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Protective Effect of Phosphatidylcholine on Restoration of Ethanol-Injured Hepatocytes Related with Caveolin-1

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Abstract The absorption of phospholipid may improve the fluidity of membrane and enzyme activities. Phospholipids also play a role in promoting Caveolae formation and membrane synthesis. Caveolin-1 has a significant effect on signaling pathways involved in regulating cell proliferation and stress responsiveness. Thus, we can speculate that Caveolin-1 could affect the sense of environmental stress. We use Chang liver cell line to investigate the ability of Caveolin-1 to modulate the cellular response to ethanol injury. Caveolin-1 downregulate cells (Cav-$1^{-/-}$) were established by stable transfecting with psiRNA-CAV1 plasmids, which were more sensitive to toxic effects of ethanol than the untransfected parental cells (WT). Releasing of ALT and electric conductivity were changed significantly in Cav-$1^{-/-}$ cells compared with WT. Caveolin-1 gene silencing could obviously down-regulate the activities of protein kinase C-α (PKC-α) and phospho-p42/44 MAP kinase, indicating cell proliferation and self-repairing abilities were inhibited. However, the levels of Caveolin-1 and PKC-α were increased by phosphatidylcholine administration. The results indicated that the inhibition of lipid peroxidation by phosphatidylcholine could lead to the prevention of membrane disruption, which closely correlated with the level of Caveolin-1. Since the protective effects of phosphatidylcholine against ethanol-induced lipid peroxidation might be regulated by phospholipid-PKC-α signaling pathway, related with Caveolin-1, the potential effects of phosphatidylcholine on membranes need to be verified.

Keywords Phosphatidylcholine · Caveolin-1 · Ethanol injury · Protective effect

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Introduction

Phospholipids are the major constituents of the biologic membranes, and the two fatty acyl chains yield a roughly cylindrical molecule (the hydrocarbon region) that can be easily packed in parallel to form extended sheets of membranes. Phospholipids allow materials to diffuse or move across the cell membrane into the cell for uptake by the cell. Phospholipids can improve cell membrane, increase membrane fluidity and enzyme activity (Bishop and Bell 1988), and also promote lipoprotein synthesis and protection of cells, mitochondria and microsomal membrane from injury, to prevent the structural stability of cell membrane degeneration and to promote recovery (Kidd 2000; Morrison et al. 2012).

Ethanolic liver injury results largely from oxidative stress generated by ethanol metabolism, which is caused by

the generation of reactive oxygen species during ethanol metabolism. The reflecting of the oxidative stress, such as lipid peroxidation and cell membrane damage, induced gradual cell loss and eventually resulted in liver cell apoptosis (Zhou et al. 2002; Stewart et al. 2004; Fataccioli 2004). There is increasing evidence that oxidative stress plays an important etiologic role in the development of ethanolic liver disease.

Caveolin-1, an essential element of Caveolae, is a protein that has the distinct capability to create these highly ordered domains at the cell surface (Kurzchalia and Parton 1999; Williams and Lisanti 2005). Recently, it was reported that liver regeneration results from the coordination of cell activation, lipid metabolism, and cell division. Caveolin-1 has been connected with the regulation of each one of these processes. In the absence of Caveolin-1, hepatocytes did not accumulate lipid droplets (Ito et al. 2002; Cohen et al. 2004; Fernandez et al. 2006).

Stress signaling cascades share many early signaling components with those regulating the response to growth factors anchored in Caveolae microdomains. The ERK and the phosphatidylinositol-3 kinase (PI3 K)/Akt survival pathways are two such cascades, and their activation is often found to be a critical step for a favorable cellular outcome upon stressful stimulation (Cao et al. 2002a, b).

Our previous works showed that phospholipid can promote the translocation of Caveolin-1, which had significant protective effects on the experimental liver injury.

Phosphatidylcholine is the major component for Caveolae formation. The aim of our study was to evaluate the protective mechanism of phosphatidylcholine, which may resist oxidative damages of hepatocytes induced by ethanol, regulating the functions of Caveolin-1 and Caveolae.

Materials and Methods

Materials

Chang liver line was purchased from ATCC (CCL-13^{TM}). Stable clones were transfected into Chang liver cells with psiRNA-CAV1 plasmid (designated as Cav-1$^{-/-}$) (Ren et al. 2008).

RPMI 1640 and bovine calf serum were from Gibco (USA). Primary antibodies were from Cell signaling (USA). ECL Western blotting detection reagents were purchased from Amersham (Pharmacia Biotech, Inc.).

Separation and Purification of Phosphatidylcholine in Swine Liver

The classical Folch method was used to separate the total liver lipids (Folch 1957). Phosphatidylcholine in total phospholipids was separated and purified using Al_2O_3 column chromatography with 95 % alcohol as eluent (Liang et al. 2006). The purity was determined by thin layer chromatography (TLC) on GF254 silica plate using chloroform–methanol–water (65:25:4 v/v) as developing agent, colored by Iodine vapor.

Separation and Purification of Plasmenylcholine and Nonpalsmenylcholine

The purified phosphatidylcholine was applied to HPTLC plate and developed, determined by TLC with chloroform–methanol–water (60:35:8 v/v) as developing agent, and visualized by I_2 vapor and 5 nmol/L HgCl.

The Extracellular ALT Release Assay

Cells were seeded at a density of 5×10^4 cells/well for 24 h and incubated with different concentrations of ethanol (0–500 mmol/L) for 10 h. At the end of the treatment, one aliquot of medium was taken out for extracellular ALT release assay. All samples were analyzed for ALT production using ALT assay kit (Nanjing Jiancheng Bioengineering Institute, China).

Cell Conductivity Assay

Chang liver cells and Cav-1$^{-/-}$ cells were seeded at a density of 5×10^5 cells/well and then incubated with 200 mmol/L ethanol for 10 h. At the end of the treatment, one aliquot of medium was taken out for cell conductivity assay. Electrical conductance of effuses was measured by the conductivity meter (DDS211A, INESA Scientific Instrument Co., Ltd.).

MTT Assay

Chang liver cells were plated in a 96-well dish (six replicates per sample), then treated with ethanol for 10 h, simultaneously and after the different concentrations of phosphatidylcholine (50, 75, and 100 nmol/L) were incubated for 24 h. 50 μl of MTT (5 mg/ml in PBS) (Sigma, USA) was added to each well. MTT-containing medium was removed, and 200 μl of DMSO was added. Plates were analyzed using a Wallac plate reader at an absorbance of 490 nm.

Immunoblot Analysis

Cells were washed, scraped into saline containing a protease inhibitor mixture, and homogenized by ultrasonic disruption. The homogenate was boiled for 5 min, analyzed by SDS-PAGE with 10 % acrylamide, and then

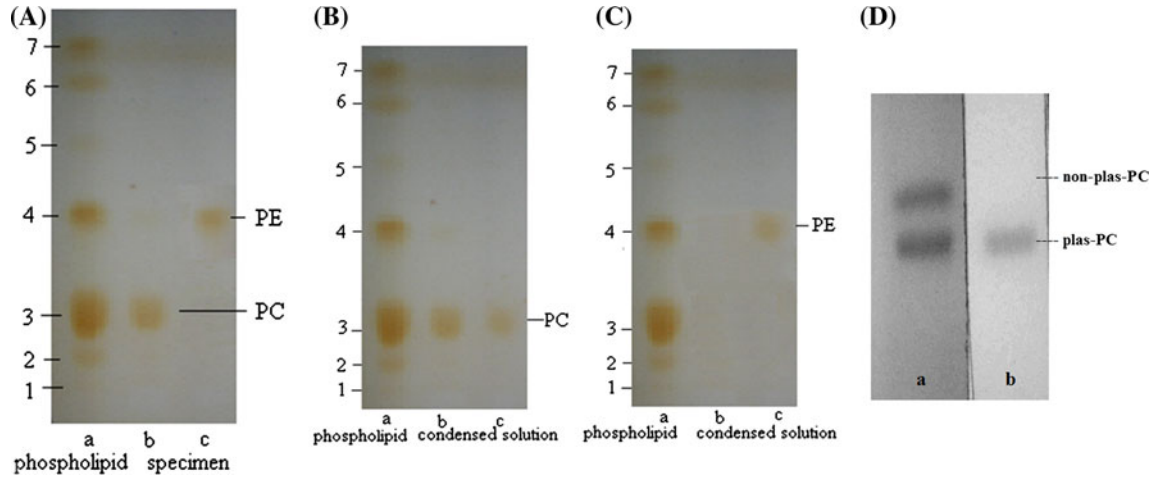

Fig. 1 Thin-layer chromatogram of separation and purification of phospholipids from swine liver. **a** TLC of the total phospholipids. The livers were subjected to Folch partition, the lower layer was applied to the TLC plate and developed by the mixture (chloroform: methanol: H_2O = 65:25:4; v/v), Temperature: 20 °C, and visualized by I_2 vapor. *a* The total phospholipids of the liver, *b* and *c*. Standard marker. (PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine). **b–c** PC and PE determination of the liver eluting components from the column by TLC. **b** The determination of PC: The early eluting components from the condensed samples of 0–225 ml (*line b*) and of 0-425 ml (*line c*). **c** The determination of PE: The late eluting components from the condensed sample of 425–750 ml (*line c*). *a* The total phospholipids of the liver. (The developing system was same as Fig. 1 shown). **d** HPTLC of the nonplasmenylcholine and plasmenylcholine. The purified phosphatidylcholine was applied to HPTLC plate and developed, the sample of band *a* was visualized by I_2 vapor, and the sample of band *b* was visualized by 5 nmol/L HgCl. *a* and *b* are both purified phosphatidylcholine. nonplas-PC: nonplasmenylcholine. plas-PC: plasmenylcholine

electroblotted to PVDF membranes. The membrane was blocked for 1 h in Tris-buffered saline containing 5 % nonfat dry milk and probed with primary antibodies (produced by Cell Signaling Technology) at a dilution of 1:2,000 in blocking buffer with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5,000) as the secondary antibodies. Analysis was by enhanced chemiluminescence (Amersham Pharmacia, USA).

Statistical Analysis

Results with repeated measurements were expressed as mean ± SD. Statistical analysis was by one-way ANOVA with statistical significance set at $P < 0.05$.

Results

Separation and Purification of Phosphatidylcholine and Plasmenylcholine

The total phospholipids were extracted from the liver tissue by the mixture of chloroform and methanol (Fig. 1a). Phosphatidylcholine in crude phospholipids from swine liver was separated and purified using Al_2O_3 column chromatography with 95 % ethanol as fluent. The purity was determined by thin layer chromatography on GF254 silica plate and with chloroform–methanol–water as developing agent. The results showing that the purity and yield rate of the PC, separated from phosphatidylethanolamine (PE) completely, reached a degree of more than 90 and 80 %, respectively, when eluted with 225 ml of 95 % ethanol, 87.6 and 87.3 % with a volume of 425 ml (Fig. 1b, c).

Plasmalogens are a type of ether phospholipid characterized by the presence of a vinyl ether linkage at the sn-1 position and an ester linkage at the sn-2 position. To determine the plasmenylcholine (plas-PC), the purified phosphatidylcholine was applied to the HPTLC plate and developed. Band B was plas-PC (Fig. 1d).

Effect of Phosphatidylcholine Against Ethanol-Induced Hepatotoxicity

Oxidative damage to lipids, proteins, and DNA after ethanol intoxication of the liver is very well described. Lipid peroxidation plays an important role in ethanol metabolism in hepatocytes. In our previous work, we found that the production of MDA and the activity of ALT in cells were inhibited when treated with different kinds of phospholipids (Supplementary Fig. S1).

To evaluate protective action of phosphatidylcholine in resistance to ethanol damage, we monitored the survival rate of cells with cotreatment and pretreatment by phosphatidylcholine. Survival rates of pretreated groups were

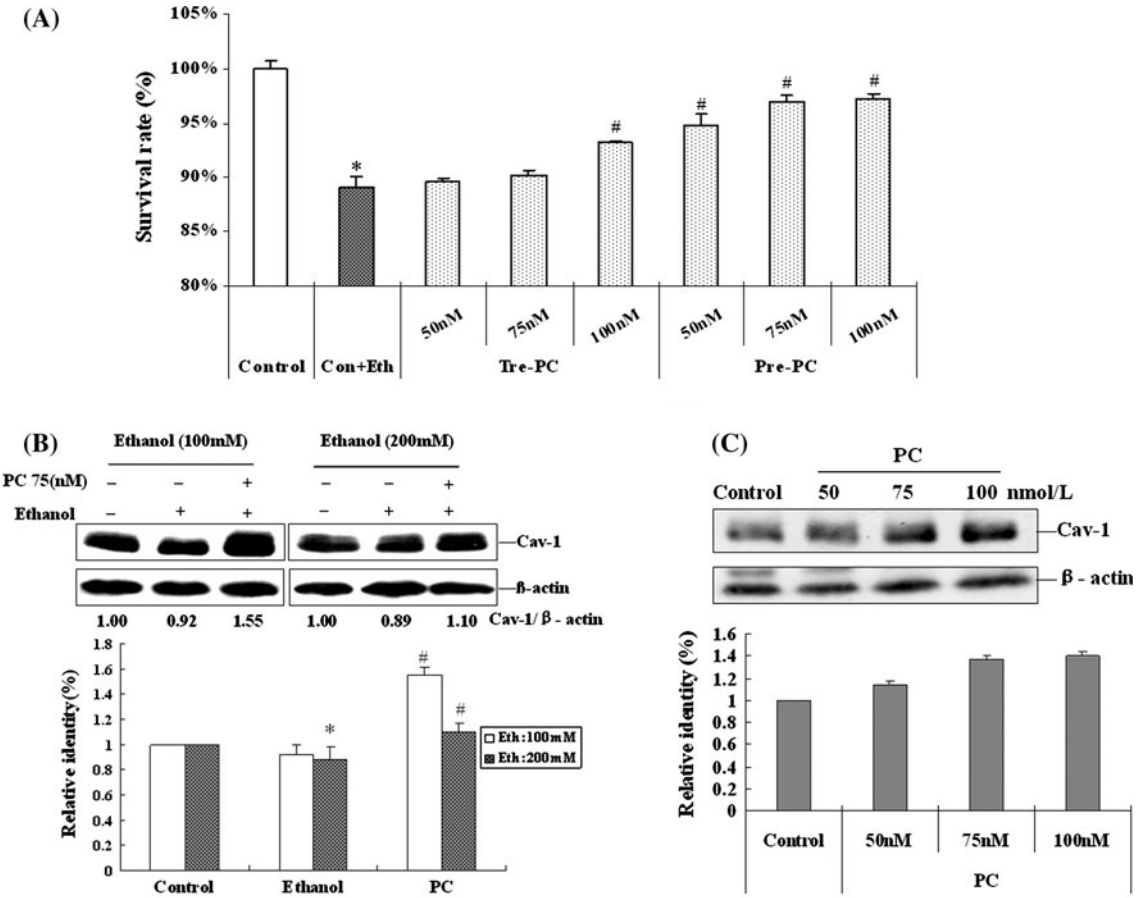

Fig. 2 Pretreatment with phosphatidylcholine increased cell survival capability and expression of Caveolin-1. **a** Tre-PC group, cells were simultaneously treated with ethanol (200 mmol/L) and PC (50, 75, and 100 nmol/L); Pre-PC, cells were pretreated with PC (50, 75, and 100 nmol/L) and then incubated with 200 mmol/L ethanol. The survival rates were tested by MTT. Data are presented as the mean ± SD $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Ethanol. **b** Chang liver cells were pretreated with 75 mmol/L of phosphatidylcholine and then incubated with 100 or 200 mmol/L of ethanol; we detected the expression of Caveolin-1, compared with control. Western blot detected the expression of Caveolin-1. Data are presented as the mean ± SD $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Ethanol. **c** The expression of Caveolin-1 in Chang liver cells. Cells were incubated with PC (50, 75, and 100 nmol/L) for 24 h alone. Western blot detected the expression of Caveolin-1. Data are presented as the mean ± SD

significantly raised in dose-dependent tendency. But it didn't show any marked difference in cotreatment groups, except higher concentration-treated phosphatidylcholine (Fig. 2a). It suggested that phosphatidylcholine plays preprotective effect on cellular defense against ethanol damages.

Treatment of Phosphatidylcholine Increased Expression of Caveolin-1

Caveolin-1 has the distinct capability to create highly ordered domains at the cell surface. Alcohol can literally dissolve lipids from the membrane, thereby inactivating the membrane proteins that depend on the lipids for activity and weakening the membrane to the point of rupture. This process may destroy the integrity of the membranes both within and surrounding the cell, seriously compromising cell function.

The free radicals may interact destructively with Caveolae and reduce the expression of Caveolin-1. PC as an antioxidant could defense against the ethanol injury. We found that the pretreatment of Chang liver cells with PC increased the level of Caveolin-1 then incubated with ethanol. The protective effect of PC-induced Caveolin-1 expression increased even higher than the normal (Fig. 2b). It suggested that the molecular fragments in PC metabolic processes could enhance the level of Caveolin-1, which prevented cells from the ethanol injury. In addition, in the absence of ethanol incubation, PC treatment alone caused a significant increase in the expression of Caveolin-1 (Fig. 2c).

Pretreatment by phosphatidylcholine is more effective on protective from ethanol injury referring to lipid peroxidation. The preprotective function is involved in elevation of Caveolin-1 level in cell line and liver tissue (data not

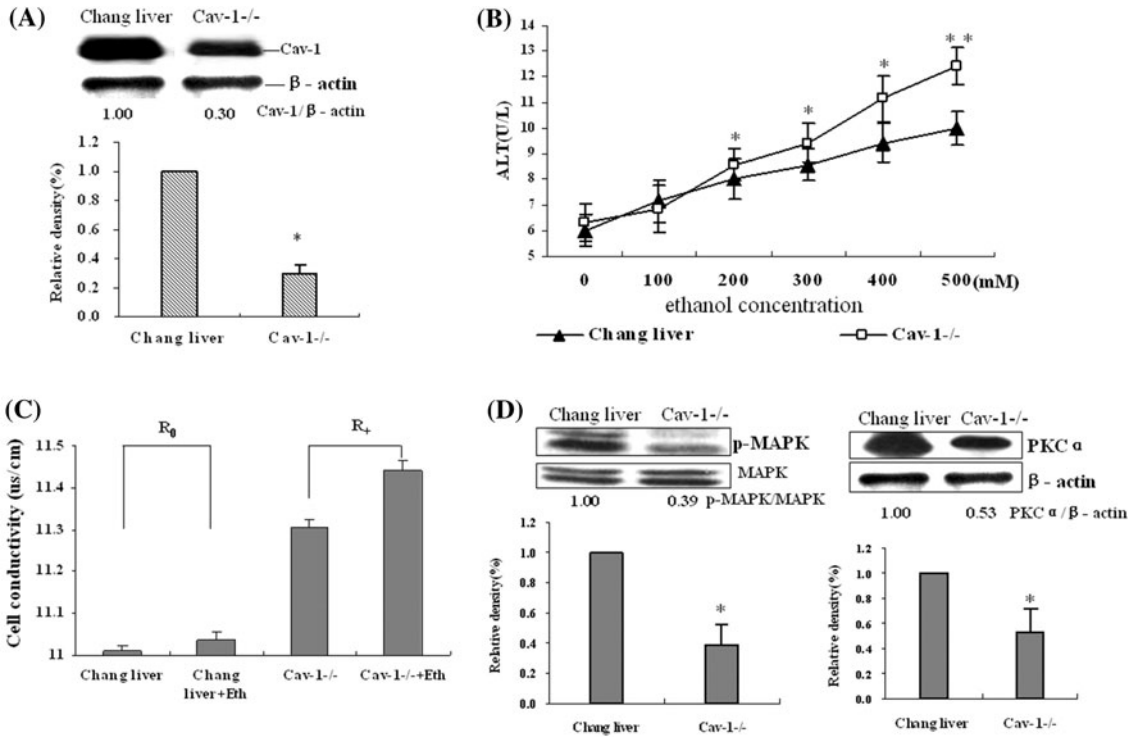

Fig. 3 Caveolin-1 down-regulated induced more susceptibility to ethanol toxicity, and induced p-MAPK and PKC-α decreased. **a** Caveolin-1 down-regulated by a siRNA approach in Chang liver cells (Cav-$1^{-/-}$ cells).**b** Cav-$1^{-/-}$ cells were incubated with 0 to 500 mmol/L of ethanol for 10 h. The leakage of intracellular ALT was detected, compared with Chang liver cells.**c** Chang liver cells and Cav-$1^{-/-}$ cells were incubated with 200 mmol/L of ethanol for 10 h. The electric conductivity of cells was detected, compared with control. Data are presented as the mean ± SD $^{*}P < 0.05$; $^{**}P < 0.01$ vs. Control. **d** The expression of PKC-α, p-MAPK, and MAPK in Cav-$1^{-/-}$ and Chang liver cells. Data are presented as the mean ± SD $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Ethanol

shown). So we assumed that Caveolin-1 is an important factor against liver injury.

Down-Regulation of Caveolin-1 Induced More Susceptibility to Ethanol Toxicity

Loss of Caveolin-1 leads the cells to be more sensitive when responding to chemical, traumatic, and infectious injuries. Caveolin-1 deficiency induces the apoptosis progress of intestinal stem cells in response to radiation. Here, we hypothesize that hepatocytes are more sensitive to ethanol injury because of loss of Caveolin-1. WT- and Caveolin-1-down-regulated cells (Cav-$1^{-/-}$) were incubated with different concentrations of ethanol for 10 h. Figure 3b showed that the level of extracellular ALT released increased either in WT or Cav-$1^{-/-}$ cells, which was dose dependent of ethanol. However, compared to WT, Cav-$1^{-/-}$ cells exhibited the markedly elevated slope of curve when incubated with ethanol concentrations of more than 200 mmol/L. Therefore, it was demonstrated that down-regulation of Caveolin-1 induced cells to be more sensitive to ethanol, while WT cells could be resistant to injury to some extent.

Electrical conductance was documented to represent the permeability of cell membrane (Sergent et al. 2005). In Fig. 3c, it was shown that down-regulation of Caveolin-1-induced cell conductivity significantly changed in comparison with WT ($P < 0.01$). Ethanol-induced peroxidation could increase fluidity and permeability of cell membrane and thus elevate the conductivity. The relative conductivity (R) was represented by the electrical conductance changes of cells after being incubated with ethanol, compared to control ($R+ = 1.194$ %, the conductivity changes of Cav-$1^{-/-}$ cells; $R0 = 0.227$ %, the conductivity changes of WT cells). When cells were incubated with ethanol, it showed more marked difference in Cav-$1^{-/-}$ cells, compared to WT.

Together, these results indicated that Caveolin-1 deficiency reduced cell membrane's fluidity. Loss of Caveolin-1 induced cell membrane dysfunction and reduced cell proliferation and survival ability that could inhibit the abilities of resistance to damages.

Moreover, we also investigated the protective effect by phosphatidylcholine on Cav-$1^{-/-}$ cells. Ethanol incubation could significantly decrease the Caveolin-1 expression in Cav-$1^{-/-}$ cells; yet, Caveolin-1 expression was increased

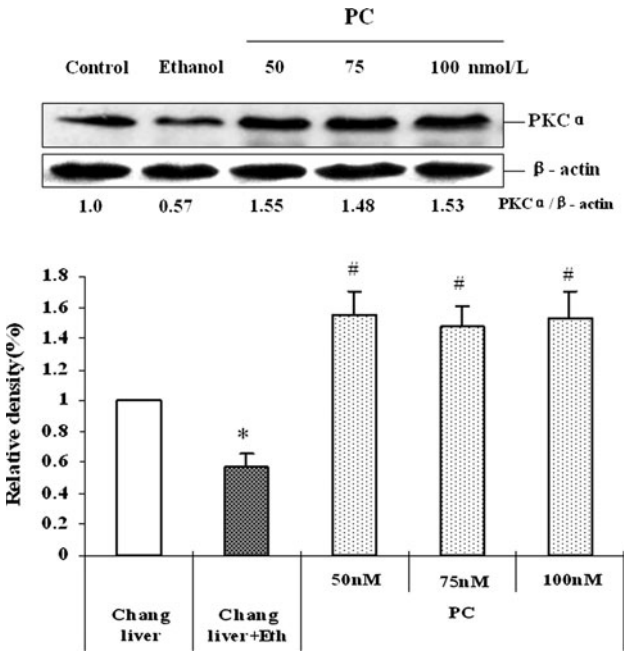

Fig. 4 Phosphatidylcholine increased expression of PKC-α. The expression of PKC-α in ethanol-injury and phosphatidylcholine preprotected group. Cells were pretreated with PC (50, 75 and 100 nmol/L) and then incubated with 200 mmol/L ethanol. Data are presented as the mean ± SD $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Ethanol

when cells were preprotected by phosphatidylcholine (Supplementary Fig. S2).

Down-Regulation of Caveolin-1 Decreased p-MAPK and PKC-α Expression

Usually, Ras-Erk1/2 pathway is considered to be associated with cell proliferation and growth. Many studies report that a number of components of Ras-p42/44 (Erk1/2) pathway are localized on Caveolae. However, silencing of Caveolin-1 gene decreased downstream signal molecules which interact with Caveolin-1. We detected phospho-p42/44 MAP kinase and p42/44 MAP kinase expression in Cav-$1^{-/-}$ cells. Caveolin-1 down-regulated displays lower level of phospho-p42/44 MAP kinase, which indicated that Caveolin-1 deficiency inhibits Ras-Erk1/2 signaling pathway (Fig. 3d).

Protein kinase C (PKC) is known to activate MAPKs. PKC-α translocating by the Caveolin scaffolding domain peptide indicates that Caveolins may be involved in the membranous recruitment of key signaling molecules, with acting downstream receptor activation. Our results showed that level of PKC-α was lower when Caveolin-1 was absent in Cav-$1^{-/-}$ cells (Fig. 3d).

These results support the notion that phosphorylation of p42/44 MAP kinase and activity of PKC-α decreased, because as Caveolin-1 down-regulated, it further impacted on the PKC-MAPK signaling pathway. It was suggested that loss of Caveolin-1 would affect the recovery process after ethanol injury.

Phosphatidylcholine Increased Expression of PKC-α

The phospholipids metabolite pathway is one of the most important pathways in lipid metabolic, which could activate PKC signaling pathway. PKC plays a pivotal role in controlling numerous cellular functions, including cell proliferation and cell injury-repaired. As shown, the expression of PKC-α decreased after ethanol-incubated (Fig. 4). While, it increased notably when cells were preincubated by phosphatidylcholine, which suggested that phospholipids metabolized might activate the PKC signaling pathway through Caveolin-1-mediated which played a protective effect.

Moreover, we detected the effect of phosphatidylcholine on PKC pathway in vivo and obtained the same tendency as in vitro. Preprotected by phosphatidylcholine would inhibit ethanol-induced PKC-α expression (data not shown).

Discussion

On the basis of previous work, the classical Folch method was selected to establish a system for simple separation of liver phospholipids (PL). In our study, a simplified method has been established, separating phosphatidylcholine (PC) in swine liver by Al_2O_3 column chromatography. The purity and recovery of PC were both more than 90 % by the method of Al_2O_3 column. Then, the plasmalogens were purified by means of HPTLC with different developing systems. A simple method to purify and identify phospholipids and plasmalogens was built.

Phosphatidylcholine (PC) is a phospholipid nutrient that is a major building block for all known cells. PC is the most abundant constituent of cell membranes, the thin and delicate yet dynamic surfaces on which cells carry out most of their activities. At present, the research and development of phospholipids is more concentrated in soybeans while the report of liver phospholipids is singularly both at home and abroad (Regente 2008). However, compared with the phospholipids from plant, animal liver phospholipids contain higher unsaturated fatty acids and a number of unique functional molecules. In addition to CDP-choline pathway for phosphatidylcholine biosynthesis, there is a special pathway catalyzed by PEMT2 (Zou et al. 2002; Tessitore et al. 2003). A question deserved to be asked here is: "Is there any special physiologic function of phosphatidylcholine on the signaling pathway that leads to protective function and cell proliferation in the liver?"

Much of the direct cell damage that occurs during alcoholic liver disease is believed to be caused by free

radicals. Free radicals are highly reactive molecular fragments that frequently contain oxygen. However, antioxidants are the cell's defense against free radicals. Our previous works suggested that phospholipids have significant protective effects of free radicals damage such as CCl_4 or ethanol metabolism (Hou et al. 2010; Yu et al. 2008a, b). Here, we use the ethanol-induced hepatic injury model in vitro to study the protective effect by phosphatidylcholine against ethanol injury on hepatocytes. A large amount of oxygen free radicals produced by ethanol metabolism in hepatocytes caused lipid peroxidation and cell membrane permeability changes in the leakage of intracellular ALT (Yu et al. 2008b; Aleynik et al. 1999; Aleynik and Lieber 2001). Our results showed that along with different concentrations of ethanol induced the release of ALT increased and cell membrane damaged significantly. Membrane phospholipids and their associated fatty acids also can be damaged or destroyed by the highly reactive acetaldehyde, which can do as much damage as many free radicals.

However, the antioxidant activity of phospholipids can inhibit free radical attack on hepatocyte membrane and reduce cell damage and apoptosis (Zhe et al. 2008a, b; Mak et al. 2003). The results demonstrate that the preprotection of PC against ethanol-induced toxicity is more effective than therapy. It suggests that the protection of PC is not only due to the role in protecting from free radicals, but it also more benefits from byproducts of various metabolic processes.

Interestingly, adding phosphatidylcholine would increase Caveolin-1 expression in Chang liver cells. Pretreatment with PC makes the level of Caveolin-1 higher than the normal when cells were damaged by low concentration of ethanol. We could help thinking that PC increased the expression of Caveolin-1 in vivo, making these cells more resistant to ethanol toxicity. Moreover, Caveolin-1 expression increased when we treated the CBRH-7919 cells with plas-PC in our previous works (data not shown). As meanwhile, we have demonstrated that administration of PC alone also induced Caveolin-1 expression in Chang liver cells.

Caveolin-1 as a key element associated with the lipid droplets and new cell membrane structure formation, plays an important role in supplying energy in the progress of liver regeneration (Cohen et al. 2004; Fernandez et al. 2006). Michael et al. have demonstrated that Caveolin-1 was involved in both lipid storage and breakdown in vivo. Caveolin-1 plays a dual role in lipid droplet metabolism, affecting both lipid droplet accumulation and breakdown. Overall, loss of Caveolin-1 leads to a decrease in lipid accumulation and, thus, progressive white adipose tissue atrophy. Toyoshi Fujimoto as well as others showed that Caveolins can exist in the lipid droplet surface (Fujimoto et al. 2001; Tauchi-Sato et al. 2002; Ostermeyer et al. 2001; Pol et al. 2001). Caveolins, i.e., Caveolin-1, 2, 3, are membrane proteins that are incorporated to the sphingolipid/cholesterol-enriched membrane microdomain and form the framework of Caveolae (Parton 2007). The lipid droplet surface is indeed a hemi-membrane, or a phospholipid monolayer, and the fatty acid composition of the lipid droplet phospholipids is distinct from that of rough ER and cholesterol/sphingolipid-rich microdomain.

Caveolin-1 gene expression did not change significantly during treatment with PC. In fact, increased Caveolin-1 has been described as a cause for the promotion of Caveolins vesicles form and transport, further it making Caveolin-1 anchor in Caveolae for stability of cell structure.

Michael reported that Caveolin-1 deficient mice exhibited a higher apoptotic rate in their crypt stem cells. Caveolin-1 deficient mice are much more sensitive to radiation and display decreased survival rates in response to high doses of γ-radiation (Li et al. 2005). Thus, Caveolin-1 deficiency leads to increased susceptibility to ethanol injury. As we have shown, Cav-$1^{-/-}$ cells exhibit a more significant ALT release and conductivity change than WT. Down-regulation of Caveolin-1 caused changes in the membrane permeability and induced cytoplasmic leakage. Caveolin-1 depletion in Chang liver cells inhibits the ability of cellular defense against ethanol damage. It is demonstrated that when cell membrane was damaged by lipid peroxidation, Caveolin-1 is involved in rebuilding new membranes to repair damaged membranes. In the mean time, phospholipids and cholesterol transporting were also involved in this process.

In addition, damaged liver can quickly initiate cell proliferation, leading to the function returning to normal. But the defective of Caveolin-1 will inhibit hepatocytes proliferation. Our studies showed that PKC-α and phospho-p42/44 MAP kinase expression decreased in Cav-$1^{-/-}$ cells which might slow cell proliferation and damage repair capability down. A key signaling cascade that might be activated by PKC in Caveolae is the ERK subfamily of mitogen-activated protein kinases (Seo et al. 2004; Wensheng 2006). The decreasing of proliferate rate caused by Cav-1 down-regulated inactivated PKC and MAPK signaling cascade, which maybe the potential mechanism.

As the results we have shown, phospholipids treated could increase Caveolin-1 expression in cell membrane, and the level of Caveolin-1 in membrane is associated with sensitivity to toxicity and the injury-repair.

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