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PKC ϵ contributes to chronic ethanol-induced steatosis in mice but not inflammation and necrosis

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Abstract

Background/Aims—Protein kinase c-epsilon (PKC ϵ) has been shown to play a role in experimental steatosis by acute alcohol. The 'two-hit' hypothesis implies that preventing steatosis should blunt more advanced liver damage (e.g., inflammation and necrosis). However, the role of PKC ϵ in these pathologies is not yet known. The goal of this current work was to address this question in a model of chronic alcohol exposure using antisense oligonucleotides (ASO) against PKC ϵ .

Methods—Accordingly, PKC ϵ ASO- and saline-treated mice were fed high-fat control or ethanol-containing enteral diets for 4 weeks.

Results—Chronic ethanol exposure significantly elevated hepatic lipid pools as well as activated PKC ϵ . The PKC ϵ ASO partially blunted the increases in hepatic lipids caused by ethanol. Administration of PKC ϵ ASO also completely prevented the increase in the expression of fatty acid synthase (FAS) and TNF α caused by ethanol. Despite these protective effects, the PKC ϵ ASO was unable to prevent the increases in inflammation and necrosis caused by chronic ethanol. These latter results correlated with an inability of the PKC ϵ ASO to blunt the upregulation of plasminogen activator inhibitor-1 (PAI-1) and the accumulation of fibrin. Importantly, PAI-1 has been previously shown to more robustly mediate inflammation and necrosis (versus steatosis) after chronic ethanol exposure.

Conclusions—This study identifies a novel potential mechanism where ethanol, independent of steatosis, can contribute to liver damage. These results also suggest that PAI-1 and fibrin accumulation may be at the center of this PKC ϵ -independent pathway.

Keywords

Alcohol; Alcoholic liver disease; Steatosis; Inflammation; Protein kinase C

Alcoholic liver disease (ALD) is a serious concern for the world's population, causing millions of deaths per year (Grant *et al.*, 1988). Whereas the pathological steps of ALD are well understood, our knowledge of the mechanisms involved in causing the disease is lacking; as a result, there is no FDA-approved therapy to treat or reverse ALD. Steatosis is a critical stage in the pathology of alcoholic liver disease. Although steatosis was once thought to be a benign pathology of ALD, more recent evidence has indicated that blunting or blocking steatosis could help prevent the progression of ALD (Teli *et al.*, 1995; Day and James 1998). Therefore, pharmacologically targeting the cause(s) of ethanol-induced steatosis is potentially a promising therapy for ALD.

Recent evidence has suggested that protein kinases c- ϵ (PKC ϵ), can contribute to steatosis in experimental non-alcoholic fatty liver disease (NAFLD) (Samuel *et al.*, 2004). Work from this group showed that PKC ϵ also plays a causal role in acute ethanol-induced steatosis in mice (Kaiser *et al.*, 2009). Results from that study support the working hypothesis that the inhibition of β -oxidation of fatty acids caused by ethanol metabolism increases the fatty acid supply for diacylglycerol (DAG) synthesis. This increase in DAG allosterically activates PKC ϵ , which then contributes to the increase in hepatic triglycerides caused by ethanol by inducing insulin resistance (Kaiser *et al.*, 2009). Thus, PKC ϵ activation may be a shared mechanism of hepatic steatosis in ALD and NAFLD.

Whereas acute bolus ethanol causes a robust steatotic response in the liver (Kaiser *et al.*, 2009), there is little or no inflammation or necrosis caused by this dose regimen of ethanol in rodents. PKC ϵ has been shown to contribute to inflammation in response to various insults (e.g. LPS), which suggests that PKC ϵ may also contribute to more advanced stages of ALD (e.g. steatohepatitis) (Aksoy *et al.*, 2004). Furthermore, as mentioned above, blunting steatosis caused by alcohol may in and of itself prevent later phases of ALD. This potential role of PKC ϵ in these later stages of experimental ALD has not yet been directly studied. Therefore, the purpose of this study was to determine the role of PKC ϵ in chronic ethanol-induced liver damage.

Experimental Procedures

Animals and Treatments

Animals 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 University of Louisville Institutional Animal Care and Use Committee. 8 week old male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Food and tap water were provided ad libitum prior to experimentation. Surgical implantation of the intragastric cannula and enteral feeding was performed as described previously (Bergheim *et al.*, 2006). PKC ϵ ASO was a kind gift of Brett Monia from Isis Pharmaceuticals (Carlsbad, CA). Half of the mice received PKC ϵ ASO (25 mg/kg, i.p.) or vehicle (saline) twice a week

(Kaiser *et al.*, 2009). In preliminary studies, administration of a scrambled control ASO did not affect pathology or indices of inflammation in comparison to mice injected with vehicle (not shown). For sacrifice, animals were anesthetized with ketamine/xylazine (100/15 mg/kg, i.m.) and blood was collected from the vena cava just prior to sacrifice. Citrated plasma was stored at -80°C until further analysis. Portions of liver tissue were frozen immediately in liquid nitrogen, while others were fixed in 10% neutral buffered formalin or embedded in frozen specimen medium (Tissue-Tek OCT compound, Sakura Finetek, Torrance, CA).

Histological 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.* (Nanji *et al.*, 1989). Micro- and macrovesicular fat droplets were defined as $<50\%$ and $>50\%$ of total cell volume, respectively. Plasma levels of aminotransferases (ALT and AST) were determined using standard kits (Thermotrace, Melbourne, Australia). Urine was collected daily and urine alcohol concentrations measured using routine spectrophotometric techniques (McKim *et al.*, 2002). Neutrophil accumulation in the liver was assessed by staining tissue sections for chloroacetate esterase (CAE), using a naphthol AS-D chloroacetate esterase kit (Sigma, St. Louis, MO) (Guo *et al.*, 2004). The accumulation of fibrin matrices was determined immunofluorometrically and quantitated by image-analysis (Beier *et al.*, 2008). For hepatic lipid staining, frozen sections of liver (10 μm) were stained with oil red O (Sigma Chemical Co., St. Louis, MO) and counter-stained with hematoxylin; staining was quantitated by image-analysis (Kaiser *et al.*, 2009).

Determination of individual free fatty acid species by gas chromatography

Samples were processed as described by Lepage and Roy (Lepage and Roy 1986). Briefly, 30 mg of liver homogenate was weighed in glass tubes. While stirring, 200 μl of acetyl chloride was slowly added. Tridecanoic acid (13:0) was used as internal standard. The tubes were tightly closed with Teflon-lined caps and subjected to methanolysis at 100°C for 1 hr. After adding 5 ml of 6% K_2CO_3 , the fatty acid esters were extracted with hexane and an aliquot of the hexane upper phase was injected into the chromatograph. FFA were chromatographed as methyl esters on an Agilent 6890 N GC equipped with an HP-5 capillary column (50 m $\times$ 0.2 mm i.d. $\times$ 0.5 μm phase thickness) coupled to a 5973 detector for mass spectrometry analysis. Helium was used as carrier gas. The split ratio was 20:1. The injection port temperature was 280°C and the detector was 250°C . The GC column temperature was set at 150°C for 3 min, increased by $5^{\circ}\text{C}/\text{min}$ until 260°C and then by $20^{\circ}\text{C}/\text{min}$ until 300°C and kept at this temperature for 4 min.

Biochemical analyses

Western Blotting for PKC ϵ was performed as described previously (Kaiser *et al.*, 2009). RNA extraction and real-time RT-PCR were performed as described previously (Bergheim *et al.*, 2006). Primers for PKC ϵ were purchased from Applied Biosystems (Foster City, CA). For the determination of hepatic lipid levels, hepatic lipids were extracted by an aqueous

solution of chloroform and methanol (Bligh and Dyer 1959). DAG was quantified by mass spectroscopy and normalized to mg wet weight of liver tissue (Kaiser *et al.*, 2009). Colorimetric assessment of non-esterified fatty acids (NEFA) was carried out using a standard kit (Roche, Penzberg, Germany) (Kaiser *et al.*, 2009). Triglycerides, cholesterol, and phospholipids were measured colorimetrically using a Cobas Mira chemistry analyzer with appropriate calibrated standards. Values for lipids (e.g. triglycerides, cholesterol, etc.) were normalized to protein in homogenate prior to extraction, as determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Statistical analyses

Results are reported as means $\pm$ SEM. ANOVA with Bonferroni's post-hoc test was used for the determination of statistical significance among treatment groups. For comparison of pathological scores, the Mann-Whitney rank sum test was used. A *p* value less than 0.05 was selected before the study as the level of significance. Summary data are represented as means $\pm$ SEM (*n* = 4–8); ^a, *p* < 0.05 compared to the absence of ethanol. ^b, *p* < 0.05 compared to the absence of ASO.

Results

Body weight and urine ethanol concentrations

Throughout the duration of this study, all mice gained ~0.5 g/week, with no differences between dietary and treatment groups. Daily urine alcohol concentrations cycled between 0 and 500 mg/dl in ethanol-exposed mice, as observed previously (McKim *et al.*, 2003). PKC ϵ ASO- and vehicle-treated mice receiving ethanol had similar patterns of urine alcohol cycling, with mean alcohol levels not significantly different (284 ± 78 mg/dl versus 217 ± 44 mg/dl for vehicle and ASO groups, respectively). In saline-treated mice fed control diet, liver weights (as percent of body weight) were 5.9 ± 0.5 , whereas ethanol-fed mice had liver weights of 9.7 ± 0.3 . This doubling in liver weight was not affected by knocking down PKC ϵ .

Effect of chronic ethanol exposure on plasma and histological indices of liver damage

Figure 1A shows representative photomicrographs depicting liver pathology (H+E stain, left column), fat accumulation (oil red O stain, middle column) and neutrophil infiltration (CAE stain, right column). Figure 1B summarizes quantitation of the staining, as well as plasma transaminase values. Administration of PKC ϵ ASO to animals fed control diet did not affect liver pathology or plasma transaminases (Figure 1B, supplemental Figure 1). Chronic exposure to ethanol diet for 4 weeks increased fat accumulation, inflammation, and cell death (Figure 1A, middle row, Figure 1B) as well as increased plasma transaminases (ALT and AST) (Figure 1B, right panel). Coadministration of the PKC ϵ ASO partially blunted the hepatic fat accumulation caused by ethanol (Figure 1A, bottom row, Figure 1B); specifically, the PKC ϵ ASO blunted the increase in macrovesicular fat caused by ethanol, but not the increase in microvesicular fat. Despite these protective effects on lipid accumulation, the PKC ϵ ASO did not significantly attenuate the increases in indices of inflammation and liver damage caused by ethanol (Figure 1). Indeed, the increase in neutrophils caused by ethanol was significantly enhanced (~2-fold) by ASO administration

(Figure 1B, left panel). In line with these findings, the PKC ϵ ASO did not prevent the increase in transaminases caused by ethanol (Figure 1B, right panel).

Effect of ethanol on hepatic DAG and PKC ϵ activation

Some of the major activators of PKC ϵ are diacylglycerols (DAG) (Petersen and Shulman 2006). Therefore, the effect of chronic ethanol on hepatic DAG content was determined. Figure 2A shows representative mass chromatograms from mice fed control- and ethanol-containing diet. In general, most DAG species were increased by ethanol. For example, chronic ethanol significantly increased hepatic content of DAG 36:4 from 129 ± 26 pmoles/mg wet weight to 346 ± 66 pmoles/mg wet weight. Likewise, ethanol exposure increased the amount of DAG 36:3 from 72 ± 4 pmoles/mg wet weight to 289 ± 63 pmoles/mg wet weight. Figure 2 also shows the effect of chronic ethanol on protein and mRNA levels of PKC ϵ . As stated previously, ethanol increases the translocation of PKC ϵ to membrane from the cytosol of PKC ϵ (Domenicotti *et al.*, 1998; Kaiser *et al.*, 2009), which is indicative of increased activation (Souroujon *et al.*, 2004). Specifically, ethanol has been shown to increase translocation of PKC ϵ . Therefore, the effect of chronic ethanol on this index of PKC ϵ activation was determined (Figure 2B). Ethanol exposure significantly increased the membrane:cytosol ratio of PKC ϵ ~5-fold (Figure 2C). Ethanol did not significantly change the total amount of PKC ϵ mRNA (Figure 3C) or protein (Figure 3C). These results are analogous to previous findings by others in a model of non-alcoholic fatty liver disease (Samuel *et al.*, 2004). As expected, administration of the PKC ϵ ASO decreased mRNA ~5-fold (Figure 2C), as well as corresponding protein levels (Figure 2B). The ASO decreased levels of PKC ϵ mRNA more effectively in alcohol-exposed livers compared to controls, albeit only slightly.

Effect of knocking down PKC ϵ on changes in hepatic lipids caused by ethanol

Histological indices of lipid accumulation (see Figure 1) indicated that ethanol-induced steatosis was blunted by the PKC ϵ ASO. However, such general staining yields no information on the effect of the ASO on specific lipid pools increased by ethanol exposure. The levels of hepatic triglycerides, FFA, cholesterol, and phospholipids were therefore determined biochemically (Figure 3). Chronic ethanol significantly increased hepatic content of all of these pools (Figure 3). The PKC ϵ ASO partially increased cholesterol and phospholipids in control-fed mice (Figure 3C and D). ASO treatment significantly prevented the ethanol-induced increases in triglycerides, FFA, and phospholipids by ~50% (Figure 3), but had no effect on the increase in cholesterol levels caused by ethanol exposure.

In addition to different types of lipid pools, the effect of the ASO on specific subtypes of lipids may be critical. Specifically, it has been shown that some species of fatty acid (i.e. palmitic acid) may be critical in mediating lipotoxicity in liver cells (Listenberger *et al.*, 2003). Therefore, although the ASO blunted the increase in total FFA caused by ethanol (Figure 3B), these data yield no information on the effect of the ASO on specific species, which may be critical for ethanol-induced hepatotoxicity. The effect of ethanol and the PKC ϵ ASO on the spectrum of fatty acids was therefore determined by gas chromatography/mass spectrometry (Figure 4). Analogous to findings with the total FFA measurements (Figure 3B), ethanol robustly increased levels of all species of fatty acids analyzed

compared to control-fed animals. The ASO significantly attenuated the increases in most of these species caused by ethanol. For example, the PKC ϵ ASO blunted (~50%) the increases in palmitic acid (16:0) caused by ethanol (Figure 4). Therefore, the effect of the ASO on specific species of FFA (Figure 4) mirrored the results on the total FFA pool (Figure 3B).

Effect of ethanol on key genes

Figure 5 summarizes the effect of ethanol the PKC ϵ ASO on key indices of pathways shown to be critical for experimental alcohol-induced liver injury. For example, one of the major genes responsible for lipogenesis after chronic alcohol exposure is fatty acid synthase (FAS) (Muramatsu *et al.*, 1981). Likewise, TNF α has been identified as a critical proinflammatory component of experimental ALD (Yin *et al.*, 1999), whose production from macrophages is blunted by inhibiting PKC ϵ (Castrillo *et al.*, 2001). Lastly, recent studies have also identified a role of PAI-1, and its effect on fibrin metabolism, on experimental liver damage caused by chronic ethanol (Bergheim *et al.*, 2006). Therefore, the effect of chronic ethanol and the PKC ϵ ASO on the expression of these indices was determined (Figure 5). As expected, ethanol significantly increased the expression of all 3 genes 3–4 fold (Figure 5). Whereas the PKC ϵ ASO completely attenuated the increase in FAS (Figure 5A) and TNF α (Figure 5B) expression caused by ethanol exposure, this ASO did not significantly affect the increase in PAI-1 caused by ethanol under these conditions.

Effect of chronic ethanol on hepatic fibrin deposition

Previous work has demonstrated that the inhibition of fibrin degradation by PAI-1 contributes to hepatic inflammation (Luyendyk *et al.*, 2004; Beier *et al.*, 2009). Since the induction of PAI-1 was not attenuated by the PKC ϵ ASO (Figure 5C), the effect of chronic ethanol on hepatic fibrin deposition was determined. Figure 6 comprises representative confocal photomicrographs depicting immunofluorescent detection of fibrin deposition. Chronic enteral ethanol increased fibrin deposition in sinusoidal spaces of the liver lobule (Figure 6). In line with PAI-1 expression results (Figure 5C), PKC ϵ ASO treatment did not affect this increase in fibrin deposition caused by ethanol (Figure 6).

Discussion

Results of several studies suggested that the accumulation of lipids in the liver is critical to the development of ALD [for review see (Teli *et al.*, 1995)]. Animal models resembling conditions of early stage ALD in humans are therefore useful tools to investigate mechanisms underlying the effects of ethanol on the liver. Acute and chronic alcohol-induced liver injury appear to share similar mechanisms [for review see (Thurman *et al.*, 1998)]. For example, several compounds previously shown to protect against chronic alcohol-induced liver damage also protected rat liver against acute ethanol induced steatosis [see (Massey and Arteel 2012) for review]. Therefore, acute alcohol exposure can also be used to mimic very early effects of ethanol in the progression of chronic liver damage. Previous work by this group demonstrated that knocking down PKC ϵ prevented steatosis caused by 1 dose of bolus ethanol in mice (Kaiser *et al.*, 2009), which suggested that this protein may be critical in the early development of ALD.

Whereas acute models of alcohol exposure are useful tools to identify new mechanisms and/or to screen new therapies for ALD, it should be emphasized that acute ethanol by no means mimics all effects of chronic ethanol on the liver. As mentioned in the Introduction, although the acute bolus model of ethanol exposure causes hepatic steatosis, this model is almost completely devoid of more severe damage (e.g., inflammation and necrosis), which may or may not be mechanistically related to the steatotic effect of ethanol. Given the importance of these later pathologies to the development of ALD in humans, positive results with an acute model is insufficient for translation to the human disease.

Knocking down PKC ϵ indeed prevented chronic ethanol-induced steatosis (Figure 1), as had been observed in the acute model (Kaiser *et al.*, 2009). Ethanol exposure causes steatosis by increasing lipid synthesis as well as by decreasing in lipid catabolism. For example, ethanol induces FAS expression and activity (synthetic) and concomitantly inhibits the β -oxidation (catabolic) of fatty acids (Tremolieres *et al.*, 1972). Here, the increase in FAS expression caused by ethanol was almost completely attenuated in ASO-treated mice that received ethanol (Figure 5). This effect by the ASO correlated with a blockade of the increase in lipid pools (i.e. triglycerides, FFA, and phospholipids) accumulation caused by ethanol (Figure 3). Therefore, these data suggest the increase in lipid accumulation caused by ethanol is mediated, in part, by the indirect upregulation of FAS via PKC ϵ . There is no evidence that PKC ϵ directly influences FAS expression. However, it has been shown that TNF α increases FAS levels in rodent liver (Grunfeld *et al.*, 1988) and that PKC ϵ can mediate the upregulation of TNF α expression (Comalada *et al.*, 2003). Therefore, the PKC ϵ ASO may be blunting FAS induction indirectly by preventing the induction of TNF α (Figure 5B). Interestingly, administration of PKC ϵ ASO increased hepatic content of cholesterol and phospholipids in mice fed control diet, albeit not to the same extent as ethanol diet (Figure 3). The mechanisms by which blocking PKC ϵ mediated this effect are unclear, but previous studies have indicated that PKCs (including PKC ϵ) contribute to phospholipid trafficking and cholesterol secretion by cells [e.g., (Mehta *et al.*, 2002)]. These effects of the PKC ϵ ASO on these lipid pools may therefore represent disruptions in these processes.

The protective effect of PKC ϵ on steatosis was incomplete, and microvesicular fat deposits were largely unaffected by PKC ϵ administration (Figure 1); the latter pattern of steatosis is associated with a worse prognosis in fatty liver disease and may represent damage to mitochondria (Begriche *et al.*, 2011). Hepatomegaly caused by ethanol exposure is hypothesized to be caused by both hyperplasia and hypertrophy of hepatocytes. The hypertrophy caused by ethanol is hypothesized to involve not only lipid accumulation, but also protein accumulation (Baraona *et al.*, 1975). The finding here that the PKC ϵ ASO did not significantly blunt the increase in liver size caused by ethanol could therefore be a result of incomplete prevention of steatosis, or a lack of protection against the hyperplastic or other hypertrophic changes caused by ethanol that contribute to liver weight.

The 'two-hit' hypothesis implies that preventing steatosis should blunt more advanced liver damage (e.g., inflammation and necrosis) (Yang *et al.*, 1997; Day and James 1998). Since the inhibition of PKC ϵ prevented acute ethanol-induced steatosis, it was hypothesized a priori that this effect on steatosis would also protect against inflammation and necrosis caused by chronic ethanol exposure. Furthermore, previous studies have demonstrated that

PKC ϵ contributes to macrophage activation (Castrillo *et al.*, 2001), and apoptosis caused by ethanol (Zhang *et al.*, 2007). It was therefore surprising that whereas PKC ϵ ASO-treatment indeed blunted steatosis and TNF α production caused by chronic ethanol, it provided no protection against inflammation or necrosis (Figure 1).

Yamaguchi *et al.* (Yamaguchi *et al.*, 2007) showed that knocking down diacylglycerol acyltransferase 2 (DGAT2) prevented steatosis in a methionine-choline deficient mouse model, but actually exacerbated inflammatory liver damage. Those results were attributed to toxic free fatty acids accumulating in the liver because of the blockade of triglyceride synthesis caused by DGAT2 knockdown (Yamaguchi *et al.*, 2007). Here, such an increase in fatty acids was not observed in the PKC ϵ ASO group. Indeed, there was a general decrease in this lipid pool (Figure 4). More detailed analysis of specific free fatty acid species (Figure 5) also showed that the ASO blunted the increase in hepatotoxic [e.g. palmitic acid (Ji *et al.*, 2005)] free fatty acids caused by ethanol (Figure 5). Therefore, although a disconnect between preventing steatosis and more severe liver damage is not without precedent in animal models, it is unlikely that the effects observed here can be explained an increase in toxic free fatty acids, as observed in that previous study (Ji *et al.*, 2005; Yamaguchi *et al.*, 2007).

Another potential mechanism by which inflammation can be maintained under these conditions is via elevated hepatic cholesterol (Arteel 2012). For example, LDLR-deficient mice that overexpressed apolipoprotein E2 and were fed a high fat cholesterol diet did not develop steatosis, but did develop a robust hepatic inflammatory response (Wouters *et al.*, 2008). A similar hepatotoxic role of cholesterol was identified in *Alms1* mutant (*foz/foz*) mice, which develop metabolic syndrome and fatty liver injury (Van Rooyen *et al.*, 2011). Mitochondrial cholesterol can be hepatotoxic when it accumulates (Fernandez *et al.*, 2008). These mechanisms could in principle provide an explanation for the results obtained here, as the PKC ϵ ASO did not blunt the increase in cholesterol caused by ethanol (Figure 3C). However, the amount of cholesterol required to cause liver damage in those studies (Wouters *et al.*, 2008; Van Rooyen *et al.*, 2011) was far greater than the <2-fold increase observed here (Figure 3C). Nevertheless, the potential role of cholesterol mediating the effects observed here (i.e., inflammatory liver damage in the absence of steatosis) cannot be completely dismissed.

Work by this group has demonstrated that PAI-1 is involved in hepatic inflammation caused by chronic ethanol exposure (Bergheim *et al.*, 2006). It was also shown that the sensitization of ethanol preexposure to LPS-induced inflammation and liver damage is mediated in part by PAI-1 induction (Beier *et al.*, 2009). The main function of PAI-1 is to impair fibrinolysis, which causes fibrin matrices to accumulate in the extracellular space. It is proposed that this increase in fibrin matrices is responsible for the proinflammatory effect of PAI-1 (Luyendyk *et al.*, 2004; Beier *et al.*, 2009). The results of the current work suggest that ethanol exposure upregulates PAI-1 expression (Figure 5) via mechanisms independent of PKC ϵ , and that this upregulation contributes to hepatic fibrin accumulation (Figure 6), inflammation and liver damage (Figure 1). These effects of ethanol also appear to be mediated via pathways that are at least partially distinct from the steatotic effect of ethanol, PKC ϵ knockdown was effective at blunting steatosis (Figure 1) and lipid accumulation (Figures 3–4) caused by ethanol. It

was previously shown that PAI-1 deficient mice are almost completely protected against inflammation and necrosis caused by chronic enteral ethanol, but only mildly protected against steatosis (Bergheim *et al.*, 2006). Taken together, these results support the hypothesis that PAI-1 mediates ethanol-induced hepatotoxicity mostly via inflammatory mechanisms and less via steatosis.

Interestingly, administration of the PKC ϵ increased the number of neutrophils found in the liver after alcohol exposure by almost of factor of 2 (Figure 1). Previous studies have demonstrated that neutrophil accumulation plays a damaging role in experimental alcohol-induced liver injury. For example, knocking out the adhesion molecule, ICAM-1, significantly blunted inflammatory liver damage in enteral alcohol model (Kono *et al.*, 2001). It is somewhat surprising, therefore, that the enhancement of ethanol-induced neutrophil accumulation by the PKC ϵ ASO did not translate to an increase in liver damage (e.g., transaminases; Figure 1). This may be because although the total number of neutrophils increased in the liver, the apparent number of extravasated (i.e., actively involved in inflammation) did not.

In summary, the results of this work provide further support for the role of PKC ϵ in ethanol-induced steatosis. Furthermore, this study determined that knocking down PKC ϵ does not prevent inflammation, most likely by failing to prevent the induction of PAI-1 caused by ethanol. These results also challenge somewhat the assumption that lipid accumulation, per se, is directly responsible for enhancing inflammation caused by ethanol, and suggest that other mechanisms that are induced in parallel with steatosis may be critical. Therefore, potential therapies that may be effective at blocking steatosis caused by ethanol may be ineffective in preventing liver injury, if they do not also regulate inflammatory mediators, such as PAI-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effect of PKC ϵ deficiency on chronic ethanol-induced liver damage

Panel A, representative photomicrographs of hematoxylin and eosin [(H+E, left panel, 100 $\times$) (inserts are 400 $\times$)], oil red O [(middle panel, 100 $\times$) (inserts are 400 $\times$)] and chloroacetate esterase (CAE, right panel, 100 $\times$) staining are shown. Panel B summarizes quantitation of histologic and plasma indices of liver damage. Data are means $\pm$ SEM (n=4–6) and are reported as fold of control values. ^a, p<0.05 compared to the absence of ethanol. ^b, p<0.05 compared to the absence of ASO as determined by 2-way ANOVA.

Figure 2. Effect of chronic ethanol on hepatic DAG levels and the activation of PKC ϵ in mouse liver

Panel A: Positive electron spray ionization mass spectrometry chromatograms are shown from liver extracts. Since Na⁺ adducts were used to detect DAG species, the m/z value represents the molecular weight of the respective DAG plus the weight of a sodium molecule (M+Na⁺). Representative chromatograms of livers from control (upper panel) and ethanol-exposed (lower panel) mice are shown. Panel B: Representative Western blots are shown depicting the membrane and cytosolic fractions of PKC ϵ . Panel C: Real-time rtPCR results (left panel) were normalized to β -actin and Western analyses (right panel) were quantitated by image-

analysis. Data are means $\pm$ SEM (n=4–6) and are reported as fold of control values. ^a, p<0.05 compared to the absence of ethanol. ^b, p<0.05 compared to the absence of ASO as determined by 2-way ANOVA.

Figure 3. Effect of chronic ethanol on indices of hepatic lipid accumulation

Quantitations of lipids were determined in hepatic extracts via colorimetric assays. Data are normalized to mg protein. Data are means $\pm$ SEM (n=4–6) and are reported as fold of control values. ^a, $p < 0.05$ compared to the absence of ethanol. ^b, $p < 0.05$ compared to the absence of ASO as determined by 2-way ANOVA.

Fatty Acid Amount (µg/mg tissue)	Retention Time (mins)	Control		EtOH	
		+Vehicle	+ASO	+Vehicle	+ASO
Internal Standard 13:0	6.89	N/A	N/A	N/A	N/A
Palmitic 16:0	12.50	14 ± 2	20 ± 5	51 ± 8 ^a	27 ± 3 ^{a,b}
Linoleic 18:2 (n-6)	15.60	14 ± 3	15 ± 3	213 ± 47 ^a	117 ± 20 ^a
Oleic 18:1 (n-9)	15.71	9 ± 1	9 ± 5	108 ± 26 ^a	45 ± 3 ^{a,b}
Stearic 18:0	16.17	12 ± 2	12 ± 1	38 ± 9 ^a	28 ± 6 ^a
Arachidonic 20:4 (n-6)	18.46	11 ± 3	8 ± 1	27 ± 5 ^a	18 ± 2 ^a
Docosohexacanoic 22:6 (n-3)	21.59	6 ± 1	10 ± 0.2	14 ± 3 ^a	11 ± 1 ^a

Figure 4. Effect of chronic ethanol on specific free fatty acid species

Representative gas-liquid chromatography profiles of fatty acid methyl esters are shown from liver extracts after direct transesterification. Labeled peaks by retention times (RT) have been identified to a known free fatty acid. Summary data is shown in tabular format for select species (lower panel). N/A refers to non-applicable. Data are means ± SEM (n=4–6) and are reported as fold of control values. ^a, p<0.05 compared to the absence of ethanol. ^b, p<0.05 compared to the absence of ASO as determined by 2-way ANOVA.

Figure 5. Effect of ethanol on expression of key genes

The effect of ethanol and ASO on the expression of fatty acid synthase (FAS; Panel A), tumor necrosis factor α (TNF α ; Panel B), and plasminogen activator inhibitor-1 (PAI-1; Panel C) was determined by real-time rtPCR. Data are means $\pm$ SEM (n=4–6) and are reported as fold of control values. ^a, p<0.05 compared to the absence of ethanol. ^b, p<0.05 compared to the absence of ASO as determined by 2-way ANOVA.

Figure 6. Effect of ethanol on fibrin deposition

Upper panels: representative confocal photomicrographs (400×) depicting immunofluorescent detection of hepatic fibrin are shown. Lower panel: quantitative image-analysis of fibrin accumulation are summarized. Data are means±SEM (n=4–6). ^a, p<0.05 compared to the absence of ethanol. ^b, p<0.05 compared to the absence of ASO as determined by 2-way ANOVA.