Fatty liver is commonly associated with alcohol ingestion and abuse. While the molecular pathogenesis of these fatty changes is well understood, the biochemical and pharmacological mechanisms by which ethanol stimulates these molecular changes remain unknown. During ethanol metabolism, adenosine is generated by the enzyme ecto-5′-nucleotidase, and adenosine production and adenosine receptor activation are known to play critical roles in the development of hepatic fibrosis. We therefore investigated whether adenosine and its receptors play a role in the development of alcohol-induced fatty liver. WT mice fed ethanol on the Lieber-DeCarli diet developed hepatic steatosis, including increased hepatic triglyceride content, while mice lacking ecto-5′-nucleotidase or adenosine A1 or A2B receptors were protected from developing fatty liver. Similar protection was also seen in WT mice treated with either an adenosine A1 or A2B receptor antagonist. Steatotic livers demonstrated increased expression of genes involved in fatty acid synthesis, which was prevented by blockade of adenosine A1 receptors, and decreased expression of genes involved in fatty acid metabolism, which was prevented by blockade of adenosine A2B receptors. In vitro studies supported roles for adenosine A1 receptors in promoting fatty acid synthesis and for A2B receptors in decreasing fatty acid metabolism. These results indicate that adenosine generated by ethanol metabolism plays an important role in ethanol-induced hepatic steatosis via both A1 and A2B receptors and suggest that targeting adenosine receptors may be effective in the prevention of alcohol-induced fatty liver.

Introduction

Fatty liver is the most common and earliest response of the liver to heavy alcohol consumption and may develop into alcoholic hepatitis and fibrosis. Although fatty liver is a very common medical problem and the molecular events involved in the pathogenesis of fatty liver are well understood, the connection between ethanol ingestion and metabolism and the activation of the events involved in the development of hepatic steatosis is not well understood.

Conflict of interests: Z. Peng and B.N. Cronstein have submitted an application for a patent on the use of adenosine A1 and A2B receptor antagonists to treat fatty liver. B.N. Cronstein’s other intellectual property interests include patents on use of adenosine A1 receptor antagonists to promote wound healing and use of A2B receptor antagonists to inhibit fibrosis, a patent on the use of adenosine A1 receptor antagonists to treat osteoporosis and other diseases of bone, and an application for a patent on the use of adenosine A2B receptor antagonists to prevent prosthesis loosening. B.N. Cronstein has received equity in Can-Fite BioPharma Ltd. for his services on the Scientific Advisory Board. B.N. Cronstein receives grant support from the NIH, King Pharmaceuticals, and the Vliek Foundation and serves on the Board of Trustees of the Vliek Foundation, the Arthritis Foundation — New York Chapter and the SLE Lupus Foundation Inc. The remaining authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; A1KO mice, adenosine A1 receptor knockout mice; AMPK, AMP-activated kinase; AST, aspartate aminotransferase; CD73KO mice, CD73-knockout mice; CPA, N′-cyclopentyladenosine; CPT, carnitine palmitoyltransferase; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; FAS, fatty acid synthase; MRE 2029F20, kinase; AST, aspartate aminotransferase; CD73KO mice, CD73-knockout mice; CPA, N′-cyclopentyladenosine; CPT, carnitine palmitoyltransferase; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; FAS, fatty acid synthase; MRE 2029F20, kinase; ZM 241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4] triazolol[3,4-a][1,3,5-triazin-5-ylamino][ethy]phenol.

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Ethanol is sequentially metabolized to acetaldehyde and acetate by the actions of alcohol dehydrogenase and aldehyde dehydrogenase, respectively. Acetate is further metabolized to acetyl-CoA accompanied by the catabolism of ATP to AMP. Ethanol is well known to stimulate increased extracellular adenosine concentration in vitro through its action on the nucleoside transporter, and ethanol ingestion increases purine release into the bloodstream and urine in normal volunteers (1–3) and into the extracellular space in liver slices from ethanol-treated mice and those from cultured hepatocyte cell line (HepG2) (4, 5). Adenosine is present in and released from nearly all mammalian tissues and organs, and increased adenosine concentrations result from either increased export of adenosine, diminished uptake of adenosine, or cellular release of adenosine nucleotides, which are dephosphorylated extracellularly to adenosine (6). Increasing evidence indicates that most extracellular adenosine is derived from adenosine nucleotides released from cells, extracellular ATP and ADP are dephosphorylated to AMP by the action of nucleoside triphosphate phosphohydrolase (CD39) or other phosphatases, and AMP is further dephosphorylated to adenosine by ecto-5′-nucleotidase (CD73) or alkaline phosphatase (7, 8). Extracellular adenosine regulates a variety of physical processes (9) and adenosine’s effects are mediated by a family of 4 G protein–coupled receptors, A1, A2A, A2B, and A3, each of which has a unique pharmacological profile, tissue distribution, and effector coupling (10).

Because prior studies have demonstrated a role for adenosine and its receptors in the regulation of hepatic fibrosis (4, 5), hepatic ureagenesis (11, 12), and glycogen metabolism (13, 14) as well as peripheral lipid metabolism (15, 16), we determined whether adenosine and its receptors play a role in the pathogenesis of hepatic
steatosis induced by ethanol ingestion. Here, we report evidence that ethanol-mediated increases in extracellular adenosine, acting via adenosine A_1 and A_2B receptors, link the ingestion and metabolism of ethanol to the development of hepatic steatosis.

**Results**

**Deletion of ecto-5′-nucleotidase prevents the development of ethanol-induced fatty liver in mice.** We have previously demonstrated that livers from mice that have been exposed to ethanol release more adenosine ex vivo than livers of mice that were not exposed to ethanol, and the increased adenosine release depends on extracellular dephosphorylation of AMP to adenosine by CD73 (5). We therefore determined whether CD73-dependent adenosine accumulation plays a role in development of ethanol-induced hepatic steatosis. WT mice developed severe hepatic steatosis after chronic ethanol ingestion but CD73-knockout mice (CD73KO mice) suffered only minimal fatty change (Figure 1, A and C). Consistent with the histological appearance, the hepatic triglyceride levels were much lower in ethanol-fed CD73KO mice than in WT mice (Figure 1G). Serum aspartate aminotransferase (AST) and triglyceride levels were significantly lower in CD73KO mice versus control WT mice as well (Table 1 and Figure 1E and F).

**Deletion of adenosine A_1 or A_2B receptors protects mice from developing ethanol-induced fatty liver.** Adenosine and its receptors play a role in...
The pharmacologic effects of ethanol (reviewed in refs. 17–19), so we studied ethanol-induced hepatic steatosis in mice lacking adenosine A<sub>1</sub>, A<sub>2a</sub>, and A<sub>2b</sub> receptors. As shown in Figure 2A, adenosine A<sub>1</sub> and A<sub>2a</sub> but not A<sub>2b</sub> receptor deletion prevents ethanol-induced hepatic steatosis (Figure 2, A and B). Interestingly, the liver/body weight ratio did not differ among ethanol-treated WT mice and adenosine A<sub>1</sub> receptor knockout mice (A1KO mice) or A2BK0 mice (Figure 2D). Hepatic triglyceride content and steatosis grade was lower in the A1KO and A2BK0 but not A2AKO mice (Figure 2, C and G), and serum triglyceride and AST levels were significantly lower in the A1KO and A2BK0 but not the A2AKO mice as well (Table 1 and Figure 2, E and F).

Blockade of adenosine A<sub>1</sub> or A<sub>2a</sub> receptors protects mice from developing ethanol-induced fatty liver. Serum triglyceride and AST levels were elevated in the ethanol-treated WT mice, and AST and triglyceride levels were significantly lower in both enprofylline-treated (A2b receptor antagonist) and 1,3 dipropyl-8-cyclopentylxanthine-treated (DPCPX-treated) (A1 receptor antagonist) mice, although there was a greater decrease in serum triglyceride levels in the enprofylline-treated mice (Table 1 and Figure 3, D and E). Similar to the knockout mice, there were significant reductions in hepatic triglyceride levels in the antagonist-treated mice compared with the vehicle-treated mice, although the levels did not return to those of mice not exposed to ethanol (Figure 3F). As with the hepatic triglyceride level, the hepatic steatosis grade was significantly decreased in all of the antagonist-treated mice compared with vehicle-treated mice (Figure 3B). Interestingly, the reduction in steatosis grade was greater in the receptor knockout and antagonist-treated mice than in CD73KO mice (Figure 1B and Figure 3B).

Ethanol ingestion increases hepatic expression of mRNA for ecto-5′-nucleotidase and adenosine A<sub>1</sub>, A<sub>2a</sub>, and A<sub>2b</sub> receptors. Hepatic adenosine receptor mRNA expression increases after chronic CCL4 treatment (4, 5). We therefore asked whether chronic ethanol treatment also regulates expression of CD73 and adenosine receptors. Chronic ethanol ingestion leads to a significant increase in CD73 and adenosine A<sub>1</sub>, A<sub>2b</sub>, and A<sub>2a</sub> receptor mRNA (AIR, A2bR, and A2aR) expression as compared with corresponding control mice, respectively (Figure 4A). Interestingly, the increase in mRNA for CD73, AIR, and A2bR was greater than that for A2aR (Figure 4A).

Ethanol ingestion modulates expression of hepatic transcription factors SREBP1 and PPAR<sub>α</sub> and hepatic enzymes and transporters involved in fatty acid synthesis, metabolism, and transport. Increased or decreased expression/function of specific transcriptional regulators plays a role in the development of hepatic steatosis: SREBP1 increases expression of genes/proteins involved in lipid synthesis (including ATP citrate lyase [ACL] and fatty acid synthase [FAS]) (20–22), PPAR<sub>α</sub> regulates proteins involved in fatty acid oxidation (e.g., acetyl-CoA carboxylase [ACC] and carnitine palmitoyltransferase [CPT]) (23–25), and PPAR<sub>γ</sub> mainly regulates lipogenesis (26–29). We therefore measured expression of mRNA for Srebp1, Pparg, Ppard, A2aR, and A1R. Chronic ethanol ingestion increased mRNA expression of Srebp1, Pparg, Ppard, and Acca in livers from WT, A2BK0, and enprofylline-treated mice, but adenosine A<sub>1</sub> receptor deletion or blockade (DPCPX treatment) prevented the ethanol-induced increase in expression (Figure 4, B–D). In contrast, ethanol ingestion reduced mRNA expression of Ppara, Cpt1a1, and Acca in the livers of WT, A1KO, and DPCPX-treated mice, but deletion or blockade of A<sub>2b</sub> receptors abrogated the ethanol-induced reduction in expression (Figure 4, C, G, and H).

A<sub>1</sub> and A<sub>2b</sub> adenosine receptor agonists promote lipid accumulation in a cultured murine hepatocyte cell line. To better understand how adenosine A<sub>1</sub> and A<sub>2b</sub> receptors are involved in the formation of fatty liver in vivo, we examined the effect of selective A<sub>1</sub> and A<sub>2b</sub> receptor agonists and antagonists on development of steatosis in an hepatocyte cell line (AML-12). AML-12 cells express mRNA for all 4 adenosine receptors (data not shown). Following treatment with the A<sub>1</sub> receptor agonist N<sup>6</sup>-cyclopentyladenosine (CPA) or the nonselective adenosine receptor agonist 5′-N-ethylcarboxamidoadenosine (NECA) at a concentration that activates A<sub>2b</sub> receptors, AML-12 cells accumulated lipid, as demonstrated by Oil Red O staining, and increased their intracellular triglyceride levels in a dose-dependent fashion (Figure 5, A–C). Addition of the adenosine A<sub>1</sub> receptor antagonist MRS1706, respectively, abrogated the effects of A<sub>1</sub> and A<sub>2b</sub> receptor agonists on hepatocyte triglyceride accumulation, which neither A2AKO nor A<sub>1</sub> adenosine receptor blockade reversed the effects of either CPA or NECA on triglyceride accumulation (Figure 5, A, D, and E).

Adenosine A<sub>1</sub> and A<sub>2b</sub> receptors regulate nuclear SREBP1, PPAR<sub>α</sub>, and PPAR<sub>γ</sub> protein levels and transcriptional activity in AML-12 cells. We studied the effects of adenosine A<sub>1</sub> and A<sub>2b</sub> receptor agonists and antagonists on nuclear SREBP1, PPAR<sub>α</sub>, and PPAR<sub>γ</sub> protein levels and the transcriptional activities of PPAR<sub>α</sub> and PPAR<sub>γ</sub> in AML-12 cells. Nuclear SREBP1 and PPAR<sub>γ</sub> but not PPAR<sub>α</sub> protein levels were significantly increased after CPA treatment, effects that were completely blocked by DPCPX (Figure 6, A, B, and D). In contrast, nuclear PPAR<sub>α</sub> protein levels were markedly diminished following NECA treatment, and the selective A<sub>2b</sub> receptor antagonist MRS1706 abrogated this decrease (Figure 6, A and C). A<sub>1</sub> receptor activation increased PPAR<sub>γ</sub> but not PPAR<sub>α</sub>.

<table>
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<tr>
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<tr>
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</table>

Mice were fed an isocaloric liquid Lieber-DeCarli diet containing either ethanol or maltose, as described in Methods. ALT, alanine aminotransferase; enpro, enprofylline.

<sup>†</sup><sup>‡</sup><sup>§</sup> P < 0.01, ethanol versus control; <sup>†</sup><sup>‡</sup> P < 0.01, DPCPX or enprofylline versus WT ethanol; <sup>§</sup> P < 0.01, DPCPX or enprofylline versus WT control or WT ethanol, respectively.

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Table 1

Mouse body weight changes and serum ALT and AST levels

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Figure 2
Deletion of adenosine A₁ or A₂B receptors protects mice from developing ethanol-induced fatty liver. Eight-week-old male mice (WT, A1KO, A2BKO, A2AWT, and A2AKO mice) were fed a liquid diet containing ethanol or an equal caloric diet containing maltose for 6 weeks and then sacrificed. The livers and bodies of the mice were weighed on the day of sacrifice and the liver/total body weight ratio was calculated. The serum AST and triglyceride and hepatic tissue triglyceride levels were also measured, as described in Methods. The hepatic steatosis grade was based on the percentage of steatotic hepatocytes in the H&E-stained liver sections. (A) H&E-stained liver sections from maltose- and ethanol-treated A1KO, A2BKO, A2AWT, and A2AKO mice (original magnification, ×400). (B) Oil Red O–stained liver sections from ethanol-treated A1KO and A2BKO mice (original magnification, ×400). (C) Hepatic steatosis grades of ethanol-treated WT, A1KO, A2BKO, A2AWT, and A2AKO mice. Steatosis grades in maltose-treated WT, A1KO, A2BKO, A2AWT, and A2AKO mice were 0. (D) Liver/body weight ratio. (E) Serum AST levels. (F) Serum triglyceride levels. (G) Hepatic tissue triglyceride levels. *P < 0.01, A1KO mice or A2BKO mice versus WT mice, respectively; **P < 0.01, ethanol mice versus control mice in different groups, respectively; ***P < 0.01, ethanol KO mice versus control KO mice or control WT mice or ethanol WT mice, respectively; n = 5–10 for each group of mice.
transcriptional activity, whereas A<sub>2B</sub> receptor activation increased PPARα transcriptional activity (Figure 6, E and F). These results are consistent with the hypothesis that adenosine A<sub>1</sub> and A<sub>2B</sub> receptors regulate hepatic fat metabolism by increasing or decreasing appropriate transcriptional regulators.

Adenosine A<sub>1</sub> and A<sub>2B</sub> receptors regulate enzymes involved in fatty acid synthesis and oxidation downstream of the transcriptional regulators that are regulated by adenosine receptor occupancy. The expression of the fatty acid synthetic enzymes ACL and FAS is regulated by SREBP1 and PPARγ, whereas expression of the fatty acid metabolic enzyme ACCα and the fatty acid transporter CPTI is regulated by PPARα. Cellular ACL and FAS protein levels were significantly increased after A<sub>1</sub> receptor activation, without any effect on ACCα or CPTI protein levels, while the A<sub>1</sub> antagonist DPCPX completely reversed this increase (Figure 7).

In contrast, A<sub>2B</sub> receptor activation diminished ACCα and CPTI protein levels but did not affect ACL or FAS levels, and A<sub>2B</sub> receptor blockade completely abrogated this effect (Figure 7). These results are consistent with the effects of ethanol and deletion or blockade of adenosine A<sub>1</sub> or A<sub>2B</sub> receptors on expression of mRNA for these proteins observed in vivo.

Adenosine A<sub>2B</sub> receptors regulate the phosphorylation of a critical signaling molecule controlling the pathways of hepatic fatty acid oxidation. AMP-activated kinase (AMPK) is a critical signaling molecule controlling hepatic fatty acid oxidation (30, 31). Therefore, we determined whether adenosine receptor occupancy regulates the phosphorylation and, presumably, activation of this critical signaling enzyme. Following ethanol treatment, phosphorylated/total AMPK ratio was significantly decreased in the hepatic tissues of WT mice, A1KO mice, and DPCPX-treated mice (Figure 8). In contrast, the phosphorylated/total AMPK ratio was not nearly as diminished in the A2BKO and enprofylline-treated mice although the ratio did not return to the levels observed in the mice on a control diet (Figure 8, A and B). Similarly, when studied in vitro, NECA decreased phosphorylated/total AMPK ratio in AML-12 cells, and the specific adenosine A<sub>2B</sub> antagonist MRS1706 blocked this effect (Figure 8, C and D). These results clearly demonstrate that hepatic adenosine A<sub>2B</sub> receptors alter the phosphorylation and, by inference, the activity of AMPK, a critical signaling intermediate in the regulation of fatty acid oxidation.
Adenosine A1, A2A, A2B, and A3 receptors are present in human liver, and there are more A2A and A2B receptors in steatotic and cirrhotic livers. Because adenosine receptor expression in mice may not reflect expression levels in humans, we studied the binding characteristics of human adenosine receptor subtypes in healthy or pathologic human liver tissue by radioligand binding studies. Adenosine A1, A2A, A2B, and A3 receptors are present in healthy human liver membranes. In plasma membrane preparations from cirrhotic and steatotic livers, there were significantly more adenosine A2A and A2B receptors, without any change in numbers of A1 or A3 receptors (Table 2).

Discussion

The results reported here demonstrate what we believe to be a novel biochemical and pharmacological mechanism for alcohol-induced fatty liver, a common medical problem. Our results demonstrate that, consistent with results of prior in vitro and in vivo studies in animals and humans, ethanol promotes hepatic adenosine nucleotide release (1, 2), which is subsequently dephosphorylated extracellularly to adenosine by the action of CD73 (5). As previously reported, adenosine levels are further increased as a result of diminished adenosine uptake in the liver (32, 33). Chronic alcohol-stimulated adenosine release stimulates adenosine A1 and A2B receptors, which promote the development of fatty liver, since blockade or deletion of these receptors in vivo and blockade of these receptors in vitro diminishes hepatic triglyceride accumulation and development of fatty liver.

Adenosine and its receptors regulate a variety of hepatic and hepatocellular functions, including glucose release (34, 35), protein synthesis (36), glutathione synthesis (37), hepatic regulation of renal Na+ and water excretion, and portal blood flow (18, 38–40). Moreover, ethanol- and acetate-induced adenosine release mediates many of these effects. Thus, although the effects of adenosine (whether exogenous or released in response to either ethanol or acetate) and its receptors on hepatic triglyceride metabolism have...
not previously been explored, the demonstration that adenosine and its receptors mediate ethanol-induced changes in hepatic function is not without precedent.

Previous studies provide indirect support for a role for adenosine and its receptors in the pathogenesis of fatty liver. Muroyama et al. (41) found that ingestion of a mixture of caffeine, arginine, thiamine, and citric acid reduced body fat, triceps skinfold thickness, and serum triglyceride levels in healthy human subjects with a high percentage of body fat and was effective in reducing visceral fat, including liver fat, in obese subjects. Caffeine, a nonselective adenosine receptor antagonist, in combination with vitamins and arginine, significantly suppressed an increase in hepatic lipid content in fasted and refed diabetic KK mice (42, 43). Thus, adenosine receptors may also play a role in the pathogenesis of nonalcoholic fatty liver as well.

Figure 5
Adenosine A<sub>1</sub> and A<sub>2B</sub> agonists promote fat accumulation in a cultured murine hepatocyte cell line (AML-12 cells). Cells were treated with adenosine receptor agonists or antagonists or their combination (A<sub>1</sub> receptor agonist CPA, 1 μM; DPCPX, 1 μM; A<sub>2A</sub> receptor antagonist ZM 241385 [ZM], 1 μM; nonselective and A<sub>2B</sub> receptor agonist NECA, 10 μM; A<sub>2B</sub> receptor antagonist MRS1706, 1 μM; A<sub>3</sub> receptor antagonist 3-ethyl-5-benzyl-2-methyl-4-phenylethylnyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate [MRS1191], 1 μM) for 24 hours, then stained with Oil Red O, or collected and the cellular triglyceride content was measured. (A) Oil red O staining of AML-12 hepatocytic cells (original magnification, ×400). (B) Curve of cellular triglyceride content of AML-12 cells with CPA concentration. (C) Curve of cellular triglyceride content of AML-12 cells with NECA concentration. (D) Cellular triglyceride content of AML-12 cells after CPA or antagonists or CPA combined with antagonist treatment. (E) Cellular triglyceride content of AML-12 cells after NECA or NECA combined with antagonist treatment. The data are expressed as percentages of control (mean ± SD) from 4 independent experiments. *P < 0.01, CPA or CPA plus ZM, MRS1706, or MRS1191 or NECA, NECA plus ZM or MRS1191 versus control, respectively; **P < 0.01, CPA plus DPCPX versus CPA; ***P < 0.01, NECA plus DPCPX versus control or NECA, respectively; ****P < 0.01, NECA plus MRS1706 versus NECA or NECA plus DPCPX, respectively.
Recently, Osei-Hyiaman et al. (44) found that endocannabinoid activation of hepatic cannabinoid receptors (CB\(_1\) receptors) is associated with development of diet-induced hepatic steatosis in mice, and others have reported that ethanol ingestion leads to stellate cell production of endocannabinoids, which activate hepatic CB\(_1\) receptors on hepatocytes, leading to hepatic steatosis (45). Treatment of rats with cannabinoid receptor antagonists prevents the development of fatty liver in animal models as well (46, 47). In the CNS, adenosine A\(_1\) receptors are tightly linked to CB\(_1\) receptors and these receptors cross-activate and cross-desensitize each other (48–51), although their interaction in the liver has not been explored. The results reported here are consistent with and expand upon the known link between cannabinoid receptors and adenosine receptors and further suggest that adenosine receptors play a role in nonalcoholic hepatic steatosis.

Prior studies, as well as the results reported here, clearly demonstrate that adenosine A\(_1\), A\(_2A\), A\(_2B\), and A\(_3\) receptors are expressed on hepatocytes (11–14, 52, 53) and chronic ethanol ingestion increases A\(_1\), A\(_2A\), and A\(_2B\) receptor expression in the liver. Adenosine receptors are expressed ubiquitously and other cell types in the liver express adenosine A\(_1\), A\(_2A\), and A\(_2B\) receptors (4, 5, 54); Kupffer cells and sinusoidal endothelial cells express adenosine A\(_2A\) receptors (55).

Since ethanol has a variety of CNS effects, many of which are due to increased CNS adenosine levels with resulting A\(_2A\) receptor activation (56, 57), it is also possible that extrahepatic effects of adenosine may lead to hepatic steatosis by, for example, production of neuroendocrine mediators or increased food intake. However, the demonstration that adenosine A\(_1\) and A\(_2B\) receptors directly stimulate steatosis in AML-12 hepatocytic cells is more consistent with the hypothesis that adenosine receptors directly regulate hepatocyte metabolism.

Alterations in 3 major regulatory pathways in the hepatocyte contribute to the development of fatty liver: stimulation of SREBP1 activation, inhibition of AMPK, and diminished PPAR\(_\alpha\) activation/increased PPAR\(_\gamma\) activation. SREBP1 is a member of the basic helix-loop-helix leucine zipper (bHLH-ZIP) family of transcription factors that is synthesized as a 125-kDa precursor attached to the nuclear envelope and endoplasmic reticulum (58, 59). In sterole-depleted cells, the membrane-bound precursor is cleaved to generate a soluble NH\(_2\)-terminal fragment that translocates to the nucleus (60). SREBP1 plays an active role in regulating the transcription of genes involved in hepatic triglyceride synthesis (including ACC, FAS, stearyl-CoA desaturase-1, ACL, and l-\(\alpha\)-glycerophosphate acyltransferase; refs. 61, 62). Ethanol and its metabolites activate SREBP1 (63), and alcohol-induced fatty liver
correlates with activation and induction of SREBP1 (30, 63, 64). In our studies ethanol and adenosine A1 receptor occupancy significantly increased nuclear SREBP1 and downstream expression of ACL and FAS mRNA expression, both deletion and antagonism of adenosine A1 receptors significantly diminished their expression in vivo and in vitro (Figures 4 and 7). These results are consistent with the hypothesis that adenosine A1 receptors regulate SREBP1 expression and activation to increase expression of fatty acid synthetic enzymes (Figure 9).

PPARα, -β/δ, and -γ belong to the nuclear receptor superfamily (65). PPARα is activated by sterols and is translocated to the nucleus, in which it stimulates the transcription of a variety of enzymes and transporters that promote fatty acid oxidation (23–25). Ethanol prevents the activation and nuclear translocation of PPARα (66–69), effects abrogated by deletion or blockade of adenosine A2B receptors. In contrast to PPARα, PPARγ appears to play a direct role in the development of fatty liver (46, 70–73). PPARγ is expressed at very low levels in the liver, and overexpression in the liver leads to hepatic steatosis with the expression of several adipogenic genes (26–29). Conversely, PPARγ agonists have been used to treat nonalcoholic fatty liver (74), possibly by increasing expression of the receptor for adiponectin (75). Ethanol increases Pparg mRNA expression and expression of lipid synthetic enzymes ACL and FAS in mouse liver, and deletion or blockade of adenosine A1 receptors decreased expression of these genes consistent with the results of the in vitro hepatocyte experiments. These results suggest the hypothesis that adenosine A1 and A2B receptors regulate PPARγ and PPARα activation and expression to promote hepatic steatosis (Figure 9).

AMPK also plays a key role in the regulation of cellular metabolism. Once activated by phosphorylation of threonine-172 or by AMP, AMPK strongly activates ACC, 3-hydroxy-3-methyl-glutaryl-CoA reductase, and other targets, leading to fatty acid oxidation and diminished cholesterol synthesis (31, 76–78). Ethanol inhibits AMPK activation, leading to accumulation of fatty acids within the hepatocyte (30). Alcohol ingestion diminished AMPK phosphorylation in mouse liver, an effect reversed by deletion or blockade of adenosine A2B receptors but not A1 receptors. Studies carried out in vitro provided parallel results. Thus, adenosine A2B receptors are also likely to promote hepatic triglyceride accumulation by diminishing AMPK phosphorylation and activity as well as by diminishing PPARα transcriptional activity (Figure 9).

We demonstrated, for the first time to our knowledge, that all 4 adenosine receptors are present in healthy human liver plasma membranes. The number and affinity of adenosine A1 and A3 receptors does not change in cirrhotic and fatty livers but the number of A2A and A2B receptors increases in cirrhotic and fatty livers. Prior studies have demonstrated a number of factors that may regulate the expression of adenosine A2A receptors, including such inflam-

Figure 7
Adenosine A1 and A2B receptors regulate enzymes involved in fatty acid synthesis and oxidation. AML-12 hepatocytes were treated with adenosine receptor agonists or antagonists or their combination (CPA, 1 μM; DPCPX, 1 μM; NECA, 10 μM; MRS1706, 1 μM) for 24 hours, cells were lysed, and the protein levels were assessed by Western blotting, quantitated by densitometry, and normalized to β-actin. (A) Representative Western blot of ACL, FAS, ACCα, and CPTI. (B) ACL protein levels. (C) FAS protein levels. (D) ACCα protein levels. (E) CPTI protein levels. The data are expressed as percentage of control (mean ± SD) from at least 3 independent experiments. *P < 0.01, versus control or other treatment groups, respectively.
matory cytokines as IL-1 and TNF (79, 80) and endotoxin (81). Interferon-γ, which stimulates increased expression of A2B receptors (82), may diminish A1A and A2B receptor signaling (79, 80, 83).

The results reported here demonstrate what we believe to be a novel pathogenic mechanism for the development of fatty liver following chronic ethanol ingestion: ethanol-induced adenosine release stimulates hepatic steatosis via activation of A1 and A2B receptors. It is also possible that adenosine and its receptors play a role in the pathogenesis of nonalcoholic fatty liver disease as well. Moreover, these results suggest that adenosine receptor antagonism may provide a novel approach for the development of agents for the treatment and prevention of alcoholic and, possibly, non-alcoholic fatty liver disease.

Methods

Reagents. A1 receptor agonist CPA (84), A2B receptor antagonist DPCPX (84), nonselective adenosine receptor agonist NECA (84), A1A antagonist 3-propylthamine (enprofylline) (85, 86), and A1 antagonist, 3-ethyl-5-benzyl-2-methyl-4-phenylethyl-6-phenyl-1,4-(-)dihydropyridine-3,5-dicarboxylate (MRS1191) (84) were obtained from Sigma-Aldrich. A more potent and selective A2B receptor antagonist MRS1706 (84) and A1A antagonist 4-(2-[(7-amino-2-(2-furyl))|1,2,4|triazolo[2,3-a]|1,3,5|triazin-5-ylamino]ethyl)pheno|le|5(84) were purchased from Tocris Cookson. [H]-DPCPX (specific activity, 120 Ci/mmol) was obtained from Perkin Elmer Life and Analytical Sciences. [H]-ZM 241385 (specific activity, 17 Ci/mmol) was obtained from Tocris Cookson Ltd. N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl]-acetamide) ([H]-MRE 2029F20, A2B receptor antagonist; specific activity, 123 Ci/mmol) and 5-N-(4-methoxyphenyl-carbamoy|l|amino-8-propyl-2(2furyl)-pyra|zol|o|4,3-e,1,2,4-triazole |1,5-c| pyrimid|i|ne ([H]-MRE 3008F20, A2B receptor antagonist; specific activity, 67 Ci/mmol) were derived from Amersham International Chemical Laboratories.

Animals. C57BL/6 WT mice were purchased from The Jackson Laboratory. CD73KO mice were provided as a gift by Linda Thompson (Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA) (87). A1KO mice were a gift of Bertil Fredholm (Karolinska Institutet, Stockholm, Sweden) (88). CD73 and A1 receptor knockout mice were bred onto a C57BL/6 background (>10 back-crosses) in the New York University School of Medicine Animal Facility. A2BKO mice (C57BL/6 background) have been previously described (89). A2AKO mice (S129 mixed background) and their corresponding WT littermates (90) were bred in the New York University School of Medicine Animal Facility. All experimental mice were 6- to 8-week-old male mice. All experimental procedures were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the School of Medicine of New York University.

Mouse model of fatty liver and treatment. Mice were fed an ethanol-containing liquid Lieber-DeCarli (BioServ Inc.) diet or a calorie equivalent Lieber-DeCarli diet supplemented with maltose (BioServ Inc.) for 6 weeks. This diet provides 35.5% of calories from ethanol (or maltose), 35% of calories from fat, 18% of calories from protein, and 11.5% of calories from carbohydrate. For the first 2 weeks, animals were gradually introduced to the ethanol-containing liquid diet, including liquid diet acclimation for 4 days, 0.75% ethanol (w/v) for 3 days, 1.50% ethanol (w/v) for 3 days, 3.75% ethanol (w/v) for 4 days, and then 5.00% ethanol (w/v) diet for 4 weeks (91, 92). Animal cages were placed on heating pads to maintain body temperature. Mice were weighed before and after experiments, and measurements of daily food consumption were recorded. A1 receptor antagonist DPCPX (50 mg/kg/d) or A2B receptor antagonist enprofylline (50 mg/kg/d) were administered to WT mice in the liquid diet, and all animals had free access to the liquid diet throughout the experimental period. There was no significant difference in food intake among the different groups of mice (data not shown) and weight gain was similar for all of the mice studied (Table 1),
Cells were de-identified, and Oil Red O staining of hepatocytes was carried out according to the manufacturers’ instructions. The extracted nuclear protein was used for Western blotting or transcription factor assay. ACL, FAS, and phospho-AMPK antibodies were purchased from Cell Signaling Technology; AMPKα, β, and γ antibodies were from Santa Cruz Biotechnology Inc.; and SREBP1, SREBP2, PPARα, PPARγ, and PPARβ/δ antibodies were from Diagenode. Western blot analyses were performed as previously described (79).

Table 2

<table>
<thead>
<tr>
<th>Human hepatic membranes</th>
<th>Adenosine receptors</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>A2A</td>
</tr>
<tr>
<td>Control subjects (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Km</td>
<td>2.11 ± 0.17</td>
<td>1.67 ± 0.15</td>
</tr>
<tr>
<td>Bmax</td>
<td>33 ± 3</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>Cirrhotic patients (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Km</td>
<td>2.02 ± 0.19</td>
<td>3.42 ± 0.22A</td>
</tr>
<tr>
<td>Bmax</td>
<td>31 ± 3</td>
<td>240 ± 21A</td>
</tr>
<tr>
<td>Steatotic patients (n = 10)</td>
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<td></td>
</tr>
<tr>
<td>Km</td>
<td>1.95 ± 0.15</td>
<td>4.09 ± 0.27A</td>
</tr>
<tr>
<td>Bmax</td>
<td>34 ± 3</td>
<td>363 ± 23A</td>
</tr>
</tbody>
</table>

Bmax, maximal binding, is measured in fmol/mg protein; Km is measured in nM. *P < 0.01, versus control subjects.
Data were expressed as mean ± SD and were analyzed by Student’s t test or ANOVA analysis, as appropriate, with SPSS software (SigmaStat) 10.0. P values of less than 0.05 were considered to be statistically significant.

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