The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis

Zhaoyu Li,[1] Luis B. Agellon,[1] Theresa M. Allen,[2] Masato Umeda,[5] Larry Jewell,[3] Andrew Mason,[4] and Dennis E. Vance[1,*]

[1] Department of Biochemistry and Canadian Institutes of Health Research Group on the Molecular and Cell Biology of Lipids
[2] Department of Pharmacology
[3] Department of Laboratory Medicine and Pathology
[4] Department of Medicine
University of Alberta, Edmonton, Alberta T6G 2S2 Canada
[5] Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan
*Correspondence: dennis.vance@ualberta.ca

Summary

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are major phospholipids in mammalian membranes. In liver, PC is synthesized via the choline pathway or by methylation of PE via phosphatidylethanolamine *N*-methyltransferase (PEMT). *Pemt*$^{-/-}$ mice fed a choline-deficient (CD) diet develop rapid steatohepatitis leading to liver failure. Steatosis is observed in CD mice that lack both PEMT and multiple drug-resistant protein 2 (MDR2), required for PC secretion into bile. We demonstrate that liver failure in CD-*Pemt*$^{-/-}$ mice is due to loss of membrane integrity caused by a decreased PC/PE ratio. The CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice escape liver failure by maintaining a normal PC/PE ratio. Manipulation of PC/PE levels suggests that this ratio is a key regulator of cell membrane integrity and plays a role in the progression of steatosis into steatohepatitis. The results have clinical implications as patients with nonalcoholic steatohepatitis have a decreased ratio of PC to PE compared to control livers.

Introduction

The rapid rise in obesity has caused an epidemic of nonalcoholic fatty liver disease associated with insulin resistance and the metabolic syndrome. Little is known, however, concerning the requirements for progression of steatosis to steatohepatitis (Cortez-Pinto et al., 2006). This paper provides insight into how a change of phospholipid composition in the liver can lead to steatohepatitis and liver failure.

The reason membrane bilayers have a defined phospholipid composition is not clear. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are two major phospholipids that are asymmetrically distributed in the plasma membrane: the majority of PC is localized to the outer leaflet, whereas PE is enriched in the inner leaflet (Devaux, 1991). PC homeostasis in the liver is regulated by PC anabolism and catabolism, as well as by PC secretion and uptake (Figure 1A). Hepatic PC is made from choline via the CDP-choline pathway and also by PE *N*-methyltransferase (PEMT), which converts PE to PC via 3 methylation reactions (Vance, 2002). PC secretion into bile is mediated by a PC-specific flippase, multiple drug-resistant protein 2 (MDR2), in the hepatic canalicular membrane (Smit et al., 1993). Mice lacking PEMT develop steatohepatitis and hyperacute liver failure within 3 days of starting a choline-deficient (CD) diet (Walkey et al., 1998). However, mice lacking both MDR2 and PEMT are resistant to liver failure and steatohepatitis observed in the CD-*Pemt*$^{-/-}$ mice and survive for at least 3 months with steatosis when fed the CD diet (Li et al., 2005). *Mdr2*$^{-/-}$ mice showed intrahepatic cholestasis and liver damage at ~35 weeks after birth (Smit et al., 1993), whereas *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice did not for up to 15 weeks after birth.

We report that *Pemt*$^{-/-}$ mice experience liver failure when fed the CD diet because the ratio of PC to PE is decreased by >50%, which leads to loss of membrane integrity with ballooning hepatocytes, and the resultant cell damage recruits an inflammatory response typical of steatohepatitis. This mouse model appears relevant to human disease since we found that patients with nonalcoholic steatohepatitis (NASH) also have a diminished PC/PE ratio within the liver. These results have broad implications for management of patients with acute liver failure or chronic liver disease associated with steatohepatitis.

Results

Liver failure is associated with a decreased ratio of PC to PE

Rapid depletion of hepatic PC by 50% via biliary secretion was originally suggested as the cause of hyperacute liver failure in CD-*Pemt*$^{-/-}$ mice (Walkey et al., 1998). However, CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice also have an ~50% reduction in hepatic PC (Figure 1B) but do not develop liver failure. Triacylglycerols accumulate in livers of CD-*Pemt*$^{-/-}$ mice and to a greater extent in CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice (Table 1). Thus, liver damage is independent of the decrease in hepatic PC and fat accumulation.

An important difference between *Pemt*$^{-/-}$ mice and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice is the hepatic PE content during choline deprivation. In *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice, the CD diet decreased hepatic PC and PE to a similar extent (Figure 1C). In contrast, in *Pemt*$^{-/-}$ mice, the CD diet decreased hepatic PC by 50%, whereas PE remained unchanged (Figure 1B). Thus, the CD diet reduced the PC:PE ratio by 57% in *Pemt*$^{-/-}$ mice but by only 17% in

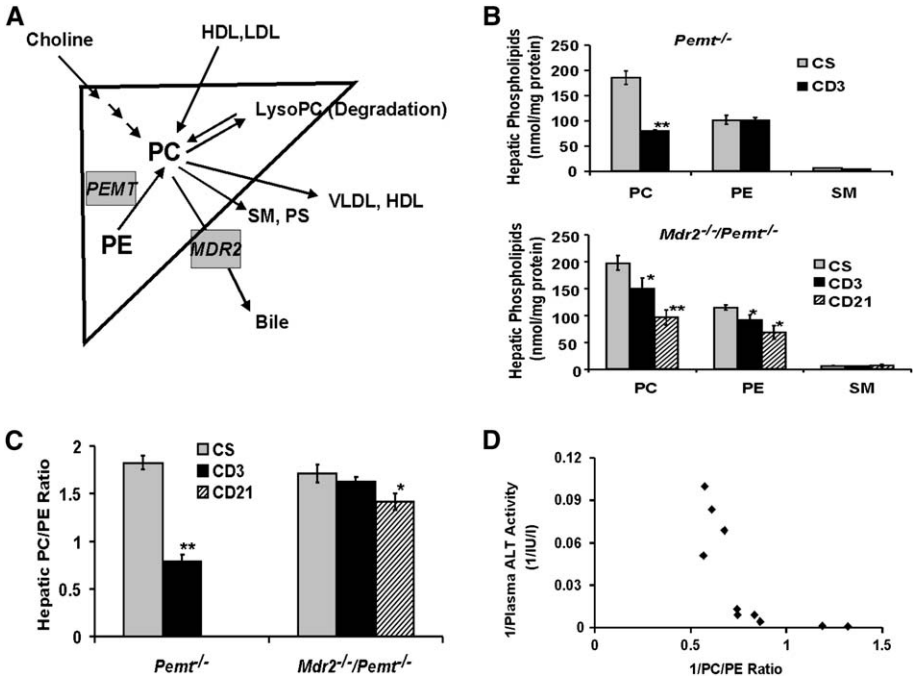

Figure 1. Hepatic failure is not associated with a decreased PC level but with a decreased PC/PE ratio

A) Phosphatidylcholine (PC) homeostasis in the liver. Four PC acquisition pathways are indicated. In the liver, 70% of PC is biosynthesized from choline via the CDP-choline pathway, whereas 30% of PC is derived from phosphatidylethanolamine (PE) via the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway. PC can also be derived from reacylation of lysoPC. High-density lipoproteins (HDL) and low-density lipoproteins (LDL) transport not only neutral lipids but also phospholipids (mainly PC) into the liver. PC provides choline for sphingomyelin (SM) synthesis and is a precursor of phosphatidylserine (PS). A major loss of hepatic PC occurs from biliary secretion mediated by multiple drug-resistant protein 2 (MDR2). In addition to the degradation of PC by phospholipases, hepatic PC can also be secreted as an important component of very low-density lipoproteins (VLDL) and HDL.

B) Levels of PC, PE, and sphingomyelin (SM) in liver homogenates obtained from *Pemt*$^{-/-}$ mice and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice fed a CS diet for 24 hr and then fed a choline-deficient (CD) diet for 3 (CD3) or 21 (CD21) days. Values are averages ± SD; *, $p < 0.05$; **, $p < 0.001$, comparing CS to CD groups.

C) Molar ratio of PC to PE in CS and CD mice. Values are averages ± SD; *, $p < 0.05$; **, $p < 0.001$.

D) Correlation between hepatic PC/PE ratio and plasma ALT activity. Each point represents averages of 6–8 mice.

Mdr2$^{-/-}$/*Pemt*$^{-/-}$ mice (Figure 1C). The decrease in PC/PE ratio in CD-*Pemt*$^{-/-}$ mice coincided with an increase in the plasma level of alanine aminotransferase (ALT) (Li et al., 2005). The strong correlation (r = 0.95) between the PC/PE ratio and plasma ALT activity (Figure 1D) suggests that liver damage in CD-*Pemt*$^{-/-}$ mice might be associated with a decreased PC/PE ratio. Hepatic cholesterol (Table 1) and sphingomyelin (SM) (Figure 1B), major plasma membrane lipids, did not change in either mouse model fed the CD diet. Moreover, no liver damage was observed in wild-type mice fed the CD diet for up to 21 days, and the hepatic PC/PE ratio was unaffected, although hepatic PC and PE levels both decreased by ~28% (data not shown).

One explanation for the induction of liver damage by the CD diet is that increased plasma bile acids might have been detrimental to plasma membrane integrity. However, plasma bile acid levels were not increased by the CD diet in either *Pemt*$^{-/-}$ or *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice (Table 1). Another explanation for the liver damage might be that alterations in phospholipid levels in the plasma membrane had reduced plasma membrane integrity. Plasma membranes were isolated from hepatocytes of CD-*Pemt*$^{-/-}$ and *CD-Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice. Specific activities of 5′-nucleotidase in plasma membrane fractions and total liver homogenates were 56.4 and 3.8 μmol/h/mg protein, respectively. Specific activities of the ER marker NADPH:cytochrome C reductase in plasma membrane fractions and total liver homogenates were 0.4 and 48.5 nmol/min/mg protein, respectively. Thus, there was significant purification of plasma membrane. The concentrations of PC, PE, and SM were determined (Figures 2A and 2B). The relative amount of SM compared to PC is also consistent with purified plasma membranes. A striking correlation (r = 0.97) was observed between the PC/PE ratio in plasma membranes and plasma ALT activity (Figure 2C). Neither the genotype nor the CD diet affected the plasma membrane level of SM (Figure 2A). Although the CD diet decreased the PC content of livers of *Pemt*$^{-/-}$ mice by ~50%, the amount of total lipids in the hepatocyte plasma membranes was not significantly affected (Table 1).

A change in membrane fluidity might cause variations in membrane integrity and functions of membrane proteins (Lang et al., 1998). We assessed the fluidity of hepatocyte plasma membranes using a fluorescent probe, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-tolunesulfonate and found that the CD diet reduced the polarization value from 0.23 to 0.17 ($P < 0.05$) of hepatocyte plasma membranes in the *Pemt*$^{-/-}$ but not in *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ *mice*, consistent with the decreased PC/PE ratio (Figure 2B).

Table 1. Factors potentially influencing membrane integrity

	Pemt$^{-/-}$, CS	*Pemt*$^{-/-}$, CD3	*Mdr2*$^{-/-}$/ *Pemt*$^{-/-}$, CS	*Mdr2*$^{-/-}$/ *Pemt*$^{-/-}$, CD21
Hepatic TG (μg/mg protein)	105.3 ± 41.9	578.9 ± 122.5*	17.3 ± 9.2	962.2 ± 171.7*
Hepatic Ch (nmol/mg protein)	14.9 ± 0.9	15.4 ± 2.9	14.2 ± 2.3	17.2 ± 2.2
Plasma Memb. Ch (nmol/mg protein)	158.7 ± 14.2	173.3 ± 1.3	146.4 ± 15.3	177.5 ± 12.6
Plasma Memb. Total Lipids (PL + Ch) (nmol/mg protein)	431.3 ± 23.7	431.2 ± 28.3	413.7 ± 17.3	380.3 ± 9.6
Plasma Bile Acids (μmol/l)	10.0 ± 7.4	16.1 ± 6.9	17.9 ± 8.1	13.8 ± 3.8

Plasma bile acids were analyzed. Neutral lipids in liver homogenates and plasma membrane were extracted and analyzed by gas-liquid chromatography. TG, triacylglycerol; Ch, cholesterol; PL, phospholipid; Memb., membrane; CS, choline-supplemented; CD, choline-deficient for 3 or 21 days. *, $p < 0.05$; Data = mean for 6–8 mice ± SD.

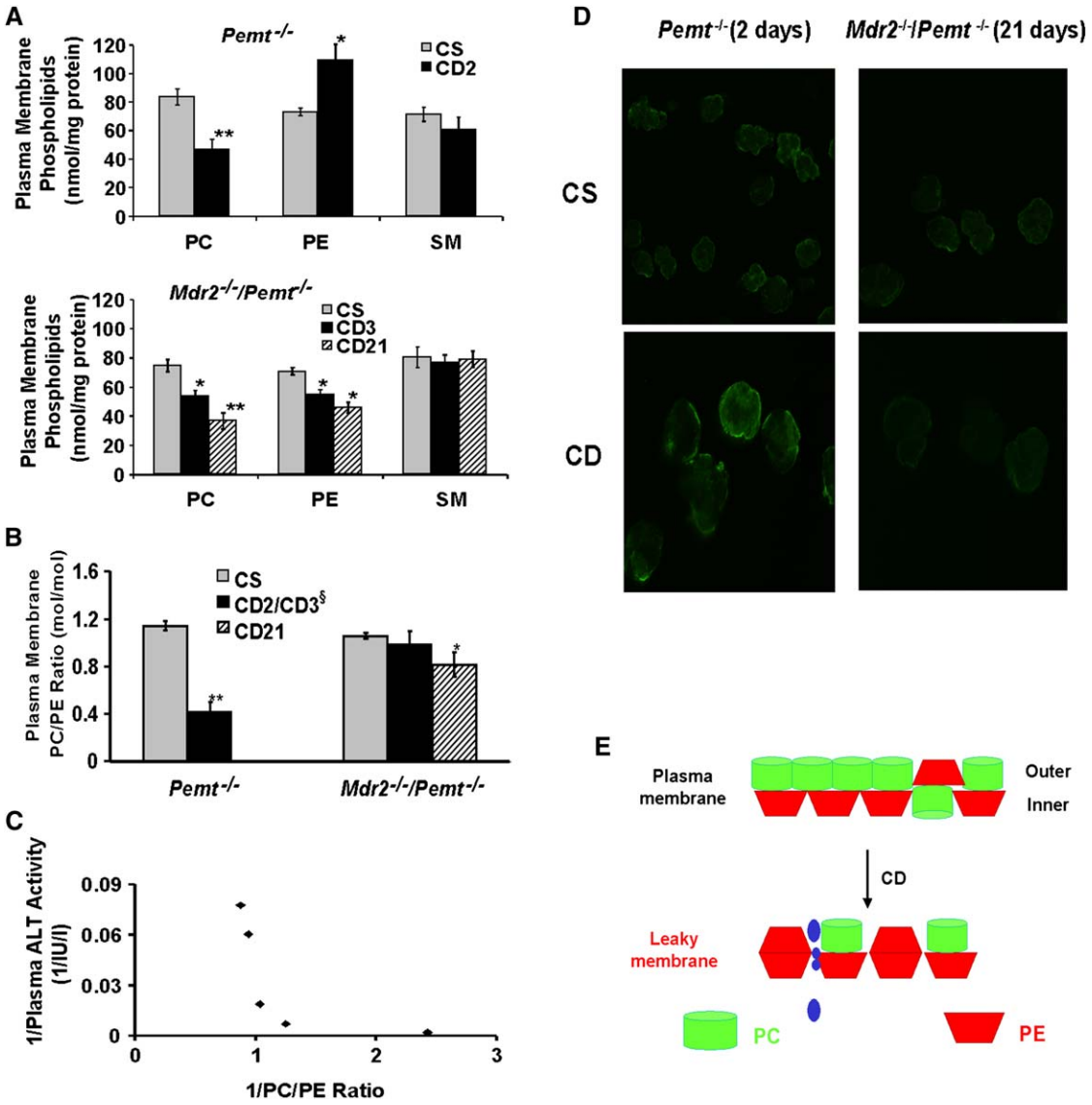

Figure 2. Dependence of plasma membrane integrity on PC/PE ratio of hepatocytes

A) Phospholipid content of hepatocyte plasma membranes from *Pemt*$^{-/-}$ mice and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice fed the CS diet for 24 hr and then fed the choline-deficient (CD) diet for 2, 3, or 21 (CD2, CD3, or CD21) days. Data are averages ± SD from 3 mice in each group. *, $p < 0.05$; **, $p < 0.01$, for CS versus CD mice.

B) The PC/PE ratio in hepatocyte plasma membranes. §, *Pemt*$^{-/-}$ mice fed the CD diet for 2 days and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice fed the CD diet for 3 days. Data are averages ± SD from 3 mice in each group. *, $p < 0.05$; **, $p < 0.01$, for CS versus CD mice.

C) Correlation between plasma membrane PC/PE ratio and plasma ALT activity. Each point represents averages of 6–8 mice.

D) PE exposure on the cell surface. Hepatocytes from 3 *Pemt*$^{-/-}$ mice and 3 *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice fed the CS or CD diet were incubated with biotinylated PE-specific binding peptide, Ro98-019, at 4ºC for 30 min and subsequently with FITC-conjugated streptavidin for 30 min and then viewed with a fluorescence microscope.

E) Hypothetical mechanism for liver failure in CD-*Pemt*$^{-/-}$ mice. PCs form cylindrical structures and PEs form conical structures. If the proportion of these two lipids is changed significantly with an increase in conical structures, the membrane might leak.

Since the PC/PE ratio is strikingly lower in CD-*Pemt*$^{-/-}$ mice than in CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice, we determined if more PE were exposed on the cell surface of CD-*Pemt*$^{-/-}$ hepatocytes. Figure 2D shows a 3-fold increase in relative fluorescent intensity of PE in hepatocytes from *Pemt*$^{-/-}$ mice fed the CD diet for 2 days ($P < 0.05$), but no change in *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice fed the CD diet for 3 (data not shown) or 21 days.

These data suggest a strong link between liver damage and the PC/PE ratio in the plasma membrane. We hypothesize that changes in membrane structure induced by increased PE exposure on the cell surface caused a loss of membrane integrity (Figure 2E). An increased content of the cone-shaped, nonbilayer-forming lipid, PE, in the outer monolayer of the plasma membrane may result in membranes that are not as closely packed as those formed by PC, which has a cylindrical shape (Dowhan and Bogdanov, 2002). The maintenance of a normal PC/PE ratio of ~1.5 to 1.8 in the livers of CD-*Mdr2*$^{-/-}$ /*Pemt*$^{-/-}$ would favor retention of membrane integrity, whereas a ratio of <1.0, as found in CD-*Pemt*$^{-/-}$ mice, would result in disruption of membrane integrity.

Mechanism for maintenance of the PC/PE ratio in CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice

We investigated the mechanism for the decline in PE when the *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice were fed the CD diet. The rate of [^{3}H]glycerol incorporation into PE was ~1.8-fold higher in hepatocytes from CD- compared to CS-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice. In contrast, the rate of catabolism of [^{3}H]PE was enhanced ~3-fold by the CD diet (Figure 3A). Thus, the decreased content of PE in CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice likely resulted from an imbalance between PE biosynthesis and degradation.

When mice of both genotypes were fed the CD diet, the levels of hepatic PC decreased. Hepatic PC is secreted into bile, into lipoproteins, and is metabolized intracellularly (Figure 1A). We attributed the rapid decline in hepatic PC in CD-*Pemt*$^{-/-}$ mice to PC secretion into bile (Li et al., 2005). However, since no PC is secreted into bile by *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice, we investigated the mechanism for the decrease of PC upon feeding the *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice the CD diet. The amount of hepatic PC derived from HDL is similar to the amount of PC secreted in VLDL (Jeong et al., 1998). The rate of VLDL-PC secretion is decreased by choline deprivation in hepatocytes isolated from both mouse models, but the rate of HDL-PC uptake is not changed by the CD diet (Figure 3B). Thus, choline deprivation does not enhance PC loss from hepatocytes via lipoprotein metabolism.

Another possibility for a decreased PC/PE ratio in *Pemt*$^{-/-}$ mice is that PE biosynthesis was not decreased in response to choline deprivation, whereas it might be in *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice. PE is made in mammalian cells by the CDP-ethanolamine pathway and by the decarboxylation of phosphatidylserine (Vance, 2002). The activity of CTP:phosphoethanolamine cytidylyltransferase (ET), the rate-limiting enzyme in the CDP-ethanolamine pathway, was the same in *Pemt*$^{-/-}$ and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ hepatocytes (3 nmol/min/mg protein) and was unaffected by the CD diet. Thus, the rate of PE biosynthesis from the CDP-ethanolamine pathway appears to be independent of the genotype and dietary choline status of the mice.

A likely mechanism for the decrease in PC content in hepatocytes from CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice is increased PC catabolism. We previously found that phospholipase A_2 (PLA_2) activity and PC degradation are increased by choline deprivation of *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice, but not *Pemt*$^{-/-}$ mice (Li et al., 2005). We now show that the activities of both calcium-dependent PLA_2 ($cPLA_2$) and calcium-independent PLA_2 ($iPLA_2$) are enhanced by choline deprivation in *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ but not in *Pemt*$^{-/-}$ hepatocytes (Figure 3C). Indeed, there is a striking decrease in $cPLA_2$ in *Pemt*$^{-/-}$ hepatocytes. The increase in PLA_2 activity in the livers of CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice was diminished by the PLA_2 inhibitors, BEL (an iPLA2-specific inhibitor) and ATK (an inhibitor of both $iPLA_2$ and $cPLA_2$) (data not shown), confirming that the activity measured was PLA_2. Therefore, rapid depletion of hepatic PC in CD-*Pemt*$^{-/-}$ mice resulted from continued secretion of PC into bile (Li et al., 2005), whereas the loss of hepatic PC in CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice can be attributed to increased PC catabolism. Hence, different regulatory mechanisms for depletion of PC from the liver alter PE metabolism in *Pemt*$^{-/-}$ mice and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ *mice*. As a result, *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice maintain the PC/PE ratio and membrane integrity in hepatocyte plasma membranes, escape liver failure, and adapt to choline deprivation by balancing the catabolism of PC and PE.

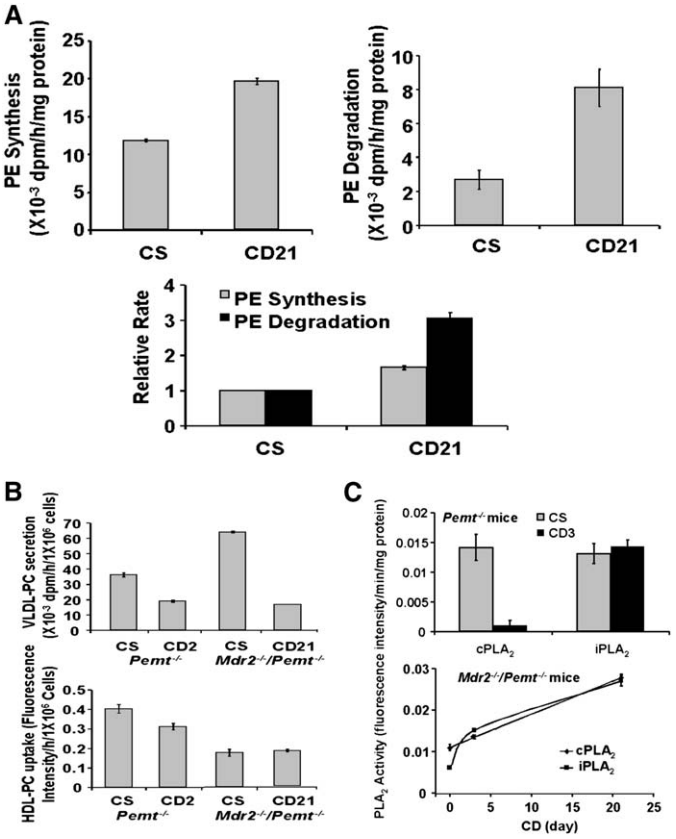

Figure 3. The mechanism by which a normal PC/PE ratio is maintained in hepatocytes from CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice

A) The rates of PE synthesis and degradation were estimated from pulse-chase labeling with [^{3}H]glycerol of primary cultured hepatocytes from 3 *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice fed the CS diet for 24 hr and then fed the choline-deficient (CD) diet for 21 days (CD21). Relative rates are based on comparisons between CS and CD mice. Values are averages ± SD.

B) VLDL-PC secretion and HDL-PC uptake by hepatocytes. *Pemt*$^{-/-}$ and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice were fed the CS diet for 24 hr and then fed the choline-deficient (CD) diet for 2 (*Pemt*$^{-/-}$) or 21 (*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$) days. VLDL-PC secretion: hepatocytes were labeled with [^{3}H]choline for 2 hr and then the radiolabel was chased for 2 hr. Medium was collected and [^{3}H]PC was isolated from VLDL. HDL-PC uptake: hepatocytes were incubated for 2 hr with mouse plasma HDL that had been incubated with BODIPY-PC (fluorescent PC). HDL-PC uptake was determined by measurement of intracellular fluorescence intensity. The values are averages from 3 preparations of hepatocytes.

C) In vitro assay of calcium-dependent PLA_2 ($cPLA_2$) and calcium-independent PLA_2 ($iPLA_2$) activities in liver homogenates from *Pemt*$^{-/-}$ and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice fed the CD diet, using as a substrate PED6 [(*N*-(6-(2,4-dinitrophenyl) amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)]-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine.The values are averages from 6–8 mice ± SD, $p < 0.05$.

The ratio of PC to PE is a key regulator of membrane integrity

To determine if a decreased PC/PE ratio leads to loss of membrane integrity, we prepared large unilamellar vesicles with different PC/PE ratios. A fluorescent molecule, calcein, is quenched when entrapped in vesicles. Fluorescence is observed upon calcein leakage from the vesicles. A decreased PC/PE ratio enhanced the release of calcein from the vesicles (Figure 4A), indicating that the low PC/PE ratio in CD-*Pemt*$^{-/-}$ hepatocytes might be responsible for inducing membrane permeability.

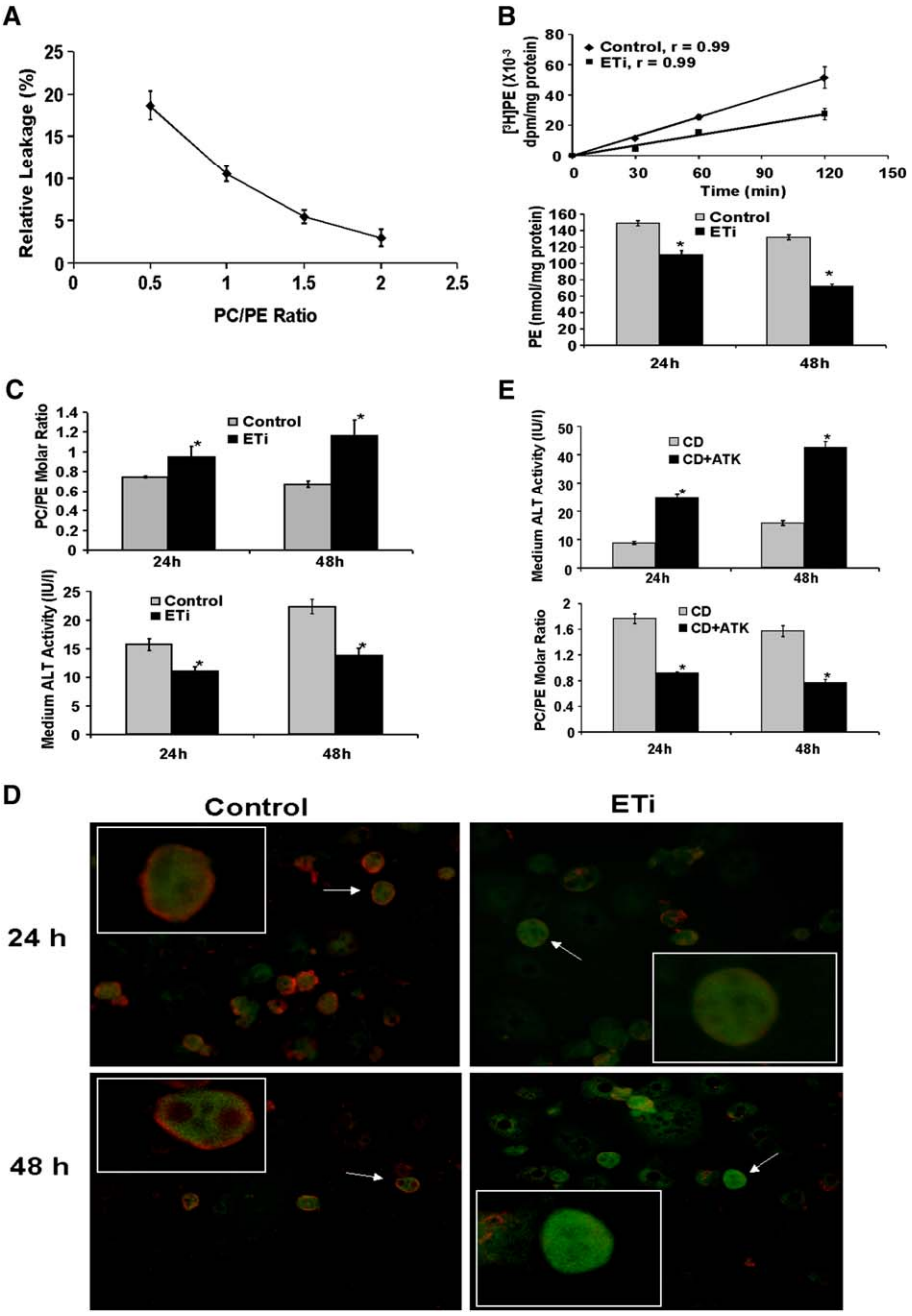

Figure 4. The PC/PE ratio influences membrane integrity of large unilamellar vesicles and hepatocytes

A) Permeability assays of large unilamellar vesicles containing different PC/PE ratios. Vesicles (100 nm in diameter) were prepared with PC/PE ratios of 0.5 to 2.0. The ratio among the content of hepatic (PC + PE), sphingomyelin, and cholesterol was 86:1:9. Leakage was measured by the release of entrapped calcein and normalized to the amount of calcein released by TritonX-100. Three independent experiments were performed. Data are averages ± SD.

B) Attenuation of PE biosynthesis by RNA interference targeted to CTP: phosphoethanolamine cytidylyltransferase (ET). Hepatocytes from CD-*Pemt*$^{-/-}$ mice were transfected with ETi or control vectors and were cotransfected with green fluorescent protein vectors (ratio, 4:1) for 24 or 48 hr. (Lower panel) PE levels in hepatocytes. Data are averages ± SD from 3 experiments. *, $p < 0.05$. (Upper panel) PE biosynthesis in hepatocytes. Hepatocytes from CD-*Pemt*$^{-/-}$ mice were labeled with [^{3}H]ethanolamine after transfection with ETi for 24 hr and incorporation of radiolabel into PE was measured. Data are averages ± SD from 3 experiments.

C) The PC/PE ratio and cell damage in hepatocytes treated with interfering RNA targeted to CTP: phosphoethanolamine cytidylyltransferase (ETi). ETi vector was cotransfected with green fluorescent protein vector (ratio, 4:1) for 24 or 48 hr into hepatocytes isolated from *Pemt*$^{-/-}$ mice fed the CD diet for 2 days. The medium was replaced with fresh medium for an additional 4 hr. The hepatocytes were harvested and the amounts of PC and PE determined. ALT activity in the medium was measured as an indicator of cell damage. Data are averages ± SD from 3 experiments. *, $p < 0.05$.

D) PE exposure on the surface of hepatocytes treated with ETi. After transfection for 24 or 48 hr, hepatocytes from CD-*Pemt*$^{-/-}$ mice were incubated with biotinylated PE-specific binding peptide, Ro98-019, at 4°C for 30 min and subsequently with Texas Red-conjugated streptavidin for 30 min. Cells were examined by fluorescence microscopy. Green fluorescence protein (green); PE binding fluorescence (red). Data are from one experiment, representative of three similar experiments.

E) Inhibition of PE catabolism-induced cell damage in CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mouse hepatocytes. Arachidonyl trifluoromethyl ketone (ATK), a PLA_2-specific inhibitor, was added to CD medium (CD+ATK) for 24 hr and 48 hr. The medium was replaced with fresh medium every 2 hr during the first 8 hr to remove secreted PC. After 4 hr incubation with fresh medium, the cells and medium were harvested. (Upper panel) ALT activity in the medium was measured as an indicator of cell damage. (Lower panel) PC and PE in the hepatocytes were measured and the ratio calculated. Data are averages from 3 independent experiments. *, $p < 0.01$.

We next investigated if hepatocyte damage could be reduced in CD-*Pemt*$^{-/-}$ mouse hepatocytes when PE synthesis was attenuated. We used RNA interference (RNAi) with a vector targeted to ET (ETi) to inhibit PE biosynthesis. Hepatocytes were isolated from *Pemt*$^{-/-}$ mice fed the CD diet for 2 days. Four hours after plating, the hepatocytes were transfected with a vector encoding green fluorescent protein and either the control vector or the ETi vector for 24 hr or 48 hr. ETi effectively decreased ET mRNA (by 71% after 24 hr and by 75% after 48 hr) and ET activity (by 55% after 24 hr and by 37% after 48 hr (data not shown), and reduced the incorporation of [^{3}H]ethanolamine into PE by 46% after transfection with control or ETi vectors (Figure 4B). The concentration of PE in hepatocytes from CD-*Pemt*$^{-/-}$ mice was also reduced after transfection with ETi by 25% after 24 hr and by 45% after 48 hr, $P < 0.05$ (Figure 4B) with a corresponding increase in the PC/PE ratio after 24 hr and 48 hr (Figure 4C). The increased PC/PE ratio correlated with both a decreased exposure of PE on the hepatocyte surface (Figure 4D) and a decreased leakage of ALT into the medium (Figure 4C). Thus, in hepatocytes from CD-*Pemt*$^{-/-}$ mice,

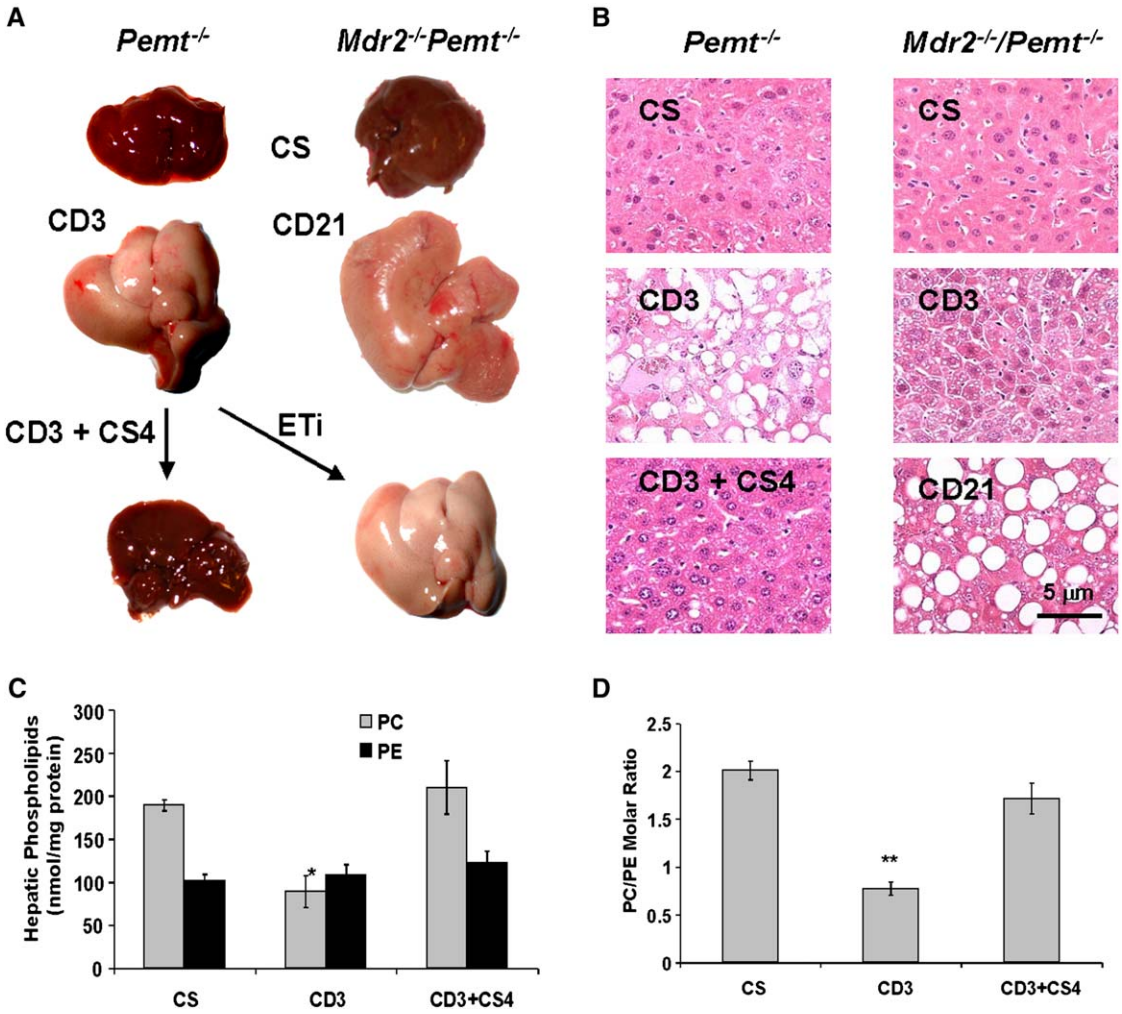

Figure 5. Choline supplementation reverses steatosis and steatohepatitis induced in CD-*Pemt*$^{-/-}$ mice

Pemt$^{-/-}$ mice and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice were fed the CS diet for 24 hr and then fed the choline-deficient (CD) diet for 3 (CD3) or 21 (CD21) days. Some CD3-*Pemt*$^{-/-}$ mice were subsequently fed the CS diet for 4 days (CD3 + CS4).

A) Livers from *Pemt*$^{-/-}$ mice and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice.

B) Liver histology as indicated by hemotoxylin and eosin staining.

C and D) Hepatic PC and PE levels and PC/PE molar ratios. Values are averages ± SD; *, $p < 0.05$; **, $p < 0.01$, for CS versus CD mice, 3–8 mice in each group.

inhibition of PE biosynthesis increased the PC/PE ratio and increased membrane integrity.

In an alternative approach, we increased the PE content of hepatocytes from CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice. An inhibitor of PLA_2 (arachidonyl trifluoromethyl ketone) was added to the medium to block PC and PE degradation. Hepatocytes were cultured in CD medium and secreted PC removed at various times. Figure 4E shows that the PLA_2 inhibitor lowered the PC/PE ratio and stimulated ALT release during 24 and 48 hr. Thus, attenuation of PE catabolism in CD-*Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ hepatocytes decreases the PC/PE ratio and increases hepatocyte damage.

The PC/PE ratio regulates the evolution of steatosis into steatohepatitis

Multiple "hit" models have been proposed for the pathogenesis of nonalcoholic fatty liver disease, but no distinct mechanism has been shown to promote the change from the relatively benign disorder, steatosis, into progressive liver disease associated with steatohepatitis (Koteish and Diehl, 2001; Koteish and Mae Diehl, 2002; Cortez-Pinto et al., 2006). The modulation of PC/PE ratio is a potential candidate for this process since the diminished ratio differentiates steatosis from steatohepatitis and liver injury. Patients with NASH disease are evaluated histologically by grading the extent of hepatocyte ballooning, steatosis, and lobular inflammation (Brunt, 2001; 2004); the modified Brunt criteria were used to grade the steatohepatitis in *Pemt*$^{-/-}$ mice.

Pemt$^{-/-}$ mice fed the CD diet died of hyperacute liver failure with panlobular 3+ macrovesicular steatosis, 2+ hepatocyte ballooning, Mallories hyaline and 3+ panacinar inflammation (Figures 5A and 5B) (Walkey et al., 1998). In contrast, *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice developed 2+ microvesicular steatosis in zone 3 after being fed the CD diet for 3 days, and 3+ macrovesicular steatosis in all zones without lobular inflammation (0) or hepatocyte ballooning (0) by 21 days (Figures 5A and 5B). Thus, the decreased PC/PE ratio (Figure 1C) correlated with grade 2 and grade 3 steatohepatitis (NASH), but not grade 1 steatosis.

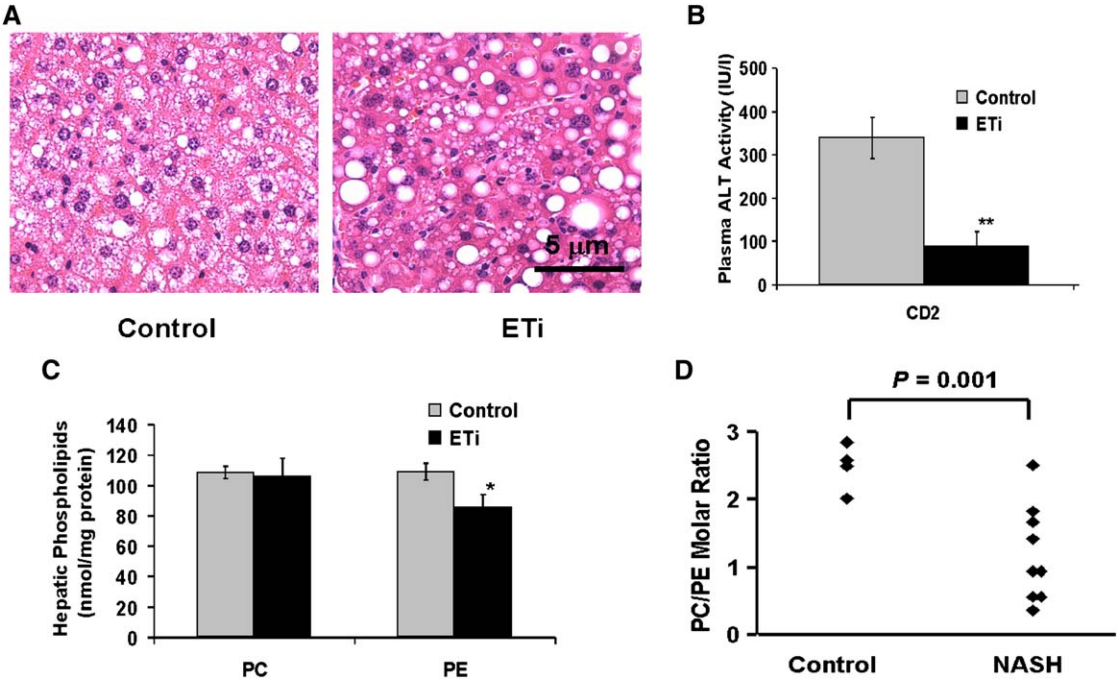

Figure 6. An increased PC/PE ratio reverses steatohepatitis, but not steatosis, induced by choline deficiency in *Pemt*$^{-/-}$ mice

Saline (control) or ETi vectors (ETi) (50 mg/kg), were injected three times into tail veins of *Pemt*$^{-/-}$ mice over a 24 hr period. The mice were fed the CD diet for 2 days. Data for each vector are from 3 mice and are the averages ± SD.

A) Histological staining of livers by hemotoxylin and eosin.

B) Hepatic damage as assessed by plasma ALT activity. Values are averages ± SD; **, $p < 0.01$.

C) Hepatic PC and PE levels; Values are averages ± SD; *, $p < 0.05$.

D) Decreased hepatic PC/PE ratios in NASH patients. Liver biopsy samples were collected from four healthy persons (control) and nine NASH patients, and PC and PE were quantified. p = 0.001.

A normal PC/PE ratio was maintained in wild-type mice fed the CD diet for 21 days, where steatosis developed without inflammation or ballooning degeneration (data not shown).

In *Pemt*$^{-/-}$ mice, a CS diet reversed the liver damage caused by choline deficiency (Waite et al., 2002). The steatohepatitis in CD-*Pemt*$^{-/-}$ mice was markedly attenuated and the PC/PE ratio returned to normal when CD-*Pemt*$^{-/-}$ mice were fed choline (Figures 5C and 5D). Moreover, normalization of the PC level (Figure 5C) coincided with reversal of panlobular macrovesicular fat deposition to normal as indicated by histology (Figures 5A and 5B), and normalizaton of hepatic triacylglycerol levels and plasma ALT activity (data not shown).

Figure 4 shows that transfection with ETi increases the PC/PE ratio and attenuates cell damage in CD-*Pemt*$^{-/-}$ hepatocytes by decreasing the amount of PE. To determine if a similar result were observed in mice, we injected the ETi vector via the tail-vein into CD-*Pemt*$^{-/-}$ mice using a hydrodynamic protocol that results in efficient delivery of the vector to the liver (Kobayashi et al., 2004; Song et al., 2002). Mice were injected 3 times over a 24 hr period and then fed the CD diet for 2 days prior to sacrifice. The ETi prevented steatohepatitis but not steatosis as indicated by Figures 5A, 6A, and 6B and caused no change in the level of triacylglycerol (~500 μg/mg hepatic protein). The PC/PE ratio in the liver was increased by the ETi injections but the PC level did not change (Figure 6C), consistent with the idea that fat accumulation in the liver depends on hepatic PC levels. These data suggest that an increased PC/PE ratio in vivo can reverse steatohepatitis, but not steatosis. However, an increased amount of hepatic PC can attenuate steatosis.

The potential relevance of these studies to human disease is indicated in a pilot study of NASH patients. We found that hepatic PC/PE ratios in NASH patients were significantly lower than those in healthy human subjects (P = 0.001) (Figure 6D).

Discussion

Our results suggest that the PC/PE ratio is a critical modulator of membrane integrity. A gradual decrease in hepatic PC in wild-type and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice upon choline deprivation provides sufficient time for PC and PE metabolism to be modified so that a normal PC/PE ratio is maintained and liver failure is prevented. On the other hand, in *Pemt*$^{-/-}$ mice, the CD diet causes a decline in hepatic PC that is too rapid for metabolic adjustments to be made. Consequently, the PC/PE ratio decreases and liver damage occurs. Support for our hypothesis comes from studies in which choline deprivation similarly decreased the amount of PC, but not PE, in PC12 cells, resulting in cell death (Yen et al., 1999). Moreover, an increase in PE, but not PC, damaged heart myocytes as indicated by leakage of the cytosolic enzyme lactate dehydrogenase (Post et al., 1995). It would, therefore, be intriguing to know if variations in the PC/PE ratio affect membrane integrity in other cell types.

A current model of NASH is to feed rodents a methionine- and choline-deficient (MCD) diet (Koteish and Mae Diehl, 2002; Weltman et al., 1996). Although no previous reports have noted a correlation between the PC/PE ratio and steatohepatitis, livers from these animals had a decreased content of PC levels but no change or a minor increase in the amount of PE (Beare-Rogers,

1971; Lyman et al., 1973; Yost et al., 1985). Therefore, the PC/PE ratio was decreased. Livers from MCD murine models have a reduced rate of PC and choline biosynthesis and mimic CD-*Pemt*$^{-/-}$ mice. Since *S*-adenosylmethionine is derived from methionine and is a substrate of PEMT, dietary deprivation of methionine attenuates the PEMT pathway. Although liver failure and steatohepatitis occurred within 3 days in CD-*Pemt*$^{-/-}$ mice, a similar degree of steatohepatitis did not develop until ~3 months in MCD mice (Koteish and Mae Diehl, 2002; Weltman et al., 1996), probably because the PEMT pathway was incompletely blocked. Moreover, methionine can be made de novo from betaine or methyltetrahydrofolate (Finkelstein and Martin, 1986). Therefore, the PC/PE ratio was decreased gradually over 3 months in MCD-wild-type mice as compared with acutely (over 3 days) in CD-*Pemt*$^{-/-}$ mice (Beare-Rogers, 1971; Lyman et al., 1973; Yost et al., 1985).

The current data certainly support the hypothesis that a decreased ratio of PC/PE is important for the evolution of steatosis into steatohepatitis. A decreased PC/PE ratio adversely affects membrane integrity, resulting in liver damage. We cannot be sure if the change in the PC/PE ratio initiates or is the end point of inflammation. Since we were able to reverse the decrease in PC/PE ratio and thereby attenuate liver damage, we speculate that this ratio is fundamentally important in the disease process. A decreased PC/PE ratio correlates with a small decrease in membrane potential of hepatocyte plasma membranes (data not shown). A change of membrane potential was suggested to initiate inflammation since the flow of ions (such as calcium) and ion radicals varies during changes in membrane potential (Curry, 1992; Kmiec, 2001). A loss of membrane integrity not only leads to the release of cellular contents, but also increases the influx of extracellular components including cytokines (Curry, 1992; Kmiec, 2001). In addition, C-reactive protein, an acute inflammatory response protein, binds more avidly to cell membranes in which the PC/PE ratio is decreased (Mold, 1989). Therefore, a decreased PC/PE ratio might initiate inflammation.

The factors that promote hepatocyte damage and inflammation in the progression of steatosis to steatohepatitis have yet to be resolved in humans. Our initial results with human subjects indicate that the PC/PE ratio is altered in many patients with NASH. It will be important to determine if the PC/PE ratio is also decreased in patients with other chronic liver disorders, such as alcoholic liver disease as well as those presenting with fatty liver and acute liver failure associated with pregnancy and drug toxicity. The mouse models used in our research suggest mechanisms that determine what regulates the transition from steatosis to steatohepatitis in humans. Our results not only provide a clue to the mechanisms involved in the development of steatohepatitis but also suggest that boosting hepatic PC levels and/or reducing the hepatic PE level may provide novel avenues for the investigation and management of steatohepatitis.

Experimental procedures

Animals

Mdr2$^{-/-}$/*Pemt*$^{-/-}$ mice were produced by breeding *Pemt*$^{-/-}$ mice (C57BL/6; 129/J background) (Walkey et al., 1997) with *Mdr2*$^{-/-}$mice (FVB; 129/J background) (Smit et al., 1993). The mice were fed a CD diet (*ICN,* Cat#0290138710) or a CS diet, the CD diet supplemented with 0.4% (w/w) choline chloride. At the age of 10–12 weeks, *Pemt*$^{-/-}$ and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice were fed the CS diet for 24 hr then fed the CD diet for 3, 21, or 90 days. *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice did not develop intrahepatic cholestasis nor show any liver damage in the first 5 months after birth, which covered the period of this experiment (10–12 weeks of age + 3 weeks treatment). In choline refeeding experiments, after being fed the CD diet for 3 days, *Pemt*$^{-/-}$ mice were fed the CS diet for an additional 4 days. In experiments in which ET was suppressed by RNAi (ETi), chow diet-fed *Pemt*$^{-/-}$ mice were hydrodynamically injected via the tail with 1 ml saline (control) or ETi vectors (50mg/kg) at 0, 8, and 24 hr. All mice were fasted for 12 hr before sacrifice. Three to eight mice were used for each time point in each experiment with assays in duplicate. All data are means ± SD.

Sample collections

Livers were frozen in liquid N_2 after dissection. Blood was collected by cardiac puncture with instruments pretreated with EDTA. Plasma was separated by centrifugation at 2000 rpm for 20 min in a refrigerated bench-top centrifuge. Bile was collected from the intact gall bladders. All samples were stored at −70ºC before use.

Analysis of phospholipid composition in human livers

Nine liver samples from NASH patients (age 41 ± 12) were used. Controls were four healthy individuals (age 50 ± 13). The pathological diagnosis of NASH after liver biopsy was in accordance with Kleiner et al. (2005). In all subjects, the diagnosis of NASH was made following the exclusion of causes of chronic hepatitis including hepatitis B, hepatitis C, α-1 antitrypsin deficiency, autoimmune hepatitis, Wilson's disease, drug toxicity, total parenteral nutrition, and chronic alcohol intake. Phospholipids in the liver homogenates were measured as indicated below. The protocol was approved by the Health Research Ethics Board of the University of Alberta.

Genotyping

As PCR templates, genomic DNA from mice tails was extracted with a DNeasy Tissue Kit (Qiagen). PCR was used to distinguish between wild-type and targeted alleles containing a neomycin resistance gene (*neo*). Primer sequences: for the *Pemt* gene were, forward primer (F): 5′-GAGCGC AATGGTACTCACCACATTCC, reverse primer (R): 5′- GATCTTGTCTTCAGA GCCACAG; for the *Pemt* knockout allele, F: 5′- GAGCGCAATGGTACTCACC ACATTCC, R: 5′-CTCGACGTTGTCGAAG; for the *Mdr2* allele, F: 5′-GCTG AGATGGATCTTGAG, R: 5′-GTCGAGTAGCCAGATGATGG; for the *Mdr2* knockout allele, F: 5′-CGGCGAGGATCTCGTCGTGACCCA, R: 5′-GCGAT ACCGTAAAGCACGAGGAAG.

Preparation of primary hepatocytes

Primary hepatocytes were isolated (Noga et al., 2002) from *Pemt*$^{-/-}$ and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice fed the CS diet for 24 hr and then fed the choline-deficient (CD). *Pemt*$^{-/-}$ mice were fed the CD diet for 2 days and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice were fed the CD diet for 3 or 21 days. Hepatocytes were cultured in either Dulbecco's modified Eagle's medium (DMEM) containing 4 mg/l choline (CS medium) or CD medium (the same medium without choline). Ethanolamine (4 mg/l) was added to both CS and CD medium. Three preparations of hepatocytes were used in each experiment.

Lipid analyses

Livers were homogenized with a Polytron in 5 vol 10 mM Tris-HCl, (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1:100 protease inhibitor cocktail (Sigma, Cat#P8340). Homogenates were centrifuged for 5 min at 600 × g and supernatants collected. Protein was quantified (Bradford, 1976) and total lipids were extracted from liver homogenates (Bligh and Dyer, 1959). Phospholipids were separated by high-performance liquid chromatography and quantified with an electron-light scattering detector (McCluer et al., 1986). Phosphatidyldimethylethanolamine was used as an internal standard for quantification. Neutral lipids, including triacylglycerols, cholesterol and cholesteryl esters, were measured by gas-liquid chromatography (Lohninger et al., 1990).

Enzyme assays

Plasma aspartate and alanine aminotransferases (AST/ALT) were assayed with a GPT/GOT Kit (Sigma, Cat#P505). ET activity was measured in total cell homogenates (Tijburg et al., 1992). The substrate for the ET assay, [^{3}H]phosphoethanolamine, was made from [^{3}H]ethanolamine by phosphorylation with choline/ethanolamine kinase. Phospholipase A_2 (PLA_2) activities

were measured in liver homogenates and primary hepatocytes with PED6 [(*N*-(6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)]-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Molecular Probes), a self-quenching fluorescent analog of PE and a PLA_2-specific substrate (Farber et al., 2001). The specificity of PLA_2 activity was further confirmed by in vitro assay in the presence of 50 μmol/l PLA_2 inhibitors, BEL (an iPLA2-specific inhibitor) (Hazen et al., 1991) or ATK (an inhibitor of both $iPLA_2$ and $cPLA_2$) (Street et al., 1993).

Preparation of hepatocyte plasma membranes and assessment of purity

Hepatocytes were incubated with colloidal silica and plasma membranes were isolated by differential centrifugation (Chaney and Jacobson, 1983; Garver et al., 2002). Purity of plasma membrane fractions was assessed by enrichment of the plasma membrane marker (5′-nucleotidase). The degree of contamination by endoplasmic reticulum membranes was assessed by determination of NADPH:cytochrome C reductase activity (Croze and Morre, 1984).

Assay of fluidity and potential of plasma membrane

Membrane fluidity in the plasma membrane of hepatocytes was assessed by incubation of hepatocytes with 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-tolunesulfonate (Molecular Probes) at 4°C for 10 min and subsequently analyzed with a fluorimeter within 1 min. The fluorescence polarization value was calculated with the formula of p = (F parallel − F perpendicular) / (F parallel + F perpendicular) (Ferretti et al., 1993; McKinley and Hazel, 2000; Yamada et al., 2001). Membrane potential was assayed by incubation of hepatocytes with bis-(1,3-dibutylbarbituric acid)trimethine oxonol (3) at 4°C for 30 min. Fluorescence was analyzed with a fluorimeter within 1 min and fluorescence intensity per cell was determined (Ferretti et al., 1993; Yamada et al., 2001).

Detection of PE on the cell surface of hepatocytes

Primary cultured hepatocytes were incubated with the biotinylated PE-specific binding peptide, Ro98-019, at 4°C for 30 min and subsequently with FITC-conjugated streptavidin for 30 min. Fluorescence was visualized by fluorescence microscopy (Emoto et al., 1997). Relative fluorescent intensity was measured by flow cytometry.

Measurement of PE synthesis and degradation

Primary hepatocytes from *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice were incubated with [^{3}H]glycerol for 2 hr and then radioactivity was chased for 2 hr. The rates of PE synthesis and degradation were calculated as described previously (Ko et al., 1986). PE biosynthetic rate = (dpm in PE at end of the pulse/mg protein) / (2 hr × nmol PE/mg protein at the beginning of the pulse). PE degradation rate = (decrease in dpm in PE between the end of the pulse and 2 hr of chase/mg protein) / (2 hr × nmole of PE/mg protein). The relative rates are compared between CS and CD.

VLDL-PC secretion and HDL-PC uptake

VLDL-PC secretion assay: CS and CD hepatocytes from *Pemt*$^{-/-}$ and *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice were labeled with [^{3}H]choline for 2 hr and then radioactivity was chased for 2 hr. Cell culture medium was harvested and VLDL were isolated by sucrose gradient centrifugation (Chung et al., 1980). Total lipids were extracted from VLDL (Bligh and Dyer, 1959), and [^{3}H]PC was separated by thin-layer chromatography. The rate of VLDL-PC secretion (dpm/h/1 × 10^6 cells) was calculated from four time points during the chase.

HDL-PC uptake assay: HDL were isolated from plasma of wild-type mice by sucrose gradient centrifugation and preincubated with fluorescence-labeled PC (BODIPY-PC) from Molecular Probes (Chung et al., 1980; Jeong et al., 1998). Primary cultured hepatocytes were incubated with [BODIPY-PC]HDL for 2 hr and then cells were washed three times. Intracellular fluorescence intensity was measured by a fluorimeter. The rate of HDL-PC uptake (change in fluorescence intensity/h/1 × 10^6 cells) was calculated from four time points during the 2 hr incubation.

Permeability assays of large unilamellar vesicles

Hepatic PC and PE (Avanti) were used to make large unilamellar vesicles with PC/PE ratios of 0.5, 1.0, 1.5, and 2.0. [PC + PE] were mixed with sphingomyelin and cholesterol in a ratio of 86:1:9. Lipids were hydrated in Tris-HCl buffer (pH 9.0) in the presence of calcein (50 mM), a concentration at which the fluorescence of calcein is highly quenched. Large unilamellar vesicles were prepared at 55°C with an extruder (Lipex Biomembranes) and 100 nm Nuclepore filters (Allen et al., 1985; Haque et al., 2001). Large unilamellar vesicles were separated from untrapped calcein by chromatography over a Sephadex G-75 column. The diameter of the vesicles was measured by a light-scattering photometer and was 100 nm (Allen et al., 1985; Haque et al., 2001). The permeability assay measured the fluorescence of calcein accompanying its de-quenching upon release from the vesicles in Tris-HCl buffer (pH 7.4). The fluorescence intensity corresponding to 100% leakage was determined by adding 10% Triton X-100 to solubilize the membranes (Allen et al., 1985; Haque et al., 2001).

RNA interference (RNAi) of ET in cultured hepatocytes

RNAi sequence, 5′-AAGCACAACTGTGACTTCTCT-3′, targeted to the mRNA of mouse ET, was inserted into p*Silencer* 4.1-CMV vectors (Ambion) to produce ETi vectors. A control sequence from ETi, 5′-AATGACAGCTAGCACT GAGCT-3′, was inserted into the p*Silencer* 4.1-CMV vector to produce an ETi control vector. Lipofectamine 2000 (Invitrogen) was used as carrier for transfection. ETi and control vectors were cotransfected for 24 or 48 hr with GFP vectors (ratio, 4:1) into cultured hepatocytes isolated from CD-*Pemt*$^{-/-}$ mice. Hepatic RNA was extracted and mRNA levels of ET were quantitated relative to cyclophilin mRNA by SYBR Green assays using real-time qPCR. Primer sequences for ET: forward primer, 5′-AGCTAGCCAAGAGGCCCTAC-3′; reverse primer, 5′-CACCAGGTCCACCTTGAAGT-3′. Primer sequences for cyclophilin: forward primer, 5′-TCCAAAGACAGCAGAAAACTTTCG-3′; reverse primer, 5′-TCTTCTTGCTGGTCTTGCCATTCC-3′. After transfection for 20 or 44 hr, cells were incubated with fresh medium and cultured for 4 hr.

Inhibition of PE catabolism by arachidonyl trifluoromethyl ketone (ATK)

Primary hepatocytes from *Mdr2*$^{-/-}$/*Pemt*$^{-/-}$ mice were cultured in medium that contained 17% delipidated fetal bovine serum in Dulbecco's modified Eagle's medium that was choline-deficient. The medium was changed every 2 hr during the first 8 hr to remove any secreted PC since secreted PC might have been reused. Arachidonyl trifluoromethyl ketone (ATK), a PLA_2-specific inhibitor (50 μmol/l) was added to the CD medium for 24 or 48 hr to block the degradation of phospholipids (including PE). After 20 or 44 hr, medium was replaced with fresh medium and cells were cultured for 4 hr.

Histological and pathological analyses

Liver tissues from three mice per experiment were fixed in 10% formalin and stained with hemotoxylin and eosin. Coded sections were evaluated by a pathologist and a hepatologist. The modified Brunt criteria were used to grade the steatosis, hepatocyte ballooning and lobular inflammation in a scale of 0 to 3 and reported as median score for each parameter as well as overall grade (Brunt, 2001; 2004). Consecutive liver biopsy samples were derived and frozen at −80°C from patients with a histological diagnosis of NASH, once other viral, autoimmune, toxic, genetic, and miscellaneous hepatic diagnoses were excluded.

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