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Acetaldehyde accumulation suppresses Kupffer cell release of TNF-α and modifies acute hepatic inflammation in rats

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Background. Alcohol-related diseases have multiple and varied associations with acetaldehyde, a highly toxic product of ethanol oxidation that accumulates in the absence of active aldehyde dehydrogenase (ALDH). This study was designed to clarify the role of acetaldehyde in liver injury, specifically in vivo and in vitro effects on Kupffer cell release of the inflammatory cytokine tumor necrosis factor-alpha (TNF-α). ***Methods.*** Rats pretreated overnight with the ALDH inhibitor disulfiram (or saline control) were ethanol loaded and challenged with lipopolysaccharide (LPS), and their blood and histological parameters were examined 3 h later. Similarly, isolated rat Kupffer cells were pretreated with disulfiram or cyanamide incubated in ethanol (1 h), then challenged with LPS and evaluated 2 h later for TNF-α and acetaldehyde levels in the culture medium. TNF-α release from Kupffer cells after LPS challenge was also evaluated following incubation in acetaldehyde and acetate for comparison with ethanol loading. ***Results.*** Higher blood acetaldehyde concentration following disulfiram pretreatment significantly attenuated acute hepatic inflammation in the ethanol-loaded, LPS-challenged rat (18 ± 2.9 vs 30 ± 3.7 polymorphonuclear cells/portal area; $P = 0.01$). After LPS challenge, ALDH inhibitor pretreatment attenuated Kupffer cell release of TNF-α in the presence of disulfiram at 5063 ± 151 pg/ml and cyanamide at 4390 ± 934 pg/ml, versus no inhibitor, 5869 ± 265 pg/ml ($P < 0.01$), but not in the absence of ethanol. Acetaldehyde significantly suppressed Kupffer cell TNF-α release ($P < 0.05$), but acetate treatment did not. ***Conclusions.*** Acetaldehyde accumulation suppresses macrophage function, at least suppressing TNF-α release, which plays a role in modifying acute hepatic inflammation in rats.

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Introduction

Alcohol is one of the most important factors that can modify the course of human diseases. Despite recent evidence that drinking an appropriate quantity of alcohol can decrease the incidence of ischemic heart disease and stroke,[1] alcohol abuse injures many organs, most commonly the liver, where it can result in acute or chronic liver injury and eventually lead to cirrhosis. Although the pathogenesis of alcoholic liver injury remains obscure, both hepatic macrophages known as Kupffer cells and endotoxin (lipopolysaccharide [LPS]) are known to play major roles. Adachi and colleagues showed that the inactivation of Kupffer cells using gadolinium chloride ($GdCl_3$) prevents early alcohol-induced liver injury.[2] They also showed that intestinal sterilization with antibiotics (polymixin B and neomycin) can prevent alcohol-induced liver injury by reducing intestinal bacterial counts and lowering the risk for endotoxinemia.[3]

Several reports have suggested that alcoholic hepatitis is milder in Japan than in the United States and most Western countries.[4] Although some investigators attribute this circumstance to the difference in the eating and drinking habits of these countries, e.g., the quantity of alcohol consumed and/or the amount of fat in the diet,[5] the underlying reason for these differences is not clear. Another possible reason for these differences may be associated with an excessive accumulation of acetaldehyde in the blood after drinking. The difference between acetaldehyde accumulation in Asians and others is readily apparent: because of their low aldehyde dehydrogenase (ALDH) activity, about half of Asians have high acetaldehyde levels after drinking.[6] Such

Table 1. In vivo experiments

Pretreat (overnight)	ALDH inhibitor ($n = 20$)			Saline ($n = 20$)		
Treat (rapid injection to 4-h infusion)	Ethanol ($n = 10$)	Saline ($n = 10$)		Ethanol ($n = 10$)	Saline ($n = 10$)	
Challenge (1 h after start of treatment)	LPS ($n = 10$)	LPS ($n = 5$)	Saline ($n = 5$)	LPS ($n = 10$)	LPS ($n = 5$)	Saline ($n = 5$)

Rats ($n = 40$)
Control (pretreatment, treatment, challenge) saline
Aldehyde dehydrogenase (ALDH) inhibitor pretreatment = disulfiram, 75 mg/kg body weight, by gastric tube
Treatment = ethanol injection, 1.75 g/kg i.v., followed by ethanol infusion, 250 mg/kg/h
Challenge = lipopolysaccharide (LPS), 1 mg/kg i.v.

genetic differences in alcohol metabolism may affect the progression of alcohol-related illnesses.

Acetaldehyde, a highly reactive product of alcohol metabolism, forms adducts with cytochrome P450 2E1, collagen fibrils, albumin, hemoglobin, microtubules, and DNA.[7-12] The accumulation of acetaldehyde may influence various genomic and protein functions as well as cellular function. Our previous research has shown that Kupffer cells possess the alcohol-metabolizing enzymes cytochrome P450 2E1 and ALDH2; consequently, these cells are capable of oxidizing ethanol to acetaldehyde and acetate.[13] Kupffer cells produce various mediators (e.g., cytokines, eicosanoids, proteases, and oxygen radicals) that participate in inflammation, usually during the acute phase, and contribute toward later immune responses.[14]

Acute inflammation in many tissues is associated with the activation of macrophages and monocytes through the production of various mediators during the acute phase.[15,16] One important monocytic mediator of acute inflammation is tumor necrosis factor-alpha (TNF-α), a cytokine produced by numerous cells, including monocytes, natural killer cells, B cells, T cells, basophils, eosinophils, neutrophils, and nonimmune cells (e.g., mast cells, Kupffer cells, astrocytes, granulosa cells, epithelial cells, keratinocytes, and glial cells). In the liver, the major source of TNF-α is the resident macrophage or Kupffer cell.[17]

The pathogenesis of liver injury with alcohol consumption remains obscure, and few studies have addressed the role of acetaldehyde in that regard.[18] In this study to clarify the effect of acetaldehyde on Kupffer cell activity during the acute phase of liver injury, we used ALDH inhibitors and ethanol to evaluate the in vitro and in vivo effects of acetaldehyde accumulation on liver injury caused by LPS, as demonstrated by the release of TNF-α from Kupffer cells.

Materials and methods

Animals

Male Wistar rats weighing about 250 g (Orientalkoubo, Tokyo, Japan) were used for the experiments. All animal care protocols were approved by the Laboratory Animal Users Committee at Keio University.

In vivo experiment

Forty rats were divided into two groups of 20 each for overnight pretreatment with or without an ALDH inhibitor, disulfiram (75 mg/kg; Sigma, St. Louis, MO, USA) by gastric tube, and treatment with or without ethanol and/or LPS (Table 1). Each rat was anesthetized with pentobarbital (30 mg/kg i.p.; Abbott, North Chicago, IL, USA), and a 24-gauge Teflon catheter (Terumo, Tokyo, Japan) was inserted into the left femoral vein for the rapid injection of 1.75 g/kg ethanol, followed immediately by ethanol infusion (250 mg/kg/h) or saline, respectively, for 4 h. One hour after the initial ethanol injection, to create endotoxinemia, we injected 1 mg/kg LPS in saline into the femoral vein of half of each group of rats; those not treated with LPS received sham injections of saline.

Three hours after LPS injection, the rat livers and serum were examined to determine the degree of liver injury. Each liver was evaluated by light microscopy, using hematoxylin and eosin (HE) staining to facilitate the counting of infiltrating polymorphonuclear inflammatory (PMN) cells (10 portal area per liver for each of 10 rats per group). The serum samples were used to measure the levels of two liver enzymes that indicate organ damage, alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), as well as TNF-α and blood acetaldehyde.

In vitro experiment

Ten rats were killed by pentobarbital injection and Kupffer cells were isolated from their excised livers by a

densitometric procedure[19] using metrizamide (Sigma). The isolated cells were seeded in six-well plastic plates (5×10^6 cells/well) and divided into three groups for incubation in RPMI 1640 medium (Gibco, Grand Island, NY, USA) fortified with 10% heat-inactivated fetal calf serum (Gibco), 50 μM kanamycin (Sigma), and one of two ALDH inhibitors (2 μM disulfiram or 200 μM cyanamide; Sigma) or neither (control). After 1-h incubation with or without an ALDH inhibitor, ethanol was added to each well to achieve a concentration of 50 mM. One hour later, 100 ng/ml LPS (Sigma) in saline was added to each well to stimulate the Kupffer cells. In another set of experiments, we added 50 mM ethanol and one of its oxidation products, acetaldehyde (50 μM–20 mM) or acetate (20 mM), to media containing Kupffer cells for 1 h, and then incubated the media with LPS (100 ng/ml) for 2 h to estimate TNF-α release (Table 2).

Two hours after LPS challenge, the Kupffer cells were collected for Northern blot analysis to examine TNF-α mRNA expression, using a TRIzol reagent (Gibco) according to the manufacturer's instructions to obtain the total cellular mRNA hybridized with rat TNF-α cDNA probes generated by polymerase chain reaction (PCR). Two hours after the LPS challenge we also collected the culture media and analyzed the levels of acetaldehyde and TNF-α. To measure the acetaldehyde concentration in medium by headspace gas chromatography, we mixed perchloric acid (Sigma) with the culture solution as quickly as possible in a procedure described by Eriksson et al.[20] The amount of TNF-α released by Kupffer cells into the culture medium was measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA). We observed no difference between the ELISA results for control TNF-α levels in the absence and presence of acetaldehyde (20 mM).

Statistical analysis

We expressed all data as the mean ± standard deviation (SD). We analyzed differences between groups using Student's unpaired t test. A value of $P < 0.05$ was considered to be statistically significant.

Results

In vivo study

After 3-h LPS challenge, PMN cells infiltrated the livers, predominantly in the portal areas (Fig. 1A), of the ethanol-loaded rats that had not received disulfiram. In

Table 2. In vitro experiments

	ALDH inhibitor ($n = 50$)								No ALDH inhibitor ($n = 102$)			
Pretreatment (1 h before treatment)	Disulfiram ($n = 25$)				Cyanamide ($n = 25$)							
Treatment	Ethanol ($n = 15$)		Saline ($n = 10$)		Ethanol ($n = 10$)		Saline ($n = 15$)		Ethanol ($n = 25$)		Saline ($n = 25$)		Acetate ($n = 12$)	Acetaldehyde ($n = 40$)
Challenge (1 h after treatment)	LPS ($n = 15$)	Saline ($n = 10$)	LPS ($n = 10$)	Saline ($n = 15$)	LPS ($n = 15$)	Saline ($n = 10$)	LPS ($n = 15$)	Saline ($n = 10$)	LPS ($n = 15$)	Saline ($n = 10$)	LPS ($n = 15$)	Saline ($n = 10$)	LPS ($n = 12$)	LPS ($n = 40$)

Cultures in 152 wells
Control = saline for both inhibitor pretreatment and ethanol treatments, and for LPS challenge
Pretreatment = disulfiram, 2 μM; cyanamide, 200 μM
Treatment = ethanol, 50 mM; acetate, 20 mM; acetaldehyde (four treatments, $n = 10$ each), 50 μM, 500 μM, 5 mM, 20 mM
Challenge = lipopolysaccharide (LPS), 100 ng/ml

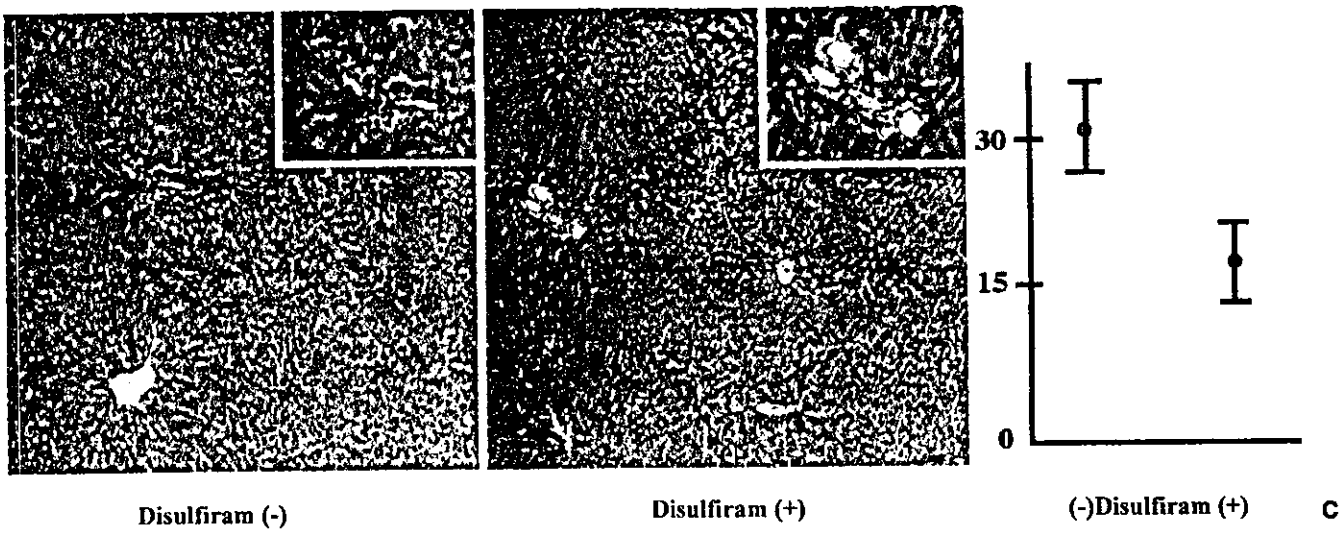

Fig. 1. Polymorphonuclear (PMN) cell infiltration following ethanol treatment and 3-h lipopolysaccharide (LPS) challenge, as shown by hematoxylin and eosin (HE) stain: **A** Without disulfiram pretreatment (×40); **B** with disulfiram pretreatment (×40); **C** PMN cells/portal area of individual liver specimens (n = 10 rat livers ×10 portal areas each; P = 0.01)

Table 3. Effects of ALDH inhibitor pretreatment, ethanol, and LPS challenge on the liver enzymes ALT and LDH and on TNF-α in rat serum

Inhibitor Pretreatment/ethanol Treatment/LPS challenge	No. rats	ALT (IU/l)	LDH (IU/l)	TNF-α (pg/ml)
None/none/none*	5	22 ± 5	372 ± 15	1.7 ± 1.78
Disulfiram/none/none*	5	29 ± 4	372 ± 84	1.8 ± 1.74
None/none/LPS**	5	115 ± 52	961 ± 184	495 ± 138
Disulfiram/none/LPS**	5	103 ± 67	1000 ± 308	506 ± 161
None/ethanol/LPS***	10	127 ± 102	1080 ± 334	481 ± 198
Disulfiram/ethanol/LPS***	10	42 ± 21	680 ± 279	255 ± 150

ALT, alanine aminotransferase; LDH, lactate dehydrogenase; TNF-α, tumor necrosis factor-alpha
*Disulfiram vs. no ALDH inhibitor: no significant difference in effect on any of the three parameters
**LPS with and without disulfiram: no significant difference in effect on any of the three parameters
***Ethanol and LPS, with and without disulfiram: statistically significant difference in effect for all three parameters ($P < 0.05$)

contrast, the livers of the disulfiram-pretreated rats had significantly fewer infiltrating cells (Fig. 1B,C) (with disulfiram 18 ± 2.9 vs without disulfiram 30 ± 3.7 cells/portal area, P = 0.01). The serum levels of ALT, LDH, and TNF-α in disulfiram-treated ethanol-loaded rats were also attenuated, but in the absence of ethanol, disulfiram pretreatment did not alter the levels of ALT, LDH, or TNF-α (Table 3). In ethanol-loaded rats, the serum ethanol concentration was 72 ± 14.8 mM. As expected, the acetaldehyde concentration in the serum after 4 h ethanol infusion was significantly higher in the ethanol-infused rats pretreated with disulfiram than in the rats receiving no disulfiram (Table 4). Thus, the livers of rats in which acetaldehyde accumulation was evoked by ethanol plus an ALDH inhibitor had reduced acute hepatic inflammation after LPS challenge.

In vitro study

As demonstrated in vivo, 3 h after the addition of 50 mM ethanol to the culture media, the acetaldehyde concentrations were significantly higher in the disulfiram-pretreated cultures than in the cultures receiving no ALDH inhibitor (see Table 4). The release of TNF-α from Kupffer cells after ethanol plus LPS challenge was significantly reduced in cultures pretreated with an ALDH inhibitor (disulfiram or cyanamide), but the inhibitors alone (without ethanol) did not suppress TNF-α release (Table 5). The addition of acetaldehyde alone, in lieu of ethanol, decreased the release of TNF-α from LPS-challenged Kupffer cells in dose-dependent fashion, whereas substituting acetate for ethanol did not produce this effect (Table 5). Northern blot analysis showed TNF-α mRNA expression in Kupffer cells after

Table 4. Effects of ALDH inhibitor (Disulfiram) pretreatment and ethanol treatment on acetaldehyde levels in vivo and in vitro

Source	*n*	Acetaldehyde concentration		
		Without disulfiram* (μM)	With disulfiram* (μM)	*P* value
Blood	6	6.2 ± 2.6	42.3 ± 5.2	<0.01
Kupffer cell culture medium	10	3.2 ± 1.2	14.1 ± 2.5	<0.01

*Disulfiram, 75 mg/kg body weight in vivo, 2 μM in vitro; no-disulfiram control = saline

Table 5. In vitro effect of LPS challenge on TNF-α concentration in Kupffer cell cultures with and without ALDH inhibitor pretreatment and ethanol or ethanol metabolic product treatment

Pretreatment/treatment/LPS challenge	*n*	TNF-α (pg/ml)	*P* value*
None/ethanol/LPS	15	5869 ± 265	—
None/saline/LPS	15	5761 ± 668	N.S.
None/saline/none	10	32 ± 3.05	<0.001
None/ethanol/none	10	34 ± 3.20	<0.001
Disulfiram/ethanol/LPS	15	5063 ± 151	<0.01
Disulfiram/saline/LPS	10	6033 ± 1171	N.S.
Cyanamide/ethanol/LPS	10	4390 ± 934	<0.01
Cyanamide/saline/LPS	15	5653 ± 1036	N.S.
None/acetate (20 mM)/LPS	12	5641 ± 1095	N.S.
None/acetaldehyde (50 μM)/LPS	10	5197 ± 397	<0.05
None/acetaldehyde (500 μM)/LPS	10	4536 ± 614	<0.01
None/acetaldehyde (5 mM)/LPS	10	2429 ± 205	<0.001
None/acetaldehyde (20 mM)/LPS	10	31 ± 12.0	<0.001

*Reference value represents no ALDH inhibitor, ethanol loading, LPS challenge; N.S., not significant

ethanol plus LPS challenge demonstrated no significant differences between cells with and without disulfiram pretreatment (Fig. 2A). TNF-α mRNA expression in LPS-challenged Kupffer cells was not significantly changed by adding 500 μM acetaldehyde (Fig. 2B), in contrast to decreased TNF-α release associated with the addition of only 50 μM acetaldehyde (see Table 5).

Discussion

Despite several reports indicating that the inflammation is suppressed by ethanol[21,22] or acetaldehyde,[18,23] the precise progression of hepatic inflammation and the function of hepatic macrophages, which are closely associated with liver diseases including alcoholic hepatitis,[2,3] have not yet been elucidated with ethanol and acetaldehyde loading. The results of our experiments, demonstrating that ALDH inhibitor disulfiram attenuates acute hepatic inflammation induced by LPS with ethanol loading, suggest one reason for the suppression of TNF-α release in the blood: the accumulation of acetaldehyde. This response, observed in vivo, also held for the in vitro experiments, in which acetaldehyde accumulated in response to treatment with ethanol and ALDH inhibitors suppressed Kupffer cell release of TNF-α. Additional experiments proved that acetaldehyde, but neither ethanol nor acetate alone, significantly attenuates TNF-α release from LPS-challenged Kupffer cells. The degree to which the disulfiram plus ethanol treatment suppressed both in vivo and in vitro TNF-α levels after LPS challenge was similar to the extent of the suppression of hepatic inflammation in vivo.

In our in vivo experiments, polymorphonuclear cells infiltrated the liver within 3 h of initiating the LPS challenge. Disulfiram in the presence of ethanol, but not disulfiram alone, attenuated this infiltration. Moreover, the serum levels of the liver enzymes ALT and LDH showed that disulfiram suppresses liver injury caused by LPS and ethanol loading. Because the serum acetaldehyde concentrations in our study were markedly elevated by disulfiram plus ethanol treatment, we speculated that acetaldehyde accumulation could be linked to hepatic protection. In vitro, administration of ethanol following either disulfiram or cyanamide had suppressed TNF-α release by Kupffer cells, an effect not produced by either ALDH inhibitor alone. Moreover, the acetaldehyde concentration in the Kupffer cell culture medium was elevated by disulfiram plus ethanol. In fact, additional experiments showed that acetaldehyde, but neither ethanol nor acetate alone, signifi-

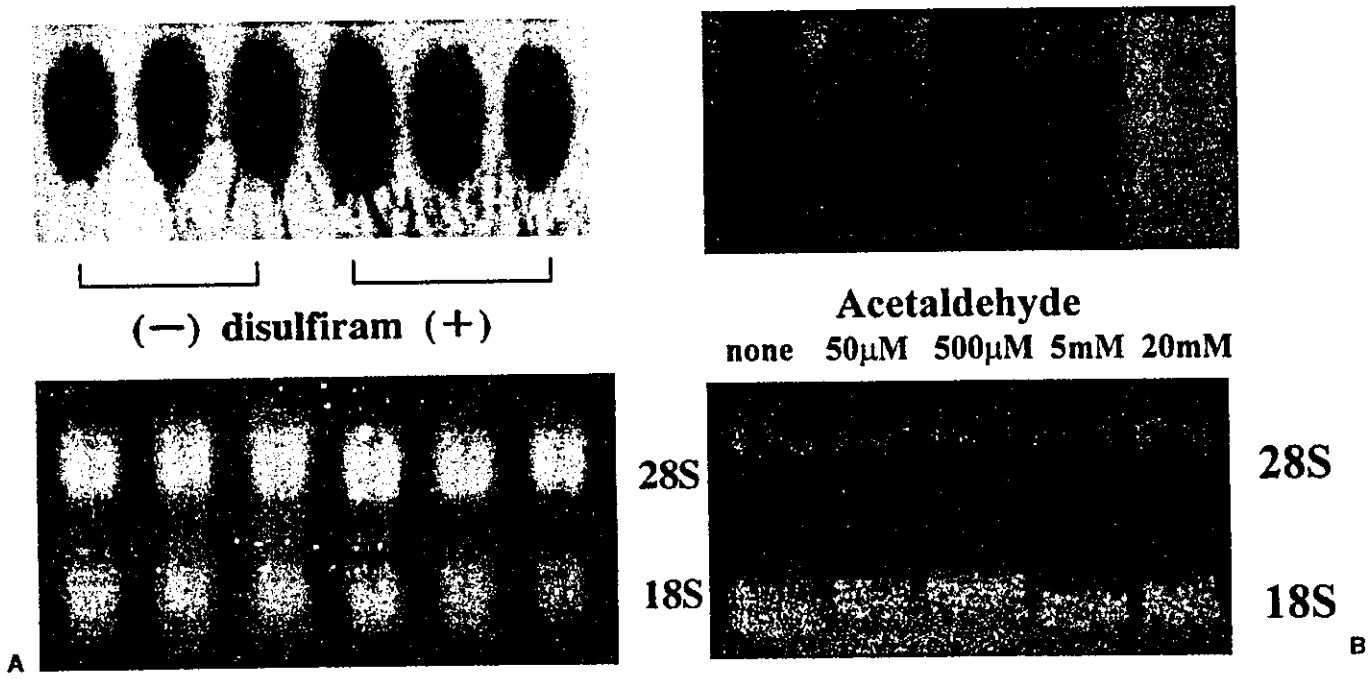

Fig. 2. Northern blot analysis showing Kupffer cell expression of tumor necrosis factor-alpha (TNF-α) mRNA as the effects of (**A**) ethanol treatment plus LPS challenge following pretreatment with or without disulfiram and (**B**) LPS challenge in the presence of various concentrations of acetaldehyde

cantly attenuates TNF-α release from LPS-challenged Kupffer cells.

Two clinical situations in which acetaldehyde accumulation could affect the internal organs, including the liver, are (1) medical treatment that inhibits ALDH activity and (2) the presence of a genotype in which the inactive ALDH2*2 allele readily incurs the accumulation of high levels of acetaldehyde (a condition especially prevalent in Asians). In addition to disulfiram and cyanamide, drugs that have an ALDH inhibitory effect include cephalosporins,[24] chlorpropamide,[25] and metronidazol.[26] These drugs might have the ability to affect macrophage function and hepatic injury after drinking alcohol. The proposed protective role for acetaldehyde could help to explain why Asians have milder alcoholic hepatitis than people in the United States and most Western countries.[4] Observations concerning acetaldehyde accumulation may help to answer some of the molecular and biochemical questions about acute hepatic inflammation with alcohol consumption.

The degree to which the disulfiram/ethanol treatment suppressed both in vivo and in vitro TNF-α levels after LPS challenge in this investigation was similar to the extent of suppression of the hepatic inflammation. Although treatment with an ALDH inhibitor before ethanol loading induced a greater accumulation of acetaldehyde and suppressed TNF-α release from Kupffer cells, our Northern blot experiments did not demonstrate significant suppression in TNF-α mRNA levels in Kupffer cells, with or without disulfiram pretreatment. Moreover, TNF-α mRNA expression from LPS-challenged Kupffer cells was not significantly affected by the addition of 500μM acetaldehyde, in contrast to the decrease in TNF-α release when we added only 50μM acetaldehyde. This finding suggests the likelihood that posttranscriptional inhibition is involved in this process.

Recent experiments by Lindros et al.[18] and Jokelainen et al.[23] indicated that acetaldehyde inhibits and suppresses hepatic inflammation in ethanol-fed rats by preventing nuclear factor kappa-B (NFκ-B) activation through the suppression of TNF-α mRNA and its release. However, the Lindros group incubated Kupffer cells with acetaldehyde for 4h in vitro, and examined the in vivo effects of treatment with an ALDH inhibitor for 1 month, whereas in our study we examined the effects of ethanol loading for only 1h (i.e., after 1h of acetaldehyde accumulation) in vitro, and overnight in vivo. The differences in duration of exposure to accumulated acetaldehyde (1 month versus overnight in vivo, 4h versus 1h in vitro) may explain the differences between our results and theirs. Some additional parameters, the concentrations of ALDH inhibitor and ethanol, are differed between the Lindros study and ours.

Acetaldehyde decreases the activity of enzymes such as sucrase, maltase, and chymotrypsin.[27,28] There is considerable evidence of the influence with acetaldehyde on numerous proteins, microtubules, hemoglobin, globulin, collagen, and DNA.[7–12] Thus, it is not surprising that no difference in the expression of TNF-α mRNA was found in our study, although the differences in mature TNF-α protein levels were significant. Among the many processes that must influence the reduction of TNF-α release to be considered are the translation by ribosomes within the endoplasmic reticulum, the intracellular movements within microtubules, the conversion of pro-TNF-α to mature TNF-α, and the surrounding conditions after TNF-α release. Our data appear to support those of Zhang and colleagues,[29] who demonstrated that ethanol inhibits the processing of pro-TNF-α to TNF-α by TNF-α-converting enzyme (TACE). Posttranscriptional inhibition such as TACE activity could offer a clue concerning acute acetaldehyde effects, but this possibility requires further investigation.

Although 500 μM acetaldehyde significantly reduced actual Kupffer cell release of TNF-α whereas 50 μM acetaldehyde decreased the response by up to 10%, disulfiram plus ethanol suppressed TNF-α secretion much more effectively when the level of acetaldehyde detected was less than 50 μM. These differences in effect were achieved following different periods of exposure to acetaldehyde. Because the boiling point of acetaldehyde is 20.1 °C, most of the acetaldehyde added to media and incubated at 37 °C would evaporate immediately. Further, in exposing Kupffer cells to 50 μM acetaldehyde, without an ALDH inhibitor, we showed a more rapid breakdown of acetaldehyde than we observed following disulfiram pretreatment and 50 mM ethanol loading. The accumulation of acetaldehyde during 3 h incubation in a 14-μM concentration of acetaldehyde would likely have had a much greater effect on Kupffer cell function than a bolus 50-μM dose of acetaldehyde, which evaporated and metabolized readily in the presence of active ALDH during a 3-h incubation.

In conclusion, this study has provided evidence that acetaldehyde accumulation resulting from simultaneous ALDH inhibition and ethanol loading suppresses LPS-induced acute hepatic inflammation in rats. One mechanism of this effect might involve the reduction in TNF-α release from hepatic macrophages. The person who has a low-activity ALDH subtype or whose ALDH activity is impaired (e.g., due to medication) would thus experience the protective effect of acute alcoholic hepatitis because of accumulated acetaldehyde. Further studies are needed to clarify the role of acetaldehyde accumulation in acute hepatic inflammation and to address the obscure role of acetaldehyde accumulation in chronic liver injury with alcohol consumption.

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Pioglitazone Prevents Alcohol-Induced Fatty Liver in Rats Through Up-regulation of c-Met

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Background & Aims: Treatment of steatosis is important in preventing development of fibrosis in alcoholic liver diseases. This study aimed to examine if pioglitazone, an antidiabetic reagent serving as a ligand of peroxisome proliferator-activated receptor gamma (PPARγ), could prevent alcoholic fatty liver. *Methods:* Rats fed with an ethanol-containing liquid diet were given the reagent at 10 mg/kg per day intragastrically for 6 weeks. Hepatic genes involved in actions of the reagent were mined by transcriptome analyses, and their changes were confirmed by real-time polymerase chain reaction and Western blotting analyses. The direct effects of pioglitazone on primary-cultured hepatocytes were also assessed in vitro. *Results:* Pioglitazone significantly attenuated steatosis and lipid peroxidation elicited by chronic ethanol exposure without altering insulin resistance. Mechanisms for improving effects of the reagent appeared to involve restoration of the ethanol-induced down-regulation of c-Met and up-regulation of stearoyl-CoA desaturase (SCD). Such effects of pioglitazone on the c-Met signaling pathway resulted from its tyrosine phosphorylation and resultant up-regulation of the apolipoprotein B (apoB)-mediated lipid mobilization from hepatocytes through very low–density lipoprotein (VLDL) as well as down-regulation of sterol regulatory element binding protein (SREBP)-1c and SCD levels and a decrease in triglyceride synthesis in the liver. *Conclusions:* Pioglitazone activates c-Met and VLDL-dependent lipid retrieval and suppresses triglyceride synthesis and thereby serves as a potentially useful stratagem to attenuate ethanol-induced hepatic steatosis.

Chronic consumption of excess alcohol is hepatotoxic in humans and produces an accumulation of hepatic triglycerides to cause steatosis. These changes are pathologically characterized by macrovesicular fatty degeneration and occur in pericentral regions. Recent clinical studies provided evidence that such an accumulation of triglycerides in the liver is not benign but could lead to fibrosis and cirrhosis with effective treatment remaining to be established.[1,2] Because the hepatic steatosis often coincides with hyperinsulinemia and insulin resistance, treatment that renders patients sensitive to the hormone could be beneficial. Such a possibility was well supported by previous studies showing that metformin, an agent improving insulin resistance of the liver, improved steatosis, hepatomegaly, and the release of transaminases in insulin-resistant ob/ob mice with nonalcoholic fatty liver; mechanisms for such beneficial effects of metformin appear to involve down-regulation of tumor necrosis factor-α (TNF-α) and TNF-inducible genes such as SREBP-1 and uncoupling protein-2[3] as well as increased phosphorylation and activation of AMP-activated protein kinase (AMPK).[4] The fact that the histological features and natural history of alcoholic fatty liver are similar to those of nonalcoholic fatty liver suggests that unidentified common mechanisms for pathogenesis of hepatic steatosis could be involved in these 2 disease conditions. However, utilization of metformin is unlikely to be applicable to treat alcoholic steatosis because of its side effect of lactic acidosis, an important complication of clinical alcoholic liver injury.

Such circumstances led us to examine if another class of antidiabetic agents such as thiazolidinediones are effective to treat hepatic steatosis caused by chronic ethanol administration. These reagents are ligands of perox-

Abbreviations used in this paper: ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; apoB, apolipoprotein B; AST, aspartate aminotransferase; BADGE, bisphenol A diglycidyl ether; HGF, hepatocyte growth factor; 4-HNE, 4-hydroxynonenal; HOX, heme oxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPAR, peroxisome proliferator-activated receptor; PCR, reverse-transcriptase polymerase chain reaction; TG, triglyceride; TNF-α, tumor necrosis factor-α; VLDL, very low-density lipoprotein; ZDF fa/fa, Zucker diabetic fatty.

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isome proliferator-activated receptor γ (PPARγ), which is expressed at high levels in adipocytes, forms a heterodimeric DNA-binding complex with retinoid X receptor, and acts as a transcriptional regulator of genes involved in adipocyte lipid metabolism. One such reagent, troglitazone, was reported to reduce the excessive islet triglyceride (TG) content of Zucker diabetic fatty (ZDF fa/fa) rats, which led to the restoration of β-cell function and the prevention of lipoapoptosis of β cells.[5,6] Troglitazone has also been shown to reduce TG content in liver and heart of prediabetic ZDF rats[6] or to reduce hepatic TG contents concurrently with improvement of plasma levels of TG and insulin in ZDF fa/fa rats suggesting restoration of SREBP-1 gene expression.[7] To examine effectiveness of thiazolidinediones on the experimental hepatic steatosis caused by chronic ethanol administration, we have herein chosen pioglitazone, another derivative of the antidiabetic reagents; this compound has never been reported so far to cause lactic acidosis or to increase transaminases, whereas troglitazone was withdrawn from the market after a case report of severe hepatic failure.[8] Our attempt to examine the hypothesis in this study has not only shown the effectiveness of pioglitazone but also shed light on significance of the c-Met–mediated signaling pathway to regulate synthesis and removal of triglycerides as a potential therapeutic target for treatment of ethanol-induced hepatic steatosis and injury.

Materials and Methods

Animal Feeding

The Lieber–DeCarli diets were purchased from Bio-Serv, Inc (Frenchtown, NJ).[9] Four-week-old male Sprague–Dawley rats housed in temperature- and light-controlled rooms were randomly divided into 3 groups: (A) rats fed ethanol-containing liquid diet for 6 weeks (n = 9), (B) rats pair fed ethanol-containing liquid diet for 6 weeks during which they were given pioglitazone (10 mg/kg body weight per day) once every 24 hours intragastrically (n = 9), and (C) rats pair fed isocaloric liquid diet without ethanol for 6 weeks (n = 9). Pioglitazone was dissolved in methylcellulose before use. Maeshida et al.[10] reported that pioglitazone was well absorbed from the gastrointestinal tract at an extent of 96% in rats. The plasma concentration of pioglitazone peaked at 4 hours after dosing and declined with a half-life of 2.6 hours. They also showed the concentration of radioactivity in rat tissues at 0.5, 2, 6, 10, 24, and 72 hours after oral administration of [^{14}C]pioglitazone.[10] The radioactivity of liver tissue was higher than that of plasma, and it peaked at 6 hours. The concentration of pioglitazone in liver at 24 hours after oral administration is still about one third of the concentration at 30 minutes. There is another report describing that 1 dose of 10 mg/kg of pioglitazone every 24 hours ameliorated insulin resistance associated with diabetes in rats.[11] Based on these previous papers, we think it reasonable to expect that 1 dose of 10 mg/kg of pioglitazone every 24 hours is sufficient to sustain effective concentration. The rats in groups A and C were given methylcellulose once every 24 hours intragastrically for 6 weeks in the same amount as their corresponding litter mates in group B. The rats in group B and group C were pair fed daily on an isoenergetic basis with the corresponding litter mates fed the ethanol-containing diet (group A). To investigate whether another thiazolidinedione, troglitazone, could prevent alcoholic fatty liver, we next gave troglitazone (200 mg/kg body weight/day) to 8-week-old male SD rats (n = 6), instead of pioglitazone, in the same way as our last examination of pioglitazone. Troglitazone was also well absorbed from the gastrointestinal tract, and the highest uptake by the liver was shown in rats.[12] All animals received humane care in compliance with the National Research Council's criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the U.S. National Academy of Sciences and published by the U.S. National Institutes of Health.

Biochemical and Histological Analysis

Hepatic triglyceride contents were measured as previously described.[13] For histological analysis, liver tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Alternatively, hepatic lipids were stained by an oil red O method (Nacalai Tesque Inc., Kyoto, Japan). For protein or RNA analysis, tissue was frozen in liquid nitrogen and stored at −80°C until used. Apoptosis was analyzed with TUNEL staining (Apoptosis Detection System, Promega Corp., Madison, WI) according to the manufacturer's instructions. At least 2 individual livers were examined in each group by counting the density of the positive-staining hepatocytes in 5 random × 200 fields/liver. Serum concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride, total cholesterol, phospholipids, total proteins, albumin, and free fatty acids were measured with a standard clinical autoanalyzer (Hitachi 7170; Hitachi Ltd., Tokyo, Japan). Plasma glucose was measured by the glucose oxidase method with a glucose analyzer II (Beckman Instruments, Brea, CA). Plasma TG levels were measured with a GPO-Trinder triglyceride kit (Sigma, St. Louis, MO). Plasma leptin and insulin were assayed with Linco leptin and insulin assay kits (Linco Research Immunoassay, St. Charles, MO). Serum low-density lipoprotein cholesterol was measured by the direct enzymatic method[14] with Cholestest LDL (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). Serum very low–density lipoprotein (VLDL) cholesterol was separated by a modification of the method of Hatch and Lees.[15] A commercially available enzyme-linked immunosorbent assay kit was used to determine serum tumor necrosis factor-α (TNF-α).

Table 1. Primer and Taqman Probe Sequences for Real-Time PCR

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	Taqman probe (5'-3')
c-Met	5'-CGACATTCAGTCCGAGGTTCA-3'	5'-GGGACACTGGCCTGACTCTTC-3'	5'-TGCATGTTCTCCCCACTTGCGG-3'
HGF	5'-GACATGTCTTGCCTGATTCTGTGT-3'	5'-AGTCTGTGACATTCCTCAGTGTTCA-3'	5'-TCACCGTTGCAGGTCATGCATTCA-3'
HOX1	5'-CACCTTCCCGAGCATCGA-3'	5'-AGGCGGTCTTAGCCTCTTCTGT-3'	5'-CTCGCATGAACACTCTGGAGATGACC-3'
MT1	5'-CTGCTCCACCGGCGG-3'	5'-GCCCTGGGCACATTTGG-3'	5'-CTCCTGCAAGAAGAGCTGCTGCTCCT-3'
MT2	5'-TCCTGTGCCACAGATGGATC-3'	5'-GTCCGAAGCCTCTTTGCAGA-3'	5'-AAAGCTGCTGTTCCTGCTGCCCC-3'
SREBP1a	5'-ACACAGCGGTTTTGAACGACA-3'	5'-GCATCAAATAGGCCAGGGAA-3'	5'-CATGCTTCAGCTCATCAACAACCAAG-3'
SREBP1c	5'-GGAGCCATGGATTGCACATT-3'	5'-GCATCAAATAGGCCAGGGAA-3'	5'-CATGCTTCAGCTCATCAACAACCAAG-3'
SCD1	5'-CCTCATCATTGCCAACACCAT-3'	5'-AGCCAACCCACGTGAGAGAA-3'	5'-TTCTCTGAGACACACGCCGACCCTC-3'
SCD2	5'-ACCGCTGGCACATCAACTTC-3'	5'-GGACACCCTCTTCCGGTCAT-3'	5'-CCACGTTCTTCATCGACTGCATGGC-3'

Real-Time Quantitative PCR Analysis

Total RNA was extracted from the liver with ISOGEN (Nippon Gene, Tokyo, Japan) according to the method of Chomczynski and Sacchi, as previously described.[16] For the reverse-transcriptase reaction, TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) were used. Briefly, the reverse-transcriptase reaction (final volume of 50 μL) was conducted for 60 minutes at 37°C followed by 25°C for 10 minutes using random hexamers. Polymerase chain reaction (PCR) amplification was performed with TaqMan Universal Master Mix (Applied Biosystems). In brief, reactions were performed in duplicate containing 2× Universal PCR master mix, 2 μL of template cDNA, 900 nmol/L of primers, and 250 nmol/L of probe in a final volume of 50 μL and were analyzed in a 96-well optical reaction plate (Applied Biosystems). Primers and probes were synthesized by Applied Biosystems custom oligo synthesis service. Sequences of primers and probes are shown in Table 1. Probes include a fluorescent reporter dye, FAM, on the 5′ end and labeled with fluorescent quencher dye, TAMRA, on the 3′ end to allow direct detection of the PCR product. Reactions were amplified and quantified using an ABI 7700 sequence detector and manufacturer's software (Applied Biosystems). The threshold cycle (Ct) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The relative quantity of target messenger RNA (mRNA) was obtained using the comparative Ct method and was normalized using predeveloped TaqMan assay reagent rat 18S ribosomal RNA as an endogenous control (Applied Biosystems) (for details, see user Bulletin 2 for the ABI PRISM 7700 Sequence Detection System under www.appliedbiosystems.com/support/tutorials). Briefly, the TaqMan software (Applied Biosystem) was used to calculate a Ct value for each reaction, where the Ct value is the point in the extension phase of the PCR reaction that the product is distinguishable from the background. The Ct values were then normalized for amplification by subtracting the Ct value calculated for 18S ribosomal RNA, an endogenous control for the amount of mRNA from the same sample, to obtain a Ct using the following equation: Ct target − Ct 18S ribosomal RNA = Ct. The fold induction was calculated relative to the Ct value obtained in the control rats or cells. The normalized expression was calculated as fold changes in a quantity of mRNA. TNF-α mRNA was measured using predeveloped TaqMan assay reagent rat TNF-α (Applied Biosystems) (for details, see user Bulletin 2 for the ABI PRISM 7700 Sequence Detection System under www.appliedbiosystems.com/support/tutorials).

Isolation and Primary Culture of Rat Hepatocytes

Primary cultured hepatocytes were prepared from livers of ethanol-fed rats through a collagenase perfusion method in group A as described elsewhere.[17] The isolated hepatocytes were cultured on gelled pig tendon collagen (Cellmatrix, Nitta zerachin, Ltd., Osaka, Japan), and the second layer of collagen gel was spread over the cells after 1 day of incubation as previously described.[17] A PPARγ-selective agonist AD4833 (pioglitazone hydrochloride, Takeda, Ltd., Osaka, Japan)[18] and troglitazone (Sankyo, Ltd., Tokyo, Japan)[5] was dissolved in dimethyl sulfoxide (DMSO) and added to the hepatocytes at various doses in a volume at 0.05% of the medium. In the control, the medium was supplemented with the same volume of DMSO.

Transcriptome Analyses by DNA Microarray

Transcriptome analyses were performed by using DNA microarrays (Atlas cDNA expression arrays, Clontech). Preparation of ^{33}P-labeled cDNA samples, hybridization, and washing were carried out with total RNA of the liver tissue according to the manufacturer's manual. All data sets were normalized to the signal density of housekeeping genes, such as the gene encoding glyceraldehyde-3-phosphate dehydrogenase, and total radioactivity, which represents the total amount of cDNA hybridized to the microarrays. The threshold for determining the significance of changes in the level of gene expression was established by using an algorithm that requires both a significant absolute and significant fold change.[19,20]

Western and Immunoprecipitation Analysis

Total cellular protein (50 μg) collected from snap-frozen liver was subjected to Western blot analysis for c-Met, mature hepatocyte growth factor (HGF), and apolipoprotein B (apoB)100. For immunoblot analyses, primary antisera were used at the concentration of 1:100. The membranes were

probed with antirat c-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-rat HGFα antibody (Santa Cruz Biotech), and anti-rat apoB antibody (Santa Cruz Biotech). For immunoprecipitation, lysates (10 mg of protein) were incubated with protein A-agarose beads (Pierce, Rockford, IL) to precipitate the antigen-antibody complex. Samples were separated by SDS-PAGE on 10% polyacryamide gels. After electrophoresis, the gel was transferred to polyvinylidine difluoride sheets, which were subsequently probed with anti-phosphotyrosine antibody (PY-20; ICN Biomedicals Inc., Aurora, OH) or antirat c-Met antibody. Then, they were incubated with a chemiluminescence substrate (ECL reagent: Amersham Life Science, Buckinghamshire, UK) and exposed to Hyperfilm-MP (Amersham). Densitometric analysis was performed with National Institutes of Health Image Data analysis software.

Immunohistochemistry

For detection of 4-hydroxynonenal (4-HNE) protein adducts, paraffinized sections were deparaffinized, rehydrated, treated with normal horse serum, and incubated with mAb anti-4-HNE (0.625 μg/mL) (Nippon Rouka Seigyo Kenkyuujo, Tokyo, Japan) overnight at 4°C. After several washes with phophate-buffered saline, the sections were stained with biotinylated antimouse IgG for 1 hour (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA). To prevent endogenous peroxidase reactions, the samples were pretreated with 0.3% H_2O_2 in cold methanol for 30 minutes and were subsequently incubated with avidin and horseradish peroxidase (HRP)-conjugated biotin for 30 minutes, followed by detection with 3,3'-diaminobenzidine solution containing 0.003% H_2O_2.

Measurement of DNA Synthesis

DNA synthesis was measured by bromodeoxyuridine (BrdU) incorporation with a Cell Proliferation ELISA, BrdU kit (Roche Molecular Biochemicals, Mannheim, Germany). In brief, hepatocytes were suspended in William's medium E supplemented with 10% fetal bovine serum (FBS), 10 nmol/L insulin, 10 nmol/L dexamethasone, penicillin (5 U/mL), and streptomycin (50 μg/mL) and seeded at a density of 5×10^4 cells/cm^2 on collagen-coated dishes. The culture medium was exchanged after 4 hours for William's medium E supplemented with 10% FBS. After incubation in William's medium E with 10% FBS for approximately 20 hours, the culture medium was exchanged for William's medium E containing 10% FBS with HGF/SF, pioglitazone, or both. They were then cultured for another 24 hours with the addition of BrdU and were harvested in determining the BrdU incorporation into cellular DNA according to the manufacturer's instructions.

Analyses for Effects of Pioglitazone on Ethanol Metabolism in Rats

Four-week-old male Sprague–Dawley rats were randomly divided into 2 groups: rats fed the Lieber–DeCarli control liquid diet for 10 days (n = 16) and those pair fed isocaloric control liquid diet for 10 days during which they were given pioglitazone (10 mg/kg body weight/day) once every 24 hours intragastrically (n = 16). After overnight starvation, rats were given ethanol intragastrically at 4 g/kg body weight on the 10th day. Before and 2, 12, and 24 hours after the ethanol administration, 4 rats in each group were sacrificed to collect heparinized blood samples which were immediately deproteinized to determine ethanol and acetaldehyde concentrations through gas chromatography.[21]

Statistical Analysis

All data are expressed as the mean ± standard error. Statistical analysis was performed by using the unpaired Student t test or by 1-way analysis of variance. When the analysis of variance analyses were applied, differences in mean values among groups were examined by Fisher's multiple comparison test.

Results

Pioglitazone Attenuates Hepatic Steatosis Caused by Chronic Ethanol Administration

The hepatic triglyceride concentration in rats given the isocaloric pair fed liquid diet was 9.67 (±4.34) mg/g liver weight, as opposed to 78.24 (±11.25) mg/g liver weight in the rats given the ethanol-containing diet for 6 weeks (Table 2). Histological analysis showed that lipid droplets accumulated in the hepatocytes in pericentral regions but not in those in periportal regions in the ethanol-fed rats. On the other hand, such changes were not notable in the control liver even with the lipid-specific oil red staining (Figure 1). Pioglitazone (10 mg/kg per day) markedly decreased accumulation of lipid droplets in both perivenular and the periportal regions with the total body weight remaining unchanged (Figure 1). In good agreement with these results, hepatic triglyceride levels became as low as in the pair-fed control rats (Table 2). Treatment with methylcellulose as a vehicle had no effect on either triglyceride contents or histology in the liver (data not shown). The ethanol feeding caused an increase in the liver weight/body weight ratio and a decrease in the epididymal fat weight/body weight ratio, whereas pioglitazone treatment prevented these changes (Table 2).

Analyses of serum AST and ALT activity to assess the effect of pioglitazone on liver function showed that pioglitazone suppressed the elevation of serum concentrations of AST and ALT in ethanol-fed rats (Table 2), and this improvement was associated with improvement in histological findings in the liver. Ethanol feeding caused elevations of the serum concentrations of free fatty acids,

Table 2. Body Weight and Serum Biochemical Parameters in the Treated Groups

	Ethanol (n = 8)	Ethanol + Pioglitazone (n = 8)	Control (n = 8)
Liver weight/body weight (%)	[b]3.57 (0.34)	3.08 (0.24)	2.35 (0.25)
Epididymal fat weight/body weight (%)	[a]0.574 (0.044)	0.808 (0.065)	0.806 (0.062)
Liver triglyceride (mg/g liver weight)	[a]78.24 (11.25)	14.36 (3.72)	9.67 (4.34)
AST (IU/L)	[a]362.4 (25.4)	195.7 (23.3)	38.1 (10.6)
ALT (IU/L)	[a]174.2 (13.4)	50.3 (4.9)	33.5 (5.2)
Phospholipid (mg/dL)	159.2 (10.8)	146.7 (14.2)	110.0 (7.3)
FFA (mEQ/L)	0.562 (0.126)	0.550 (0.240)	[a]0.448 (0.066)
Total cholesterol (mg/dL)	116.0 (8.9)	130.8 (20.9)	[a]72.7 (7.1)
LDL (mg/dL)	82.4 (15.8)	98.0 (22.3)	45.4 (7.9)
VLDL (mg/dL)	7.25 (0.23)	7.96 (0.35)	6.94 (0.28)
Triglyceride (mg/dL)	21.67 (3.68)	20.35 (2.76)	[a]14.58 (2.64)
Leptin (ng/mL)	0.62 (0.25)	1.55 (1.25)	0.82 (0.46)
TNF-α (pg/mL)	22.26 (4.48)	23.32 (3.87)	25.02 (4.87)

NOTE. Results (and standard error of the mean) from 8 rats/group at the end of feeding period.
[a]$P < 0.05$ versus both other groups.
[b]$P < 0.05$ versus the control group.

triglyceride, phospholipid, total cholesterol, and LDL, and pioglitazone caused elevation of serum VLDL levels, but no significant changes were found in serum TNF-α or leptin levels (Table 2). We did not find any significant changes in total protein, albumin, glucose, insulin, lactic acid, or choline esterase levels (data not shown).

Transcriptome Analyses for Mining Genes Responsible for Antisteatotic Effects of Pioglitazone

To elucidate mechanisms by which pioglitazone reduces hepatic lipid contents in alcoholic fatty liver, we

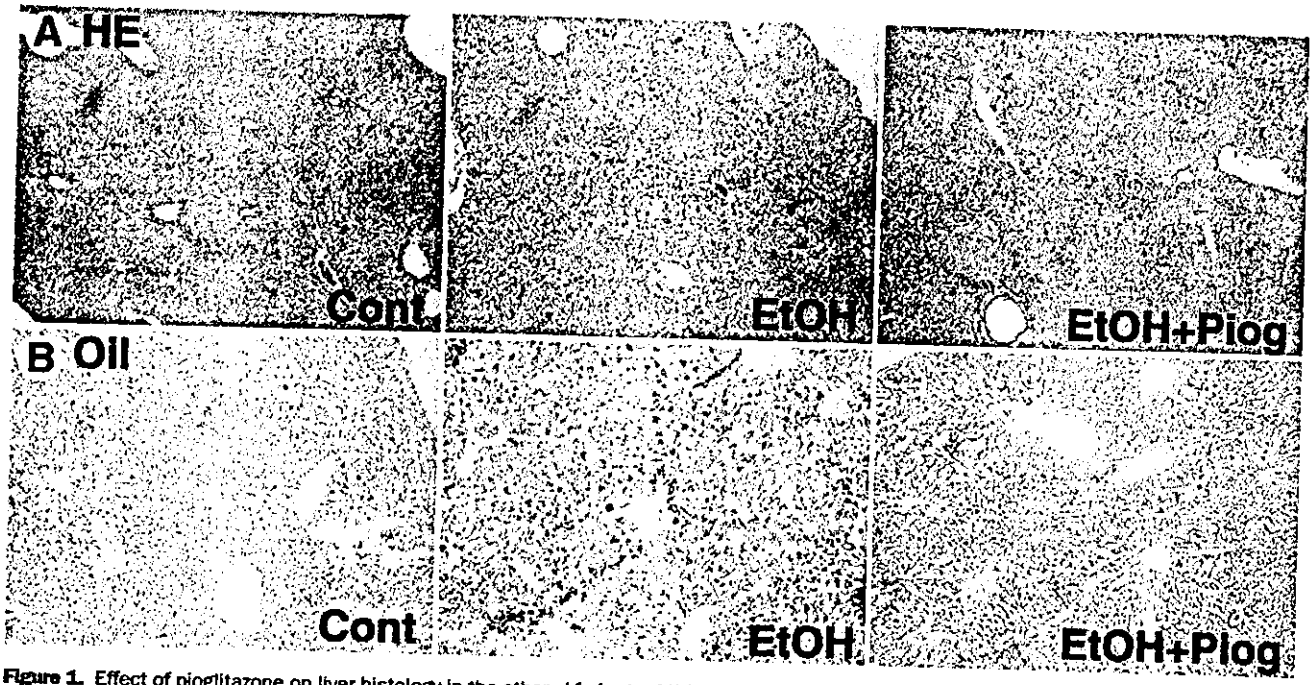

Figure 1. Effect of pioglitazone on liver histology in the ethanol-fed rats. (*A*) H&E-stained sections of representative liver samples of control rats (Cont), ethanol-fed rats (EtOH), and ethanol-fed rats given pioglitazone (EtOH+Piog) (×40). Minimal steatosis or no change was seen in the livers of the pair-fed control rats. Severe macrovesicular steatosis was observed preferentially in the perivenular areas in the ethanol-fed rats. Pioglitazone markedly decreased accumulation of lipid droplets in both the perivenular and the periportal areas. Only minimal steatosis was left in the livers of ethanol-fed rats given pioglitazone. (*B*) Oil red–stained sections of representative liver samples of control rats, ethanol-fed rats, and ethanol-fed rats given pioglitazone (×40). Specific staining of lipid accumulation was shown. Pioglitazone markedly decreased accumulation of lipid droplets.

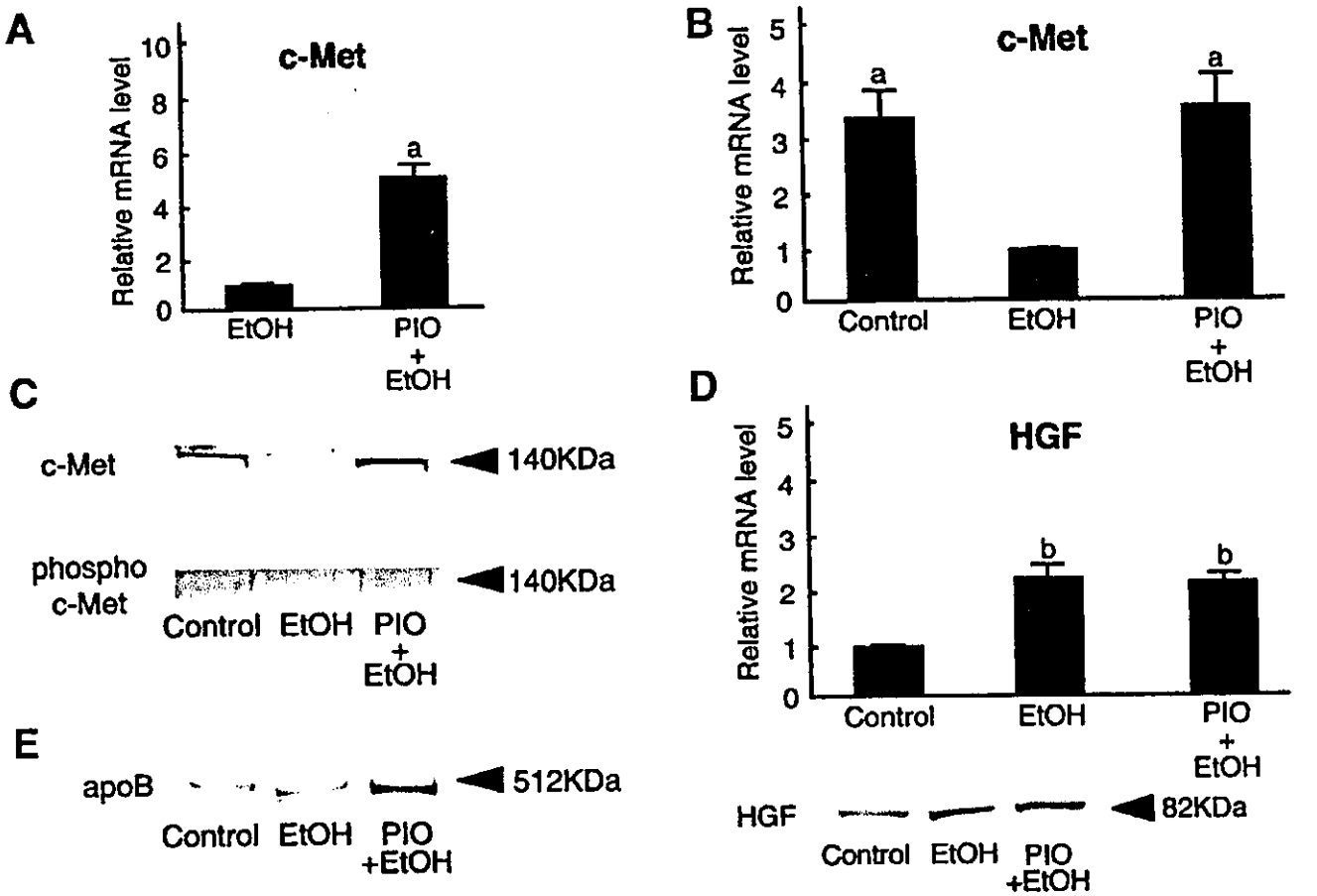

Figure 2. Hepatic c-Met, HGF, and apoB expression. (*A*) DNA microarray analysis showed that pioglitazone increased hepatic c-Met mRNA expression. Results (and standard error of the mean) from 3 rats/group at the end of feeding period. $^{a}P < 0.05$ versus the ethanol-fed group. EtOH, ethanol-fed rats; PIO+EtOH, ethanol-fed rats given pioglitazone. (*B*) Real-time PCR analysis showed that chronic ethanol feeding decreased hepatic c-Met expression and that pioglitazone prevented this decrease. Results (and standard error of the mean) from 6 rats/group at the end of feeding period. All real-time quantitative PCR reactions were carried out in duplicate. $^{a}P < 0.05$ versus the ethanol-fed group. (*C*) Western immunoblot analysis of expression of c-Met (*upper*) and tyrosine phosphorylation of c-Met (*lower*) in liver homogenates (containing 50 μg of total protein each). (*D*) Real-time PCR analysis (*upper*) and Western blotting analysis (*lower*) showed that chronic ethanol feeding increased hepatic HGF expression and pioglitazone had no effect on this. Results (and standard error of the mean) from 6 rats/group at the end of feeding period. All real-time quantitative PCR reactions were carried out in duplicate. $^{b}P < 0.05$ versus the control group. (*E*) Western immunoblot analysis of expression of apoB in liver homogenates (containing 50 μg of total protein each). Control, rats pair-fed isocaloric liquid diet without ethanol; EtOH, ethanol-fed rats; PIO+EtOH, ethanol-fed rats given pioglitazone.

applied DNA microarrays to reveal differential mRNA expression between the livers exposed to chronic ethanol feeding with and without pioglitazone treatment. The transcriptome analysis allowed us to reveal up-regulation of c-Met (Figure 2*A*) and down-regulation of SCD-1 and -2, lipogenic enzymes in the liver (data not shown). Pioglitazone had no effect on the hepatic levels of TNF-α, UCP-2, CYP4A2 (cytochrome P450 4A2), CYP2E1, PPARγ, AMPK-catalytic subunit (α1 and α2), microsomal triglyceride transfer protein (MTTP), hepatic enzymes contributing to fatty acid oxidation such as carnitine palmitoyltransferase-1 or acyl-CoA oxidase (data not shown). These results were confirmed by real-time PCR analysis (Table 3). Chronic ethanol feeding significantly increased CYP2E1 mRNA and decreased PPARγ mRNA (Table 3).

Pioglitazone Increased Hepatic c-Met Expression and Induced Tyrosine Phosphorylation of c-Met Leading to Increased Hepatic apoB Expression

Alterations in c-Met gene expression were also confirmed at the protein levels. Moderate expression of c-Met protein was observed in the liver of the control rats, and the ethanol diet decreased the expression of c-Met protein (Figure 2*C*). The decrease in c-Met protein

Table 3. The Level of Expression of Genes Known to Play a Role in the Formation of Steatosis and Steatohepatitis Among the Treated Groups

Gene	Ethanol	Ethanol + Pioglitazone
TNF-α	1.12 ± 0.22	1.17 ± 0.23
UCP2	1.21 ± 0.30	1.18 ± 0.21
CYP2E1	*1.81 ± 0.32	*1.76 ± 0.24
CYP4A2	1.07 ± 0.36	1.11 ± 0.23
PPAR-α	*0.46 ± 0.12	*0.50 ± 0.09
AMPKα1	0.89 ± 0.31	0.95 ± 0.19
AMPKα2	1.14 ± 0.15	1.08 ± 0.17
Acyl-CoA oxidase	0.90 ± 0.28	0.95 ± 0.21
CPT-1	1.06 ± 0.17	1.10 ± 0.23
MTTP	0.94 ± 0.27	0.95 ± 0.26

NOTE. Results (and SEM) from 8 rats/group at the end of feeding period. Quantifications were normalized for RNA from the liver in the control group.

*$P < 0.05$ versus the control group.

in the liver of the ethanol-fed rats was restored by pioglitazone administration (Figure 2*C*). The level of tyrosine phosphorylation of c-Met was also reduced in the ethanol-fed liver, whereas the pioglitazone treatment attenuated this change (Figure 2*C*). DNA array and real-time PCR analyses also showed that ethanol administration decreased hepatic c-Met expression and that pioglitazone prevented this decrease (Figure 2*A, B*). Chronic ethanol feeding significantly increased HGF at both mRNA and protein levels, whereas the pioglitazone treatment did not alter these levels (Figure 2*D*). Western blotting analysis showed that the level of apoB 100 protein in the liver was increased by pioglitazone in the ethanol-fed rats (Figure 2*E*).

Stimulatory Effects of Pioglitazone on c-Met Expression, Its Tyrosine Phosphorylation, DNA Synthesis, and apoB Expression in Primary-Cultured Hepatocytes

To examine the direct effect of pioglitazone on expression of c-Met, rat primary cultured hepatocytes were incubated with various doses of pioglitazone and troglitazone for 24 hours (from 24 hours to 48 hours after inoculation). Ten μM pioglitazone markedly increased c-Met expression. The same concentration of troglitazone also increased c-Met expression, and the addition of bisphenol A diglycidyl ether (BADGE), a PPARγ antagonist,[22] inhibited this reaction (Figure 3*A*). The level of tyrosine phosphorylation was also increased by the addition of pioglitazone (Figure 3*B*). Stimulation of hepatocytes with both 5 ng/mL HGF and pioglitazone led to a greater increase in DNA synthesis than with either alone. In primary-cultured hepatocytes, HGF at 5 ng/mL and pioglitazone at 10 μmol/L maximally stimulated BrdU incorporation into DNA (Figure 3*C*). Pioglitazone also mimicked stimulatory effects of HGF on the expression of apoB in primary-cultured hepatocytes as seen in Figure 3*D* and *E*.

Troglitazone Mimicked the Actions of Pioglitazone In Vivo

In the in vivo study for troglitazone, we found that troglitazone significantly reduced fat accumulation and improved serum transaminase level in ethanol-fed rats. Troglitazone also enhanced hepatic c-Met expression (Figure 4).

Pioglitazone Decreased Hepatic SCD-1, 2, and SREBP-1c Expressions, Mimicking Effects of HGF

The real-time PCR analysis showed that chronic ethanol feeding conspicuously increased hepatic SCD-1 and 2 expressions, and that pioglitazone treatment suppressed them, consistent with the results of DNA array (Figure 5). Pioglitazone significantly decreased hepatic SREBP-1c expressions, although it had no effect on hepatic SREBP-1a level (Figure 5). Such effects of pioglitazone were also reproducible in primary-cultured hepatocytes. The HGF treatment dramatically decreased SREBP-1c and SCD-2 expressions in primary-cultured hepatocytes (Figure 6*A*). Pioglitazone treatment also decreased SREBP-1c and SCD-2 expressions upon the application of HGF, although the reagent had no effect on them without it (Figure 6*B, C*).

Pioglitazone Attenuates Hepatic Lipid Peroxidation Elicited by Chronic Ethanol Feeding

4-HNE-adducted protein, as a product of lipid peroxidation reaction, was intensely detected in centrilobular regions of ethanol-fed rats and colocalized with accumulated lipid droplets. The pioglitazone treatment decreased the products of lipid peroxidation (Figure 7*A*). We found by real-time PCR analysis that the hepatic level of stress response proteins such as metallothionein (MT) -1, -2, and heme oxygenase (HOX)-1 dramatically increased in ethanol-fed rats (group A). Pioglitazone prevented these stress responses induced by ethanol (Figure 7*B*). Of importance in the current study is that neither the pioglitazone treatment nor chronic ethanol feeding induced any notable apoptosis, as judged by the TUNEL method seen in Figure 7*C*.

In this study, all rats in the groups were pair fed daily on an isoenergetic basis, and the rats in group A and B were daily given the same amounts of ethanol. We then inquired

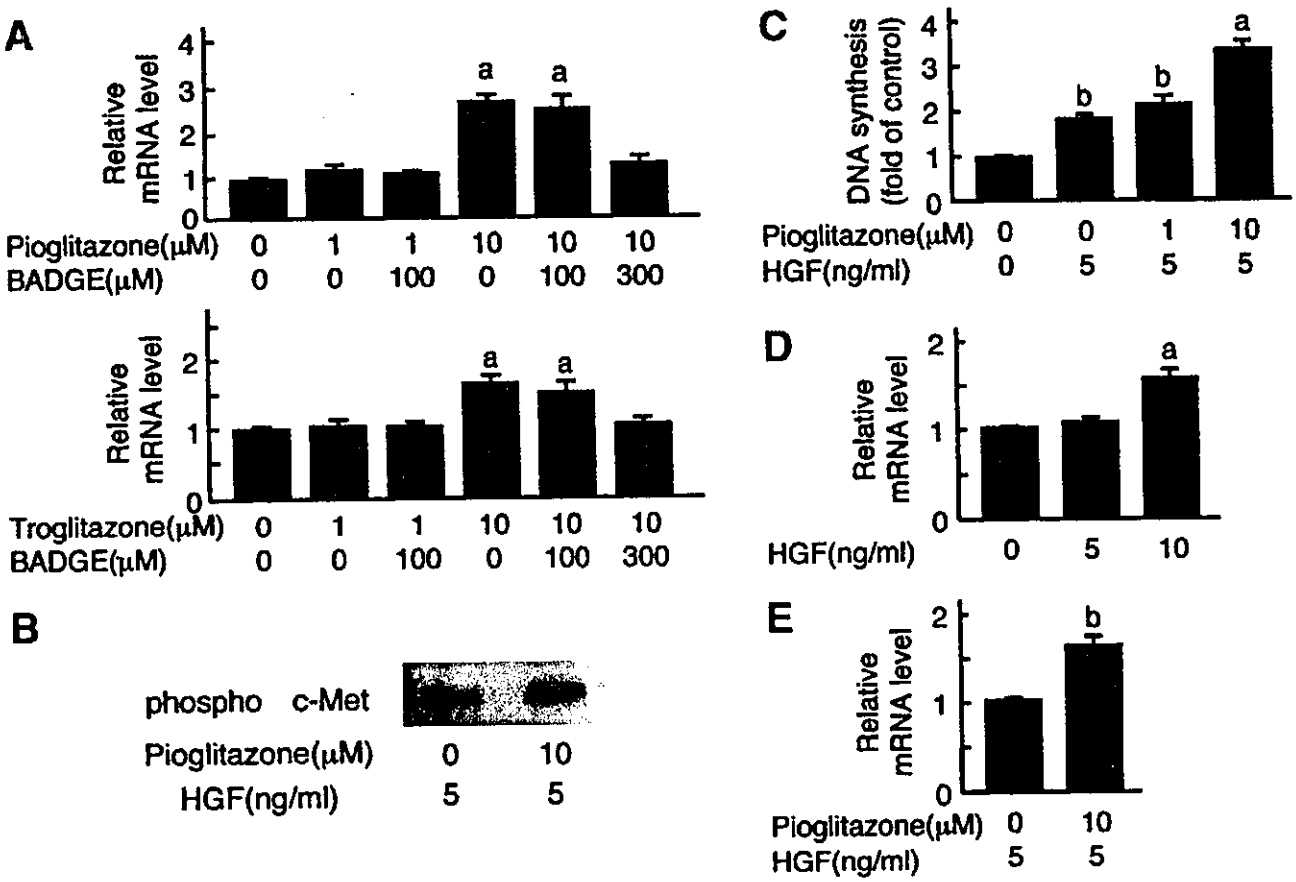

Figure 3. Direct effect of pioglitazone on expression and tyrosine phosphorylation of c-Met, expression of apoB, and on DNA synthesis in rat primary cultured hepatocytes. (*A*) Rat primary-cultured hepatocytes were incubated with various doses of pioglitazone or troglitazone for 24 hours (from 24 to 48 hours after inoculation). In the control, the medium was supplemented with the same volume of DMSO. Real-time quantitative PCR analysis of c-Met showed that 10 μmol/L of pioglitazone treatment dramatically increased c-Met expression. Ten μmol/L of troglitazone treatment also significantly increased c-Met expression. Addition of 300 μmol/L of BADGE inhibited this increase. All real-time quantitative PCR reactions were carried out in duplicate. Results (and standard error of the mean) from 4 individual experiments. $^{a}P < 0.05$ versus the groups with 0 or 1 μmol/L of pioglitazone or troglitazone treatment. (*B*) Tyrosine phosphorylation of c-Met in homogenates of primary-cultured hepatocytes stimulated or not with 5 ng/mL HGF or pioglitazone. Addition of 10 μmol/L pioglitazone induced tyrosine phosphorylation of c-Met. (*C*) Effect of pioglitazone on DNA synthesis of primary cultured hepatocytes. BrdU incorporation into cellular DNA was determined 24 hours after the addition of HGF and/or pioglitazone. $^{a}P < 0.05$ versus the other groups. $^{b}P < 0.05$ versus the groups without any addition of HGF or pioglitazone. Next, real-time PCR analysis of primary-cultured hepatocytes was used to assess apoB gene expression (*D*, *E*). (*D*) Rat primary-cultured hepatocytes were incubated with various doses of HGF for 24 hours. $^{a}P < 0.05$ versus the group without addition of HGF. (*E*) Incubation with pioglitazone with addition of 5 ng/mL of HGF for 24 hours. $^{b}P < 0.05$ versus the group without any addition of pioglitazone. All real-time quantitative PCR reactions were carried out in duplicate. Results (and standard error of the mean) from 4 individual experiments.

whether the treatment with pioglitazone by itself could alter ethanol metabolism in rats. Table 4 shows that blood levels of ethanol and acetaldehyde did not differ significantly irrespective of the presence or absence of pioglitazone in ethanol-fed rats. This result suggests that pioglitazone has little, if any, effect on ethanol metabolism and thus is not a likely mechanism for the attenuating effect of this reagent on hepatic steatosis.

Discussion

This study first suggested that pioglitazone serves as a potentially therapeutic tool to attenuate hepatic steatosis caused by chronic administration of ethanol. Furthermore, several lines of evidence provided in this study suggest that the ability of this antidiabetic to significantly alter synthesis and redistribution of lipids is ascribable to therapeutic potential against the ethanol-induced hepatic steatosis. The epididymal fat weight/body weight ratio significantly decreased in the ethanol-fed rats compared with that in the pair fed rats. Distinct from nonalcoholic hepatic steatosis, which induced by chronic alcohol abuse often coincided with energy wasting and inhibition of adipose tissue accumulation. These events are likely to result in the fact that alcoholics are

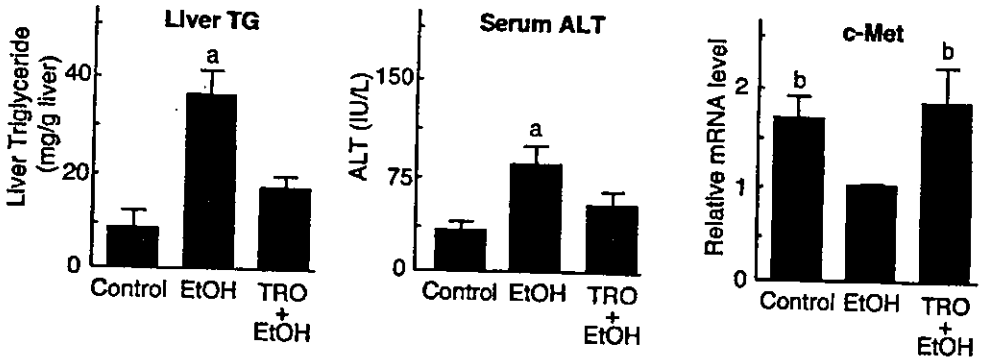

Figure 4. Troglitazone mimicked the actions of pioglitazone in vivo. Eight-week-old male SD rats fed ethanol-containing liquid diet were given troglitazone (200 mg/kg body weight per day) once every 24 hours intragastrically for 6 weeks. In this study, we divided the rats into 3 groups in the same way as our last examination of pioglitazone (n = 6). Troglitazone decreased accumulation of lipid droplets and suppressed the elevation of serum concentrations of ALT in ethanol-fed rats. Real-time PCR analyses showed that ethanol administration decreased hepatic c-Met expression and that troglitazone prevented this decrease. Results (and standard error of the mean) from 6 rats/group at the end of feeding period. All real-time quantitative PCR reactions were carried out in duplicate. $^{a}P < 0.05$ versus the other groups. $^{b}P < 0.05$ versus the ethanol-fed group. Control, rats pair-fed isocaloric liquid diet without ethanol; EtOH, ethanol-fed rats; TRO+EtOH, ethanol-fed rats given troglitazone.

not obese despite a high total energy intake.[23] Pioglitazone prevented the decrease in amount of adipose tissue, probably by mobilizing fat from the liver to adipose tissue.

It has been believed until now that impaired mitochondrial β-oxidation of fatty acids could be a major cause of triglyceride accumulation in alcoholic fatty liver.[24] In the current study, however, pioglitazone did not alter the expression of hepatic enzymes contributing to fatty acid oxidation such as carnitine palmitoyltransferase-1 or acyl-CoA oxidase (AOX) or the expression of hepatic TNF-α, uncoupling protein-2, PPARγ, or AMPK-catalytic subunit (α1 and α2) levels; plasma TNF-α, glucose, leptin, insulin levels, or insulin resistance remained unchanged. These results suggest that other mechanisms play central roles in pioglitazone's effect on alcoholic fatty liver. Differential

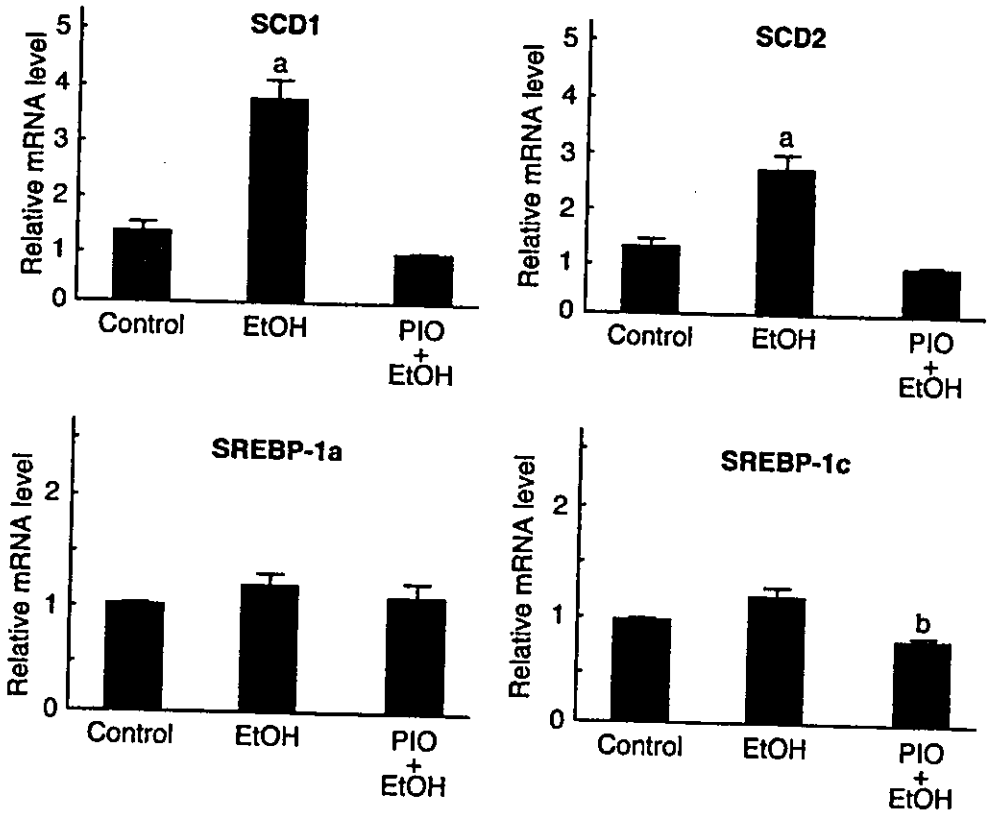

Figure 5. Hepatic SCD1, SCD2, SREBP-1a, and SREBP-1c expression. Real-time PCR analysis was carried out to quantitate hepatic mRNA levels of SCD1, SCD2, SREBP-1a, and SREBP-1c in liver homogenates among the groups. Results (and standard error of the mean) from 4 rats/group at the end of feeding period. All real-time quantitative PCR reactions were carried out in duplicate. $^{a}P < 0.05$ versus the other groups. $^{b}P < 0.05$ versus the ethanol-fed group. Control, rats pair-fed isocaloric liquid diet without ethanol; EtOH, ethanol-fed rats; PIO+EtOH, ethanol-fed rats given pioglitazone.

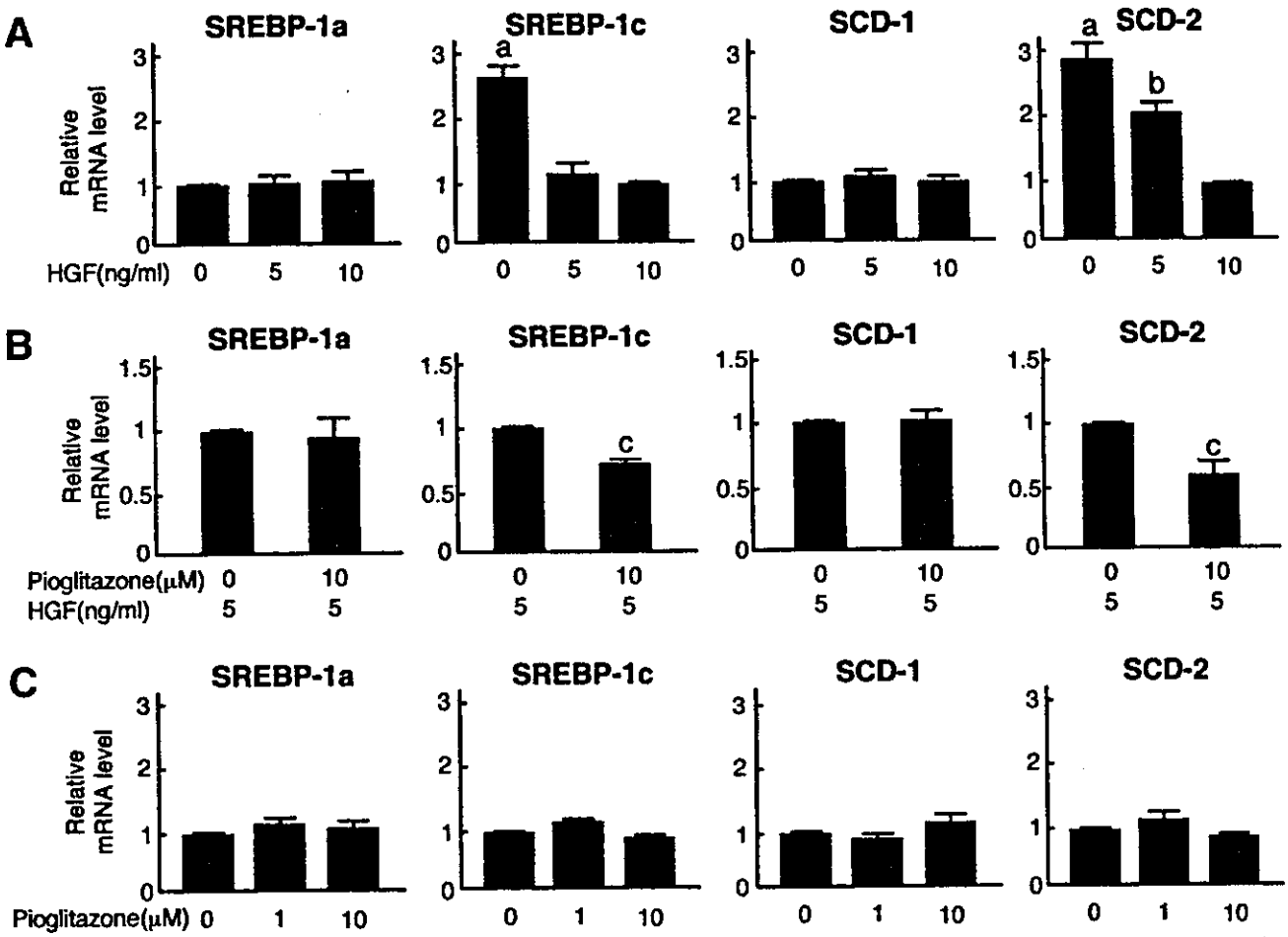

Figure 6. Effects of pioglitazone and HGF on expression of SREBP-1a, c, and SCD-1, 2 mRNA in hepatocyte cultures. Real-time PCR analysis was used to assess SREBP-1a, SREBP-1c, SCD-1, and SCD-2 gene expression. (*A*) Rat primary-cultured hepatocytes were incubated with various doses of HGF for 24 hours. [a]$P < 0.05$ versus the other groups. [b]$P < 0.05$ versus the group with addition of 10 ng/mL of HGF. (*B*) Incubation with pioglitazone with addition of 5 ng/mL of HGF for 24 hours. [c]$P < 0.05$ versus the group without any addition of pioglitazone. (*C*) Incubation with various doses of pioglitazone for 24 hours. All real-time quantitative PCR reactions were carried out in duplicate. Results (and standard error of the mean) from 4 individual experiments.

transcriptome analyses comparing mRNA expression in the chronic ethanol-exposed livers with and without the pioglitazone treatment led us to pinpoint a critical role of the hepatic c-Met signaling pathway, and the results suggest that the reagent facilitates HGF-induced intracellular signaling without altering hepatic HGF levels.

HGF is the most potent stimulator of hepatocyte proliferation.[25] HGF has multiple biological properties in the liver, including mitogenic, antifibrotic, antiapoptotic, and cytoprotective activities.[26–28] Such multiple biological responses elicited by HGF are transferred through the cytoplasmic domain of c-Met, a specific cell surface transmembrane tyrosine kinase receptor.[26] Observation that pioglitazone directly enhanced both c-Met and its activated form in rat primary-cultured hepatocytes from ethanol-fed rats suggests that this reagent serves as the first clinically available tool that directly up-regulates c-Met expression in hepatocytes. Several data in the current study suggested that PPARγ is involved in mechanisms by which the reagent up-regulates c-Met, inasmuch as this event is mimicked by troglitazone, another PPARγ ligand, and is canceled by BADGE, a PPARγa antagonist. Further studies are necessary to determine the whole mechanisms for exploring direct actions of the reagent on c-Met expression.

HGF is known to stimulate apoB secretion in hepatocytes and to induce cell maturation during liver regeneration.[29] The HGF administration has recently been shown to improve alcoholic fatty liver by enhancing apoB synthesis and subsequent mobilization of lipids from hepatocytes with fatty changes.[13] These observations led us to examine if pioglitazone could serve as a

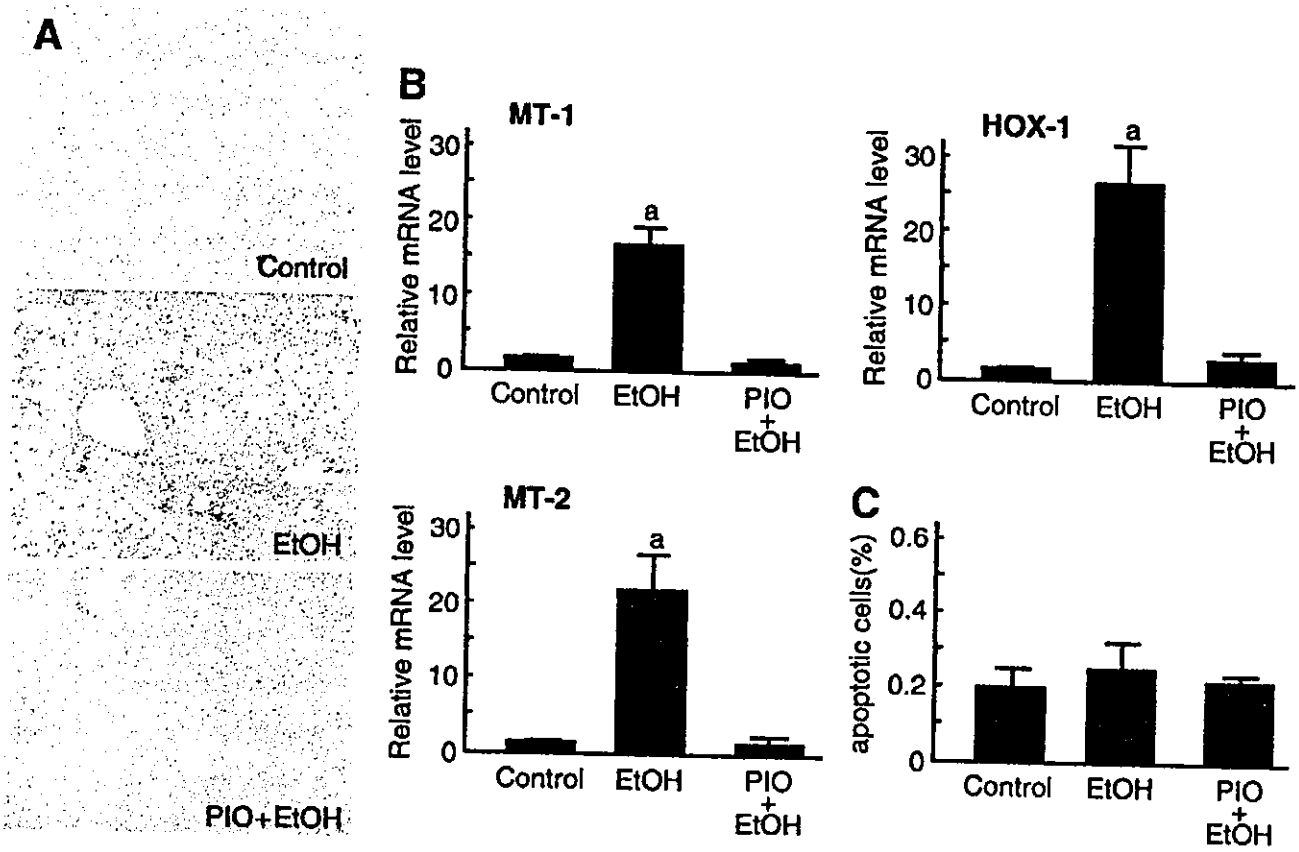

Figure 7. Effect of pioglitazone on hepatic lipid peroxidation, apoptosis, and expression of stress response proteins. (*A*) Immunohistochemistry for 4-HNE. Representative liver samples of control rat (Cont), ethanol-fed rats (EtOH), and ethanol-fed rats given pioglitazone (EtOH+Piog) (×100). (*B*) Real-time PCR analysis was used to assess stress response proteins such as MT-1, MT-2, and HOX-1 mRNA expression in liver homogenates among the groups. Results (and standard error of the mean) from 4 rats/group at the end of feeding period. All real-time quantitative PCR reactions were carried out in duplicate. $^{a}P < 0.05$ versus the other groups. (*C*) TUNEL results of liver apoptosis among the treated groups. The apoptotic cells and hepatocytes were counted from 5 lower microscopic fields for each animal.

substitute reagent that triggers lipid mobilization from the liver through stimulation of the c-Met pathway. As one might expect, the current results showed that pioglitazone mimics such effects of HGF to stimulate hepatic apoB synthesis and resultant VLDL secretion through HGF/c-Met intracellular signaling; this event could greatly contribute to mobilization of triglycerides from the liver undergoing chronic ethanol exposure.

Table 4. Blood Concentration of Ethanol and Acetaldehyde in Pioglitazone-Treated and Control Rats After Ethanol Administration (4 g/kg)

		Control	Pioglitazone Treated
Ethanol (mg/dL)	0 h	0 ± 0	0 ± 0
	2 h	184 ± 27	198 ± 26
	12 h	82 ± 22	84 ± 35
	24 h	3 ± 1	4 ± 2
Acetaldehyde (mg/L)	0 h	0 ± 0	0 ± 0
	2 h	1.9 ± 0.5	2.0 ± 0.4
	12 h	0.2 ± 0.1	0.2 ± 0.1
	24 h	0 ± 0	0 ± 0

Besides its action on lipid mobilization from the liver, pioglitazone obviously exerts its anti-steatotic actions through multiple mechanisms as judged by alterations in expression of genes responsible for triglyceride synthesis. SREBPs and stearoyl-CoA (SCD) are such genes responding to pioglitazone. Ntambi et al.[30] recently reported that a lipogenic diet fed to mice with a null mutation in the SCD1 gene (SCD1-/-) failed to induce the synthesis of triglycerides in liver, despite the induction of expression of SREBP-1 and its target genes. Cohen et al.[31] also reported that SCD-1 is required for the fully developed obese phenotype of leptin-deficient mice, including fatty liver. SCD-1 and SCD-2 catalyze the same reaction, and SCD-2 is reported to be expressed at higher levels in livers of mice overexpressing the truncated nuclear form of SREBP-1.[32] These observations suggest that induction of triglyceride synthesis is highly dependent on SCD