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Polyenephosphatidylcholine prevents alcoholic liver disease in PPARα-null mice through attenuation of increases in oxidative stress☆

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***Background/Aims*: Alcoholic liver disease (ALD) is one of the leading causes of cirrhosis and yet efficient therapeutic strategies are lacking. Polyenephosphatidylcholine (PPC), a major component of essential phospholipids, prevented alcoholic liver fibrosis in baboons, but its precise mechanism remains uncertain. We aimed to explore the effects of PPC on ALD using ethanol-fed peroxisome proliferator-activated receptor α (*Ppara*)-null mice, showing several similarities to human ALD.**

***Methods*: Male wild-type and *Ppara*-null mice were pair-fed a Lieber-DeCarli control or 4% ethanol-containing diet with or without PPC (30 mg/kg/day) for 6 months.**

***Results*: PPC significantly ameliorated ethanol-induced hepatocyte damage and hepatitis in *Ppara*-null mice. These effects were likely a consequence of decreased oxidative stress through down-regulation of reactive oxygen species (ROS)-generating enzymes, including cytochrome P450 2E1, acyl-CoA oxidase, and NADPH oxidases, in addition to restoration of increases in Toll-like receptor 4 and CD14. PPC also decreased Bax and truncated Bid, thus inhibiting apoptosis. Furthermore, PPC suppressed increases in transforming growth factor-β1 expression and hepatic stellate cell activation, which retarded hepatic fibrogenesis.**

***Conclusions*: PPC exhibited anti-inflammatory, anti-apoptotic, and anti-fibrotic effects on ALD as a result of inhibition of the overexpression of ROS-generating enzymes. Our results demonstrate detailed molecular mechanisms of the antioxidant action of PPC.**

***Keywords*: Cytochrome P450 2E1; Acyl-CoA oxidase; NOX-4; MCP-1; TLR-4**

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Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; AOX, acyl-coenzyme A oxidase; AP-1, activator protein-1; ASK1, apoptosis signal-regulating kinase 1; COL1A1, collagen type 1 α1; COX-2, cyclo-oxygenase 2; CYP2E1, cytochrome P450 2E1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx, glutathione peroxidase; 4-HNE, 4-hydroxynonenal; ICAM-1, intercellular adhesion molecule-1; IκB, inhibitor of NF-κB; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MDA, malondialdehyde; MPT, mitochondrial permeability transition; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; NOX, nonphagocytic oxidase; PI3K, phosphatidylinositol-3 kinase; PPC, polyenephosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; αSMA, α smooth muscle actin; SD, standard deviation; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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1. Introduction

Chronic alcohol consumption can cause a wide spectrum of liver abnormalities that ranges from simple steatosis to hepatitis, cirrhosis, and hepatocellular carcinoma. It has been reported that alcoholic liver disease (ALD) remains the most common cause of liver cirrhosis in Western countries [1]. Since the appearance of hepatitis is a predictor of progression to cirrhosis and liver cancer, appropriate therapeutic intervention at this point is important in treating ALD.

Numerous data on the pathogenesis of ALD have been obtained from animal studies [1–3]. Chronic alcohol consumption induces hepatic oxidative stress due to increased generation of reactive oxygen species (ROS) and/or reduced anti-oxidant capacity. Oxidative stress causes further lipid peroxidation, which can directly damage the membranes of cells and organelles and lead to release of reactive aldehydes with potent pro-inflammatory and pro-fibrotic properties. Chronic alcohol intake also increases gut-derived lipopolysaccaride (LPS) concentration in portal blood, which binds to Toll-like receptor 4 (TLR4)/CD14 complexes and activates nuclear factor-kappa B (NF-κB), triggering pro-inflammatory responses such as induction of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Furthermore, ethanol is singularly so toxic that it can induce hepatocyte apoptosis by itself. These mechanisms are all presumed to contribute to human ALD to varying degrees.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily. Three isoforms of PPARs exist, designated as PPARα, PPARβ/δ, and PPARγ. Of these, PPARα is associated with the control of fatty acid transport and metabolism primarily in the liver [4]. A close relationship between PPARα and the development of ALD is believed to exist since chronic alcohol consumption decreases hepatic PPARα expression and suppresses the transcriptional activity of PPARα-regulated genes [5,6]. We previously reported that PPARα-null (*Ppara*$^{-/-}$) mice fed a 4% ethanol-containing Lieber-DeCarli diet for 6 months exhibited hepatomegaly, macrovesicular steatosis, hepatocyte apoptosis, mitochondrial swelling, hepatitis, and hepatic fibrosis, all of which resembled the clinical and pathological features of patients with ALD [7]. These abnormalities appeared with very high reproducibility and stressful surgical procedures to increase alcohol levels, such as gastric tube insertion, were not required. Therefore, *Ppara*$^{-/-}$ mice are regarded as a useful animal model to investigate the pathogenesis of human ALD.

Essential phospholipids are highly-purified phosphatidylcholine fractions containing linoleic acid and other unsaturated fatty acids. Polyenephosphatidylcholine (PPC), a major active ingredient in essential phospholipids, has a high bioavailability and affinity for cellular and subcellular membranes and maintains membrane fluidity and function. Several experiments have demonstrated the hepatoprotective effects of PPC [8–10]. However, the precise molecular mechanism of PPC action against ethanol toxicity has not been fully elucidated *in vivo*, which prompted us to evaluate the effects of PPC on ALD in greater detail using *Ppara*$^{-/-}$ mice.

2. Methods

2.1. Mice and treatment

Generation of *Ppara*$^{-/-}$ mice on a Sv/129 genetic background was described previously [11]. The mice were housed in an environment controlled for temperature, humidity, and light (25 °C, 12-h light/dark cycle) and maintained with standard laboratory chow and tap water *ad libitum* until 12 weeks of age. Male 12-week-old Sv/129 wild-type or *Ppara*$^{-/-}$ mice ($n = 24$ in each genotype) weighing 31–35 g were selected, randomly divided into 4 groups, and pair-fed the following diet for 6 months: (1) control Lieber-DeCarli liquid diet ($n = 6$); (2) control liquid diet with PPC (30 mg/kg/day) ($n = 6$); (3) 4% ethanol-containing Lieber-DeCarli liquid diet ($n = 6$); and (4) 4% ethanol-containing liquid diet with PPC (30 mg/kg/day) ($n = 6$). The ethanol-containing diet consisted of 19.4% (per weight basis) protein as casein, 51.0% carbohydrate as sucrose, 13.2% olive oil, 3.6% corn oil, 4.5% cellulose, 2.9% mineral mix, 1.1% vitamin mix, 0.3% choline bitartrate, and 4% ethanol (Oriental Yeast Co., Ltd, Tokyo, Japan). The control diet was replaced ethanol with isocaloric sucrose. PPC was provided from Alfresa (Osaka, Japan), and mixed in with the diet. Ethanol concentrations were raised gradually from 2% to 4% over the first month and maintained at 4% for the remainder of the administration period. Dietary intake was recorded every day and body weight was measured once a week. Six months after commencing treatment, all mice were sacrificed under anesthesia. After obtaining portal blood, livers were removed and weighed. All animal experiments were conducted in accordance with animal study protocols outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and approved by Shinshu University School of Medicine.

2.2. Measurement of plasma ethanol concentrations

To ascertain the amount of ethanol intake, blood was obtained from a tail vein at the same time (AM 10:00) of the first day of each month, and plasma ethanol concentration was determined using the vial equilibration method, as described elsewhere [7].

2.3. Preparation of nuclear and cytosolic fractions

Nuclear and cytosolic fractions were prepared as described previously [12].

2.4. Immunoblot analysis

Protein concentrations were measured colorimetrically with a BCA™ Protein Assay kit (Pierce, Rockford, IL, USA). For analysis of NF-κB, nuclear fractions (100 μg of protein) were subjected to 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). For analysis of other proteins, whole lysate or cytosolic fractions (50–100 μg of protein) were subjected to 8–10% SDS–PAGE [12–15]. The samples were obtained from all *Ppara*$^{-/-}$ mice ($n = 6$ in each group). In each electrophoresis assay, samples from three different mice in the same group were loaded. After electrophoresis, the proteins were transferred