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Down-regulation of LPCAT expression increases platelet-activating factor level in cirrhotic rat liver: Potential antiinflammatory effect of silybin

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ABSTRACT

Cholestasis is one of the major causes of liver diseases. A chronic accumulation of toxic bile acids in the liver, which occurs in this condition, can induce fibrosis and cirrhosis. Inflammation is a fundamental component of acute and chronic cholestatic liver injury.
Platelet-activating factor (PAF) is a proinflammatory lipid which may be generated by two independent pathways called the de novo and remodeling pathway being the last responsible for the synthesis of PAF during inflammation. In recent years a key role in PAF remodeling has been attributed to lysophosphatidylcholine acyltransferase (LPCAT) enzymes. Although the knowledge on their characteristic is growing, the exact mechanism of LPCAT in pathological conditions remains still unknown.
Here, we reported that the level of lyso-PAF and PAF significantly increased in the liver of cirrhotic vs. control rats together with a significant decrease in both mRNA abundance and protein level of both LPCAT1 and LPCAT2. Acyltransferase activities of both LPCAT1 and LPCAT2 were parallel decreased in the liver of cirrhotic animals. Interestingly, treatment with silybin strongly decreased the level of both pro-inflammatory lipids and restored the activity and expression of both LPCAT1 and LPCAT2 of cirrhotic liver. Silybin effect was specific for LPCAT1 and LPCAT2 since it did not affect LPCAT3 mRNA abundance of cirrhotic liver.

1. Introduction

Cholestasis is a pathological condition caused by injury to the bile ducts induced by immunologic, toxic, or genetic causes [1]. Cholestasis results in intrahepatic accumulation of potentially toxic bile acids leading to hepatocyte apoptosis and necrosis [1]. Moreover, the combined effects of progressive inflammation and toxicity of bile trapped within hepatocytes can culminate in cirrhosis [2]. Excessive bile accumulation into the liver has been reported to markedly alter the expression of various genes involved in cholesterol and phospholipid homeostasis [3].

Platelet-activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine or PAF) and alkyl-lysophosphatidylcholine (lyso-PAF) are phosphatidylcholine-derived molecules with important roles in inflammatory processes [4]. It has been demonstrated that PAF and lyso-PAF are hyper-produced in many inflammatory conditions such as diabetes [5] and nervous system diseases [6] where they activate inflammatory process and oxidative stress.

Recently, several observations have indicated increased plasma [9] and liver [10] PAF levels in cirrhotic rats suggesting a crucial role of PAF in liver diseases progression [7–9].

PAF can be synthesized through two different pathways: the de novo and a recovery pathway [4]. The latter involves the hydrolysis of alkyl-PC at *sn*-2 position by PLA2 with the production of lyso-PAF which can be acetylated, in presence of acetyl-CoA, in a reaction catalyzed by the key enzyme lyso-PAF:acetyl-CoA acetyltransferase (PAF-AT, EC 2.3.1.67) [11]. While the de novo synthesis is linked to a constitutive production of PAF, the remodeling pathway seems to be involved in inflammatory responses of PAF [12]. Interestingly, the two biosynthetic pathways are associated to distinct activities thus allowing targeting antiinflammatory intervention to specific enzymes [13].

The catabolism of PAF is normally made by PAF-acetylhydrolases (PAF-AH) which catalyze the degradation of PAF during antiinflammatory response [14]. In fact, treatment of diabetic rats with recombinant PAF-AH reduces insulitis and the frequency of diabetes [15].

In addition to the well characterized PAF-AT and PAF-AH, a growing number of enzymes have been recognized, in the last few years, in

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the remodeling pathway of PAF. Four different isoforms of lysophosphatidylcholine acyltransferases (LPCAT 1–4) are now considered to be involved in PAF remodeling during inflammation [16–20].

Although a significant progress has been made in the study of LPCAT enzymes, their role in pathological conditions remains still unknown.

Aim of the present study was to investigate the role of LPCATs in a rodent model of secondary biliary cirrhosis. The effect of silybin, a well known hepatoprotective compound [21–23] on the activity and expression of LPCAT was also evaluated.

The most relevant results were that the level of proinflammatory lyso-PAF and PAF dramatically increased and the mRNA expression of LPCAT1, LPCAT2 and LPCAT3 significantly reduced in the liver of cirrhotic rats. Silybin was able to restore normal level of inflammatory lipids and to normalize gene expression of both LPCAT1 and LPCAT2 but not LPCAT3. A role for LPCAT1 and LPCAT2 in the antiinflammatory effect of silybin, in the liver, was then discussed.

2. Materials and methods

2.1. Materials

All lipids, acyl-CoAs, 4-amidinophenylmethanesulfonyl fluoride (APMSF), 7-diethylaminocoumarin-3-carbonylazide, 7-methoxycoumarin-4-acetic acid, 1,2-dimethoxyethane, N,N′-dicyclohexylcarbodiimide, phospholipase C (*Bacillus cereus*, type IXV) were purchased from Sigma. [^{14}C]acetyl-CoA, [^{14}C]oleoyl-CoA, [^{14}C]palmitoyl-CoA and [^{14}C]arachidonyl-CoA were supplied by PerkinElmer (PerkinElmer, Monza, Italy). 4N3OBA was from Vinci-Biochem s.r.l., LPCAT2 and LPCAT1 antibodies were from DBA Italy s.r.l. RNAlater solution and SV Total RNA Isolation System kit from Promega. SuperScriptTM III RNase H-Reverse transcriptase was from Invitrogen. Primers were purchased from Sigma. All other chemicals and reagents were of analytical grade.

2.2. Animal model

Male Wistar rats, weighing 200–250 g, supplied by Harlan Italy Srl (S. Pietro al Natisone, UD), housed at 22 ± 1 °C, have been used throughout the study. The animals were maintained and sacrificed according to the Italian Official Statement N.116/92. The animals were divided into three groups: a group of rats subjected to bile duct ligation (BDL), a group of control rats (SHAM), subjected to the entire surgical procedure except for the ligation, and finally a group of BDL rats treated with silybin (BDL + Sil). The duct ligation was performed as previously described by Serviddio et al. [1]. The induction of chronic cholestasis was demonstrated by increases in the concentration of bilirubin in the serum of rats BDL which was found 7.3 ± 3.3 mg/dl vs. 0.5 ± 0.3 mg/dl of the controls (Fig. 1). Histological analysis after 4 weeks of bile duct ligation in rats BDL shows that the liver developed the characteristic of liver cirrhosis, with an inhomogeneous structure due to the presence of fibrous septa highlighted by Masson's trichrome (Fig. 1).

2.3. Histological analysis

Small blocks of liver tissue from all mice were immediately fixed in 4% neutral formalin and embedded in paraffin. Sections (4 μm thick) were stained by the hematoxylin and eosin or Masson's trichrome method. At least three discontinuous liver sections were evaluated for each mouse.

2.4. Biochemical analysis

Serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured with AST, ALT and ALP assay kits, respectively (Catachem, Bridgeport, CT).

2.5. Isolation of microsomal fraction from rat liver

For microsome isolation liver were homogenized in a cold buffer containing 100 mM Tris–HCl (pH 7,4), 250 mM sucrose, 5 mM 2-mercaptoethanol, 20 μM 4-APMSF and a cocktail of protease inhibitors as reported in [17]. Microsomes were isolated by differential centrifugation as reported in [24].

2.6. Phospholipase A2 activity

Phospholipase A2 (PLA2) activity was assayed as reported in [25] in liver homogenates by using 4N3OBA as substrate. Reaction mixture contained 150 mM KCl, 10 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.5 and 4N3OBA 2 mM. The reaction was started by addition of samples and after 1 h, at room temperature; the absorbance was read at two different wavelengths (425 nm and 600 nm to correct for any turbidity in the sample). Specific activity was expressed as μmol/h × mg protein.

2.7. Assay of lyso-PAF acyltransferase activity

The acyltransferase activity of LPCAT1 was assayed essentially as reported in [18]. Briefly, 5 μg of microsomal fractions was used to start the reaction in an assay mix containing 100 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mg/ml PC, 20 μM lyso-PAF (1-O-palmityl-*sn*-glycero-3-phosphocholine), 25 μM [^{14}C]oleoyl-CoA (2.035 GBq/mmol) or 25 μM [^{14}C]palmitoyl-CoA (293 MBq/mmol). After 5 min at 30 °C reaction was stopped adding 600 μl of a cold mixture of chloroform/methanol 1:2 (v/v). LPCAT2 acyltransferase activity was measured as in [19] incubating 5 μg of microsomal proteins in a buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM $CaCl_2$, 1 mg/ml PC, 20 μM APMSF, 5 mM 2-mercaptoethanol, 20 μM lyso-PAF (1-O-palmityl-*sn*-glycero-3-phosphocholine) and 20 μM [^{14}C]arachidonyl-CoA (1.11–2.035 GBq/mmol). After 10 min at 37 °C reaction was stopped adding 600 μl of a cold mixture of chloroform/methanol 1:2 (v/v). After extraction, lipids were separated by thin layer chromatography. Bands at positions corresponding to expected product were scraped from the plate and radioactivity counted using liquid scintillation.

2.8. Assay of LPCAT1 and LPCAT2 acetyltransferase activities

Both LPCAT1 and LPCAT2 have been demonstrated to catalyze acetyltransferase reaction [26]. The acetyltransferase activity of LPCAT1 was assayed as reported in [17]. Briefly, 5 μg of microsomal protein was incubated in a buffer containing 20 mM Tris–HCl (pH 7.4), 5 mM 2-mercaptoethanol, 20 μM APMSF, 1 mg/ml PC, 1 mM EDTA, 20 μM lyso-PAF and 200 μM [^{3}H]acetyl-CoA. Acetyltransferase activity of LPCAT2 was assayed following the procedure described in [17]. In this case microsomal proteins were incubated in a buffer containing 20 mM Tris–HCl (pH 7.4), 5 mM 2-mercaptoethanol, 20 μM APMSF, 1 mg/ml PC, 2 mM $CaCl_2$, 20 μM lyso-PAF and 100 μM [^{3}H]acetyl-CoA. After 10 min at 37 °C reactions were stopped by chloroform/methanol 1:2 (v/v). After lipid extraction and separation by TLC radioactivity associated to the radiolabeled products was measured by liquid scintillation.

2.9. Assay of liver lyso-PAF acetyltransferase activity

We also assayed the lyso-PAF acetyltransferase activity as in [27]. In this case, 50 μg of liver homogenate was incubated in a buffer containing 250 mM Tris–HCl, 25 mM BSA at pH 6.9 in presence of 20 μM [^{14}C]acetyl-CoA and 20 μM lyso-PAF. Reaction lasted 20 min at 37 °C and was terminated by adding chloroform/methanol 1:2 (v/v). Lipid extracted was loaded on TLC plate and after separation the band