Research Article

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The synthetic bile acid–phospholipid conjugate ursodeoxycholyl lysophosphatidylethanolamide suppresses TNFα-induced liver injury

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Background & Aims: Excessive apoptosis and leukocyte-dependent inflammation mediated by pro-inflammatory cytokines, such as TNFα, are cardinal features of acute liver injury. This study evaluated the ability of the newly designed bile acid–phospholipid conjugate ursodeoxycholyl lysophosphatidylethanolamide (UDCA-LPE) to protect from hepatocellular injury in comparison to the known hepatoprotectant ursodeoxycholic acid (UDCA) and phosphatidylcholine (PC).

Methods: Anti-apoptotic and anti-inflammatory properties of UDCA-LPE were evaluated after TNFα treatment of embryonic human hepatocyte cell line CL48 as well as of primary human hepatocytes. Acute liver injury was induced in C57BL/6 mice with D-galactosamine/lipopolysaccharide (GalN/LPS) in order to determine *in vivo* efficacy of the conjugate.

Results: UDCA-LPE inhibited TNFα-induced apoptosis and inflammation in hepatocytes *in vitro* and markedly ameliorated GalN/LPS-mediated fulminant hepatitis in mice, whereas UDCA or PC failed to show protection. The conjugate was able to decrease injury-induced elevation of phospholipase A_2 activity as well as its product lysophosphatidylcholine. Analysis of hepatic gene expression showed that UDCA-LPE treatment led to favourable inhibitory effects on expression profiles of key pro-inflammatory cytokines and chemokines, which are crucial for leukocyte recruitment and activation thereby inhibiting chemokine-mediated aggravation of parenchymal damage.

Conclusions: Thus, UDCA-LPE as a synthetic bile acid–phospholipid conjugate may represent a potent anti-inflammatory agent that is more effective than UDCA and PC for treatment of liver diseases.

Keywords: UDCA-LPE; TNFα; Endotoxin; Liver inflammation; Phospholipid homeostasis.

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Abbreviations: CHX, cyclohexamide; CMC, caboxymethylcellulose; GalN, D-Galactosamine; KC, Kupffer cells; LPC, 1-oleoyl lysophosphatidylcholine; LPE, 1-oleoyl lysophosphatidylethanolamine; LPS, lipopolysaccharide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PHH, primary human hepatocytes; PLA_2, phospholipase A_2; UDCA, ursodeoxycholic acid; UDCA-LPE, ursodeoxycholyl lysophosphatidylethanolamide.

Introduction

Fulminant hepatitis independent of possible underlying causes is due to massive leukocyte-dependent inflammation and extensive apoptosis of hepatocytes. TNFα, a cytokine released by hepatocytes and cells of the monocyte/macrophage lineage, is known to be critically involved in the pathogenesis of hepatic injury. Thus, it seems reasonable that novel therapeutic approaches with the objective of alleviating fulminant hepatitis should be capable of blocking TNFα-mediated liver injury.

Phospholipids such as phosphatidylcholine (PC) have been shown to inhibit TNFα-induced upregulation of pro-inflammatory cytokines [48] and to exert general anti-inflammatory effects in the liver e.g. by reducing LPS-induced Kupffer cell activation [39]. As an important component of cellular membranes, PC restores membrane integrity [28] resulting in an inhibition of mitochondrial depolarization and apoptosis. Indeed, there is evidence showing that phospholipid homeostasis is altered during liver injury [29], whereby hepatic PC levels are decreased during apoptosis and supplementation of PC inhibits cell death [11]. Additionally, excessive levels of lysophosphatidylcholine (LPC), a PC cleavage product generated by increased activity of phospholipase A_2 (PLA_2), have been implicated in hepatocellular apoptosis [18]. However, to date therapeutic efforts to utilize cytoprotective phospholipids in the context of liver diseases remained disappointing. This may be due to limited efficacies of PC uptake *in vivo* as demonstrated by the lack of effectiveness in a clinical trial using dilinoleoyl PC for the treatment of alcoholic steatohepatitis [30].

Thus, for specific targeting to the liver and improvement of therapeutic efficacies, we have rationalized that the hydrophilic bile acid ursodeoxycholic acid (UDCA) should be chemically coupled with a phospholipid or a PC precursor. UDCA itself has well-defined immunomodulating and anti-apoptotic action properties [6,20,42]. Additionally, former studies proved that conjugation of UDCA with another protective molecule was a useful strategy to improve efficacies in ameliorating liver disease. Therefore, UDCA has been employed for successful targeting of hepatocytes to deliver anti-inflammatory molecules such as a nitric oxide donor [14] and salicylic acid [17].

We had taken our first step in performing an amide coupling of UDCA with the PC precursor lysophosphatidylethanolamine (LPE) to form the conjugate UDCA-LPE [9]. It was plausible that this compound could be cytoprotective by releasing UDCA and PC upon cellular metabolism as well as through the action of the intact

compound. Our initial studies revealed that UDCA-LPE was indeed capable of inducing cell survival signaling pathways as well as inhibiting apoptosis in HepG2 cells and that cytoprotection was conferred by the intact conjugate but not by its metabolites.

The aim of this study was to delineate and define mechanisms of anti-inflammatory effects of UDCA-LPE during TNFα-induced liver injury. Because the hepatoma cell line HepG2 may not necessarily reveal the physiologic reaction pattern of native hepatocytes, we first tried to reproduce the inhibition of apoptosis by UDCA-LPE in two other *in vitro* hepatocellular culture systems, the non-transformed embryonic liver cell line CL48 [10] and primary human hepatocytes (PHH). For the evaluation of UDCA-LPE *in vivo*, we used the model of D-galactosamine/lipopolysaccharide(GalN/LPS)-induced fulminant hepatitis in mice [16]. Here, the endotoxin LPS initiates inflammatory response in the liver by activating Kupffer cells, which leads to the liberation of cytokines, such as TNFα, IL-1β, and IL-6 [32,43]. This model was suitable to complement the *in vitro* experiments as TNFα is the critical mediator of hepatocellular apoptosis and inflammation in this experimental setting [44].

Materials and methods

Materials

Custom synthesis of UDCA-LPE was performed by ChemCon GmbH. 18:1 LPE, 18:1 LPC were obtained from Avanti Polar Lipids. 18:1 and 18:1 PC were from Lipoid KG. Agonistic anti-Fas antibody (CH11) was from Upstate Direct. All other chemicals were from Sigma, unless stated otherwise.

Cultures

Human embryonic liver cell line CL48 was from ATCC. CL48 cells were maintained in DMEM containing 10% FCS. Media and supplements were from PAA. Primary human hepatocytes (PHH) and Hepatocyte Basal Medium were obtained from Lonza.

Animal model

Male C57BL/6 mice (Charles River Laboratories) at 8 weeks received i.p. injection with GalN 700 mg/kg in PBS with LPS at 10 μg/kg. For UDCA-LPE treatment groups 1 h prior to GalN/LPS animals received i.p. injection of UDCA-LPE or other test compounds at 30 mg/kg in 0.5% carboxymethylcellulose (CMC); details are in Supporting Materials. All experiments were approved by the Animal Care and Use Committee of the University of Heidelberg.

Measurement of ALT, AST, and LDH activity

ALT, AST, and LDH activities were determined immediately after serum preparation using commercial kits from Randox.

Measurement of PLA_2 activity

Enzymatic activity of PLA_2 was measured in lysates of CL48 cells and liver tissue as well as in serum samples using a commercial kit (Axxora); details are in Supporting Materials.

Measurement of phospholipids

LPC concentration of mitochondrial lipid extracts or cell lysates or liver tissue was determined using an enzymatic assay previously reported [22]. Lipids were extracted by Folch's method [15] with chloroform/methanol (2:1 vol/vol). Detailed description of the enzymatic assay is in Supporting Material.

NFκB luciferase reporter assay

NFB transcription factor activity was measured with the Cignal™ Reporter Assay Kit (SA Bioscience) according to the manufacturer's instructions; details are in Supplementary Material. Cell lysates were assayed for luciferase activity employing the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol, *Renilla* reporter was used for internal normalization.

Immunofluorescence

We performed immunofluorescence staining of NFB in CL48 cells according to standard protocols as described previously in detail [49]. Stained cells were analyzed using a spectral, confocal laser-scanning microscope (Leica, TCS SL).

Gene expression analysis by quantitative RT-PCR

TaqMan Gene Expression Assays™ (Applied Biosystems) were used as recommended by the manufacturer. Specific assays, details of RNA isolation and cDNA synthesis are listed in Supplementary Material.

Statistical analysis

Statistical analysis was performed with Prism Software version 4.0 (GraphPad). Significance of differences between two groups was determined by unpaired two-tailed student's *t*-test. Comparison of multiple groups was determined by one-way analysis of variance with Dunett's post test. Results are mean ± *SEM*. unless stated otherwise. A p value <0.05 was considered significant.

Note: Additional methods are described in "Supplementary Material & Methods".

Results

Inhibition of apoptosis by UDCA-LPE

Death receptor-mediated apoptosis was studied by using TNFα and cyclohexamide (CHX) to investigate the anti-apoptotic capacity of UDCA-LPE as compared with its components UDCA and LPE as well as LPC which has shown to be anti-apoptotic [11]. CL48 cells were susceptible towards TNFα as evidenced by ~45% apoptotic cells (Fig. 1A) and 7-fold increase of caspase 3/7 activity, following TNFα/CHX exposure compared to untreated cells (Fig. 1B). In contrast, preincubation with UDCA-LPE, 1 h prior to TNFα/CHX challenge decreased sensitivity towards TNFα resulting in the reduction of apoptosis rates by 90% and the inhibition of caspase 3/7 activities by 50% (Fig. 1A and B). Anti-apoptotic functions of UDCA-LPE were superior to LPC and LPE, which showed only marginal effects, and UDCA, which did not protect against TNFα-induced cell death (Fig. 1A and B). Notably, we confirmed a significant inhibition of TNFα-mediated activation of caspase 3/7 and 8 due to UDCA-LPE pretreatment in PHH (Fig. 1C and D), whereas UDCA, LPE, and LPC showed no protection. Potential desensitization of CL48 cells against death-inducing ligands was additionally studied by employing anti-Fas antibody. Consistently, apoptosis rates (Supplementary Fig. 1A) and caspase 3/7 activity (Supplementary Fig. 1B), following incubation with an agonistic Fas antibody, showed that only UDCA-LPE, but not UDCA, LPC, or LPE protected the cells in a significant manner. In addition to the effects on extrinsic apoptosis, the conjugate further modified proteins involved in the intrinsic pathway at the level of mitochondria. Accordingly, UDCA-LPE was capable of reducing TNFα-mediated accumulation of pro-apoptotic factors such as cytochrome c and tBid (Fig. 1E and F). Furthermore, the conjugate increased anti-apoptotic proteins such as