Ursodeoxycholy Lysophosphatidylethanolamide Inhibits Lipoprotection by Shifting Fatty Acid Pools toward Monosaturated and Polysaturated Fatty Acids in Mouse Hepatocytes

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Received June 18, 2013; accepted August 23, 2013

ABSTRACT

Ursodeoxycholy lysophosphatidylethanolamide (UDCA-LPE) is a hepatoprotective in inhibiting apoptosis, inflammation, and hyperlipidemia in mouse models of nonalcoholic steatohepatitis (NASH). We studied the ability of UDCA-LPE to inhibit palmitate (Pal)-induced apoptosis in primary hepatocytes and delineate cytoprotective mechanisms. We showed that lipoprotection by UDCA-LPE was mediated by cAMP and was associated with increases in triglycerides (TGs) and phospholipids (PLs). An inhibitor of cAMP-effector protein kinase A partially reversed the protective effects of UDCA-LPE. Lipidomic analyses of fatty acids and PL composition revealed a shift of lipid metabolism from saturated Pal to monounsaturated and polyunsaturated fatty acids, mainly, oleate, docosapentaenoate, and docosahexaenoate. The latter two \( \omega-3 \) fatty acids were particularly found in phosphatidylcholine and phosphatidylserine pools. The catalysis of Pal by stearoyl-CoA desaturase-1 (SCD-1) is a known mechanism for the channeling of Pal away from apoptosis. SCD-1 protein was upregulated during UDCA-LPE lipoprotection. SCD-1 knockdown of Pal-treated cells showed further increased apoptosis, and the extent of UDCA-LPE protection was reduced. Thus, the major mechanism of UDCA-LPE lipoprotection involved a metabolic shift from toxic saturated toward cytoprotective unsaturated fatty acids in part via SCD-1. UDCA-LPE may thus be a therapeutic agent for treatment of NASH by altering distinct pools of fatty acids for storage in TGs and PLs, and the latter may protect lipotoxicity at the membrane levels.

Introduction

Nonalcoholic steatohepatitis (NASH) is the most common form of chronic liver disease and is associated with metabolic syndrome and obesity (Diehl, 1999). NASH is defined as lipid accumulation with cellular damage, inflammation, and different degrees of fibrosis, and it is considered a serious condition as 25% of NASH patients can progress to cirrhosis, portal hypertension, and a high risk of hepatocellular carcinoma. Numerous advances in understanding its pathogenesis have been made, thus providing a rationale for translation into clinical trials. Besides dietary modification and bariatric surgery, pharmacological interventions have been...
tested, including insulin sensitizers, peroxisome proliferator–
activated nuclear receptor-γ agonists, tumor necrosis factor-α
antagonists, lipid-lowering agents, as well as antioxidants
and hepatoprotectants (Satapathy and Sanyal, 2010). Clinical
trials using insulin sensitizers, such as metformin and
glitzones, have revealed ineffective or only partial efficacy
(Ratziu et al., 2010). Histologic improvement of disease, at least in some patients, is observed treatment with vitamin
E (Satapathy and Sanyal, 2010) and ursodeoxycholic acid
(UDCA) (Ratziu et al., 2011). High-dose UDCA (28–35 mg/kg
daily) has been shown to improve aminotransferase levels,
serum fibrosis markers, and selected metabolic parameters
(Ratziu et al., 2011).

UDCA is known to be a hepatoprotective (Rodrigues et al.,
1998), anti-inflammatory (Zhang et al., 2010), and antifibrotic
agent (Zhang et al., 2010), and it is approved for the treatment
of cholestatic liver disease (Tschatzis et al., 2009). UDCA is
efficiently taken up by bile acid transport proteins (Maeda
et al., 2006), and the coupling of UDCA at C24 with drugs such
as, 5-aminosalicylic acid (Goto et al., 2001) and cisplatin (Briz
et al., 2002) renders efficient uptake by these transporters
(Balakrishnan et al., 2006). Moreover, the C23 homolog of
UDCA, which lacks one methylene group in its side chain,
called norUDCA, has been shown to be a better hepatoprotect-
ant than UDCA in the treatment of experimental sclerosing cholangitis (Fickert et al., 2006) and NASH (Beraza
et al., 2011). We rationalized that the efficacy of UDCA could
be improved by coupling UDCA with a phospholipid because
phospholipids are known to increase hepatocyte membrane
integrity (Li et al., 2006). We performed a coupling at C24
of UDCA with lysophosphatidylethanolamine (LPE; 18:1n-9
lysophosphatidylethanolamine) to generate UDCA lysophos-
phatidylethanolamidol (UDCA-LPE), the chemical structure
of which is shown in Fig. 1A (Chamulitrat et al., 2009). UDCA-
LPE was shown to be cytoprotective as an intact compound.
The superiority of UDCA-LPE to UDCA has been demon-
stated in terms of inhibition of tumor necrosis factor-α–
induced apoptosis and protection against acute liver injury
(Chamulitrat et al., 2009; Pathil et al., 2011). Furthermore,
we have shown that UDCA-LPE administration to mice fed
with a high-fat diet could lower systemic and hepatic
hyperlipidemia concomitant with significant inhibition of
hepatocyte apoptosis and inflammation (Pathil et al., 2012).

The hallmark of NASH includes increases in hepatocellular
saturated fatty acids and subsequent lipoapoptosis. In the
present study, we demonstrated that UDCA-LPE could
inhibit apoptosis induced by palmitate (Pal) in mouse hepa-
tocytes. We further investigated whether the mechanisms of
lipoprotection could be mediated by cAMP (Kwon et al., 2004)
and by pathways associated with the accumulation of
triglycerides (TGs) (Listenberger et al., 2003) and phospholi-
pids (PLs) (Collins et al., 2010). By using inhibitors and
performing knockdown experiments, we showed the latter to
be the major mechanism involving the action of stearyl-CoA
desaturase-1 (SCD-1), an enzyme that converts saturated
fatty acids (SFAs) to monounsaturated fatty acids (MUFAs).
Lipidomic data revealed that UDCA-LPE was able to induce
changes in fatty acid composition in lowering cytoxic SFA
and Pal while increasing MUFA and polyunsaturated fatty
acids (PUFAs), including docosapentaenoate (DPA) and doco-
se-hexaenoate (DHA). Thus, the major mechanism for UDCA-LPE
lipoprotection in vitro appeared to involve alterations of
fatty acid composition.

Materials and Methods

Reagents. The synthesis of UDCA-LPE was reported previously
(Chamulitrat et al., 2009). For UDCA-LPE used in this study, the
same synthesis procedure was performed by ChemCon (Freiburg,
Germany). Intracellular cAMP was determined by enzyme-linked
immunosorbent assay kit (BT-730) from Hyultec GmbH (Beueltbach,
Germany). Palmitate, bovine serum albumin (BSA), neonatal calf
serum, and N-TER Nanoparticle small interfering RNA (siRNA)
transfection system were obtained from Sigma-Aldrich (Taufkirchen,
Germany). Brefeldin A and 8-(4-chlorophenylthio)-2′-O-methyladenosine
cAMP (8-CPT-2′-O-Me-cAMP) were obtained from Biomol (Hamburg,
Germany). cAMP, N6,O2′-dibutyryl (dibutyryl-cAMP), 8-bromo-cAMP,
KT5720 (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-
1-oxo-9,12-epoxy-1H-diindol[1,2-f:3,2′-g:3′,2′′]-1′-k[pyrrolo[3,4-][1,6]benzodiazepine-10-carboxylic acid, hexyl ester), and protease inhibitor
cocktails were obtained from Calbiochem (Darmstadt, Germany).
The following were our sources of primary antibodies: SCD-1 (clone CD.E10)
and fatty acid elongase (Elov16) (ab68937) were from Abcam (Cam-
bridge, UK), cleaved poly(ADP-ribose) polymerase (PARP-1; clone E51)
was from Epitomix (Hamburg, Germany), and Bim and cleaved
caspase-3 (clone SA1E) were from Cell Signaling Technologies
(Frankfurt, Germany).

Hepatocyte Isolation. Hepatocytes were isolated from 7- to
10-week-old male C57BL/6 mice (Charles River Laboratories, Sulzfeld,
Germany) by using a two-step collagenase perfusion technique and
were purified by Percoll. Freshly isolated hepatocytes were plated
and cultured for 4 hours in M199 medium containing Hank’s salts and
l-glutamine (PAA, Cölbe, Germany) supplemented with 1% penicillin
and streptomycin, 100 nM dexamethasone, 0.5 nM insulin, and 4%
neonatal calf serum. Dead hepatocytes were removed, and the
adhered cells were treated with freshly prepared Pal with or without
UDCA-LPE in serum-free M199 medium on the same day of isolation.

Palmitate Preparation and Caspase-3 Assay. Pal stock solu-
tion in BSA was prepared according to a published procedure
(Rahman et al., 2009). Briefly, 250 µl of 200 mM Pal in ethanol
was mixed with 4.5 ml of 27% BSA in phosphate-buffered saline (PBS).
The total 5-ml volume was adjusted to pH 7.4 with 0.1 N NaOH until
the mixture became clear. After treatment for the indicated time,
hepatocytes were washed with PBS and lysed with 1% Triton X-100 in
PBS. Cell lysates after centrifugation were subjected to determination
of protein (Bio-Rad protein kit; Bio-Rad, Hercules, CA) and caspase-3/-7
activity using Caspase-3/-7Glo kit (Promega, Mannheim, Germany).
Luminescence was measured with a Fluostars Optima (BMG Labtech
Germany). Brefeldin A and 8-(4-chlorophenylthio)-2
lipid extractions, fatty acid, and phospholipid analyses.

Lipid Extraction, Fatty Acid, and Phospholipid Analyses.
Lipid extraction of 100-
mL of 200 mM Pal in ethanol was
mixed with 4.5 ml of 27% BSA in phosphate-buffered saline (PBS).
The total 5-ml volume was adjusted to pH 7.4 with 0.1 N NaOH until
the mixture became clear. After treatment for the indicated time,
hepatocytes were washed with PBS and lysed with 1% Triton X-100 in
PBS. Cell lysates after centrifugation were subjected to determination
of protein (Bio-Rad protein kit; Bio-Rad, Hercules, CA) and caspase-3/-7
activity using Caspase-3/-7Glo kit (Promega, Mannheim, Germany).
Luminescence was measured with a Fluostars Optima (BMG Labtech
Germany). For solvent controls, wells without Pal contained
0.5% BSA and 0.1% ethanol.

Immunoblotting. After treatment, mouse hepatocytes plated in
six-well collagen coated plates were lysed and centrifuged at 13,000g,
4°C for 15 minutes. Cell lysates were separated by gel electrophoresis
and transferred onto polyvinylidene fluoride membranes. Blots were
treated with a primary antibody followed by a secondary antibody.
Protein bands were visualized by using Luminata Forte ECL system
(Millipore, Darmstadt, Germany).

Lipid Extraction, Fatty Acid, and Phospholipid Analyses.
Lipid extraction of 100-µl lysates of treated mouse hepatocytes was
performed according to Folch’s method by using 10 volumes 2:1
chloroform/methanol. After removal of protein precipitates, chlo-
roform was collected and evaporated to complete dryness. Lipids
were dissolved in 50 µl 3:2 hexane/isopropanol. TG levels nor-
malized to milligrams of protein were determined with LabAssay
Tandem TLC kit (Wako GmbH, Neuss, Germany) using a micro-
plate reader Mutisaskan Astep (ThermoFisher Scientific, Schwerte,
Germany). Treated mouse hepatocytes were taken up into 200 µl of
PBS and lysed by freeze-thawing. After centrifugation, lipoa

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subjected to fatty acid analyses by gas chromatography coupled to mass spectrometry (GC-MS) as previously described (Ecker et al., 2012), and quantification of phospholipids by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive mode was done using the setup described previously (Liebisch et al., 2004).

**Analyses of UDCA-LPE and UDCA.** Cell lysates were subjected to lipid extraction in the presence of D₄-UDCA as an internal standard. UDCA-LPE and UDCA concentrations in samples and standards were determined using a liquid chromatography mass spectrometer (Waters 2695 interfaced with a Quattro Micro; Waters, Milford, MA) (Chamulitrat et al., 2012).
Lipoprotection by UDCA-LPE

Gene Expression by Reverse Transcription-Polymerase Chain Reaction. Total RNA of treated mouse hepatocytes was isolated using Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2 μg of RNA using a Maxima First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). The mRNA expression was analyzed in quadruplets by real-time polymerase chain reaction (PCR) using Applied Biosystems TaqMan gene expression assays with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and run on an Applied Biosystem 7500 Fast real-time PCR machine by using Assay-On-Demand TaqMan primers. The expression level of targets in quadruplets was calculated using the Δ-Ct transformation method and determined as a ratio of target gene normalized to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. PCR results were obtained from three or four independent experiments except for Fig. 5D, which shows representative results from two experiments.

siRNA and Transfection. SCD-1 siRNAs were designed and synthesized by Ribobxx GmbH (Radebeul, Germany). Two siRNA pairs were used in our study: SCD-1 siRNA_1 (antisense: UUUA-CUUAAAGA CACCAAGCCCCC and sense: GGGGCCCUGGU CUUUAAGU AAA) and SCD-1 siRNA_2 (antisense: UAUAAUAGAU- CAUUCACUGGCCCCC and sense GGGGCCCC AUGAAUGUA- CUAUAUA). Negative control siRNAs were obtained from Eurogentec (Seraing, Belgium). After allowing mouse primary hepatocytes to adhere for 4 hours, cells were transfected with 50 nM control or SCD-1 siRNAs using N-TER Nanoparticle siRNA transfection reagent for 4 hours. Cells were subsequently treated with Pal with or without UDCA-LPE in serum-free M199 medium for an additional 9 or 20 hours.

Data Analysis. Results were expressed as mean ± S.D. from at least two independent experiments performed at least in triplicates. For data in the figures, significance using analysis of variance with at least two independent experiments performed at least in triplicates.

Effects of UDCA-LPE on Triglycerides and Fatty Acid Composition during Lipoprotection. Treatment of cultured cells with MUFA, such as oleate (18:1n-9) and palmitoleate (16:1n-7) used at 200 μM, is associated with accumulation of TGs (Listenberger et al., 2003; Li et al., 2009) and PLs (Collins et al., 2010) concomitant with protection against lipotoxicity. We therefore determined whether UDCA-LPE could protect lipoproteinoma by modulating TG and PL levels. Treatment of mouse hepatocytes with Pal for 20 hours elevated TG levels, which were further elevated by UDCA-LPE cotreatment (Fig. 3A). Such further TG elevation was not observed on treatment with 60 μM UDCA-LPE, individually added UDCA + LPE, oleate, or palmitoleate (Fig. 3B). Notably, treatment of hepatocytes with 50 μM UDCA-LPE could cause a moderate increase in TG levels (Fig. 3A).

Results

UDCA-LPE Inhibits Lipopapoptosis in Part by cAMP/Protein Kinase Signaling. Treatment of mouse hepatocytes with 300 μM Pal for 20 hours induced significant apoptosis as evident by increased expression of proapoptotic BCL-2 family Bim, caspase-3, and cleaved PARP-1 proteins (Fig. 1B). Representative data from three experiments show that cotreatment of Pal with 60 μM UDCA-LPE significantly inhibited the expression of proapoptotic proteins (Fig. 1B) and caspase-3 activities (Fig. 1C) by >90%. IC₅₀ for UDCA-LPE lipoprotection was determined to be ~32 μM (Fig. 1D). The addition of UDCA-LPE 2 or 4 hours post Pal addition decreased the extent of lipoprotection, indicating that UDCA-LPE cotreatment showed a trend for inhibition of these increases concomitant with a trend for further increases of monounsaturated, more than two unsaturated as well as 18–20 versus 18–19 and 22–24 carbon fatty acids. Detailed analysis shown in Table 1 revealed that UDCA-LPE cotreatment reduced the levels of Pal (16:0) by ~27 nmol/mg protein (219.7 ± 20 versus 192.7 ± 8.1 for Pal and Pal + UDCA-LPE, respectively). Concomitantly, the levels of oleate (18:1n-9) were further increased by ~21 nmol/mg protein (74.5 ± 9.2 versus 95.7 ± 4.4 for Pal and Pal + UDCA-LPE, respectively). We investigated further whether the UDCA-LPE molecule (which by itself contains oleate on the LPE moiety) could account for the observed increases in oleate. Intracellular concentrations of UDCA-LPE were ~0.5 nmol/mg protein (Fig. 3D), much lower...
Fig. 2. Lipoprotection by UDCA-LPE in mouse hepatocytes in part involves cAMP/PKA signaling. (A) After 30-minute treatment, 25, 75, or 100 μM UDCA-LPE stimulated intracellular cAMP levels, whereas 100 μM UDCA had a weak effect. (B) Cotreatment with 100 μM dibutyryl or 800 μM 8-bromo-cAMP for 20 hours markedly inhibited Pal-induced caspase-3 activities in a manner similar to 60 μM UDCA-LPE. (C) Inhibition of lipoprotection by 60 μM UDCA-LPE or 100 μM dibutyryl cAMP was partially blocked by PKA inhibitor KT5720, which, at 5 μM, was pretreated 30 minutes before Pal addition. (D) UDCA-LPE inhibited Pal-induced upregulation of cleaved caspase-3 and cleaved PARP-1 proteins after 15 hours. This inhibition was partially blocked by 30-minute pretreatment with 10 μM KT5720. (E) An EPAC activator CPT-2-Me-cAMP used at 20 μM did not inhibit Pal-induced apoptosis after 20 hours. An EPAC inhibitor brefeldin A used at 100 μM with 1 hour of pretreatment did not reverse protective effects by UDCA-LPE. Data were mean ± S.D., N = 6; *P < 0.05; ***P < 0.001 versus con; †P < 0.05 versus Pal; ‡P < 0.05 versus Pal + UDCA-LPE or Pal + dibutyryl cAMP, con, control.
than those of the increased oleate. With UDCA-LPE treatment alone, intracellular concentrations of UDCA (Fig. 3D) and total LPE (Table 2) were similar to those of controls of ~0.3 and 1.55 nmol/mg protein, respectively, and the latter again could not account for the observed increased oleate. Thus, our data indicated an absence of UDCA-LPE hydrolysis to UDCA and LPE, which is consistent with our previous study using fluorescently labeled UDCA-LPE (Chamulitrat et al., 2009). These data were consistent with the observed optimal lipoprotection by intact UDCA-LPE, but not by its metabolite UDCA or LPE (Fig. 1F). Together with oleate (18:1n-9), UDCA-LPE cotreatment showed a trend to increase even further the levels of mono- and unsaturated fatty acids, as well as saturated and 15–17 carbon fatty acids. UDCA-LPE cotreatment showed a trend for inhibition of saturated and 15–17 carbon fatty acids concomitant with further increases of monosaturated fatty acids, and more than two unsaturated fatty acids, with the total fatty acid levels being increased in Pal treatment, and these levels were not altered by UDCA-LPE cotreatment (Fig. 3E). Rather than by...
lowering the total fatty acid contents, UDCA-LPE protected lipoprotection by altering fatty acid composition. These alterations include the decreases in toxic SFA concomitant with marked increases in cytoprotective MUFA and, to a lesser extent, ω-3 PUFAs, including DPA and DHA.

Role of SCD-1 in UDCA-LPE Lipoprotein. The mechanism for increases in TGs during lipoprotein has been demonstrated to involve conversion of SFA to MUFA by SCD-1 (Listenberger et al., 2003; Collins et al., 2010). In the liver (Li et al., 2009), MUFA provides metabolic adaptation for lipoprotein by incorporating SFA and MUFA into TGs and PLs. We therefore investigated a possible role of SCD-1 during UDCA-LPE lipoprotein. We observed time-dependent regulation of SCD-1 on the mRNA and protein levels (Fig. 4A). Compared with Pal, UDCA-LPE cotreatment caused further increases in SCD-1 mRNA after 4 hours and SCD-1 protein after 9 hours, which may indicate an early response for MUFAs synthesis to elicit protection during apoptosis observable in 20 hours. Treatment of mouse hepatocytes with Pal for 20 hours markedly decreased SCD-1 mRNA expression, which was not rescued by UDCA-LPE cotreatment, likely the result of inhibition of SCD-1 transcription by PUFAs (Ntambi, 1999), which had an increased trend after UDCA-LPE (Fig. 4B). Because the cAMP-PKA pathway could in part play a role in UDCA-LPE lipoprotein (Fig. 2), PKA inhibitor KT5720 was used to test whether there was a cross-talk between cAMP and SCD-1...

### TABLE 1

Total fatty acid composition during lipoproteinosis and protection by UDCA-LPE

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Con</th>
<th>Pal</th>
<th>Pal + UDCA-LPE</th>
<th>UDCA-LPE</th>
<th>Changes Pal + UDCA-LPE vs. Pal in nmol/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauric acid (12:0)</td>
<td>4.1 ± 0.6</td>
<td>3.5 ± 0.1</td>
<td>4.0 ± 0.3</td>
<td>2.6 ± 1.0*</td>
<td>ns</td>
</tr>
<tr>
<td>Myristic acid (14:0)</td>
<td>3.5 ± 0.27</td>
<td>5.1 ± 0.6</td>
<td>0.0 ± 0.0*</td>
<td>3.9 ± 2.3</td>
<td>↓ 5.0</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>113.6 ± 6.8</td>
<td>219.7 ± 20*</td>
<td>1927 ± 8.1*</td>
<td>952 ± 57.3</td>
<td>↓ 27</td>
</tr>
<tr>
<td>Sapienic acid (16:1-10)</td>
<td>0.6 ± 0.1</td>
<td>1.0 ± 0.2*</td>
<td>0.9 ± 0.00*</td>
<td>0.6 ± 0.2</td>
<td>↓ 0.2</td>
</tr>
<tr>
<td>Palmitoleic acid (16:1-7)</td>
<td>10.2 ± 0.9</td>
<td>24.1 ± 3.2*</td>
<td>20.3 ± 1.1*</td>
<td>7.7 ± 6.8</td>
<td>↓ 3.9</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>61.2 ± 2.5</td>
<td>62.0 ± 2.1</td>
<td>64.1 ± 3.6</td>
<td>51.5 ± 11.5*</td>
<td>ns</td>
</tr>
<tr>
<td>Oleic acid (18:1n-9)</td>
<td>68.8 ± 5.9</td>
<td>74.5 ± 0.2</td>
<td>95.7 ± 4.4*</td>
<td>89.2 ± 12.1*</td>
<td>↑ 21.2</td>
</tr>
<tr>
<td>Vercenic acid (18:1n-7)</td>
<td>6.3 ± 0.4</td>
<td>7.1 ± 0.6</td>
<td>6.7 ± 0.3</td>
<td>5.0 ± 1.2</td>
<td>ns</td>
</tr>
<tr>
<td>Linoleic acid (18:2n-6)</td>
<td>54.4 ± 3.7</td>
<td>59.5 ± 3.9</td>
<td>63.1 ± 2.4</td>
<td>44.4 ± 13.7</td>
<td>ns</td>
</tr>
<tr>
<td>Linolenic acid (18:3n-3)</td>
<td>3.1 ± 0.3</td>
<td>3.3 ± 0.4</td>
<td>3.7 ± 0.2</td>
<td>2.3 ± 0.9</td>
<td>ns</td>
</tr>
<tr>
<td>Arachidic acid (20:0)</td>
<td>3.63 ± 0.4</td>
<td>1.3 ± 0.07*</td>
<td>1.4 ± 0.0*</td>
<td>2.9 ± 1.0*</td>
<td>ns</td>
</tr>
<tr>
<td>Gondoic acid (20:1n-9)</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.1*</td>
<td>1.3 ± 0.1</td>
<td>2.2 ± 0.6*</td>
<td>ns</td>
</tr>
<tr>
<td>Homo-glycinic acid (20:3n-6)</td>
<td>4.9 ± 0.3</td>
<td>5.1 ± 0.2</td>
<td>5.6 ± 0.3</td>
<td>4.8 ± 1.0</td>
<td>ns</td>
</tr>
<tr>
<td>Arachidonic acid (20:4n-6)</td>
<td>33.3 ± 1.8</td>
<td>37.7 ± 1.6</td>
<td>41.4 ± 2.8*</td>
<td>33.0 ± 8.1</td>
<td>ns</td>
</tr>
<tr>
<td>Docosapentanoic acid (22:5n-3)</td>
<td>2.4 ± 0.3</td>
<td>2.7 ± 0.2</td>
<td>3.1 ± 0.2*</td>
<td>2.1 ± 0.7</td>
<td>ns</td>
</tr>
<tr>
<td>Docosahexanoic acid (22:6n-3)</td>
<td>20.3 ± 2.0</td>
<td>22.4 ± 1.3</td>
<td>25.8 ± 1.6*</td>
<td>21.9 ± 4.7</td>
<td>ns</td>
</tr>
</tbody>
</table>

Con, control; ns, not significant.

*P < 0.05, vs. Pal + UDCA-LPE vs. Pal in nmol/mg Protein.

### TABLE 2

Fatty acid saturation of phospholipids during lipoproteinosis and protection by UDCA-LPE

<table>
<thead>
<tr>
<th>PL</th>
<th>Fatty Acid Saturation</th>
<th>Con</th>
<th>Pal</th>
<th>Pal + UDCA-LPE</th>
<th>UDCA-LPE</th>
<th>Changes Pal + UDCA-LPE vs. Pal in nmol/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>SFA</td>
<td>1.87 ± 0.10</td>
<td>6.81 ± 0.24*</td>
<td>5.33 ± 0.12*</td>
<td>1.04 ± 0.13*</td>
<td>↓</td>
</tr>
<tr>
<td>MUF</td>
<td>11.37 ± 0.32</td>
<td>15.34 ± 0.40*</td>
<td>18.22 ± 0.59*</td>
<td>9.61 ± 1.21*</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>PUFA</td>
<td>42.71 ± 1.05</td>
<td>46.54 ± 1.92*</td>
<td>50.32 ± 2.15*</td>
<td>39.97 ± 5.61</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Total PC</td>
<td>57.53 ± 1.22</td>
<td>70.47 ± 2.48*</td>
<td>75.74 ± 2.91*</td>
<td>51.61 ± 7.00</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>SFA</td>
<td>0.15 ± 0.01</td>
<td>1.80 ± 0.05*</td>
<td>1.11 ± 0.03*</td>
<td>0.10 ± 0.01</td>
<td>↓</td>
</tr>
<tr>
<td>MUF</td>
<td>0.43 ± 0.01</td>
<td>1.50 ± 0.02*</td>
<td>1.56 ± 0.02*</td>
<td>0.40 ± 0.06</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>PUFA</td>
<td>29.03 ± 0.67</td>
<td>32.39 ± 1.63</td>
<td>34.90 ± 1.53*</td>
<td>32.67 ± 4.67</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Total PE</td>
<td>32.84 ± 0.75</td>
<td>38.61 ± 1.80*</td>
<td>40.92 ± 1.68*</td>
<td>37.83 ± 5.39</td>
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<tr>
<td>PS</td>
<td>MUF</td>
<td>0.19 ± 0.08</td>
<td>0.09 ± 0.01*</td>
<td>0.08 ± 0.01*</td>
<td>0.06 ± 0.01*</td>
<td>ns</td>
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<tr>
<td>PUFA</td>
<td>6.69 ± 0.23</td>
<td>7.40 ± 0.19*</td>
<td>9.00 ± 0.33*</td>
<td>8.57 ± 0.99*</td>
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<td>Total PS</td>
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<td>7.49 ± 0.20</td>
<td>9.09 ± 0.32*</td>
<td>8.63 ± 1.00*</td>
<td>↑ 1.6</td>
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<td>PI</td>
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<td>0.10 ± 0.01*</td>
<td>0.10 ± 0.01*</td>
<td>0.06 ± 0.01*</td>
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<tr>
<td>MUF</td>
<td>0.13 ± 0.01</td>
<td>0.29 ± 0.01*</td>
<td>0.44 ± 0.02*</td>
<td>0.13 ± 0.02</td>
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<tr>
<td>PUFA</td>
<td>24.57 ± 0.28</td>
<td>26.00 ± 2.00</td>
<td>25.05 ± 1.68</td>
<td>24.79 ± 3.04</td>
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<td>24.78 ± 0.28</td>
<td>26.40 ± 1.99</td>
<td>25.59 ± 1.68</td>
<td>24.98 ± 3.06</td>
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<td>LPE</td>
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<td>1.52 ± 0.25</td>
<td>2.14 ± 0.08*</td>
<td>2.45 ± 0.11*</td>
<td>1.25 ± 0.17</td>
<td>↑</td>
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<tr>
<td>MUF</td>
<td>0.18 ± 0.039</td>
<td>0.21 ± 0.012</td>
<td>0.28 ± 0.012*</td>
<td>0.18 ± 0.03</td>
<td>↑</td>
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<tr>
<td>PUFA</td>
<td>0.12 ± 0.023</td>
<td>0.14 ± 0.012</td>
<td>0.18 ± 0.013*</td>
<td>0.12 ± 0.017</td>
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<tr>
<td>Total LPE</td>
<td>1.82 ± 0.30</td>
<td>2.49 ± 0.10</td>
<td>2.91 ± 0.12*</td>
<td>1.55 ± 0.21</td>
<td>↑ 0.4</td>
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Con, control; ns, not significant.

*P < 0.05, vs. Pal + UDCA-LPE vs. Pal in nmol/mg Protein.
Fig. 4. Role of SCD-1 during lipoprotection by UDCA-LPE in mouse hepatocytes. (A) UDCA-LPE and Pal cotreatment increased SCD-1 mRNA expression after 4 hours (left) and SCD-1 protein after 9 hours (right). (B) After 9 hours of treatment, LPE or individually added UDCA + LPE did not upregulate SCD-1 protein (left). UDCA-LPE’s ability to upregulate SCD-1 protein (middle) and mRNA (right) after 9 and 4 hours of cotreatment, respectively, was not significantly modified by 30 minutes of pretreatment with 5 or 10 μM KT5720. (C) After 9 hours of treatment, SCD-1 siRNA-transfected cells showed decreased SCD-1 protein compared with con siRNA-transfected cells; this was concomitant with increased cleaved caspase-3 and cleaved PARP-1 protein expression in the corresponding treatment group. (D) The ability of UDCA-LPE to inhibit lipoapoptosis (two left panels) and to increase triglycerides (two right panels) was decreased on SCD-1 knockdown using SCD-1 siRNA-1 and SCD-1 siRNA-2. Data are mean ± S.D., N = 4; *P < 0.05; ***P < 0.001 versus con; †P < 0.05 versus Pal or con siRNA. con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
pathways. We found that KT5720 treatment did not significantly block UDCA-LPE–dependent upregulation of SCD-1 protein detectable at 9 hours or SCD-1 mRNA detectable at 4 hours (Fig. 4B). This finding indicated that SCD-1 upregulation by UDCA-LPE cotreatment was unlikely to be mediated by cAMP and PKA.

We performed SCD-1 knockdown by using siRNAs to determine a possible role of SCD-1 on UDCA-LPE lipoprotection (Fig. 4C). In our hands, siRNA knocked down SCD-1 protein by only ∼50% after 9 hours of treatment. Pal and UDCA-LPE cotreatment significantly increased SCD-1 protein expression in control siRNA-transfected cells but failed to do so in SCD-1 siRNA-transfected cells. Compared with control siRNA-transfected cells, Pal treatment of SCD-1 knockdown cells caused markedly increased apoptosis, as seen by increased cleaved caspase-3 and cleaved PARP-1 protein expression (Fig. 4C), which was consistent with previous reports (Li et al., 2009). In SCD-1–knockdown cells, the ability of UDCA-LPE to inhibit apoptosis became weaker, as quantitatively demonstrated by caspase-3 activity assay (Fig. 4D, left). UDCA-LPE inhibited apoptosis by ∼80% in control siRNA-transfected cells and, to a lesser extent, by ∼40% in SCD-1 siRNA– or siRNA-2–transfected cells. Concomitantly, UDCA-LPE and Pal cotreatment significantly further increased TG levels in control siRNA-transfected cells (Fig. 4D, right), and the ability of UDCA-LPE to further increase TG became less effective with SCD-1 knockdown. Thus, SCD-1 may in part contribute to UDCA-LPE lipoprotection for increases of TGs.

To explore the possible mechanisms for increases of more than two unsaturated (Fig. 3C), as well as an increased trend of DPA (22:5n-3) and DHA (22:6n-3) (Table 1), we measured mRNA expression of fatty acid desaturase (Fads) and Elovl (Moon et al., 2009; Green et al., 2010) genes. Similar to the earlier observation of SCD-1 upregulation at 4 hours, UDCA-LPE cotreatment further upregulated mRNA expression of Fads 1 (∆6 desaturation) (Fig. 5A), as well as Elovl5 and 6 (Fig. 5B). Expression of Fads 2 (∆5 desaturation) was not affected by Pal or UDCA-LPE treatment. Expression of de novo lipogenesis gene fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC1) was further increased by UDCA-LPE cotreatment after 20 hours of treatment (Fig. 5C). Furthermore, expression of TG synthesis, lipolysis, β-oxidation, and metabolism transcription factor genes was measured in samples with 20 hours of treatment (Fig. 5D). Pal treatment decreased the expression of diacylglycerol acyltransferase 1 (DGAT1) and liver X factor (LXR), which was rescued by UDCA-LPE cotreatment. UDCA-LPE cotreatment did not do so in the expression of DGAT2, adipocyte triglyceride lipase, acyl-CoA oxidase, peroxisome proliferator–activated receptor activator (PPAR)α, PPARγ, and SREBP1c. Taken together, the addition of SCD-1 (∆9 desaturation) UDCA-LPE protection against Pal was thus associated with the rescues of Fads1, Elovl5/6, DGAT1, and LXR by UDCA-LPE cotreatment, and this may account for increased polyunsaturated fatty acid (PUFA) contents (Fig. 3C; Table 1).

**UDCA-LPE Protects Lipoapoptosis by Modulating the Composition of Phospholipids.** It has been shown that Pal (16:0) is significantly incorporated into PL (Collins et al., 2010), and we further characterized the molecular species of PLs in our samples by using ESI-MS/MS. Pal treatment increased total phosphatidylcholine (PC) and phosphatidylethanolamine (PE) levels (Table 2). UDCA-LPE cotreatment further increased the total PC (with a trend), and the total phosphatidylserine (PS) and LPE levels of ∼5, 1.6, and 0.4 nmol/mg protein, respectively. Pal treatment increased the levels of SFA and MUFA in PC, PE, and phosphatidylinositol (PI) (Table 2). UDCA-LPE cotreatment inhibited the increases in SFA while further increasing MUFA levels in PC, PE, and PI, supporting an SCD-1 mechanism for protection. Pal treatment increased PUFA levels in PC and PS, whereas UDCA-LPE cotreatment further increased PUFA in PS and with a trend in PC. LPE containing all SFA, MUFA, and PUFA classes was increased during lipoprotection by UDCA-LPE, likely by an SCD-1–independent mechanism, which may additionally contribute to apoptosis inhibition, shown to be mediated by MAPK (Nishina et al., 2006).

Further detailed analyses of PL molecular species shown in Table 3 revealed that UDCA-LPE lipoprotection was associated with decreases of PL species containing 14:0, 16:0, and 18:0, concomitant with increased levels of PC, PE, PS, PI, and LPE containing SFAs and MUFAs or PUFAs. MUFAs were mainly 18:1n-9, and PUFAs were mainly arachidonic acid (AA) (20:4n-6), eicosapentaenoate (20:5n-3), DPA (22:5n-3), and DHA (22:6n-3). During protection, the predominant species most affected by UDCA-LPE were PC 34:1 (PC 16:0,18:1) and PC 34:2 (PC 16:0,18:2), indicating an efficient incorporation of 16:0 into PC. During lipoprotection by UDCA-LPE, decreases of 16:0 and 18:0 fatty acids were also observed in ceramides and plasmalogen-based PE (Supplemental Table 1). During protection by UDCA-LPE, long-chain fatty acids (i.e., 22:0, 20:4, 20:5, 22:5, and 22:6) were also increased in ceramides, sphingomyelin, plasmalogen-based PE, and cholesterol.
Because LPE (Fig. 1F) and cAMP (Fig. 2B) were able to inhibit lipoapoptosis, we further compared alterations of fatty acid composition among UDCA-LPE, UDCA, LPE, or cAMP by using GC-MS. Reported as the percent of mol fatty acids, we found that treatment with UDCA-LPE or LPE for 20 hours decreased cellular SFA while increasing MUFA levels (Fig. 6A), consistent with the observed effects of UDCA-LPE shown in Table 1. Treatment with UDCA-LPE or LPE was able to increase oleate (18:1n-9) levels (Fig. 6B, left), suggesting a similar effect of LPE in stimulating MUFA synthesis, which

Fig. 5. Alterations of lipid metabolism genes during lipoprotection by UDCA-LPE. Mouse hepatocytes were treated with 300 μM Pal with cotreatment with 60 μM UDCA-LPE for 4 or 20 hours. (A) Fatty acid desaturase (Fads) 1, but not Fads 2 mRNA, expression was increased after 4 hours of treatment. (B) Expression of Elovl6 mRNA was elevated by UDCA-LPE and Pal cotreatment after 4 and 20 hours. (C) Pal treatment of 20 hours increased FAS and acetyl-CoA carboxylase (ACCL) expression, which was further increased by UDCA-LPE cotreatment. (D) Pal treatment of 20 hours lowered the expression of DGAT 1 and LXR, which was rescued by UDCA-LPE cotreatment. UDCA-LPE cotreatment with Pal did not modify DGAT2, adipocyte triglyceride lipase (ATGL), acyl-CoA oxidase (Acox), PPARγ, PPARα, and SREBP1c after 20 hours of treatment. Data were mean ± S.D., N = 3–4, except data in D (quadruplet PCRs) were representatives from two experiments; *P < 0.05; **P < 0.01 versus con; †P < 0.05 versus Pal. con, control.

Because LPE (Fig. 1F) and cAMP (Fig. 2B) were able to inhibit lipoapoptosis, we further compared alterations of fatty acid composition among UDCA-LPE, UDCA, LPE, or cAMP by using GC-MS. Reported as the percent of mol fatty acids, we found that treatment with UDCA-LPE or LPE for 20 hours decreased cellular SFA while increasing MUFA levels (Fig. 6A), consistent with the observed effects of UDCA-LPE shown in Table 1. Treatment with UDCA-LPE or LPE was able to increase oleate (18:1n-9) levels (Fig. 6B, left), suggesting a similar effect of LPE in stimulating MUFA synthesis, which
could account for partial protective effects of LPE (Fig. 1F). However, UDCA-LPE was the only agent that increased the levels of more than two unsaturated fatty acids as seen by the increases in AA (20:4n-6) and DHA (22:6n-3) (Fig. 6B, right). These increases corresponded well with the observed upregulation of Fads1, Elov6 and 5 mRNA by UDCA-LPE treatment alone (Fig. 5, A and B). Treatment with UDCA did not cause any alterations in fatty acid composition, whereas that of 8-bromo-cAMP decreased oleate, AA, and DHA levels. The action of cAMP as a mediator of lipoprotection (Fig. 2) could be the type of fat contributes to the onset and progression from steatosis to NASH. The liver plays a central role in whole-body lipid metabolism and responds rapidly to changes in dietary fat composition. Strategies for the development of therapeutic agents should involve lowering hepatic toxic SFA and at the same time inhibiting key deleterious events occurring in NASH. We here demonstrated that UDCA-LPE inhibited Pal-induced apoptosis in mouse hepatocytes while altering the composition of fatty acids such that total contents of TGs and PLs were accumulated. Lipidomic data revealed that this protection was accompanied with upregulation of Fads1, Elovl6 and 5 mRNA by UDCA-LPE, thereby contributing to the observed effects on fatty acid composition.

### Discussion

It is accepted that not only the quantity of dietary fat but also the type of fat contributes to the onset and progression from steatosis to NASH. The liver plays a central role in whole-body lipid metabolism and responds rapidly to changes in dietary fat composition. Strategies for the development of therapeutic agents should involve lowering hepatic toxic SFA and at the same time inhibiting key deleterious events occurring in NASH. We here demonstrated that UDCA-LPE inhibited Pal-induced apoptosis in mouse hepatocytes while altering the composition of fatty acids such that total contents of TGs and PLs were accumulated. Lipidomic data revealed that this protection was accompanied with increases of mainly oleate (18:1n-9), DPA (22:5n-3), and 22:6n-3 (DHA). Our study delineated molecular therapeutic pathways in UDCA-LPE’s ability to inhibit lipoproteins by modulating composition of fatty acids in part via the SCD-1.
pathway and as a minor mechanism by inducing cAMP/PKA signaling (Fig. 6C).

UDCA-LPE was active as an intact compound in inhibiting Pal-induced apoptosis, which is in line with our previous studies (Chamulitrat et al., 2009). This exemplifies the significance of UDCA and LPE conjugation rendering its superiority over unconjugated bile acid (Pellicciari et al., 2004) and a preference of PL for conjugation (Miller et al., 2007). Conjugated bile acids have been shown to activate receptor tyrosine kinases and intracellular signaling
lipid remodeling and protect cells by increasing membrane fluidity (Stubbs and Smith, 1984).

AA (20:4n-6), DPA (22:5n-3), and DHA (22:6n-3) were identified as the main PUFAs, which were increased by UDCA-LPE treatment alone in a similar way to those fatty acids found during UDCA-LPE lipoprotection (Table 3). These increases in PUFAs may be due to the ability of UDCA-LPE to upregulate Fads1, Elovl6, FAS, and PPARγ mRNA expression (Fig. 5). These increases were uniquely specific for UDCA-LPE as its metabolite UDCA, LPE, or cAMP with lesser effectiveness in apoptosis inhibition did not increase these PUFAs. UDCA-LPE alone increased DPA (22:5n-3) and DHA (22:6n-3) contents in PS, thus supporting the importance of these species to play a role in cytoprotection (Kim et al., 2010). DHA is a precursor of potent anti-inflammatory signaling molecules (Moon et al., 2009; Kim et al., 2010). As ω-6 and ω-3 PUFAs are key components of membrane PL, their levels are known to be decreased in livers of NASH patients (Puri et al., 2007). The administration of DHA (22:6n-3) in NASH mice (Depner et al., 2013) and in children with fatty liver (Nobili et al., 2011) has been shown to be beneficial for treatment of this liver disease. The ability of UDCA-LPE to increase DHA bolsters its therapeutic use by strengthening PL membranes, increasing cell membrane fluidity, and replenishing the depleted DHA in NASH livers. Under in vivo conditions, TGs and PLs may be hydrolyzed by lipases and phospholipases to release free ω-3 PUFA, which can be subjected to β-oxidation (Hall et al., 2010). AA (20:4n-6) and DHA (22:6n-3) have been shown to suppress nuclear SREBP-1c (Ntambi, 1999; Jump et al., 2008; Moon et al., 2009), which in turn leads to decreased transcription of de novo lipogenesis genes in vivo. Consistently, we found that de novo lipogenesis gene expression in the livers of mice fed with a high-fat diet was markedly inhibited by chronic treatment with UDCA-LPE (Pathil et al., 2012).

In conclusion, UDCA-LPE protected lipoapoptosis by inducing a shift in fatty acid content toward MUFAs and PUFAs for incorporation into TGs and PLs concomitant with decreased Pal and SFA and rendering them unavailable for apoptosis. UDCA-LPE protected apoptosis by uniquely increasing PUFAs in PC and PS for cell membrane remodeling and stabilization. Our in vitro results provide mechanistic insights of a drug candidate UDCA-LPE for treatment of NASH by its unique metabolic reprogramming that minimizes damage brought on by excessive SFA.

Acknowledgments

The authors thank Nenad Katava for technical assistance and Dr. W. Hartmann for statistical analyses.

Authorship Contributions

Participated in research design: Chamulitrat, Xu, Pathil, Stremmel.

Conducted experiments: Xu, Gan-Schreier, Liebisch, Pathil.

Contributed new reagents or analytic tools: Liebisch, Schmitz, Pathil, Gan-Schreier, Stremmel.

Performed data analysis: Chamulitrat, Xu, Liebisch, Gan-Schreier.

Wrote or contributed to the writing of the manuscript: Chamulitrat, Liebisch, Xu, Stremmel.

References


Balagh et al., 2010. Associated with protection, increases in PUFAs, such as, AA, DPA, and DHA, were observed not only in PL but also in PE-based plasmalogen pools (Wallner and Schmitz, 2011). These PUFA-containing lipids may alter membrane fluidity (Stubbs and Smith, 1984).


