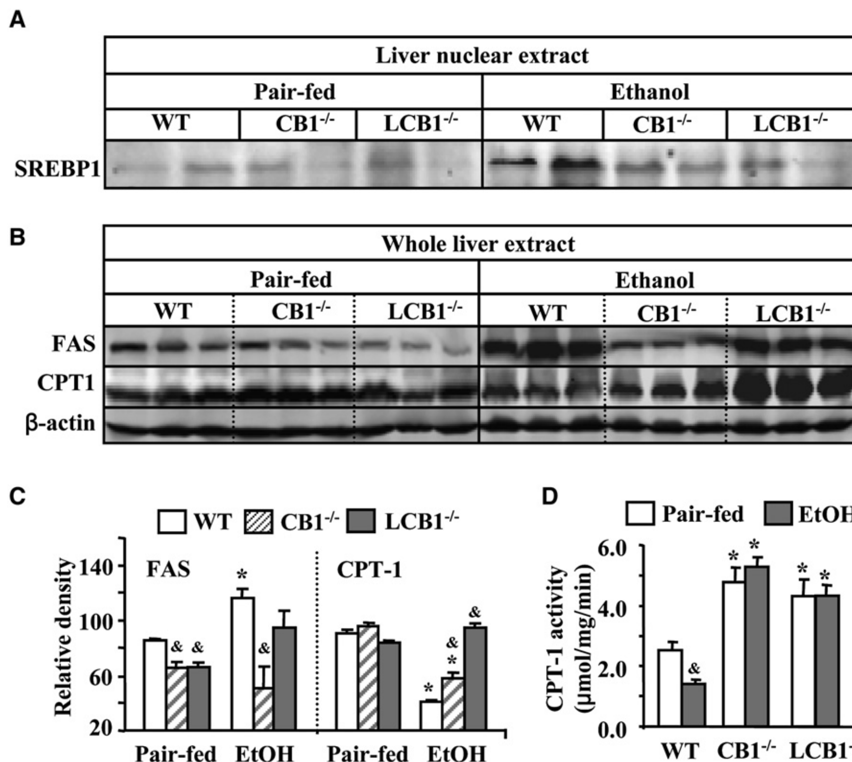

Figure 3. Ethanol Upregulates Hepatic Lipogenic Gene Expression and Inhibits Fatty Acid Oxidation via Activation of Hepatic CB_1 Receptors

(A) Chronic ethanol exposure increases nuclear levels of SREBP-1c protein in the liver of wild-type (WT) but not $CB_1^{-/-}$ or $LCB_1^{-/-}$ mice, as detected by western blotting.

(B) The alcohol-induced increase in FAS and decrease in CPT1 protein levels in the liver of WT mice are blunted, absent, or reversed in $CB_1^{-/-}$ and $LCB_1^{-/-}$ mice.

(C) Densitometric analysis of data in (B). Labeling of columns as in Figure 2A.

(D) Hepatic CPT1 activity is inhibited by chronic EtOH in the presence, but not in the absence, of hepatic CB_1 receptors.

Columns and error bars represent means and SEM, respectively; n = 3 per group. *p < 0.05 versus wild-type, &p < 0.05 versus pair-fed control.

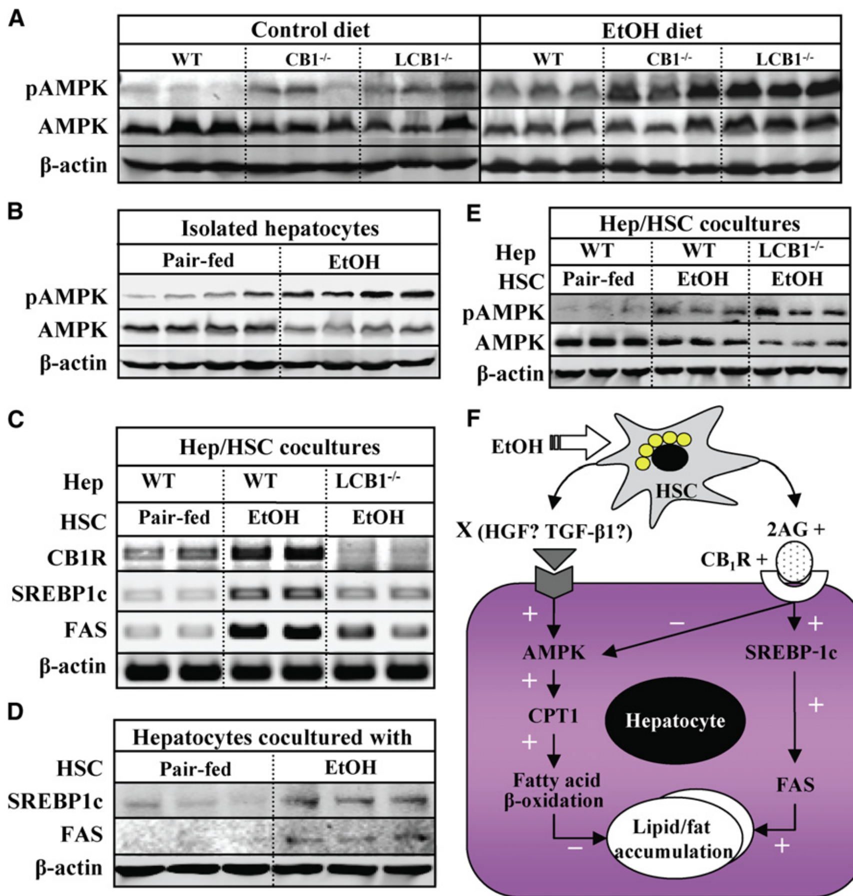

Figure 4. Increased Hepatic AMPK Phosphorylation in the Absence of CB_1 Receptors in Ethanol-Treated Mice

(A) Chronic ethanol-induced activation of hepatic AMPK is potentiated in $CB_1^{-/-}$ and $LCB_1^{-/-}$ mice, as indicated by increased phosphorylation of AMPK (pAMPK) in liver tissue detected by western blotting.

(B) Activation of AMPK in hepatocytes isolated from ethanol-fed versus pair-fed mice.

(C) Upregulation of *CB₁* receptor, *SREBP-1c*, and *FAS* gene expression in hepatocytes from pair-fed wild-type (WT) mice cocultured with hepatic stellate cells from ethanol-fed versus pair-fed mice (middle two lanes versus left two lanes). In $LCB_1^{-/-}$ hepatocytes cocultured with hepatic stellate cells from ethanol-fed mice (right two lanes), CB_1 mRNA is absent and the induction of *SREBP-1c* and *FAS* expression is blunted compared to in WT hepatocytes. mRNA levels were determined by RT-PCR.

(D) Increased SREBP-1c and FAS protein levels as determined by western blotting in wild-type control hepatocytes cocultured with hepatic stellate cells from ethanol-fed (right lanes) versus pair-fed mice (left lanes).

(E) In hepatocyte/hepatic stellate cell cocultures, the presence of hepatic stellate cells from ethanol-fed mice results in increased phosphorylation of AMPK in hepatocytes, which is more pronounced in $LCB_1^{-/-}$ than WT hepatocytes.

(F) Scheme of paracrine regulation of hepatic lipogenesis via hepatic stellate cell-derived endocannabinoids acting on CB_1 receptors in hepatocytes.

of the gene encoding the CB_1 receptor as well as the lipogenic transcription factor SREBP-1c and its target FAS in the wild-type hepatocytes (middle two lanes versus left two lanes). SREBP-1c and FAS protein levels were also markedly increased (Figure 4D). In similar cocultures using $LCB_1^{-/-}$ hepatocytes exposed to stellate cells from ethanol-fed mice, CB_1 receptor expression was absent, and the induction of *SREBP-1c* and *FAS* was less pronounced than in wild-type hepatocytes (Figure 4C, right two lanes). This suggests that ethanol upregulates hepatic CB_1 receptors through a paracrine mechanism involving one or more stellate cell-derived mediators and that activation of these receptors by endocannabinoids, including stellate cell-derived 2-AG, contributes to the lipogenic action of ethanol. Stellate cell-derived mediators may also be involved in the endocannabinoid-independent activation of AMPK by ethanol, described above. The presence of stellate cells from ethanol-fed mice in cocultures resulted in a more pronounced increase in the pAMPK/AMPK ratio in $LCB_1^{-/-}$ hepatocytes than in wild-type hepatocytes (Figure 4E).

We also tested whether the effect of ethanol feeding on stellate cell 2-AG levels is a direct result of ethanol, acetaldehyde, or reactive oxygen species or whether paracrine mechanisms requiring the presence of neighboring cells may be involved. Incubation of freshly isolated hepatic stellate cells with 100 mM ethanol, 0.2 mM acetaldehyde, or 0.5 mM H_2O_2 for 24 hr resulted in no change in the cellular 2-AG content (Figure S2).

DISCUSSION

The present study provides evidence for the involvement of endocannabinoids acting at hepatic CB_1 receptors in the development of alcohol-induced fatty liver. Several findings support this notion. First, chronic ethanol feeding resulted in the activation of the hepatic endocannabinoid system, as reflected in the upregulation of both *CB₁* receptor expression and 2-AG levels in the liver. Second, chronic treatment of mice with a CB_1 receptor antagonist protected against ethanol-induced steatosis. Third, mice with either global or hepatocyte-specific knockout of CB_1 receptors were resistant to ethanol-induced steatosis and the effects of ethanol on de novo lipogenesis and fatty acid oxidation. Fourth, ethanol feeding selectively increased 2-AG levels in hepatic stellate cells, and coculture of ethanol-activated stellate cells with control hepatocytes resulted in a CB_1 receptor-dependent increase in the expression of the lipogenic genes *SREBP-1c* and *FAS* in the latter.

A surprising finding was the selectivity of the effect of ethanol in terms of both the nature of the endocannabinoid affected, 2-AG, and its source, the hepatic stellate cell. The biosynthesis and degradation of anandamide and 2-AG occur through distinct pathways (Pacher et al., 2006), so the ethanol-induced selective increase in 2-AG levels in stellate cells is most likely due to the increased expression of the gene encoding the 2-AG biosynthetic enzyme DAGLβ in these cells, whereas the rate of

degradation of 2-AG is probably unaffected in view of the unchanged cellular levels of *MGL* mRNA and protein (Figures 1D and 1E). Although the anandamide-degrading enzyme fatty acid amidohydrolase (FAAH) can also hydrolyze 2-AG under in vitro conditions, the unchanged tissue levels of 2-AG in *FAAH* knockout mice (Osei-Hyiaman et al., 2005b) argue against its involvement in the observed increase in hepatic 2-AG levels. In contrast to the source of 2-AG, the functionally relevant upregulation of CB_1 receptors most likely occurs in hepatocytes, as also suggested by the effect of coculture on hepatocyte CB_1 receptor expression (Figure 4C). Whether 2-AG itself or another stellate cell-derived signal is involved in the upregulation of CB_1 receptors in hepatocytes is not yet known. Induction of 2-AG may be a general response of stellate cells to injury, as treatment of mice with CCl_4 also results in a selective increase in hepatic 2-AG levels (Siegmund et al., 2007). The paracrine, lipogenic action of 2-AG on adjacent hepatocytes may also be a general phenomenon, as one of the earliest actions of both ethanol and CCl_4 treatment is increased lipid accumulation in the liver (Cunnane, 1987). Steatosis is also commonly found in chronic hepatitis C cases, and recent observations indicate that daily marijuana use is an independent predictor of steatosis severity in such individuals (Hézode et al., 2008). These latter findings, together with studies cited in the introduction, suggest that endocannabinoids and hepatic CB_1 receptors are part of a common pathway involved in the development of hepatic steatosis of varied etiologies, including high-fat diet, ethanol, and viral hepatitis.

Ethanol feeding resulted in a selective increase in 2-AG levels in stellate cells and a parallel induction of CB_1 receptor-mediated steatosis, which is known to occur in hepatocytes. This suggested a paracrine mechanism for ethanol-induced steatosis, which was further supported by the results of coculture experiments, demonstrating that stellate cells from ethanol-fed mice induce lipogenic gene expression in cocultured wild-type but not CB_1-deficient hepatocytes (Figure 4C). Hepatic stellate cells are thought to play a key role in tissue repair and fibrogenesis, but their possible involvement in steatosis has not been previously contemplated. In addition to the CB_1 receptor-mediated increase in lipogenic gene expression likely mediated by stellate cell-derived 2-AG, other stellate cell-derived mediators may be involved in compensatory mechanisms aimed at counteracting steatosis. Cytokines produced by activated stellate cells, such as TGF-β or HGF, are known to activate AMPK in hepatocytes (Suzuki et al., 2005; Martinez-Chantar et al., 2006), and HGF has been shown to promote recovery from alcohol-induced fatty liver (Tahara et al., 1999). Release of such a cytokine (or cytokines) may account for the activation of hepatic AMPK by chronic ethanol feeding (see Figure 4A). Indeed, the presence of ethanol-activated hepatic stellate cells in cocultures stimulated AMPK phosphorylation in hepatocytes, a result which, similar to the findings in whole liver, was more pronounced in $LCB_1^{-/-}$ than wild-type hepatocytes (Figure 4E). Thus, stellate cells may play a key role in both the endocannabinoid-dependent lipogenic action of ethanol, which involves decreased AMPK phosphorylation, and the endocannabinoid-independent increase in fatty acid oxidation in CB_1-deficient mice, which is triggered by an increase in AMPK phosphorylation.

In contrast to the robust increase induced in stellate cell 2-AG by in vivo ethanol treatment, incubation of freshly isolated stellate cells with ethanol, acetaldehyde, or H_2O_2 failed to affect cellular 2-AG content. This argues against a direct action by ethanol, its metabolite, or reactive oxygen species and rather suggests an indirect, paracrine mechanism. Although the specific mechanism of 2-AG induction remains to be established, ethanol is known to increase the circulating levels of bacterial endotoxin (Thurman, 1998; Tamai et al., 2002), which has been shown to stimulate endocannabinoid production in LPS-sensitive cells such as macrophages and platelets (Varga et al., 1998; Liu et al., 2003). Endotoxin can also directly affect hepatic stellate cells (Paik et al., 2003), and it remains to be established whether it can stimulate them to produce 2-AG.

Endocannabinoids are lipophilic substances that act at or close to their site of release, and their plasma levels remain well below concentrations required for a hormone-like action at distant receptors (Pacher et al., 2006). Therefore, the present findings strongly suggest that the steatogenic action of chronic ethanol intake is mediated through a paracrine mechanism, by endocannabinoids produced by and released from hepatic stellate cells that activate CB_1 receptors on adjacent hepatocytes to increase lipogenesis and decrease fatty acid oxidation in the liver (Figure 4F). As discussed above, the ethanol-induced elevation of 2-AG levels suggests its primary role, although anandamide may also be involved in spite of its unchanged tissue levels, in view of the observed upregulation of hepatic CB_1 receptor expression in ethanol-treated mice.

CB_1 receptors are also expressed in hepatic stellate cells, where their activation by endocannabinoids has recently been implicated in liver fibrogenesis (Teixeira-Clerc et al., 2006). Alcoholic fatty liver is a known risk factor for cirrhosis (Reuben, 2006); thus, endocannabinoids acting at CB_1 receptors in different types of liver cells may be involved in both the steatogenic and the fibrogenic effects of ethanol. 2-AG at high micromolar concentrations has been found to induce stellate cell apoptosis in vitro, which would be antifibrogenic (Siegmund et al., 2007). However, this is unlikely to occur at the lower concentrations detected in vivo in the present model, as an ethanol diet identical to the one used here inhibits rather than promotes stellate cell apoptosis (Jeong et al., 2008), and ethanol is also known to promote stellate cell proliferation (Friedman, 1999).

Although alcoholic fatty liver is reversible in its early stages by cessation of drinking, this is often not feasible. The present findings suggest that treatment with a CB_1 antagonist may slow the development of steatosis and thus prevent or delay its progression to more severe and irreversible forms of liver disease. Importantly, our finding that the steatogenic effect of ethanol specifically involves CB_1 receptors expressed in hepatocytes suggests that selective targeting of peripheral CB_1 receptors may be effective in this pathology, thereby reducing the potential for centrally mediated adverse effects of CB_1 blockade, such as anxiety and depression (Pacher et al., 2006). The additional antifibrogenic effect of CB_1 blockade could add to the benefit of such treatment. Rimonabant has recently been introduced in Europe for the treatment of visceral obesity and the metabolic syndrome, which themselves are known risk factors for steatosis and cirrhosis (Marceau et al., 1999). Clinical trials testing the effectiveness of CB_1 receptor blockers in the treatment of both alcoholic and nonalcoholic fatty liver and their more severe sequelae may be warranted.

EXPERIMENTAL PROCEDURES

Mice
C57BL/6J mice were purchased from the National Cancer Institute. Male mice (8 to 10 weeks old) were used in all experiments and were cared for in accordance with National Institutes of Health guidelines. $CB_1^{+/+}$ and $CB_1^{-/-}$ littermates were obtained by breeding heterozygotes that had been backcrossed to a C57BL/6J background, as described previously (Wang et al., 2003). Mice with hepatocyte-specific knockout of CB_1 receptors were generated by crossing mice homozygous for the CB_1 floxed allele ($CB_1^{f/f}$), which were on a predominantly C57BL/6N background (7–8 crosses) (Marsicano et al., 2003), with mice expressing the bacterial Cre recombinase driven by the mouse albumin promoter (*TG[Alb-cre]21Mgn*, from the Jackson Laboratory), which had been backcrossed to a C57BL/6J background to obtain $CB_1^{f/f}$ × $CB_1^{f/f;AlbCre}$ breeding pairs. The littermates obtained were therefore on a mixed C57BL/6N × J background. All experiments with knockout mice used the corresponding homozygous wild-type (+/+) littermates as controls. Genotyping by PCR for the *Cre* transgene was performed as described previously (Marsicano et al., 2003). Individually caged mice were placed on a Lieber-DeCarli low-fat liquid diet (Dyets) containing 1 kcal/ml, of which 18% was derived from protein, 12% from fat, and either 70% from carbohydrate (control diet) or 43% from carbohydrate and 27% from ethanol (ethanol diet). In some experiments, mice had free access to the diet, and food intake and body weight were monitored daily; in other experiments, mice were pair-fed with the control versus ethanol diet, as indicated. The mice were on these diets for a total of 26 days; ethanol was introduced gradually by increasing the content by 1% (v/v) each day until the mice were consuming a diet containing 5% (v/v) ethanol and was then continued for 3 more weeks. During ethanol feeding, wild-type mice were injected every other day with 10 mg/kg rimonabant (SR141716, obtained from the NIDA Drug Supply Program) or vehicle for 3 weeks. Because CB_1 receptor-deficient mice have increased sensitivity to the hypothermic effect of ethanol (Naassila et al., 2004), these animals were maintained on a heating pad at 37°C throughout the ethanol feeding period. At the end of this period, mice were sacrificed and liver tissues and trunk blood were collected. Although rimonabant is known to significantly reduce ethanol intake in C57BL/6 mice in a two-bottle free-choice paradigm with free access to solid food (Wang et al., 2003), the lack of a significant reduction per g of body weight in the present experiments is probably due to the fact that the liquid alcohol diet was the only nutrient or liquid available to the animals.

Blood Chemistry and Histology
Serum ALT and ethanol levels were assayed using kits from Drew Scientific and BioAssay Systems, respectively. Blood ethanol levels were measured in blood drawn via tail clips at 8 a.m. For histological documentation of fat accumulation in liver, 5 μm sections of paraffin-embedded tissue blocks were stained with hematoxylin and eosin and 10 μm frozen sections were stained with oil red O (Vector Laboratories) as described previously (Osei-Hyiaman et al., 2005a). For quantitation of fat content, six randomly selected oil red O-stained sections from each liver were scanned at 600× magnification using NIH ImageJ software (http://rsb.info.nih.gov/ij/).

Tissue Levels of Lipids and Endocannabinoids
For measuring triglyceride, cholesterol, and endocannabinoid levels in liver, mice were sacrificed and their livers were removed and extracted. Total hepatic triglyceride and cholesterol were measured as described previously (You et al., 2004). Anandamide and 2-AG levels were determined by liquid chromatography/mass spectrometry as described previously (Wang et al., 2003).

Western Immunoblots
Hepatocytes were isolated from liver using collagenase perfusion and then separated from nonparenchymal cells using density gradient centrifugation, as described previously (Sun et al., 2005). Proteins obtained from whole liver were quantified by Bradford assay (Bio-Rad), and nuclear extracts of liver were obtained using a nuclear/cytosol fractionation kit (BioVision). Primary antibodies used for blotting were anti-FAS, anti-AMPKα, anti-pAMPKα (Cell Signaling), anti-β-actin (Sigma), anti-CPT1, anti-CB_1 receptor (Alpha Diagnostics), and anti-SREBP-1c (US Biological). Immunoreactive bands were visualized on nitrocellulose membranes using alkaline phosphatase-linked anti-mouse or anti-rabbit antibody and the ECF detection system (Amersham Pharmacia). Band density was quantified by digital imaging using NIH ImageJ software.

RT-PCR and Real-Time PCR
mRNAs for CB_1 receptors, *SREBP-1c*, *FAS*, *MGL*, *DAGLα*, and *DAGLβ* were quantified using RT-PCR or real-time PCR. Primers for *SREBP-1c* and *FAS* were as described in detail elsewhere (Osei-Hyiaman et al., 2005a). Relative mRNA levels of *MGL*, *DAGLα*, and *DAGLβ* were quantified by real-time PCR of cDNA from whole liver, hepatocytes, or stellate cell mRNA from ethanol-fed (n = 4) or pair-fed (n = 4) mice. The predesigned QuantiTect Primer Assay (QIAGEN) containing gene-specific forward and reverse primer pairs was used for *MGL* (catalog number QT01163428), *DAGLα* (catalog number QT00167706), and *DAGLβ* (catalog number QT00173453).

CPT1 Assay
CPT1 activity in mitochondrial protein isolated from whole liver was quantified by the incorporation of [^{3}H]methylcarnitine into carnitine palmitoyl-CoA, as described previously (Bremer, 1981).

Isolation of Pure Fractions of Hepatocytes and Hepatic Stellate Cells
Mouse hepatic stellate cells were isolated via in situ collagenase perfusion and differential centrifugation on OptiPrep (Sigma) density gradients as described in detail previously (Jeong et al., 2006).

Coculture of Isolated Hepatocytes and Hepatic Stellate Cells
Isolated hepatocytes were resuspended in RPMI 1640 medium containing penicillin, streptomycin, and 10% FBS, plated onto six-well plates at a density of 5×10^5 cells per well in 1.5 ml culture medium, and cultured for 6 hr. The isolated hepatocytes were then cultured in serum-free medium overnight (serum starvation), coincubated with hepatic stellate cells isolated from ethanol-fed or pair-fed mice for 24 or 48 hr, and loaded onto cell-culture inserts of 3 μm pore size (Corning) in order to keep the two types of cells separate, as described previously (Nieto et al., 2002). The ratio of hepatocytes to hepatic stellate cells was 5:1, similar to the ratio of parenchymal to nonparenchymal cells in liver.

Statistical Analyses
Data are expressed as means ± SEM. To compare values obtained from two groups, Student's t test or one-way ANOVA was performed as appropriate. $p < 0.05$ was considered significant.

SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/7/3/227/DC1/.

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REFERENCES

Arakawa, M., Taketomi, S., Furuno, K., Matsuo, T., and Iwatsuka, H. (1975). Metabolic studies on the development of ethanol-induced fatty liver in KK-Ay mice. J. Nutr. *105*, 1500–1508.

Basavarajappa, B.S., Saito, M., Cooper, T.B., and Hungund, B.L. (2000). Stimulation of cannabinoid receptor agonist 2-arachidonylglycerol by chronic ethanol and its modulation by specific neuromodulators in cerebellar granule neurons. Biochim. Biophys. Acta *1535*, 76–86.

Bergheim, I., Guo, L., Davis, M.A., Lambert, J.C., Beier, J.I., Duveau, I., Luyendyk, J.P., Roth, R.A., and Arteel, G.E. (2006). Metformin prevents alcohol-induced liver injury in the mouse: critical role of plasminogen activator inhibitor-1. Gastroenterology *130*, 2099–2112.

Bisogno, T., Howell, F., Williams, G., Minassi, A., Cascio, M.G., Ligresti, A., Matias, I., Schiano-Moriello, A., Paul, P., Williams, E.J., et al. (2003). Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. J. Cell Biol. *163*, 463–468.

Blüher, M., Engeli, S., Klöting, N., Berndt, J., Fasshauer, M., Bátkai, S., Pacher, P., Schön, M.R., Jordan, J., and Stumvoll, M. (2006). Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. Diabetes *55*, 3053–3060.

Bremer, J. (1981). The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. Biochim. Biophys. Acta *665*, 628–631.

Côté, M., Matias, I., Lemieux, I., Petrosino, S., Alméras, N., Després, J.P., and Di Marzo, V. (2007). Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. Int. J. Obes. (Lond.) *31*, 692–699.

Cunnane, S.C. (1987). Hepatic triacylglycerol accumulation induced by ethanol and carbon tetrachloride: interactions with essential fatty acids and prostaglandins. Alcohol. Clin. Exp. Res. *11*, 25–31.

Després, J.P., and Lemieux, I. (2006). Abdominal obesity and metabolic syndrome. Nature *444*, 881–887.

Dinh, T.P., Carpenter, D., Leslie, F.M., Freund, T.F., Katona, I., Sensi, S.L., Kathuria, S., and Piomelli, D. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc. Natl. Acad. Sci. USA *99*, 10819–10824.

Friedman, S.L. (1999). Stellate cell activation in alcoholic fibrosis–an overview. Alcohol. Clin. Exp. Res. *23*, 904–910.

Garcia-Villafranca, J., Guillen, A., and Castro, J. (2007). Ethanol consumption impairs regulation of fatty acid metabolism by decreasing the activity of AMP-activated kinase in rat liver. Biochimie. Published online October 5, 2007. 10.1016/j.biochi.2007.09.019.

Gary-Bobo, M., Elachouri, G., Gallas, J.F., Janiak, P., Marini, P., Ravinet-Trillou, C., Chabbert, M., Cruccioli, N., Pfersdorff, C., Roque, C., et al. (2007). Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker fa/fa rats. Hepatology *46*, 122–129.

Hézode, C., Zafrani, E.S., Roudot-Thoraval, F., Costentin, C., Hessami, A., Bouvier-Alias, M., Mekour, F., Pawlostky, J.M., Lotersztajn, S., and Mallat, A. (2008). Daily cannabis use, a novel risk factor of steatosis severity in patients with chronic hepatitis C. Gastroenterology *134*, 432–439. Published online November 27, 2007. 10.1053/j.gastro.2007.11.039.

Jeong, W.I., Park, O., Radaeva, S., and Gao, B. (2006). STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. Hepatology *44*, 1441–1451.

Jeong, W.I., Park, O., and Gao, B. (2008). Abrogation of the antifibrotic effects of natural killer cells/interferon-gamma contributes to alcohol acceleration of liver fibrosis. Gastroenterology *134*, 248–258. Published online September 29, 2007. 10.1053/j.gastro.2007.09.034.

Ji, C., Chan, C., and Kaplowitz, N. (2006). Predominant role of sterol response element binding proteins (SREBP) lipogenic pathways in hepatic steatosis in the murine intragastric ethanol feeding model. J. Hepatol. *45*, 717–724.

Kola, B., Hubina, E., Tucci, S.A., Kirkham, T.C., Garcia, E.A., Mitchell, S.E., Williams, L.M., Hawley, S.A., Hardie, D.G., Grossman, A.B., et al. (2005). Cannabinoids and ghrelin have both central and peripheral metabolic and cardiac effects via AMP-activated protein kinase. J. Biol. Chem. *280*, 25196–25201.

Lieber, C.S., and Schmid, R. (1961). The effect of ethanol on fatty acid metabolism: stimulation of hepatic fatty acid synthesis in vitro. J. Clin. Invest. *40*, 394–399.

Lieber, C.S., Spritz, N., and DeCarli, L.M. (1966). Role of dietary, adipose and endogenously synthesized fatty acids in the pathogenesis of the alcoholic fatty liver. J. Clin. Invest. *45*, 51–62.

Lin, J., Yang, R., Tarr, P.T., Wu, P.H., Handschin, C., Li, S., Yang, W., Pei, L., Uldry, M., Tontonoz, P., et al. (2005). Hyperlipidemic effects of dietary saturated fats mediated through PGC-1β coactivation of SREBP. Cell *120*, 261–273.

Liu, J., Batkai, S., Pacher, P., Harvey-White, J., Wagner, J.A., Cravatt, B.F., Gao, B., and Kunos, G. (2003). Lipopolysaccharide induces anandamide synthesis in macrophages via CD14/MAPK/phosphoinositide 3-kinase/NF-kappaB independently of platelet-activating factor. J. Biol. Chem. *278*, 45034–45039.

Marceau, P., Biron, S., Hould, F.S., Marceau, S., Simard, S., Thung, S.N., and Kral, J.G. (1999). Liver pathology and the metabolic syndrome X in severe obesity. J. Clin. Endocrinol. Metab. *84*, 1513–1517.

Marsicano, G., Wotjak, C.T., Azad, S.C., Bisogno, T., Rammes, G., Cascio, M.G., Hermann, H., Tang, J., Hofmann, C., Zieglgänsberger, W., et al. (2002). The endogenous cannabinoid system controls extinction of aversive memories. Nature *418*, 530–534.

Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., Azad, S.C., Cascio, M.G., Gutiérrez, S.O., van der Stelt, M., et al. (2003). CB1 cannabinoid receptors and on-demand defense against excitotoxicity. Science *302*, 84–88.

Martinez-Chantar, M.L., Vázquez-Chantada, M., Garnacho, M., Latasa, M.U., Varela-Rey, M., Dotor, J., Santamaria, M., Martínez-Cruz, L.A., Parada, L.A., Lu, S.C., et al. (2006). S-adenosylmethionine regulates cytoplasmic HuR via AMP-activated kinase. Gastroenterology *131*, 223–232.

Matias, I., and Di Marzo, V. (2007). Endocannabinoids and the control of energy balance. Trends Endocrinol. Metab. *18*, 27–37.

Muoio, D.M., Seefeld, K., Witters, L.A., and Coleman, R.A. (1999). AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. Biochem. J. *338*, 783–791.

Naassila, M., Pierrefiche, O., Ledent, C., and Daoust, M. (2004). Decreased alcohol self-administration and increased alcohol sensitivity and withdrawal in CB1 receptor knockout mice. Neuropharmacology *46*, 243–253.

Nieto, N., Friedman, S.L., and Cederbaum, A.I. (2002). Stimulation and proliferation of primary rat hepatic stellate cells by cytochrome P450 2E1-derived reactive oxygen species. Hepatology *35*, 62–73.

Osei-Hyiaman, D., DePetrillo, M., Pacher, P., Liu, J., Radaeva, S., Bátkai, S., Harvey-White, J., Mackie, K., Offertáler, L., Wang, L., et al. (2005a). Endocannabinoid activation of hepatic CB_1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. J. Clin. Invest. *115*, 1298–1305.

Osei-Hyiaman, D., DePetrillo, M., Harvey-White, J., Bannon, A.W., Cravatt, B.F., Kuhar, M.J., Mackie, K., Palkovits, M., and Kunos, G. (2005b). Cocaine- and amphetamine-related transcript is involved in the orexigenic effect of endogenous anandamide. Neuroendocrinology *81*, 273–282.

Pacher, P., Bátkai, S., and Kunos, G. (2006). The endocannabinoid system as an emerging target for pharmacotherapy. Pharmacol. Rev. *58*, 389–462.

Paik, Y.H., Schwabe, R.F., Bataller, R., Russo, M.P., Jobin, C., and Brenner, D.A. (2003). Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. Hepatology *37*, 1043–1055.

Pocai, A., Obici, S., Schwartz, G.J., and Rossetti, L. (2005). A brain-liver circuit regulates glucose homeostasis. Cell Metab. *1*, 53–61.

Ravinet Trillou, C., Arnone, M., Delgorge, C., Gonalons, N., Keane, P., Maffrand, J.P., and Soubrie, P. (2003). Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. *284*, R345–R353. Published online October 24, 2002. 10.1152/ajpregu.00545.2002.

Ravinet Trillou, C., Delgorge, C., Menet, C., Arnone, M., and Soubrié, P. (2004). CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. Int. J. Obes. Relat. Metab. Disord. *28*, 640–648.

Reuben, A. (2006). Alcohol and the liver. Curr. Opin. Gastroenterol. *22*, 263–271.

Sampath, H., Miyazaki, M., Dobrzyn, A., and Ntambi, J.M. (2007). Stearoyl CoA-desaturase-1 mediates the pro-lipogenic effects of dietary saturated fat. J. Biol. Chem. *282*, 2483–2493.

Savage, D.B., Choi, C.S., Samuel, V.T., Liu, Z.X., Zhang, D., Wang, A., Zhang, X.M., Cline, G.W., Yu, X.X., Geisler, J.G., et al. (2006). Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. J. Clin. Invest. *116*, 817–824.

Siegmund, S.V., Qian, T., de Minicis, S., Harvey-White, J., Kunos, G., Vinod, K.Y., Hungund, B., and Schwabe, R.F. (2007). The endocannabinoid 2-arachidonoyl glycerol induces death of hepatic stellate cells via mitochondrial reactive oxygen species. FASEB J. *21*, 2798–2806.

Sun, R., Jaruga, B., Kulkarni, S., Sun, H., and Gao, B. (2005). IL-6 modulates hepatocyte proliferation via induction of HGF/p21^{cip1}: regulation by SOCS2. Biochem. Biophys. Res. Commun. *338*, 1943–1949.

Suzuki, A., Kusakai, G., Shimojo, Y., Chen, J., Ogura, T., Kobayashi, M., and Esumi, H. (2005). Involvement of TGF-β1 signaling in hypoxia-induced tolerance to glucose starvation. J. Biol. Chem. *280*, 31557–31563.

Tahara, M., Matsumoto, K., Nukiwa, T., and Nakamura, T. (1999). hepatocyte growth factor leads to recovery from alcohol-induced fatty liver in rats. J. Clin. Invest. *103*, 313–320.

Tamai, H., Horie, Y., Kato, S., Yokoyama, H., and Ishii, H. (2002). Long-term ethanol feeding enhances susceptibility of the liver to orally administered lipopolysaccharides in rats. Alcohol. Clin. Exp. Res. *26*, 75S–80S.

Teixeira-Clerc, F., Julien, B., Grenard, P., Tran Van Nhieu, J., Deveaux, V., Li, L., Serriere-Lanneau, V., Ledent, C., Mallat, A., and Lotersztajn, S. (2006). CB_1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. Nat. Med. *12*, 671–676.

Thurman, R.G. (1998). II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. Am. J. Physiol. *275*, G605–G611.

Tomita, K., Tamiya, G., Ando, S., Kitamura, N., Koizumi, H., Kato, S., Horie, Y., Kaneko, T., Azuma, T., Nagata, H., et al. (2005). AICAR, an AMPK activator, has protective effects on alcohol-induced fatty liver in rats. Alcohol. Clin. Exp. Res. *29*, 240s–245s.

Varga, K., Wagner, J.A., Bridgen, D.T., and Kunos, G. (1998). Platelet- and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension. FASEB J. *12*, 1035–1044.

Wang, L., Liu, J., Harvey-White, J., Zimmer, A., and Kunos, G. (2003). Endocannabinoid signaling via cannabinoid receptor 1 is involved in ethanol preference and its age-dependent decline in mice. Proc. Natl. Acad. Sci. USA *100*, 1393–1398.

Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., et al. (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat. Med. *8*, 1288–1295.

You, M., Fischer, M., Deeg, M.A., and Crabb, D.W. (2002). Ethanol induces fatty acid synthesis pathways by activation of sterol response element-binding protein (SREBP). J. Biol. Chem. *277*, 29342–29347.

You, M., Matsumoto, M., Pacold, C.M., Cho, W.K., and Crabb, D.W. (2004). The role of AMP-activated protein kinase in the action of ethanol in the liver. Gastroenterology *127*, 1798–1808.