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Metformin Prevents Alcohol-Induced Liver Injury in the Mouse: Critical Role of Plasminogen Activator Inhibitor-1

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Abstract

Background & Aims—The biguanide drug metformin has recently been found to improve steatosis and liver damage in animal models and in humans with non-alcoholic steatohepatitis.

Methods—The aim of the present study was to determine whether metformin also prevents steatosis and liver damage in mouse models of acute and chronic alcohol exposure.

Results—Acute ethanol exposure caused a >20-fold increase in hepatic lipids, peaking 12 hours after administration. Metformin treatment significantly blunted the ethanol effect by >60%. Although metformin is a known inducer of AMP kinase (AMPK) activity, the hepatoprotective property of metformin did not correlate with activation of AMPK or of AMPK-dependent pathways. Instead, the protective effects of metformin correlated with complete prevention of the upregulation of plasminogen activator inhibitor (PAI)-1 caused by ethanol. Indeed, a similar protective effect against acute alcohol-induced lipid accumulation was observed in PAI-$1^{-/-}$ mice. Hepatic fat accumulation caused by chronic enteral ethanol feeding was also prevented by metformin or by knocking out PAI-1. Under these conditions, necroinflammatory changes caused by ethanol were also significantly attenuated.

Conclusions—Taken together, these findings suggest a novel mechanism of action for metformin and identify a new role of PAI-1 in hepatic injury caused by ethanol.

Alcoholic liver disease (ALD) ranks among the major causes of morbidity and mortality in the world.[1] Fat accumulation in the liver is 1 of the first characteristics of the onset of ALD.[2] Originally thought to be a pathologically inert histologic change, more recent work indicates that steatosis may play a critical role not only in the initiation, but also in the progression of ALD.[3] Indeed, results of clinical studies indicate that patients with fatty liver are more vulnerable to developing later stages of the disease (eg, fibrosis and cirrhosis[4]). Because of an incomplete understanding of the mechanism(s) involved in the disease process, a universally accepted therapy to prevent or reverse ALD in humans is lacking. Therefore, better understanding of the biochemical and pathologic changes that cause alcohol-induced fatty liver may lead to therapies that not only prevent steatosis, but may also have therapeutic effects on later stages of ALD.

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Steatosis resulting from alcohol has historically been considered the direct result of alcohol metabolism on β-oxidation of fatty acids. However, other factors may also contribute. For example, a number of drugs and genetically altered mice have a decreased steatotic response to alcohol without any apparent effect on alcohol metabolism (see Arteel[5] for a review). There is a strong association between hepatic insulin resistance and fatty liver as well as steatohepatitis in humans.[6,7] In support of this hypothesis, the results of several animal and human studies suggested that the insulin-sensitizing biguanide, metformin, confers protection in nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitits (NASH).[8-10] The natural history of NAFLD displays similar features to that of ALD. Indeed, studies indicate that fatty liver disease, be it alcoholic or nonalcoholic, may share similar mechanisms and pathologic features.[11,12] Given the protective effect of metformin in NAFLD and NASH, it has been suggested that metformin may also be effective in the treatment of ALD. Although the mechanism(s) by which metformin confers protection in fatty liver diseases is unclear, it has been proposed that a downregulation of tumor necrosis factor (TNF)-α–mediated signaling[9] as well as an activation of AMP-kinase (AMPK) and AMPK-dependent pathways may mediate this effect; specifically, the enhancement of β-oxidation of fatty acids caused by AMPK-dependent signaling has been proposed to be critical.[13]

Animal models of ethanol exposure have made it possible to produce pathologic changes in rodent liver that resemble early alterations in human ALD. Furthermore, these models provide an important tool to determine the effect of specific genetic alterations in transgenic or knockout mice. For example, fat accumulation caused by chronic alcohol ingestion was completely blocked in mice deficient in TNF-α receptor 1 (TNFR1).[14] Here, using mouse models of acute and chronic alcohol exposure, the hypothesis that metformin protects against alcohol-induced lipid accumulation and hepatic damage was tested, and the potential role of plasminogen activator inhibitor-1 (PAI-1) in this effect were determined.

Materials and Methods

Animals and Treatments

Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and procedures were approved by the local Institutional Animal Care and Use Committee. Six-week-old C57BL/6J, TNF knockout (B6.129-Tnfrsf1atm 1 Mak/J; TNFR1$^{-/-}$), and PAI-1 knockout (B6.129S2-Serpine 1 tm 1 Mlg/J; PAI-1$^{-/-}$) mice were obtained from Jackson Laboratory (Bar Harbor, ME). All knockout mice used in this study were back-crossed at least 10 times onto C57BL/6, avoiding concerns regarding genetic differences between wild-type strain and the knockouts at nonspecific loci. Food and tap water were allowed ad libitum prior to experimentation.

Acute alcohol exposure model—1,1-Dimethylbiguanide hydrochloride (metformin; Sigma, St. Louis, MO) or vehicle (saline) was given once a day (200 mg/kg IP) for 4 days prior to ethanol administration; this dosage was based on preliminary range-finding studies and previous work published by others.[15] One hour after the last metformin dose, mice received ethanol (6 g/kg IG) or isocaloric/isovolumetric maltose-dextrin solution. This model was slightly modified from Enomoto et al[16] and has been proposed as a predictive/screening tool for therapies against liver damage due to chronic alcohol intake. With this dose of alcohol, mice were sluggish, but conscious and regained normal behavior within ~6 hours of alcohol feeding. This dose caused no mortality.

Chronic enteral alcohol exposure model—Surgical implantation of the intragastric cannula and enteral feeding was performed as described previously.[17] Briefly, a liquid diet described by Thompson and Reitz[18] supplemented with lipotropes as detailed by Morimoto

et al[19] was prepared daily. Either ethanol-containing or isocaloric maltose-dextrin (control) diet was fed for 4 weeks as described elsewhere. The initial rate of ethanol delivery (16 g/kg per day) was increased in a stepwise manner 1 g/kg every 2 days until the end of the first week and then 1 g/kg every 4 days until the end of the experiment. In parallel studies, additional wild-type mice fed enteral diet received metformin (350 mg/kg per day in the diet) during the course of the study. The dose of metformin was based on previous studies investigating the effect of chronic metformin on dietary steatosis in mice,[9] and shown to be well-tolerated in preliminary experiments with alcohol.

At sacrifice, animals were anesthetized with sodium pento-barbital (75 mg/kg IP) 0.5, 1, 2, 3, 6, or 12 hours after ethanol administration. Blood was collected from the vena cava just prior to sacrifice by exsanguination and plasma was stored at −80°C for further analysis. Portions of liver tissue were frozen immediately in liquid nitrogen, whereas others were fixed in neutral-buffered formalin, or frozen-fixed in OCT mounting media (Tissue Tek, Hatfield, PA) for subsequent sectioning and mounting on microscope slides.

Cell Culture and Treatment

AML-12 cells (alpha mouse liver 12, American Type Culture Collection, Manassas, VA) were grown in DMEM/HAM's F12 media (Gibco, Carlsbad, CA) supplemented with 10% FBS, 40 ng/mL dexamethasone, 0.005 mg/mL insulin, and 5 ng/mL selenium (Gibco) in a humidified 5% CO_2 atmosphere at 37°C until they were 80% confluent. Cells were serum starved for 18 hours and treated with 500 μmol of metformin for 7 hours, analogous to previous work by others.[13,20] Medium was subsequently removed and replaced with fresh serum-free medium with or without metformin (500 μmol). Parallel groups were also incubated in the presence and absence of the insulin receptor kinase inhibitor tyrphostin-AG1024 (300 μmol; Sigma). After 20 minutes, 5 ng/mL of TNF-α (mouse TNF-α; Sigma) was added. Cells were incubated for 3 hours, rinsed with phosphate-buffered saline, and lysed with RNA-STAT 60 Tel-Test (Ambion, Austin, TX) for later RNA isolation and analysis by real-time reverse transcriptase polymerase chain reaction (RT-PCR; see below).

Immunoblots

All buffers used for protein extraction contained protease, tyrosine phosphatase, and serine/threonine phosphatase inhibitors purchased from Sigma. To prepare total hepatic protein, snap-frozen liver samples were homogenized with lysis buffer (20 mmol MOPS, 150 mmol NaCl, 1 mmol EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) containing protease, tyrosine phosphatase, and serine/threonine phosphatase inhibitors (as described). To prepare cytosolic fractions, livers were homogenized in Dignum A buffer (10 mmol HEPES, 1.5 mmol $MgCl_2$, 5 mmol KCl, 0.5 mmol DTT), and then extracted with Dignam C (10 mmol HEPES, 50% glycerol, 84 mmol NaCl, 1.5 mmol Mg_2Cl, 0.2 mmol EDTA, 0.5 mmol DTT) buffer to obtain nuclear proteins. Respective lysates (40−75 μg protein per well) were separated on either 5% or 8% SDS-polyacrylamide gel. Proteins were transferred to Hybond-P polyvinylidene difluoride membranes using a semidry electroblotter. The resulting blots were then probed with antibodies against phospho-c-Met (Cell Signaling Technology, Beverly, MA), apolipoprotein B100 and B48, sterol regulator element binding protein 1 (SREBP-1; both Santa Cruz, Santa Cruz, CA), phospho-AMPK (Cell Signaling Technology), or phospho-acetyl CoA carboxylase (ACC; Upstate, Waltham, MA) and bands were visualized using Amersham Biosciences ECL plus kit (Amersham Biosciences, Piscataway, NJ). To ensure equal loading, all blots were stained with Ponceau red; haptene signals were normalized to β-actin using a commercially available antibody (Sigma).

Microsomal Triglyceride Transfer Protein Activity

Frozen mouse livers were homogenized in extraction buffer (10 mmol Tris, pH 7.4, 150 mmol NaCl, 2 mmol EDTA) centrifuged at 7000 ×*g* for 5 minutes. Fluorescent (λ_{ex} = 465 nm, λ_{em} = 535 nm) measurement of microsomal triglyceride protein activity was performed with a commercially available kit according to the instructions of the manufacturer (Roar Biomedical Inc., New York, NY). One hundred micrograms of total protein was utilized in each reaction.

Very Low-Density Lipoprotein Extraction

Plasma samples were mixed with OptiPrep (12% final concentration, AXIS-SHIELD PoC AS, Oslo, Norway), over layered with HEPES-buffered saline (0.85% NaCl; 10 mmol HEPES, pH 7.4), and centrifuged at 350,000 ×g for 3 hours at 16°C. Immediately after centrifugation, samples were collected in 20 μL fractions starting from the bottom of the centrifugation tube. To ascertain appropriate fractionation of lipoproteins, 2 μL from each fraction was separated in an agarose lipoprotein gel (Beckman Coulter, Fullerton, CA), fixed (60% ethanol, 10% glacial acetic acid), and stained overnight (55% ethanol, 0.1 % Paragon Lipo Stain [Beckman Coulter]). Gels were destained (45% ethanol) and dried. The remaining very low-density lipoprotein (VLDL) fraction was stored at 4°C for later detection of triglyceride levels.

Triglyceride Determinations

Mouse livers were homogenized in 2× phosphate-buffered saline. Tissue lipids were extracted with methanol: chloroform (1:2), dried in an evaporating centrifuge, and re-suspended in 5% fat-free bovine serum albumin. Colorimetric assessment of hepatic and plasma triglyceride levels was carried out using Sigma Diagnostics Triglyceride Reagent (Sigma). Values were normalized to protein in homogenate prior to extraction determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Oil Red O Staining

Frozen sections of liver (10 μm) were stained with oil Red O (Sigma) for 10 minutes, washed, and counterstained with hematoxylin for 45 seconds (DAKO, Carpinteria, CA).[21] A Metamorph image acquisition and analysis system (Chester, PA) incorporating a Nikon microscope (Nikon, Melville, NY) was used to capture and analyze the oil Red O stained tissue sections at 200× magnification.[22] The extent of labeling in the liver lobule was defined as the percent of the field area within the default color range determined by the software. Data from each tissue section (5 fields per section) were pooled to determine means.

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted from liver tissue samples by a guanidium thiocyanate-based method (RNA STAT 60 Tel-Test, Ambion). RNA concentrations were determined spectrophotometrically, and 1 μg total RNA was reverse transcribed using an AMV reverse transcriptase kit (Promega, Madison, WI) and random primers. PCR primers for PAI-1 and β-actin were designed using primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA). Primers were designed to cross introns to ensure that only cDNA and not genomic DNA was amplified (Table 1). The fluorogenic MGB probe was labeled with the reporter dye FAM (6-carboxyfluorescein). TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used to prepare the PCR mix. The 2× mixture was optimized for TaqMan reactions and contains AmpliTaq gold DNA polymerase, AmpErase, dNTPs with UTP, and a passive reference. Primers and probe were added to a final concentration of 300 and 100 nmol, respectively. The amplification reactions were carried out in the ABI Prism 7700 sequence detection system (Applied Biosystems) with initial hold steps (50°C for 2 minutes, followed by 95°C for 10 minutes) and 50 cycles of a 2-step PCR (92°C for 15 seconds, 60°C for 1 minute). The fluorescence intensity of each sample was measured at each temperature change

to monitor amplification of the target gene. The comparative CT method was used to determine fold differences between samples. The comparative CT method determines the amount of target, normalized to an endogenous reference (β-actin) and relative to a calibrator ($2^{-\Delta\Delta Ct}$). The purity of PCR products were verified by gel electrophoresis.

Histologic Analysis and Clinical Chemistry

Formalin-fixed and paraffin-embedded sections (6 μm) were cut and stained with hematoxylin and eosin for pathologic assessment after chronic enteral alcohol feeding. Pathology was scored as described by Nanji et al.[23]: steatosis <5%, 1; 25%−50%, 2; 50%−75%, 3; >75%, 4; 1 or 2 for one or more inflammatory or necrotic foci. Plasma alanine aminotransferase (ALT) activity was determined using a commercially available kit (Thermo Electron, Melbourne, Australia). Urine was collected daily and urine alcohol concentrations measured using routine spectrophotometric techniques.[24]

Statistical Analyses

Results are reported as means ± SEM (n = 4−6). ANOVA with Bonferroni's post hoc test was used for the determination of statistical significance among treatment groups. For comparison of pathologic scores, the Mann–Whitney rank-sum test was used. A P value < .05 was selected before the study as the level of significance.

Results

Metformin Prevents Fat Accumulation After Acute Alcohol

Figure 1 shows representative photomicrographs depicting lipid accumulation in mouse livers after acute ethanol ingestion, as determined by oil Red O staining, and Figure 2*A* shows quantitation of oil Red O staining by image analysis. Figure 2*B* shows quantitation of hepatic triglycerides after acute ethanol. Lipid staining (Figures 1 and 2*A*) and triglyceride content (Figure 2*B*) in livers from maltose-dextrin–treated animals were minimal regardless of other treatments and did not differ from staining in livers from naive control mice; results with wild-type mice are shown to represent these groups. In contrast, ethanol treatment caused significant >20-fold accumulation of lipid droplets in livers of wild-type animals, peaking 12 hours after administration (Figures 1*C* and 2*A*); this effect was significantly blunted in wild-type mice treated with metformin (Figure 1*E* and 2*A*). As has been observed previously with chronic ethanol,[14] lipid accumulation was dramatically blunted in $TNFR1^{-/-}$ mice (Figures 1*D* and 2*A*); lipid accumulation in $TNFR1^{-/-}$ mice was similar to wild-type mice treated with metformin (Figures 1*E* and 2*A*). Pretreatment with metformin did not enhance protection from lipid accumulation caused by ethanol in $TNFR1^{-/-}$ mice (Figures 1*F* and 2*A*). Similar patterns were observed for hepatic triglyceride accumulation in these groups (Figure 2*B*).

Effect of Acute Ethanol and Metformin on Phosphorylation of AMPK and ACC, and Nuclear Localization of SREBP-1 in Mouse Liver

Results of in vitro studies have indicated that the inhibition of lipid accumulation caused by metformin could, in part, be mediated through an activation of AMPK, leading to phosphorylation and inactivation of ACC[25]; this inhibition of ACC could subsequently result in a decreased formation of malonyl CoA, a known allosteric inhibitor of carnitine palmitoyl-transferase (CPT)1, and subsequent mitochondrial hepatic β-oxidation. Furthermore, it has been suggested that the alcohol-induced loss of AMPK activity might also result in an activation of SREBP-1, one of the key regulators of fat metabolism.[26] Therefore, phosphorylation of AMPK and ACC and nuclear translocation of SREBP-1 were determined by Western blot after acute alcohol ingestion (Figure 3). Figure 3 depicts representative results (*A*) and densitometric analysis (*B*) of these immunoblots. Under these conditions, acute ethanol

did not significantly alter the phosphorylation status of AMPK and did not have an apparent effect on nuclear SREBP-1 levels at any time point tested (Figure 3), including 0, 1, and 2 hours after ethanol administration (data not shown). Acute ethanol caused a significant increase in the phosphorylation status of ACC with values ~2-fold higher 6 hours after ethanol ingestion. The effect of metformin pretreatment in vivo on the changes in these parameters was also determined (Figures 3*A* and 3*B*). Metformin neither significantly altered the phosphorylation status of AMPK and ACC, nor the nuclear localization of SREBP-1, in control (maltose-dextrin)-treated mice (data not shown). Analogous to ethanol alone (Figures 3*A* and 3*B*), the phosphorylation status of AMPK and nuclear localization of SREBP-1 were unaffected by metformin under these conditions. Metformin administration also did not significantly alter the increase in ACC phosphorylation caused by ethanol alone.

Metformin Prevents Induction of PAI-1 Expression in Liver Tissue Caused by Acute Alcohol

Metformin confers other effects in the cell in addition to activating AMPK. For example, it is also a potent inhibitor of PAI-1 expression.[27] To determine if PAI-1 expression was altered by acute alcohol ingestion, PAI-1 mRNA levels were determined by real-time PCR in liver samples 0−12 hours after acute alcohol ingestion (Figure 4*A*). Acute alcohol ingestion caused a biphasic increase in hepatic PAI-1 mRNA levels, peaking 2 and 12 hours after ethanol ingestion, with values ~5- and ~10-fold over controls, respectively (Figure 4*A*). In parallel experiments, the effect of metformin on the induction of PAI-1 were determined 12 hours after ethanol ingestion (Figure 4*B*). Metformin had no effect on basal expression of PAI-1 in the absence of ethanol in wild-type mice. Furthermore, basal levels of PAI-1 mRNA in TNFR1$^{-/-}$ mice were not different from wild-type mice. The results with wild-type mice treated with maltosedextrin are shown as a summary of these groups in Figure 4*B*. However, metformin treatment significantly blunted the induction of PAI-1 expression caused by ethanol in wild-type mice, with values in this group similar to maltose-dextrin controls (Figure 4*B*). Analogous to findings with lipid accumulation (see Figure 1), the increase in PAI-1 expression caused by ethanol was also prevented in TNFR1$^{-/-}$ mice.

Hepatic Lipid Levels After Acute Alcohol Ingestion: Effect of Knocking Out PAI-1

Figure 2 also summarizes the effect of ethanol on the levels of hepatic lipid accumulation (*A*) and triglyceride content (*B*) in PAI-1 knockout (PAI-1$^{-/-}$) mice. The increase in hepatic lipid levels caused by acute ethanol was also significantly blunted in PAI-1$^{-/-}$ mice (see Figure 2), with values similar to those of ethanol treated wild-type mice given metformin or TNFR1$^{-/-}$ mice. As with TNFR1$^{-/-}$ mice, metformin treatment conferred no additional protective effect against acute ethanol-induced lipid accumulation in PAI-1$^{-/-}$ mice (Figure 2).

Metformin Prevents TNFα-Induced PAI-1 Expression in AML-12 Cells

As described, a correlation between TNF-α, lipid accumulation (Figures 1 and 2), and the expression of PAI-1 (Figure 4) was observed after acute ethanol in vivo. Therefore, the effect of TNF-α and metformin on the expression of PAI-1 was determined in AML-12 cells (see Materials and Methods). Incubation of AML-12 cells with TNF-α (5 ng/mL) for 3 hours resulted in a significant increase of PAI-1 mRNA expression by ~3-fold (Figure 5). In cells concomitantly treated with metformin (500 μmol), the induction of PAI-1 expression caused by TNF-α was significantly suppressed by ~40%; this protective effect was also prevented by concomitant administration of the insulin receptor tyrosine kinase inhibitor, tyrphostin AG1024 (see Figure 5). Furthermore, the protective effect of metformin was prevented if normal insulin found in the culture medium was excluded (data not shown).

Impaired Hepatic c-Met Signaling and Lipid Secretion Caused by Acute Alcohol Is Prevented by Metformin and in PAI-1$^{-/-}$ Mice

Previous studies have indicated that in the liver, hepatic growth factor (HGF)/c-Met signaling and subsequent lipoprotein synthesis is impaired by alcohol ingestion, which may in part mediate hepatic triglyceride accumulation.[28,29] Therefore, the effect of ethanol and metformin on c-Met activation was evaluated. Figure 6 depicts a representative Western blot (*A*) and quantitative analysis (*B*) of this protein. The phosphorylation status of c-Met in liver was similar in the absence of ethanol, regardless of additional treatment or mouse strain (Figure 6*A*, lane 1 is representative for all control groups). Acute ethanol caused no significant alteration in the ratio of phospho:total c-Met 12 hours after alcohol ingestion (Figures 6*A* lane 2, and 6*B*). However, pre-treatment with metformin caused a significant ~3-fold increase in the ratio of phospho:total c-Met after acute ethanol administration. Similarly, c-Met phosphorylation was enhanced following acute administration of ethanol in PAI-1$^{-/-}$ and TNFR1$^{-/-}$ mice. In line with the data on lipid accumulation (Figure 2), metformin had no additional effect on c-Met phosphorylation in these strains (data not shown).

Previous studies have shown that c-Met activation can increase VLDL synthesis in liver. Therefore, hepatic apolipoprotein B100 and B48 protein (Figures 6*C* and 6*D*) levels and the activity of the microsomal triglyceride transfer protein (MTTP; Figure 6*E*) were determined 12 hours after acute alcohol ingestion. Levels of apolipoprotein B100 in livers of wild-type animals exposed to acute alcohol did not differ from those of controls (Figures 6*C* and 6*D*). However, in livers of animals pretreated with metformin, as well as in PAI-1$^{-/-}$ and TNFR1$^{-/-}$ mice, levels of apolipoprotein B100 were significantly increased by approximately 4-fold in comparison to wild-type animals exposed to ethanol. No differences were found between wild-type, PAI-1$^{-/-}$ and TNFR1$^{-/-}$ mice treated with maltose-dextrin and saline or maltosedextrin and metformin (Figure 6*C*, lane 1 shown to represent these groups). Whereas ethanol had no apparent effect on the basal level of apolipoprotein B48 in wild-type animals, concomitant metformin treatment also led to an increase in this protein after ethanol administration (Figures 6*C* and 6*D*). A similar increase in levels of this protein was observed in TNFR1$^{-/-}$ and PAI-1$^{-/-}$ mice given ethanol.

MTTP activity in maltose-dextrin treated animals was similar to those of chow fed mice, irrespective of strain or additional treatment (eg, metformin); results for wild-type mice given maltose-dextrin are therefore shown to represent these groups. Acute alcohol ingestion had no effect on hepatic MTTP activity in wild-type mice (Figure 6*E*). However, similar to apolipoprotein B100 and B48 levels, in livers of metformin pretreated wild-type mice as well as TNFR1$^{-/-}$ and PAI-1$^{-/-}$ mice ethanol ingestion resulted in a significant induction of MTTP activity in comparison with controls. Specifically, MTTP activity in livers of these animals was ~35% higher in comparison with controls (Figure 6*E*). Furthermore, acute ethanol ingestion resulted in a ~60% decrease in the amount of triglycerides (normalized to total protein) in the plasma VLDL fraction compared to controls (data not shown); this decrease was not observed in wild-type mice treated with metformin and values were not significantly different from wild-type animals treated with maltose dextrin.

Effect of Metformin and Deletion of PAI-1 on Chronic Alcohol-Induced Liver Damage

Although studies with acute ethanol (Figures 1-6) implicated a potential role of PAI-1 in atosis owing to ethanol, whether or not metformin or knocking out PAI-1 will prevent chronic liver damage owing to ethanol remained unclear. Therefore, the effect of metformin or knocking out PAI-1 on liver damage owing to chronic enteral alcohol feeding was determined. All animals gained weight during the course of the study; there were no significant differences in body weight gains between the groups studied, indicating that animals were adequately nourished. As reported previously by several groups,[14,30] daily urine alcohol concentrations

fluctuated between 0 and 500 mg/dL, which is caused by fluctuations in hormones from the hypothalamic–pituitary–thyroid axis.[31] Wild-type mice (±metformin) and PAI-1$^{-/-}$ mice have similar patterns of urine alcohol cycling and average mean and peak urine alcohol levels were not significantly different between the groups with values of ~200 and ~450 mg/dL, respectively.

In wild-type mice fed control diet, liver weights (as percent of body weight) were 5.3 ± 0.2; chronic enteral ethanol nearly doubled liver weight in wild-type mice, with values of 9.8 ± 0.5. Although not significantly affecting the liver weight in animals fed control diet, metformin administration or knocking out PAI-1 significantly attenuated the increase in liver size caused by ethanol by 30%−40%, with values of 8.4 ± 0.3 and 7.8 ± 0.1 for wild-type mice given metformin and PAI-1$^{-/-}$ mice, respectively. Figure 7 shows representative photomicrographs of livers from wild-type (±metformin) and PAI-1$^{-/-}$ mice fed enteral diet with and without ethanol for 4 weeks. Figure 8 depicts corresponding ALT values (*A*) and pathologic scores (*B*) in these groups. There were no significant pathologic changes after 4 weeks of high-fat control diet in wild-type (±metformin) or in PAI-1$^{-/-}$ mice (Figure 7*A* shown to represent these groups) ALT levels were normal with values ~25 IU/L (Figure 8*A*). As expected, enteral ethanol alone for 4 weeks caused severe fatty infiltration (Figure 7*B*), with focal inflammation (Figure 7*E*) and necrosis (Figure 7*F*) in wild-type mice, as is summarized by the pathology scores (Figure 8*B*). Enteral ethanol also increased ALT levels significantly ~5-fold over control values in this group (Figure 8*A*) in wild-type mice.

In wild-type mice, metformin administration prevented liver damage caused by ethanol (Figure 7*C*), as quantitated by a significant blunting of the increase in ALT (Figure 8*A*) and pathology scores (Figure 8*B*). Similar to the effect of metformin in wild-type mice, PAI-1$^{-/-}$ mice were also protected against liver damage due to chronic ethanol (Figures 7*D* and 8). Interestingly, the protection against steatosis due to chronic ethanol with metformin or knocking out PAI-1 (Figure 8*B*, upper left panel), was not as robust as observed with these treatments after acute ethanol (Figures 1 and 2). However, inflammation and necrosis due to ethanol were almost completely blunted in these groups (Figure 8*B*, upper right and lower left panels).

Table 2 summarizes the effect of ethanol, metformin, or knocking out PAI-1 on some indices determined above with acute ethanol. As observed with acute ethanol (Figure 4), the expression of PAI-1 was significantly induced in wild-type mice fed chronic ethanol (Table 2); however, the magnitude of induction was not as robust as observed after acute ethanol. Metformin treatment prevented the induction of PAI-1 caused by ethanol; indeed, expression of PAI-1 in this group was actually only ~30% of that of wild-type mice fed control diet (Table 2). As expected, PAI-1 mRNA was not detectable in PAI-1$^{-/-}$ mice. As had been observed with acute ethanol under these conditions (Figure 3), the phosphorylation status of AMPK was not significantly altered in any group after chronic enteral ethanol feeding (Table 2). Furthermore, in wild-type mice fed chronic ethanol, the phosphorylation of ACC was not significantly altered. Whereas metformin had no significant effect on the phosphorylation status of ACC in wild-type mice fed control diet, the status was significantly decreased in metformin-treated mice fed ethanol in comparison with wild-type mice fed ethanol in the absence of metformin (Table 2). The relative phosphorylation status of ACC was significantly lower in PAI-1$^{-/-}$ mice in comparison with wild-type mice fed in the absence of metformin, but ethanol had no effect on this strain.

Discussion

Results of several studies have identified accumulation of lipids in the liver to be a critical early stage in the development of ALD (for review see Teli et al[32] and Wanless and Shiota[33]). However, therapies to prevent the initiation of this process are limited; the mechanisms

involved are still poorly understood. Animal models resembling conditions of early stage ALD in humans have been found to be useful tools to investigate mechanisms and pathophysiology underlying the effects of ethanol on the liver. Here, an acute model of alcohol exposure consisting of 1 bolus dose of alcohol was used to mimic these early changes. Acute and chronic alcohol-induced liver injury seem to share similar mechanisms (for review see Thurman et al[34]). Therefore, acute alcohol exposure can also be used to mimic very early effects of ethanol in the progression of chronic liver damage. Although it should be emphasized that acute ethanol by no means mimics all effects of chronic ethanol on the liver (eg, inflammation), this model could potentially serve as a screening tool for new therapies. For example, Enomoto et al[16] showed that compounds previously shown to protect against chronic alcohol-induced liver damage[35-37] also protected rat liver against acute ethanol-induced steatosis. In the present study, the hypothesis that metformin protects against early alcohol-induced fat accumulation was first tested in a model of acute alcohol ingestion. Indeed, pretreatment with metformin significantly blunted lipid accumulation in the liver of mice owing to acute alcohol exposure (see Figures 1*D* and 5).

Metformin Confers Protection Against Ethanol Independent of AMPK Signaling

One mechanism by which alcohol is proposed to cause lipid accumulation is by inhibition of β-oxidation of fatty acids. Previous in vitro studies with metformin indicate that this drug may prevent such an inhibition. Specifically, metformin was shown in vitro to activate AMPK, leading to phosphorylation and inhibition of ACC[13]; malonyl CoA, the product of ACC, is an allosteric inhibitor of CPT1, a key component of β-oxidation of fatty acids. Furthermore, it has been suggested that the alcohol-mediated impairment of AMPK activity might result in an activation of SREBP-1, a key regulator of fat metabolism. In the present study, a decrease in AMPK/ACC phosphorylation and/or nuclear translocation of SREBP-1 was not observed with acute alcohol ingestion; indeed, acute alcohol administration led to an increase in the phosphorylation status of ACC. After chronic enteral alcohol exposure, the phosphorylation status of AMPK and ACC were also not decreased (see Table 2). Despite conferring a protective effect against alcohol-induced lipid accumulation, metformin did not alter AMPK and ACC phosphorylation or nuclear translocation of SREBP-1 under these conditions. It may be that dosages of metformin required to induce AMPK are higher than those used here. Taken together, these data suggest that the protective effect of metformin under these conditions is independent of AMPK activation and its dependent pathways. These results do not preclude a role of AMPK signaling in alcohol-induced liver injury, but rather suggest that additional pathways may contribute to lipid accumulation.

Ethanol Induces Hepatic PAI-1 Expression: Prevention With Metformin

In addition to activating AMPK, metformin has been shown to suppress induction of PAI-1 expression.[20,27] Here, acute alcohol ingestion led to a biphasic induction of hepatic PAI-1 mRNA expression, peaking at 2 and 12 hours after alcohol ingestion; this increase was blunted by metformin pretreatment (Figure 5*B*). PAI-1 was also observed to be induced in the liver by chronic ethanol ingestion (Table 2). To further investigate the role of PAI-1 under these conditions, lipid accumulation after acute and chronic alcohol administration was determined in PAI-$1^{-/-}$ mice (Figures 1, 2, 7, and 8). PAI-$1^{-/-}$ mice were protected against lipid accumulation owing to ethanol (Figures 1 and 2). Importantly, this effect was not further enhanced by metformin pretreatment in this mouse strain. Although less effective relative to acute studies with ethanol, metformin or knocking out PAI-1 also significantly attenuated steatosis owing to chronic ethanol administration (Figures 7 and 8).

Previous studies have indicated a link between hepatic PAI-1 expression and TNF-α.[38,39] Indeed, both in increases hepatic lipid content (Figures 1 and 2) and PAI-1 expression due to ethanol (Figure 4) were significantly blunted in TNFR$1^{-/-}$ mice relative to wild-type mice.

Furthermore, metformin did not confer additional protective effects against lipid accumulation and PAI-1 induction in this strain of mice. In line with a link between PAI-1 and TNF-α, addition of TNF-α to AML-12 cells caused an increase in PAI-1 expression (Figure 6), similar to what has been observed by others.[40] The mechanism by which TNF-α induces PAI-1 expression is not completely understood. However, it was shown here that metformin prevented TNF-α–induced PAI-1 expression. This protective effect of metformin was abrogated by either removing insulin from the culture media, or by blocking insulin receptor signaling (tyrphostin AG1024; Figure 6), suggesting a link between the protective effects of PAI-1 and insulin signaling under these conditions.

How Does PAI-1 Mediate Alcohol-Induced Lipid Accumulation?

PAI-1 is an acute phase protein expressed under conditions of inflammation. PAI-1 is a major inhibitor of both tissue-type plasminogen activator and urokinase-type plasminogen activator (uPA), thereby playing a major regulatory role in fibrinolysis.[41] However, recent work has indicated that PAI-1 may have other functions, namely, in lipid metabolism. For example, Ma et al[42] found in a model of high-fat/high-carbohydrate diet induced obesity that PAI-$1^{-/-}$ mice were protected from hepatic lipid accumulation. Furthermore, Alessi et al[43] reported that circulating plasma PAI-1 levels in humans are closely related to the degree of liver steatosis. The findings of the current work indeed support the hypothesis that PAI-1 is causally involved in hepatic steatosis.

Although pharmacologic (metformin) or genetic (TNFR$1^{-/-}$ and PAI-$1^{-/-}$) inhibition of PAI-1 induction clearly prevented acute alcohol-induced fat accumulation, the mechanisms of this inhibitory effect were unclear. In addition to the inhibition of β-oxidation of fatty acids, ethanol also inhibits the synthesis and excretion of VLDL lipids,[44,45] which can also lead to fat accumulation. Although uPA is mainly known for its function in proteolytic cleavage of plasminogen to plasmin, it has also been shown to activate pro-HGF,[46] which binds to the c-Met receptor with high affinity.[47] Results of in vitro studies performed by Kaibori et al[48] suggested that activation of c-Met by HGF stimulates lipoprotein secretion in hepatocytes. Indeed, chronic ethanol treatment down-regulates hepatic c-Met and apolipoprotein expression.[49] Furthermore, HGF administration enhances the rate of recovery from experimental alcohol-induced fatty liver and is associated with increased synthesis and secretion of apolipoprotein B and subsequent formation of VLDL.[28,49]

Although acute ethanol administration did not significantly decrease c-Met phosphorylation, hepatic apolipoprotein levels, and MTTP activity per se in wild-type animals, pretreatment with metformin caused a signifi-cant increase in all of these parameters. In support of the hypothesis that TNF-α and PAI-1 are involved in this process, TNFR$1^{-/-}$ and PAI-$1^{-/-}$ mice had a similar response to ethanol as did metformin treated wild-type mice. Importantly, metformin pretreatment did not enhance these parameters in either knockout strain, further supporting the hypothesis that TNF-α and PAI-1 signaling involve a shared pathway. Taken together, these data suggest that the protective effect of metformin against alcohol-induced steatosis is mediated at least in part by prevention of the activation of PAI-1 by TNF-α and subsequent up-regulation of VLDL synthesis through HGF-mediating signaling. These data suggest that the protective effect under these conditions represents a compensatory increase in VLDL synthesis to account for the greater amount of lipids in the hepatocyte after ethanol exposure. With chronic ethanol administration, other factors that can contribute to lipid accumulation may surpass the importance of PAI-1, thereby explaining the less effective inhibition under these conditions.

Does PAI-1 Play a Causal Role in Alcoholic Liver Disease?

A very interesting finding in studies of PAI-$1^{-/-}$ mice in the chronic enteral ethanol model is that it appears that genetic inhibition of PAI-1 also conferred anti-inflammatory effects. Specifically, although knocking out PAI-1 significantly blunted the steatotic changes caused by ethanol, this strain had almost a complete abrogation of inflammatory changes caused by ethanol under these conditions (Figure 8). A similar anti-inflammatory effect of knocking out PAI-1 was observed by Kitching et al[50] in a mouse model of glomerulonephritis; PAI-$1^{-/-}$ mice had fewer infiltrating leukocytes and less severe damage to the glomeruli in their model. Two potential mechanisms identified in the literature by which PAI-1 could mediate a proinflammatory response include impaired fibrinolysis, leading to fibrin accumulation (eg, Holdsworth et al[51] and Loike et al[52]), or inhibition of the proteolytic activation/deactivation of inflammatory/anti-inflammatory cytokines by PAI-1.[53-55] These potential mechanisms are of course not mutually exclusive and may all contribute to liver damage caused by ethanol (mediated by PAI-1).

The results of the present study suggest a novel pathway in alcohol-induced steatosis and liver damage involving the induction of PAI-1 expression in the liver via a metformin sensitive, TNF-α–dependent pathway. Furthermore, these results also suggest a new mechanism by which metformin protects the liver from fat accumulation at the level of VLDL synthesis. Although treatment of ALD in humans with metformin may be limited due to the possible risk of lactic acidosis,[56,57] these results suggest that therapies that more specifically target PAI-1 expression or enhance uPA activity might be useful therapies to treat ALD or prevent its progression. This hypothesis is further bolstered by the possibility that inhibition of PAI-1 may also be anti-inflammatory and antifibrotic in liver.[58,59]

Acknowledgements

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Abbreviations used in this paper

ACC, acetyl CoA carboxylase
ALD, alcoholic liver disease
AMPK, AMP kinase
CPT, carnitine palmitoyl-transferase
HGF, hepatic growth factor
MTTP, microsomal triglyceride transfer protein
NAFLD, nonalcoholic fatty liver disease
NASH, non-alcoholic steatohepatitis
PAI-1, plasminogen activator inhibitor 1
SREBP-1, sterol regulator element binding protein 1
TNF-α, tumor necrosis factor alpha
TNFR1, tumor necrosis factor alpha receptor 1
uPA, urokinase-type plasminogen activator
VLDL, very low-density lipoprotein

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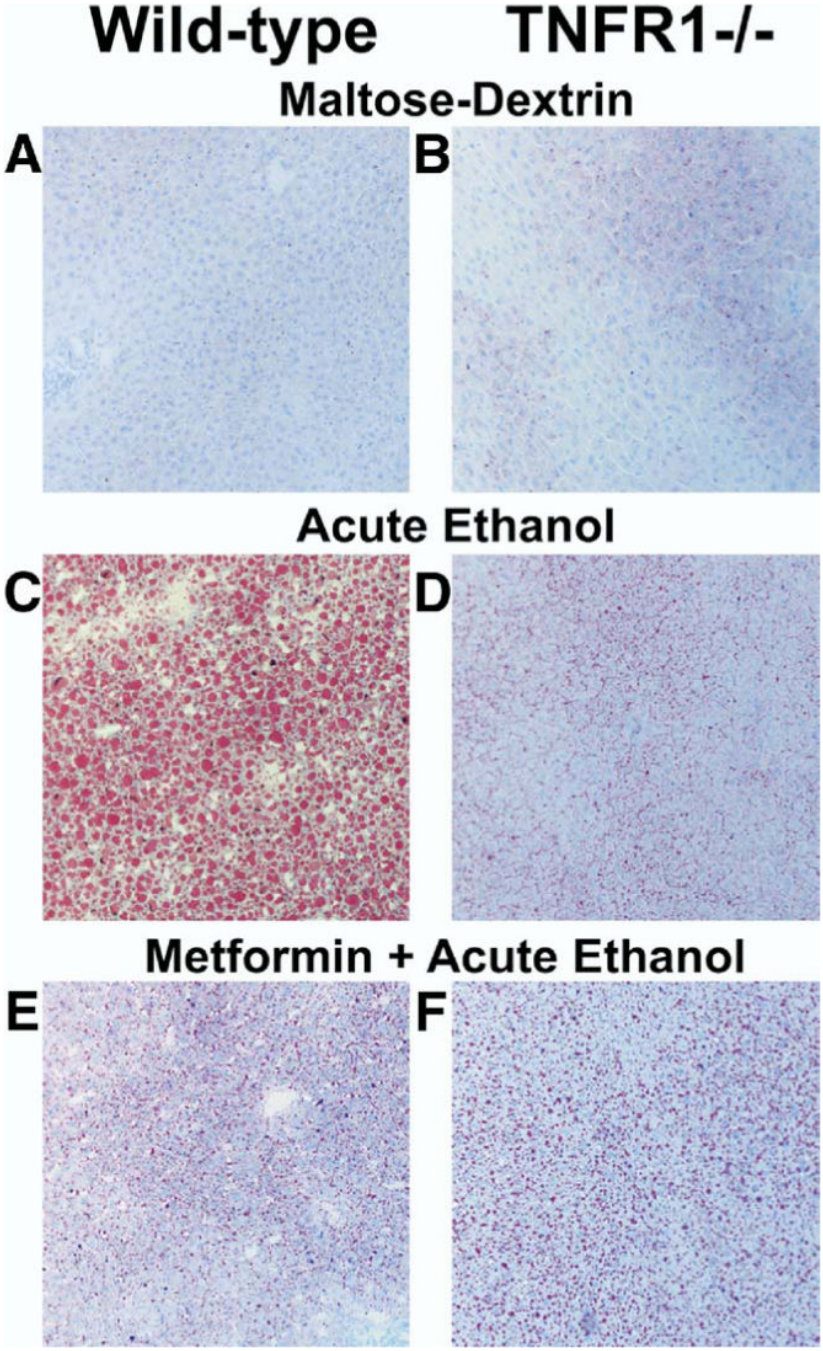

Figure 1.

Effect of metformin on hepatic fat accumulation after acute ethanol ingestion in mice. Representative photomicrographs are shown depicting oil Red O staining of liver sections (200×) of wild-type and (*E*, *F*) TNFR1$^{-/-}$ mice given ethanol (EtOH) or isocaloric maltose-dextrin. Animals were either injected with 200 mg/kg metformin or saline prior to ethanol (maltose dextrin) administration. See Materials and Methods for details.

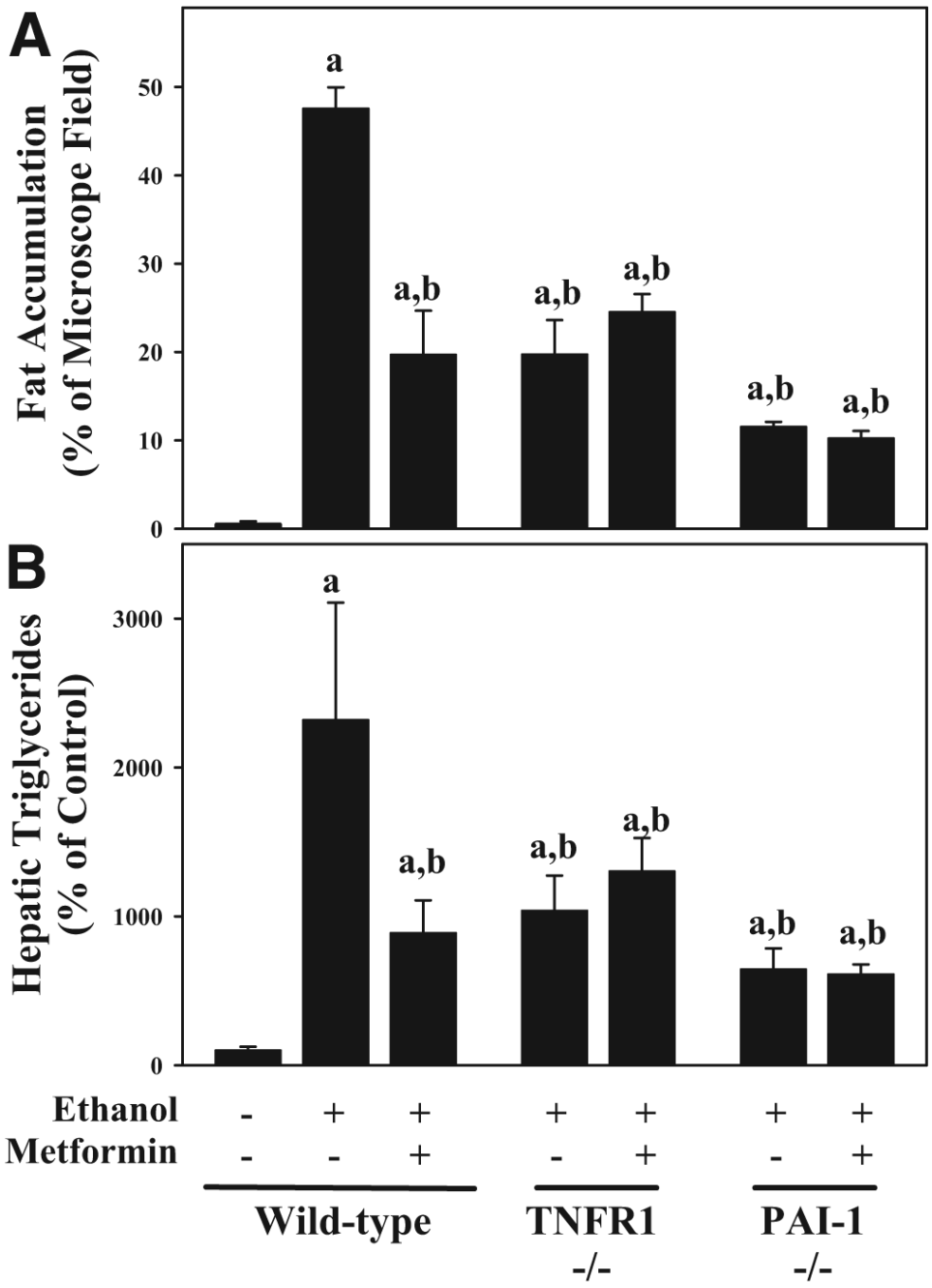

Figure 2.

Quantitation of triglycerides and hepatic fat accumulation 12 hours after acute alcohol ingestion in mice. Animals and treatments are as described in Materials and Methods. Image analysis of lipid accumulation (*A*; see also Figure 1) and determination of hepatic triglycerides (*B*) were performed as described in Materials and Methods. Data are means ± SEM (n = 4−6) and are normalized to percent of control (*A*) or as percent of microscope field (*B*). [a]$P < .05$ compared with wild-type control; [b]$P < .05$ compared with wild-type ethanol-treated.

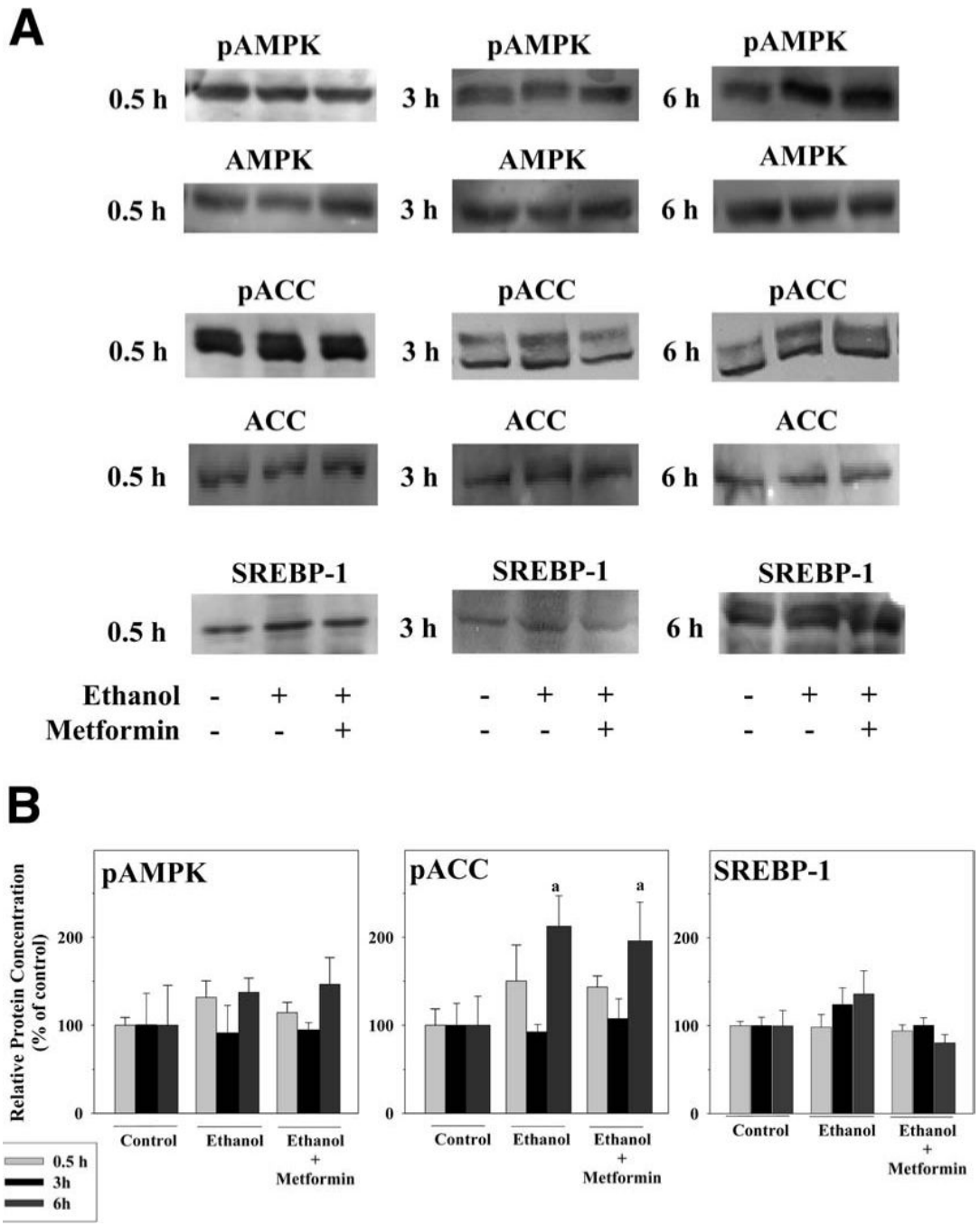

Figure 3.

Effect of ethanol and metformin on the phosphorylation status of AMPK and ACC, as well as nuclear translocation of SREBP-1 after acute alcohol ingestion. Animals and treatments are as described in Materials and Methods. (*A*) Representative Western blots depicting phosphorylated and total AMPK and ACC, as well as nuclear localization of SREBP-1 0−6 hours after acute ethanol administration. (*B*) Results of quantitative analysis. Data are presented as mean values ± SEM and are normalized to percent of control (n = 4−6). [a]*P* < .05 compared with wild-type mice administered maltose dextrin.

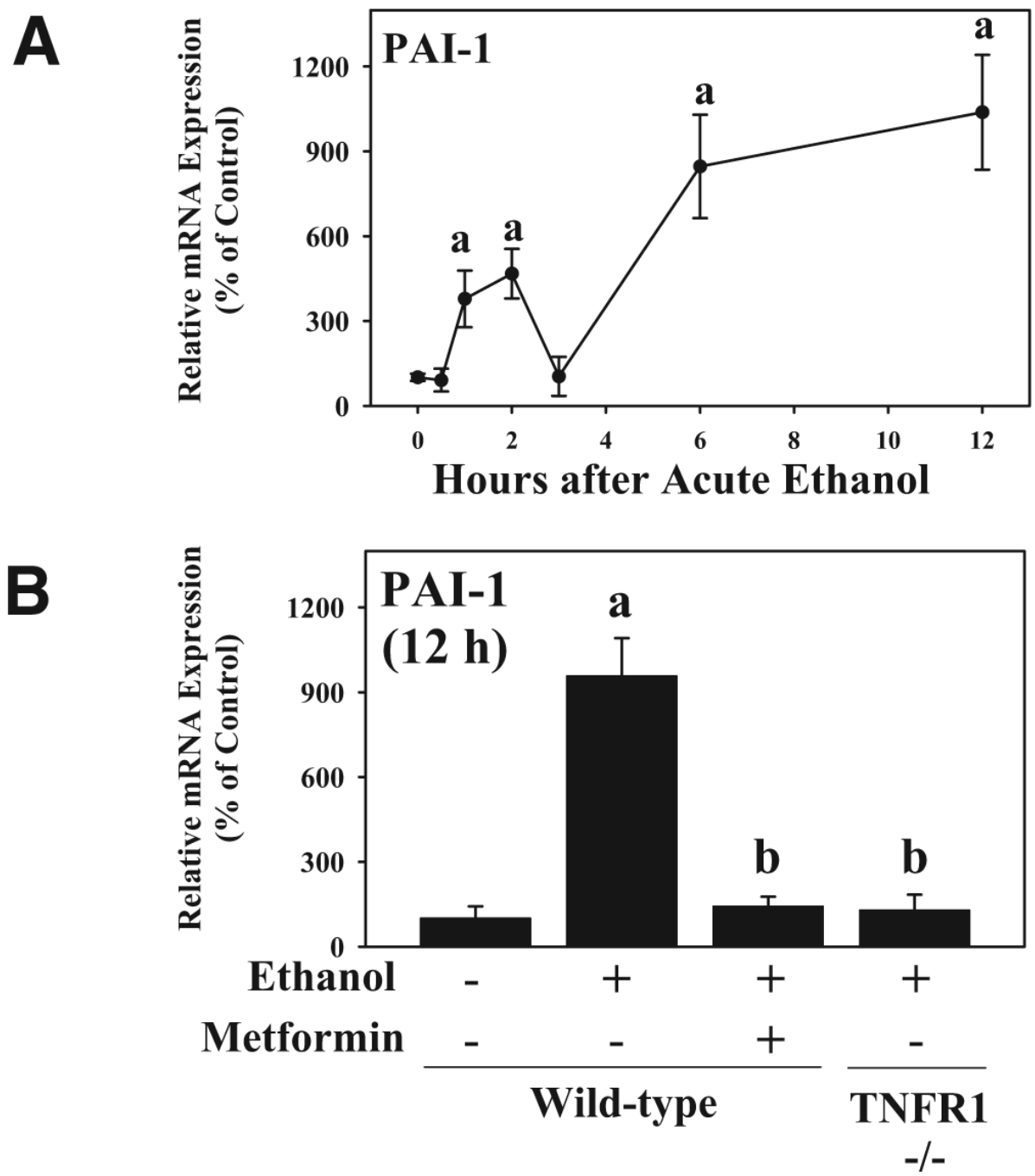

Figure 4.

Effect of ethanol and metformin on the expression of PAI-1 mRNA in liver. Animals and treatments are as described in Materials and Methods. Expression of PAI-1 mRNA was determined by real-time RT-PCR as described in Materials and Methods. (*A*) Expression of PAI-1 mRNA 0−12 hours after acute alcohol ingestion in wild-type mice. [a]$P < .05$ compared with $t = 0$ hours. (*B*) Effect of metformin or knocking out TNFR1 ($TNFR1^{-/-}$) on the increase in PAI-1 expression caused by ethanol 12 hours after administration. Data are mean values ± SEM (n = 4−6). [a]$P < .05$ compared with wild-type mice administered maltose dextrin. [b]$P < .05$ compared with wild-type given ethanol.

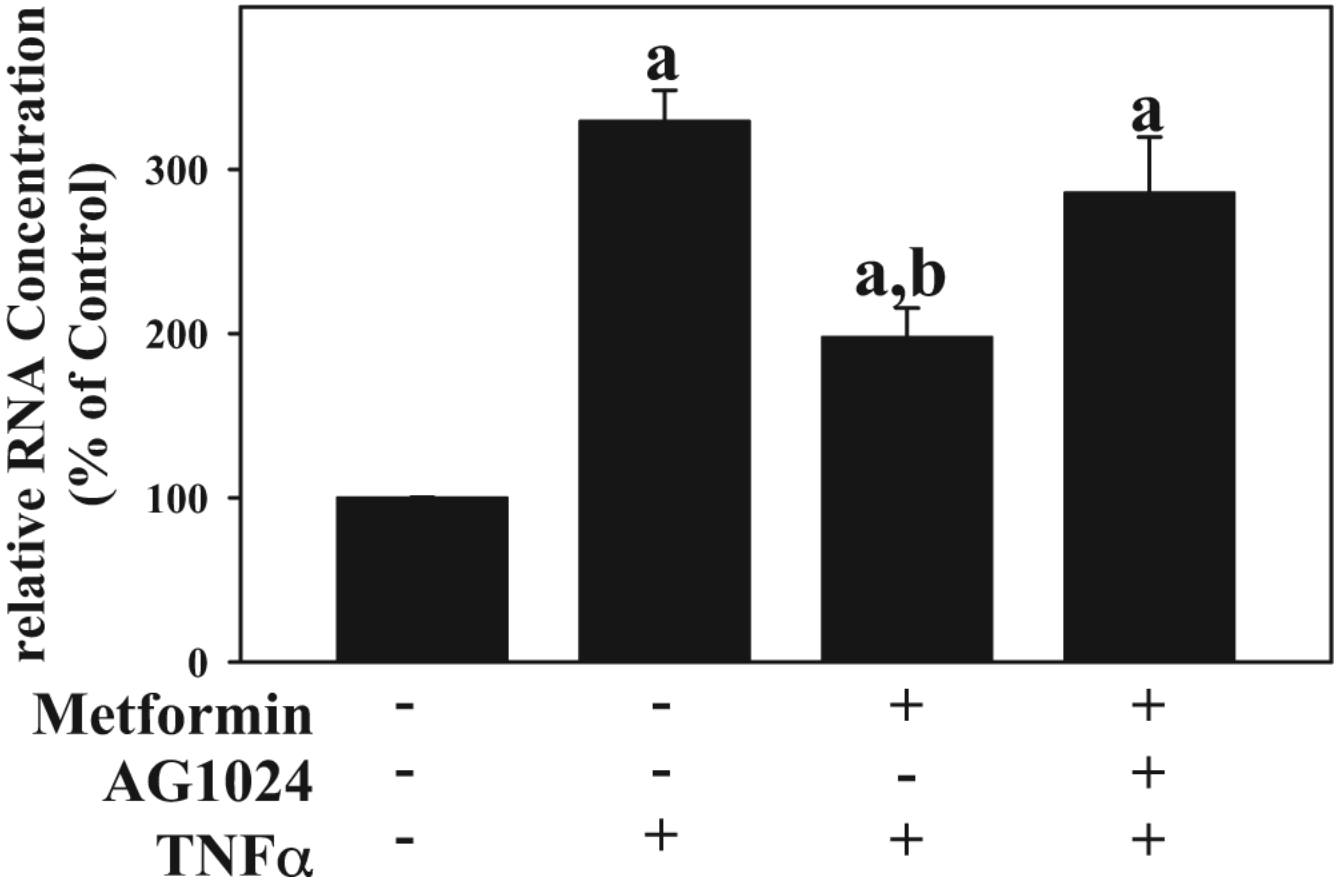

Figure 5.

Effect of metformin and tyrphostin-AG1024 on TNF-α–induced PAI-1 mRNA levels in AML-12 cells. AML-12 cells were pre-treated as described in Materials and Methods. PAI-1 mRNA levels were determined in cell culture samples using real-time RT-PCR. Data are mean values ± SEM and are normalized to percent of control. Quantitative analysis of 3 separate experiments. [a]$P < .05$ compared to control. [b]$P < .05$ compared with TNF-α–treated cells.

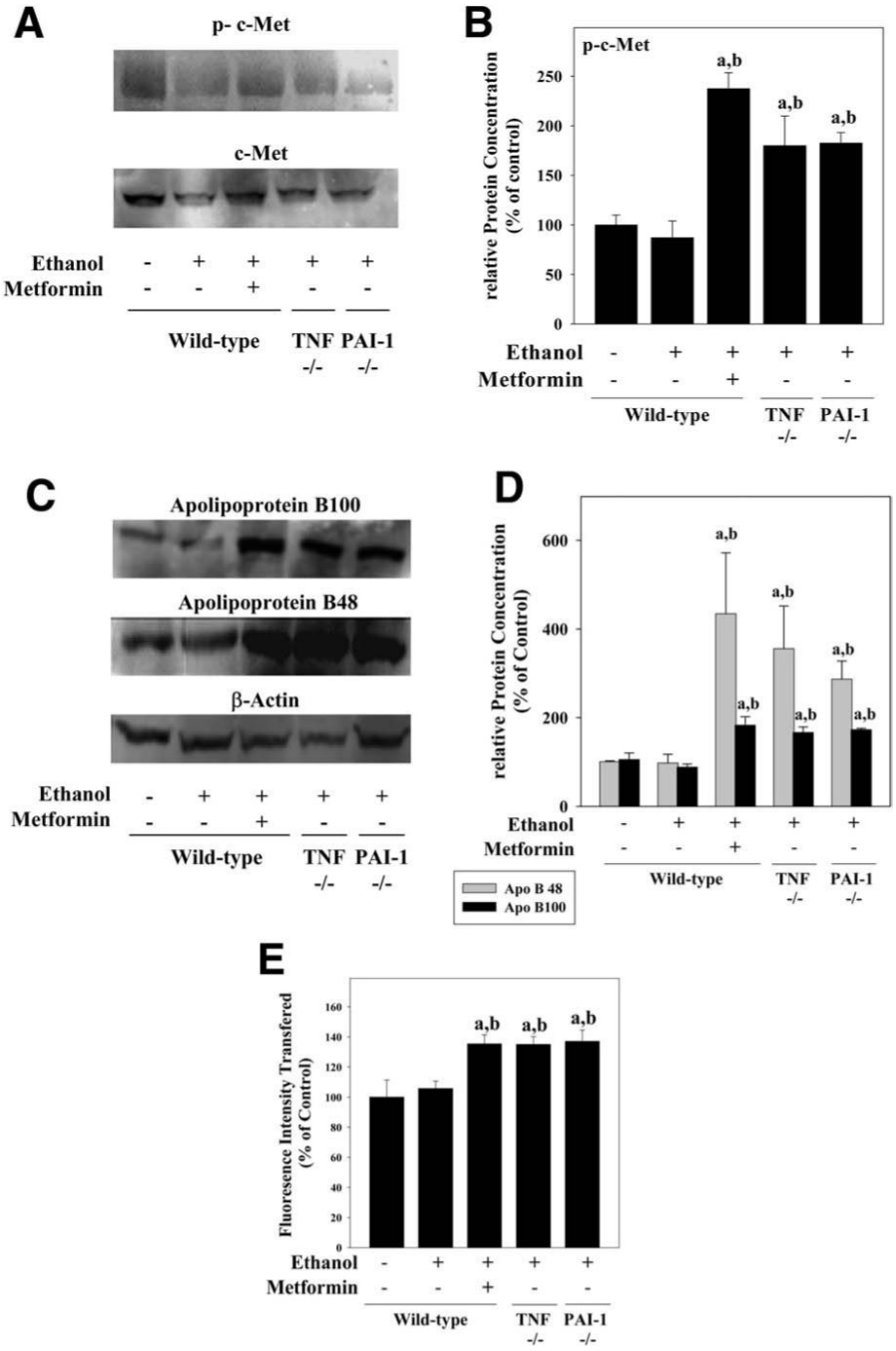

Figure 6.

Effect of ethanol and metformin on phosphorylation status of c-Met, hepatic levels of ApoB (100 and 48), and hepatic MTTP activity after acute alcohol ingestion. Animals and treatments are as described in Materials and Methods. (*A*) Representative Western blots of phosphorylated and total c-Met. (*B*) Quantitative analysis of blots. (*C*) Representative Western blots of ApoB100 and B48. (*D*) Quantitative analysis of blots. Data for ApoB100 and B48 were normalized to β-actin. (*E*) Results of determination of hepatic MTTP activity. Data are mean values ± SEM (*B*, *D*, and *E*) and are normalized to percent of control (n = 4−6). [a]*P* < .05 compared with wild-type mice administered maltose dextrin. [b]*P* < .05 compared with wild-type mice administered ethanol.

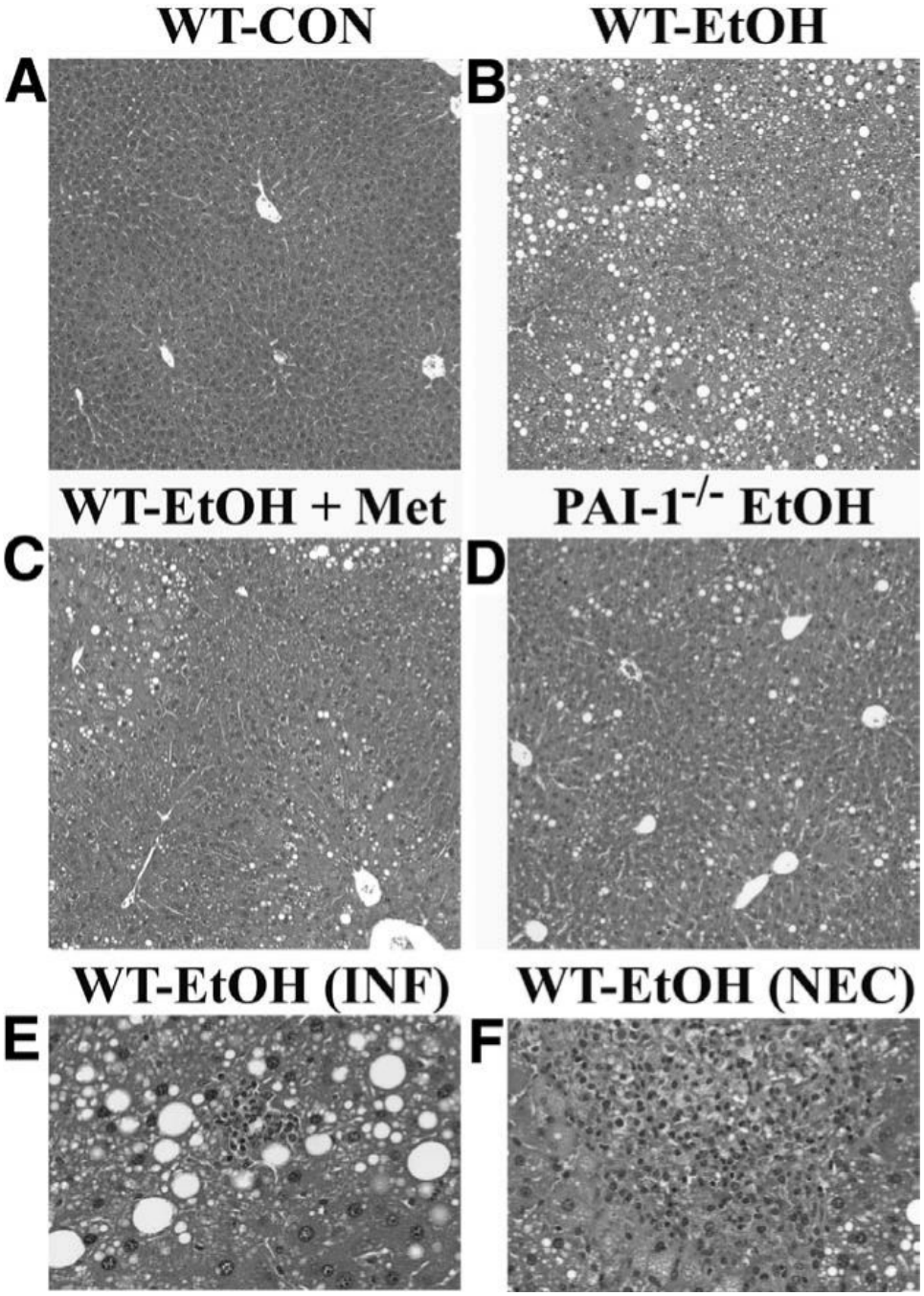

Figure 7.

Photomicrographs of livers following chronic ethanol treatment. Animals were fed high-fat control or high-fat ethanol-containing diets enterally for 4 weeks as described in Materials and Methods. Representative photomicrographs (100×) of livers from wild-type mice fed control diet (*A*), wild-type mice fed ethanol diet (*B*), wild-type mice fed ethanol diet containing metformin (*C*), and PAI-1 knockout mice fed ethanol diet (*D*) are shown. Additional panels (200×) show focal inflammatory (*E*) and necrotic (*F*) changes observed in wild-type mice fed chronic ethanol-containing diet.

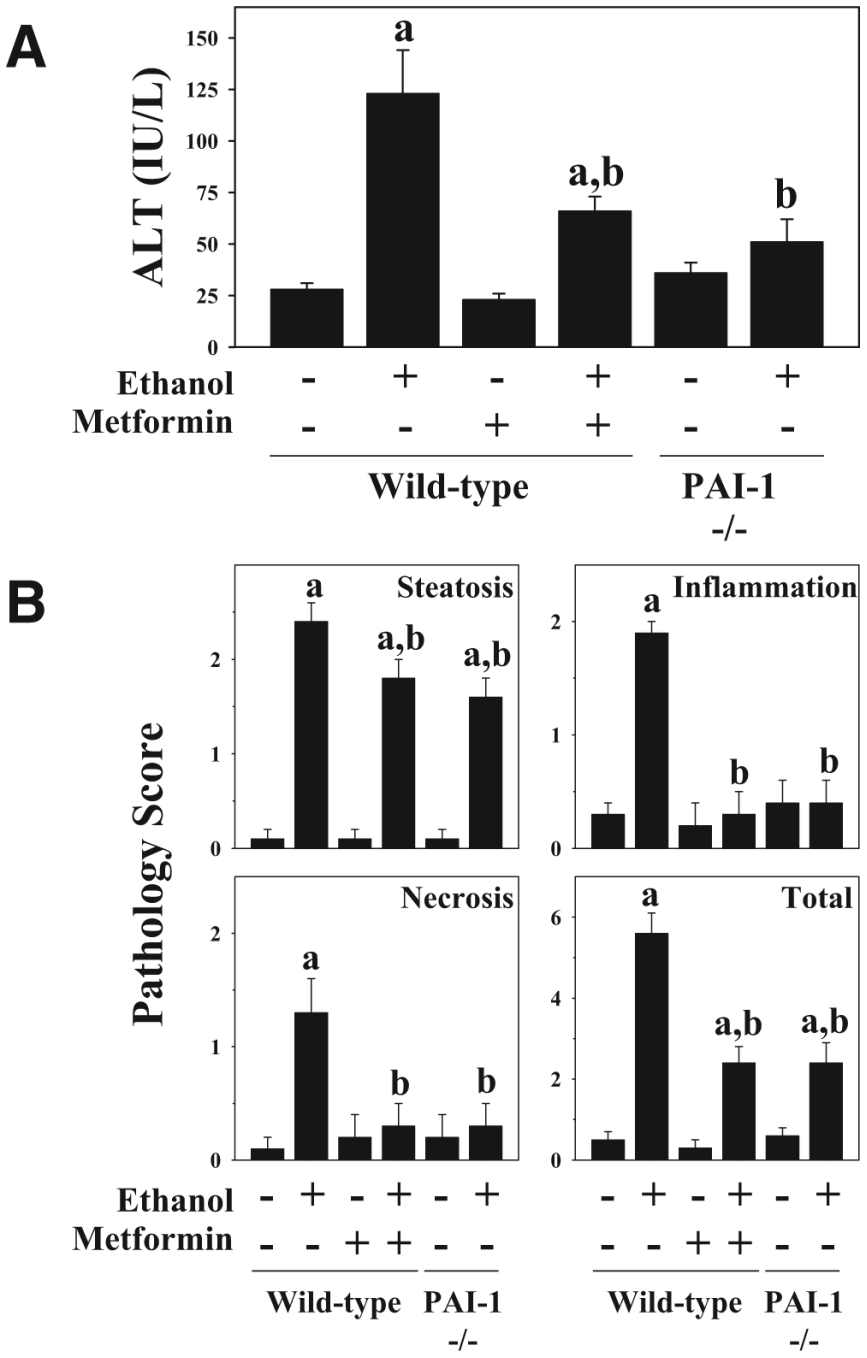

Figure 8.

Effect of ethanol and metformin on alanine aminotransferase activity and pathology scores. Animals were fed high-fat control or high-fat ethanol-containing diets enterally for 4 weeks as described in Materials and Methods. Alanine aminotransferase (ALT; *A*) activity and pathology scores (*B*) were determined as described in Materials and Methods. Data represent means ± SEM (n = 4−6). [a]*P* < .05 compared to mice fed high-fat control diet; [b]*P* < .05 compared to wild-type mice fed high-fat ethanol diet.

Table 1

Primers and Probes Used for Real-Time RT-PCR Detection of PAI-1 and β-Actin

	Forward (3′–5′)	Reverse (3′–5′)	Probe (3′–5′)
PAI-1	CACCAACATTTTGGACGCTGA	TCAGTCATGCCCAGCTTCTCC	CCA GGC TGC CCC GCC TCC TC
β-Actin	GGCTCCCAGCACCATGAA	AGCCACCGATCCACACAGA	AAGATCATTGCTCCTCCTGAGCGCAAGTA

PAI-1, plasminogen activator inhibitor 1; RT-PCR, reverse transcriptase polymerase chain reaction.

Table 2

Effect of Chronic Ethanol on Select Parameters in Mice

	Wild-type mice		Wild-type mice + metformin		PAI-1$^{-/-}$ mice	
	High fat control	**High fat ethanol**	**High fat control**	**High fat ethanol**	**High fat control**	**High fat ethanol**
PAI-1 mRNA	100 ± 18	476 ± 86	164 ± 79	33 ± 6[b]	n/d	n/d
p-AMPK:t-AMPK	100 ± 24	124 ± 27	95 ± 17	99 ± 12	152 ± 37	113 ± 29
p-ACC:t-ACC	100 ± 19	93 ± 14	93 ± 24	79 ± 15[b]	68 ± 14[b]	78 ± 9[b]

Feeding of chronic enteral diet and metformin treatment are as described in Materials and Methods. Expression of PAI-1 was determined by real-time RT-PCR. The ratio of phosphorylated AMPK and ACC to total protein was determined by Western blot, as described in Materials and Methods. Data are mean values ± SEM and are normalized to percent of wild-type mice fed control diet (n = 4–6). n/d, not detectable.

[a] $P < .05$ compared with wild-type mice administered maltose dextrin.

[b] $P < .05$ compared with wild-type mice administered ethanol.